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Full Length Research Paper

Diversity of methanogens in the hindgut of grower and finisher pigs

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This study examined the diversity of the methanogens in the hindgut of two different weight groups of pigs and correlated it with the amount of digested organic carbon (OC) and various components of dietary fiber. Five grower (58.9 ± 1.15 kg) and five finisher (89.4 ± 0.85 kg) Duroc × Landrace × Large Yorkshire female pigs were allocated into two groups and individually housed in cages. During the experiment, feed intake and fecal output were recorded for determination of apparent digestibility of OC, crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF). At the end of the digestibility trial, pigs were sacrificed, and the contents of five segments of hindgut were sterilely collected to determine diversity of methanogens. Total microbial DNA of the hindgut contents was used as template for amplification of the methanogen16S rRNA gene, and the PCR products were further subjected to denaturing gradient gel electrophoresis (DGGE) analysis. Results show that the number of DGGE bands and Shannon diversity index for the 90 kg pigs were higher (P<0.05) than those for the 60 kg pigs. Methanogen communities did not alter along the different segments of the hindgut for the two weight groups. In addition, the amount of OC, CF, NDF and ADF digested (g/d) for the 90 kg pigs (1018.77, 23.11, 268.86 and 99.16, respectively) was higher (P<0.05) than the respective values for the 60 kg pigs (669.27, 13.77, 222.31 and 69.07), indicating that the higher diversity of methanogens in the former group was related to the higher quantity of fiber materials fermented in the hindgut. The positive correlation (p<0.05) between number of DGGE bands and Shannon diversity index with quantity of digested OC and ADF further reaffirmed the above suggestion.

Key words: Methanogen, pig, Shannon diversity index, polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

INTRODUCTION

There has been an intense interest in rumen methanogenic archaea because hydrogen is used by methanogen to reduce carbon dioxide (CO_2) to methane (CH_4) gas. It is well documented that diet influenced diversity and population of a wide range of bacterial species (Tajima et al., 1999, 2000; Kocherginskaya, et al., 2001) and methanogen (Zhou et al., 2007; Zhou et al., 2010) in the rumen. Due to its contribution as a greenhouse gas and loss of dietary energy for the host animals, enteric CH_4 production from ruminant livestock has been extensively studied (Johnson and Johnson, 1995; Lassey et al., 1997; Moss et al., 2000; Lassey, 2007; Andy Thorpe, 2009). On the other hand, studies on enteric CH_4 emission from pigs are scarce (Jørgensen, 2007; Ji et al., 2011). However, due to the large population of pigs, particularly in China, enteric CH_4 emission, which has been estimated to represent a 1.2% loss of the ingested energy (Monteny et al., 2001) from hindgut fermentation in pigs, cannot be ignored. In addition, pigs

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Abbreviations: OC, Organic carbon; **CF,** crude fiber; **NDF,** neutral detergent fiber; **ADF,** acid detergent fiber; **PCR,** polymerase chain reaction; **DGGE,** denaturing gradient gel electrophoresis.

are an appropriate animal model for gastrointestinal micro-ecological studies of monogastric animals including humans.

Butine and Leedle, (1989) quantified methanogens in cecal and colonic contents of pigs using ruminal fluid-based broth medium and reported that the quantity of methanogens in colonic sample was 30 folds higher than those in the cecum without determining their diversity. Although methanogenic archaea community in pig feces and in anaerobic bioreactors fed with pig feces were studied using different molecular techniques (Ufnar et al., 2007; Liu et al., 2009; Zhu et al., 2011; Mao et al., 2011), we do not know of any published data on diversity of methanogens in different segments of larger intestine (the major site of feed fermentation in pigs) of pig using polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE).

Methanogens are phylogenetically placed exclusively as members of the domain archaea (Woese, 1987). Due to the fact that methanogens are strictly anaerobes and difficult to isolate and culture, phenotypic characters are often insufficient for their identification (Woese et al., 1990) and thus molecular ecology techniques, such as 16S rRNA gene clone libraries, 16S rRNA gene fingerprinting including PCR-DGGE, quantitative real-time PCR, fluorescent *in situ* hybridization (FISH) and DNA microarray have been used in studies of gastrointestinal microbial communities (Amann et al., 1992).

PCR-DGGE technology was used for the first time to analyze microbial diversity in soil by Muyzer, et al. (1993), and then applied in micro-ecological studies of animal gastrointestinal tract (Tannock et al., 2000; McCracken et al., 2001; Donskey et al., 2003). The advantage of PCR-DGGE is its simplicity, that is, it can rapidly monitor the spatial-temporal variability of microbial populations by analyzing bands that migrates separately on DGGE gel to study the structure and composition of intestinal microbes without using the conventional cultivation procedures.

