DIVERSITY OF BACTERIA AND ARCHAEA IN THE MULTI-CHAMBERED STOMACH OF *ODOCOILEUS VIRGINIANUS* (WHITE-TAILED DEER)

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ABSTRACT

DIVERSITY OF BACTERIA AND ARCHAEA IN THE MULTI-CHAMBERED STOMACH OF *ODOCOILEUS VIRGINIANUS* (WHITE-TAILED DEER)

by Chengsheng Zhu

Ruminants have four-chambered stomachs in which they process their food. Stomach microorganisms are crucial to animals as they decompose cellulose, yet they are not well characterized. I collected the stomachs of fifteen white-tailed deer harvested by hunters in 2007 and nineteen in 2008 in Michigan. I analyzed the bacterial and archaeal community in the four chambers of the deer stomachs (rumen, reticulum, omasum and abomasum) using PCR-DGGE and sequencing of the 16S rRNA gene. Archaeal communities were dominated by a few types and Archaeal sequences were identified as uncultured microorganisms closely related to uncultured strains of *Methanobrevibacter* and *Methanosphaera* (>97% similarity). Bacterial communities were more diverse and bacterial sequences were similar to uncultured rumen bacterial clones related to *Prevotella*-like *Bacteroides, Firmicutes, Proteobacteria* and *Spirochaetes*. In addition, each individual deer showed a unique gut microflora (both archaea community and bacteria community), indicating a strong host impact.

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CHAPTER I

LITERATURE REVIEW

Introduction to gut microbiology of herbivores and rumen microbiology

The gastrointestinal microbial flora is an incompletely studied, complex microbial ecosystem whose diversity is not well defined yet (Hooper & Gordon, 2001). It starts to develop soon after an animal's birth and is beneficial for the animal's health in many ways. The normal microflora is part of the first line defense as it competes with incoming foreign microorganisms for space and resources. This prevents potential pathogens from growing (Tortora, *et al.*, 2008). Experimental animals which have been fed and maintained in a sterile environment since their birth (germ-free animals) usually have immunological abnormalities, such as greatly reduced numbers of plasma cells and poorly formed B- and T-cell zones. However, if germ-free animals are allowed to be colonized for several weeks, all of those abnormalities can be reversed (Macpherson & Harris, 2004). The normal flora is thus considered crucial for the development of the immune system. In addition, it has a large effect on animal nutrition and normally promotes the harvest of energy from the diet and its storage in the host. Backhed *et al.* (2004) compared the growth of germ-free mice and germ-free mice colonized by normal microflora. Colonized mice produced 60% more body fat despite their reduced food intake.

The normal microflora is especially important for herbivores. The main carbon source in plant material is cellulose, a polysaccharide that animals cannot digest by themselves. Microorganisms living in the gastrointestinal tract of herbivores break down the ingested cellulose to monosaccharides and subsequently ferment those

monosaccharides to short-chain fatty acids, which the host can process. In return, the microorganisms are provided with a rich food source and a stable anaerobic environment. This kind of relationship between gut microorganisms and their host is a form of mutualism – both partners experience increased fitness (Backhed, *et al.*, 2005). Some herbivores, like horse, harbor microorganisms in an enlarged cecum, which is located in the hind gut, hence they are considered hindgut fermenters. Most animals can only absorb water in their colon. In contrast, hindgut fermenters usually have a long colon so that absorption of the nutrients generated in the cecum can occur in it. Some hindgut fermenters also practice coprophagy (feces eating) because the feces exiting the gastrointestinal system for the first time contain a considerable amount of undigested fiber (Hobson & Wallace, 1982).

The digestive process in hindgut fermenters is not very effective because microbial fermentation occurs after the acidic stomach. Alternatively, some herbivores (such as deer), known as ruminants, have evolved a very specific digestive system with foregut fermentation. Ruminants have a complex stomach which is made of four linearly connected chambers: rumen, reticulum, omasum and abomasum. Food first enters the rumen, an anaerobic sac where cellulose digestion occurs. The flow of food through the rumen is mediated partly by ingested water but much more by copiously produced saliva. The saliva contains salts such as bicarbonates and phosphates which buffer the rumen fluid against the fermentation acids to a pH of about 6.5. This pH allows growth of the commensal microorganisms (bacteria, archaea and protozoa), which are responsible for the process of cellulose fermentation (Hobson & Wallace, 1982).

Rumen fermentation has been extensively studied. First, carbohydrate fermenters such as *Ruminococcus* and *Fibrobacter* degrade cellulose to volatile fatty acid, and CO_2. This process occurs in three stages (Russell *et al.*, 1981). The initial stage includes attachment of microorganism to the plant particles and the disassociation of carbohydrate polymers from plant cells. According to Cheng and McAllister (1997), the rumen microbial community can be divided into three groups: i) those in the rumen fluid, ii) those attached to food particles and iii) those associated with the rumen wall. Microbes attached to food particles were found to be numerically dominant, making up 70-80% of the total microbial population (Craig *et al.*, 1987). Those microbes were estimated to be responsible for 80% of total rumen polysaccharide degradation (Minato *et al.*, 1966). Using electron microscopy, Akin (1980) evaluated different morphological types of rumen bacteria associated with Bermuda grass and fescue and showed that the ratio of encapsulated cocci to irregularly shaped bacteria was different. Koike *et al.* (2003) revealed that three representative cellulolytic bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus*, could colonize food particles within 10 min, which was then followed by bacterial growth and fibrolytic action. Recently, Sun *et al.* (2008) used PCR-DGGE to study the dynamics of bacteria associated with food particles during the incubation in the rumen. Samples were collected from rumen of goats fed on different diets by using nylon bags. Their results showed that samples collected from 10 min to 6 h after incubation had similar DGGE patterns, with up to 24 predominant bands to each sample, including 14 common bands to all samples. This suggested a rapid and stable colonization by a diverse bacterial community. Samples collected at 12 and 24 h showed similar DGGE patterns, however they were significantly different from those collected at 10 min to 6 h.

This indicates an apparent shift in the bacterial community after 6 hours incubation. Diet differences had little effect in this study.

The second fermentation stage is the hydrolysis of the released polysaccharides to small saccharides. Releases polysaccharides include cellulose, starch, xylan and pectin, but "cellulose complexes" are the predominating type. The hydrolysis is catalyzed by different extracellular enzymes, such as cellulase, amylase, xylanase and pectinase, accordingly (Russell *et al.*, 1981). Different bacteria are able to produce different enzymes (usually more than one), therefore multiple types of bacteria are usually involved in this stage. For example, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* are the most common cellulolytic bacteria. Among them, *Fibrobacter succinogenes* is also amylolytic, while *Ruminococcus flavefaciens* and *Ruminococcus albus* are capable of xylan degradation (Russell *et al.*, 1981). Besides, *Bifidobacteria* are known to ferment xylose (Biavati & Mattarelli, 1991), members of the genus *Treponema* are known to be fructanolytic (Piknova, *et al.*, 2008) and *Quinella* spp. are known to perform a special fermentation of mannitol (Krumholds *et al.*, 1993). *Streptococcus* spp. can grow on many different carbon sources and tolerate acidic environments (Ghali, *et al.*, 2004). Similarly, *Prevotella* spp. can use multiple carbon sources and although they can't degrade cellulose, they have been found to contribute to this process by acting synergistically with cellulolytic bacteria (Avgustin, *et al.*, 1994).

The final fermentation stage is the intracellular metabolism of small saccharides (Russell *et al.*, 1981). The major biochemical pathway employed by ruminal bacteria for hexose degradation is the Embden-Meyerhof pathway, during which bacteria first ferment hexose to pyruvate (Russell *et al.*, 1981). Pyruvate is then converted to a variety of

fermentation products through different pathways. In some cases, multiple steps are involved and products from one bacterium can be utilized and fermented by another bacterium. For example, succinate, a common intermediate fermentation product from pyruvate, rarely accumulates in the rumen because organisms such as *Selenomonas ruminantium* are able to convert it to propionate (Scheifinger & Wolin, 1973). The final products of the fermentation process include acetate, buyrate, H_2, CO_2 and propionate (Russell *et al.*, 1981).

In addition to bacteria, the rumen contains methanogenic archaea and protozoa. After bacterial fermentation, methanogenic archaea in the rumen such as *Methanobrevibacter* and *Methanosarcina* can use the end product of the fermenters (CO_2, H_2 or acetate) and poduce CH_4, which is later released from the animal through eructation. Though the metabolism of archaea is not as complex as that of bacteria, it is of great importance. The removal of H_2 by methanogens makes the bacterial fermentation of cellulose more thermodynamically favorable. Protozoa found in the rumen mainly feed on ruminal bacteria, but they are also capable of fermenting all major plant materials to acetate, butyrate, lactate, CO_2 and H_2 (Weimer, 1992).

The digestion of cellulose described above occurs relatively slowly. To speed the fermentation process up ruminants regurgitate the partly digested food back up to the mouth and chew it again, so that the food particles can become smaller and therefore easier to digest. This process is called rumination and usually takes about 8 hours (Hobson & Wallace, 1982).

After rumination, food flows into the reticulum, which is the chamber immediately following the rumen. The contents of the reticulum are usually intermixed with those in

rumen (Hobson & Wallace, 1982). The omasum is the third chamber where salt, fatty acids and some water are absorbed. The more concentrated solid particles move to abomasum, a true stomach with a low pH, where normal food digestion occurs (Hobson & Wallace, 1982).

Among the four parts of stomach, rumen is the most studied because of its special fermentation and methane generation. Methane's global warming potential is 23 times greater than that of carbon dioxide and methane accounts for 16% of all green house gas emissions globally (Iqbal *et al*., 2008). It has been calculated that 71% of Australia's anthropogenic agricultural methane emissions (or about 62.1 million tons of carbon dioxide equivalents annually) is from livestock (sheep and cattle). If not wasted as emitted methane, this amount of carbon would represent an energy gain of 4-15% to the animal (Ouwerkerk, *et al.*, 2008). Therefore, a thorough understanding of the microorganisms involved in cellulose digestion, along with the effort to reduce methanogenesis in rumen, can directly benefit ruminant livestock productivity.

