DEVELOPMENT AND COMPOSITIONAL ANALYSIS OF MICROBIAL COMMUNITIES SUITABLE FOR ANAEROBIC DIGESTION OF CARROT POMACE.

by

SARAHI LORENA GARCIA GARCIA

(Under the direction of Keshav Das)

ABSTRACT

Depletion of energy and environmental pollution are two of the major problems the world is currently facing. Anaerobic digestion of organic wastes offers a solution to both problems because it converts the organic waste material into methane which is a combustible gas and can be a renewable energy source. However, knowledge about the microorganisms involved in the process is still very limited. In this study a microbial community suitable for anaerobic digestion of carrot pomace was developed from inocula obtained from natural environmental sources. The changes along the process were monitored using pyrosequencing of the 16S rDNA gene. As the community adapted from a very diverse natural community to a specific community with a definite function, the diversity decreased drastically. The bacterial population in an anaerobic reactor was found to be more diverse than the archaeal population. Major bacterial groups in the anaerobic digestion were *Bacilli* (31% - 45.3%), *Porphyromonadaceae* (12.1% - 24.8%) and *Spirochaetes* (12.5% - 18.5%). The archaeal population was mainly represented by an OTU that is 99.7% similar to *Methanosarcina mazei*. Failures in the methane production were related to shifts in bacterial populations and loss of methanogens.

INDEX WORDS: Anaerobic digestion, Carrot pomace, Microbial community, 16S rDNA libraries

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

In the Unites States, food waste has progressively increased from nearly 30% of the available food supply in 1974 to almost 40% in recent years (9). This percentage includes the 18% of vegetables lost in the farm due to deterioration, neglect and processing and the 17 tons of fruits and vegetables wasted annually at the supermarket (14). Many of the fruits and vegetables produced are never consumed and go to waste. This kind of waste represents a potential pollutant and a loss of biomass that could have other applications. The use of this biomass to produce energy would lead to a more sustainable economy.

Fruit and vegetable waste contains 8-18% total solids (TS), with a total volatile solids (VS) content of 86-92%. Anaerobic digestion of fruits and vegetables waste generally permits the conversion of 70-95% of the organic matter to methane. Efficient systems can produce methane up to 420 L/kg of VS added (3). Taking in consideration these numbers, the amount of fruits and vegetables wasted in every supermarket would yield up to 1 million liters of methane per year. This is the equivalent to 350 gallons of gasoline in terms of energy content.

Anaerobic digestion, a process that involves three major groups of microorganisms (1), is a conversion of organic wastes into biogas, mainly methane (CH_4 60% - 70%) and carbon dioxide (CO_2 30% - 40%) (4). The more methane produced the more efficient the process becomes because it decreases environmental pollution and increases the production of combustible energy (10). Although scientists have been working on anaerobic digestion since the early 20th century, commercial anaerobic digestion

is far below its potential. Furthermore, digestion of vegetable waste has an added difficulty due to the presence of rapidly hydrolyzable components and consequent acidification and inhibition of methanogenesis (30). In addition, monitoring and controlling the anaerobic digestion from a biological perspective has not been fully possible due to the complexity of the microbial community and lack of understanding of the biochemical reactions and interactions within the community (21, 24).

This project conducts a deeper study of the anaerobic digestion of carrot waste starting with small volume reactors and natural sources of microorganisms. Information from the small scale digesters will be used to develop a mature microbial community. The study includes the scaling of reactors to test the community's stability.

LITERATURE REVIEW

Anaerobic digestion is a process where microorganisms convert organic wastes into biogas. This process simultaneously degrades waste while producing energy in the form of methane. This energy can be used for electricity or heat production (1).

Three major groups of microorganisms are involved in the degradation process (Figure 1):

1. Hydrolyzing and fermenting microorganisms, which organisms break down the polymers and monomers present in the waste and produce acetate, hydrogen gas and volatile fatty acids.
2. Obligate hydrogen-producing bacteria, that convert volatile fatty acids into hydrogen gas and acetate.
3. Methanogens that are composed of two subgroups. The first producing methane from hydrogen (hydrogenotrophic methanogens) and the second from acetate (acetotrophic methanogens).

There is a close relationship between the obligate hydrogen-producing bacteria and hydrogenotrophic methanogens. This relationship is called syntrophy. This means that the consumption of the fatty acids (especially butyrate), formed as intermediates by the hydrolyzing microorganisms, is thermodynamically unfavorable unless coupled with the consumption of hydrogen by hydrogenotrophic methanogens (28).

There are different kinds of feedstocks for anaerobic digestion (30). Likewise, the versatility of the process is due to the different types of microorganisms that can grow on the components of the organic matter. A summary of the different microbes found in various types of reactors and substrates is shown in Table 1.

Vegetable wastes are mainly characterized by high moisture, low total solids and high volatile solids. This kind of waste is easily degraded, but the rapid hydrolysis of polymers and monomers leads to acidification and, in many cases, the delay or inhibition of methanogenesis (3, 30). In the juice industry, thousands of tons of carrot pomace are produced after juice extraction. This waste, generally disposed of as animal feed (11), could be used for methane and subsequently electricity production if the right microbial population can be found.

Previous studies have examined the feasibility of using vegetable or fruit waste as a feedstock for methane production. One such study was conducted by Clark and his colleagues (5).The digestion of banana waste was studied relying entirely on its natural microbial consortia. Approximately 80% of the volatile solids in bananas, like in many other fruits and vegetables, are composed of easily degradable carbohydrates. Their results showed a drop in pH during the startup phase. This acidification is related to hydrogen, acetate and butyric acid accumulation. The acidic conditions continued up to 40 days. After this time, the pH started to rise and hydrogen and volatile fatty acids concentrations started to diminish upon the onset of methane production. In conclusion, banana waste has poor presence of microorganisms for rapidly initiating methanogenic conditions. Clarke's study demonstrates that

vegetable and fruit waste are prone to acidification and highlights the importance of using a proper inoculum.

Starting a bioprocess such as anaerobic digestion can be achieved by inoculating with a consortium of microorganisms or by adding specific microorganisms such as in bioaugmentation (1). All of these microbes occur naturally in anaerobic ecosystems such as sediments, paddy fields, water-logged soils and in the rumen (32). In general, the inocula for reactors are usually mixed communities of unknown composition. Mesophilic sludge from wastewater treatment plant was used as inoculum in a study by Forster-Carneiro et al. In their study the acclimatization took up to 60 days until the methane production reached its maximum (7). Nair et al. used rumen fluid to digest grass observing a 50% degradation in 4 days (22). Kaparaju et al. used material from a biogas fermenter treating manure and industrial waste as inoculum for the treatment of manure (15). Since many different microbes with different roles in the overall process are needed, there are a wide variety of anaerobic sources that can be used.

Bioaugmentation, the addition of specific microbes for the desired process, can be used as well. This concept relies on the ability of microorganisms to adapt to environmental conditions established during the process. In 2007 Nielsen et al., reported that adding two types of bacteria specialized in degrading cellulose and xylose improved anaerobic digestion of cattle manure (23). Savant and Ranade added an acid-tolerant methanogen to an acidogenic digester to a 8 day experiment (26). Both studies found a significant increase in methane yield.

Even when microbes are added, monitoring and controlling the anaerobic digestion process from a biological perspective has not been fully possible (24) due to the complexity of the microbial community and lack of understanding of the biochemical reactions and interactions among the microbes within this community (21). There have been many studies aiming to elucidate the microbial communities in anaerobic digesters (6, 8, 13, 16-20, 27, 29, 31) (Table 1). However, no previous studies

were found characterizing microbial communities performing anaerobic digestion using carrot as a substrate.

Polymerase chain reaction amplification of the 16S rRNA gene is used to assist in the understanding of community structures (2). This technique can also be used to elucidate population dynamics of bacteria and archaea through time. Furthermore, it has been shown that full length small subunit rRNA and hypervariable regions V3 and V6 provide equivalent measures of relative abundance (12). Pyrosequencing, a DNA sequencing technique that allows a large number of samples to be analyzed in one run (25), increases the number of organisms that can be sampled allowing a cost-effective exploration of changes in microbial community structure (12). All these techniques would be helpful in the study of the anaerobic digester's microbial communities and their changes during their acclimatization to a specific substrate.

It can be inferred from the literature that the startup and success of a reactor depends on more than just having waste in an anaerobic reactor. By adding a random source of microorganisms as inocula, the certainty of having the right microorganisms decreases. Microorganisms selected should be specific to degrade the desired organic waste and tolerate the conditions they will be exposed to.

Carrot waste is composed of easily hydrolysable polymers that may acidify the waste and delay methane production. Starting a reactor with substrate acclimatized microorganisms, and elucidating the composition of the communities, would lead to a better understanding of the process within the reactors and their stability.

OBJECTIVES

The main objective of this study was to develop a natural microbial community capable of converting carrot pomace into methane. The adapted consortium was developed from several

environmental samples through a series of enrichments with the selected waste. Specific objectives included the following:

- Elucidating the adapted communities' composition.

  Molecular analysis was performed on samples taken from the microbial community at defined times during the enrichment process, and their composition was determined. This provided insight into the adaptation process and the microorganisms present in the communities that oxidize organic matter into methane.

- Scale up the adapted community and test its stability in a continuous reactor.

  The microbial community was scaled up, and its stability was tested in a 3.45 liter upflow anaerobic reactor. This was to further demonstrate the stability of the community and the capability of adaptation in a continuous flow reactor.

This project was the first step towards the understanding of microbial communities' adaptation in methane producing reactors.

HYPOTHESIS

The individually developed microbial communities will possess microorganisms that have functionally equivalent characteristics even though the sources of inoculum are different. It is expected that groups of carbohydrate-utilizing bacteria, amino acid utilizing bacteria, syntrophic bacteria, and aceticlastic and hydrogenotrophic methanogens will develop in consortia regardless of the source of inoculum.

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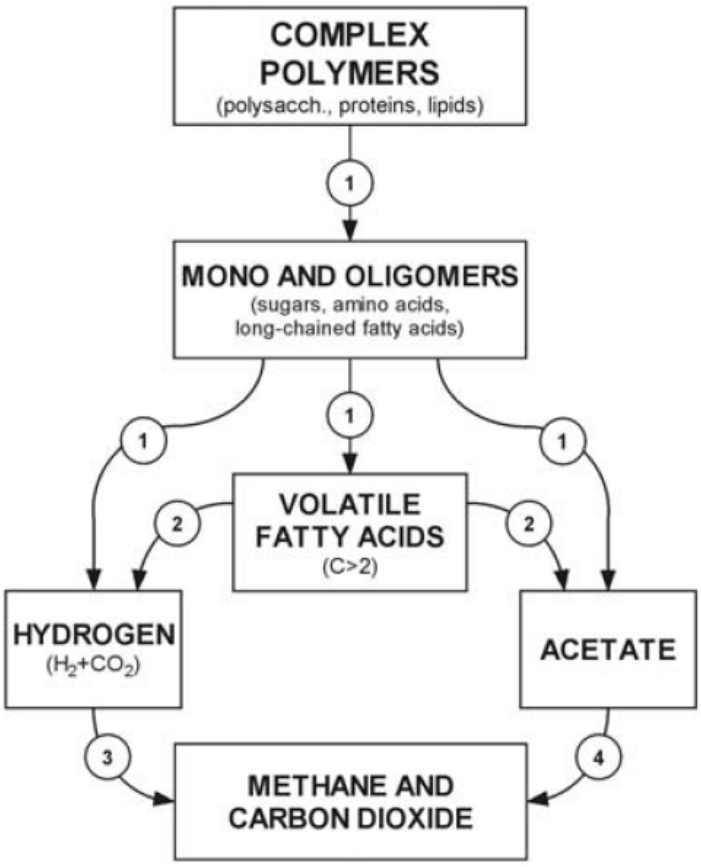

Figure 1. The anaerobic digestion process according to Ahring (1). Numbers in the figure are described. 1.Hydrolyzing and fermenting microorganisms. 2.Obligate hydrogen-producing bacteria. 3. Hydrogenotrophic methanogens. 4. Acetotrophic methanogens.

Table 1. Summary of microbes present in digestion of different types of organic matter

Organic substrate	Type of reactor	Most abundant bacteria*	Most abundant methanogens*	Molecular tool used to identify the microbe	Reference
Glucose	Continuously stirred reactor	Spirochete group *Eubacterium* genus *Propionibacterium* genus	*Methanobacterium formicicum* *Methanisarcina mazei* *Methanobacterium bryantii*	ARDRA	Fernández et al 1999 (6)
Municipal wastewater	UASB	10 uncultured *Desulforhabdus amnigena*	*Methanosaeta concilii*	DGGE	Ying et al 2005 (31)
Fodder beet silage	CSTR	*Sedimentibacter* *Desulfotomaculum* *Peptococcus*	*Methanosarcina* *Methanosaeta concilii* *Methanobacterium formicium*	Clone library and ARDRA	Klocke et al 2007 (16)
Maize silage, green rye and chicken manure	Nonspecified	*Sedimentibacter* *Syntrophomonas* *Acetivibrio* *Clostridium*	*Methanoculleus*	16S rDNA gene pyrosequencing	Krause et al 2008 (18)
Maize silage, green rye and chicken manure	Nonspecified	*Clostridium thermocellum* *Thermosinus carboxydivorans* *Halothermothrix orenii*	*Methanoculleus marisnigri*	Metagenomics and pyrosequencing	Schlüter et al 2008 (27)
Liquid pig manure, thermally pretreated food, plant cuttings, grass silage and corn silage	CSTR	N/A	*Methanoculleus bourgensis* *Methanobrevibacter* *Methanobacterium formicicum* *Methanosarcina thermophila*	ANAEROCHIP microarray 16S rRNA gene cloning and sequencing	Franke-Whittle et al 2009(8)
Pig manure	Nonspecified	Uncultured anaerobic *Alkaliflexus imshenetskii* *Petrimonas sulfuriphila*	*Methanoculleus bourgensis* *Methanosarcina barkeri* *Methanospirillum hungatei*	DGGE and sequencing	Liu et al 2009 (20)
Swine wastewater	UASB	*Acidobacterium sp.* *Deltaproteobacteria* *Syntrophobacter sp.* *Tissierella sp.*	N/A	PCR-DGGE and ARDRA	Li et al. 2010 (19)
Beet silage and beet juice	Nonspecified	N/A	*Methanobacteriales*	FISH, cloning and ARDRA	Krakat et al 2010 (17)
Swine wastewater	Anaerobic batch reactor	N/A	*Methanoculleus bourgensis* *Methanosarcina acetivorans*	DGGE, cloning and real-time PCR	Hwang et al 2010 (13)
Cow manure and grass silage	CSTR	*Bacteroidetes* *Clostrideacea*	N/A	T-RFLP and DGGE	Wang et al 2010 (29)

* Organisms found in the studies were reported in terms of their closest homologues.

CHAPTER 2

MATERIALS AND METHODS

Samples

Six samples were collected from different natural environment sources (Table 2). Samples A, B, E and F, are all sediments collected underwater, from the bottom of the natural sites, where anaerobic conditions were confirmed by biogas production. Sample C was collected from a grass fed cow's rumen using a hose and a vacuum pump. Sample D was collected from a grain-fed, fistulated cow by manually extracting the contents of the rumen and squeezing them to extract the liquid portion.

The samples were stored in sealed glass bottles at room temperature in darkness until the day of inoculation. Each sample was used to inoculate a separate enrichment. A seventh enrichment was inoculated using a mixture comprised of equal volumes of each sample. DNA was extracted from each sample on the day of inoculation. DNA was stored at -80°C.

Cultivation media

A volume of 1.5 kg of carrot was subjected to juice extraction in a clean juice extractor. Pomace, 102 g, together with 850 mL of filtered water were transferred to a clean glass blender. The water and the waste were blended for 5 min at the highest setting and the resulting material was evenly distributed into 14 sterile bottles of 160 mL to be stored in the freezer at -20°C.

