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ORIGINAL ARTICLE

EFFECT OF HUMAN GUT MICROBIOTA ON THE METHANE PRODUCTION

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Abstract Background and aim: The aim of this study is to investigate the relationship between methane production and gut microbiota in a general population.

Methods: Total of 697 subjects (58 ± 13 years, 261 men and 436 women) have participated in this study. Their breath methane and hydrogen concentration were measured after an overnight fasting using gas chromatography, and their gut bacterial component was analyzed using T-RFLP (Terminal restriction fragment length polymorphism) method. All gut microbiota were divided into 28 operational taxonomic units (OTUs) according to its predominant bacterial groups. The breath methane concentration of above 10ppm and breath hydrogen concentration of above 20ppm were considered positive. Subjects were divided into four groups; (1) Both negative: both methane and hydrogen were negative (2) CH_4 only: only methane was positive (3) H_2 only: only hydrogen was positive (4) Both positive: both hydrogen and methane were positive

Results: OTU317 (*Prevotella*) was significantly higher in CH_4 only than both negative and H_2 only. And OTU940 (*Clostridium* subcluster XIVa and Enterobacteriales) was significantly lower in CH_4 only than both negative and H_2 only.

Conclusions: OTU317 and OTU940 might affect the bacterial metabolism of methanogens.

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Key words: Breath methane concentration; terminal restriction length polymorphism; gut Microbiota; large intestine.

原　著

腸管内メタン産生に影響を与える腸内細菌叢検索

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抄録　一般住民697人(58 ± 13歳，男性261人，女性436)の腸内細菌叢とメタン産生の関係を調査した．一晩の絶食の後，呼気中メタン濃度と呼気中水素濃度測定，T-RFLP を用いた腸内細菌叢の測定を行った．腸内細菌は遺伝子長ごとに28の分類群(OTUs)に分類した．また呼気中メタン濃度が10 ppm より大きい者をメタン陽性，呼気中水素濃度が20 ppm より大きい者を水素陽性とし Both negative，CH_4 only，H_2 only，Both positive と群分けした．CH_4 only 群では Both negative 群と H_2 only 群に比べて OTU317(*Prevotella*)の比率が有意に高く，OTU940(*Clostridium* subcluster XIVa, Enterobacteriales)の比率は有意に低かった．以上より OTU317 と OTU940 はメタン産生に関わっている可能性が考えられた．

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キーワード：呼気メタン濃度；terminal restriction length polymorphism；腸内細菌；大腸．

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Introduction

The large intestine contains a complex and dynamic microbial ecosystem known as the gut Microbiota[1-2] with number of up to 10^{12} organisms within the gut. The first study of gut bacteria was conducted by Leeeuwenhock in 1674, where he found organisms in a gut sample by using a microscope he had developed by himself. Two hundred years later, Theodor Escherich cultured *Escherichia coli* in 1886. In the 1950s, further studies on gut bacteria were conducted, and anaerobes belonged to lactobacilli and bifidobacteria were cultured[3]. These studies revealed that an individual contains approximately 300-500 different species of bacteria, and the number of microbial cells within the gut lumen is about 10 times larger than the number of eukaryotic cells in the human body[4]. These bacteria play a major role in maintaining health, affecting and controlling immunity, nutrition, and disease[5-6].

It has been estimated that only 20 to 30% of total bacteria in large intestine can be cultivated in the laboratory[7]. Recently, many researchers have used various culture-independent analysis[8-9]. Terminal restriction fragment length polymorphism (T-RFLP) is an alternative molecular approach that allows the assessment of a diversity of complex bacterial communities. Nagashima et al [10] used 16rDNA clone library and T-RFLP to analyze human gut microbiota, and showed that they are predominantly composed of approximately ten phylogenetic bacterial groups. This method allows us to easily analyse the gut microbiota of large subject number, and thus was used in the current study.

In humans, hydrogen and carbon dioxide are generated from fermentation of dietary fiber and resistant starch by the gut microbiota. Hydrogen generated in the large intestine is expelled either as flatus or is absorbed and excreted as a breath via lungs[11].

