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ESTABLISHMENT, IDENTIFICATION, QUANTIFICATION OF METHANOGENIC ARCHAEA IN CHICKEN CECA AND METHANOGENESIS INHIBITION IN IN VITRO CHICKEN CECA BY USING NITROCOMPOUNDS

A Thesis

by

SUWAT SAENGKERDSUB

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2006

Major Subject: Food Science and Technology

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Approved by:

Co-Chairs of Committee,	Steven C. Ricke
	Jimmy T. Keeton
Committee Member,	Robin C. Anderson
Chair of Food Science and Technology Faculty,	Rhonda K. Miller

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ABSTRACT

Establishment, Identification, Quantification of Methanogenic Archaea in Chicken Ceca
and Methanogenesis Inhibition in in vitro Chicken Ceca by
Using Nitrocompounds. (May 2006)

Suwat Saengkerdsub, B.S., Chulalongkorn University;
M.S., Chulalongkorn University

Co-Chairs of Advisory Committee: Dr. Steven C. Ricke
Dr. Jimmy T. Keeton

In the first phase of this study, the diversity of methanogenic bacteria in avian ceca was found to be minimal. Based on 16S rDNA clone libraries, a common phylotype, designated CH101, ranged between 92.86 to 100 % of the total clones whereas less than 1% of the other phylotypes were found. On the basis of the sequence identity, all of the sequences, except sequence CH1270, are related from 98.97 to 99.45% to 16S rDNA *Methanobrevibacter woesei* GS. Sequence CH1270 is 97.62% homologous to the sequence identified to uncultured archaeon clone ConP1-11F. Clearly, the predominant methanogen found to reside in the chicken ceca was *M. woesei*. By using a MPN enumeration method, methanogen counts were found to be in the range of 6.38 to 8.23 $\log_{10}$ organisms per gram wet weight. The 16S rDNA copy number per gram wet weight in the samples was between $\log_{10}$ 5.50 and 7.19.

The second phase of the study was conducted to observe the effects of selected nitrocompounds and two different feedstuffs on in vitro methane production in chicken

cecal contents and rumen fluid. Initially, one of the three nitrocompounds was added to incubations containing cecal contents from laying hens supplemented with either alfalfa or layer feed. Both feed materials influenced volatile fatty acids (VFA) production and also fostered methane production in the incubations although methane was lower ($P < 0.05$) in incubations with added nitrocompound, particularly nitroethane. Secondly, nitroethane was examined in incubations of bovine or ovine rumen fluid or cecal contents containing either alfalfa or layer feed. Unlike cecal contents, layer feed significantly ($P < 0.05$) supported in vitro methane production in incubations of both rumen fluids. The results show that nitroethane impedes methane production, especially in incubations of chicken cecal contents.

The final phase of this study was carried out to determine the methanogenic establishment in the chicken ceca by the cultural method with the quantitative PCR. The results suggested that methanogens colonized in chicken ceca at a few days after birth. Litter and house flies could be potential sources for methanogenic colonization in broiler chicks.

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CHAPTER I

INTRODUCTION

Methane-producing archaea (known as methanogens) are a distinct group of organisms which are a normal component of the animal gastrointestinal microbial ecosystem. Methanogens residing in the animal gastrointestinal tract belong to the genera *Methanobacterium*, *Methanobrevibacter*, *Methanosphaera*, *Methanomicrobium*, *Methanogenium*, and *Methanosarcina* (Miller and Wolin, 1986; Boopathy, 1996; Jarvis et al., 2000; Miller, 2001a; Miller, 2001b; Miller and Lin, 2002). Most methanogenic research has focused on humans and ruminants whereas studies of methanogens present in monogastic animals such as the chicken remain minimal (Miller, 1995; Lange et al., 2005). For avian species, only one study has reported the isolation of methanogens from chicken, turkey, and goose feces (Miller et al., 1986). Based on bacterial cell wall composition, two methanogenic strains isolated from chicken and turkey belong to the genus *Methanogenium* (König, 1986) while one isolate from goose feces was *Methanobrevibacter woesei* (Miller and Lin, 2002).

The gastrointestinal tract harbors a wide variety of microbial species. Most of these microorganisms are anaerobes with many species able to hydrolyze polysaccharides to short-chain volatile fatty acids, hydrogen, and carbon dioxide as the primary fermentation products (Wolin et al., 1997). Most methanogens obtain energy

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This thesis follows the style of Poultry Science.

by reduction of carbon dioxide to methane by using hydrogen as the electron donor. This process maintains the low partial pressure of hydrogen in the rumen and promotes the production of hydrogen and other products by the non-methanogenic, fermentative microbial community (Wolin et al., 1997). In humans, Belay et al. (1990) found methanogenic activity in patients with bacterial vaginosis. Recently, by using quantitative PCR, it has been suggested that methanogens might indirectly promote periodontal disease (Lepp et al., 2004). However, the effect of methanogenic colonization in the chicken cecum is still unknown. Characterization of the community structure of gastrointestinal microflora is the first important step in studying this ecosystem (Zoetendal et al., 2004a). Unfortunately, the 16S rRNA sequence of methanogens isolated from chicken feces remains unidentified.

Methanogenesis accounts for 2 - 12% of the dietary energy loss in ruminants (Johnson and Johnson, 1995); therefore, there has been considerable interest in limiting methane production and avoiding energy loss. The addition of nitrocompounds showed that they not only reduced methanogenesis but also lowered foodborne pathogen colonization (Anderson et al., 2001; Anderson et al., 2003; Jung et al., 2004a; Jung et al., 2004b).

Generally, when an animal is born, the intestinal tract is sterile and becomes successively colonized by microorganisms acquired from the mother and surrounding environment (Conway, 1997). Microbial diversity in the gastrointestinal tract becomes more complex as the host gets older. Morvan et al. (1994) found that methanogens colonized the lamb rumen as early 30 hours after birth. Previous studies showed that

methanogens colonized animals when they were young and became established after settlement (Miller and Wolin, 1986; Maczulak et al., 1989; Skillman et al., 2004).

The first phase of this study (Chapter III) is focused on identification and quantification of methanogenic archaea in chicken ceca. The 16S rDNA methanogenic gene libraries were constructed from genomic DNA isolated directly from chicken ceca. Using this method showed a more accurate picture of the bacterial composition in the gastrointestinal tract than did culturing (Zoetendal et al., 2004a). Based on *Hae*III digestion of partial 16S rDNA genes, all different riboprints were sequenced and compared with a DNA database. Concentrations of methanogens in the ceca were measured by using the most-probable-number method (MPN) and quantitative PCR approach. Therefore, this experiment reports the presence and the number of methanogenic bacteria in the adult laying chicken ceca.

The second phase of this study (Chapter IV) investigated the effects of nitrocompounds and two different feedstuffs on in vitro methane production by chicken cecal contents and rumen fluid. First, we tested one of three nitrocompounds with one of the two types of feedstuffs in incubations of chicken cecal contents. Volatile fatty acid, hydrogen, and methane were examined after 24-hour incubations. Second, nitroethane was examined in incubations of bovine or ovine rumen fluid or chicken cecal contents containing either alfalfa or layer feed. Methanogenesis was quantified after 3, 5, and 7 hours of incubation.

The third phase of this study (Chapter V) examined the time course of methanogen establishment in the chicken ceca. One hundred and twenty, 1-day old

broilers were divided into twelve groups (10 birds/group). Fecal samples from each group were collected on days 3 to 5, 9, and 12. In addition, flies and litter were collected one time during the experiment. Methanogenic archaea in these samples were enumerated by culture in modified Balch1 medium (Balch et al., 1979; Miller and Wolin, 1982). Methanogenic DNA was also isolated from samples and quantified using quantitative PCR.

CHAPTER II

LITERATURE REVIEW

Intestinal Methanogenic Archaea in Animals

Introduction

Methanogens, a sub-group of archaea, are present in numerous species of animals (Lange et al., 2005). The unique feature of methanogens is their use of methane production as their sole energy generating mechanism. In the rumen, numerous bacteria and fungi provide hydrogen gas generated during the fermentation of cellulose and other carbohydrate substrates. The microbial ecology of methanogens has been studied often in ruminants since methane production in these animals is becoming recognized as a serious environmental issue. To reduce the amount of methane produced from ruminants, the development a new diets or feeding regimes will be likely required. Other effects of methanogen colonization in animals remain unclear. For example, one study found methanogenic activity in patients with bacterial vaginosis (Belay et al., 1990). More recently, by using quantitative PCR, the relative abundance of archaeal small subunit ribosomal RNA genes (SSU rDNA) in the subgingival crevice was related to the severity of periodontal disease (Lepp et al., 2004). These archaea, presumably methanogens, may indirectly promote periodontal disease in some patients by serving as a hydrogen sink, which may support on increase in total microbial activity (Kulik et al., 2001; Lepp et al., 2004).

This review begins with a brief overview of the diversity and abundance of methanogenic bacteria in ruminant and non-ruminant animals; this is followed with a description of the physiology of these archaea. The relationship between environmental factors and the physiology of methanogens is also examined.

The diversity and amount of methanogenic archaea in gastrointestinal tracts

The most predominant species of methanogens in the animals' intestinal tracts are related to the genus *Methanobrevibacter* (Garcia et al., 2000). A few non-*Methanobrevibacter* species, *Methanobacterium, Methanosarcina*, *Methanosphaera*, and *Methanomicrobium*, have also been isolated from animals (Pol and Demeyer, 1988; Boopathy, 1996). Only two strains of *Methanogenium* spp. have been found to originate from the turkey and chicken feces (Miller et al., 1986). Methanogens comprise approximately 0.5 to 3% of the total population of microflora in steers, cows, sheep, pigs, and goats (Lin et al., 1997).

Early studies on methanogens involved the bacteriological isolation and quantification of methanogenic bacteria (Smith and Hungate, 1958; Nottingham and Hungate, 1968; Paynter and Hungate, 1968; Miller et al., 1986; Sorlini et al., 1988; Butine and Leedle, 1989; Brusa et al., 1993; Morvan et al., 1996). Recently, molecular approaches have provided an alternative means ofinvestigating the gastrointestinal ecosystem without bacteriological culture methodology (Lin et al., 1997; Shinzato et al., 1999; Tajima et al., 2001; Whitford et al., 2001; Shin et al., 2004; Wright et al., 2004; Eckburg et al, 2005). These molecular methods have become increasingly popular due to the difficulty of isolating methanogens, long incubation times and strict anaerobic

cultivation methods needed, and incomplete knowledge of the nutritional requirements of many methanogenic bacteria (Garcia et al., 2000). It has generally been believed that methanogens are the only group of archaea living in animals. However, recent studies have demonstrated the presence of some other non-methanogenic archaea in the gastrointestinal tract. *Thermoplasma*-associated sequences have been observed in the bovine rumen (Tajima et al., 2001), pig manure storage pits (Snell-Castro et al., 2005), and the hindgut of the lower termite *Reticulitermes speratus* (Shinzato et al., 1999). Shin et al. (2004) also found non-thermophilic-Crenarchaeota and thermophilic Crenarchaeota sequences in the bovine rumen.

Ruminant animals

Based on culture method, five methanogenic species have been isolated from the rumen: *Methanobacterium formicicum*, *Methanobrevibacter ruminantium*, *Methanosarcina barkeri*, *Methanosarcina mazei*, and *Methanomicrobium mobile*.

In bovine rumen contents *Methanobrevibacter ruminantium* has been considered the predominant methanogen based on the frequency of cultural isolation and the specific molecular techniques used (Miller and Wolin, 1986; Sharp et al., 1998; Whitford et al., 2001; Shin et al., 2004). However, *Methanomicrobium mobile* may also be present at concentrations similar to that of *Methanobrevibacter ruminantium* (Paynter and Hungate, 1968; Jarvis et al., 2000). In mature bovine rumen, *Methanosarcina* spp. or *Methanomicrobium* spp. were not detected in a library of cloned methanogen 16S rRNA genes (Whitford et al., 2001). A study with hybridization probes found that less than 3%

of the archaeal DNA from the mature bovine rumen originated from the order Methanosarcinales (Sharp et al., 1998).

From the culturable and molecular data, *Methanobrevibacter* spp. and *Methanomicrobium* spp. are the most abundant methanogens in the sheep rumen while *Methanobacterium* spp., *Methanosarcina* spp. constitute minor populations (Lin et al., 1997; Yanagita et al., 2000; Skillman et al., 2004). Previous studies have shown that *Methanomicrobium mobile* is the predominant methanogen in the ovine rumen (Lin et al., 1997; Yanagita et al., 2000). By using the fluorescent in situ hybridization (FISH) method, methanogen counts were approximately 3.6% of the total rumen microflora and approximately 54% of the total methanogens were *Methanomicrobium mobile* (Yanagita et al., 2000).

The methanogenic community in the rumen is established very soon after birth. Methanogen densities reach 10^4 to 10^9 organisms per gram in rumen fluid of grazing lambs at 1 and 3 weeks of age, respectively (Skillman et al., 2004). Morvan et al. (1994) also found that methanogens colonize the lamb rumen by 30 hours after birth and reach 10^6 organisms per ml by 15 days of age. These results showed that methanogens colonized the rumen well before the diet contains forage material. A strain of *Ruminococcus flavefaciens*, a hydrogen-producing, cellulolytic bacterium which is known to form syntrophic associations with methanogens (Wolin et al., 1997), was isolated from lamb rumen one day after birth (Skillman et al., 2004). The genus *Methanobrevibacter* become established early in young lambs and seem to be more

stable than the *Methanobacterium* populations which in several instances appeared and then disappeared as the rumen developed (Skillman et al., 2004).

The levels of methanogenic archaea present in rumen contents range between 10^6 to 10^8 organisms ml^{-1} and are shown in Table 2.1. *Methanomicrobium mobile* was present at a level of at least 10^6 organisms ml^{-1} the rumen of cattle grazing on ryegrass/clover pasture (Jarvis et al., 2000) and 10^8 organisms ml^{-1} in cattle fed alfalfa hay (Paynter and Hungate, 1968). By cultural methodology, *Methanobrevibacter ruminantium* was found to be the most abundant methanogen in the bovine rumen and present at approximately 10^8 organisms ml^{-1}in cattle fed alfalfa and grass (Smith and Hungate, 1958).

Non-ruminant animals

In contrast to ruminants, bacterial fermentation in monogastric animals is concentrated on activity in the cecum and colon (Apajalahti, 2005). These parts of the gastrointestinal tract receive dietary compounds that escape host digestion and absorption; therefore, bacteria do not compete with the host when they ferment these remaining substrates. However, in the upper GI tract, competition occurs for all simple sugars and amino acids utilized by the host, which are also available for the bacteria. In poultry, equines, and rodents, the site for intense bacterial fermentation is a well-developed appendix or cecum. In other monogastric animals, such as humans and swine, the cecal appendix is diminished, and the bacterial fermentation mainly occurs in the colon.

Table 2.1 Number of methanogenic bacteria present in rumen, feces, colon contents, ceca from various animals, and references of these studies.

Species	Numbers of methanogens	Reference
Cattle	10^5 to 10^8 per ml rumen samples	Morvan et al., 1996
Cattle	10^6 per gram dry wt feces	Sorlini et al., 1988
Sheep	10^7 to 10^8 per ml rumen samples	Morvan et al., 1996
Sheep	10^8 per ml rumen samples	Yanagita et al., 2000
Buffalo	10^7 per ml rumen samples	Morvan et al., 1996
Deer	10^8 per ml rumen samples	Morvan et al., 1996
Llama	10^6 to 10^8 per ml rumen samples	Morvan et al., 1996
Horse	10^4 to 10^6 per gram wet wt ceca	Morvan et al., 1996
Rabbit	10^4 per gram dry wt feces	Sorlini et al., 1988
Pig	10^8 per gram dry wt feces	Sorlini et al., 1988
Pig	$6 * 10^6$ per gram wet wt ceca	Butine and Leedle, 1989
Pig	10^8 per gram wet wt colon contents	Butine and Leedle, 1989
Rat	10^9 per gram dry wt feces	Maczulak et al., 1989
Human	10^7 per gram dry wt feces	Sorlini et al., 1988
Human	10^3 to 10^{10} per gram dry wt feces	Miller and Wolin, 1982
Chicken	10^6 to 10^8 per gram wet wt ceca	This study

Compared to ruminants, the methanogen diversity in non-ruminants appears to be minimal. In general, methane production by monogastric animals is lower than methane production by ruminants (Jensen, 1996). By using the FISH method, Lin et al., (1997) found that archaea were more abundant in the gastrointestinal tracts of ruminants than in cecal samples of non-ruminant pigs. While only 8 phylotypes were found in a pig manure storage pit (Snell-Castro et al., 2005), Wright et al. (2004) observed 65 phylotypes in the sheep rumen. In our study, we found only 11 phylotypes in chicken ceca and one phylotype represented more than 96% of the total archaeal sequences.