The enriched mineral solution was prepared according to Balch et al. (3). It consisted of 50 mL of general salts solution, 50 mL of K_2HPO_4 (6 g/L), 10 ml of trace mineral solution, 1 mL of iron stock

solution, 5 g of $NaHCO_3$ and 0.5 g of cysteine (added after boiling). General salt solution contained K_2HPO_4 (6 g/L), $(NH_4)_2(SO_4)_2$ (6 g/L), NaCl (12 g/L), $MgSO_4.7H_2O$ (2.6 g/L), and $CaCl_2.2H_2O$ (0.16 g/L). Iron stock solution was prepared by adding 0.2 g of $Fe(NH_4)_2(SO_4)_2.6H_2O$ to a small screw top bottle with 2 drops of concentrated HCl followed by 100 mL of glass distilled water. Trace mineral solution contained nitriloacetic acid (1.5 g/L), $MgSO_4.7H_2O$ (3 g/L), $MnSO_4.2H_2O$ (0.5 g/L), NaCl (1 g/L), $Fe_2SO_4.7H_2O$ (0.1 g/L), $CoCl_2$ (0.1 g/L), $CaCl.2H_2O$ (0.1 g/L), $ZnSO_4$ (0.1 g/L), $CuSO_4.5H_2O$ (0.01 g/L), $ALK(SO_4)_2$ (0.01 g/L), H_3BO_3 (0.1 g/L) and $Na_2MoO_4.2H_2O$ (0.1 g/L).

Preliminary experimentation to determine optimal substrate concentration

Two sets of preliminary experiments were performed to determine the carrot concentration best suited for the enrichments. In the first set of enrichments, four different environmental samples were used as 10% inocula into two concentrations (1% and 10%) of simple carrot enrichments to determine at which substrate concentration methane was produced the fastest. A second set of enrichments were performed to confirm that 1% carrot enrichments and 10% inocula were the optimum substrate and inoculum concentration. Aliquots of 3 ml were taken from the two enrichments with the highest methane production and further tested at different carrot concentrations. Eight conditions were tested in duplicate. Five of those conditions were to test the carrot concentration and three were to test inoculum concentration. Aliquots of 3 mL from the selected enrichments were inoculated into 1%, 2%, 4%, 6% and 10% carrot waste. To analyse the effects of the inoculum concentration, three dilutions of the 1% enrichment were prepared. After the 1% carrot enrichment was prepared and inoculated, a series the dilutions were transferred to bottles containing 1% carrot, giving rise to enrichments that were inoculated with approximately 1%, 0.1% and 0.01% of the contents of the previous selected of enrichment. The enrichments contained deionized water, 10% inoculum (or other if stated) and the carrot concentration to be tested for a total working volume of 30 mL in a 160 mL glass bottle.

Anaerobic conditions were created by sealing the bottles, flushing the bottles with nitrogen for 30 minutes.

Enrichments

Six environmental samples were used as inocula for acclimatization to anaerobic digestion of carrot waste. A seventh acclimatization was performed using a mixture of the six sources sources. The objective of the acclimatization of the mixture was to observe how a combination of different inoculum sources would work to develop a community capable of degrading carrot waste and producing methane. In total, three successive sets of enrichments were conducted in triplicate. The first set was inoculated with 10% (vol./vol.) of an environmental sample, the second set was inoculated with 10% of the first enrichment, and the third set was inoculated with 10% of the second enrichment.

Acclimatization of the samples to carrot waste was conducted in 160 mL serum bottles. Each sample was enriched in triplicate. The enrichment consisted of 24.5 mL of enrichment mineral solution, 2.5 mL of carrot waste solution (120 g/L), and 3 mL of inoculum.

The enrichment mineral solution was added to the serum bottles in an anaerobic chamber. The gas phase of the bottles was exchanged using two cycles of 30 seconds of vacuum and 30 seconds of a mixture of gas (N_2 80% and CO_2 20%) with a pressure of 5 psi. After gas exchange, the bottles were autoclaved. Carrot waste and inoculum were transferred consecutively with a sterile syringe and needle, size 18G1½, once the bottles were cooled at room temperature.

Once the enrichments did not produce more methane, an aliquot of 3 mL was taken and inoculated into fresh carrot waste enrichment. The aliquots were taken after vigorous shaking to homogenize the enrichment. The next enrichment preparation was conducted as previously described. The successive enrichment was repeated for another generation for a total of three enrichments. After

inoculation, 0.2 mL of the enrichment products were used for analysis of chemical oxygen demand (COD) and 4.5 mL were centrifuged for DNA extraction from the pellet.

Scaling up the microbial community

The triplicates of the first generation carrot enrichment that produced the most methane were scaled up to 200 mL enrichments in a one liter bottle. The scaled enrichment consisted of 163.3 mL of enriched mineral solution, 16.67 mL of carrot waste solution (120 g/L), and 20 mL of inoculum. Methane production was monitored using a gas chromatograph (SRI 8610-C). Once the maximum amount of methane was reached, the 200 mL enrichments were scaled up into a 2 L enrichment in 4 L bottles. Since the bottles did not fit in the anaerobic chamber, the anaerobic conditions were created by flushing the bottles with a mixture of gas (N_2 80% and CO_2 20%) for 60 minutes.

Upflow anaerobic reactor

Two 3.45 L working volume reactors were used. Each reactor consisted of a glass column with an internal diameter of 10 cm and height of 50 cm, with 4 sampling ports evenly distributed along the height of the column. A peristaltic pump was set so the inlet could be at the bottom of the reactor and the outlet at the top. One reactor was set up with raw rumen fluid as the inoculum and another reactor was set up with the acclimatized inoculum from the 4 L bottle enrichments. Both inocula were analyzed for total solids (TS), volatile suspended solids (VSS) and chemical oxygen demand (COD) before starting the reactor. The reactor was started with about 1.7 L acclimatized inoculum, and the other reactor with 350 mL of rumen fluid. Both reactors were topped with enriched mineral solution and 1% carrot pomace was added. The content of the reactor was recirculated at a flow rate of 0.5 mL per minute until the COD decreased by 60%. After recirculation the flow rate was kept constant with fresh waste influent. The hydraulic retention time (HRT) at that flow rate was 5 days. The flow rate was further increased based

on the COD removal. The feedstock bucket was cleaned and feedstock was prepared daily. Biogas production was monitored.

Analytical methods

Methane production was monitored using gas chromatography on a SRI 8610-C gas chromatograph with a 80/100 Porapack Q 6ft x 1/8 inch column with nitrogen carrier gas, an oven temperature of 60°C, and a flame ionization detector. All needles and syringes used were sterile and disposable and were subjected to gas exchange with nitrogen.

COD was assessed using a colorimetric method. Samples were digested in a digital reactor, DRB 200 (Hach Company, Loveland, Colorado), using the fabricant digestion solution for COD (0 – 1500 ppm range, Hach) and optical density was observed in a spectrophotometer DR 2700 (Hach).

Community DNA extraction and pyrosequencing

Total community DNA was extracted using ZR Soil Microbe DNA Kit (Zymo research, Orange, California) and the manufacturer's protocol, with the following modifications: the starting material was a pellet from 4.5 ml of supernatant, and the Zymo-Spin™ IIC Column was incubated for 5 minutes at room temperature after the addition of DNA Elution Buffer to increase DNA yield. DNA extraction was visually verified by electrophoresis on a 1% agarose gel with ethidium bromide staining (Sigma-Aldrich, St. Louis, Missouri). PCR amplification of the V3 and V6 region of the bacterial and archaeal 16S rRNA genes, respectively, was conducted. The PCR conditions consisted of initial denaturation at 95°C for 3 minutes followed by 20 or 25 cycles for bacteria and archaea, respectively, of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 68°C for 60 s. The final extension was carried out at 68°C for 4 minutes. The bacterial primers used were 515R-M (5'-CCGCNGCKGCTGGCAC-3') modified after Acosta-Martínez et al. (1) and the sevenfold-degenerate primer 27F-YM+3 (5). The sevenfold-

degenerate primer 27F-YM+3 is four parts 27F-YM (5'-AGAGTTTGATYMTGGCTCAG-5'), plus one part each of the primers specific for the amplification of *Bifidobacteriaceae* (27F-Bif, 5'-AGGGTTCGATTCTGGCTCAG-3'), *Borrelia* (27F-Bor, 5'-AGAGTTTGATCCTGGCTTAG-3'), and *Chlamydiales* (27F-Chl, 5'-AGAATTTGATCTTGGTTCAG-3'). The archaeal primers used were 1043R-YH (5'-GGCCATGCACCWCYHCTC-3') (2) and 533F-K (5'-GTGBCAGCMGCCGCGGKAA-3') modified after Sørensen and Teske (11). The binding sites with respect to *E. coli* 16S rRNA gene (Genbank accession number U00096) are reflected in the primer name. For purposes of pyrosequencing, the primers were synthesized with an adaptor as shown: Adaptor(A)-BARCODE-(515R-M or 1043R-YH) and Adaptor(B)-(27F-YM+3 or 533F-K). The Roche Adaptor(A) (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3') and Adaptor(B) (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3') for their titanium platform were used. For each DNA sample, a specific 8-nt barcode was used. The barcode sequences were selected from Hamady et al. (2008) (6) based on GC%, melting temperature and complementarity with the primers listed above. PCR reactions were performed on a Veriti 96-well Thermal Cycler (AB Applied Biosystem, Carlsbad, california). PCR products were visualized by electrophoresis on 1% agarose gels, stained with SYBR Green Dye (Invitrogen, Carlsbad, California). The resultant images were scanned using Typhoon Trio+ Variable mode imager (GE Healthcare, Pittsburg, new Jersey). PCR bands were quantified using Image Quant 5.2 (Molecular Dynamics).

PCR amplicons obtained from replicate samples were pooled together in equimolar concentrations prior to gel elution. Pooled products were recovered using Zymoclean Gel DNA Recovery Kit (Zymo research, Orange, California). Concentration of the gel eluted DNA were assessed using capillary electrophoresis on the Experion System (Bio-Rad, Hercules, California). All samples were pooled together in equimolar concentration according to the number of sequences desired for each sample. Pooled samples were purified using the Agencourt AMpure magnetic bead purification method (Beckman Coulter, Brea, California). The purified DNA was resuspended in 40 µL of TE buffer.

Concentrations of the purified DNA were assessed using the Experion System (Bio-Rad, Hercules, California) and 500 ng of purified amplicons were submitted to Georgia Genomics Facility (http://dna.uga.edu/) for pyrosequencing.

Real-time polymerase chain reaction (qPCR)

qPCR was used to estimate the abundance of the 16S rDNA genes from Bacteria and Archaea. Gene abundance in 1 µL of extract was measured in triplicate on a iCycler iQ5 thermocycler (Bio-Rad, Hercules, California). The PCR conditions consisted of initial denaturation at 95°C for 5 minutes followed by 35 and 45 cycles for bacteria and archaea, respectively, of denaturation at 95°C for 45 s, annealing and extention at 60°C and 62°C (for bacteria and archaea, respectively) for 40 s and image at 82°C for 25 s. At the end, the reaction was heated at 95°C for 1 minutes followed by 1 minute at 56°C. The bacterial primers used were 515R (5'-CCGCNGCKGCTGGCAC-3') modified after Acosta-Martínez et al. (1) and 356F (5'-ACTCCTACGGRAGGCWGC-3') modified after Rudi et al.(9). The archaeal primers used were 515R (5'-TTMCCGCGGCKGCTGVCAC -3') modified after Sørensen and Teske (11) and 349F (5'-GYGCASCAGKCGMGAAW -3') (12). The binding sites with respect to *E. coli* 16S rRNA gene (Genbank accession number U00096) are reflected in the primer name. Abundance of *Archaea* and *Bacteria* 16S rDNA genes was determined using iQ SYBRgreen super mix (Bio-Rad, Hercules, California) to measure amplicon acuumulation. *Bacteria* standards were made from *Xanthamonas* (Genbank accession number EF665883). Standards for *Archaea* genes were amplicons cloned from environmental samples. Standard curves were performed according to Kalanetra et al. (7).

Inclusion of controls for pyrosequencing

A set of three previously cloned full length 16S rRNA gene fragments were used to prepare control amplicons to test for errors during PCR amplification and pyrosequencing. These clones were

previously prepared for the Michigan GASP dataset (Jangid et al., in prep). Plasmids were isolated from 5 ml of overnight cultures previously inoculated with a single colony of the selected clones followed by gel quantification using SYBR Green Dye (Invitrogen, Carlsbad, California) on a Typhoon Trio+ Variable mode imager (GE Healthcare, Pittsburg, New Jersey). To prepare the controls C1 to C3, 30 ng of plasmid was used as a template for three separate PCR amplifications, as identified in Table 3. For amplification 20 cycle PCR reactions were set for each control under previously described conditions. Each control reaction used a different barcode as described above. Sequence pipelines were done testing different length of controls DNA sequences and distances for clustering, as well as the use of software to evaluate the best set of conditions to analyze the enrichments amplicons sequences.

Sequence analysis pipeline

Sequences analysis was carried out using a combination of QIIME (4) and MOTHUR (10). The sequences were aligned using the SILVA database in MOTHUR and further filtered. Operational taxonomic units were clustered using the average neighbor method. Representative sequences were classified using SIMO RDP query (8).

The commands for the analysis pipeline go as follows (Step 1 to 4 were done in QIIME, 5 and 6 were done using word processing software and 7 to 15 were done using MOTHUR):

1. sffinfo *.sff > *.sff.txt
2. split_libraries.py -m *map.txt -f *.fna -q *.qual -l 400 -r -b 12 -o *output_folder*
3. denoise.py -v -i *.sff.txt -f *output_folder*/seqs.fna -o *output_folder* /denoised/ -m *map.txt
4. cat *output_folder* /denoised/centroids.fasta *output_folder* /denoised/singletons.fasta > *.fasta
5. *.names file from denoised_mapping.txt
6. *.groups file from seqs.fasta
7. align.seqs(candidate=*.fasta, template=silva.bacteria.fasta/silva.archaea.fasta)

8. screen.seqs(fasta=*.align, start=, end=, name=*.names, group=*.groups)
9. filter.seqs(fasta=*.good.align, vertical=T, trump=.)
10. unique.seqs(fasta=*.good.filter.fasta, name=*.good.names)
11. pre.cluster(fasta=*.good.filter.unique.fasta, name=*.good.filter.unique.names)
12. dist.seqs(fasta=*.good.filter.unique.precluster.fasta, cutoff=0.10)
13. read.dist(column=*.good.filter.unique.precluster.dist, name=*.good.filter.unique.precluster.names, cutoff=0.03)
14. cluster(method=average)
15. read.otu(list=*.good.filter.unique.precluster.an.list, group=*.good.groups)

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Table 2. Samples collected from natural environments to adapt for anaerobic digestion of carrot

Sample ID	Sample description	Sampling time	pH
A	Okeefenokee swamp sediments	January 2009	5.3
B	Okeefenokee swamp sediments	January 2009	4.9
C	Rumen from a cow fed with grass	March 2009	7.3
D	Rumen from a cow fed with grass and grains	March 2009	6.3
E	Lake Herrick sediments	September 2008	6.4
F	Botanical garden sediments	September 2008	6.7

Table 3. Details of the three control datasets used in the study

Control Number	Template used	Genbank accession number	Total # of sequences
C1	MA1S1_F03 *Actinobacteria (Frankineae)*	EF662369	1521
C2	MB3W2_G05 *Gamma (Xanthomonas)*	EF665883	1514
C3	MB1W1_H07 *Firmicute (Bacillus)*	EF664802	952

CHAPTER 3

RESULTS AND DISCUSSIONS

Preliminary experimentation to determine optimal substrate concentration

In the first set of enrichments, four types of samples were inoculated in duplicate to two different concentrations of carrot, 1% and 10%. The 1% and 10% carrot concentration represent 13.5 mg/L and 135 mg/L of VS respectively. At day 21, all of the 1% carrot enrichments had produced more methane than the 10% carrot enrichments (Figure 2). Vegetables, such as carrot, are easily degradable and can produce and accumulate volatile acids which are toxic to methanogenesis (1, 22). The 10% of carrot waste enrichments likely inhibited methanogenesis through volatile fatty acids accumulation.