Studies of gut microflora using cultivation and molecular-based methods

Rumen microbial communities were initially characterized using cultivation methods (Hungate, 1947). Isolated pure cultures can be tested for the ability to perform important biochemical reactions (for example, fermenting ability, fermentation products, etc.). Isolates can also be characterized in terms of their chemical composition (GC content, long-chain fatty acid composition, etc.). All of the information can ultimately be used to identify microbes and their role in the rumen ecology (Minato *et al.*, 1992). Cotta *et al.* (1986) developed an artificial media to grow *Butyrivibrio fibrisolvens*, a rumen bacterium,

under lab condition and confirmed it could utilize protein. Later on, with modification, Griswold *et al.* (1999) applied it to compare the proteolytic activities among *Prevotella*, another common rumen bacteria group. Their results revealed a great variation among different *Prevotella* species. On the other hand, many researchers have relied on rumen fluid/extract based media, which best mimics the nutrient conditions in the rumen. Rea *et al.* (2007) isolated four *Methanobrevibacter* species from bovine rumen and confirmed they could grow on formate. Ghali *et al.* (2004) isolated *Streptococcus bovis* from Rusa deer and tested its biochemical characteristics. All isolates produced L-lactate as the major fermentation end product.

So far, all major groups of microbes, including bacteria, archaea and protozoa have been discovered in the rumen based on conventional cultivation techniques (White, *et al.*, 1999). Many fibrolytic bacteria, such as *Prevotella*, *Ruminococcus* and *Streptococcus*, have been successfully isolated and their characteristics have been well studied (Russell *et al.*, 1981).

Although isolation of pure culture is the best way to study the characteristics of an unknown species, often it is hard to find out the optimum biotic and abiotic environment required for a species to grow in lab conditions. Researchers face difficulties in looking for proper isolation procedures, media chemistry and gas composition for anaerobic conditions. Recently, with the development of molecular techniques, it has been shown that only a small portion (<10% at most, often <1%) of environmental microorganisms can be cultured in the lab (Zengler, *et al.*, 2002). The development of molecular techniques has provided researchers with new ways to explore the rumen diversity. DNA-based

techniques allow researchers to retrieve and analyze genetic data directly from environmental samples.

Yu *et al.* (2008) compared different hypervariable regions (from V1 to V8) of the archaeal 16S rRNA gene for use in archaea-specific PCR-DGGE analysis. They found that PCR products of V3 region provided the most informative DGGE profiles and methanogens associated with *Methanobrevibacter* were discovered in sheep rumen. Kocherginskaya *et al*. (2001) used DGGE to study bacterial diversity in the rumen of steer. They extracted DNA from the ruminal fluid and amplified the 16S rDNA gene. Using DGGE and sequencing analysis, they recognized that the dominant bacteria in rumen essentially belong to four phyla, CFB (*Cytophaga-Flavobacteria-Bacteroides*), *Firmicutes*, *Proteobacteria* and *Spirochaetes*, among which CFB and *Firmicutes* were most dominant.

The application of molecular techniques offered the researchers a chance to get access to those uncultivable microbes, which broadened our recognition to rumen microbiology. However, in order to define these potentially new species and understand their role in rumen metabolism, phenotype information, which can only be obtained from cultivation method, is also necessary. Both approaches will be important in the future study in this field.

Factors controlling microbial communities in the rumen

Several factors have been considered that might control the diversity of microbial communities in the gut. Gut microorganisms can be affected by diet. Animals fed on different food have different gut microbial diversity. In Kocherginskaya's study (2001), rumen bacterial diversity in steer fed on hay or corn was compared. The proportion of

Proteobacteria in rumen increased vastly from 3% to 27% when diet was switched from hay to corn. Changes caused by diet were observed in protozoa as well. Regensbogenova *et al.* (2004) used DGGE to study ciliate diversity in the rumen of sheep. They extracted DNA from the ruminal content and amplified an approximately 200 bp piece of 18S rDNA gene. The major rumen ciliate populations of sheep fed on high and low protein containing diet showed significantly different DGGE profiles. As far as we known, diet might have only a minor effect on methogenic archaea in the rumen, since they live on a few simple fermentation products, such as CO_2, produced during the fermentation of cellulose (Hobson & Wallace, 1982).

Delgado *et al.* (2006) studied interindividual variations of cultivable microbial populations in the feces of eight healthy Spanish persons over a period of one year. A number of biochemical variables, such as enzyme activities, and short-chain fatty acid concentrations, which are thought to be influenced by the gastrointestinal microbiota were also analyzed. In this study, the predominant cultivable bacteria populations (*Clostridium*, *Bifidobacteria* and *Bacteroides*) remained constant over time, while other microbial populations (coliforms) were greatly different among individuals. The biochemical variables were also individual-dependent. This indicates that genetic variation in the host animal may have an impact on gut microbial diversity.

Similar research has been done on ruminants. Sadet *et al.* (2007) studied the bacterial population extracted from rumen content and attached to the rumen epithelium from two groups of 5-month-old lambs fed on different diets. Unlike the rumen content, no diet-effect was observed in the epithelium profiles. However the epithelium profiles showed a high inter-animal variation, which indicated a strong host impact. Li *et al.* (2009)

took samples from different locations of rumen (cranial dorsal, cranial ventral, central rumen, caudal dorsal and caudal ventral) at different times (3h before feeding, 3h after feeding and 9h after feeding). Their DGGE profiles showed that regardless of location and time, samples taken from the same individual were more similar with each other. These findings further confirmed that the animal host could have a strong effect on gut microflora.

Rumen microbiology of wild ruminants

Many studies on rumen microbiology have been performed with domestic ruminants, while little is known about wild ruminants. Kobayashi (2006) pointed out that since wild ruminants needed to deal with a wide range of food, the rumen of wild animals could have different microbial communities and be a reservoir of novel microorganisms that could potentially be used to improve livestock productivity. Indeed, in a study with wild Hokkaido Sika deer in Japan, Yamano *et al.* (2003) revealed that the deer rumen was dominated by bacteria in the CFB group, while other studies indicated that the most abundant rumen bacteria in domestic animals belong to the *Firmicutes* (Tajima *et al.*, 1999). In addition, the proportion of previously uncultured bacteria in Hokkaido Sika deer was as high as 96%, suggesting a great potential of obtaining novel bacteria in wild animals (Yamano *et al.* 2003).

Recently, Sundset *et al.* (2007) compared rumen bacterial diversity in two sub-species of reindeer, Svalbard reindeer and Norwegian reindeer. The former live under austere nutritional conditions on the high-arctic archipelago of Svalbard, while the latter are semi-domesticated, migrating between lush coastal summer pastures and inland winter

pastures in midland Norway. Samples were taken from three groups: Norwegian reindeer fed a pelleted concentrate; Norwegian reindeer from a lush summer pasture on the coast of Norway; and Svalbard reindeer on pasture in Svalbard. Similar to previous studies, all the reindeer rumens were dominated by CFB and *Firmicutes*, however ratios among the groups differed. For Norwegian reindeer fed on artificial pelleted concentrate, the percentages of total clones for *Firmicutes* and CFB was 91% and 7%. The numbers for Norwegian reindeer from summer pasture and Svalbard reindeer were 71% and 29%, and 55% and 42%, accordingly. This study suggests that artificial diets provided by humans stimulate the proliferation of *Firmicutes* in the rumen, while wild-fed animals even in the same species develop a more balance rumen microflora where CFB bacteria and Firmicutes make up relatively equal proportions of the community. The most commonly known cellulolytic rumen bacteria, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* (Koike *et al.*, 2003), were not found in their clone libraries. Not surprisingly, the majority of their sequences (92.5%) had <97% similarity to known species, indicating novel strains.

In another study on Norwegian reindeer (from natural pasture) by Sundset *et al.* (2009), the rumen archaeal community was explored in great detail. Unlike bacteria, the majority of archaeal clones in their study (83%) were shown to be similar (>97%) with those reported in domesticated ruminants. Among 19 OTUs (including 54 clones) identified, 14 were related to the family Methanobacteriaceae (43 clones), 1 was related to the family Methanosarcinaceae (2 clones) and 4 were related to an uncultured archaeal group (9 clones). It was shown that the reindeer rumen was dominated by archaea clones

most related to *Methanobrevibacter*, which agreed with other studies (Yu *et al.*, 2008; Wright *et al.*, 2008)

Applied aspects of rumen microbiology

Development of both cultivation and molecular methods has contributed greatly to our understanding about rumen microbes and their metabolism. This knowledge has been applied in an attempt to improve domestic animal productivity.

Mimosine is a toxic amino acid for ruminants, which will result in the production of dihydroxypridines (DHP) while being metabolized in rumen. Allison *et al.* (1990) transferred rumen fluid from mimosine-tolerant Hawaiian goats to intolerant Australian sheep, which enabled the sheep to develop tolerance to the mimosine-containing plants. *Synergistes jonesii*, a rumen bacterium able to degrade DHP, was later found responsible for the outcome (Allison *et al.*, 1990.). Similarly, tannin is another common toxic material in plant leaves produced as a natural form of defense. Some herbivores, such as koalas (Osawa, 1990) and domestic ruminants fed on tannin-rich diet (McSweeney *et al.*, 2001), have developed the ability to degrade it via gut bacteria. Characterization of isolated bacteria and genes responsible for tannin degradation (tanninase) can prove to be very helpful in animal production (Kobayashi *et al.*, 2004).