Approximately 25% of normal populations have methanogenic microorganisms[11-13]. They produce methane through the use of hydrogen and carbon dioxide as a substrate (Figure 1). Miller et al[14] cultured and isolated *Methanobrevibacter smithii* which is the representative methane producing organism in human. In 1990, Woese et al[15] proposed the "domain" *Archaea* as a new and the highest taxon. *Archaea* was a different group from *Bacteria* such as Gram-positive bacteria and Gram-negative bacteria. Meanwhile, it was revealed that some bacteria could affect the methane production indirectly. For example, acetogens[16] and sulfate reducing bacteria (SRB)[17-18] also consume hydrogen and compete against methanogens.

However, methane can not be metabolized further in human body[19]. It was estimated that 20% of methane is excreted through lungs and the rest is released as flatus[19]. Consequently, the breath methane test is used as an index of methane production in human colon[20]. Breath methane has been detected in approximately 30% of adult population[21]. Although some previous studies showed that breath methane concentration increase in patients with colon cancer[22], diverticulosis[12] and irritable bowel disease[23], its health implication is still unknown due to lack of large scale population studies.

In this study, we investigated the relationship between methane production and gut microbiota among the general populations in Japan. It was considered as the first study to examine the relationship between breath methane and gut microbiota among general populations in Japan.

Subjects and Methods

The study subjects were 697 Japanese adults (261 males and 436 females of ages between 20 and 82 years old, with mean age of 58 ± 13 (SD)) who participated in the Iwaki Health Promotion

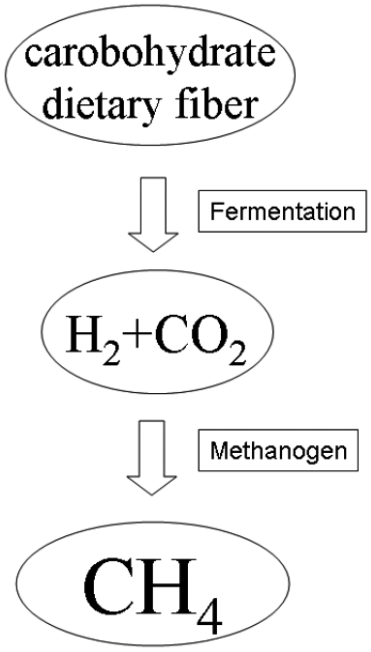

Figure 1 Metabolized pathway of methane production in the human colon. Carbohydrate and dietary fiber are consumed, and then, hydrogen and carbon dioxide are produced by colonic flora. Then methanogens produces methane by using hydrogen to reduce carbon dioxide.

Project in Aomori, northern Japan in 2007. The concentrations of methane and hydrogen levels under fasting state and gut microbiota were measured using gas chromatography and T-RFLP method. Subjects who were taking any antibiotics, and had any kinds of gastrointestinal operation were excluded from this study.

The purpose and method of the present research were thoroughly explained to the subjects prior to the investigation, and written consent was obtained from each participant. The present investigation was also approved by the Ethics Committee at the Hirosaki University Graduate School of Medicine.

Measurement of breath methane and hydrogen concentration

After an overnight fasting (about 10 hours), alveolar breath samples were obtained using a T-tube and commercial bag (Otsuka pharmaceutical, Tokyo, Japan). The first 500 ml of expired air (dead space) were discarded, and the next 200 ml of terminal expired air was captured. The methane and hydrogen concentration of each sample were determined using gas chromatography (autoanalyzer, Mitoleben Laboratory, Osaka, Japan).

Subjects with breath methane concentration above 10 ppm were considered as positive[24], and those with breath hydrogen concentration of above 20 ppm were considered positive [24-25]. Subjects were divided into four groups according to the level of each expired gas;

1. Both negative: both methane and hydrogen were negative
2. CH_4 only: only methane was positive
3. H_2 only: only hydrogen was positive
4. Both positive: both hydrogen and methane were positive

Especially, subjects whose breath methane was positive were classified as CH_4 positive; CH_4

only + both positive, and subjects whose breath methane was negative were classified as CH_4 negative; both negative + H_2 only.

DNA isolation

Total gut DNA was isolated from each sample as reported previously [26]. Briefly, gut samples were suspended in a solution containing 100 mM of Tris-HCl (pH9.0) and 40 mM of EDTA. The suspension was transferred into a 0.5 ml tube containing glass beads, and treated at 5 m/sec for 3 min in a FastPrep. DNA was then extracted from the gut sample using the Magtration system 12GC (Precision system science, Chiba, Japan).