In the second set of enrichments, bottles containing 1% and 2% carrot and an inoculum of 10% produced more methane than the other enrichments (Figure 3). Although, in sample 2 similar concentration of methane at 1% and 2% carrot loading were observed, sample 4 shows clearly that 1% carrot enrichments produced higher amounts of methane. Comparison of consecutive enrichments (Figure 2 and 3) show that at day 21, the methane production in the second set of enrichments is lower than the methane production from the first set. This could be caused by the difference in the amount of organic matter present in the samples. The first set of enrichments are inoculated with 10% natural samples, which contained significant amounts of carbon, while the second set of enrichments are inoculated with digested product from the first set. Based on these observations, it was decided that a 10% inoculums and 1% carrot waste will be used for further experiments.

Adapting natural sources of inoculum to anaerobic digestion of carrot.

In the first set of enrichments, which were inoculated with seven different environmental samples, only two showed considerable methane production (Figure 4). The enrichments were monitored until a plateau was observed. Rumen (pH 6.3) and the mixture of inocula reached its maximum methane production around day 40. On day 63 of the first set of enrichments, 3 mL of each digested enrichments were transferred to fresh medium to begin the second set of enrichments. The behavior of the first set and the second set of enrichments showed a similar trend in methane production (Figure 4 and 5). Unlike the first set, the second set of enrichments reached the maximum methane production five days earlier. This shows the adaptation of the biogas producing community to the carrot waste. The second set of enrichments produced approximately half as much methane as the first set. This phenomenon was not unexpected since the first set contained a large organic load acquired from the 10% inoculum, while the second set was inoculated with digested product from the first set which had only small amounts of organic matter.

Once the methane production from the second set of enrichments plateaued, a third set was inoculated. The methane production from the third generation of enrichments followed a similar pattern. The rumen (pH 6.3) inoculum was the highest (Figure 6). The mixture exhibited a large variance in methane production because one of the triplicates failed to produce methane. In the third set of enrichments, neither the rumen (pH 6.3) nor the mixture reached a plateau.

Additional COD removal analyses were performed on the third set of enrichments in order to estimate the loss of organics. It can be seen that rumen (pH 6.3) and the mixture of inocula were the enrichments that removed the most COD, and this correlated with the methane production observed (Figure 7). There was a larger variance in the mixed inoculum enrichment, this is related to the single enrichment that did not produce methane. Theoretically for every gram of COD removed, 0.351 L of

methane should be produced (8). On average the rumen (6.3 pH) removed 45 mg of COD but only produced 8.9 mL of methane, which represented 56% of the methane that could have been produced.

Carrot waste has a moisture content of 85%, therefore the actual amount of digestible dry weight per bottle was 0.045 g of carrot waste. Carrot pomace is mainly a source of fiber (4). If all that fiber converted to sugars and then to acetate, carbon dioxide and methane, a maximum amount of 750 micromoles of methane could be produced. Microorganisms use carbon for growing and maintenance, therefore it is impossible to reach the maximum methane production. Mixture and rumen (pH 6.3) enrichments produced larger concentrations of methane in the first enrichments; this may be due to the organic matter present in the original source of inoculum. Nevertheless, these same sources of microbes exhibit faster methane production in the later sets of enrichments which illustrates the adaptation of the microorganisms.

Influence of sulfates in the methane production.

Only two of the seven sources of inoculum produced significant amounts of methane. The mineral media used in these enrichments (3) was designed to culture methanogens. The final concentration of sulfate in the enrichments is around 83 μM. Since the carrot enrichments contained anaerobic communities, there is a chance that sulfate-reducing bacteria may be present and convert sulfate into sulfide, which could inhibit methanogenesis (9). A new medium was designed, replacing all of the sulfates with chlorides in equimolar concentrations. A set of enrichments was started which were inoculated with the six environmental samples (the mixture was not used) in triplicate. These enrichments contained either Balch medium or the modified Balch medium (replacing sulfate with chlorides). The methane production of the enrichments at day 42 showed that the presence of sulfates did not make a significant difference in five of the six cultures (Figure 8). The presence of sulfate makes a significant difference in the community present in sediments from the Botanical Garden (Athens, GA)

(letter D in Figure 8). However the amount of methane produced by this sample in the absence of sulfate was far below its potential. This eliminated the possibility that sulfate may have inhibited methanogens in the previous enrichments.

The enrichments inoculated from the rumen (pH 7.3) produced a significant amount of methane in contrast to its performance in the serial enrichments (letter C in figure 8). Although the source of inoculum was taken from the same anaerobic stored bottle, this graph shows that after a year of being stored anaerobically and at room temperature, the rumen community had transformed. Most likely shifts in the methanogenic community during starvation (7) resulted in a community more suitable for carrot digestion.

Scaling up the microbial community.

Usually anaerobic digesters are inoculated with up to 50% of volume, therefore, scaling up the enrichments to achieve a volume large enough for testing in an anaerobic reactor was necessary because. The upflow anaerobic digesters that were used have a volume of 3.45 L. This means that 1.72 L of inocula were needed.

From the first set of enrichments, 20 mL of digested product was used as a 10% inoculum for a 200 mL digestion. Based on the amount of substrate added and ignoring the carbon uptake of the microorganisms, the maximum methane formation would be 5000 µmol. Methane peaked around day 35 (Figure 9). These results are similar to its parallel experiment, the second set of enrichments. Both the second set of enrichments and the first stage scale up are derivatives of the microbial community developed in the first stage enrichment.

After the 200 mL enrichment's methane production plateaued, a second stage scale up was started. The volume of these enrichments was 2 L. These enrichments performed poorly compared to the previous enrichments (Figure 10A). The amount of substrate was 10 times higher than the first

scaled up enrichment, but the methane production was far below the expected production levels. From the triplicate, two of the enrichments produced less than 10% of the expected methane production, and the other produced 39% of its potential. Moreover, the maximum amount of methane was produced by day 73, which is twice as long as was previously observed.

The setup of these enrichments differed from previous enrichments in several aspects. Since the bottle size was 4 L, it did not fit into the anaerobic chamber so gas flushing was used to create an anaerobic environment. Cysteine was added after autoclaving, and, in general, the anaerobic environment was not maintained as well with these techniques. Presence of oxygen possibly caused the inhibition of methanogenesis. In previous setups, O_2 was completely absent. There is the chance that no microaerophilic bacteria were developed in these microbial communities, making it difficult for the community to tolerate minimum amounts of oxygen.

The microbial community of the 2 L enrichments was assessed. An aliquot of 3 mL of the digested product was taken and inoculated as previously described in the anaerobic chamber. The methane production was delayed when compared with the third set of enrichments, but after a period of acclimatization, the methane production was restored (Figure 10B). This demonstrates that the microbial community was still capable of methane production. Possibly, the presence of oxygen affected the methanogenesis in the 2 L enrichments.

Testing the community on an upflow anaerobic digester.

Two upflow anaerobic digesters were setup. One was inoculated with 50% with the digested product of the 2 L enrichment that produced higher concentration of methane; the second was inoculated with 10% of freshly collected filtered rumen fluid. Both reactors contained 1% carrot waste. A photograph of the reactors setup on day two can be seen in Figure 11.

The reactors went through a process of recirculation, in which a maximum methane production of 87.5 mmol was expected according to the amount of carrot waste added. COD removal and methane production were observed throughout the recirculation process. The methane production of the reactor with the adapted inoculum was very low, whereas the rumen showed a logarithmic methane production (data not shown). Chemical oxygen demand measurements were not accurate due to difficulty in taking representative and homogenous samples.

The fermentation process which can be scaled up without any difficulty is a rarity (12). During the setup of the reactors, the preparation and handling of the media and the containers differed from the 160 mL enrichments. The anaerobic conditions are much less controlled. Since the developed community was specialized and acclimatized to the conditions given in the small volume enrichments, all these changes in medium preparation and oxygen levels could be reasons for the failure of the reactor setup. However, the rumen sample consists of a very rich and diverse community that can adapt to these conditions with more ease than the already adapted developed community.

Analysis of 16S rDNA gene controls.

To establish the length of the amplicon used, the software parameters and distance at which the OTUs were to be clustered, three cloned 16S rDNA genes were run as controls. Since the length of the obtained sequences varied from 34 bp to 531 bp, with a median of 489 bp, a test was performed to determine the length at which the sequences should be trimmed. The sequences were analyzed using only MOTHUR (and without step #11 in the sequence analysis pipeline). At length 250 bp is when the less number of OTUs are formed, followed by length 400 bp (Table length). Among these two lengths, the percent of correctly classified sequences only differs by 1.21%. The length 250 bp includes 231 more sequences in the analysis than the length 400 bp, which translates to 34,650 base pairs more. On the other hand, the length 400 bp includes 367,800 more base pairs than the length 250 bp. Since the 400

bp length included more base pairs (information) and performed as wellas the 250 bp, it was chosen for further analyses.

Once it was established that the length of the amplicon used should be 400 bp, a comparison using different pipelines was performed. One of the pipelines tested was using only MOTHUR (and without step #11 in the sequence analysis pipeline). The other pipeline tested was using QIIME to denoise (step #3 in the sequence analysis pipeline) the data and pre.cluster (step #11 in the sequence analysis pipeline). It was evident that the use of these two tools reduced the number of OTUs and increased the percentage of correctly classified sequences especially at shorter distances (Figure 12). At longer distances such as 0.06 and 0.08 using denoise and precluster tools just increased by 0.3% the correctly classified sequences. The use of these parameters was included in the rest of further analysis.

Out of the 3987 sequences obtained from the sequencing facility, 70% passed the quality filters for inclusion in the analysis (Table 4). The quality sequences were aligned against the SILVA database in MOTHUR and clustered at different distances. The number of OTUs and the percentage of sequences correctly assigned approached a constant value at a distance of 0.03 (Figure 12). Therefore, 0.03 was selected as the distance to cluster the sequences. It is important to note that at distance 0.03, the percentage of correctly classified sequences is 96.23%. Therefore, there is an error rate of 3.77%. In addition, 38 OTUs are observed which is 35 more OTUs than expected from the three different 16S rRNA genes. Of those 35 OTUs, one contained 17 sequences distributed in the three libraries, which was probably a contamination. The representative sequence from this OTU was compared to the NCBI database and revealed 100% similarity to an *E. coli* 16S rRNA gene sequence. This information leads to the conclusion that 0.6% of the analyzed sequences came from a contaminant. The other 34 OTUs were all smaller than 10 sequences.

Community analysis.

The composition of the communities inhabiting the enrichments and the natural sources of inoculum was assessed through the amplification of a 16S rRNA gene amplified region (V3 for bacteria and V6 for archaea). The reads were aligned and clustered into operational taxonomic units (OTU). Since 5 of the inoculum sources did not produce a significant amount of methane, only the 16S rRNA genes from the two sources of inoculum and their successive enrichments that did produce significant amounts of methane were sequenced. Therefore the whole series of rumen (pH 6.3) and mixed source inocula were sequenced. One of the samples from the second generation of the mixed inoculum enrichments was lost due to degradation of DNA in the freezer.

Previous reports show that bacteria are the dominant superkingdom in the biogas reactor (10), therefore more sequences from the bacterial than the archaeal 16S RNA gene were obtained by submitting 20 times more bacterial than archaeal amplicons. Once the sequences were obtained from the sequencing facility, it was observed that the good quality sequences were about 70% (Table 5). After clustering, the archaeal sequences formed 59 OTUs, and the bacterial sequences formed 13,328 OTUs. The diversity of archaeal and bacterial communities diminished as the community became more adapted to the enrichment (Figure 13). The number of OTUs was drastically reduced after the first enrichment. Rarefaction curves were created to better illustrate how the richness of the communities decreased with the adaptation process (Figure 14). Another good estimator of diversity is the Simpson's diversity index. With this index, 0 represents infinite diversity and 1 represents no diversity. The Simpson's index of the bacterial and the archaeal gradually increases as the communities adapt (Table 6 and 7). The Shannon diversity index represents a measure of community evenness. The higher the number, the more homogeneous is a population. All these estimators confirm that anaerobic digestion has greater bacterial than archaeal diversity and that the more adapted the community becomes to a specific substrate and condition, the less diverse the community becomes.

A representative sequence from every archaeal and bacterial OTU was chosen and its closest homologue was identified. Representative sequences were matched to isolated species and environmental samples. Table 8 shows the number of bacterial and archaeal sequences and their percentage of relatedness with isolated microorganisms and environmental samples. Sequences with high similarity to database are less likely to be errors. Of the bacterial sequences, 97% have greater than 90% similarity to previously described environmental sequences, whereas 99.9% of the archaeal sequences have greater than 96% similarity to previously described environmental sequences. This reflects that bacterial communities from the environment and reactors are described less than archaeal communities in the literature. This could be because the archaeal community is less diverse, therefore, easier to sample and describe. The table also shows the number of OTUs and their percentage similarity with isolated microorganisms. At similarity values below 97.5% it is unlikely that two organisms are related at the level of species (18). Only 366 bacterial OTUs have greater than 97% similarity with previously isolated bacteria. Therefore, 97.25% of the OTUs found in the anaerobic community most likely have never been isolated. In the archaeal community, 10 OTUs show more than 97% similarity to isolated species, representing 16.9% of the total number of archaeal OTUs. The sampled archaeal community has been relatively more isolated than the sampled bacterial community.

Members from the domain Archaea are responsible for the methane production, which is the last step in the anaerobic digestion. After sequencing, 7698 quality were sequences obtained and clustered into 59 OTU using precluster followed by cluster commands in MOTHUR. Eleven OTUs out of the 59 cluster more than 20 sequences (Table 9). These eleven OTUs represent 98% of the total number of archaeal sequences obtained (Figure 15). A summary of these 11 OTUs is shown in Table 10 and Table 11. Two different trends can be observed. OTUs that were not detected in the source of inoculum were enriched and dominate the community in the digestion conditions. The most dominant OTUs in the source of inoculum were diluted out or otherwise removed during the enrichments. The distribution of

the sequences in the libraries supports that as the communities become more adapted to specific conditions, the diversity decreases. *Methanobacteriales* was the most dominant order of methanogens in both sources of inoculum (Table 12 and 13). *Methanosarcinales* was the most dominant order in the anaerobic digestion enrichments. The most abundant OTU in the enrichments was represented by a sequence that shares 99.7% similarity with *Methanosarcina mazei*. The group *Methanosarcina* is often found in anaerobic digestion (5-7, 11). Relatives to *Methanosarcina* were not detected in the rumen and hardly detected in the mixed source of inoculum.

The triplicate inoculated with the mixed source which failed to produce methane, was primarily colonized by an unclassified order of archea that is 82.5% related to *Thermogymnomonas acidicola.* Since the percent of similarity is low the phenotype of the microorganism cannot be deduced. The representative sequence of this OTU did have 97.4% similarity to a clone from a pig manure storage pit (16). Unclassified sequences closely related to members of the order Thermoplasmatales have also been found in sheep rumen (23). This shows that this kind of microorganism is well adapted to anaerobic environments and communities. As Snell-Castro discussed, it might represent a microorganism belonging to a new group of archaea.

The bacterial communities in reactors are responsible for the conversion of polymers into volatile acids, acetate, carbon dioxide and hydrogen that can be used by the methanogens to produce methane. Table 14 and 15 summarize the trend of the bacterial communities when adapted from the environment to the anaerobic digestion of carrot. Both natural communities have high abundance of *Prevotellaceae* and *Clostridia*. After the adaptation the major groups were *Spirochaetes*, *Porphyromonadaceae*, *Bacteroidaceae* and *Bacilli*. Some studies have found members of the group *Clostridia* were abundant in anaerobic reactors (5, 10, 14, 21), others have found *Bacteroidetes* (11, 21). All of these organisms anaerobically hydrolyze complex and simple sugars to produce acids (Table 16 and 17) (2, 13, 17, 20). It is also interesting to note that *Synergistetes* was not detected in the rumen and

barely detected in the mixed inoculum but increased to approximately 1% of the population in the final enrichments. *Synergistetes* is a group of microorganisms that couple the volatile fatty acids consumption with the H_2 production (15).

The enrichment that failed to produce methane shows another anomaly. While the other third generation enrichments have a low abundance of *Clostridia*, 44% of this enrichment consist of *Clostridia*. Most of these sequences are represented by OTU 9, which has 98.7% similarity to *Clostridium quinii* (Table 17). This microorganism was isolated from a UASB reactor and is a saccharolytic anaerobe that produces small amounts of butyrate during exponential growth (19). It was also observed that the number of *Porphyromonadaceae* and *Bacilli* are reduced in this enrichment. Presumably, this shift in bacterial population affected the methane production as well as the archaeal population, including the absence of the *Methanosarcina* group.