Besides providing tolerance to toxic agents, a thorough understanding of rumen microbiology will help increasing diet efficiency, especially fiber digestion. Genes important to fiber degradation have been characterized using cultivable rumen bacteria. Ha *et al.* (2001) and Jun *et al.* (2003) have explored the importance of debranching enzymes in fiber digestion by isolating several enzymes from *Fibrobacter succinogenes*. In addition,

regulation of gene expression of fibrolytic enzymes has been partially determined in *Prevotella* (Miyasaki *et al.*, 2003). All these researchers aim at finding genes responsible for fiber digestion, which can be used to raise animal productivity.

CHAPTER II

OBJECTIVES

Recently, more attention has been paid to wild ruminants since they may harbor novel gut microbes that can be applied to increase livestock productivity (Kobayashi, 2006). Several studies have focused on the gut microbial diversity of wild ruminants (Yamano *et al.*, 2003; Sundset *et al.* 2007; Sundset *et al.* 2008), however our understanding is still very limited. Therefore, I designed and performed a study with the following objectives:

1. Collect deer gut samples from wild white-tailed deer in Michigan.
2. Determine the diversity of bacterial and archaeal communities in the four chambers of each deer gut using molecular techniques.
3. Determine whether prokaryotic communities in deer gut are consistent among chambers and across different deer samples.
4. Determine the phylogeny of the bacterial and archaeal communities in deer stomachs.

CHAPTER III

METHODOLOGY

Sampling

I collected deer stomach samples from individual hunters during the hunting season in Michigan in 2007 and 2008 (Table 1). The hunters were instructed to remove the stomachs carefully right after the deer were shot so that the chambers were not broken and the contents were not contaminated. The stomachs were then frozen and sent to the lab, where they were kept at -20^{o}C until processed. A sample of the contents from all four chambers of stomachs was removed using a sterile scalpel and a sterile spatula. The subsamples were stored in sterile 50 ml centrifuge tubes at -20^{o}C until DNA extraction.

DNA extraction

DNA from stomach contents was extracted using a commercial kit (ZR Soil Microbe DNA Kit^{TM}, Zymo Research, Orange, CA), according to the manufacturer's instructions.

PCR-DGGE

I used the 16S rRNA gene as the molecular marker to explore the bacterial and archaeal diversity in deer stomachs. A single PCR was performed for the bacterial

Table 1. Michigan counties in which the deer samples were harvested.

County	Deer
Alcona	D13[1]
Calhoun	D1, D2, D7, D8, D9
Clare	D29, D30, D31
Dickinson	D23
Gratiot	D5, D6, D11, D12, D22, D24
Iosco	D3, D4, D25
Isabella	D14, D15, D16, D17, D21, D26, D32, D33, D34
Montcalm	D10
Montmorency	D18, D19, D20
Upper Penninsula (county unknown)	D27, D28

[1] – Deer 1-15 were sampled in 2007, deer 16-34 were sampled in 2008.

community with primers PRBA338f (5' AC TCC TAC GGG AGG CAG CAG 3', with a GC-clamp added to the 5' end of the primer) and PRUN518r (5' ATT ACC GCG GCT GCT GG 3') (Nakatsu, *et al.*, 2000). A standard PCR mix was used (Promega, Madison, WI) to which $MgCl_2$ (Promega, Madison, WI) was added to bring the final concentration of Mg to 3.5 mM. Samples were amplified in MyCycler™ (Biorad, Hercules, CA) using the following program: initial denaturation at 94°C for 2 minutes; 30 cycles of (i) denaturation at 92°C for 30 seconds, (ii) annealing at 56°C for 30 seconds, (iii) extension at 72°C for 30 seconds; final extension at 72°C for 7 min.

In order to explore the diversity of the archaeal community in rumen, I performed a nested PCR. This was necessary due to the low copy of archaeal DNA in the samples. In the first round PCR I amplified a long fragment (about 1200 bps) of archaeal 16S rRNA gene using primers PRA46F (5' C/TTA AGC CAT GCG/A AGT) and PREA1100R (5' T/CGG GTC TCG CTC GTT G/ACC 3') (Nakatsu, *et al.*, 2000) A standard master mix was used (Promega, Madison, WI), to which the following additions were made: (i) acetamide (Alfa Aesar, Ward Hill, MA), 7 μl of a 20% stock solution per 50 μl reaction and (ii) $MgCl_2$ (Promega, Madison, WI) to a final concentration of 3.5 mM. Acetamide was added to improve the specificity of the PCR reaction (Reysenbach, *et al.*, 1992). Samples were amplified in a MyCycler^{TM} (Biorad, Hercules, CA) using the following program: initial denaturation at 94°C for 5 minutes; 33 cycles of (i) denaturation at 94°C for 30 s, (ii) annealing at 48°C for 30 s, (iii) extension at 72°C for 1 min; final extension at 72°C for 7 min.

The products from the first round of archaeal PCR were then used as the templates in the second round PCR with primers PARCH344F (5' CC TAC GGG GC/TG CAG/C CAG 3', with a GC clamp at the 5' end of the primer) and PARCH519R (5' TTA CCG CGG CG/TG CTG 3') (Nakatsu, *et al.*, 2000). A standard master mix was used (Promega, Madison, WI) to which $MgCl_2$ (Promega, Madison, WI) was added to bring the final concentration to 1.75 mM. Samples were amplified in a MyCycler^{TM} (Biorad, Hercules, CA) using the following program: initial denaturation at 95°C for 5 minutes; 30 cycles of (i) denaturation at 95°C for 30 s, (ii) annealing at 56°C for 30 s, (iii) extension at 72°C for 1 min; final extension at 72°C for 10 min.

Denaturant Gradient Gel Electrophoresis (DGGE)

DGGE is a fast way to examine the diversity of PCR products and therefore the diversity of the microbial community from which the 16S rRNA genes has been amplified. In DGGE, a special gel is poured which contains a gradient of denaturants (from low to high). The double stranded DNA sequences travel through the gel from low concentration of denaturants to high concentration of denaturants under an electrical force. When they meet a high enough denaturant concentration, they partially denature to single-stranded molecules and stop moving. Since there are two hydrogen-bonds between adenine (A) and thymine (T) and three hydrogen-bonds between guanine (G) and cytosine (C), more energy is needed to separate GC than AT. Therefore, DNA sequences with different base composition require different denaturant concentrations to denature. Different PCR products will therefore be denatured at different denaturant concentrations and stop at different positions. The DGGE profile represents the community structure of the sample and each visible band on gel stands for organisms that make up at least 1% of the community (Muyzer *et al.*, 1993).

DGGE was performed using 8% (wt/vol) polyacrylamide gels (37.5:1 acrylamide:bisacrylamide) with denaturing gradients ranging from 30 % to 52.5% (100% denaturant contains 7M urea and 40% (vol/vol) formamide). DGGE was carried out in a DeCode machine (Biorad, Hercules, CA) at 200 V and 60°C for 5.5 hours. I used SYBR® Green I (Cambrex Bio Science, Rockland, ME) to stain the gels and images were digitized using a Gel Logic 2200 Imaging System (Kodak Eastman Company, Rochester, NY).

16S rDNA sequencing

Bacterial sequences were obtained from several of the deer samples. For bacteria, a single round of PCR was performed using primers pA (5' AGA GTT TGA TCC TGG CTC AG 3') and pH (5' AAG GAG GTG ATC CAG CCG CA 3') (Edwards *et al.*, 1989). A standard master mix was used (Promega, Madison, WI) to which $MgCl_2$ (Promega, Madison, WI) was added to bring the final concentration to 1.5 mM. The following program was used: initial denaturation at 95°C for 5 minutes; 30 cycles of (i) denaturation at 95°C for 30 seconds, (ii) annealing at 56°C for 30 seconds, (iii) extension at 72°C for 1.5 minutes; final extension at 72°C for 10 minutes. The PCR products of this primer pair are about 1500 bps long (near full length of the 16S rRNA gene).

Several deer samples were used to obtain archaeal sequences and a nested PCR was performed. In the first round PCR I amplified a long fragment (about 1200 bps) of archaeal 16S rRNA gene using primers PRA46F (5' C/TTA AGC CAT GCG/A AGT) and PREA1100R (5' T/CGG GTC TCG CTC GTT G/ACC 3') (Nakatsu, *et al.*, 2000) as described above. The products from the first round of archaeal PCR were then used as the templates in the second round PCR with primers PRA46F (5' C/TTA AGC CAT GCG/A AGT) and PARCH519R (5' TTA CCG CGG CG/TG CTG 3') (Nakatsu, *et al.*, 2000). A standard master mix was used (Promega, Madison, WI) to which $MgCl_2$ (Promega, Madison, WI) was added to bring the final concentration to 3.5 mM. Samples were amplified in a MyCycler^{TM} (Biorad, Hercules, CA) using the following program: initial denaturation at 95°C for 5 minutes; 30 cycles of (i) denaturation at 95°C for 30 s, (ii) annealing at 56°C for 30 s, (iii) extension at 72°C for 1 min; final extension at 72°C for 10 min. These PCR products (about 500 bps) were used for the sequencing analysis.

PCR products were cloned in the pGEM-T easy vector system (Promega, Madison, WI) using competent *E. coli* cells prepared with the ZymoBroth™ kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. For each archaeal library, I screened the transformants using a whole cell PCR-DGGE technique in order to ensure that all major bands in the DGGE profiles were sequenced. Briefly, a small clump of cells from an *E. coli* colony was suspended in water and used as a template in a PCR reaction with archaeal primers, PARCH344F (with a GC clump) and PARCH519R (as described above). The PCR products are then analyzed by DGGE (as described above), next to a lane which contained the original fingerprint from which the cloning was performed. *E. coli* colonies which contain inserts that produce bands in the DGGE coinciding with bands from the original fingerprint were selected for sequencing. Two to five (based on available clones) clones per band were used to ensure that related species that might co-form a DGGE band were characterized. Plasmids were isolated from these colonies using a kit (Ultra Clean™ Standard Mini Plasmid Prep Kit™, Carlsbad, CA) and sequenced in Central Michigan University and Michigan State University's DNA sequencing facility.