This study was designed to achieve three objectives: (i) To determine and compare the diversity of methanogens in hindgut between two different weight groups of pigs using PCR-DGGE technique; (ii) to examine whether methanogen communities alter along the different segments of the hindgut, and (iii) to correlate the amount of digested dietary fiber with methanogenic diversity in hindgut of pigs.

MATERIALS AND METHODS

Animals and feeding

Five grower (mean body weight of 58.9 ± 1.15 kg) and five finisher (89.4 ± 0.85 kg) Duroc × Landrace × Large Yorkshire female pigs, purchased from a commercial farm near Guangzhou, south China, were used for this study. The pigs were randomly assigned into individual cages (2.0 m long × 1.0 wide) with five animals as replicates per weight group. The experimental pigs were fed *ad libitum* with the same commercial diet as pigs in the respective weight groups in the farm, twice daily at 07.00 and 19.00 h. The composition and nutrient content of the experimental diets are shown in Table 1. The study, carried out during winter with mean outdoor temperature of 20.3°C and the indoor temperature of 23.7°C, consisted of 11 days of adaptation and three days measurement of diet digestibility. Pigs were weighed on day one and seven of the experimental period. Fresh drinking water was available at all time.

Digestibility and sampling of intestinal content and feces

During the digestibility trial, daily fecal output of each pig was collected, weighed and sampled (200 g) and stored at -20°C. Fecal samples were separately dried at 60°C for 72 h and ground through 1 mm and followed by 0.45 mm sieve and the three days fecal samples were pooled for individual pig for determination of their nutrient contents, that is, organic carbon (OC), crude fiber (CF), acid detergent fiber (ADF) and neutral detergent fiber (NDF). Nutrient apparent digestibility was calculated as:

Apparent digestibility (%) = (nutrient intake – nutrient excretion) / nutrient intake ×100%

and

The amount of digested nutrient (g/d) = (nutrient intake – nutrient excretion).

At the end of the digestibility trial, 10 pigs were sacrificed, approximately 3 h after feeding. The whole gastrointestinal tract was immediately excised; cecum, colon (proximal, medium and distal) and rectum were ligated and their contents sterilely collected separately into 50 ml sterile centrifuge tubes, and immediately stored at -20°C for later determination of diversity of methanogens using DGGE procedure.

Chemical analysis

CF, ADF and NDF were determined according to Van Soest et al. (1991) using F57 filter bag in an Ankom Fiber analyzer (Ankom220 Fiber Analyzer, ANKOM Technology, USA). OC of diet and feces was analyzed following the method of Bao (2000).

DNA extraction and PCR amplification

The total microbial DNA isolation from large intestinal contents were extracted using the E.Z.N.A. stool DNA kit (Omega Corp, USA) following the procedure provided by the manufacturer. Total DNA obtained was used as a template for PCR amplification of small subunit rRNA gene sequences from the large intestinal contents for domain archaea community.

The universal primer pair (Wu et al., 2001) A934F (5'-AGGAATTGGCGGGGGAGCA-3') and 1390R-gc(5'-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGCG GTGTGTGCAA-3', with the underlined sequences are the GC-clamp region) were used for PCR to amplify 16S rRNA gene from members of the domain archaea with a PCR C1000^{TM} thermal cycler (Bio-Rad Laboratories, Inc., USA), using the following program: Initial denaturation for 5 min at 94°C; 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 2 min; and final extension for 10 min at 72°C.

DGGE analysis of methanogens

The aforementioned PCR products were subjected to DGGE using Bio-Rad D-Code system. PCR products were separated using a

Table 1. Ingredient (%) and chemical composition of the diets for two weight groups of pigs.

The composition and nutrient content of diet	Diet 1 (60 kg grower pigs)	Diet 2 (90 kg finisher pigs)
Composition		
Corn (%)	66	69
Bean meal (%)	23	20
Rapeseed meal (%)	4	4
DDGS[a] (%)	3	3
Premix[b] (%)	4	4
Nutrient content		
Gross energy (MJ/kg)	13.81	13.39
Organic carbon (%)	47.04	46.40
Crude protein (%)	17.00	16.10
Lysine (%)	0.87	0.84
Met+Cys (%)	0.54	0.51
Calcium (%)	0.60	0.53
Phosphorus (%)	0.50	0.45
Available phosphorus (%)	0.24	0.19
Crude fiber (%)	3.90	3.77
Neutral detergent fiber ((%)	17.97	16.23
Acid detergent fiber (%)	7.99	7.04

[a]DDGS, soluble distiller's dried grains. [b]Commercial premix consists of trace elements (Fe, Cu, Zn, Mn, I and Se), vitamin (A, D, K, E, B_1, B_2, B_6, B_{12}, C, folic acid and biotin), amino acids (lysine, methionine), Ca, P and salts.