Moreover the abundance of the 16S rDNA genes was assessed. The average gene copy number per 1 µL of the bacterial communities in the enrichments was $3.58x10^6 \pm 2.36x10^6$. It was found that in reactors which produce methane, the number of bacterial 16S rDNA genes is up to 5 fold the number of archaeal genes, and this ratio increased to 16 in the reactor that failed to produce methane (Table 18). This proved the reduction of the archaeal community when the reactors failed to produce methane.

In conclusion, it was observed that when the community adapts from a very diverse natural community to a specific community with a definite function, the diversity decreased drastically. It was also be observed that the bacterial population in an anaerobic reactor was more diverse than the archeal population. A reactor failure to produce methane involved community shifts and the loss of methanogens. Further proposed studies would include amplification and sequencing of genes related to function along with the 16S rRNA gene in order to obtain more information from the communities. This information could be helpful to better understand the chemical interaction among microorganisms.

Isolation of new bacterial and archaeal species will also give further insight into the anaerobic digestion microbial communities.

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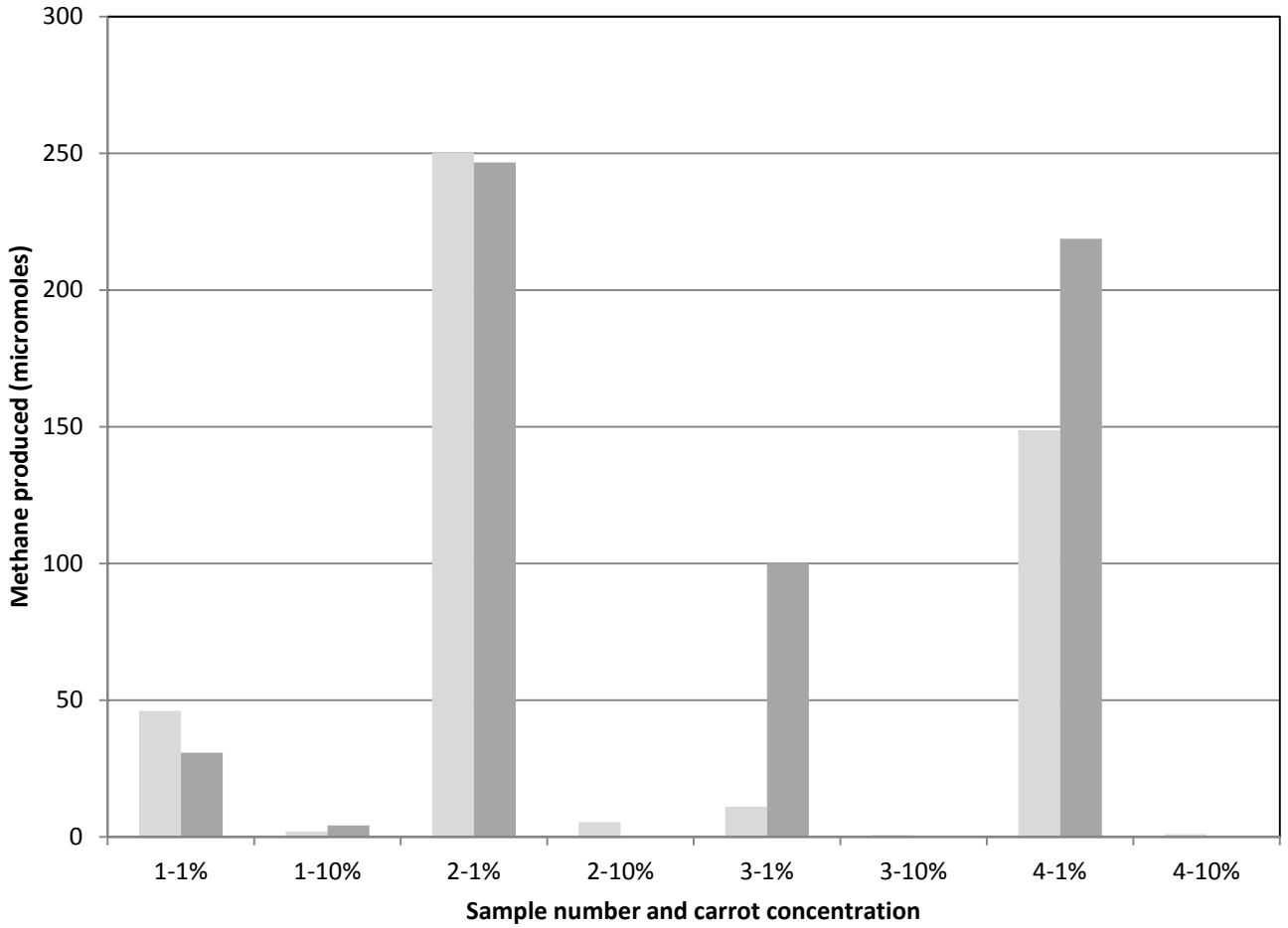


Figure 2. Micromoles of methane present in first set of preliminary enrichments on day 21. Dark grey and light grey bars represent the data from the two replicates. The sample numbers correspond to four different environmental samples that were collected, namely, 1. Okefenokee marsh (3/18/07), 2. Botanical garden (9/20/08), 3. Okefenokee wildlife refuge (3/18/07), and 4. Lake Herrick (9/20/08)

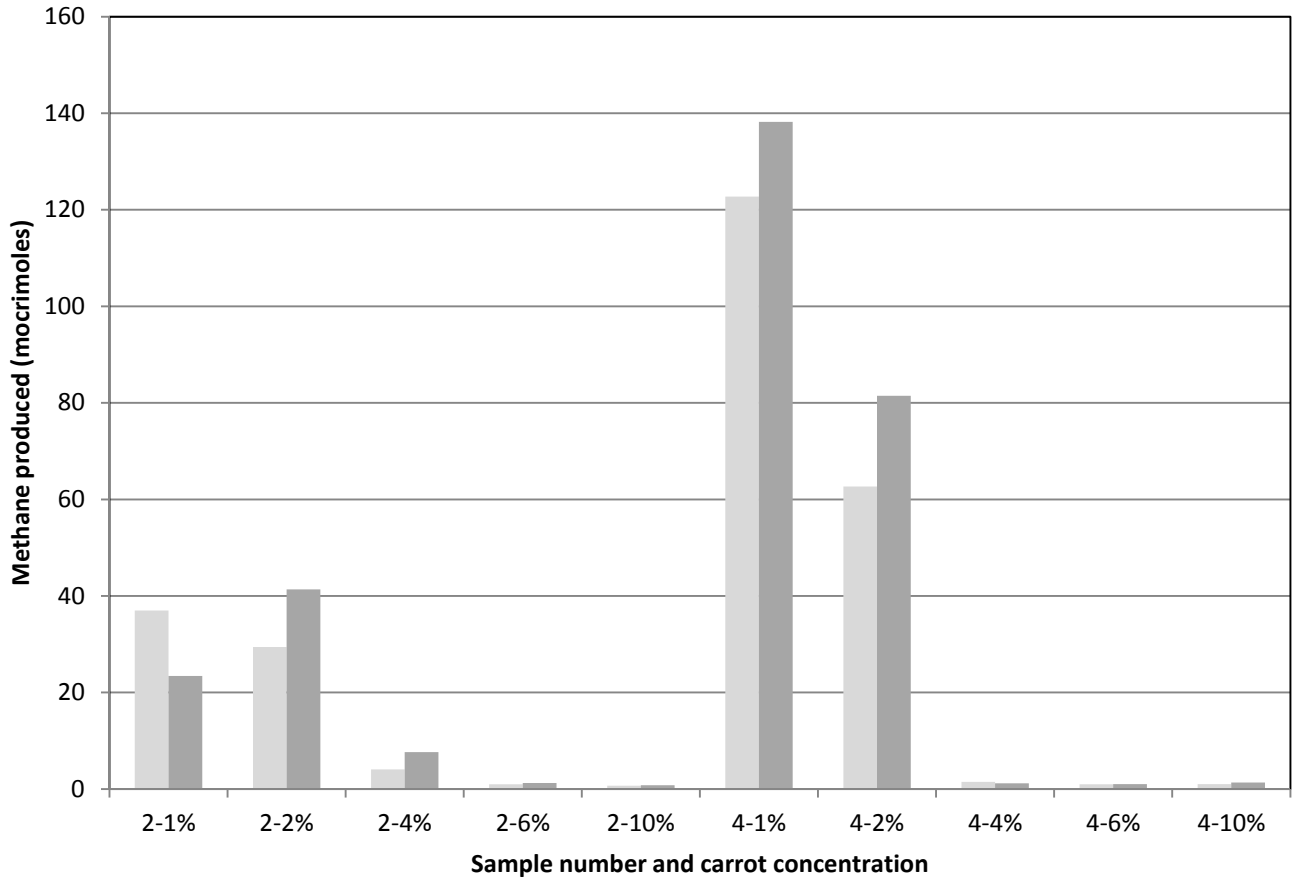


Figure 3. Micromoles of methane per bottle present in the second set of preliminary enrichments on day 21. Dark grey and light grey bars represent the data from the two replicates. The sample numbers correspond to four different environmental samples that were collected 2. Botanical garden (9/20/08), 4. Lake Herrick (9/20/08

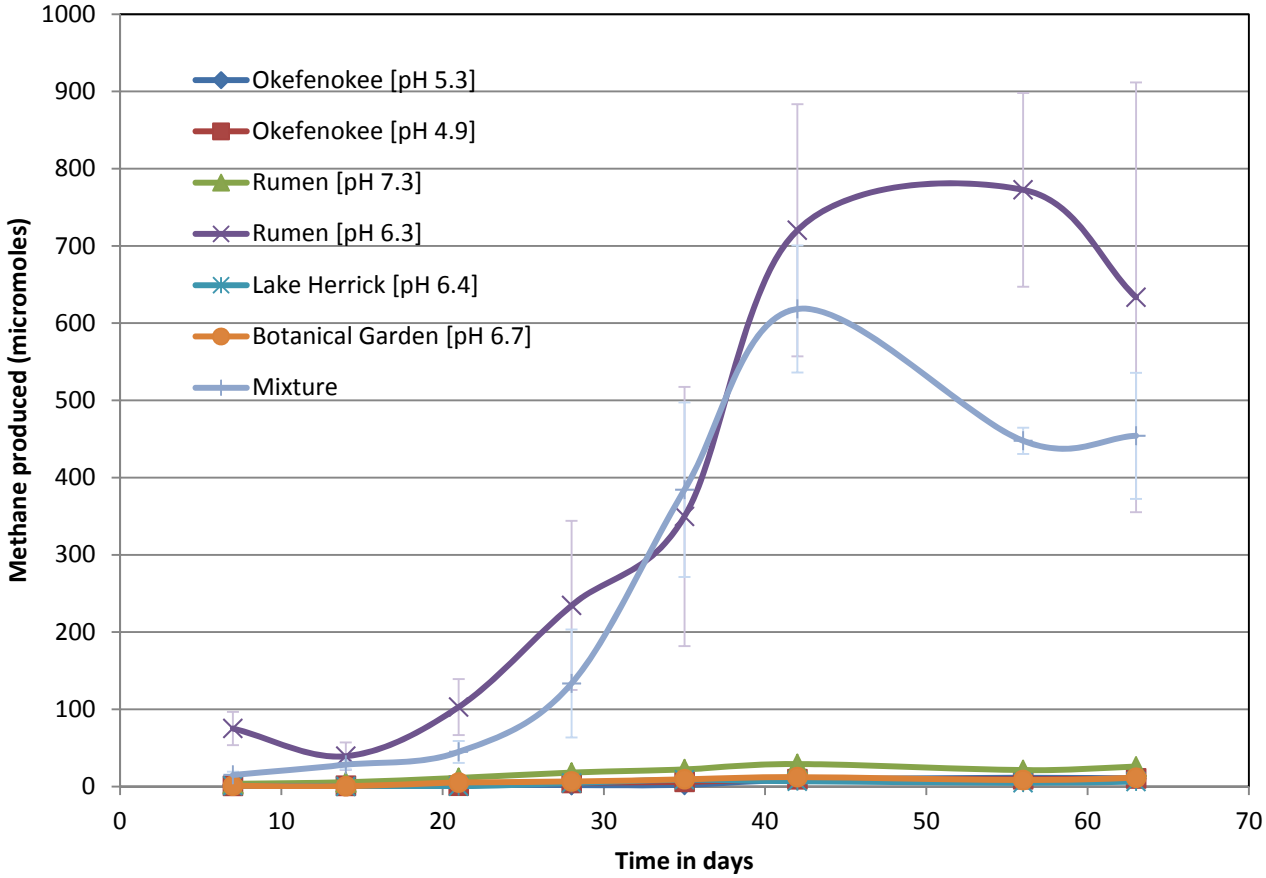


Figure 4. Comparison of methane production over the duration of enrichment after inoculation with environmental sample.

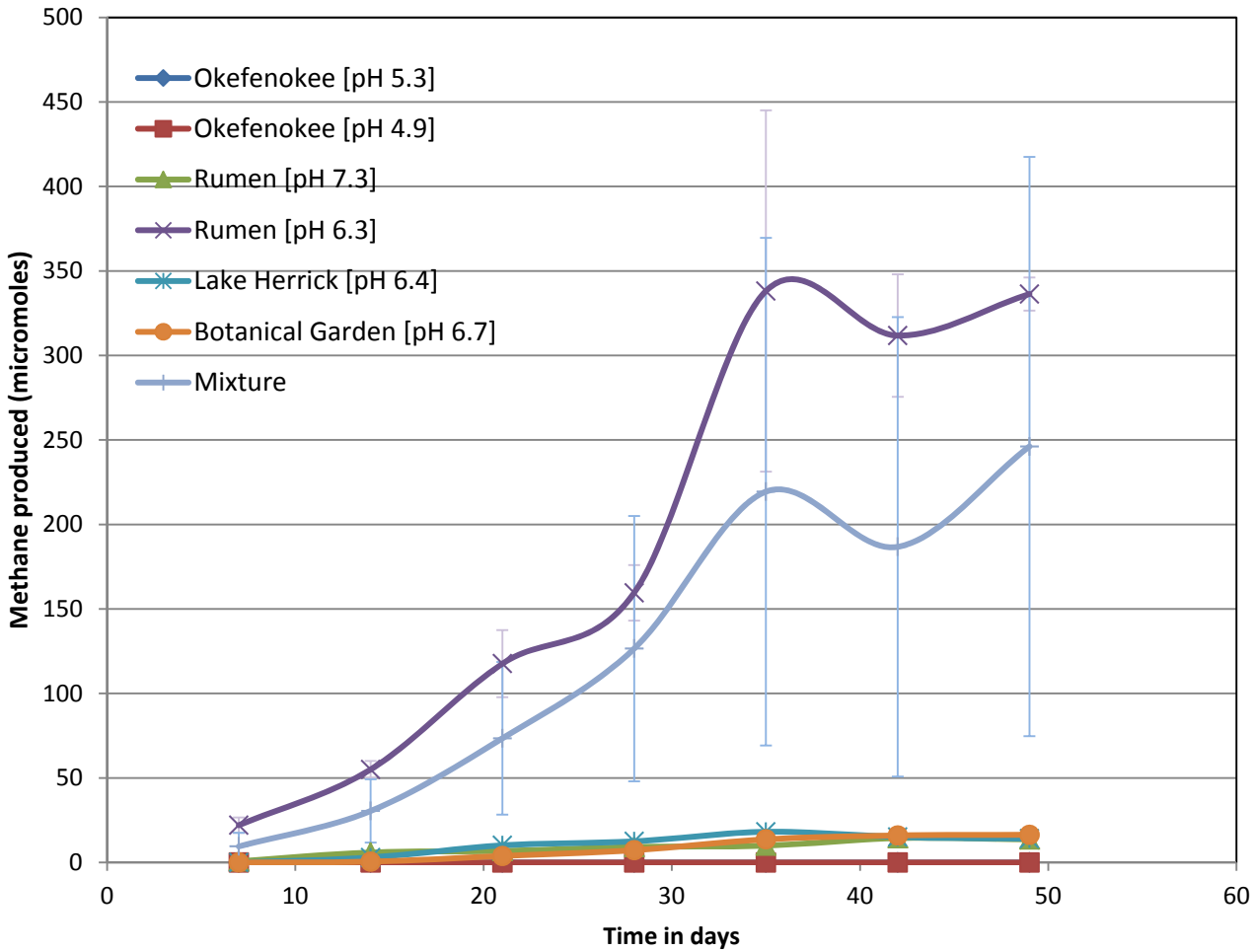


Figure 5. Comparison of methane production over the duration of enrichment after inoculation with digested product from the first generation enrichments.