Since there were a lot more bands in the bacterial DGGE profiles, they were characterized by randomly choosing and sequencing 30 clones from each bacterial library. PCR was used to amplify the inserts with primers M13F (5' GTT TTC CCA GTC ACG ACG TTG TA 3') and M13R (5' CAG GAA ACA GCT ATG ACC 3'), using cell suspensions as templates. This primer set is on the plasmid, flanking the insert. Standard master mix was used (Promega, Madison, WI) to which $MgCl_2$ (Promega, Madison, WI) was added to bring the final concentration to 1.5 mM. The following program was used: initial denaturation at 95°C for 5 minutes; 30 cycles of (i) denaturation at 95°C for 30

seconds, (ii) annealing at 56°C for 30 seconds, (iii) extension at 72°C for 1.5 minutes; final extension at 72°C for 10 minutes. The products were purified with DNA Clean & Concentrator^{TM}-5 Kit (Zymo Research, Orange, CA) and sequenced in Central Michigan University and Michigan State University's DNA sequencing facility.

Data analysis

All the DGGE gels were normalized according to the marker lanes and all the bands were assigned to band classes (GelCompar II, Applied Maths, Belgium). A 0/1 profile for each sample was obtained based on the presence/absence of bands, from which a dissimilarity matrix was calculated using Jaccard distances. Differences in community composition between chambers and deer for each year were visualized using nonmetric multidimensional scaling (NMDS), a statistical method that plots data in a two-dimensional diagram based on dissimilarity coefficients between different samples. The Euclidean distance between points in an NMDS plot is proportional to the dissimilarity of the samples. In addition I used Multiple Response Permutation Procedures (MRPP) to explore whether the gut prokaryotic diversity was significantly grouped by chambers (4 groups) or by deer (15 groups for 2007; 19 groups for 2008). MRPP works by calculating a weighed intragroup average distance and comparing it to a distribution from 1000 random permutations of our data. A small p-value (<0.05) would indicate that the observed distance was significantly smaller than the expected distance if the data were randomly assigned to groups. Therefore the observed groups would be significantly different.

I compared the obtained the 16S rDNA sequences with sequences in the GenBank database of the National Center for Biotechnology Information (NCBI) using the BLASTn

program (Altschul *et al.*, 1997). Sequences from each year were aligned with CLUSTALX2 (Larkin *et al.*, 2007) and based on the multiple sequences alignments, phylogenetic trees were generated with PAUP*, incorporated in Genetic Data Environment (GDE), using the maximum parsimony algorithm.

CHAPTER IV

RESULTS

DGGE profiles

DGGE profiles for bacteria and archaea were obtained for both years (Figure 1 & 2). Each band in DGGE gels is assumed to represent one potential dominant (more than 1% of the community) species in an environment sample. I only used intense sharp bands in our study. The average band numbers in each sample for bacteria were 7±0.3 (SE) in 2007 and 9±0.4 (SE) in 2008, while for archaea these numbers were only 5±0.2 (SE) for both years. In addition, 44 (2007) or 47 (2008) distinct band classes were required to include all of the bacteria bands, while for archaea only 11 and 17 band classes were needed. These numbers clearly indicate that bacteria had more diversity than archaea in the deer rumen.

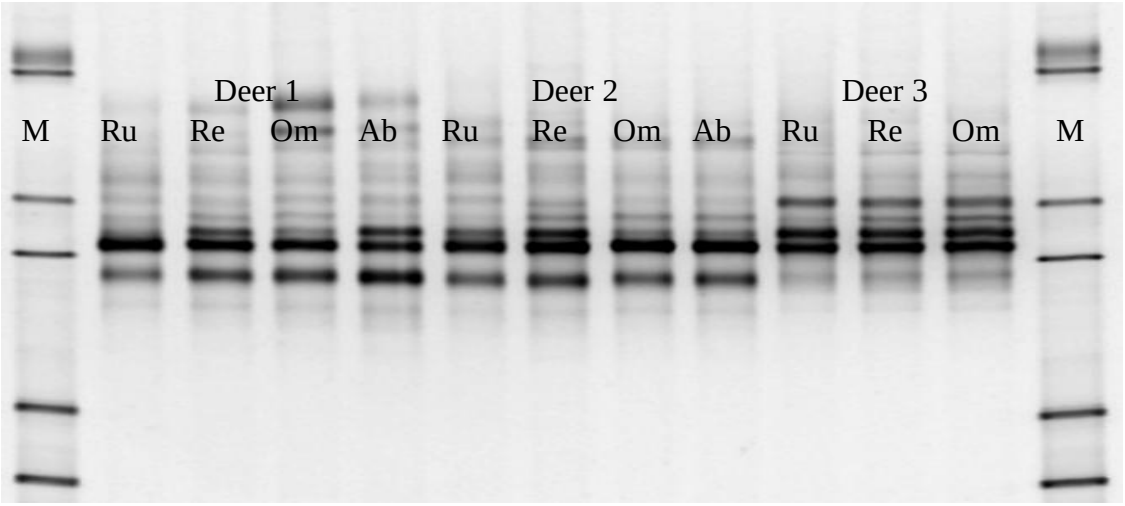

Figure 1. DGGE profiles of rumen archaeal 16S rRNA genes amplified using total DNA extracted from rumen contents from three wild white-tailed deer in Michigan in 2008. Denaturants used were from 30% to 52.5% (100% denaturant contains 7M urea and 40% (vol/vol) formamide). Chambers are as follows: Ru – rumen, Re – reticulum, Om – omasum, Ab – abomasum. M stands for marker. These three deer were chosen to be representative of all the other deer samples as they contain all the major bands.

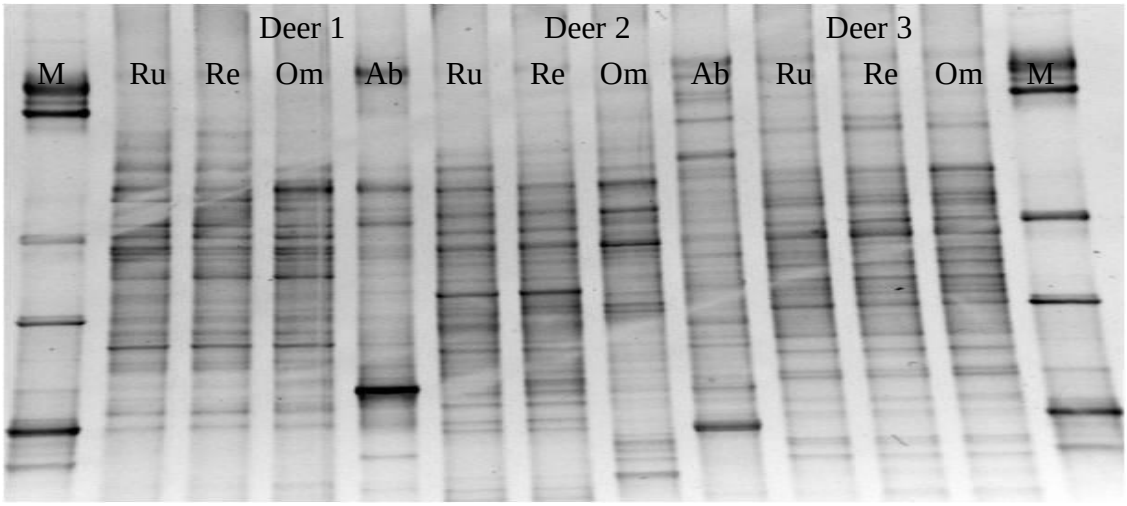

Figure 2. DGGE profiles of rumen bacterial 16S rRNA genes amplified using total DNA extracted from rumen contents from three wild white-tailed deer in Michigan in 2008. Denaturants used were from 30% to 52.5% (100% denaturant contains 7M urea and 40% (vol/vol) formamide). Chambers are as follows: Ru – rumen, Re – reticulum, Om – omasum, Ab – abomasum. M stands for marker. These three deer were chosen to be representative of all the other deer samples as they contain all the major bands.

I was able to explore two alternative hypotheses regarding microbial communities in deer stomachs. First, I determined if communities in different chambers contain the same microbial communities. Second, I was able to test whether the communities in the four chambers of an individual deer are more similar to each other than communities in the same chamber but in different animals. Significant grouping of DGGE profiles was tested with MRPP and visualized using NMDS. MRPP tests clearly indicated that bacterial/archaeal data from the same deer tightly grouped together while data from same chambers but in different animals was randomly distributed (Figure 3-10). The results of the MRPP tests were identical for both of the sampled years.

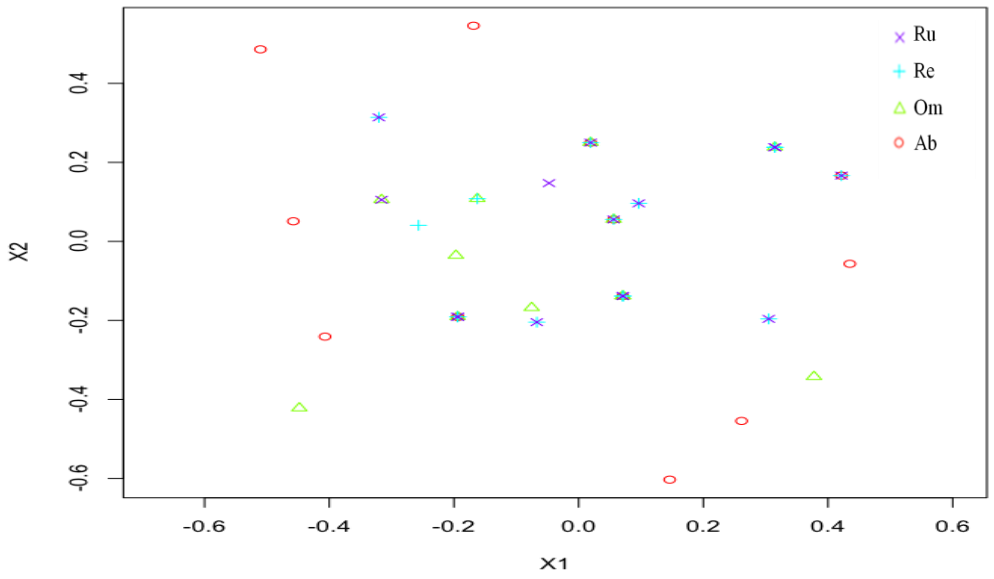

Figure 3. NMDS diagram of archaea samples grouped by chamber in 2007.
Chambers are as follows: Ru – rumen, Re – reticulum, Om – omasum, Ab – abomasum. Distances between dots represent dissimilarities between samples in term of their DGGE profiles.