6.5% polyacrylamide gel in one Tris-base, acetic acid and EDTA (TAE) buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA) with a 40 to 65% linear denaturing gradient. The gels were initiated by pre-running for 10 min at 200 V and subsequently ran at 80 V for 21 h at 60°C. Then the gels were stained for 10 to 15 min with SYBR Green and photographed using UV transillumination.

Gel images were analyzed using Labwork 4.0 image analysis software. Bands in DGGE fingerprints were automatically identified. Lanes were individually converted to filled plots by the program. After a background correction was made, the intensity of each band was measured by integrating the area under the peak and expressing the total area in the lane in percentage, and then the Shannon diversity index of different lanes were calculated. The formula of Shannon diversity index is as follow:

$$H' = -\sum_{i=1}^{S} p_i \ln p_i$$

where, S = numbers of band per lane, and P_i = ratio of intensity of each band / total bands.

Statistical analysis

Data were analyzed using SPSS 15.0 (2005). T-test was used to compare treatment means. Pearson correlation was adopted to analyze correlation of parameters, and 0.05 level of probability was used to identify differences.

RESULTS

16S rRNA gene fragments of methanogens were amplified by A934f/1390r primer using total microbial DNA as template, and all PCR products were 500 bp. PCR products were then used for DGGE analysis. PCR-DGGE profiles obtained from the large intestinal content samples of 60 and 90 kg pigs are presented in Figures 1 and 2 and Table 2.

There were 5 DGGE bands for the 60 kg pigs and the average Shannon diversity index ranged from 1.33 to 1.38 between cecum to rectum. The higher Shannon diversity index indicated higher diversity. The Shannon diversity index of distal colon was lowest (1.33), followed by rectum (1.34), proximal colon (1.35), cecum (1.36) and medium colon (1.38) the highest. However, no differences ($p>0.05$) were observed in the number of band and Shannon diversity index among the five segments, indicating no differences in diversity of methanogens throughout in the length of hindgut for the 60 kg pigs.

For the 90 kg pigs, the averaged number of DGGE bands in hindgut was 7.48 and Shannon diversity index ranged from 1.45 to 1.69 for cecum to rectum. The mean number of DGGE bands and Shannon diversity index for rectum (6.47 and 1.45) were significantly lower ($p<0.05$) than the other four segments of hindgut, which were not statistically different ($P>0.05$). Results of T-test (Table 2) show that the number of DGGE bands and Shannon diversity index of 90 kg pigs were higher ($p<0.05$) than 60 kg pigs in four segments except rectum.

Only apparent digestibility of OC was higher ($p<0.05$) for the 90 kg pigs compared to the 60 kg pigs (92.05% vs. 89.01%), while no differences were detected in the

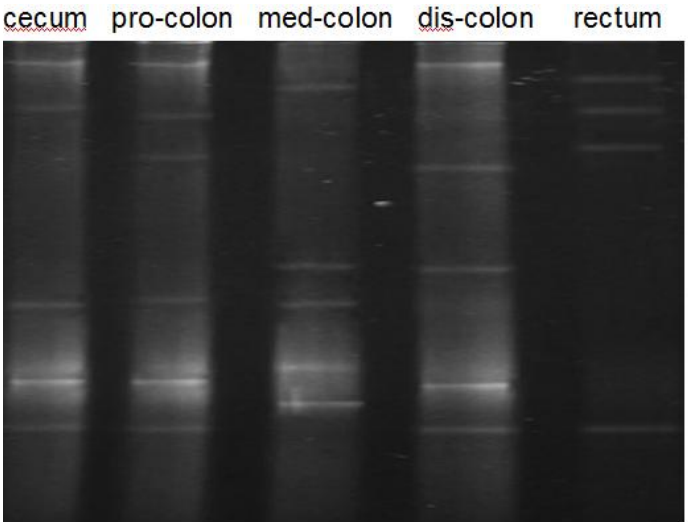

Figure 1. DGGE profiles of 60 kg pig. DGGE, Denaturing gradient gel electrophoresis.