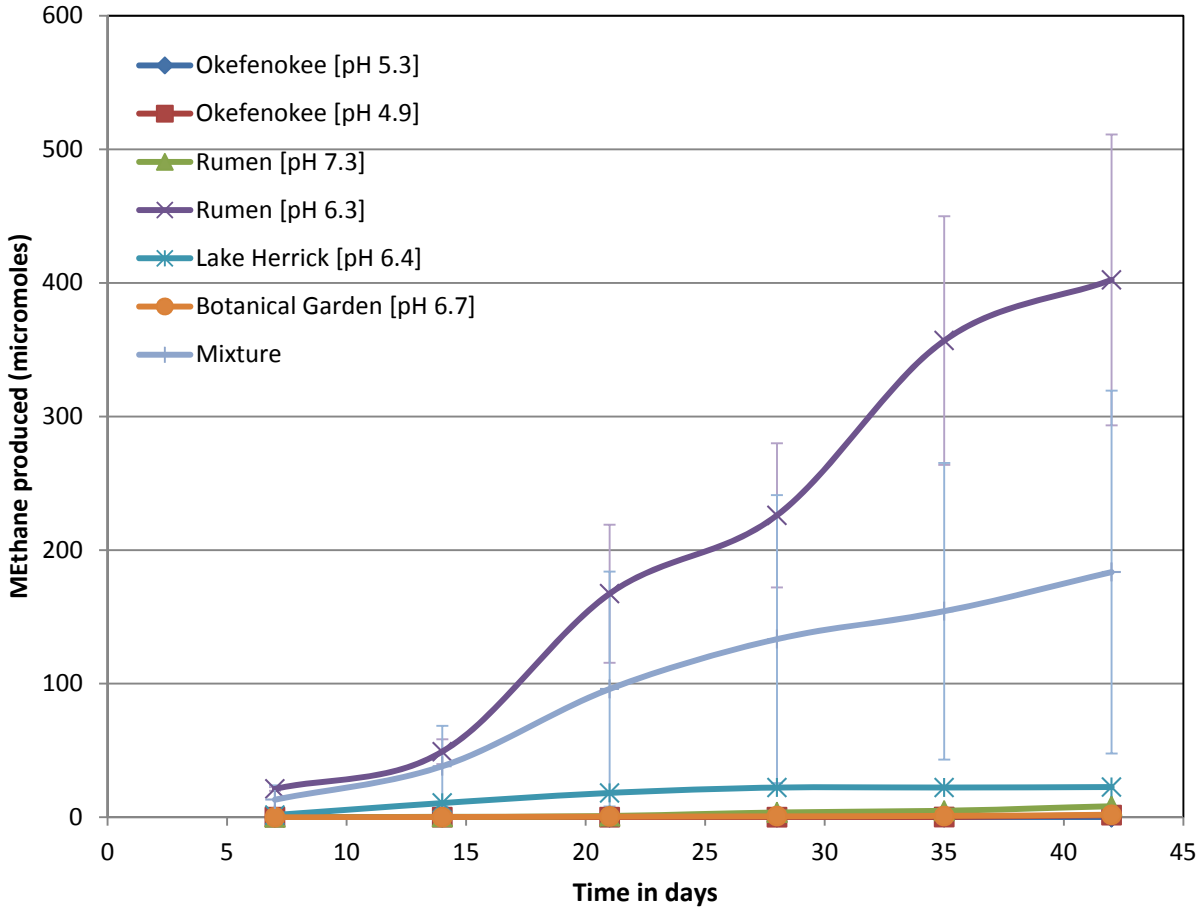


Figure 6. Comparison of methane production over the duration of enrichment after inoculation with digested product from the second generation enrichments.

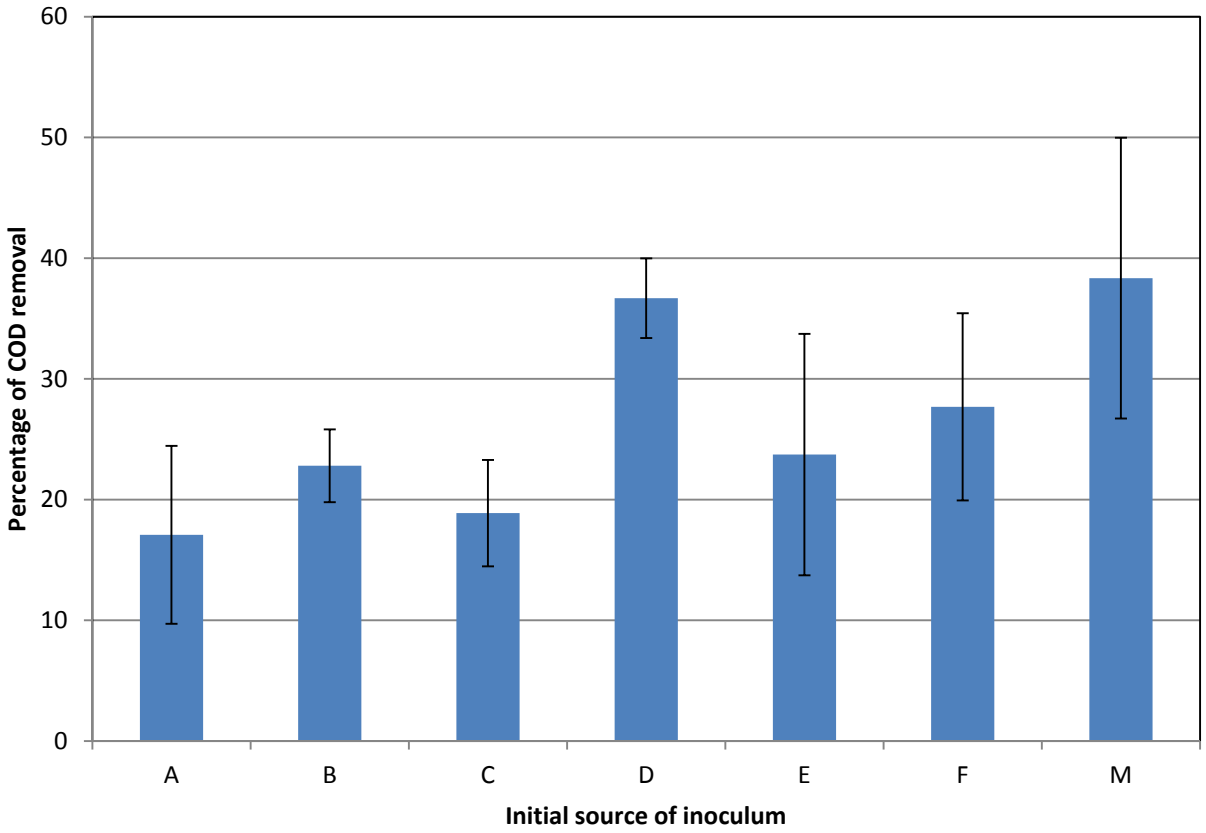


Figure 7. COD removal of the third generation enrichment. Enrichments were inoculated with digested product of a second generation enrichments. A. Okefenokee (pH 5.3). B. Okefenokee (pH 4.9). C. Rumen (pH 7.3). D. Rumen (pH 6.3). E. Lake Herrick (pH 6.4). F. Botanical Garden (pH 6.7). Initial COD was 4318 mg/L with a standard deviation of 200 mg/L.

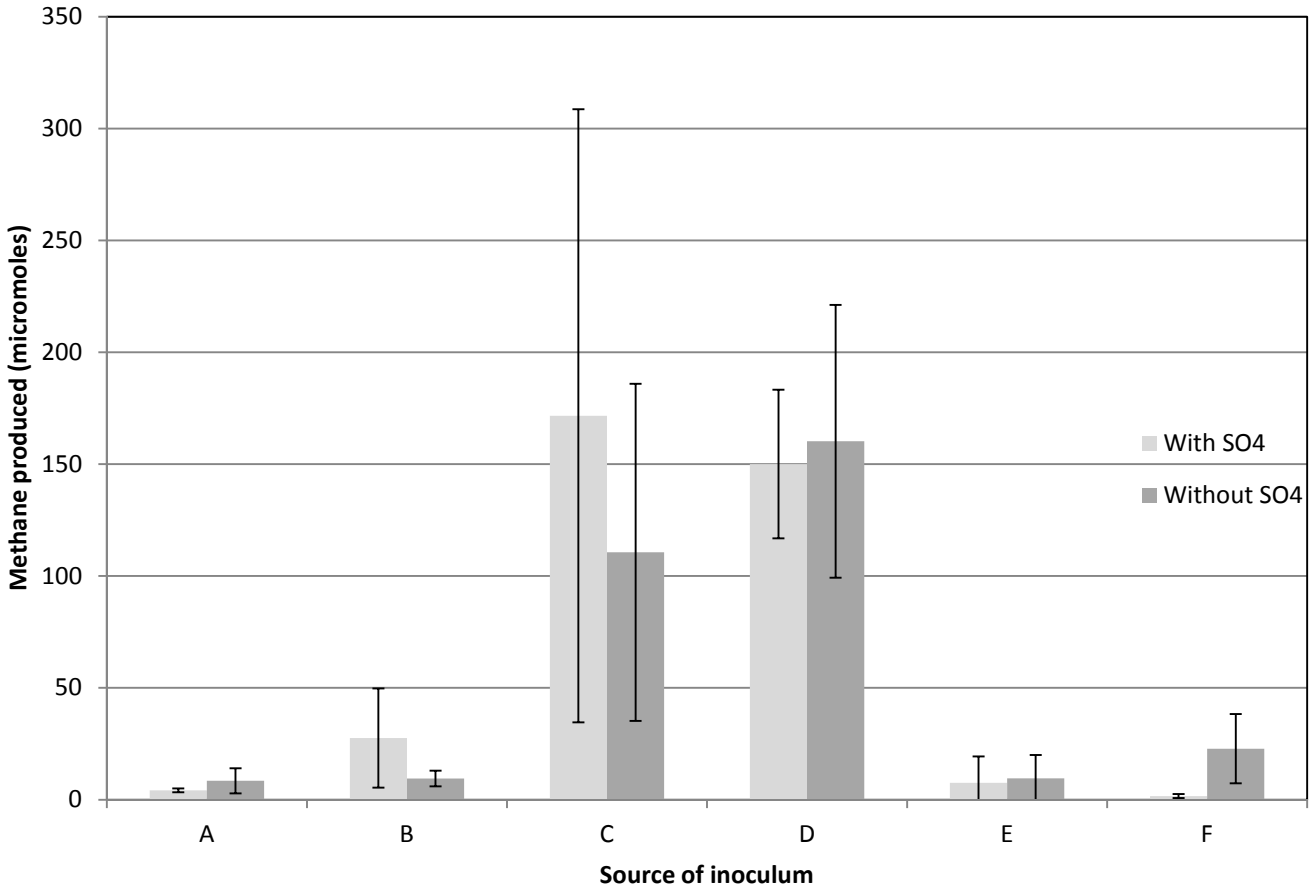


Figure 8. Influence of sulfate on the anaerobic communities producing methane on day 42. A. Okefenokee (pH 5.3). B. Okefenokee (pH 4.9). C. Rumen (pH 7.3). D. Rumen (pH 6.3). E. Lake Herrick (pH 6.4). F. Botanical Garden (pH 6.7). Inocula was stored for 7 months after the inoculation of the first generation enrichments and before inoculation of this experiment.

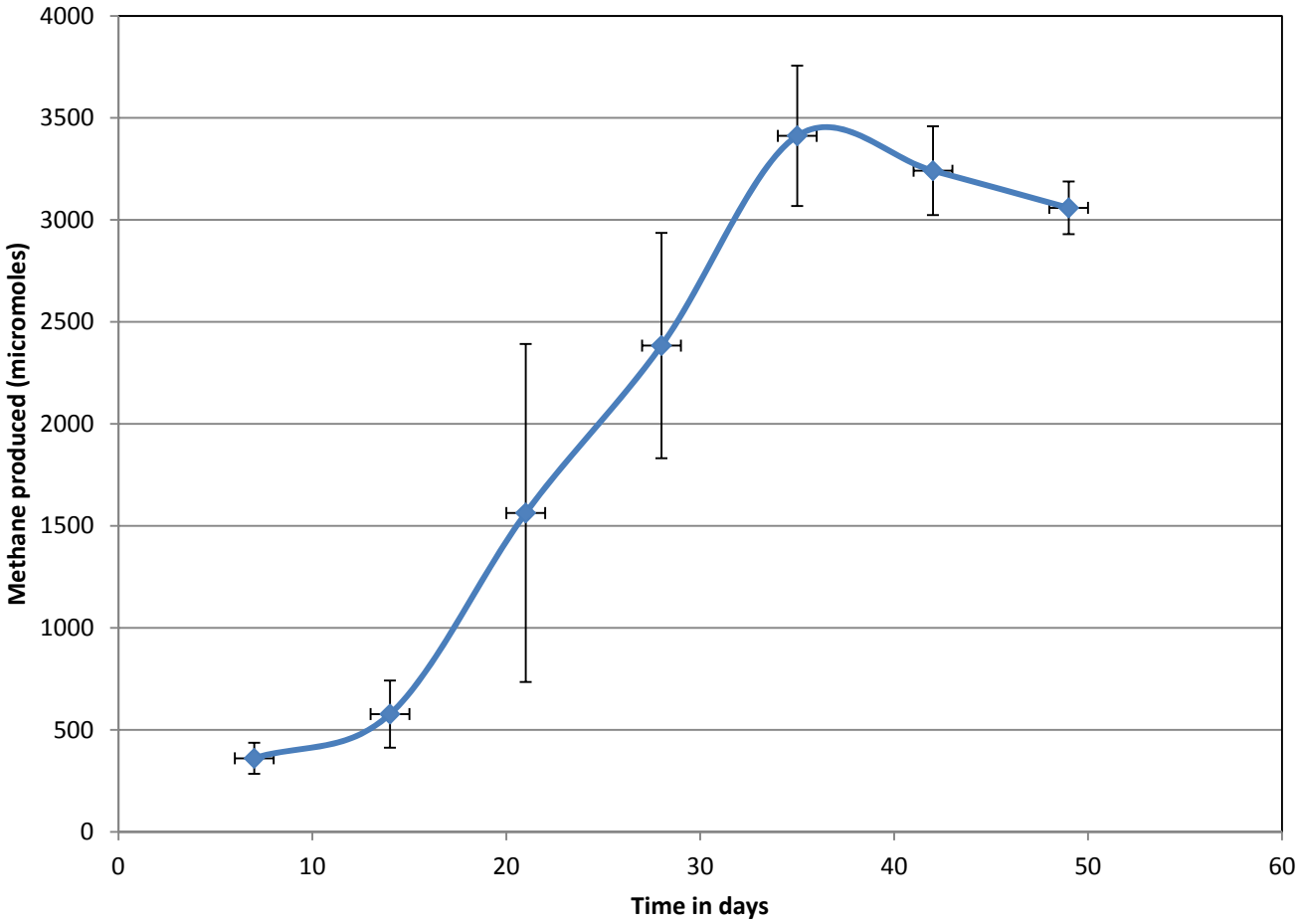


Figure 9. Methane production of the first stage of scaling from rumen (pH 6.3) enrichment.