PCR

PCR was performed using a total gut DNA, the HEX-labeled 516f' and 1510r. The amplification program was used as follows: preheating 95 C for 15 min; 30 cycles of denaturation at 95 C for 35 sec, annealing at 50 C for 30 sec, and extension at 72 C for 10 min. PCR was performed in a reaction mixture (2.5 µl) containing of 10x PCR buffer, each deoxynucleoside triphosphate at a concentration of 25 mM (2.5 µl) and MgCl2 1.5 µl, each primer at a concentration of 10 mM of gut DNA 0.2 µl and, and 5 U/µl HotStarTaq DNA polymerase (Qiagen, Tokyo, Japan) 0.2 µl. Amplified DNA was verified by 2.0% agarose gel electrophoresis. Fluorescently labeled PCR products were purified by using GFX PCR DNA and Gel Band Purification Kit. In the case of digestion with *BslI* (5'-CCNNNNN|NNGG-3'), a reaction mixture containing 1 µl of *BslI*, NEB3 buffer (NEW ENGLAND BioLabs) 1 µl, 5 µl of the PCR product (50-100 ng) from the gut DNA was incubated at 55 C for 3 hours.

T-RFLP analysis

The fluorescently labeled T-RFs were analyzed by electrophoresis on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) in GeneScan mode. The restriction enzyme digestion mixture was mixed with 0.2 µl of MapMaker X-Rhodamine Labeled 500-1000 bp size standard (BIOVENTURES, TN, USA) and 12 µl of deionized formamide, followed by denaturation at 96 C for 2 min and immediate chilling in ice. On ABI PRISM 3100 Genetic Analyzer, was used with the injection time of 30 sec and collection time of 40 min.

Operational taxonomic units (OTUs)

The length and peak areas of the T-RFs were determined with the GenScan software. They were then divided into 28 operational taxonomic units (OTUs) according to the length of T-RFs which corresponds to predominant bacterial groups in human feces; including the genera *Bacteroides, Bifidobacterium, Clostridium, Prevotella,* and the order Enterobacteriales, Lactobacillales.

PCR for the domain *Archaea*

Gut DNA from domain *Archaea* in gut samples was examined with PCR amplification and compared between samples from high CH_4 group ((=CH_4 positive) n=60) and lower methane subjects (low CH_4: 60 subjects from low breath methane concentration).

The primer set used were PARCH340F (CCCTACGGGGiGCAiCAG) and PARCH519R (TTACCGCGGCiGCTG)[27], and the PCR program was as follows; preheating at 95 C for 15 min, 30 cycles of denaturation at 95 C for 30 sec, annealing at 50 C for 30 sec, and extension at 72 C for 1 min, and finally a terminal extension at 72 C for 10 min. Amplified DNA was verified by 2.0% agarose gel electrophoresis.

Table 1a Characteristics of subjects

	Both negative (n=535)			CH_4 only (n=52)			H_2 only (n=102)			Both positive (n=8)		
Age (years)	58.04	±	12.51*	63.33	±	10.53	57.41	±	13.13*	54.78	±	11.58
Breath methane (ppm)	2.37	±	1.28	34.95	±	20.28	2.37	±	1.11	25.39	±	19.69
Breath hydrogen (ppm)	6.06	±	4.53	6.11	±	5.56	35.08	±	14.27	36.34	±	10.42

breath methane concentration >10ppm was positive.
breath hydrogen >20ppm was positive.
*: Significant difference compare to CH_4 only ($p<0.05$)

Table 1b Characteristics of subjects

	CH_4 positive (n=60)			CH_4 negative (n=637)		
Age (year)	62.2	±	11	57.9	±	12.6**
Breath methane (ppm)	33.7	±	20.3	2.4	±	1.25
Sex (male: female)	25	:	35	236	:	401

mean ± S.D.
**: $p<0.01$
CH_4 positive: breath CH_4 was positive (Both positive + CH_4 only)
CH_4 negative: breath CH_4 was negative (Both negative + H_2 only)

Table 2 Characteristics of subjects in whom gut DNA for the domain *Archaea* was examined.