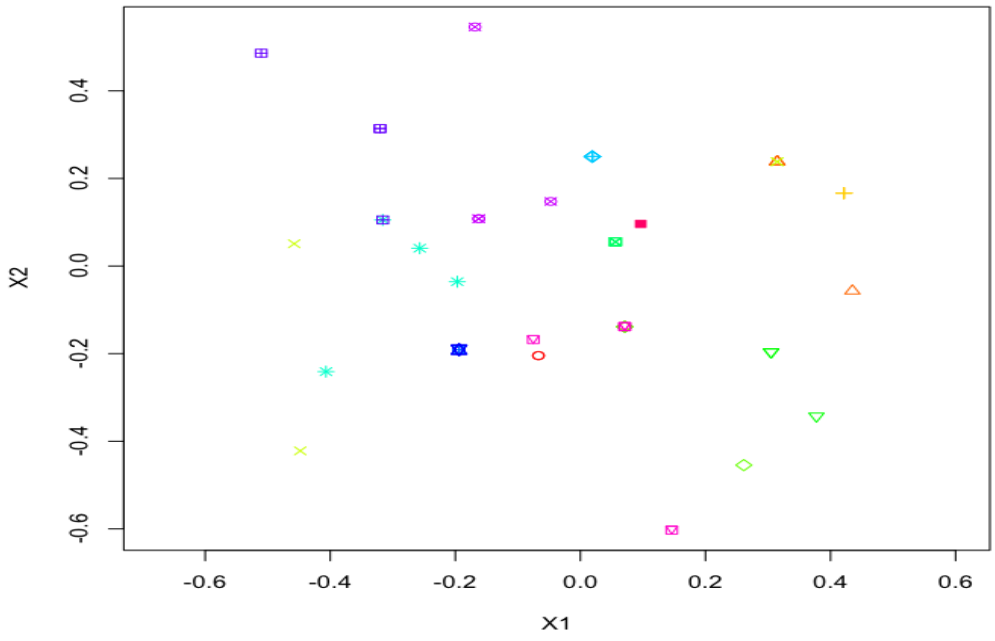

Figure 4. NMDS diagram of archaea samples grouped by deer in 2007.
The same symbols stand for the four chamber samples from the same deer. Distances between dots represent dissimilarities between samples in term of their DGGE profiles.

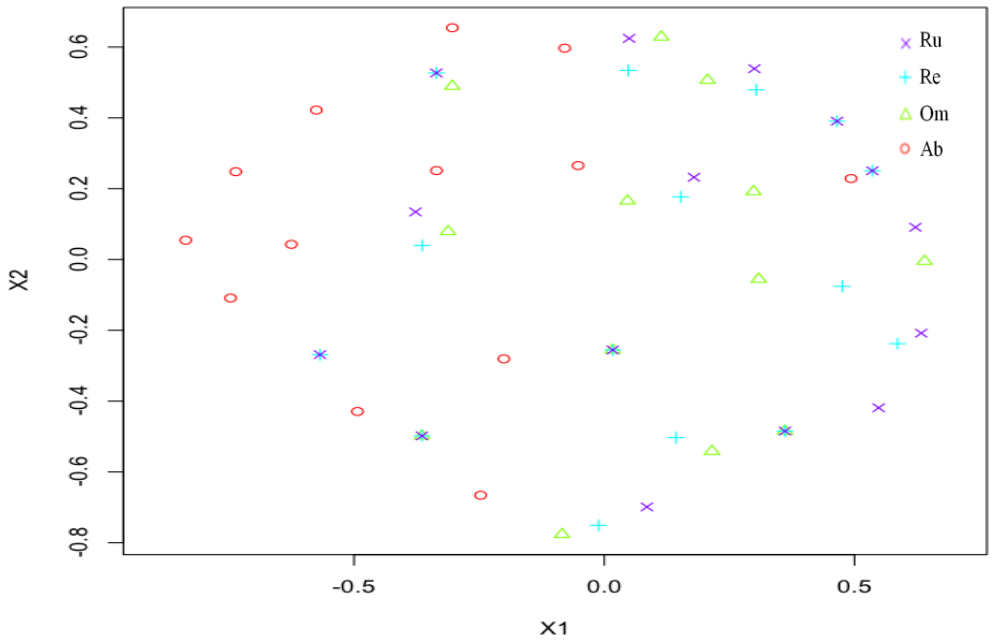

Figure 5. NMDS diagram of bacteria samples grouped by chamber in 2007.
Chambers are as follows: Ru – rumen, Re – reticulum, Om – omasum, Ab – abomasum. Distances between dots represent dissimilarities between samples in term of the DGGE profiles.

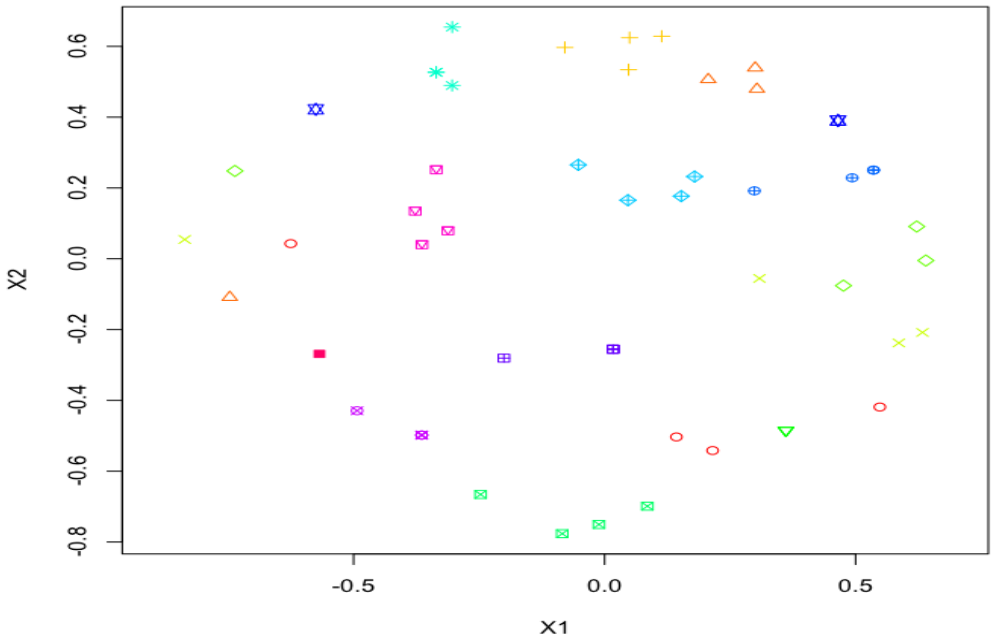

Figure 6. NMDS diagram of bacteria samples grouped by deer in 2007.
Same symbol stands for samples from the same deer. Distances between dots represent dissimilarities between samples in term of the DGGE profiles.

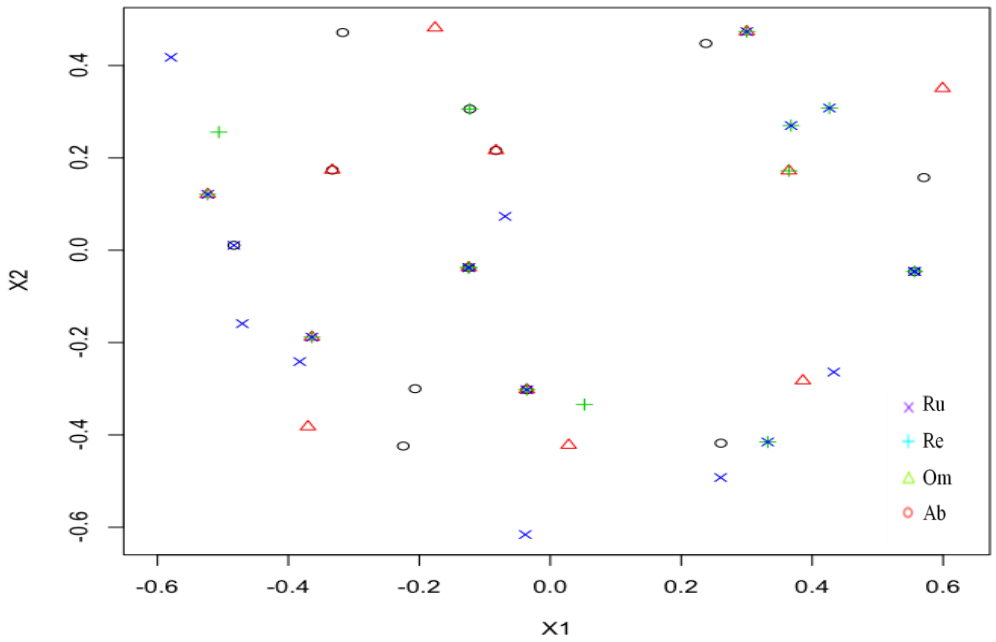

Figure 7. NMDS diagram of archaea samples grouped by chamber in 2008.
Chambers are as follows: Ru – rumen, Re – reticulum, Om – omasum, Ab – abomasum. Distances between dots represent dissimilarities between samples in term of the DGGE profiles.