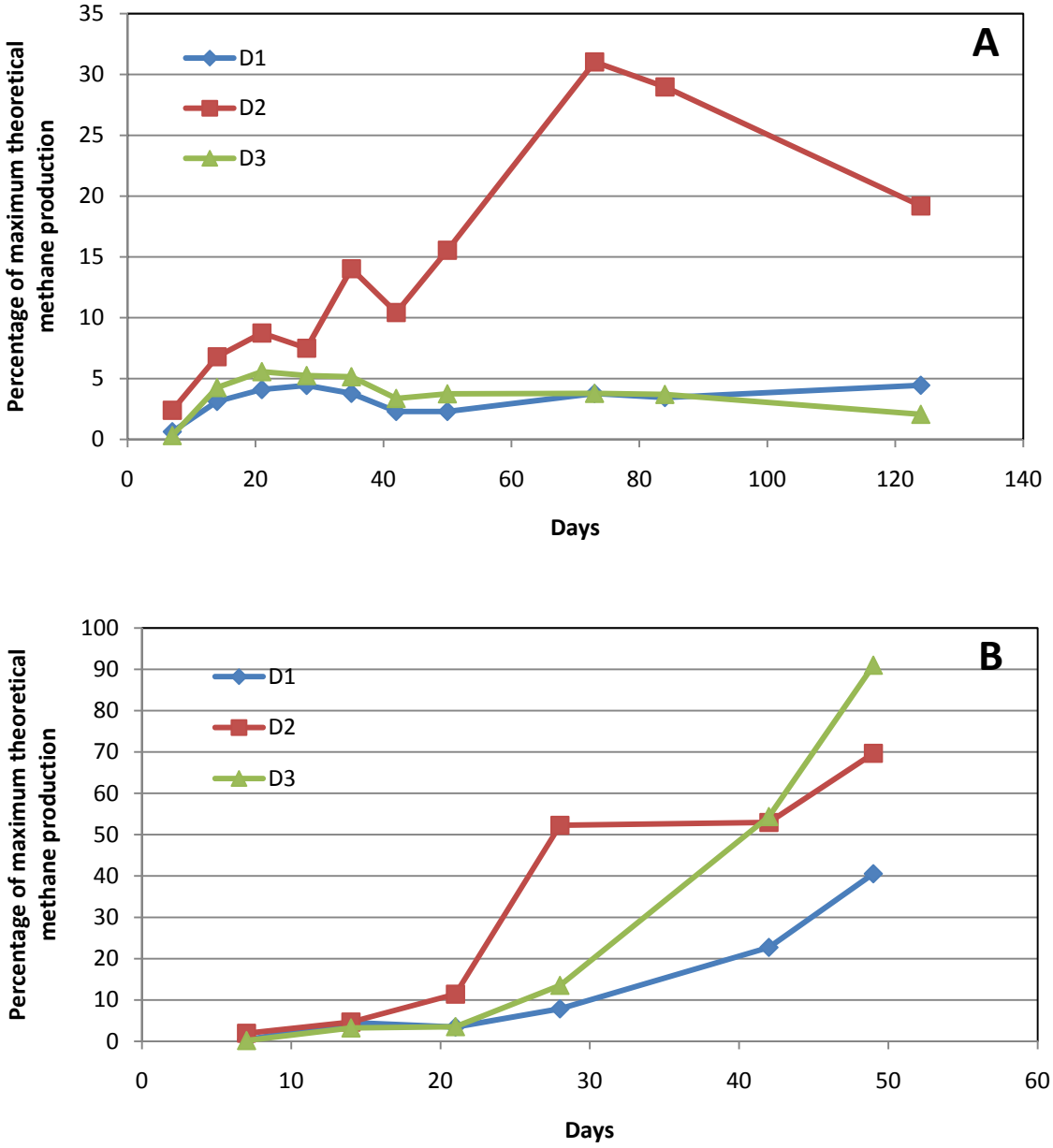


Figure 10. Percentage of maximum theoretical methane produced. A. Methane production from the second stage scale up enrichment inoculated initially with rumen (pH 6.3).100% methane would be 50,000 micromoles. B. Assessing the viability of the scaled up 2 liter microbial community. 100% methane would be 750 micromoles. (Triplicate are shown separately).

Figure 11. Upflow anaerobic digesters.

Table 3. Performance of the clustering at different lengths of sequence used. Three cloned environmental 16S rDNA genes were amplified and the sequences trimmed at the specified length and then clustered at distance 0.03

Minimum length (bp)	**100**	**150**	**200**	**250**	**300**	**350**	**400**
Number of sequences analyzed	3286	3172	3120	3068	3008	2955	2837
Number of sequences correctly classified	2864	2967	2940	2915	2767	2804	2661
Percent of correctly classified sequences	87.18	93.56	94.26	95.04	92.02	94.89	93.83
Number of OTU	119	91	80	66	74	74	69

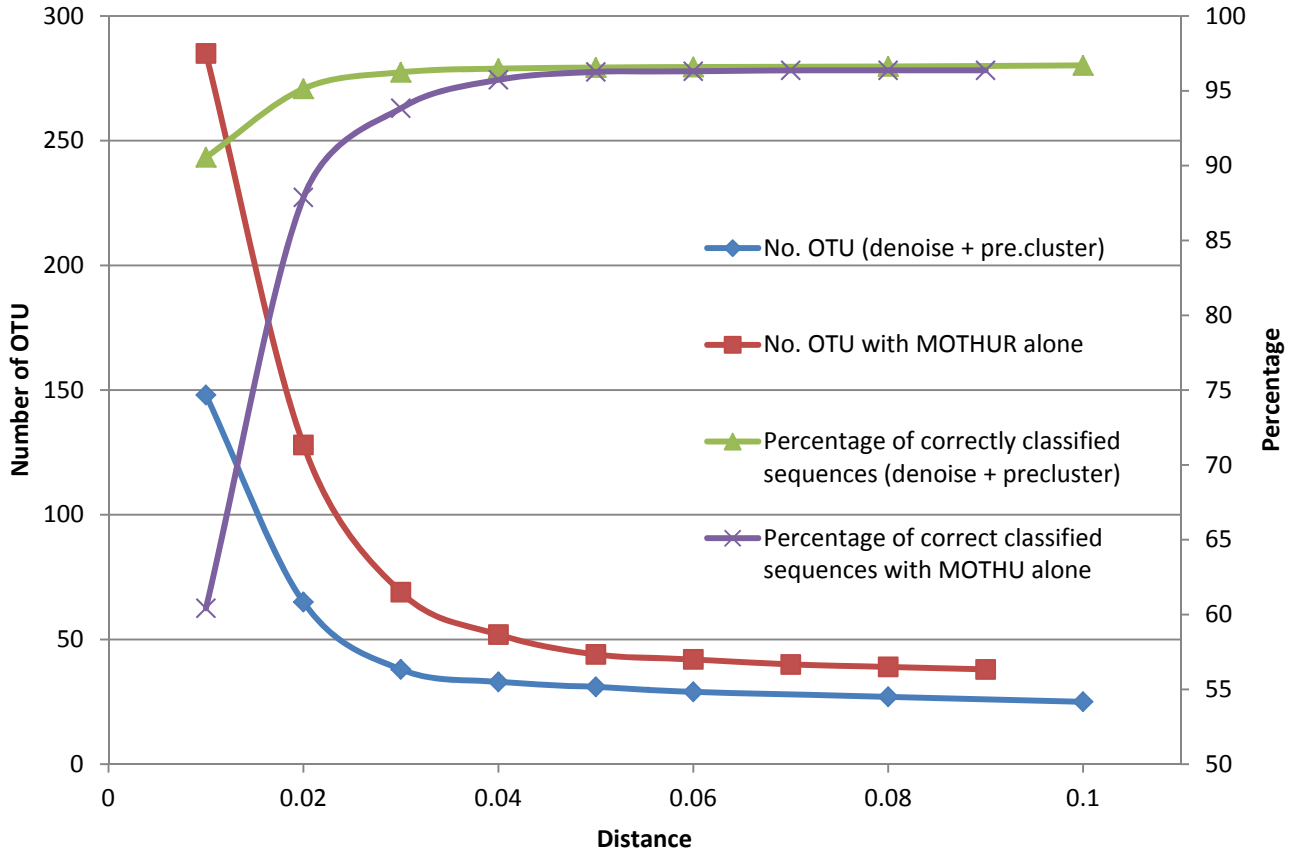


Figure 12. Summary of controls analysis. Comparison of the performance of clustering using pyronoise in QIMME and pre.cluster in MOTHUR versus MOTHUR alone. Percentage of correctly classified sequences and number of OTUs are plotted versus the distance at which those OTUs where formed.

Table 4. Sequences obtained from the controls. Raw sequences were obtained from the sequence facility and quality sequences were assessed using QIIME

	Raw sequences	**Quality sequences**
C1	1521	1011
C2	1514	1070
C3	952	737
Total	3987	2818

Table 5. Sequences obtained from the communities. On the labels, the ordinal number refers to the number in the enrichment generation, the letter refers to the source of inoculum and the last number refers to triplicate.

Labels	**Bacteria**		**Archaea**	
	Raw sequences	Quality sequences	Raw sequences	Quality sequences
Rumen	42931	27762	1833	1355
Mixture	37272	25173	1687	1203
1stD1	4305	3023	471	320
1stD2	9898	6465	569	407
1stD3	13114	9367	872	636
1stM1	15301	10426	392	273
1stM2	9169	5940	847	638
1stM3	34644	21897	299	208
2ndD1	3982	2549	346	242
2ndD2	3551	2386	63	46
2ndD3	5412	3598	198	150
2ndM2	5108	3370	1175	872
2ndM3	4936	3360	143	97
3rdD1	3721	2532	155	116
3rdD2	4498	3134	98	68
3rdD3	5563	3864	200	138
3rdM1	6898	4840	949	674
3rdM2	4637	3000	327	226
3rdM3	6596	4509	49	29
Total	**221536**	**147195**	**10673**	**7698**

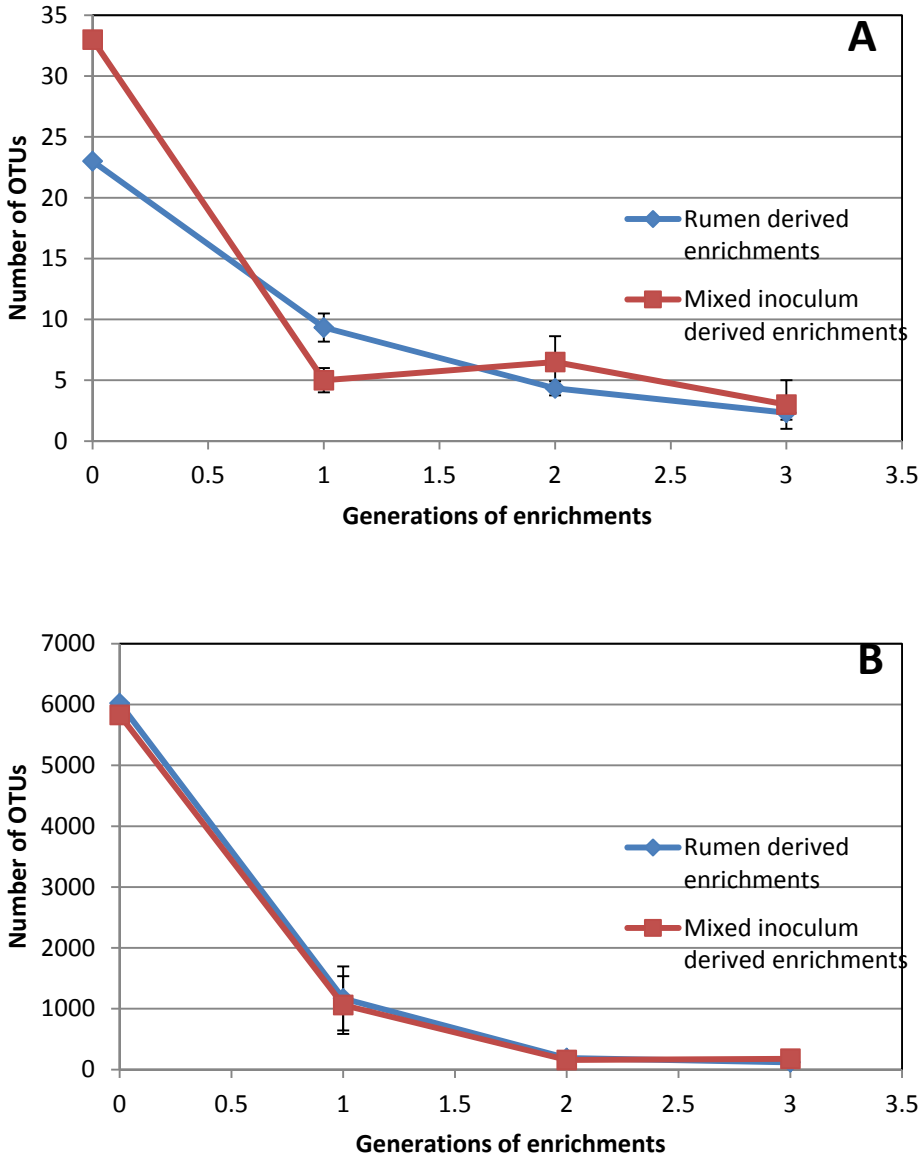
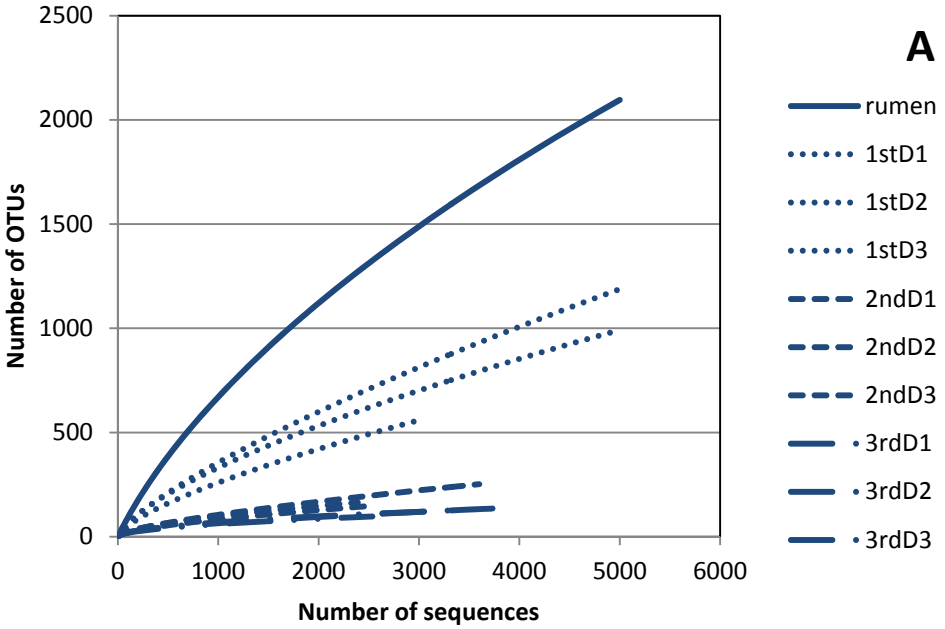