	High CH_4 (n=60)			Low CH_4 (n=60)		
Age (year)	62.2	±	11	54.3	±	14.9**
Breath methane (ppm)	33.7	±	20.3	1.4	±	0.25
Sex (male: female)	25	:	35	20	:	40

mean ± S.D. **: $p<0.01$
High CH_4: breath methane >10ppm (=CH_4 positive)
Low CH_4: 60 people in ascending order

Statistical analysis

Statistical evaluation of the OTUs between CH_4 positive and CH_4 negative was carried out using an analysis of covariance, and the data were adjusted by age and sex. Evaluation of the OTU of both negative, CH_4 only, H_2 only, and both positive were also analyzed using one way ANOVA (Games-Howell). The differences of the average values and proportion for characteristics between these groups were evaluated using the Mann-Whitney *U* test, Chi-square test and one way ANOVA (Games-Howell). Differences were considered to be significant when $p<0.05$.

Results

Table 1a shows the characteristics of subjects in four groups, each group was comprised of 535 (76.8%), 52 (7.5%), 102 (14.6%) and 8 (1.1%) subjects. Average age was significantly higher than both negative and H_2 only ($p<0.05$). Table 1b shows the characteristics of all subjects, CH_4 positive was comprised 60 of the 697 subjects (8.6%), and that of CH_4 negative was 637 (91.3%). The average age of the two groups were 62.2 ± 11.0 years vs 57.9 ± 12.6 years respectively ($p<0.01$).

Table 2 shows the characteristics of 60 subjects in high CH_4 and low CH_4 who underwent

Table 3 Result of PCR for the domain *Archaea*.

	High CH_4 (n=60)	Low CH_4 (n=60)
PCR positive	56/60(93.3%)	1/60(1.67%)

n/N (%)
High CH_4: breath methane >10ppm (=CH_4 positive)
Low CH_4: 60 people in ascending order

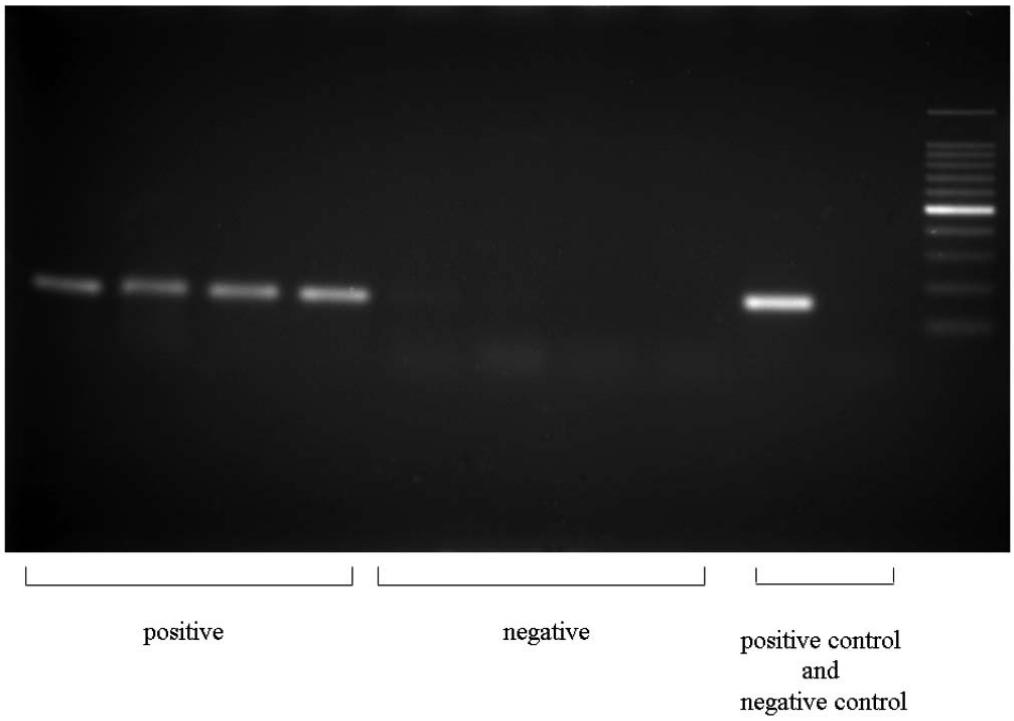

Figure 2 PCR exam for domain *Archaea*. From left, positive PCR, negative PCR and control. Positive control was *Methanosphaera stadtmaniae* (JCM11832), and negative control was sterilized water.