The predominant methanogen in non-ruminants is *Methanobrevibacter* spp. which is also found in ruminants. Eckburg et al. (2005) found that all of the 1524 archaeal sequences examined from human intestinal tracts belonged to *Methanobrevibacter smithii*. Four strains belonging to the genus *Methanobrevibacter* have been isolated from rat feces (Maczulak et al., 1989). In the cockroach hindgut, Gijzen et al. (1991) observed *Methanobrevibacter* spp. endosymbiosis with the ciliate *Nycotherus ovalis*. In the hindgut of the lower termite *Reticulitermes speratus*, more than 93% of archaea has been reported to be *Methanobrevibacter* spp. (Shinzato et al., 1999).

In humans, the genetic diversity of oral methanogens is quite low and only three phylotypes have been reported (Kulik et al., 2001). The major group of oral methanogens is *Methanobrevibacter oralis* while *Methanobrevibacter smithii* is found mainly in the colon (Miller et al., 1982; Kulik et al., 2001).

Zhu and Joerger (2003) detected microorganisms in chicken ceca of 2-day to 6-week-old broilers by using FISH and found that hybridization with the archaea probe were obtained even from samples of very young birds. Methanogens represented 0.7 to 3.3 % of the total microflora. Two methanogenic strains were isolated from turkey and chicken feces (Miller et al., 1986). Based on cell wall composition, these two strains isolated from chicken and turkey feces belong to the genus *Methanogenium* (König, 1986).

A few non-*Methanobrevibacter* spp. have been isolated or found to be present, based on detection of the 16S rDNA genes, from these animals. *Methanosarcina* sp. has also been isolated from swine manure (Boopathy, 1996). Less abundant methanogenic bacterium found in the human colon is *Methanosphaera stadtmaniae* (Miller et al., 1982).

The levels of methanogens harboring in the colon or cecum are similar to those in rumen fluid (Table 2.1). The range of methanogens in ceca has been shown to be 10^4 to 10^8 organisms per gram wet weight ceca (Table 2.1).

Physiology of methanogens

Some physiological characteristics of methanogenic archaea residing in animal gastrointestinal tracts are shown in Table 2.2. The optimal growth temperature and pH of these methanogens are similar (Table 2.2). The optimal temperature range of most methanogens, except *Methanobrevibacter curvatus*, *Methanobrevibacter filiformis*, and *Methanosphaera stadtmanae*, is 35 to 41 ^{o}C and the optimal pH of all methanogens is 7.0 (Table 2.2). Growth of methanogens isolated from the cow, rat, and sheep is

inhibited by bile salts; however, most methanogenic archaea isolated from non-ruminants tolerate the same bile salts (Table 2.2).

Most methanogens, except the genus *Methanosarcina* and *Methanosphaera*, residing in the gastrointestinal tract obtain energy by reducting CO_2 to CH_4 using H_2 and sometimes formate as the electron donor. The genus *Methanosphaera* requires methanol and hydrogen for growth (Miller, 2001a). The genus *Methanosarcina* obtain energy via formation of methane from acetate, methanol, monomethylamine, dimethylamine, trimethylamine, H_2/CO_2 and CO (Boone and Mah, 2001).

Some methanogens have extraordinary characteristics from others. For example, most methanogenic strains isolated from bovine (*Methanobrevibacter ruminantium*; *Methanobacterium formicicum*; *Methanosarcina barkeri*) or ovine (*Methanobrevibacter wolinii*) species require either coenzyme M, a heat-stable factor in rumen fluid or other growth factors present in rumen fluid (Table2) (Taylor et al., 1974). In addition, methanogens originating from geese (*Methanobrevibacter woesei* GS), the horse (*Methanobrevibacter gottschalkii* HO), and pigs (*Methanobrevibacter gottschalkii* PG) grow in a medium with salt concentrations similar to that of sea water (Blach medium 3) (Miller et al., 1986).

Table 2.2 Some phenotypic traits of the species isolated from animal intestinal tracts.

Trait	Opt. Temp. for growth (^oC)	Opt. pH	Growth with bile	Formate used for growth	Medium additions for good growth
1	38	7.2	-	+	AcH, AA, CoM, NH4, 2-MB
2	30	7.2	NK	-	NutB, RF
3	37	7.7	NK	+	AA, RF, YE
4	30	7.2	NK	-	DTT, YE
5	37	6.9-7.4	+	-	FaecX, VFA
6	38	6.9-7.4	+	+	AcH, Bvits
7	37	7	+	-	AcH, Trp+YE
8	37	7	-	-	AcH, Trp+YE
9	37	7	+	+	AcH, Trp+YE
10	37	7	-	-	AcH, CoM+VFA
11	35-41	7	-	+	NK
12	38	6.5-7	NK	+	RF, YE
13	38	6.5-7	NK	+	NK
14	40	6.5	NK	-	RF
15	37	6.8	NK	-	AcH
16	30-40	6.5-6.9	+	-	Biotin, AcH
17	35-40	6.8	NK	-	AcH

Taxa are indicated as: 1. *Methanobrevibacter ruminantium* M1 isolated from bovine rumen; 2. *Methanobrevibacter curvatus*RFM-2 isolated from hindgut content of termite *Reticulitermes flavipes* (Kollar) Rhinotermitidae); 3. *Methanobrevibacter curticularis* isolated from hindgut content of termite *Reticulitermes flavipes* (Kollar) (Rhinotermitidae); 4. *Methanobrevibacter filiformis* RFM-3 isolated from hindgut content of termite *Reticulitermes flavipes* (Kollar) (Rhinotermitidae); 5. *Methanobrevibacter oralis* ZR isolated from human subgingival plaque; 6. *Methanobrevibacter smithii* PS isolated from human feces; 7. *Methanobrevibacter gottschalkii* HO isolated from horse feces and *M. gottschalkii* PG isolated from pig feces; 8. *Methanobrevibacter thaueri* CW isolated from cattle feces; 9. *Methanobrevibacter woesei* GS isolated from goose feces; 10. *Methanobrevibacter wolinii* isolated from sheep feces; 11. *Methanobrevibacter* spp. isolated from rat feces; 12. *Methanobacterium formicicum* BRM9 isolated from grazing cattle; 13. *Methanomicrobium mobile* BRM16 isolated from grazing cattle; 14. *Methanosarcina barkeri* CM1 isolated from grazing cattle; 15. *Methanosarcina* sp. isolated from swine manure; 16. *Methanosphaera stadmanae* isolated from human feces ; 17.*Methanosphaera cuniculi* isolated from the contents of rabbit rectum; Abbreviations: NK, not known; AA, amino acid mixture; AcH, acetate; Bvits, B vitamin mixture; CoM, coenzyme M; DTT, dithiothreitol (cysteine and H_2S inhibition growth); FaecX, fecal extract; 2-MB, 2-methylbutyric acid; NutB, nutrient broth; RF, rumen fluid; Trp, Trypticase; v, strain-dependent; VFA, volatile fatty acid mixture; YE, yeast extract. Most strains use $H_2 + CO_2$ for growth; no substrate other than formate, except *Methanosarcina barkeri* CM1 and *Methanosarcina* sp. can use acetate, methanol as substrates but not formate, and *Methanosphaera* spp. are able to utilize only methanol and H_2.

Factors affecting methanogens in animal intestinal tracts

Environment and genetic attributes can affect the distribution of methanogenic archaea in the gut ecosystem. Tajima et al. (2001) mentioned that the microorganisms in rumen are highly responsive to changes in diet, age, antibiotic use, and the health of the host animal, and vary according to geographical location, season, and feeding regimen.

Diet

Recent molecular approaches have revealed that the composition of methanogens in animal intestinal tracts can vary with different diets (Wright et al., 2004). By observing 16S rRNA sequences, Wright et al. (2004) found that pasture-grazed sheep had greater methanogen diversity than sheep fed either an oaten hay or lucerne hay.

High fiber diets have been reported to increase the amount of methanogens in the rumen. Pol and Demeyer (1988) demonstrated that sheep fed a hay-concentrate diet increased the rate of methanogenesis from methanol, which is the substrate for *Methanosarcina* spp.

Cellulolytic organisms may play a role in the development of a methanogenic community in the gut by providing substrates for methanogens. Minato et al. (1992) hypothesized that cellulolytic bacteria supply hydrogen gas to methanogens. An increase in the fiber content of animal diets has been shown to result in an increase in methanogenesis in the cockroach gut (Kane et al., 1991). Robert et al. (2003) found that the presence of certain fibrolytic species (cellulolytic isolates related to *Enterococcus faecalis*, *Ruminococcus* spp) was related to the presence of methanogenic archaea. A correlation was observed between the numbers of methanogens and those of cellulolytic

microorganisms in fresh rumen samples of sheep, cattle, deer, llama, and cecal contents from horses (Morvan et al., 1996). Numbers of the ciliate *Nyctotherus ovalis*, which harbor *Methanobrevibacter* sp., and methane production increased when the cockroach was fed a high fiber diet (Gijzen et al., 1991). On the other hand, one study found that the number of methanogens associated with ciliates in sheep rumen, not the number of ciliates, is modulated by feeding. Shrimpton (1966) found that a high-fiber diet increased methane production in the chicken by nine-fold in comparison to birds given low-fiber feed. In contrast to other studies, Maczulak et al. (1993) did not observe significant differences in the numbers of methanogens in the fecal samples from rats fed a high-fiber vs. fiber-free diets.

Another reason that high-fiber diets increase either methanogenic diversity or the level of organisms is that methanol may be available in the gastrointestinal tract. *Methanosarcina* spp. in bovine and sheep rumens can utilize methanol as a methanogenic substrate. In addition, *Methanosphaera* spp., as well as *Methanosphaera stadtmanae* from the human colon and *Methanosphaera cuniculi* isolated from the contents of rabbit rectum, can utilize only methanol as the substrate for growth (Miller, 2001a). Methanol is produced by the hydrolysis of methyl esters from pectins, which are abundant polysaccharides in plants. Based on the genomic sequence of *Methanosphaera stadtmanae*, this archaeon lacks the genes for biosynthesis of molybdopterin, indicating that *Methanosphaera stadtmanae* cannot synthesize active formylmethanofuran dehydrogenase (Fricke et al., 2006).

Bile acids

Bile, produced and secreted by the liver, can act as a detergent to interact with bacterial membrane lipids. Therefore, exposure to bile is a serious challenge for intestinal microorganisms (Begley et al., 2005). Most bile acids, except those in the pig, are cholic acid or chenodeoxycholic acid as shown in Table 2.3. In mammals, primary bile acids include cholic acid and small amounts of chenodeoxycholic acid while chenodeoxycholic acid is the main bile acid in fowl (Singleton and Sainsbury, 2001). Elkin et al. (1990) reported that chenodeoxycholyltaurine and cholyltaurine are the primary bile salts in chicken and turkey (Table 2.3). In humans and chickens, more than 90% of bile acids are resorbed in the small intestine (Begley et al., 2005; Denbow, 2000). The fecal loss of bile acids in human is in the range of 0.3-0.6 g per day (Begley et al., 2005).

Most *Methanobrevibacter* spp. isolated from rumen do not have the ability to tolerate bile (Miller and Lin, 2002). Therefore, these acids may inhibit methanogens. Two methanogens isolated from horse feces and goose feces, *Methanobrevibacter gottschalkii* HO and *Methanobrevibacter woesei* GS, can grow in the presence of bile (Miller and Lin, 2002). The concentration and types of bile acids influence bacterial growth. Florin et al., (1995) found that bile acid concentrations greater than 0.05 % can inhibit human methanogenesis in vitro. Grill et al.(2000) suggested that glycine conjugated bile salts exerted a higher level of toxicity than taurine conjugated salts, and bile containing trihydroxy conjugated bile salts was less inhibitory than that containing bihydroxy conjugated bile salts. In addition, *Methanobrevibacter* spp. that can tolerate

Table2.3 The primary bile acids and conjugated molecules present in animals.

Animal	Primary free bile acid	Main conjugated molecule	References
Human	Cholic acid [a]	Glycine	Florin et al. (1995)
Chicken/Turkey	Chenodeoxycholic acid [b] Cholic acid [a]	Taurine	Elkin et al. (1990)
Cow	Cholic acid [a] (83.5%) [c]	Taurine (31.0%) [d]	Washizu et al. (1991)
Horse	Chenodeoxycholic acid [b] (68.4%)	Taurine (85.3%)	Washizu et al. (1991)
Pig	Chenodeoxycholic acid [b] (26.2) Hyodeoxycholic acid [b] (20.6%) Hyocholic acid [a] (33.8%)	Glycine (85%)	Legrand-Defretin et al. (1991)

[a] Trihydroxy bile acid

[b] Dihydroxy bile acid

[c] The percentage of the total bile acid

[d] The percentage of either glycine or taurine to the conjugated molecule

bile salts usually can grow in seawater medium (Miller and Lin, 2002). Membrane architecture and composition, especially fatty acid composition, play an important role in bile and osmotic resistance (Begley et al., 2005). Flahaut et al. (1996) showed that when *Enterococcus faecalis* ATCC19433 is subjected to an osmotic stress, it shows cross-protection against exposure to bile acids.

pH

Rumen methanogenesis is also dependent on pH with no methane production at pH values less than 6.0 (Russell, 1998). In general, the pH in rumen contents is between 5.0 to 7.0 (Cotta and Hespell, 1986). Chicken cecal pH values are in the ranges of 5.5 to 7.0 (Shrimpton, 1966; Denbow, 2000).

Host genotype

Host specificity has been demonstrated to affect the colonization ability of certain methanogens since one bacterial strain from one animal may fail to colonize another animal. In spite of same breed, some individual rats in one study resist methanogenic colonization (Florin et al., 2000). In humans, *Methanobrevibacter smithii* was found to range from a few to 10^{10} organisms per gram fecal dry weight (Miller and Wolin, 1986). Tannock et al. (1982) examined various strains of lactobacilli isolated from many kinds of animals and noted that they exhibit host specificity to squamous epithelia in the gastrointestinal tracts. However, a study of monozygotic and dizygotic twins did not find that genetics played an important role in methanogen colonization in human colon (Florin et al., 2000).

Gender

In humans, males usually carry less methanogens than females. Florin et al. (2000) mentioned that genetic traits of human males or behavioral effects, such as passage rate and difference in the enterohepatic circulation of bile acids, may influence mechanisms that control methanogenesis.

Some compounds that are present in gastrointestinal tracts are necessary for growth of methanogens. For example, *Methanomicrobium mobile* requires a heat-stable factor that is available in bovine rumen fluid (Tanner et al., 1988). Taylor et al. (1974) also found that coenzyme M, a growth factor presenting in rumen fluid, is essential for growth of *Methanobrevibacter ruminantium*.

Hackstein et al. (1999) proposed that the presence of symbiotic methanogenic archaea in the gastrointestinal tracts is a necessary requirement for the evolution of foregut- and hindgut-fermenting structures, irrespective of the nutritional preferences of the hosts. In addition, methanogenesis seems to obey Dollo's law which suggest that if it is lost in a particular phylogenetic branch, it will not reappear in any species of this branch-regardless of whether these species are herbivores or not (Hackstein et al., 1999).

Molecular Techniques in Chicken Gastrointestinal Tract Studies

Introduction

Bacterial populations as high as 10^{11} cells per gram of chicken cecum are composed of at least 38 different types of anaerobic bacteria (Barnes et al., 1972; Barnes and Impey, 1972; Barnes, 1979). Bacterial cells outnumber animal cells by a factor of 10 and have a profound influence on immunological, nutritional, and physiological processes in the host (Savage, 1977). Although it is relatively easy to obtain a total viable count, enumerating individual bacterial species by culturing methods is laborious and time consuming. Drawbacks associated with culture-based techniques are exacerbated in anaerobic habitats. Selective media are not available for most of the strict anaerobes and several hundred isolates from each specimen should be identified for reliable statistics. Only those organisms whose niche can be mimicked relatively easily in the laboratory have been isolated and identified. Thus, despite extensive culture studies performed on the gastrointestinal ecosystem using conventional techniques, it must be assumed that, as for other natural ecosystems, the fraction of well-described species is limited. Although traditional cultivation methods are generally tedious, expensive and probably more open to misinterpretation with intestinal samples, they remain the gold standard for identification. Indeed, recent estimates of culturability range from 10 to 50% (Zoetendal et al., 2004b). The reasons for this cultivation anomaly include unknown growth requirements of the bacteria, the stress imposed by the cultivation procedures, the necessity of strictly anoxic conditions, and difficulties with stimulating the interactions of bacteria with other microbes and host cells.