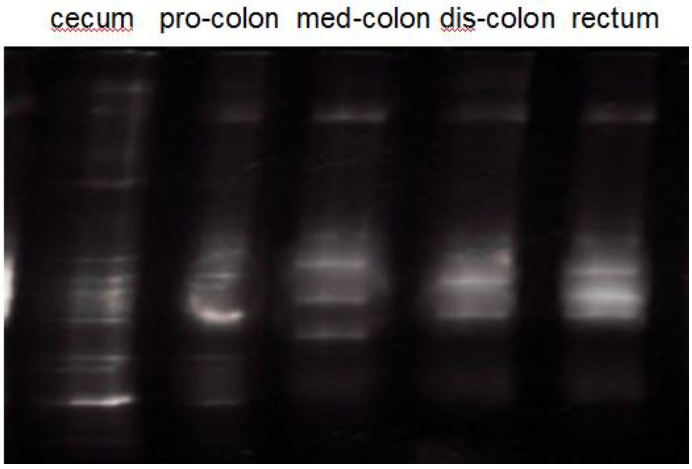

Figure 2. DGGE profiles of 90 kg pig. DGGE, Denaturing gradient gel electrophoresis.

apparent digestibility of the other nutrient between the two age groups. As expected (due to the heavier weight) the amount of digested OC, CF, NDF and ADF (g/d) for 90 kg pigs was significantly higher ($p<0.05$) than those for 60 kg pigs (Table 3). Results of correlation studies show that the quantity of digested OC ($p<0.01$) and ADF ($p<0.05$) significantly correlated with number of DGGE bands and Shannon diversity index (Table 4). However, digested NDF only correlated ($p<0.05$) to number of DGGE bands but not with Shannon diversity index for the two weight groups (Table 4).

DISCUSSION

The number of methanogenic PCR-DGGE profiles from steer fed with different diets were reported to range from 22 to 28 (Zhou et al., 2010) and 13 for swamp buffaloes fed CH_4 mitigating agents, such as coconut oil and garlic powder (Kongmun et al., 2011) while the present results recorded only between four to eight bands for pigs. Mao et al. (2011) assessed the diversity of methanogens in feces of pig by constructing the 16S rRNA gene clone libraries using primers Met86F and Met1340R and reported clones consisting of 10 phylotypes which belonged to three monophyletic groups. The lower value recorded for pigs could be because, being monogastric animals, most of the ingested feed are digested in the small intestine leaving only the undigested feed to undergo fermentation in the hindgut. This is in accordance with the much lower CH_4 production in pigs (Ji et al., 2011) compared to ruminants (Yamaji et al.,

Table 2. Number of band and Shannon diversity index between five segments of 60 and 90 kg pigs.

Segment / index	Cecum	Pro-colon	Med-colon	Dis-colon	Rectum
Number of DGGE band for 60 kg	4.33±0.33^{B}	5.13±0.13^{B}	5.26±0.18^{B}	5.07±0.27^{B}	5.33±0.38
Number of DGGE band for 90 kg	8.00±0.28^{aA}	7.67±0.61^{aA}	7.88±0.48^{aA}	7.40±0.60^{aA}	6.47±0.26^{b}
Shannon diversity index for 60 kg	1.36±0.01^{B}	1.35±0.05^{B}	1.38±0.04^{B}	1.33±0.05^{B}	1.34±0.04
Shannon diversity index for 90 kg	1.69±0.07^{aA}	1.67±0.01^{aA}	1.67±0.05^{aA}	1.59±0.04^{aA}	1.45±0.11^{b}

^{a,b}, Different superscripts within the same row differed significantly (P<0.05) . $^{A, B}$, Different superscripts within the same column differed significantly (P<0.05).

Table 3. Digestibility and daily quantity of OC, CF, NDF and ADF digested for 60 and 90 kg pigs.

Parameter (kg)	Organic carbon	Crude fiber	Neutral detergent fiber	Acid detergent fiber
Digestibility (%)				
60	89.01±0.85^{a}	22.09±2.45	77.36±3.32	54.10±3.31
90	92.05±0.60^{b}	25.71±2.99	69.46±2.66	59.06±2.88
Digested nutrients (g/d)				
60	669.27±5.94^{a}	13.77±1.54^{a}	222.31±9.97^{a}	69.07±4.08^{a}
90	1018.77±6.66^{b}	23.11±2.67^{b}	268.86±9.98^{b}	99.16±4.77^{b}

$^{a, b}$Different superscripts within the same column for the same parameter differed significantly (P<0.05).

Table 4. The correlation of number of band and Shannon diversity index with amount of digested OC, CF, NDF and ADF (g/d).