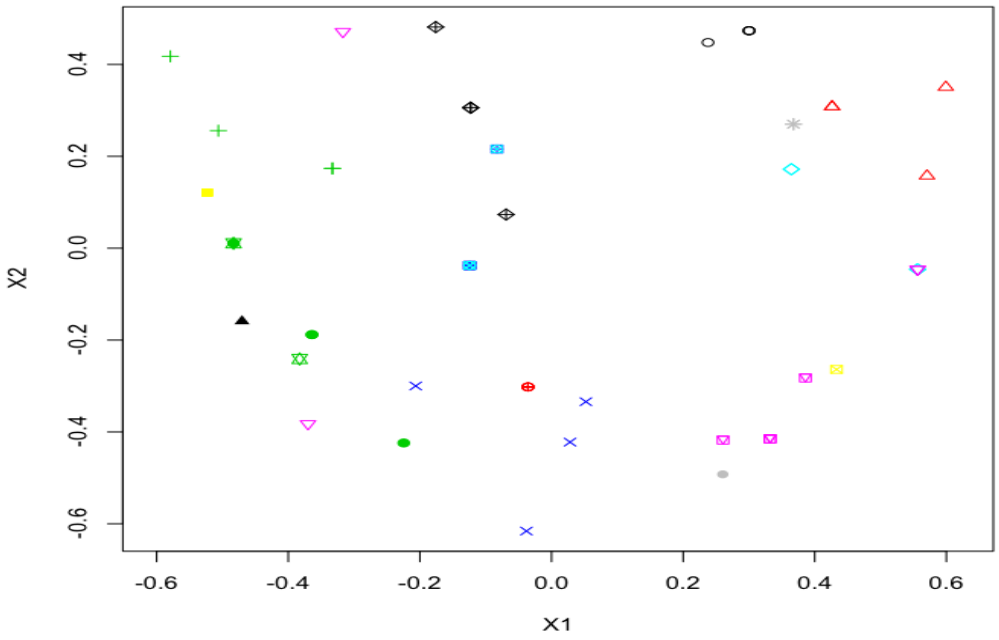

Figure 8. NMDS diagram of archaea samples grouped by deer in 2008.
Same symbol stands for samples from the same deer. Distances between dots represent dissimilarities between samples in term of the DGGE profiles.

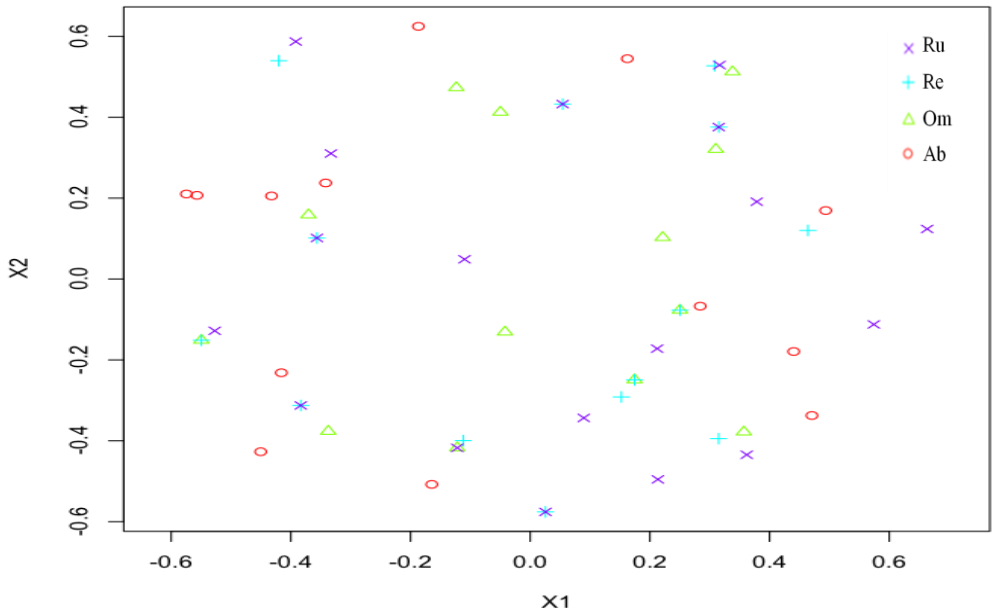

Figure 9. NMDS diagram of bacteria samples grouped by chamber in 2008.
Chambers are as follows: Ru – rumen, Re – reticulum, Om – omasum, Ab – abomasum. Distances between dots represent dissimilarities between samples in term of the DGGE profiles.

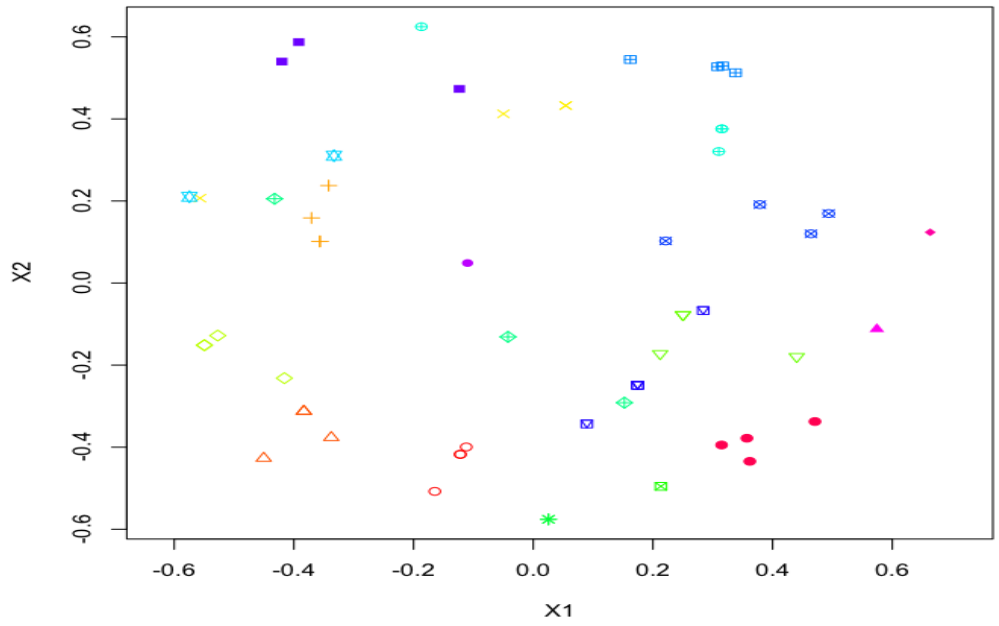

Figure 10. NMDS diagram of bacteria samples grouped by deer in 2008.
Same symbol stands for samples from the same deer. Distances between dots represent dissimilarities between samples in term of the DGGE profiles.

Random sequencing of 16S rDNA and phylogenetic analysis

I obtained sequences from a total of 147 bacterial clones (69 from 3 clone libraries in 2007 and 78 from 3 clone libraries in 2008) and 48 archaeal clones (17 from 4 clone libraries in 2007 and 31 from 4 clone libraries in 2008). Phylogenetic analysis of the sequences revealed that the majority of bacteria and archaea were most closely related to uncultured clones. The archaeal sequences were similar (>97%) with those from known species. In contrast, bacterial sequences shared low similarity (<97%) with previously cultured species, which indicated that they came from novel microbes. Based on the sequences, phylogenetic trees were generated for bacteria/archaea in each year (Figure 11-14). For both years, archaea sequences were found within two consistent phylogenetic groups which were most closely related to *Methanobrevibacter* and *Methanosphaea*, respectively (Figure 11 & 12). In 2007, sixteen of the sequences were related to *Methanobrevibacter* and one sequence was related to *Methanosphaea*, while in 2008, the numbers of sequences from those two groups was 24 and 7, respectively. The data clearly showed that the archaeal community was tightly conserved and dominated by uncultured archaea which are most related to *Methanobrevibacter*.

In sharp contrast to archaea, bacterial communities exhibited much higher diversity. In 2007, the majority of our bacterial sequences (59 out of 69) were found in a group of *Prevotella*-like *Bacteroidetes*. Based on the 16S rRNA sequence analysis, they were most closely related to *Prevotella*, yet with low similarities (<97%) which indicated they might belong to new species. In addition, I found sequences related to different phyla such as *Firmicutes* (7 clones), *Proteobacteria* (1 clone) and *Spirochaetes* (2 clones) (Fig. 13). In 2008 the *Prevotella*-like *Bacteroidetes* group was also dominant making up with 67% of

the sequences. In addition, 25 clones were found to be related to *Firmicutes* and 1 clone was not related to any known phyla (Figure 14). Phylogenetic analysis of the data confirmed that each individual animal harbors unique bacterial and archaeal species – several of the clades in the phylogenetic trees contained sequences isolated from one animal only (Figure11-14).

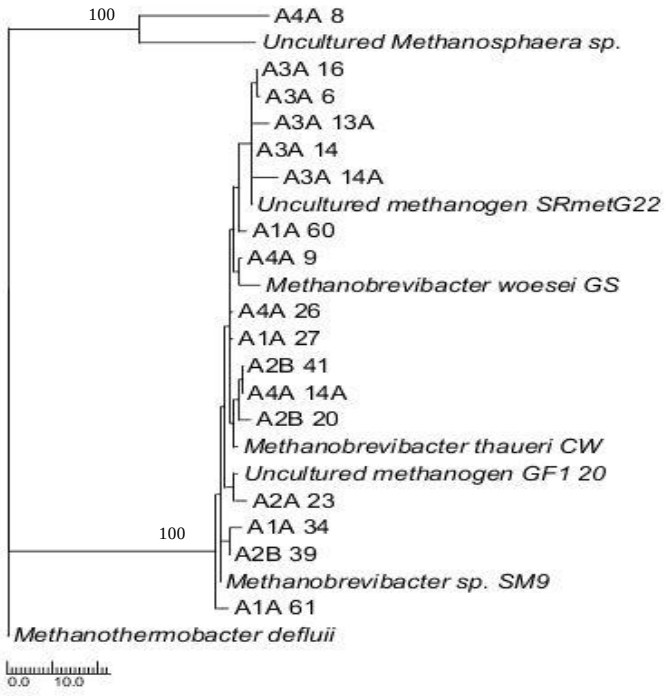

Figure 11. Parsimony tree of partial rumen archaea 16S rRNA gene sequences (about 500 bps) in 2007.
Sixteen sequences were closely related to *Methanobrevubacter*; one sequence was closely related to *Methanosphaea*.