The study of gastrointestinal microbial ecology involves investigation of the organisms present (abundance and diversity), their activity (usually determined in vitro, but ideally *in vivo* activity should be measured), and their relationship with each other and the host animals (synergistic and competitive interactions). The circumvention of cultivation limitations requires culture-independent methods. A dramatic increase in the application of approaches based on the sequence diversity of the 16S rRNA gene have been made during the past decade to explore the diversity of bacterial communities in a variety of ecosystems, including chicken gastrointestinal tract (Gong et al., 2002; Zhu et al., 2002; Lu et al., 2003b). Sequence comparisons of nucleic acids isolated from complex microbial ecosystems can be used to provide molecular characteristics, while at the same time providing a classification system, which predicts natural evolutionary relationships (Woese et al., 1990). As such, the field of molecular microbial ecology is defined as the application of molecular technology, typically based on comparative nucleic acid sequence information, to identify specific microorganisms in a particular environment, to assign functional roles to these organisms, and to assess their significance or contribution to environment processes.

Molecular characterization by rRNA analysis

The primary structure of all ribosomal sequences consists of alternating conserved and variable domains which makes them very suitable for the detection and identification of microbial species and ideal targets for species and ideal targets for specific DNA probes. By aligning the appropriate 16S rRNA sequences, genus-specific and species-specific sequences can be identified. As a result, molecular methods address

detection and classification at the same time. Comparative sequencing of the 16S rRNA molecule has become by far the most commonly used measure of environmental diversity. The 16S rRNA, consisting of about 1500 nucleotides, provides a large mount of information for phylogenetic inference and is a reasonable size for sequencing. A comprehensive set, currently greater than 79000 small subunit rRNA entries, is available in generally accessible database such as GenBank and EMBL (Vaughan et al., 2000; Zoetendal et al., 2004a).

Sequencing of 16S rDNA clone libraries

The construction of small subunit (SSU) rRNA libraries is required to inventory bacteria and archaea present in a given environment. Sequencing of SSU rRNA genes has become a standard procedure for the identification of isolates and it is now impossible to adequately describe microbial communities without SSU rRNA sequence data.

Traditionally, bacteria have been classified on the basis of phenotypic properties and only after the availability of nucleic acid based technology have SSU rDNA sequences been recognized as a standard phylogenetic classification tool in the description of bacterial strains (Stackebrandt and Goebel, 1994). While large number of cloned SSU rDNA sequences from the gastrointestinal tract of a variety of animals have been deposited in DNA databases, few examples exist of direct comparisons between SSU rDNA sequences retrieved from cloned amplicons and colony forming units from culturable gastrointestinal tract bacteria (Zoetendal et al., 2004a).

DGGE/TGGE

Denaturing or temperature gradient gel electrophoresis (DGGE/TGGE) methods have been successfully applied to the analysis of chicken intestinal tract samples (van der Wielen et al., 2002; Zhu et al., 2002). DGGE and TGGE are gel-electrophoretic separation procedures for double stranded DNA's equal size but different base-pair composition or sequence (Muyzer and Smalla, 1998). In principle, the methods are sensitive enough to separate DNA's on the basis of a single point mutation (Sheffield et al., 1989). Both techniques are gaining increased popularity in microbial ecology for analyzing the diversity of total bacterial communities.

Briefly, the 16S rRNA genes are amplified using the appropriate primer pair, one of which has a G+C clamp attached to the 5' end that prevents the two DNA strands from completely dissociating even under strong denaturing conditions. During electrophoresis through a polyacrylamide gel containing denaturants, migration of the molecule is essentially arrested once a domain in a PCR product reaches its melting temperature. Following staining of the DNA, a banding pattern emerges that represents the diversity of the rRNA gene sequences present in the sample. The intensity of an individual band is a semi-quantitative measure for the relative abundance of this sequence in the population. DGGE and TGGE of 16S rRNA amplicons are exceptional tools to study the bacterial species composition of unknown samples. Since individual bands can be excised and sequenced, the identity of the bacteria present in the sample can be determined without cultivation. DGGE has being used to monitor the bacterial succession in the chicken gastrointestinal tracts (van der Wielen et al., 2002). The

bacterial diversity remains very low during the first four days after birth and the dominant bacterial community becomes more complex when chickens age (van der Wielen et al., 2002).

T-RFLP

Terminal RFLP of PCR-amplifed DNAs is a refined fingerprinting technique based on RFLP. The general steps include PCR amplification of a conserved target of a conserved target sequence (most commonly a region of the SSU rRNA gene) followed by restriction enzyme digestion and gel fractionation of the resulting fragments. However, one of the two PCR primers is fluorescently labeled at the 5' end. This results in PCR amplification products that are tagged with a fluorescent dye at only one terminus. Following restriction enzyme digestion (usually with a tetrameric restriction enzyme), the restricted products are resolved using an automated DNA sequencer or a capillary electrophoresis system equipped with a laser-induced fluorescence detector. Only the fluorescently tagged terminal fragments are detected and quantified. T-RFLP analysis has been used to reveal some differences between bacterial populations present in the mucosa and lumen of the chicken ceca (Gong et al., 2002).

Fluorescence in situ hybridization

In contrast to most molecular methods referred to in the above sections, whole cell fluorescence in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes is quantitative on a cell by cell basis. Ultimately, enumeration of species in the intestinal tract is best addressed by this approach.

The first report report on enumeration of bacteria in the chicken intestinal tract by FISH methods indicated that an enteric group and *Clostridium leptum* subgroup were the dominant microbiota when the broilers were 2 days and 6 weeks old (Zhu and Joerger, 2003).

Quantitative (RT) PCR of 16S rRNA

Although PCR is the mot sensitive technique to detect sequences that are present in very low concentrations in the environment, many factors can influence the amplification reaction, and fingerprinting techniques alone do not provide quantitative data (Wintzingrode et al., 1997). However, it is possible to determine quantitatively SSU rDNA or rRNA using PCR. Quantitative (RT) PCR is one approach to quantify the target. Although RT-PCR still needs to be proven suitable for analyzing complex bacterial communities, this application looks promising because bacterial targets in very low concentration can be quantified, which are difficult using other approaches. Using RT-PCR, *Campylobacter jejuni* (Rudi et al., 2004) and *Clostridium perfringens* (Wise and Siragusa, 2005) were quantified in chicken gastrointestinal tract samples.

Non-16S rRNA-based profiling

Most culture-independent techniques used to describe bacterial communities have focused on the sequence diversity of 16S rRNA. However, determining G+C content has been used successfully (Apajalahti et al., 1998; Apajalahti et al., 2001; Holben et al., 2004). The advantage of these types of profiling is that they are direct without any amplification step. However, the identification of bacteria causing the shifts in the profiles is difficult, because the data lacks phylogenetic information. Therefore,

16S rRNA approaches are needed for the validation of these alternative methods. In addition, this method has not been widely applied at the moment, which may restrict researchers to use them.

CHAPTER III

IDENTIFICATION OF METHANOGENIC ARCHAEA IN CHICKEN CECA BASED ON 16S rDNA AND QUANTIFICATION BY PERFORMING MPN, REAL-TIME PCR

Introduction

Methanogens, a sub-group of the archaebacteria, have been isolated from various animals (Miller and Wolin, 1986; Miller et al., 1986). In contrast to methanogens in ruminants and human, studies of methanogenic bacteria present in monogastric animals are still scarce (Miller, 1995; Jensen, 1996; Lange et al., 2005;). For avian animals, only one report exists regarding the isolation of methanogens from chicken, goose, and turkey feces (Miller et al., 1986). In addition, a few notes have reported methanogens to harbor in chicken intestinal tracts (Shrimpton, 1966; Zhu and Joerger, 2003). Unfortunately, analysis of the 16S rRNA genes of methanogen isolated originating from turkey and chicken feces was not carried out. Based on cell wall composition, however, these two strains appeared to belong to the genus *Methanogenium* (König, 1986).

Characterizing the community structure of gastrointestinal microorganisms is the first important step in studying this ecosystem (Zoetendal et al., 2004a). It has been reported that only 10 to 50% of the gastrointestinal tract bacteria are able to be cultured (Zoetendal et al., 2004b). Molecular techniques provide improved ways to study mixed populations of gastrointestinal microorganisms (Vaughan et al., 2000; Zoetendal et al., 2004a, Zoetendal et al., 2004b). Compared to the culture-dependent approaches,

molecular methods revealed greater diversity of bacterial populations from chicken ceca (Gong et al., 2002). Several molecular methods, 16S rRNA libraries (Lu et al., 2003a), Fluorescent In Situ Hybridization (FISH) (Zhu and Joerger, 2003), terminal restriction fragment length polymorphism (T-RFLP) (Gong et al., 2002), temporal temperature gradient gel electrophoresis (TTGE) (Zhu et al., 2002), denaturing gradient gel electrophoresis (DGGE) (Knarreborg et al., 2002; van der Wielen et al., 2002; Hume et al., 2003), percent G + C content (Apajalahti et al., 1998; Apajalahti et al., 2001; Holben et al., 2004), and specific 16S rDNA primers (Amit-Romach et al., 2004), have been used to examine the intestinal microbiota of the chicken.

The cultural methods have been used to count methanogens in human and animals (Smith and Hungate; 1958; Nottingham and Hungate, 1968; Miller and Wolin, 1982; Weaver et al., 1986; Sorlini et al., 1988; Butine and Leedle, 1989; Brusa et al., 1993; Morvan et al., 1996). Molecular methods, especially quantitative real-time PCR (Q-PCR), have also been used to quantify specific bacterial populations in the chicken gastrointestinal tract (Rudi et al., 2004; Wise and Siragusa, 2005; Dumonceaux et al., 2006). The use of Q-PCR for counting methanogens has already been used to examine anaerobic treatment systems (Shigematsu et al., 2003; Tang et al., 2004; Yu et al., 2005a), sediments (Chan et al., 2005), and rice fields (Kemnitz etal., 2005).

To the best our knowledge, this is the first study to examine, quantify methanogens in chicken using 16S rDNA genes, and count using most-probable-number (MPN) enumeration. The ribosomal gene sequences were amplified by PCR with specific methanogenic archaea primers, primers Met86F and Met1340R (Wright and

Pimm, 2003) followed by riboprinting to determine the relative proportions of different methanogen groups. In addition, methanogens in these cecal contents were quantified by a MPN method based on Balch 1 medium supplemented with rumen fluid and additional NH_4Cl (Balch et al., 1979; Miller and Wolin, 1982), and by real-time PCR by using primers MBT (Yu et al., 2005b).

Materials and Methods

Source of cecal samples

Ceca were obtained from female Leghorn chickens 56 to 72 weeks of age and maintained on a layer ration. The composition of the Texas A&M University (TAMU, College Station, TX) layer ration was (%): corn 56.72; soy bean meal 31.63; vegetable oil 7.68; monocalcium phosphate 1.69; calcium carbonate 1.56; methionine (98 %) 0.17; vitamin premix 0.25; NaCl 0.25; and a trace mineral premix 0.05. A preliminary trial was performed and consisted of the ceca from one chick, and was as designed as sample 1. Twenty-four chickens were divided into four groups and were sacrificed to take cecal contents. Ceca from each individual of 6 birds were pooled and these 4 groups designed as samples 2-5.

DNA extraction

Bacterial genomic DNA was isolated by the method of Wright et al. (1997) with some modifications. Cecal contents were suspended in Tris-EDTA (TE) buffer and treated with proteinase K for 1 h at 37 ^{o}C, followed by five cycles of freezing at –80 ^{o}C for 1 h and heating in a water bath at 65 ^{o}C for 30 min. The lysate was treated with

cetryltrimethylammonium bromide/sodium chloride (CTAB/NaCl). The CTAB was extracted with an equal volume of chloroform-isoamyl alcohol (24:1), mixed, and centrifuged at 7,000 xg for 5 min. The DNA solution was transferred to a new microcentrifuge tube with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), mixed and centrifuge at 7,000 g for 5 min and isopropanol precipitation. The extracted DNA was further purified with a Dneasy® Tissue kit (Quigen, Valencia, CA). The DNA solution was stored at -20°C.

Riboprinting and sequencing

Methanogenic 16S rDNA sequences from five chicken cecal samples were amplified using methanogen-specific forward and reverse primers Met86F and Met1340R (Wright and Pimm, 2003). The sequences of primers Met86F and Met1340R are shown in Table 3.1. The PCR conditions followed the protocol of Wright et al. (2004). Cloning of the 1.2-kb PCR product was performed by using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol, and the resulting ligation product was used to transform *Escherichia coli* One Shot TOP10 competent cells (Invitrogen, Carlsbad, CA). A total of 420 clones were cultured overnight in Luria Broth with ampicillin (Sigma, St. Louis, MO) for extracting plasmids with a QIAprep spin miniprep Kit (Quigen, Valencia, CA). The 16S rDNA-inserted plasmids were reamplified by PCR using the parameters and primers described above. The PCR products were purified with a QIAquick PCR purification kit (Quigen, Valencia, CA) and digested overnight at 37 ^{o}C with *Alu*I or *Hae*III (Promega, Madison, WI). Restriction fragment were separated on agarose gels (4%, w/v) and stained with

Table 3.1 Names, sequences, application, and references of the primers used in this study.

Name	Sequence 5’ – 3’	Application	Reference
Met86F	GCT CAG TAA CAC GTG G	Cloning	Wright et al (2003)
Met1340R	CGG TGT GTG CAA GGA G	Cloning	Wright et al. (2003)
MBT857F	CGW[a] AGG GAA GCT GTT AAG T	Real-time PCR	Yu et al. (2005b)
MBT929F	AGC ACC ACA ACG CGT GGA	TaqMan	Yu et al. (2005b)
MBT1196R	TAC CGT CGT CCA CTC CTT	Real-time PCR	Yu et al. (2005b)

[a] W = A/T

ethidium bromide as shown in Fig. 3.1. Restriction fragment length polymorphisms were grouped according to their riboprint patterns and were compared to a riboprint database for identification (Wright and Pimm, 2003). Clones representing all *Hae*III restriction fragment length polymorphism patterns were bidirectionally sequenced with ABI Prism® BigDye® Primer Cycle Sequencing kits (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

The partial nucleotide sequences for the 16S rDNA cloned from chicken cecal populations were used to query Genbank. To place these chicken cecal sequences within a phylogeny of representative methanogenic archea, the following sequences from Genbank were included in the analysis: *Methanobrevibacter woesei* (U55237), *Methanobrevibacter* sp. (U55241), *Methanobrevibacter thaueri* (U55236), *Methanobacterium bryantii* (AF028688), *Methanosphaera stadtmanae* (M59139), *Methanotermobacter thermautotrophicus* (X68713), *Methanopyrus kandleri* (M59932), *Methanothermus fervidus* (M59145), uncultured archaeal symbiont (AB062309) and *Methanococcus voltae* (U38488). The alignment was generated with ClustalW (Thompson, 1994). The neighbor joining tree was constructed in Phylogenetic Analysis Using Parsimony and Other Methods (PAUP* 4.0b) (Swofford, 2002) employing a distance matrix calculated with the Jukes-Cantor correction model. The *Methanococcus voltae* (U38488) sequence served as the outgroup. The tree was subjected to 1000 replicates of bootstrapping, the percentage of replicates supporting a given node are indicated on Fig. 3.2.

Culturing methanogen and most-probable-number (MPN)

Methanobrevibacter woesei GS was cultured in a serum tube containing modified Balch 1 medium supplied with cephalothin and clindamycin and maintained as described below (Balch et al., 1979; Miller and Wolin, 1982).

Cecal samples 2-5 were transferred and mixed together in an anaerobic glove box maintained in an atmosphere of 95% N_2/ 5% H_2. One gram was added into a serum tube containing 9 ml of modified Balch 1 medium (Balch et al., 1979; Miller and Wolin, 1982). Dilutions from 10^{-5} to 10^{-12} were inoculated into five serum tubes at each dilution and the tubes were removed from the glove box after being sealed with stoppers and aluminum caps. Each tube was flushed with 80% H_2/ 20% CO_2 under 200 kPa. The bottles were incubated standing at 37 ^{o}C and mixed one time per day by hand. After 20 days, methane was determined in the headspace gas by GC (SRI, model 8610C, Torrance, CA). Tubes with methane concentrations greater than 100 ppm (µg/ml) were counted positive for the determination of methanogens by MPN. The freeware MPN calculator (VB6 version; Michael Curiale [members.ync.net/mcuriale/mpn/index.html]) was used to calculate MPN numbers. In this study, fresh bovine rumen fluid was used as a positive control and collected from a cannulated Holstein-Friesian cow maintained on a 50 % alfalfa hay, 50 % flaked corn diet. The fluid was diluted from 10^{-3} to 10^{-10} and incubated as described above. The cecal samples were stored at –80 ^{o}C until DNA extraction as described above.