Figure 13. Microbial community diversity trends through successive enrichments. A. Archaeal community trends. B. Bacterial community trends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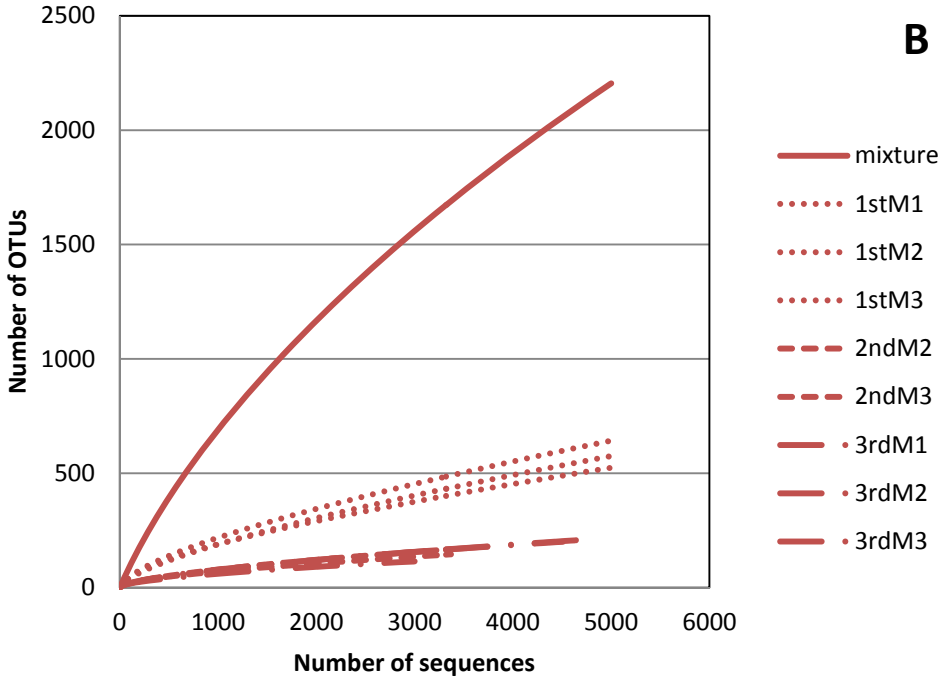
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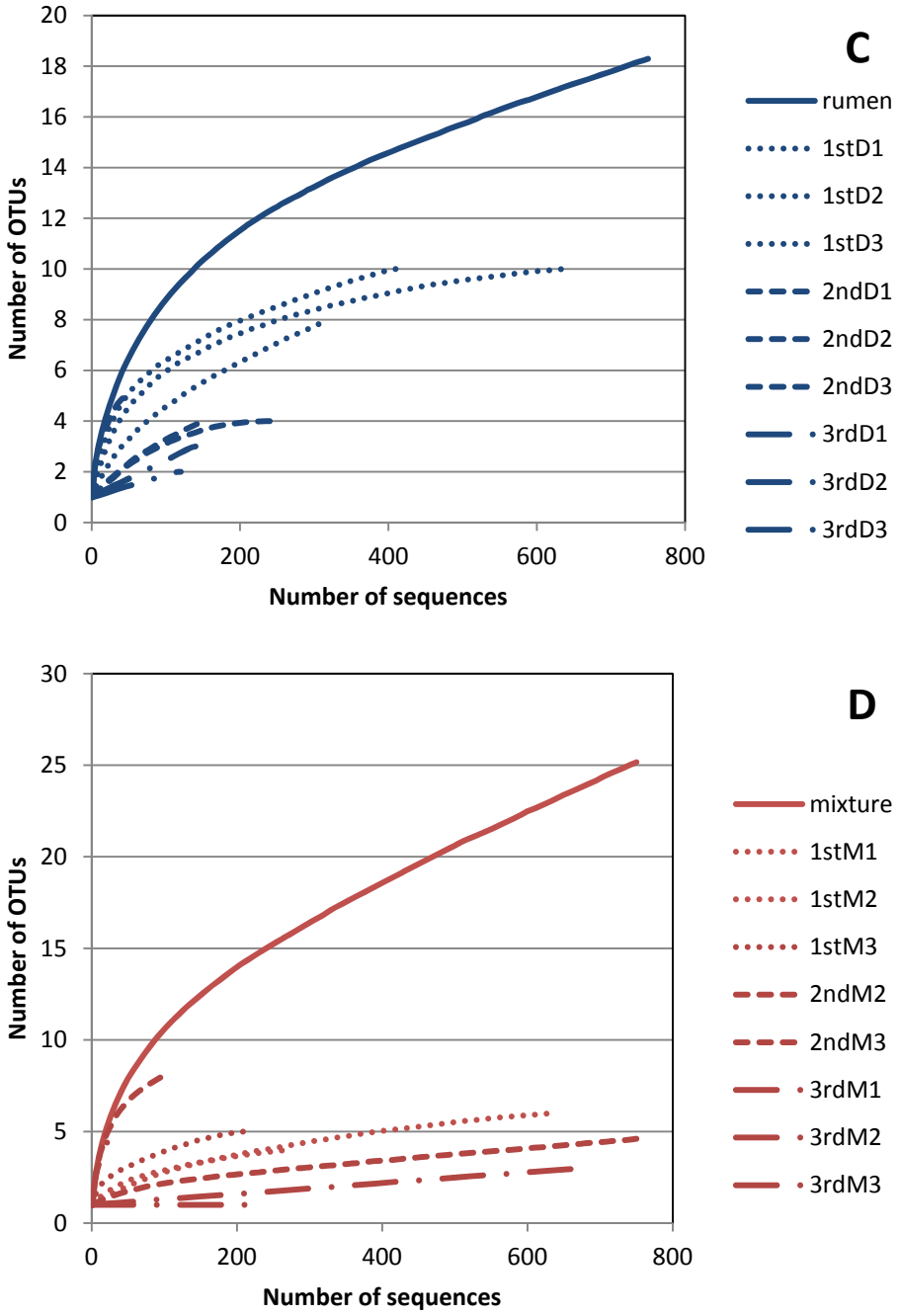

Figure 14. Rarefaction curves of the microbial communities. A. Bacterial communities from rumen (pH 6.3). B. Bacterial communities from mixed source of inoculum. C. Archaeal communities from rumen (pH 6.3). D. Archaeal communities from mixed source of inoculum. On the labels, the ordinal number refers to the number in the enrichment generation, the letter refers to the source of inoculum and the last number refers to triplicate.

Table 6. Bacterial diversity indices. On the labels of the libraries, the ordinal number refers to the number in the enrichment generation, the letter refers to the source of inoculum and the last number refers to triplicate.

Library	Number of sequences	Coverage	Shannon	Simpson	No. OTU	Chao
Rumen	27762	0.873	7.629	0.0017	6020	13693
Mixture	25173	0.872	7.715	0.0016	5829	11654
1stD1	3023	0.871	4.546	0.0856	562	1651
1stD2	6465	0.839	5.485	0.0389	1433	4526
1stD3	9367	0.893	5.587	0.0164	1510	4328
1stM1	10426	0.945	4.301	0.0593	856	2517
1stM2	5940	0.917	4.483	0.0541	723	2045
1stM3	21897	0.951	4.176	0.0657	1602	4369
2ndD1	2549	0.963	2.747	0.1383	149	412
2ndD2	2386	0.957	3.079	0.1069	164	486
2ndD3	3598	0.951	2.950	0.1509	252	994
2ndM2	3370	0.973	2.722	0.1274	146	419
2ndM3	3360	0.969	2.649	0.1594	168	396
3rdD1	2532	0.981	2.436	0.1751	97	181
3rdD2	3134	0.981	2.665	0.1492	122	258
3rdD3	3864	0.980	2.429	0.2195	138	292
3rdM1	4840	0.972	2.485	0.2131	213	530
3rdM2	3000	0.978	2.329	0.2313	115	231
3rdM3	4509	0.973	2.421	0.2106	201	421

Table 7. Archaeal diversity indices. On the labels of the libraries, the ordinal number refers to the number in the enrichment generation, the letter refers to the source of inoculum and the last number refers to triplicate.

Library	Number of sequences	Coverage	Shannon	Simpson	No. OTU	Chao
Rumen	1355	0.993	1.224	0.4681	23	32
Mixture	1203	0.983	1.462	0.4217	33	128
1stD1	320	0.988	0.407	0.8683	8	11
1stD2	407	0.993	0.777	0.6889	10	11
1stD3	636	0.997	0.606	0.7678	10	11
1stM1	273	0.996	0.166	0.9495	4	4
1stM2	638	0.997	0.225	0.9210	6	6
1stM3	208	0.995	0.484	0.7825	5	5
2ndD1	242	1.000	0.177	0.9430	4	4
2ndD2	46	0.978	1.152	0.4000	5	5
2ndD3	150	0.987	0.232	0.9345	4	5
2ndM2	872	0.997	0.126	0.9616	5	8
2ndM3	97	0.979	1.285	0.4377	8	9
3rdD1	116	0.991	0.082	0.9828	2	2
3rdD2	68	0.985	0.126	0.9706	2	2
3rdD3	138	0.986	0.141	0.9711	3	4
3rdM1	674	0.997	0.036	0.9941	3	4
3rdM2	226	1.000	0.000	1.0000	1	1
3rdM3	29	0.931	1.187	0.4606	5	6

Table 8. Distribution of the sequences according to percent similarity to database sequences.

	Bacteria			Archaea		
	Closest environmental clone	Closest isolated species		Closest environmental	Closest isolated species	
Percent similarity	No. of sequences	No. sequences	No. OTU	No. of sequences	No. sequences	No. OTU
100	1998	34	3	0	0	0
>99	90175	24300	99	7376	4892	2
>98	103564	28372	217	7641	6644	5
>97	118453	34451	366	7682	7377	10
>96	126643	36056	571	7696	7386	17
>95	131772	39338	846	7696	7393	21
>94	135441	42487	1133	7696	7400	27
>93	138577	44955	1459	7696	7402	28
>92	140535	55082	1821	7696	7402	27
>91	142003	58808	2307	7696	7402	26
>90	143578	63606	2887	7696	7402	26
Total	147195	147195	13323	7698	7698	59

Table 9. OTU size distribution

OTU size	No. of bacterial OTU	No. of archaeal OTU
>1000	14	2
>100	146	5
>50	299	7
>20	720	11
>10	1258	16
>5	2093	17
>1	5408	27
Total no. OTU	13328	59

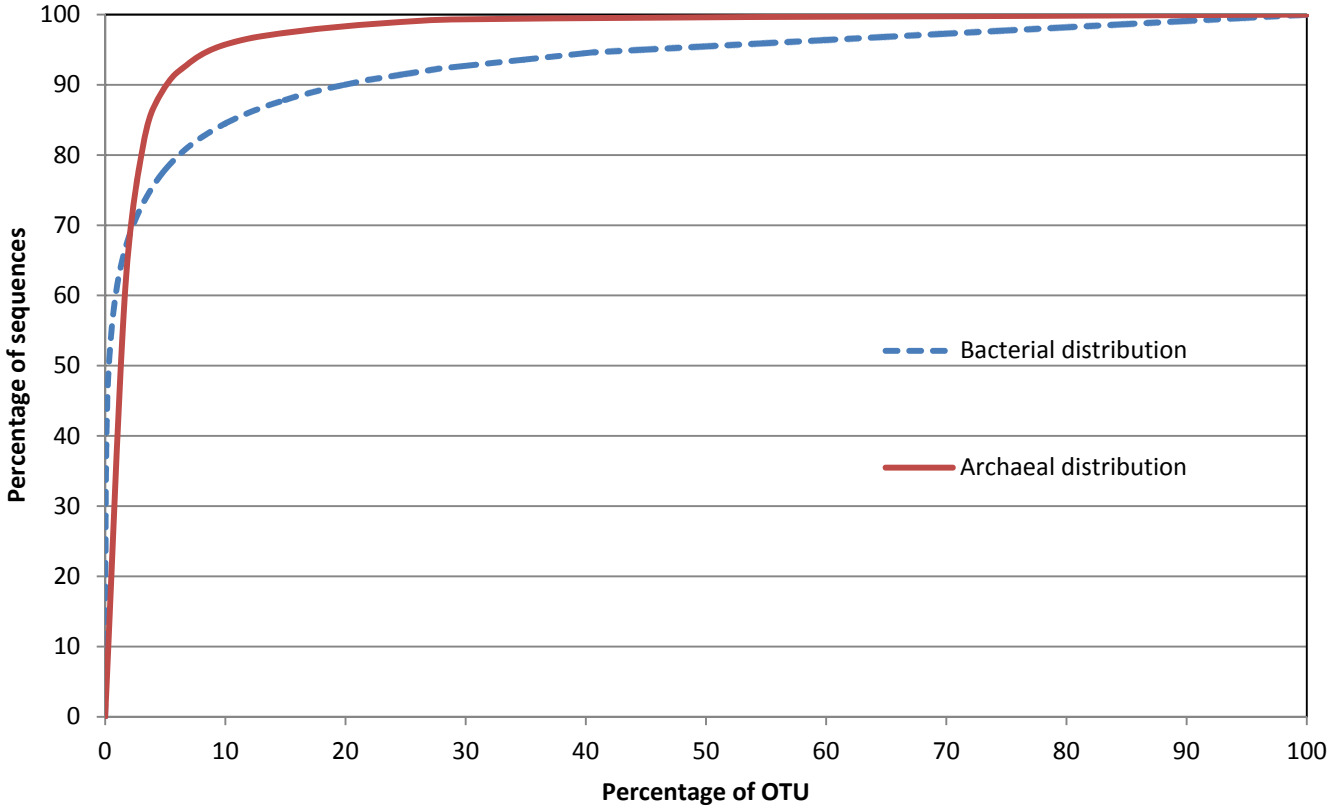

Figure 15. Distribution of the archaeal and bacterial population along all the data.

Table 10. Abundance of specific OTUs in the archaeal 16S rRNA gene libraries created from rumen (pH6.3) enrichments. [a]Total number of sequences.

OTU number	Closest homologue	Percent similarity	Libraries										N[a]
			Rumen	1st generation			2nd generation			3rd generation			
				1	2	3	1	2	3	1	2	3	
1	*Methanosarcina mazei*	99.7	0	298	336	556	235	24	145	115	67	136	1912
2	*Methanobrevibacter millerae*	98.4	879	5	17	18	2	2	1	0	0	1	925
3	*Methanobrevibacter wolinii*	97.9	285	0	0	0	0	0	0	0	0	0	285
5	*Methanoculleus submarinus*	97.1	0	11	30	32	2	17	3	0	1	1	97
4	*Methanobrevibacter olleyae*	99.4	60	0	10	13	0	2	1	0	0	0	86
6	*Thermogymnomonas acidicola*	81.4	40	0	0	3	0	0	0	0	0	0	43
8	*Methanosphaera stadtmanae*	98.7	15	0	2	1	0	0	0	0	0	0	18
11	*Thermogymnomonas acidicola*	82.9	19	0	0	0	0	0	0	0	0	0	19
7	*Thermogymnomonas acidicola*	82.5	0	0	0	0	3	0	0	0	0	0	3
13	*Thermogymnomonas acidicola*	83.9	13	0	0	0	0	0	0	0	0	0	13
14	*Thermogymnomonas acidicola*	83.5	9	0	0	1	0	0	0	0	0	0	10

Table 11. Abundance of specific OTUs in the archaeal 16S rRNA gene libraries created from mixed inoculums enrichments. [a] Total number of sequences. [b] Enrichment that failed to produce methane.

OTU number	Closest homologue	Percent similarity	Libraries									N[a]
			Mixture	1st generation			2nd generation		3rd generation			
				1	2	3	2	3	1	2	3[b]	
1	*Methanosarcina mazei*	99.7	0	266	612	183	855	15	672	226	0	2829
2	*Methanobrevibacter millerae*	98.4	744	2	2	0	0	2	0	0	0	750
3	*Methanobrevibacter wolinii*	97.9	223	0	0	0	0	0	0	0	0	223
5	*Methanoculleus submarinus*	97.1	0	1	20	20	14	62	1	0	6	124
4	*Methanobrevibacter olleyae*	99.4	59	0	0	2	0	0	0	0	0	61
6	*Thermogymnomonas acidicola*	81.4	41	0	0	0	0	0	0	0	0	41
8	*Methanosphaera stadtmanae*	98.7	39	0	0	0	0	0	0	0	0	39
11	*Thermogymnomonas acidicola*	82.9	21	0	0	0	0	0	0	0	0	21
7	*Thermogymnomonas acidicola*	82.5	0	0	0	0	0	8	0	0	19	27
13	*Thermogymnomonas acidicola*	83.9	13	0	0	0	0	0	0	0	0	13
14	*Thermogymnomonas acidicola*	83.5	16	0	0	0	0	0	0	0	0	16

Table 12. Distribution of the sequences in the rumen enrichments within the orders of the domain Archaea. Total percent of sequences in libraries is presented. [a] Number of sequences.

Phylogenetic group	Libraries									
	Rumen	1st generation			2nd generation			3rd generation		
(Order)		1	2	3	1	2	3	1	2	3
Methanobacteriales	93.0	1.6	7.1	5.0	0.8	8.7	1.3	0.0	0.0	0.7
Methanomicrobiales	0.0	3.5	7.4	5.0	0.8	37.0	2.0	0.0	1.5	0.7
Methanosarcinales	0.0	94.3	82.6	87.4	97.1	52.2	96.7	100.0	98.5	98.6
Thermoplasmatales	1.8	0.6	1.0	0.8	0.0	2.2	0.0	0.0	0.0	0.0
Unclassified	5.2	0.0	2.0	1.7	1.2	0.0	0.0	0.0	0.0	0.0
Sample size[a]	1355	318	407	636	242	46	150	116	68	138

Table13. Distribution of the sequences in the mixed inoculum enrichments within the orders of the domain Archaea. Total percent of sequences in libraries is presented. [a] Number of sequences. [b]Enrichment that failed to produce methane.