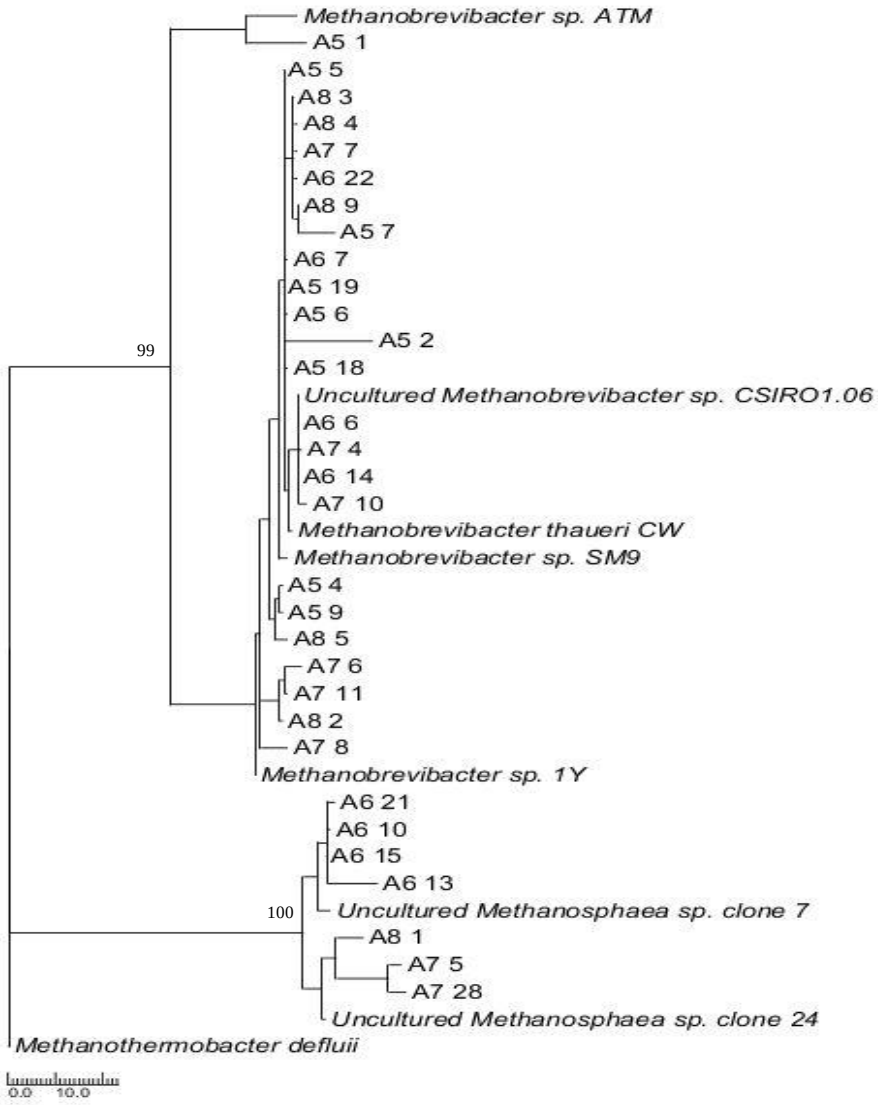

Figure 12. Parsimony tree of partial rumen archaea 16S rRNA gene sequences (about 500 bps) in 2008.
Twenty-four sequences were closely related to *Methanobrevibacter*; seven sequences were closely related to *Methanosphaea*.

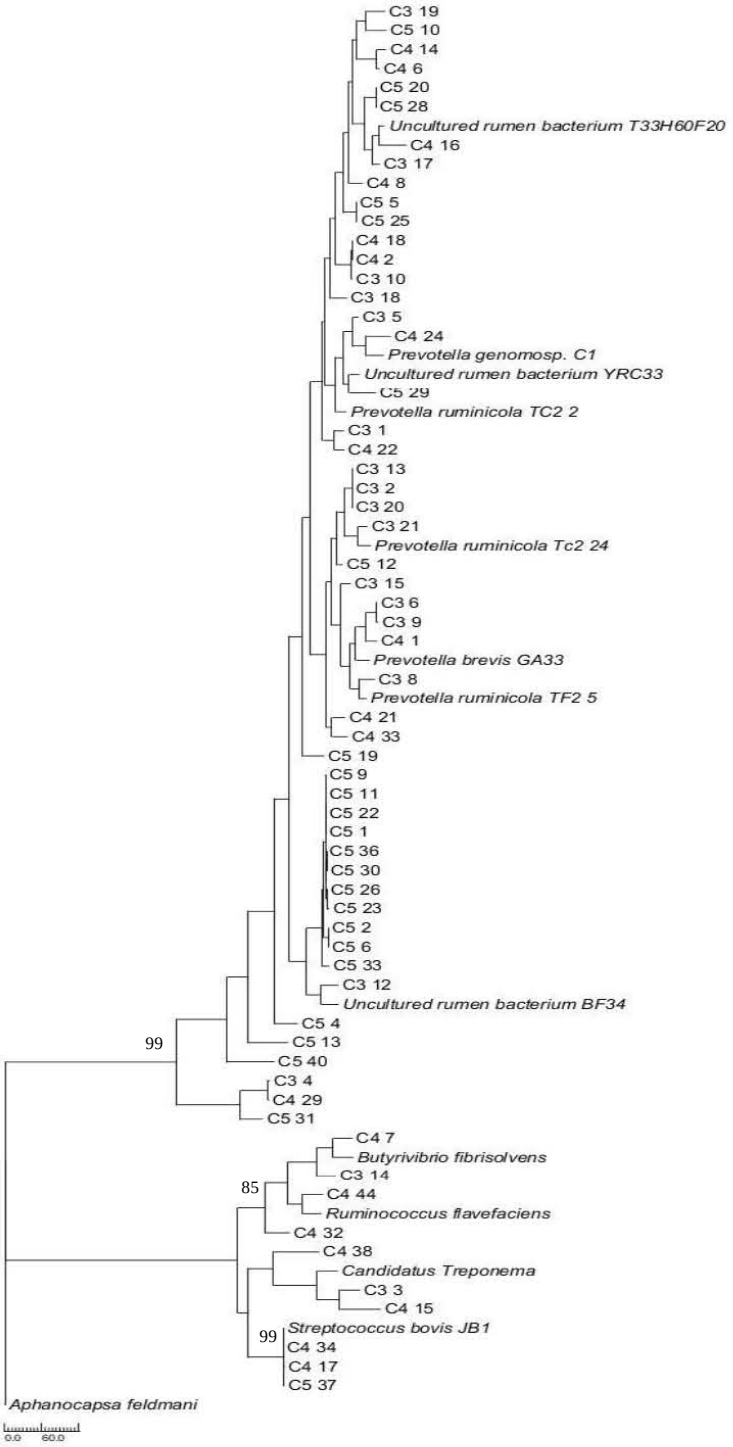

Figure 13. Parsimony tree of partial rumen bacteria 16S rRNA gene sequences (about 500 bps) in 2007.

Seven sequences were closely related to *Firmicutes*; one sequence was closely related to *Proteobacteria*; two sequences were closely related to *Spirochaetes*; fifty-nine sequences were related to *Bacteroidetes*.