Quantitative PCR assays

Calibration standards for the quantitative PCR assays were developed with a 10-fold dilution series of plasmid containing sequence CH101. Plasmid copy number was calculated from plasmid molecular weight, and plasmid concentration was measured with Picogreen (Molecular Probes, Eugene, OR) with a Spectrafluor Plus (Research Triangle Park, NC). The quantitative PCR reactions by using primers MBT857F, MBT929F, MBT1196R including the PCR conditions were followed as described in Yu et al. (2005b). The sequences of these primers are available in Table 3.1.

Results and Discussion

Methanogens have been described traditionally as strictly anaerobes found in many environmental habitats including the animal gastrointestinal tract. Most methanogen research conducted in humans and rumens has been studied, and many methanogen strains have been described (Miller 2001b; Miller and Lin, 2002). However, knowledge regarding methanogens, particularly in the chicken cecum, is extremely limited due to the difficulty of isolation, long incubation periods, strictly anaerobic cultivation, and incomplete nutritional knowledge (Garcia et al., 2000). Molecular techniques provide an alternative route to study this ecosystem without culturing the microorganisms (Vaughan et al., 2000). In this study, we identified and quantified methanogenic archaea in chicken ceca by using both culture methods and molecular approaches.

Riboprinting and phylogenetic analysis

We directly amplified methanogenic 16S rDNA sequences from 5 cecal samples using methanogen-specific primers Met86F and Met1340R (Wright and Pimm, 2003). Primers Met86F and Met1340R were used in this experiment since these primers can amplify 26 diverse strains of methanogens (Wright and Pimm, 2003). Even though this approach is more accurate than the cultural methods, it still has a bias in amplification and formation of chimeric molecules (Wintzingerode et al., 1997). These primers may not recover sequences from some archaea existing in gastrointestinal tracts. *Thermoplasma*-associated sequences have been observed in bovine rumen (Tajima et al., 2001), pig manure storage pit (Snell-Castro et al., 2005), and hindgut of the lower termite *Reticulitermes speratus* (Shinzato et al., 1999). Using the archaea-specific primers, Zhu et al. (2002) were unsuccessful in detecting methanogens in chicken ceca. This failure might be due to low specificity of the archaeal primers (Wright and Pimm, 2003).

In our study, eleven banding patterns, designed as phylotype CH101, CH103, CH138, CH194, CH1117, CH344, CH389, CH3126, CH5164, CH1254, CH1270, were observed from the total 420 clones digested by *Hae*III and are shown in Fig. 3.1 and Table 3.2. Our observation shows that the riboprint pattern of *Methanobrevibacter woesei* GS was similar to that of sequence CH101 (Fig. 3.1). The sequences ranged in size from 1256 to 1268 base pairs (bp). From the total 420 clones, 406 clones belonged to sequence CH101 while other banding patterns consisted of only two or fewer clones (Table 3.2). Despite finding eleven different sequences in chicken ceca, sequence

identity data shows that all of the sequences, except sequence CH1270, were 98.97 to 99.45 % similar to the 16S rDNA sequence of *Methanobrevibacer woesei* GS (accession number U55237), a methanogen isolated from goose feces. Sequence CH1270 had a 97.62 % sequence identity to an uncultured archaeon clone ConP1-11F (accession number AY911630.1). However, phylotype CH1270 was not identifiable to the species level. Our finding that only one out of the eleven phylotypes was dominant in this study is consistent with the finding of Snell-Castro et al. (2005), who also found only one of eight phylotypes in a pig manure storage pit that contained 61% of the total archaeal sequences.

Based on riboprinting patterns, our results show that methanogen diversity in chicken ceca is minimal. In our preliminary study, PCR products of 133 clones obtained from sample 1 were digested with *Hae*III or *Alu*I. However, we did not observe differences among PCR products digested by *Alu*I (data not shown). The percentage of sequence CH101 in sample 1 from one chick, was similar to that of the other four samples (Table 3.3). This suggests that the diversity of methanogenic archaea is not different among individual birds. From 5 cecal samples, data generated by 16S rDNA clone libraries shows that phylotype CH101 was between 92.86 to 100 % of the total clones whereas less than 1 % of the other phylotypes was found.

Clearly, phylogenetic analysis supported the conclusion that the predominant methanogenic species harboring in chicken ceca is *Methanobrevibacter woesei* while Miller et al. (1986) isolated *Methanogenium* spp. from chicken and turkey feces.

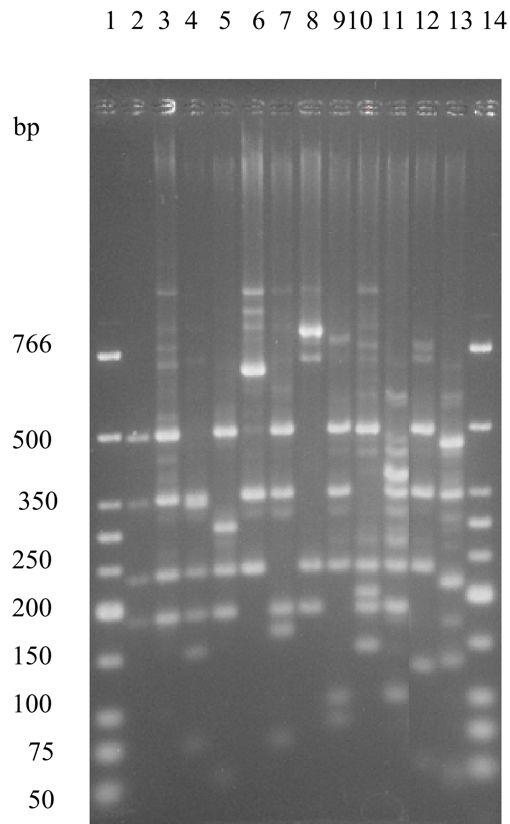

Figure 3.1 *Hae*III-riboprinting 16S rDNA of methanogenic archaea harboring in chicken cecal samples. Key: Lane 1 and 14: low molecular weight DNA ladder (New England BioLab, Beverly, MA); 2: *Methanobrevibacter woesei* GS DNA isolated from pure culture; 3 to 13: 16S rRNA riboprinting of sequences CH101, CH103, CH138, CH194, CH1117, CH344, CH389, CH3126, CH5164, CH1254, CH1270 , respectively.

Table 3.2 The 420 clones of 16S rDNA genes obtained in this study.

16S rDNA phylotype	No. of clones	Size (bp)	Nearest taxon	% Sequence identity
CH101	406	1266	*Methanobrevibacter woesei* GS	99.21
CH103	2	1264	*Methanobrevibacter woesei* GS	99.21
CH138	2	1268	*Methanobrevibacter woesei* GS	98.97
CH194	1	1266	*Methanobrevibacter woesei* GS	99.21
CH1117	2	1266	*Methanobrevibacter woesei* GS	98.97
CH344	1	1263	*Methanobrevibacter woesei* GS	98.97
CH389	1	1263	*Methanobrevibacter woesei* GS	99.45
CH3126	1	1263	*Methanobrevibacter woesei* GS	99.29
CH5164	1	1262	*Methanobrevibacter woesei* GS	99.13
CH1254	2	1264	*Methanobrevibacter woesei* GS	99.05
CH1270	1	1256	Uncultured archaeon clone ConP1-11F	97.62

It is not surprising that all sequences were very closely related to *Methanobrevibacter woesei* GS with the exception of sequence CH1270 as shown in Fig. 3.2. The primary methanogens in animal intestinal tracts are related to the genus *Methanobrevibacter* (Garcia et al., 2000). *Methanogenium* spp., on the other hand, originate from aquatic environments (Romesser, 2001). In contrast to ruminant animals, methanogen diversity in non-ruminants is minimal. In this study, 11 phylotypes were observed in chicken ceca while 8 phylotypes were found in a pig manure storage pit (Snell-Castro et al., 2005), and 65 phylotypes were found in sheep rumen (Wright et al., 2004). Eckburg et al. (2005) found that all 1524 archaeal sequences in human intestinal tracts belonged to *Methanobrevibacter smithii*. On the other hand, the *Methanomicrobiaceae* family and the *Methanobacteriaceae* family were found in cow rumen (Shin et al., 2004). In addition, by using FISH method, Lin et al. (1997) found that archaea were relatively more abundant in the GI tracts of ruminants than in the cecum samples of non-ruminant pigs. In contrast to other animals, we did not observe *Methanosphaera*- or *Methanosarcina*-related sequences. The methanogenic substrates may be the limiting factor in chicken cecum since birds are usually fed with low-fiber diets. *Methanosphaera* spp. require to and *Methanosarcina* spp. can use methanol as the substrate for growth (Boone et al., 2001; Miller, 2001a). In general, methanol is produced by the fermentation of pectins, which are abundant polysaccharides in plants.

Table 3.3 Distribution of phylotype patterns from chicken cecal samples.

Riboprinting patterns	CH101	CH103	CH138	CH194	CH1117	CH344	CH389	CH3126	CH5164	CH1254	CH1270	Total no. of the clones
Sample 1 [a] (1 chick)	129 (96.99)	1 (0.75)	1 (0.75)	1 (0.75)	1 (0.75)	0 NA[b]	0 NA	0 NA	0 NA	0 NA	0 NA	133
Sample 2 (6 chicks)	52 (92.86)	0 NA	1 (1.79)	0 NA	0 NA	1 (1.79)	1 (1.79)	1 (1.79)	0 NA	0 NA	0 NA	56
Sample 3 (6 chicks)	150 (98.04)	1 (0.65)	0 NA	0 NA	1 (0.65)	0 NA	0 NA	0 NA	1 (0.65)	0 NA	0 NA	153
Sample 4 (6 chicks)	20 (100)	0 NA	0 NA	0 NA	0 NA	0 NA	0 NA	0 NA	0 NA	0 NA	0 NA	20
Sample 5 (6 chicks)	55 (94.83)	0 NA	0 NA	0 NA	0 NA	0 NA	0 NA	0 NA	0 NA	2 (3.45)	1 (1.72)	58
Total no. of the clones [c]	406 (96.67)	2 (0.48)	2 (0.48)	1 (0.24)	2 (0.48)	1 (0.24)	1 (0.24)	1 (0.24)	1 (0.24)	2 (0.48)	1 (0.24)	420

[a] Number of strains and percentage of methanogens for all clones observed in one sample.

[b] NA, not applicable.

[c] Number of strains and percentage of methanogens in parenthesis for all 420 clones examined in this study.

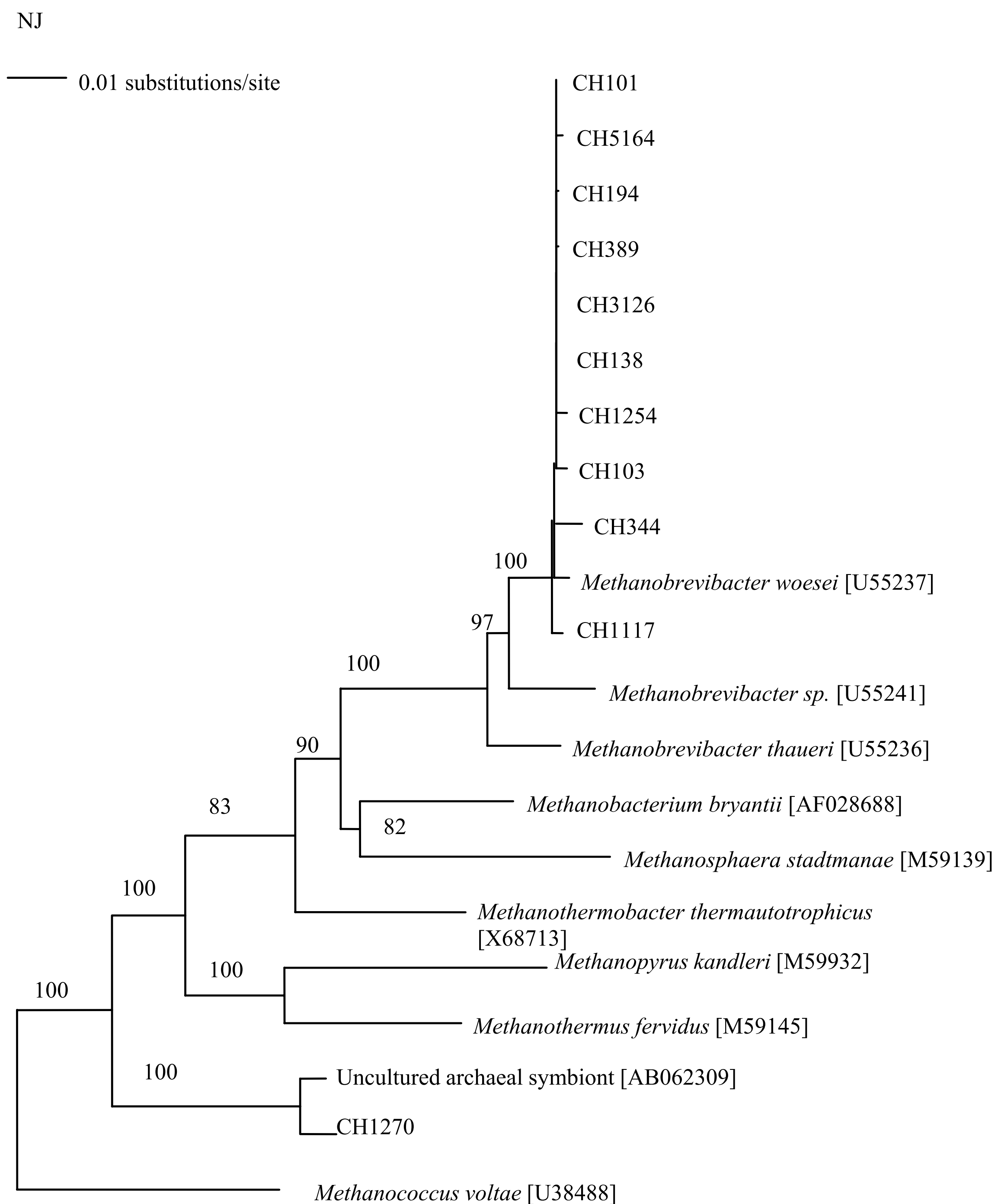

Figure 3.2 Phylogeny of partial SSU rDNA sequences from chicken ceca placed within the context of several methanogenic species within archaea. Evolutionary distances were produced by the Jukes-Cantor correction model and the phylogram was constructed using the neighbor-joining method (Swofford, 2002). Sequences harvested from Genbank are followed by accession numbers in brakets. Bootstrap support for 1000 replicates is indicated at the nodes. The scale bar represents the nucleotide substitution rate.

The physiology of *Methanobrevibacter woesei* GS is described in Miller and Lin (2002). The optimum temperature of this strain is 37 ^{o}C which corresponds to 41.5 ^{o}C, the body temperature of domestic fowl (*Gallus gallus*) (Dawson and Whittow, 2000). Chicken cecal pH values are from 5.5 to 7.0 (Denbow, 2000); the optimum pH of strain GS is 7.0 (Miller and Lin, 2002). Shrimpton (1966) also reported that the pH range of the bird cecum fresh weight content was 6.2-7.8 and that the temperature range was 36-40 ^{o}C. Unlike *Methanobrevibacter woesei* GS, optimal temperature of *Methanogenium* spp. range from 15-57 ^{o}C while the optimal temperature of *Methanogenium organophilium* CV is 30-35 ^{o}C (Romesser, 2001).

Methanobrevibacter woesei GS also grows with bile and in Balch 3 medium that has salt concentrations similar to seawater (Miller and Lin, 2002). Bile, produced and secreted by the liver, act as detergents to interact with bacterial membrane lipids. Therefore, exposure to bile is a serious challenge for intestinal microorganisms. Elkin et al. (1990) reported that chenodeoxycholyltaurine and cholyltaurine are the primary bile acids in chicken and turkey. In chickens, more than 90 % of bile acids are reabsorbed in the small intestine (Denbow, 2000); therefore, some bile acids pass through cecum and colon. Membrane architecture and composition, especially fatty acid composition, play an important role in bile and osmotic resistance (Begley et al., 2005). Flahaut et al. (1996) showed that when *Enterococcus faecalis* ATCC19433 is subjected to osmotic stress, it shows cross-protection against exposure to bile acids.