PCR examination of the gut DNA of the domain *Archaea*. The average age of the subjects in low CH_4 was 54.3 ±14.9 years, it was significantly lower than those in high CH_4 ($p<0.01$).

The PCR result on the fecal DNA of domain *Archaea* in Table 3 and Fig. 2. Fifty-six of 60 subjects in high CH_4 group were positive (93.3%). In low CH_4 group, only 1 of 60 subjects was positive (1.67%), which was significantly less than high CH_4 ($p<0.01$).

The OTUs analysis in both groups was shown in Table 4. In CH_4 positive, proportions of OTU168 (*Clostridium* cluster IV), OTU317 (*Prevotella*), OTU338 (*C.* cluster XI), OTU369 (*C.* cluster IV), OTU853 (*Bacteroides*), and OTU990 (*C.* subcluster XIVa) were significantly higher than CH_4 negative ($p<0.05$). On the other hand, proportions of the following OTUs were significantly lower in CH_4 positive group than in CH_4 negative ($p<0.05$); OTU494 (*C.* subcluster XIVa), OTU657 (Lactbacillales), OTU749 (*C.* cluster IV), OTU754 (*C.* subcluster XIVa), OTU940 (*C.* subcluster XIVa), and OTU955 (*C.* subcluster XIVa)[10] as shown in Table 4.

Table 5 shows the OTUs of both negative, CH_4 only, H_2 only and both positive. In CH_4

Table 4 Differences of OTUs between CH_4 positive and CH_4 negative.

OTU (%)	CH_4 positive (n=60)	CH_4 negative (n=637)	Most closely related genera
OTU106	0.16 ± 0.07	0.16 ± 0.02	*Clostridium* subcluster XIVa
OTU110	1.91 ± 0.41	1.76 ± 0.13	*Clostridium* cluster IX *Megamonas*
OTU124	3.62 ± 0.53	3.64 ± 0.16	*Bifidobacterium*
OTU137	-0.01 ± 0.09	0.03 ± 0.03	*Prevotella*
OTU168	2.23 ± 0.27	1.3 ± 0.08**	*Clostridium* cluster IV
OTU317	6.91 ± 0.93	4.2 ± 0.29**	*Prevotella*
OTU332	1.06 ± 0.35	1.45 ± 0.11	Lactobacillales
OTU338	2.13 ± 0.28	1.42 ± 0.09**	*Clostridium* cluster XI
OTU366	0.01 ± 0.13	0.15 ± 0.04	*Bacteroides*
OTU369	5.43 ± 0.54	3.89 ± 0.17**	*Clostridium* cluster IV
OTU423	0 ± 0.03	0.01 ± 0.01	*Clostridium* cluster XVIII
OTU443	0.06 ± 0.04	0.05 ± 0.01	Unknown
OTU469	10.48 ± 0.98	10.9 ± 0.3	*Bacteroides*
OTU494	5.91 ± 0.49	7.31 ± 0.15**	*Clostridium* subcluster XIVa
OTU505	0.8 ± 0.12	0.63 ± 0.04	*Clostridium* subcluster XIVa
OTU517	0.06 ± 0.03	0.06 ± 0.01	*Clostridium* subcluster XIVa
OTU520	0.63 ± 0.21	0.77 ± 0.07	Lactobacillales
OTU550	0.02 ± 0.01	0.03 ± 0	None
OTU650	0.24 ± 0.05	0.24 ± 0.02	*Clostridium* cluster XVIII
OTU657	4.44 ± 1.05	6.68 ± 0.32*	Lactobacillales
OTU749	7.85 ± 0.67	9.37 ± 0.20*	*Clostridium* cluster IV
OTU754	0.27 ± 0.19	0.71 ± 0.06*	*Clostridium* subcluster XIVa
OTU770	0.94 ± 0.15	0.52 ± 0.05*	None
OTU853	1.23 ± 0.11	1.01 ± 0.03*	*Bacteroides*
OTU919	4.12 ± 0.28	4.28 ± 0.09	*Clostridium* cluster XI, subcluster XIVa XIVa
OTU940	5.63 ± 0.54	7.78 ± 0.16**	*Clostridium* subcluster XIVa Enterobacteriales
OTU955	9.17 ± 0.7	12.24 ± 0.21**	*Clostridium* subcluster XIVa
OTU968	1.18 ± 0.12	0.94 ± 0.04	None
OTU990	13.51 ± 0.83	11.37 ± 0.25**	*Clostridium* subcluster XIVa
Others	10.01 ± 0.67	7.14 ± 0.21**	