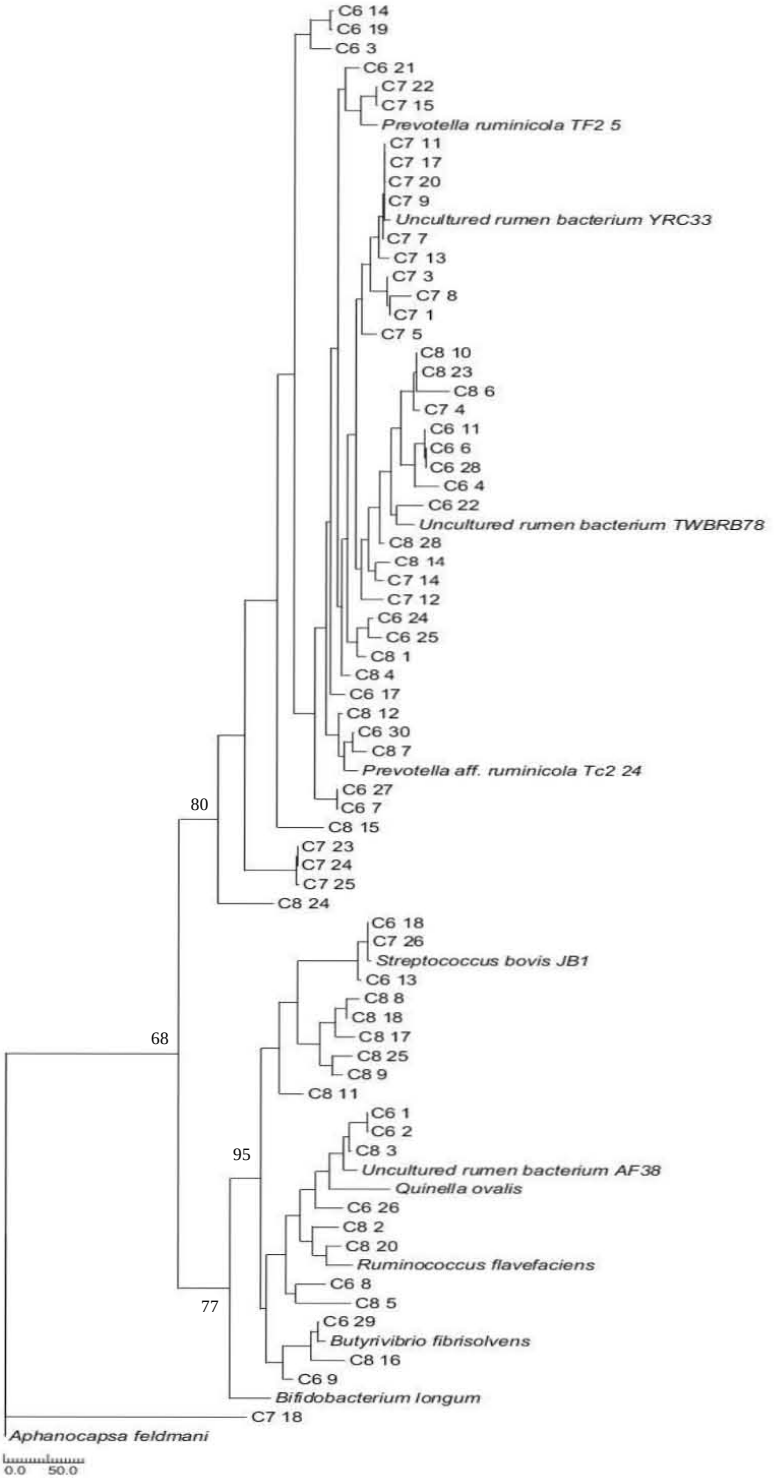

Figure 14. Parsimony tree of partial rumen bacteria 16S rRNA gene sequences (about 500 bps) in 2008.
Twenty-five sequences were closely related to *Firmicutes*; fifty-two sequences were closely related to *Bacteroidetes*; one sequence was not related to any known phyla.

CHAPTER V

DISCUSSION

In this study, I investigated the bacterial and archaeal diversity in the stomachs of white-tailed deer harvested by hunters during the hunting season in Michigan in 2007 and 2008. I retrieved the partial 16S rDNA sequences from the gut content and analyzed them with DGGE and random-sequencing approaches. The DGGE profiles clearly showed that in both years, each individual deer had a unique gut microflora, which was shared among its four chambers (Figure 2-10). This pattern was also observed in the sequencing results in the form of deer-specific clades on the constructed phylogenetic trees (Figure 11-14). This inter-animal variation could be explained by a different diet or a host impact. Many studies have shown that diet has a great effect in domestic animals and their rumen microbial communities will change considerably according to diet shifts (Kocherginskaya *et al.*, 2001; Regensbogenova *et al.* 2004). However, if the high inter-animal variation I observed was due to diet, it can be inferred that every deer in my sample must have been fed on unique food, which is very unlikely because they were all taken from the same location (Michigan) during the same time period of year (hunting season in Michigan, from October to January). Although I was unable to reliably determine the diet of the harvested animals used in my study, I was able to examine the rumen contents and distinguish some of the foods that the animals were consuming. Most of the rumens I studied contained similar remnants of grass, tree material, and corn (data not shown). Therefore, even though diet effect cannot be excluded, I assume that the main controlling factor for the inter-animal variation was a host impact on gut microbial communities. This could be attested in

domestic animal studies in which the kinship of animals are known. However, because of the general limitation in wild animal studies, I could only make assumptions.

As described in the literature review, previous studies have shown the host impact of domestic ruminants, via the host-specific gut microbial communities, regardless of diet (Sadet *et al.*, 2007; Li *et al.*, 2009). My study agrees with these findings, revealing that wild ruminants hosts also significantly impact their gut microflora. Guan *et al.* (2008) used PCR-DGGE to investigate the microbial profiles in the rumen of cattle in different feed efficiency. Their results showed that the profile from efficient steers clustered together and were clearly separated from those obtained from inefficient steers. In addition, the bacteria profiles were more likely clustered within a certain breed, indicating the host impact could be due to host genetic makeup. However the mechanism of how the host genetics affects gut microflora has not been well understood yet. Few papers have been published in this field and it is not clear if host genetics directly affect gut microbial communities, or via something else, such as the immune system or the tendency of choosing diet.

The archaeal community in the stomach of white-tailed deer was dominated by *Methanobrevibacter*-like species (>97% similarity) in both years (94% in 2007 and 77% in 2008). *Methanobrevibacter* phylotypes have been reported to be the most abundant archaea in the rumen of many domestic ruminants, such as Venezuela sheep (Wight *et al.*, 2008), Australia cattle (Ouwerkerk *et al.*, 2008) and Chinese goat (Cheng *et al.*, 2009). In a recent study on wild Norwegian reindeer (Sundset *et al.*, 2008), the dominant archaea group was also found to be *Methanobrevibacter* phylotypes. It is likely that they are dominant rumen methanogens worldwide, both in domestic and wild ruminants.

Random-sequencing revealed that in both years, the bacterial community was dominated by *Bacteroidetes* and *Firmicutes* (86% and 10% in 2007; 67% and 32% in 2008). Not surprisingly, my findings agree with a previous study on the wild Hokkaido Sika deer (Yamano *et al.* 2003): unlike domestic animals, the most predominant bacterial group in wild deer rumens was *Bacteroidetes* and not *Firmicutes.*. The large differences between domestic and wild ruminants may be due to differences in their diets. Kocherginskaya *et al*. (2001) compared rumen bacterial diversity in steer fed on hay or corn and found that hay-fed animals had more *Firmicutes* in their rumen than corn-fed animals. In addition, Sundset *et al.* (2007) revealed that artificial diets provided by humans would stimulate the proliferation of *Firmicutes* in the rumen of reindeer. While most studies (including mine) agree that wild ruminants tend to have less *Firmicutes* than domestic ruminants the mechanism behind this has not been well understood yet.

Within the *Bacteroidetes* group, most sequences were found to be closely related to *Prevotella*. *Prevotella* has long been recognized as an important genus of rumen bacteria. Some common species, such as *Prevotella ruminicola*, have been successfully cultivated and well studied (Avgustin, *et al.*, 1994). However, sequences within this group retrieved in my study were less than 97% similar to all known *Prevotella* species, indicating that these were uncultured new species. With the aid of molecular techniques, similar *Prevotella*–like, uncultured *Bacteroidetes* groups were also found in high abundance in other cellulose degrading communities, such as in reindeer rumen (Sundset *et al*., 2008) and termite guts, (Ohkuma, *et al.*, 2002). Their abundance and prevalence indicate that they must play an important role in the cellulose metabolism: *Prevotella* is known to be able to utilize different carbon source and help with cellulose degradation (Avgustin, *et al.*,

1994). In order to study these previously underrepresented rumen *Prevotella/Bacteroidetes* group and confirm whether those uncultured clones are new species, more advanced cultivation approaches would be needed.

All of my data clearly indicates that deer harbor a relatively diverse bacterial community, while their archaeal communities are composed of a few dominant phylotypes. This was evident in DGGE profiles and was confirmed by my random-sequencing approach. All retrieved archaeal sequences were closely related to two genera (*Methanobrevibacter* and *Methanosphaea*), while the bacterial sequences were spread among different phyla (*Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Spirochaetes*) (Figure 4-7). This consistent trend (found in both years) can possibly be explained by the different substrates that bacteria and archaea are adapted to. The bacterial community must be able to ferment various food sources. For example, *Fibrobacter succinogenes*, a common fibrolytic bacterium found in the rumen can hydrolyze cellulose and starch but cannot degrade xylose. Therefore in order to make xylose digestible, animals have to also harbor xylose fermenters, such as *Ruminococcus flavefaciens* in their rumen (Russell *et al.*, 1981). Some bacteria, such as *Prevotella*, are not capable of degrading cellulose. However they can make this process more feasible by acting synergistically with cellulolytic bacteria (Avgustin, *et al.*, 1994), hence are found in high abundance in rumen. A high rumen bacterial diversity is crucial for wild ruminants because they have to adapt to the changing seasons, especially in winter when the food quality is poor. On the other hand, the archaea were responsible of the transformation from CO_2, H_2 and/or acetate to CH_4, thus a high diversity might not be necessary (Hobson & Wallace, 1982).

CHAPTER VI

CONCLUSION

This PCR-DGGE based study presents data on the bacterial and archaeal diversity in the four-chambered stomach of wild white-tailed deer in Michigan. Significant inter-animal variation was observed both in bacterial and archaeal community, indicating a strong host impact on gut microflora.

The methanogenic archaea living in rumen convert CO_2 and H_2 to CH_4, which is later released from animals to atmosphere. Methane is known to have 23 times greater global warming potential than carbon dioxide and methane accounts for 16% of all green house gas emissions globally (Iqbal *et al*., 2008). Attentions have been paid to the inhibition of methane generation from domestic ruminants. (Ouwerkerk, *et al*., 2008). Though practically difficult, it will be good if the methane generation from wild ruminants is inhibited. In addition, some uncultured archaea strains discovered in the rumen of wild ruminants may potentially help with this process.

The bacteria are even more important for they have direct effect on animal nutrition and diet efficiency. Most of our bacteria sequences don't belong to any known species and the bacteria community show a different composition with domestic ruminants, suggesting wild ruminant gut to be a great reservior of novel bacteria. These novel bacteria enable wild ruminants to adapt to the changing seasons and survive the winter, when the food quality is poor. Therefore they could potentially be applied to domestic ruminants to increase stock productivity. Future work should focus on cultivating these unknown members in lab and identifying their functional role in rumen metabolism.

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