Diets, gender, and host specificity may affect methanogenic colonization in the chicken cecum. In the present study, however, all birds, fed with a corn-soy based diet,

were female, the same age, and the same Leghorn breed. By observing 16S rRNA sequences, Wright et al. (2004) found that pasture-grazed sheep had more methanogen diversity than sheep fed either an oaten hay or lucerne hay diet. High fiber diets have been reported to increase the level of methanogens in the gut. The number of the ciliate *Nyctotherus ovalis*, which harbors *Methanobrevibacter* sp., and methane production increased when the cockroach was feed with high fiber diet (Gijzen et al., 1991). Shrimpton (1966) found that the high-fiber diet increased methane production in the chicken by ninefold in comparison with birds given low-fiber feed. Florin et al. (2000) mentioned that human male genetic effects or behavioral effects, such as passage rate and the difference in the enterohepatic circulation of bile acids, may influence mechanisms that control methanogenesis. In spite of the same traits, some individual rats resist methanogenic colonization (Florin et al., 2000). In humans, *Methanobrevibacter smithii* was found in the range of a few to 10^{10} organisms per gram fecal dry weight (Miller and Wolin, 1986). Tannock et al. (1982) examined the various strains of lactobacilli isolated from many kinds of animals that exhibit host specificity to squamous epithelia in the gastrointestinal tract. However, Hackstein et al. (1999) suggested that the presence of symbiotic methanogenic archaea in the gastrointestinal tract is a necessary requirement for the evolution of foregut- and hindgut-fermenting structures, irrespective with the feeding habits of a particular host.

Methanogen counts

By using the MPN enumeration method, the log transformations of the methanogen concentrations for one bovine rumen fluid sample and four chicken cecal

samples are shown in Table 3.4. The methanogen population in bovine the rumen fluid was used as a positive control and found to be 7.15 $\log_{10}$ cells/ml. The number of methanogens in the rumen fluid was similar to those found in previous studies (Smith and Hungate, 1958; Morvan et al., 1996). From four chicken cecal samples (samples 2 to 5), composed of contents pooled from twenty-four birds, harboring methanogens in ceca between a $\log_{10}$ of 6.38 to 8.23 cells per gram wet weight and the numbers of methanogen per gram dry weight were between 7.04 to 8.88 $\log_{10}$ cells. The number of methanogens in chicken ceca closely resembled that in both horse and pig ceca and ranged from $\log_{10}$ of 4 to 6 and 6.78 per gram wet weight, respectively (Butine and Leedle, 1989; Morvan et al., 1996). In this study, we used clarified rumen fluid as the supplement. Salanitro et al. (1974) indicated that 60 % of the total bacteria in chicken ceca could be recovered with a rumen medium while 45 % of these organisms were enumerated with a liver extract and chicken fecal extract medium. In addition, clarified rumen fluid is markedly stimulatory to the growth of *Methanobrevibacter* spp. (Miller 2001b).

Bacteria in chicken ceca occur at levels as high as 10^{11} cells per gram of dry cecum (Salanitro et al., 1974). Based on the MPN enumeration results, therefore, methanogens in this study range from 0.01 to 0.76 % of the total numbers of organisms. By using the Bacterial, Eucarya, and Archaea fluorescent probes to detect microbes, archaea comprised 0.5 to 3 % of the rRNA in steer rumens, cow rumens, sheep rumens, pig gastrointestinal contents, goat rumens (Lin et al, 1997) while *Methanobrevibacter*

Table 3.4 The $\log_{10}$ numbers of methanogenic archaea in bovine rumen and chicken cecal contents.

Sample	MPN			16S rDNA copy number/ g wet wt.	Reference
	cells/ g wet wt. (or ml. bovine rumen fluid)	95 % Confidence limits			
		Lower	Upper		
Bovine rumen fluid	5 to 8	NK [a]	NK	NK	Morvan et al., 1996
Bovine rumen fluid	6 to 8	NK	NK	NK	Smith and Hungate (1958)
Bovine rumen fluid	7.15	6.72	7.58	ND [b]	This study
Chicken cecal sample 1 (1 bird)	ND	NA [c]	NA	ND	This study
Chicken cecal sample 2 (6 birds)	6.45 (7.08) [d]	6.00	6.87	5.50 ± 0.11 [e]	This study
Chicken cecal sample 3 (6 birds)	8.23 (8.88)	7.82	8.67	7.19 ± 0.09	This study
Chicken cecal sample 4 (6 birds)	6.73 (7.36)	6.23	7.23	6.76 ± 0.08	This study
Chicken cecal sample 5 (6 birds)	6.38 (7.04)	5.96	6.81	6.78 ± 0.12	This study

[a] NK, not known.

[b] ND, not determined.

[c] NA, not applicable.

[d] Log_{10} MPN/ g dry weights are given in parentheses.

[e] The mean of $\log_{10}$ 16S rDNA copy number/ g dry weights and standard deviations.

smithii was found in humans from a few up to 10% of the total anaerobic bacteria (Weaver et al., 1986). In the chicken ceca, methanogens counts using FISH ranged from 0.7 to 3.3 % of the total microbial community from 2-d-old to 6-wk-old broilers (Zhu and Joerger, 2003).

Primers MBT, designed specifically for the order Methanobacteriales (Yu et al., 2005b) were used to quantify the methanogen population. The means of log10 16S rDNA copy number per gram wet weight cecum with the corresponding standard deviations are shown in Table 3.4. The results represent two PCR replicates of DNA samples. All PCR reactions were performed in triplicate in the same run. We found that the 16S rDNA copy number per gram wet weight in the samples was between $\log_{10}$ 5.50 to 7.19. Sequence CH101 exactly matches with primer MBT857F, MBT929F, and MBT1196R. According to Yu et al., (2005b), estimates of the 16S rDNA methanogenic copy number in this study should not have false-positive or false-negative results from MBT primers. The results of this experiment revealed that the copy number of 16S rDNA in four samples, particularly samples 4 and 5, was much the same as the number observed by MPN method. The $\log_{10}$ 16S rDNA copy number per gram wet weight in samples 1 and 2 was less than the lower numbers of 95 % confidence limits counted by MPN method. Our results show that methanogens in chicken caca might have one SSU rDNA copy. In general, all methanogens have only one or two SSU rRNA genes (Fogel et al., 1999). However, a recent study showed that the genome sequence of *Methanosphaera stadtmanae* contains 4 copies of 16S rRNA genes (Fricke et al., 2006).

CHAPTER IV

EFFECTS OF NITROCOMPOUNDS AND FEEDSTUFFS ON IN VITRO METHANE PRODUCTION FROM CHICKEN CECAL CONTENTS AND RUMEN FLUID

Introduction

The gastrointestinal tract harbors a wide variety of microbial species, including *Bacteriodes* spp., *Clostridium* spp., *Enterobacteriacae* spp., *Enterococus* spp., *Eubacterium* spp., and *Lactobacillus* spp. Most of these microorganisms hydrolyze polysaccharides to short-chain volatile fatty acids (VFA), hydrogen, and carbon dioxide as the primary fermentation products (Wolin et al., 1997). Some endproducts of fermentation such as hydrogen are utilized by a second group of organisms (i.e. methanogens) to produce methane. Methanogens are members of the domain Archaea, and fall within the phylum Euryarchaeota (Woese et al., 1990). They catalyze the transfer of hydrogen and carbon dioxide into methane. In addition to the production of methane, the maintenance of a low partial pressure of hydrogen by methanogenesis has a profound influence on the production of hydrogen and other products by the non-methanogenic, fermentative microbial community (Wolin et al., 1997).

In the chicken gastrointestinal tract, the cecum has the highest microbial activity, harboring approximately 10^{10} to 10^{11} obligate anaerobes per gram (wet weight) (Mead, 1989; Jensen, 1996). In chickens, the cecum functions as a fermentor to provide VFA (Marounek et al., 1999). Józefiak et al. (Józefiak et al., 2004) concluded that 8 % of the energy requirements of chickens are derived from VFA. Surprisingly, few studies have

reported methane gas production in the chicken ceca. However, we hypothesize that altering production of cecal methane production could influence the fermentation profile from the cecal microbial population. Both in situ and in vitro studies demonstrated that different types of substrates influence methane production in the chicken ceca (Marounek et al., 1999; Shrimpton, 1966). In addition, the age of the birds may be a factor as methane gas has been reported to occur in cecal contents from two-month old birds (Marounek and Rada, 1998). By measuring fluorescent in situ hybridization, methanogens were found from cecal samples of very young chicks (Zhu and Joerger, 2003). However, only one methanogen strain (i.e., *Methanogenium* sp.) has thus far been isolated from chicken feces (Miller et al., 1986).

In contrast to chicken ceca, microbial composition in the bovine and ovine rumen and the effect of feed material on methane production have been well studied. Using molecular techniques, rumen bacteria as well as methanogen diversity are dependent on the types of feed material (Tajima et al., 2000; Tajima et al., 2001; Wright et al., 2004). Methane, a greenhouse gas, accounts for 2-12 % of dietary energy loss in ruminants (Johnson and Johnson, 1995). Consequently, there has been considerable interest in developing compounds and dietary regimes that limit methane production. From previous studies, the addition of nitrocompounds not only reduced methane production, but also lowered foodborne pathogen colonization (Anderson et al., 2001; Anderson et al., 2003; Jung et al., 2004a; Jung et al., 2004b). By administering 2-nitropropanol to broiler chicks, the concentration of *Salmonella enterica* serovar Typhimurium in the cecum was significantly reduced (Jung et al., 2004b). Moreover, these nitrocompounds

inhibited the growth of pure cultures of *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Enterococcus faecalis*, and *Yersinia enterocolitica* in vitro (Jung et al., 2004a; Anderson et al., 2005). In an in vitro study, 12 mM of nitroethane, nitroethanol, or 2-nitropropanol significantly inhibited ruminal methanogenesis (Anderson et al., 2001; Anderson et al., 2003). In addition, nitroethane reduced in vivo methane production in the ovine rumen (Anderson et al., 2004). Corn layer diets are typically fed during the egg laying cycle (Coon, 2001). During molt for shifting birds into a second egg laying cycle, high fiber diets such as alfalfa have been proposed to avoid problems associated with feed withdrawal (Landers et al., 2005a; Landers et al., 2005b; Woodward et al., 2005). The addition of alfalfa could also lead to increased methane production since alfalfa has been shown to support extensive VFA production in the ceca of molting birds (Woodward et al., 2005). The specific objective of this study was to evaluate the addition of nitrocompounds on in vitro methane production and VFA production within the chicken ceca, ovine rumen fluid, and bovine rumen fluid. In this study, we examined the effect of three nitrocompounds on in vitro methanogenesis in cultures derived from adult chicken ceca, bovine, and ovine rumen fluid incubated with either high fiber alfalfa or high-energy corn-based diet as the substrate.

Materials and Methods

Experimental design

Experiments 1 and 2 in this chapter examined the efficiency of three nitrocompounds to inhibit methanogenesis. Experiments 3-8 were designed to compare

the effect of nitroethane on in vitro methane production in chicken ceca contents and rumen fluid from cattle and sheep. VFA in experiment 1 and methane production in experiments 1-8 were subjected to statistical analyses (see section Statistical analyses) as a completed randomized design.

Source of samples

Experiment 1 and 2 in this chapter conducted with cecal mixtures mixed and pooled from 10, 56 to 72-wk old Leghorns maintained on a layer ration. The diluted cecal contents were incubated with each nitrocompound under a CO_2 and H_2 (50:50) gas mixture. Experiments 3-8 were conducted with chicken ceca, rumen fluid from cattle and sheep. Bovine rumen fluid was obtained from a cannulated Holstein-Friesian cow maintained on a 50% alfalfa hay, 50% flaked corn diets, sheep rumen fluid was obtained via stomach tube from a mature Suffolk ewe. Rumen fluid was strained through a nylon paint strainer (Leyendecker et al., 2004). To avoid excess handling of the samples and subsequent shock to the microbial population, ceca and rumen fluid were used immediately. These mixtures were incubated under a CO_2 and H_2 (20:80) gas mixture at 200 kPa pressure (Miller et al., 1986).

Nitrocompounds and in vitro cecal methane production

Cecal contents from laying hens were diluted 1:20 (cecal content: final mixture volume) concentration with a mixture (1:1) of anaerobic culture medium (Maciorowski et al., 1997) and anaerobic dilution solution (Bryant and Burkey, 1953) containing 60 mM added sodium formate. The cecal suspension (10 ml/ tube) was added to 18 x 150 millimeter crimp top culture tubes containing 0.2 g alfalfa (AF), 0.2 g layer ration (LF)

(10 ml/ tube) or no feed material (NF). The four nitrocompound treatments were: no nitrocompound (NN), 12 mM nitroethane (NE), 12 mM nitroethanol (NOH), and 12 mM 2-nitropropanol (NP). The tubes were capped and incubated at 37 ^{o}C for 24 h under a CO_2 and H_2 (50:50) gas mixture. The composition of the Texas A&M University (TAMU, College Station, TX) layer feed will be (%): corn 56.72; soy bean meal 31.63; vegetable oil 7.68; monocalcium phosphate 1.69; calcium carbonate 1.56; methionine (98 %) 0.17; vitamin premix 0.25; NaCl 0.25; trace mineral premix 0.05. In experiment 2 (Table 1), a comparative no-feedstuff incubation containing only diluted mixture and no-nitrocompound treatment was included in the experimental design and VFA was quantified in this treatment without replication. However, there were triplicates in experiment 1.

Comparison of in vitro ruminal methane production

Experiments 3-8 were designed to compare the effect of nitroethane on in vitro methane production in chicken ceca contents and rumen fluid from bovine and ovine sources. Essentially the same procedure was used as that described in experiment 1, except for the following modifications: 1) cecal contents, bovine rumen fluid, and ovine rumen fluid was inoculated in experiments 3 and 4, 5 and 6, and 7 and 8, respectively; 2) only nitroethane was added in these treatments; 3) the tubes were incubated at 37 ^{o}C for 3, 5, 7 h under a CO_2 and H_2 (20:80) gas mixture at 200 kPa pressure.

Analytical

Final concentrations of hydrogen and methane gas present in the headspace of treatments at the end of the incubation period were determined via gas chromatography (Gow Mac Instrument Company, Bethlehem, PA) equipped with a HaysepQ column (Anderson et al., 2003). Final concentrations of acetate, propionate and butyrate from the samples were collected at the end of the incubation period and determined via gas chromatography (Shimadzu GC-14A, Tokyo, Japan) equipped with a Supelco 4% Carbowax, 80/120 Carbopack BDA column (2 m x 5 mm x 3mm; Sigma-Aldrich, St. Louis, MO) (Hinton et al., 1990).

Statistical analyses

Standard errors are presented in the subsequent tables and figures, and these values represent the average of triplicate measurements. The statistical tests for treatment effects were performed using an analysis of variance (ANOVA) procedure of Statistix® 8 Analytical Software (Tallahassee, FL). Means were further separated using least significant difference (LSD) multiple comparisons.

Results and Discussion

Effects of feedstuff and nitrocompounds on in vitro chicken cecal fermentation

The corn-based layer feed and 100 % alfalfa diets were described in Donalson et al. (Donalson et al., 2005). The levels of VFA production, hydrogen, and methane are

shown in Tables 4.1 and 4.2. The results show that alfalfa or corn-based diets provided substrates for cecal microorganisms to produce more VFA and that the nitrocompounds shifted the VFA production pattern. The type of substrate is the most importance factor influencing the microbial activity in the gastrointestinal tract of monogastric animals (Jensen, 1996; Apajalahti et al., 2001). In experiment 1 (Table 4.1), acetic acid concentration in alfalfa and layer feed supplement with and without nitrocompounds was not ($P > 0.05$) different. In both experiment 1 and 2, acetic acid was the primary VFA product. Acetic acid has been shown to be the major VFA produced in vivo in ceca from hens fed either alfalfa or corn-based diets (Woodward et al., 2005; Park et al., 2004; Ricke et al., 2004). However, different acetic acid producing microorganisms could be present in different diets. By using molecular techniques and cultural methods, several microorganisms have been identified as acetic acid-producing bacteria in chicken ceca (Barnes et al., 1972; Salanitro et al., 1974; Salanitro et al., 1978; Donalson et al., 2005). Salanitro et al. (1978) isolated acetic acid-producing bacteria from chicken ceca, namely *Eubacterium* spp., *Clostridium* spp., and *Bacteroides* spp. In general, the saccharolytic bacteria in the chicken ceca are the primary producers of acetic acid (Mead, 1989). Barnes et al. (1980) found *Clostridium* spp. harbored in chicken ceca as high as 10^8 per gram wet weight. However, chicken ceca also contain cellulolytic bacteria that are capable of producing acetate. Based on 16S rDNA analysis, 19% of the cecal bacteria were *Ruminococcus* spp. which produce acetic and formic as their primary products (Apajalahti et al., 2001).

Table 4.1 Effect of three nitrocompounds and feedstuffs on in vitro chicken cecal fermentation during 24 h incubation.