Phylogenetic group	Libraries								
	Mixture	1st generation			2nd generation		3rd generation		
(Order)		1	2	3	2	3	1	2	3[b]
Methanobacteriales	90.1	0.7	0.5	1.0	0.0	3.1	0.1	0.0	0.0
Methanomicrobiales	0.2	0.4	3.1	9.6	1.6	64.9	0.1	0.0	20.7
Methanosarcinales	0.3	97.4	95.9	88.0	98.1	15.5	99.7	100.0	0.0
Thermoplasmatales	2.2	1.5	0.5	1.0	0.2	5.2	0.0	0.0	6.9
Unclassified	7.1	0.0	0.0	0.5	0.1	11.3	0.0	0.0	72.4
Sample size[a]	1203	273	638	208	872	97	674	226	29

Table 14. Phylogenetic distribution of sequences in the rumen enrichments. Total percent of sequences in libraries is presented. Shading represents the classes and families within the phylum above. [a] Number of sequences. [b] Includes *Deinococcus Thermus, Gemmatimonadetes, OP10, SR1, Thermotogae,* and *TM7*.

Phylogenetic group	Libraries									
	Rumen	1st generation			2nd generation			3rd generation		
		1	2	3	1	2	3	1	2	3
Actinobacteria	0.7	0.5	0.6	4.0	0.1	1.2	0.0	0.1	0.3	0.0
Bacteroidetes	31.9	15.5	21.2	28.9	48.8	60.3	29.9	36.6	35.8	23.3
Bacteroidia	29.6	9.7	16.2	26.2	47.7	57.7	29.1	36.4	34.5	21.9
Bacteroidaceae	0.1	1.6	1.1	1.6	11.4	8.0	4.3	10.5	17.7	3.3
Porphyromonadaceae	1.0	5.5	4.1	19.4	35.9	42.8	23.5	24.8	14.6	17.4
Prevotellaceae	26.1	0.7	0.5	1.4	0.0	0.0	0.0	0.0	0.0	0.0
Sphingobacteria	0.2	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Unclassified	2.1	5.8	4.9	2.7	1.1	2.6	0.8	0.2	1.3	1.4
Chloroflex	0.5	0.2	0.4	0.2	0.1	0.1	0.1	0.0	0.4	0.1
Fibrobacteres	2.3	0.2	0.7	2.8	0.0	0.0	2.3	0.0	0.0	1.9
Firmicutes	44.4	25.9	31.5	32.9	25.5	13.0	37.7	37.6	35.8	46.2
Bacilli	0.2	0.4	0.2	2.5	21.9	8.5	33.0	32.9	31.0	43.0
Clostridia	41.6	24.6	30.3	29.5	3.6	4.4	4.7	4.6	4.8	3.1
Erysipelotrichi	0.6	0.7	0.8	0.3	0.0	0.0	0.1	0.1	0.0	0.2
Unclassified	2.0	0.1	0.2	0.5	0.0	0.0	0.0	0.0	0.0	0.0
Fusobacteria	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Lentisphaerae	2.3	1.8	1.3	1.7	0.2	0.3	0.2	0.0	0.0	0.0
Planctomycetes	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Proteobacteria	3.9	8.0	7.3	3.6	1.6	2.4	1.2	4.6	4.7	2.5
Spirochaetes	0.9	35.5	27.3	12.4	15.9	13.5	17.1	17.6	18.5	17.7
Synergistetes	0.0	1.6	1.4	1.0	1.5	1.5	1.9	1.1	1.2	1.4
Tenericutes	1.3	2.9	1.7	2.7	4.5	5.7	4.3	2.2	2.8	5.2
Verrucomicrobia	2.4	4.2	2.3	1.1	0.0	0.1	0.1	0.0	0.0	0.0
Unclassified bacteria	8.6	3.4	3.7	8.5	1.8	1.9	5.2	0.3	0.5	1.7
Minor phylums[b]	0.7	0.1	0.4	0.1	0.0	0.1	0.0	0.0	0.0	0.0
Sample size[a]	27762	3023	6465	9367	2549	2386	3598	2532	3134	3864

Table 15. Phylogenetic distribution of sequences in the mixed inoculum enrichments. Total percent of sequences in libraries is presented. Shading represents the classes and families within the phylum above.[a] Number of sequences. [b] Enrichment that failed to produce methane. [c] Includes *Bacteria incertae sedis, BRC1, Caldiserica, Chlamydiae, Chlorobi, Nitrospira, Deinococcus Thermus, Gemmatimonadetes, OD1, OP10, SR1, Thermotogae, WS3* and *TM7*.

Phylogenetic group	Libraries								
	Rumen	1st generation			2nd generation		3rd generation		
		1	2	3	2	3	1	2	3[b]
Acidobacteria	0.1	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0
Actinobacteria	0.7	0.4	0.6	0.5	0.3	0.3	0.1	0.1	0.2
Bacteroidetes	31.3	21.9	21.7	41.4	43.5	43.4	33.6	27.1	22.3
Bacteroidia	28.4	18.4	18.6	25.6	42.5	42.2	33.4	25.9	22.0
Bacteroidaceae	0.1	3.6	4.2	10.3	5.3	6.0	17.2	10.6	15.1
Porphyromonadaceae	1.5	10.8	11.1	12.6	36.0	35.2	13.7	12.1	3.9
Prevotellaceae	24.0	0.1	0.2	0.2	0.0	0.0	0.0	0.0	0.0
Flavobacteria	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0
Sphingobacteria	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Unclassified	2.7	3.5	3.0	15.8	1.0	1.1	0.2	1.2	0.3
Chloroflexi	0.6	0.2	0.5	0.4	0.0	0.0	0.0	0.0	0.0
Cyanobacteria	0.1	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.0
Fibrobacteres	1.6	0.4	0.4	0.3	0.0	0.0	0.0	0.0	0.0
Firmicutes	45.4	39.4	36.3	22.3	20.1	31.0	46.7	54.5	62.6
Bacilli	1.1	1.9	1.2	1.1	16.0	22.9	41.3	45.3	18.6
Clostridia	41.8	36.8	34.4	20.9	4.0	8.1	5.3	9.1	44.0
Erysipelotrichi	0.8	0.7	0.6	0.2	0.1	0.1	0.0	0.1	0.0
Unclassified	1.9	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0
Fusobacteria	0.1	0.3	0.1	0.1	0.0	0.0	0.0	0.0	0.0
Lentisphaerae	1.8	1.8	0.8	0.7	0.1	0.3	0.0	0.0	0.0
Planctomycetes	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Proteobacteria	4.9	11.3	12.1	11.9	4.5	5.1	2.4	1.8	1.9
Spirochaetes	0.9	19.5	20.3	17.3	21.7	14.7	13.5	12.5	10.7
Synergistetes	0.1	1.1	0.9	0.7	2.1	1.4	1.2	0.8	0.9
Tenericutes	1.0	0.8	2.8	1.1	5.2	2.4	2.1	2.4	1.1
Verrucomicrobia	2.6	0.7	0.5	0.3	0.0	0.0	0.0	0.0	0.0
Unclassified bacteria	8.1	1.8	2.6	2.5	2.6	1.4	0.4	0.8	0.2
Minor phylums[c]	0.7	0.2	0.1	0.1	0.0	0.0	0.0	0.0	0.0
Sample size[a]	25173	10426	5940	21897	3370	3360	4840	3000	4509

Table 16. Abundance of specific OTUs in the bacterial 16S rRNA gene libraries created from rumen inoculum enrichments. [a]Total number of sequences.

OTU Number	Closest homologue	Percent similarity	Libraries									N[a]
			Rumen	1st generation			2nd generation			3rd generation			
				1	2	3	1	2	3	1	2	3	
2	*Trichococcus flocculiformis*	99.0	0	2	1	3	542	200	1141	810	966	1631	5296
1	*Paludibacter propionicigenes*	89.3	0	35	81	53	604	351	346	520	293	504	2787
3	*Spirochaeta coccoides*	86.1	1	832	1036	682	331	230	549	331	262	530	4784
6	*Bacteroides graminisolvens*	99.5	0	8	40	67	267	186	126	259	544	120	1617
7	*Treponema brennaborense*	97.1	0	195	549	266	30	40	26	67	203	62	1438
12	*Acholeplasma morum*	99.3	0	4	6	2	111	122	88	55	82	180	650
8	*Parabacteroides goldsteinii*	92.1	0	61	50	368	203	558	414	40	102	116	1912
13	*Treponema denticola*	87.2	0	38	98	98	32	37	22	44	102	58	529
77	*Salmonella enterica*	99.3	0	0	0	0	2	7	4	44	41	48	146
15	*Desulfovibrio desulfuricans*	99.3	0	24	60	24	1	4	1	60	38	27	239
14	*Capnocytophaga cynodegmi*	80.9	1	2	266	14	1	148	38	8	64	37	579
20	*Paludibacter propionicigenes*	89.9	0	14	28	17	47	58	17	40	29	25	275
53	*Aminobacterium mobile*	87.9	0	9	29	16	10	10	38	12	18	44	186
35	*Fibrobacter succinogenes*	86.8	0	7	42	244	0	0	78	0	0	71	442
60	*Escherichia fergusonii*	99.5	4	0	0	0	0	7	0	2	57	0	70
25	*Dokdonia donghaensis*	79.6	0	79	61	96	26	34	23	5	4	40	368
5	*Anaerophaga thermohalophila*	83.2	0	35	125	70	1	21	2	0	36	12	302
514	*Acetobacterium malicum*	99.2	0	0	0	0	0	1	0	0	44	1	46
112	*Blautia hydrogenotrophica*	93.0	0	5	3	2	22	0	0	43	0	0	75
11	*Carnobacterium pleistocenium*	72.1	0	22	24	27	25	32	149	1	11	28	319
61	*Clostridium clariflavum*	82.7	0	81	82	128	13	11	21	10	12	11	369
43	*Paludibacter propionicigenes*	89.0	0	0	2	0	14	6	16	15	5	11	69

Table 17. Abundance of specific OTUs in the bacterial 16S rRNA gene libraries created from mixed inoculum enrichments. [a]Total number of sequences. [b] Enrichment that failed to produce methane.

OTU number	Closest homologue	Percent similarity	Libraries									N[a]
			Mixt.	1st generation			2nd generation		3rd generation			
				1	2	3	2	3	1	2	3[b]	
2	*Trichococcus flocculiformis*	99.0	0	95	59	191	535	752	1948	1331	801	5712
6	*Bacteroides graminisolvens*	99.5	0	77	4	506	173	181	805	311	657	2714
9	*Clostridium quinii*	98.7	0	0	0	0	0	145	0	2	1733	1880
3	*Spirochaeta coccoides*	86.1	0	1914	1097	3675	687	484	507	342	421	9127
1	*Paludibacter propionicigenes*	89.3	0	137	32	498	441	138	499	82	47	1874
8	*Parabacteroides goldsteinii*	92.1	0	713	490	1602	640	949	93	142	84	4713
44	*Clostridium saccharobutylicum*	98.7	5	31	26	28	2	6	0	210	82	390
14	*Capnocytophaga cynodegmi*	80.9	0	157	81	98	35	31	85	71	76	634
12	*Acholeplasma morum*	99.3	0	43	15	150	171	80	99	71	48	677
20	*Paludibacter propionicigenes*	89.9	0	30	17	97	98	59	30	117	26	474
13	*Treponema denticola*	87.2	0	64	50	23	30	3	47	30	49	296
15	*Desulfovibrio desulfuricans*	99.3	0	120	94	280	9	2	35	10	39	589
60	*Escherichia fergusonii*	99.5	1	59	1	7	7	6	18	34	31	164
7	*Treponema brennaborense*	97.1	0	24	34	0	8	0	78	1	0	145
112	*Blautia hydrogenotrophica*	93.0	0	38	21	23	0	0	67	1	5	155
56	*Pyramidobacter piscolens*	93.8	0	23	35	64	35	11	25	5	17	215
25	*Dokdonia donghaensis*	79.6	0	16	56	207	17	12	2	26	8	344
61	*Clostridium clariflavum*	82.7	0	77	45	101	13	14	21	4	11	286
50	*Pedobacter composti*	84.3	0	71	30	105	1	0	7	11	16	241
237	*Citrobacter farmeri*	99.0	0	0	0	1	24	11	33	0	1	70
244	*Anaeromusa acidaminophila*	97.9	0	10	2	11	0	0	22	0	12	57
34	*Aminiphilus circumscriptus*	87.1	0	76	13	61	28	24	12	13	8	235

Table 18. Number of copies of bacterial and archaeal 16S rDNA gene per 1 µL of DNA extracted from the samples and the enrichments. On the labels, the ordinal number refers to the number in the enrichment generation, the letter refers to the source of inoculum and the last number refers to triplicate. [a]Mean of 3 replicates. SD was below 10% of the mean in most of the samples. [b]Enrichment that failed to produce methane.

Labels	DNA (ng/µL)	Number of copies of 16S rDNA gene[a]		Ratio Bac/Arc
		Bacteria	Archaea	
Rumen	104.9	4.02E+06	9.30E+05	4.32
1stD1	254.4	1.39E+06	2.68E+06	0.52
1stD2	259.3	2.97E+06	2.57E+06	1.15
1stD3	171.9	3.79E+06	4.13E+06	0.92
2ndD1	758.1	5.25E+06	2.04E+06	2.58
2ndD2	104.1	3.51E+06	6.80E+05	5.16
2ndD3	629.8	9.90E+06	6.65E+06	1.49
3rdD1	Unknown	1.42E+06	2.68E+05	5.29
3rdD2	3.5	2.63E+06	8.45E+05	3.11
3rdD3	4.6	2.87E+06	6.70E+05	4.28
Mixture	Unknown	2.37E+06	3.66E+05	6.48
1stM1	36.8	1.09E+06	1.37E+06	0.80
1stM2	43.8	6.25E+05	8.10E+05	0.77
1stM3	56.3	1.57E+06	9.95E+05	1.57
2ndM2	198.1	5.90E+06	3.74E+06	1.58
2ndM3	307.2	5.41E+06	1.19E+06	4.55
3rdM1	5.2	5.95E+06	1.40E+06	4.25
3rdM2	5.0	2.35E+06	2.19E+06	1.07
3rdM3[b]	3.1	4.25E+06	2.59E+05	16.39

CHAPTER 4

CONCLUSIONS

This study performed the development of microbial communities to anaerobic digestion of carrot pomace. After the adaptation, the composition of the communities was assessed based on the 16S rDNA gene. The following conclusions were reached:

1. Not all the anaerobic natural sources of microbes were suitable inocula for anaerobic digestion. In this study, this was mainly due to a low microbial load and probably the absence of key organisms.
2. The decrease in time for methane production in the enrichments illustrated the adaptation of the microbial community to the new environment.
3. Sampled bacterial populations are less described in the literature compared to archaeal populations. Probably because of the large diversity of bacterial population.
4. In anaerobic digestion showed a greater bacterial diversity than archaeal diversity.
5. As the community became more adapted to specific conditions, its diversity decreased.
6. The microorganisms that were most abundant in the adapted community were mostly not detectable in the DNA extracted from the natural sources of inocula.
7. The majority of the microorganisms present in the natural source of inoculum were not detected after the first process of adaptation to new environmental conditions. This mostly implied the death of the microorganisms.

8. The adapted microbial compositions performed very similarly in terms of composition and methane production even when the inocula were not identical.
9. *Methanosarcinales* was the most abundant archaeal order in the anaerobic digestion of carrot pomace performed in the enrichments that did produce methane.
10. *Spirochaetes, Porphyromonadaceae, Bacteroidaceae* and *Bacilli* were the main bacterial groups enriched in a community that produces methane using carrot pomace as substrate.
11. Minor changes in the environment caused a community to favor the growth of certain microorganisms, shifting the microbial population composition and metabolism.