Parameter	Digested OC (g/d)	Digested CF (g/d)	Digested NDF (g/d)	Digested ADF (g/d)	Number of band	Shannon diversity index
Digested OC (g/d)	1.000					
Digested CF (g/d)	0.740*	1.000				
Digested NDF (g/d)	0.757*	0.796**	1.000			
Digested ADF (g/d)	0.882**	0.648*	0.696*	1.000		
Number of band	0.910**	0.603	0.740*	0.814*	1.000	
Shannon diversity index	0.868**	0.652*	0.582	0.669*	0.878**	1.000

*p<0.05; **p<0.01. OC, Organic carbon; CF, crude fiber; NDF, neutral detergent fiber; ADF, acid detergent fiber.

2003; IPCC, 2007; Zhou et al., 2007). Liu et al. (2009) studied structure of the bacterial and archaeal community in a biogas digester using pig manure as substrate obtained from nine archaeal bands in DGGE profile. The aforementioned finding further reaffirmed the lower diversity of methanogens in pigs compared to ruminants.

We must emphasize that this study did not carry out gene sequence analysis of the DGGE bands to identify the species of methanogens in the large intestinal content of pigs, thus the diversity of methanogens could have been over-or underestimated. However, we believe that the simultaneous use of DGGE band and Shannon diversity index procedures is sufficient for the primary objectives of this study; that is, to compare the diversity of methanogens in the hindgut between two different weight groups of pigs as well as whether methanogen communities alter along the different segments of their hindguts.Fermentation of dietary fiber leads predominantly to the production of volatile fatty acids (VFA), gases (CO_2, H_2, and CH_4), ammonia and heat. Methanogens obtained their energy by reducing CO_2 to CH_4 using H_2 (produced by catalyzing the terminal step in this anaerobic digestion) as the electron donor. Results from our study suggest that the diversity of methanogens in hindgut of 90 kg pigs was higher (P<0.05) than that of 60 kg pigs. Based on the higher quantities of CF, NDF and ADF digested (g/d) in the heavier pigs (Table 3), the present results seem to suggest that the higher methanogens diversity in the 90 kg pigs was related to the larger quantity of fermented fiber materials in their hindgut. This is further supported by the fact that the number of DGGE bands and Shannon diversity index were significantly correlated (p<0.05) with the amount of digested ADF and OC (Table 4) for the two weight

groups. The aforementioed assumption is in agreement with previous study (Noblet and Goff, 2001) which reported that the ability of the pig to digest dietary fiber improved with the age and live weight and mainly due to changes in the composition of microbial population (without any reference to methanogens) of its hindgut. Similarly, Zhu et al. (1993) and Jensen and Jørgensen (1994) reported that the amount of CH_4 production increased with increasing fiber content in the diet. Recent study from our laboratory (Ji et al., 2011) showed that daily enteric CH_4 production from 90 kg (2.01 g/pig) was higher ($P<0.05$) than that from 60 kg (1.13 g/pig). Available information from the literature seems to support our view that diversity of methanogenic archaea increased with body weight and/or quantity of fermented materials in the high gut of pigs.

No regular pattern of change in the number of DGGE bands and Shannon diversity index among the different segments of the hindgut in the two weight groups was detected, thus indicating no differences in the diversity of methanogens along the hindgut of the two weight groups. It has been reported that the gastrointestinal tract bacterial community structure is susceptible to changes by the diet of the host animal (Durmic et al., 1998; Moore et al., 1987). For instance, bacterial community can adapt to the introduction of high levels of dietary fiber by increased growth of bacteria with cellulolytic and xylanolytic activities (Varel et al., 1987). However, Jensen and Jørgensen (1994) found that the density of microorganisms was quite constant throughout the cecum and hindgut for pigs received high and low fiber diets, and they suggested that CH_4 production from pig increased with increasing amounts of feed intake and dietary fiber in the diet because greater amount of undigested material would reached the hindgut and provided more substrates for the microorganisms to utilize. None of the afore-mentioned studies specifically referred to methanogens and thus could not be used for direct comparison with the present study.

In conclusion, the number of DGGE band and Shannon diversity index for 90 kg pigs were higher ($P<0.05$) than those for 60 kg pigs, thus suggesting higher diversity of methanogen in the hindgut of the heavier finisher pigs compared to the lighter grower pigs. However, no differences in the diversity of methanogen among the different sections of the hindgut were detected in both weight groups of pigs. The amount of digested OC, CF, NDF and ADF of the 90 kg pigs were higher ($P<0.05$) than those of the 60 kg group, indicating that the higher diversity of methanogen in the former group was due to the higher quantity of fiber materials fermented in the hindgut. The significant correlations ($p<0.05$) between number of DGGE band and Shannon diversity index with quantity of digested OC and ADF further reaffirmed the above suggestion. We do not know of any published data on diversity of methanogenic archaea in the different segments of large intestine of pigs for direct comparison with the results of the present study.

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