Treatment	Experiment 1: Concentration of fermentation product[a] (μmol/g cecal content)					Experiment 2: Concentration of fermentation product[a] (μmol/g cecal content)				
	Acetate	Propionate	Butyrate	Total[b]	Acetate: propionate	Acetate	Propionate	Butyrate	Total[b]	Acetate: propionate
Alfalfa	198.7±14.2 (85.9)	23.9±1.0^{d} (10.3)	8.7±0.4^{f,g} (3.8)	231.3	8.3^{c}	73.5 (85.7)	9.0 (10.5)	3.3 (3.8)	85.8	8.2
Alfalfa-nitroethane	186.5±11.9 (85.7)	22.9±1.2^{d} (10.7)	8.2±0.4^{g} (3.8)	217.6	8.1^{c}	83.9 (86.8)	9.6 (9.9)	3.2 (3.3)	96.7	8.7
Alfalfa-nitroethanol	209.2±16.9 (79.8)	39.9±3.4^{c} (15.2)	13.2±1.0^{c} (5.0)	262.3	5.2^{d}	58.6 (85.4)	7.4 (10.8)	2.7 (3.9)	68.6	7.9
Alfalfa-2-nitropropanol	204.4±10.0 (81.5)	34.9±1.7^{c} (13.9)	11.4±0.5^{e} (4.5)	250.7	5.9^{d}	71.3 (83.2)	10.6 (12.4)	3.8 (4.4)	85.7	6.7
Layer feed	219.1±17.3 (82.9)	27.5±2.5^{d} (10.4)	17.8±1.5^{d} (6.7)	264.4	8.0^{c}	73.1 (82.6)	9.2 (10.4)	6.2 (7.0)	88.5	7.9
Layer feed-nitroethane	194.5±8.5 (85.8)	21.0±1.4^{d} (9.3)	11.2±1.3^{d,e} (4.9)	226.7	9.3^{c}	67.4 (87.4)	6.7 (8.7)	2.9 (3.8)	77.1	10.0
Layer feed-nitroethanol	172.4±4.2 (76.9)	34.3±1.2^{c} (15.3)	17.5±0.1^{d} (7.8)	224.2	5.0^{d}	57.7 (86.4)	6.2 (9.3)	3.0 (4.5)	66.8	9.3

Table 4.1 Continued.

Treatment	Experiment 1: Concentration of fermentation product[a] (μmol/g cecal content) Acetate	Propionate	Butyrate	Total[b]	Acetate: propionate	Experiment 2: Concentration of fermentation product[a] (μmol/g cecal content) Acetate	Propionate	Butyrate	Total[b]	Acetate: propionate
Layer feed-2-nitropropanol	198.1±6.2 (77.0)	38.6±1.3[c] (15.0)	20.5±1.0[c] (8.0)	257.2	5.1[d]	66.3 (79.7)	11.1 (13.3)	5.9 (7.1)	83.2	6.0
No-feedstuff and no-nitrocompound (control)	ND[h]	ND	ND			64.8 (94.0)	3.0 (4.4)	1.1 (1.6)	68.9	21.9

[a] Values in parenthesis represent molar proportion as a percent of total.

[b] Total = sum of acetate + propionate + butyrate.

[c,d,e,f,g] Means within same columns with unlike superscripts differ ($P < 0.05$).

[h] ND, not determined.

Incubations containing either corn-based layer ration or alfalfa had increased propionate concentrations approximately three times higher than the incubation containing no-feedstuff and no-nitrocompound as a control in experiment 2 (Table 4.1). However, the levels of propionate were not different ($P > 0.05$) between incubations containing added feedstuffs in experiment 1 (Table 4.1). Incubations in experiment 1 containing added nitroethanol or 2-nitropropanol had higher ($P < 0.05$) propionate concentrations than incubations containing no nitrocompound or containg added nitroethane supplement (Table 4.1). However, incubations cotaining nitroethanol and incubations containing mixtures between layer feed and nitroethane addition exhibited lower values than others in experiment 2 (Table 4.1). Some clostridia, such as *Clostridium propionicum* can oxidize lactate to carbon dioxide, propionic and acetic acids via the acrylate pathway. *Clostridium* spp. have typically been identified as one microbial group in chicken ceca without distinction for fermentation capabilities. Based on 16S rDNA analysis, Apajalahti et al. (Apajalahti et al., 2004) determined that approximately 7% of cecal bacteria are clostridia. In contrast, Zhu et al. (2002) found that more than 50% of total bacteria belonged to *Clostridium* spp. (Zhu and Joerger, 2003) and Lu et al. (2003) found that 65% of 16S rRNA gene clones had *Clostridiaceae*-related sequences.

Adding nitroethane was associated with an increase in the ratio of acetic acid to propionic acid suggesting that this compound might affect some gram-negative propionic-producing bacteria. In general, the phospholipid-rich cell wall in gram-negative bacteria is more susceptible to damage by lipid solvents, alcohol, and acetone

(White, 2000). The 2-nitropropanol supplement tended to increase the percentage of propionic acid. However, this compound is more effective against gram-negative bacteria and may not inhibit *Clostridium propionicum* (Jung et al., 2004b). By using a strict anaerobic isolation method, Salanitro et al. (1978) found that 18% of cecal microorganisms were *Propionibacterium acnes*, a gram-positive, acetic and propionic-producing bacterium.

Results from experiment 1 indicated that incubations containing layer feed produced more ($P < 0.05$) butyrate than incubations containing alfalfa (Table 4.1). In addition, alfalfa and layer feed supplements increased butyric acid concentration 3 and 6 times, respectively, as compared to the incubation containing no-feedstuff and no-nitrocompound in experiment 2 (Table 4.1). With the alfalfa supplement, adding nitroethanol and 2-nitropropanol increased ($P < 0.05$) butyrate producing, whereas nitroethane treatment decreased ($P < 0.05$) butyrate production during experiment 1 (Table 4.1). In experiment 2, approximately 3 to 4 μmol butyrate/ g cecal content were produced in incubations a containing combination of alfalfa and nitrocompounds (Table 4.1). In the layer feed supplement, only nitropropanol treatment yielded a significantly ($P < 0.05$) higher value in butyrate production and nitroethane treatment decreased ($P < 0.05$) butyrate production in experiment 1 (Table 4.1). In experiment 2 (Table 4.1), butyrate production of incubations containing either nitroethane or nitroethanol plus the layer feed treatment was lower numerically than nitropropanol treatment. Like propionic acid production, *Bacteroides* spp., *Fusobacterium* spp., and *Gemmiger* spp., gram-negative and butyric-acid producing bacteria, might be influenced by adding

nitroethane. Salanitro et al. (1978) found that. *Bacteroides* spp., *Fusobacterium* spp., and *Gemmiger* spp. comprised 12.8%, 6.2%, and 3.4% of the cecal microorganisms, respectively. By analysis of partial 16S rRNA gene sequences, 5% and 14% of the sequences belonged to *Bacteroides* spp., and *Fusobacterium* spp., respectively (Lu et al., 2003). As mentioned previously, gram-negative bacteria are likely more susceptible to 2-nitropropanol. This compound may promote growth of gram-positive bacteria, especially *Clostridium* spp. Some clostridia, *C. perfringens* (formerly *C. welchii*), *C. innocum*, *C. sporogenes* isolated from chicken ceca, are saccharolytic, acetic and butyric-producing bacteria (Barnes et al., 1972).

The mechanism of inhibition by these nitrocompounds is currently unknown. Further studies to more fully elucidate the spectrum of inhibitory activity and the mode of action of these nitrocompounds are needed to determine the specific effects on different fermentative cecal microbial populations.

Effects of feedstuffs and nitrocompounds addition on in vitro chicken cecal methane formation

Compared to an earlier study on ruminal fermentation (Anderson et al., 2003), it was also observed in this study that hydrogen gas accumulated in the fermentation flasks containing nitrocompounds (Table 4.2). These results indicate that microbial interspecies-hydrogen transfer may not have been optimized under these incubation conditions (Anderson et al., 2003).

Methane production was obviously increased in feedstuff only supplemented incubations ($P < 0.05$) as shown in Table 4.2. In experiment 2, methane production in

incubations containing added feedstuff increased 2 to 6 times (19.1 to 59.1 μmol/g cecal content) as compared to incubations without added feedstuff (10.7 μmol/g cecal content). Alfalfa addition supported more methane production than layer feed addition. Shrimpton (1966) found that the rate of methane production of in situ was higher in chickens offered a fiber-based diet consisting of wheat, barley, oats, and maize. However, in vitro production of methane in cecal contents was not significantly different between starch and pectin as substrates (Marounek et al., 1999). Maczulak et al. (1993) found that no difference in concentration of methanogens in rats fed a high-fiber diet or a fiber-free diet, but a higher level of methanogens in fecal output of rats fed high-fiber diet caused higher excretion of methanogens. Several studies have shown that these nitrocompounds reduce methane production in ruminants (Anderson et al., 2001; Anderson et al., 2003). In vitro methane production was also significantly reduced ($P < 0.05$) in incubations with added nitrocompound additions (Table 4.2).

Comparison of nitroethane supplement on in vitro ruminal and chicken cecal methane production

Since only nitroethane completely inhibited methane production in in vitro chicken cecal fermentation in experiments 1 and 2 (Table 4.2), it was chosen for comparative effects on in vitro methane production in rumen fluid from cattle and sheep (Figs. 4.1 to 4.4) and in chicken ceca (Fig. 4.5 to 4.6). Each rumen fluid or cecal incubation was repeated twice. Bovine rumen fluid, ovine rumen fluid, and chicken cecal incubations were designed as experiments 3-4, 5-6, and 7-8, respectively. The results of in vitro bovine ruminal methane production are shown in Figs. 4.1 and 4.2.

Table 4.2 Effect of three nitrocompounds and feedstuff source on in vitro chicken cecal methane production during 24 h incubation (Experiments 1 and 2).

Treatment	Concentration of hydrogen and methane [a] (µmol/g cecal content)					
	Experiment 1			Experiment 2		
	Hydrogen	Methane	Methane reduction (%)	Hydrogen	Methane	Methane reduction (%)
Alfalfa	14.7±2.6[d]	32.0±0.2[c]	-	7.6 ± 6.9[d]	59.1 ± 3.1[b]	-
Alfalfa-nitroethane	30.3±0.5[c]	0.7±0.0[e]	97.9 ± 0.0	49.3 ± 2.3[b]	0.2 ± 0.0[e]	99.7 ± 0.0
Alfalfa-nitroethanol	34.9±0.0[b]	7.3±0.4[d]	77.2 ± 1.2	14.4 ± 0.7[c,d]	0.5 ± 0.3[e]	99.2 ± 0.6
Alfalfa-2-nitropropanol	35.7±0.1[b]	9.7±2.8[d]	69.8 ± 8.6	28.4 ± 16.4[c]	31.9 ± 5.0[c]	46.6 ± 5.7
Layer feed	18.6±0.2[d]	38.0±0.4[b]	-	5.0 ± 2.9[d]	19.1 ± 0.9	-
Layer feed-nitroethane	30.8±0.5[c]	0.7±0.0[e]	98.3 ± 0.0	52.1 ± 2.2[b]	0.2 ± 0.0[e]	99.0 ± 0.1
Layer feed-nitroethanol	37.4±0.2[b]	2.7±0.1[e]	92.8 ± 0.1	51.9 ± 0.6[b]	0.2 ± 0.0[e]	99.0 ± 0.1
Layer feed-2-nitropropanol	35.5±0.9[b]	7.3±0.4[d]	80.9 ± 0.9	55.0 ± 2.0[b]	27.2 ± 23.0[c]	-
No-feedstuff and no-nitrocompound (control)	ND [g]	ND	-	10.3 ± 0.2	10.7 ± 0.3	-

[a] Values in parenthesis represent percentage of methane reduction. (Methane production in alfalfal or layer feed supplement – methane production in each nitrocompound supplement)/ methane production in alfalfal or layer feed supplement x 100.

[b,c,d,e,f] Means within same columns with unlike superscripts differ ($P < 0.05$).

[g] ND, not determined.

At 3 h incubation, methane production was not significantly ($P > 0.05$) different between treatments during experiment 3 (Fig 4.1). However, nitroethane addition significantly suppressed in vitro methane production during experiment 4 (Fig. 4.2). At 5 h, the nitroethane supplement also ($P < 0.05$) inhibited methane production in both experiments (Figs 4.1 and 4.2). At 7 h, feed material influenced ($P < 0.05$) in vitro methane production. Layer feed enhanced ($P < 0.05$) and nitroethane decreased ($P < 0.05$) in vitro methane production in both experiments (Figs. 4.1 and 4.2).

The results of in vitro ovine ruminal methane production are shown in Figs. 4.3 and 4.4. In experiment 5, nitroethane completely inhibited methane production (Fig. 4.3). In experiment 6, nitroethane supplement significantly ($P < 0.05$) inhibited in vitro methane production after 5 h incubation. Like in vitro bovine ruminal methane production, layer feed significantly ($P < 0.05$) increased and nitroethane significantly ($P < 0.05$) inhibited methane accumulation in headspaces (Figs. 4.3 and 4.4).

Only nitroethane inhibited ($P < 0.05$) in vitro chicken cecal methane production at 5 and 7 h incubation (Figs. 4.5 and 4.6). Neither alfalfa or layer feed significantly influenced mean methane production by the in vitro chicken ceca incubations ($P < 0.05$). Feedstuffs had an impact on methane generation in vitro rumen fluid (Figs 4.1 to 4.4), particularly at 7-hour incubation. By using 16S rDNA identification, Wright et al. (Wright et al., 2004) reported that the types of feed material affect methanogen diversity in the ovine rumen. Based on preliminary work in our laboratory, the diversity of methanogens in the chicken ceca appears to be minimal compared to the rumen population (Saengkerdsub et al., 2005).

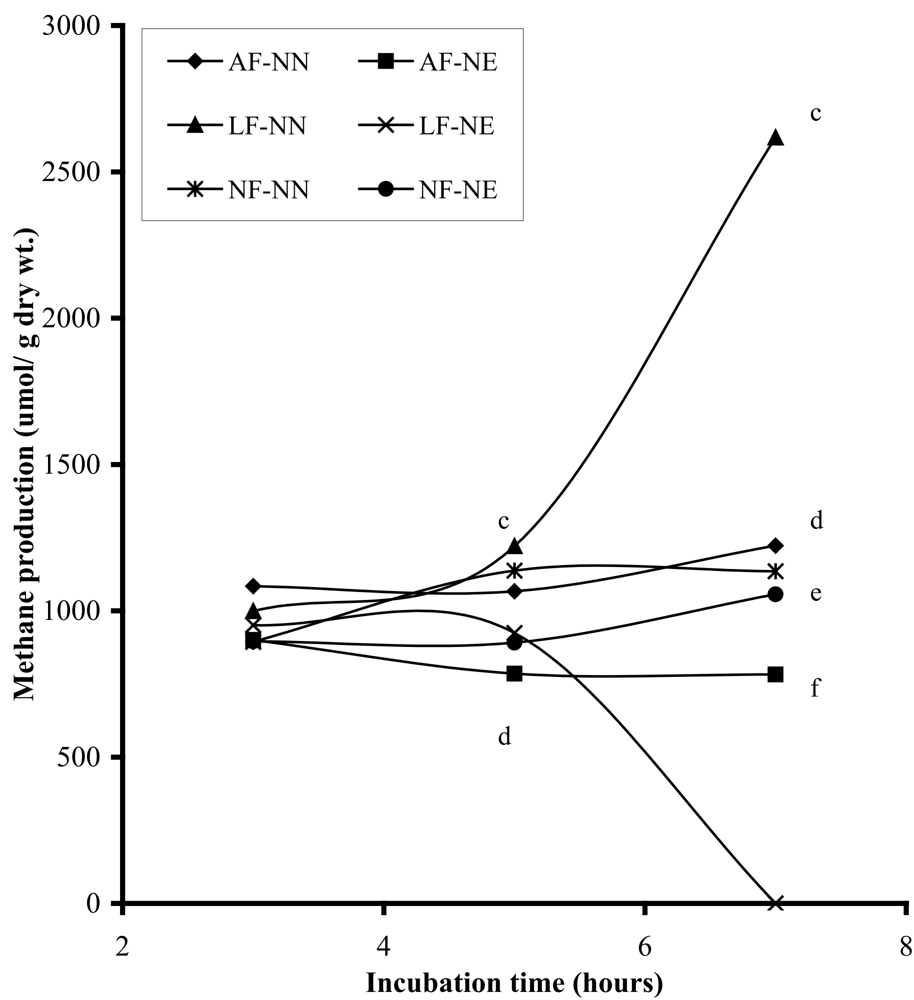

Figure 4.1 Methane production in in vitro bovine rumen fluid (Experiment 3). Key: alfalfa (AF); layer feed (LF); nitroethane (NE); no feed material (NF); no nitrocompound (NN). [c,d,e,f] Means within same columns with unlike superscripts differ ($P < 0.05$).

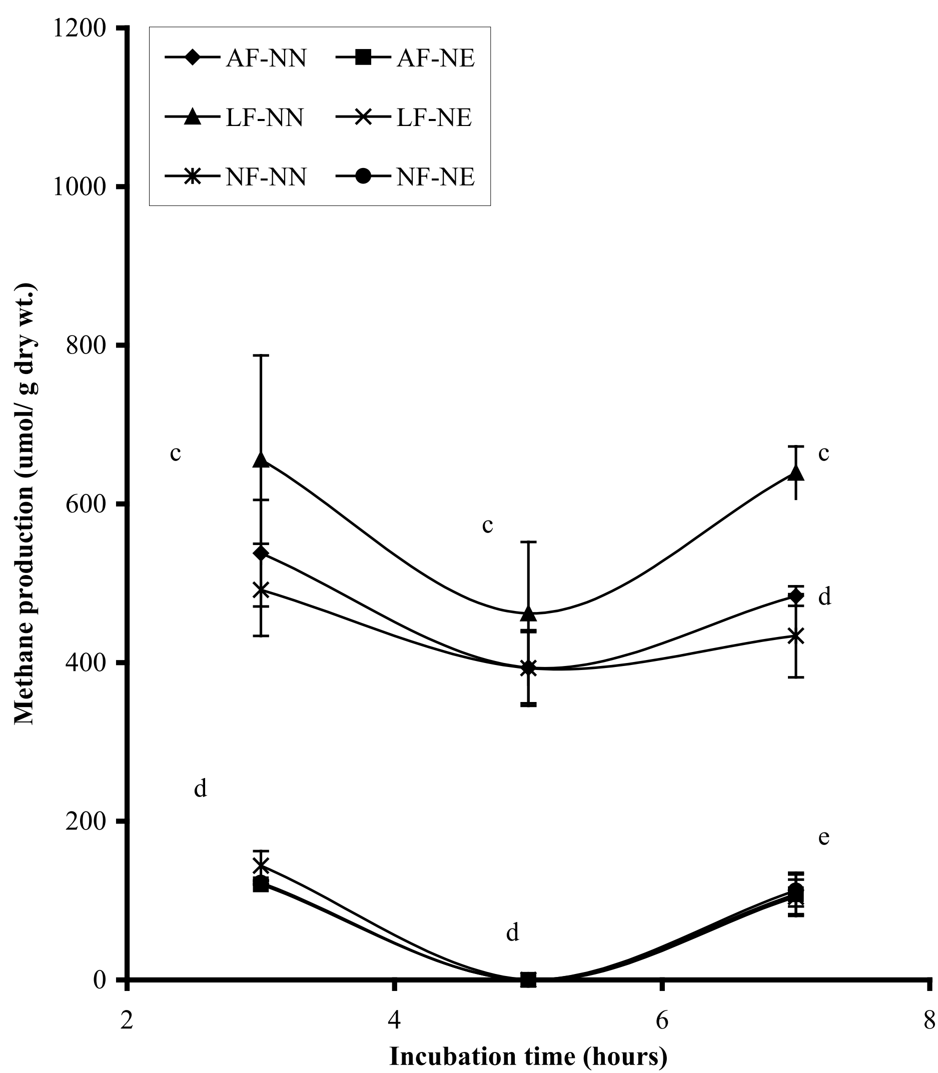

Figure 4.2 Methane production in in vitro bovine rumen fluid (Experiment 4). Key: alfalfa (AF); layer feed (LF); nitroethane (NE); no feed material (NF); no nitrocompound (NN). [c,d,e] Means within same columns with unlike superscripts differ ($P < 0.05$).

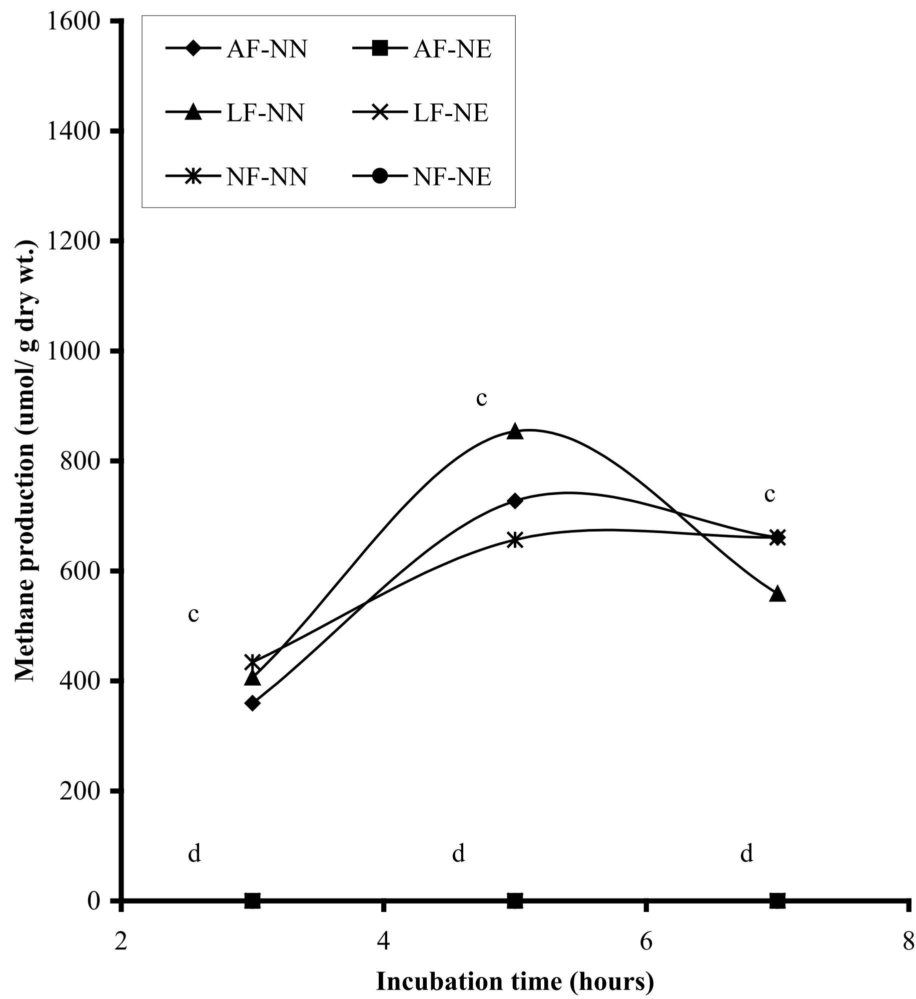

Figure 4.3 Methane production in in vitro ovine rumen fluid (Experiment 5). Key: alfalfa (AF); layer feed (LF); nitroethane (NE); no feed material (NF); no nitrocompound (NN). [c,d] Means within same columns with unlike superscripts differ ($P < 0.05$).

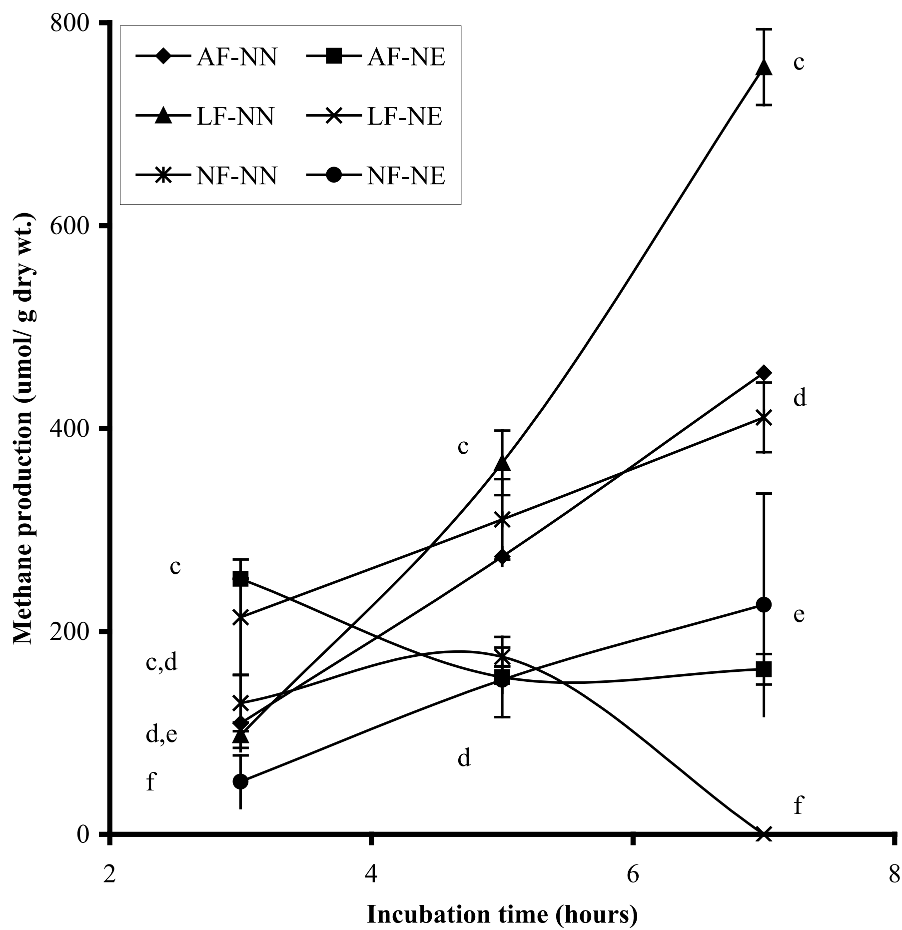

Figure 4.4 Methane production in in vitro ovine rumen fluid (Experiment 6). Key: alfalfa (AF); layer feed (LF); nitroethane (NE); no feed material (NF); no nitrocompound (NN). [c,d,e,f] Means within same columns with unlike superscripts differ ($P < 0.05$).

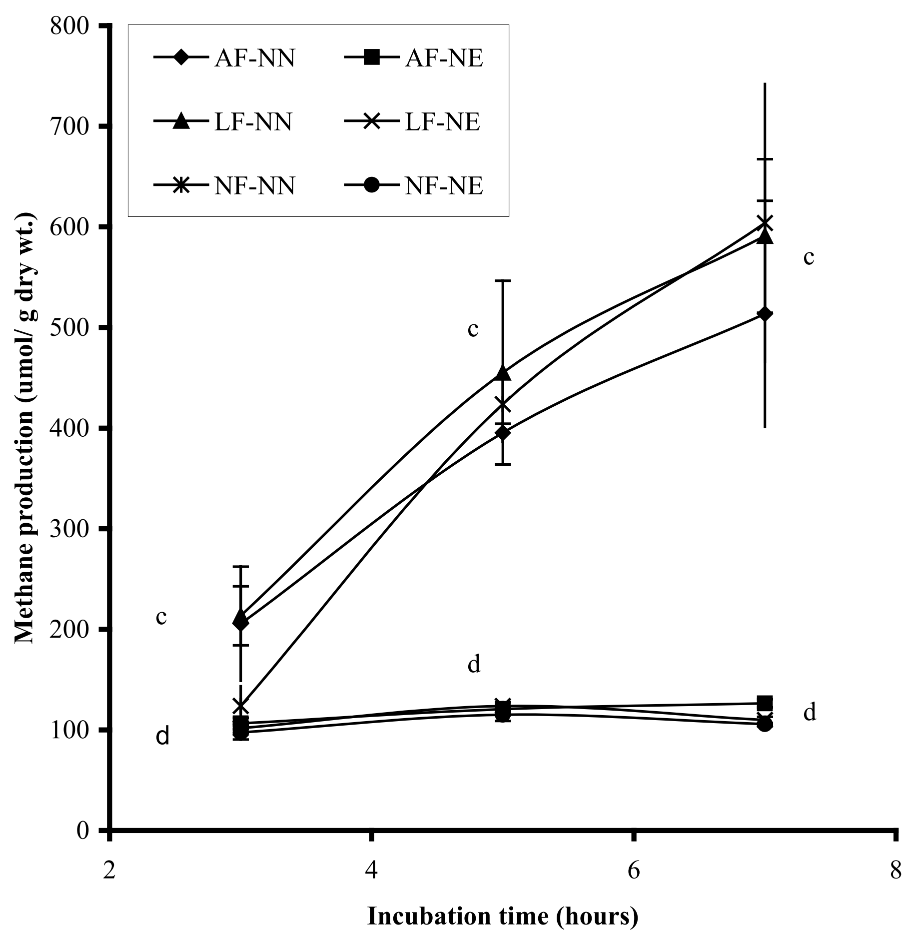

Figure 4.5 Methane production in in vitro chicken ceca (Experiment 7). Key: alfalfa (AF); layer feed (LF); nitroethane (NE); no feed material (NF); no nitrocompound (NN). [c,d] Means within same columns with unlike superscripts differ ($P < 0.05$).

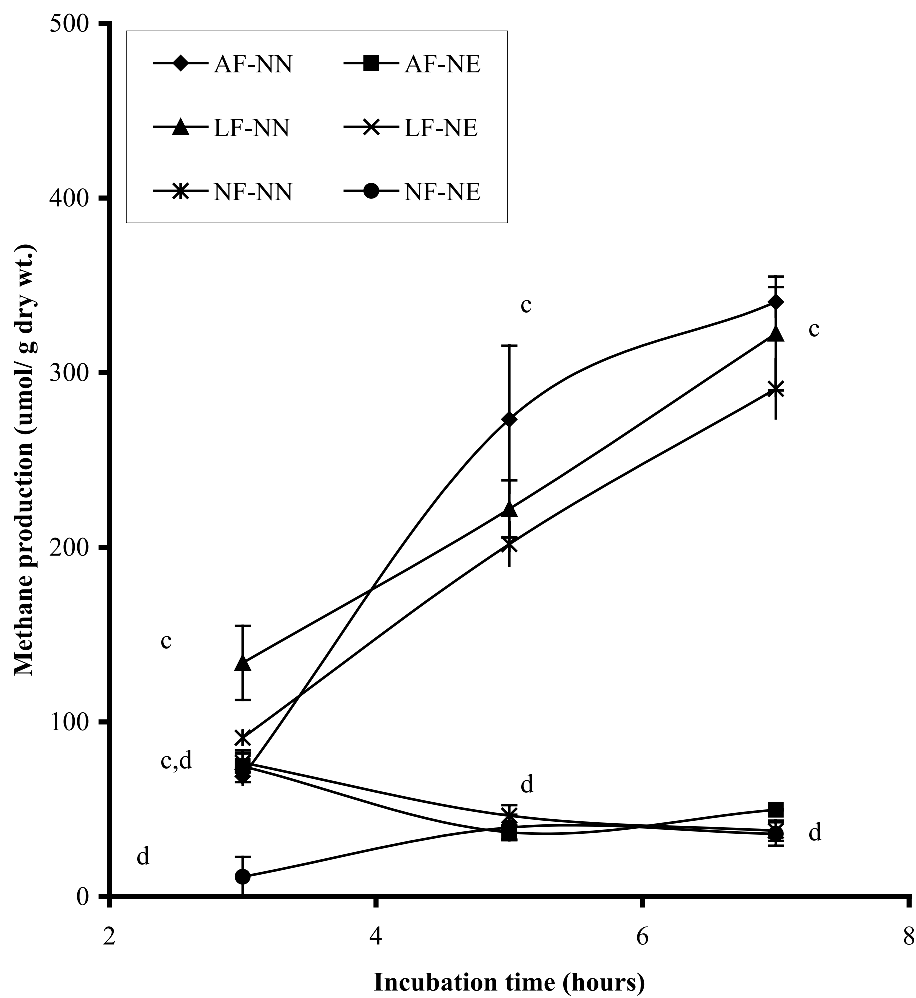

Figure 4.6 Methane production in in vitro chicken ceca (Experiment 8). Key: alfalfa (AF); layer feed (LF); nitroethane (NE); no feed material (NF); no nitrocompound (NN). [c,d] Means within same columns with unlike superscripts differ ($P < 0.05$).

Among these three animal species, in vitro methane production in chicken ceca was the lowest. In general, methane production by monogastric animals is lower than methane production by ruminants (Jensen, 1996).

Nitroethane clearly impeded methane production, especially in incubations of chicken cecal contents. One reason is that the microbial diversity harbored in chicken ceca is minimal. From our preliminary work (Saengkerdsub et al., 2005), only 16S rDNA of *Methanobrevibacter woesei* was obtained from chicken ceca, while 65 phylotypes belonging to order *Methanobacteriales* have been sequenced from ovine rumen (Wright et al., 2004). Moreover, in the bovine rumen, three clusters of methanogens have been identified (Whitford et al., 2001).

Cell wall permeability in gram-negative bacteria is a mechanism that protects them from bile acid (White, 2000). The archael cell wall of *Methanobrevibacter woesei* may behave in a similar fashion to gram-negative eubacteria cell walls since *Methanobrevibacter woesei* can tolerate bile acid (Miller and Lin, 2002). As previously discussed, gram-negative bacteria are more susceptible to nitroethane and this may be a reason why nitroethane can more efficiently reduce methane in in vitro incubations of chicken cecal contents.

Three nitrocompounds can reduce in vitro methane production in chicken ceca. However, nitroethane gave the best results. Therefore, this nitrocompound was chosen for comparative studies. For comparison, three gastrointestinal microbial sources (i.e. chicken ceca, bovine rumen, and ovine rumen) were used to study the ability of nitroethane to reduce methane production. However, only chicken ceca results showed

complete inhibition. One reason might be the methanogens harbored in chickens are not as diverse as those found in ruminants. Moreover, the mechanism of nitroethane inhibition is currently unknown. Further studies will be necessary to more fully elucidate the toxicity, the spectrum of inhibitory activity, and the mode of action of nitroethane. In addition, in vivo studies are needed to determine if methane formation is inhibited in the bird by addition of these nitrocompounds and whether shifts in fermentation influence bird physiology.

CHAPTER V

ESTABLISHMENT OF METHANOGENIC ARCHAEA IN CHICKEN CECA

Introduction

A complex diverse microbial community plays an important role in the health and well-being of the host and harbors in the gastrointestinal (GI) tracts of animals. When animals are born, the intestinal tracts are sterile and are successively colonized by microorganisms from the mother and the surrounding environment. Microbial diversity in intestinal tracts becomes more complex as the host gets older. Previous reports using culture and molecular techniques indicates that the cecal flora evolve from a simple to a more complex mixture as a chicken ages. Barnes et al. (1972) and Salanitro et al. (1974) observed that the microbial community structure in chicken ceca varies with age. In chickens, the gastrointestinal tract becomes rapidly colonized by bacteria with maximum bacterial densities achieved within the first 5 d after hatching. During the following weeks, the composition of microflora changes markedly (Apajalahti, 2005). Recently, molecular approaches have provided ways to directly observe microbial diversity in the gastrointestinal tract without culture. Hume et al. (2003), using DGGE method, found 8 and 26 major bands from chicken cecal samples at 2 and 32 days of age.

Methanogens, a sub-group of the archaebacteria, have also been isolated from various animals (Miller and Wolin, 1986; Miller, Wolin, and Kusel, 1986). Several studies showed that methanogens establish in intestinal tracts when animals are young. Methanogen densities reach 10^4 and 10^9 per gram in rumen fluid of grazing lambs at 1

and 3 weeks of age, respectively (Skillman et al., 2004). Morvan et al. (1994) found that methanogens colonize in the rumen of lambs 30 hours after birth and reach 10^6 per ml at 15 days. In the rat, the concentration of methanogens increases from 10^5 per gram dry weight at 3 weeks of age (shortly after weaning) to 10^9 per gram dry weight at 96 weeks of age (shortly before the end of the life span) (Maczulak et al., 1989). Rutili et al. (1996) showed that methanogens in fecal samples obtained from children under 27 months were not detected and found 40 and 60% of fecal samples from the 3 and 5 years old children, respectively.

In contrast to other anaerobes in the chicken ceca, studies showing of the presence of methanogens are still scarce. One in vitro ceca study observed methane gas when the chicks were 2 months old (Marounek and Rada, 1998). Zhu and Joerger (2003), using FISH method, found that methanogens become established in ceca of very young chicks.

In this study, we describe methanogen colonization in the cecal ecosystem of broiler chicks that were fed a corn-soy diet during 1 to 26 days of age. We detected methanogens in fecal samples based on culture method by using Balch 1 medium supplemented with rumen fluid and additional NH_4Cl (Balch et al., 1979; Miller and Wolin, 1982) and quantified 16S rDNA copy number of methanogenic archaea by using real-time PCR based on primers MBT (Yu et al., 2005). An understanding of the development of methanogen community may allow us to manipulate fermentation characteristics and ecological balance in chicken ceca.

Materials and Methods

Animal sampling

One hundred and twenty 1-day-old commercial broiler chicks, were placed on sawdust bedding and used as the source of bacteria for DNA extractions. These chicks were maintained on a layer feed ration. The composition of the Texas A&M University (TAMU, College Station, TX) layer feed ration was (%): corn 56.72; soy bean meal 31.63; vegetable oil 7.68; monocalcium phosphate 1.69; calcium carbonate 1.56; methionine (98 %) 0.17; vitamin premix 0.25; NaCl 0.25; trace mineral premix 0.05. All chicks were divided into twelve groups designated as groups 1-12. Each group was composed of ten chicks and feces from all 12 groups were collected on days 5, 9, and 12. The fecal specimens were also subjected to bacteriological cultivation and real-time PCR for the detection of methanogens. Only samples from groups 3,5,and 7 were collected on days 3 and 4 for methanogen detection using real-time PCR. Houseflies (*Musca domestica*) and litter in the bird house were collected one time to measure methanogens using the same method as fecal samples. Dust and layer feed ration were also collected for bacterial cultivation.

DNA extraction

Bacterial genomic DNA was isolated by the method of Wright et al. (1997) with some modifications. Fecal samples were suspended in TE buffer and treated with proteinase K for 1 h at 37 ^{o}C, followed by five cycles of freezing at –80 ^{o}C for 1 h and heating in a water bath at 65 ^{o}C for 30 min. The lysate was treated with CTAB/NaCl. The CTAB was extracted with an equal volume of chloroform-isoamyl alcohol (24:1),

mixed, and centrifuged at 7,000 g for 5 min. The DNA solution was transferred to a new microcentrifuge tube with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), mixed and centrifuged at 7,000 xg for 5 min and isopropanol precipitation. The extracted DNA was further purified with a Dneasy® Tissue kit (Quigen, Valencia, CA). The DNA solution was stored at −20°C.

Methanogenesis detection

Fecal samples were transferred and mixed together in an anaerobic glove box maintained in an atmosphere of 95% N_2/ 5% H_2. The fecal samples were added into a serum tube contained 9 ml of modified Balch 1 medium (Miller and Wolin, 1982). The tubes were removed from the glove box after being sealed with stoppers and aluminum caps. Each tube was flushed with 80% H_2/ 20% CO_2 under 200 kPa. The bottles were incubated standing at 37 ^{o}C and mixed one time per day by hand. After 20 days, methane was determined in the headspace gas by GC (SRI, model 8610C, Torrance, CA). Tubes with methane concentrations greater than 100 ppm (μg/ml) were counted positive for the presence of methanogens in fecal samples. The fecal samples were stored at –80 ^{o}C until extracted DNA as described above.

Quantitative PCR assays

Calibration standards for the quantitative PCR assays were developed with a 10-fold dilution series of plasmid containing sequence CH101 closely related to *M. woesei* GS. Plasmid copy number was calculated from plasmid molecular weight, and plasmid concentration was measured with Picogreen (Molecular Probes, Eugene, OR) with a Spectrafluor Plus (Research Triangle Park, NC). The quantitative PCR reaction and

condition were followed as described in Yu et al. (2005). All PCR reactions were performed in triplicate in the same run.

Results and Dicussion

In the present study, methanogenic archaea were recovered during culture in BRN medium (Balch et al., 1979; Miller and Wolin, 1982) and methane was found to occur later in the headspace of the cultures. Concentrations of methane greater than 100 ppm (μg/ml) in the headspace were considered positive for the presence of methanogens in fecal samples (Table 5.1). The copy number of methanogenic16S rDNA present in samples, as measured by using primers MBT specifically designed to the order Methanobacteriales (Yu et al., 2005). From our previous study, we isolated bacterial genomic DNA directly from chicken ceca and found that the predominant methanogen was *Methanobrevibacter woesei*, which belongs to the order Methanobacteriales. The plasmid containing 16S rDNA gene of the primary phylotype in chicken ceca was used as the standard in real-time PCR reactions. All PCR reactions were performed in triplicate in the same run (Table 5.1).

The copy number of methanogenic 16S rDNA gene in chicken feces ranged from $\log_{10}$ 4.19 to 5.05 per gram wet weight when the broilers were 3, 4, 5, 9, and 12 days of age (Table 5.1). Methanogens in litter and house flies collected in the bird house were $\log_{10}4.94 \pm 0.10$ and 5.51 ± 0.11 16S rDNA copy number per gram wet weight, respectively.

Table 5.1 The log_{10} copy numbers of 16S rDNA methanogenic archaea in chicken fecal samples.

Group	Day				
	3	4	5	9	12
1	ND[a]	ND	4.60 ± 0.01 [b] (-) [c]	4.62 ± 0.05 (-)	5.34 ± 0.14 (+)
2	ND	ND	4.63 ± 0.03 (-)	4.68 ± 0.05 (+)	4.54 ± 0.14 (+)
3	4.53 ± 0.03	5.03 ± 0.02	4.85 ± 0.07 (-)	4.30 ± 0.04 (+)	4.61 ± 0.05 (+)
4	ND	ND	4.97 ± 0.02 (+)	4.59 ± 0.09 (+)	4.73 ± 0.07 (+)
5	4.51 ± 0.02	4.57 ± 0.02	4.87 ± 0.02 (-)	4.86 ± 0.11 (+)	4.72 ± 0.06 (+)
6	ND	ND	4.92 ± 0.04 (+)	4.50 ± 0.06 (+)	4.89 ± 0.03 (+)
7	4.50 ± 0.06	4.62 ± 0.06	4.70 ± 0.02 (-)	4.52 ± 0.04 (-)	4.80 ± 0.06 (+)
8	ND	ND	4.82 ± 0.12 (-)	4.42 ± 0.04 (-)	4.96 ± 0.04 (+)
9	ND	ND	5.03 ± 0.04 (-)	5.05 ± 0.18 (+)	4.81 ± 0.01 (+)
10	ND	ND	4.37 ± 0.05 (-)	4.19 ± 0.05 (+)	4.72 ± 0.06 (+)
11	ND	ND	4.19 ± 0.04 (+)	4.48 ± 0.14 (-)	4.86 ± 0.11 (+)
12	ND	ND	4.27 ± 0.08 (-)	4.80 ± 0.06 (+)	4.75 ± 0.01 (+)

[a] ND, not determined.

[b] Values are the mean ± standard deviation of triplicate in the same RT-PCR reactions.

[c] +/- in the parenthesis represent methane production based on BRN medium.

Methane gas was also observed in headspace of the tubes inoculated with litter and flies. However, incubations with dust and layer feed ration collected from the house were not produced methane gas in headspace. Methanogenesis was observed in culture tubes of 25, 67, and 100 % of the samples at 5, 9, and 12 days of age, respectively. We found methane gas in headspace from 5-day fecal samples and the percentage of positive results was dramatically increased when the broilers were 9 and 12 days of age. Results of this study showed that methanogens rapidly colonized in chicken ceca. The results from one study agreed with this study that methanogens establish in ceca of very young chicks (Zhu and Joerger, 2003). In addition, obligate anaerobes become dominant in the chicken cecum after the first few days of life (Mead and Adams, 1975). One in vitro ceca study, on the other hand, observed methane gas when the chicks were 2 months old (Marounek and Rada, 1998). The failure to detect methane gas in in vitro cecal fermentation from one-month chicks might have been due to the lack of hydrogen gas during incubation atmosphere that would support the reducing CO_2 to CH_4. In addition, the chicken cecal samples were incubated for only 20 h. According to Nottingham and Hungate (1968), methane could be detected from the lowest dilution of human fecal samples after 2 days of incubation; but 20 to 30 days were required for it to appear in easily measurable amounts in the highest positive dilution.

An important finding in this study is that methanogens colonized in chicken ceca before the full development of the gastrointestinal tract. Generally, the gastrointestinal tract of the chick is sterile when the bird is hatched. However, bacterial colonization occurs with in a few hours and the dominant bacterial community becomes more

complex as the chickens age (van der Wielen et al., 2002). According to van der Wielen et al. (2002) and Lu et al. (2003) almost microflora in chicken ceca were similar to those in ilea in 3-day broilers.

Enterococcus spp. and *Ruminococcus* spp. might play an important role in methanogenic settlement in the chicken ceca. Morvan et al. (1996) and Robert et al. (2003) suggested that these cellulolytic organisms might play a role in the development of a methanogenic community in the gut by providing substrates for methanogens. These microorganisms were found in cecal samples when the chicks were 3-day old (Lu et al., 2003a). In addition, the abundance of *Ruminococcus* spp. was increased from 3-14 days, and after 14 days the percentage of *Ruminococcous* spp. was 16% of the total clones in chicken ceca (Lu et al., 2003a). In contrast, only *Enterococcus* spp. were found when the chicks were 3 days of age in ileal samples, and after 21 days of age, *Enterococcus* spp. was only 3% of the total flora (Lu et al., 2003a). A strain of *Ruminococcus flavefaciens*, a hydrogen-producing, cellulolytic bacterium which is known to form synthrophic associations with methanogens (Wolin et al., 1997), was isolated from the rumen of lamb one day after birth (Skillman et al., 2004). In humans, the presence of certain fibrolytic species (cellulolytic isolates related to *Enterococcus faecalis*, *Ruminococcus* spp.) was related to the presence of methanogens (Robert and Bernalier-Donadille, 2003).

Litter and flies might be vehicles for transmitting methanogenic archaea in these birds. Bacteria in cultures of poultry litter were to be as higher as 10^9 CFU/g of material and some primary microorganisms were microaerobic bacteria (Lu et al., 2003b). The presence of methanogens in litter might be due to the residual presence of these

organisms from previous flocks. The presence of methanogens in fly samples might be due either to methanogen contamination from chick feces or methanogen colonization in this insect. House flies carry heterogeneous mixtures of organism and have been considered to be a source of *Campylobacter* colonization in broiler chickens (Newell et al., 2003; Ekdahl et al., 2005; Nichols, 2005). Methanogens, however, might be normal flora in the house fly. *Methanobrevibacter* spp. have been isolated from the cockroach hindgut (Gijzen et al., 1991) and the hindgut content of termite *Reticulitermes flavipes* (Kollar) (Rhinotermitidae) (Miller, 2001). Further studies are needed to more fully elucidate the source(s) of methanogens that serve as consistent inoculation reservoirs for chickens.

CHAPTER VI

CONCLUSIONS

The results of this study provide evidence indicating that the methanogenic diversity in chicken ceca is minimal. Methanogenic communities were composed of eleven phylotypes; however, only one phylotype belonging to *Methanobrevibacter woesei* GS was dominant. By using a MPN enumeration method, the numbers of methanogens in chicken ceca were found to be approximately that in horse and pig ceca. These numbers were also similar to those obtained from quantitative PCR. From quantitative PCR data, the results suggested that methanogens in chicken ceca may have only one SSU rDNA copy per cell. This study reports the first identification of the methanogenic archaea 16S rDNA gene in chicken ceca and shows that the primary methanogen in chicken ceca is *Methanobrevibacter woesei*. In addition, the amount of methanogens in this ecosystem is as high as found in other animals.

Based on in vitro studies, feed material influenced VFA production and acetic was the primary component. We also found that incubations with nitroethanol and 2-nitropropanol had significantly ($P < 0.05$) higher propionate concentrations than incubation with added nitroethane. Layer feed supplement produced more butyrate than alfalfa addition. These feed materials fostered, while nitrocompounds retarded, in vitro methanogenesis in chicken ceca. Unlike cecal contents, layer feed significantly ($P < 0.05$) supported in vitro methane production in incubations of both rumen fluids. The

results show that nitroethane impedes methane production, especially in incubation of chicken cecal contents.

Also, I found that methanogenesis in culture tubes presented in fecal samples when broilers were 5 days old and the number of positive tubes increased when the birds aged. However, the copy number of 16S rDNA methanogens was similar for 3- to 12-day old broilers. The results of this study showed that methanogens rapidly colonize in young chicken. An important finding in this study is that methanogens colonized before the full development of gastrointestinal tract. Litter and house flies collected from the broiler house contained 16S rDNA methanogenic copy numbers similar to those in fecal samples. Litter and flies may be the potential source of methanogenic colonization in chicken cecum.

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VITA

NAME: Suwat Saengkerdsub

Email Address: saengsuwat@yahoo.com

EDUCATION:

B.S. in Marine Science, Chulalongkorn University, Thailand, 1997.

M.S. in Industrial Microbiology, Chulalongkorn University, Thailand, 2000.

Certification in Microbiology and Biotechnology, Osaka University and Tohoku University, Japan, 2001.

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