adjusted mean ± S.E. OTU : operational taxonomic units * : $p<0.05$ ** : $p<0.01$
All data were adjusted by age and sex.

only, proportion of OTU168 was significantly higher than in both negative ($p<0.05$). Additionally, OTU317 of CH_4 only was higher than both negative and H_2 only ($p<0.05$). In contrast, OTU494 and OTU955 of CH_4 only were significantly lower than in both negative ($p<0.05$). Also, OTU940 of CH_4 only was lower than both negative and H_2 only ($p<0.05$).

Discussion

The demography of methane production in healthy subjects has been studied extensively. In previous studies[20], several factors increasing the probability that a subject will have highly gut methan-producing microorganisms have been identified. Age is clearly a factor as breath methane cannot be detected in infant[28]. Namely, the methane production increase with age and reaches adult levels after 10 years of age, and its number tends to increase up until they become adult. On reaching adulthood the prevalence of methane producers does not increase with age[29]. On the other hand, two studies[28, 30] found an increasing percentage of methane producers throughout adulthood. In this study, subjects

Table 5 Differences of OTUs among four groups.

OTU(%)	Both negative (n=535)	CH_4 only (n=52)	H_2 only (n=102)	Both positive (n=8)
OTU106	0.17 ± 0.02	0.17 ± 0.07	0.14 ± 0.05	0.12 ± 0.18
OTU110	1.73 ± 0.14	2.10 ± 0.44	1.89 ± 0.31	0.65 ± 1.11
OTU124	3.49 ± 0.18	3.65 ± 0.57	4.36 ± 0.41	3.43 ± 1.45
OTU137	0.03 ± 0.03	-0.02 ± 0.09	0.01 ± 0.07	0.02 ± 0.23
OTU168	1.30 ± 0.09*	2.13 ± 0.29	1.31 ± 0.20	2.83 ± 0.73
OTU317	4.41 ± 0.31*	7.33 ± 1.00	3.08 ± 0.71**	4.26 ± 2.54
OTU332	1.47 ± 0.12	1.03 ± 0.38	1.34 ± 0.27	1.28 ± 0.96
OTU338	1.34 ± 0.09	2.16 ± 0.30	1.83 ± 0.22	1.92 ± 0.77
OTU366	0.15 ± 0.04	0.01 ± 0.13	0.16 ± 0.10	0.00 ± 0.34
OTU369	3.96 ± 0.18	5.23 ± 0.58	3.57 ± 0.41	6.68 ± 1.48
OTU423	0.00 ± 0.01	0.00 ± 0.03	0.05 ± 0.02	0.00 ± 0.07
OTU443	0.05 ± 0.01	0.06 ± 0.04	0.04 ± 0.03	0.05 ± 0.10
OTU469	10.96 ± 0.33	10.91 ± 1.06	10.57 ± 0.75	7.75 ± 2.68
OTU494	7.29 ± 0.16*	5.74 ± 0.53	7.37 ± 0.37	6.99 ± 1.34
OTU505	0.60 ± 0.04	0.82 ± 0.13	0.78 ± 0.09	0.67 ± 0.32
OTU517	0.05 ± 0.01	0.06 ± 0.04	0.08 ± 0.03	0.04 ± 0.09
OTU520	0.78 ± 0.07	0.67 ± 0.23	0.71 ± 0.16	0.38 ± 0.58
OTU550	0.03 ± 0.00	0.03 ± 0.01	0.02 ± 0.01	0.00 ± 0.03
OTU650	0.25 ± 0.02	0.28 ± 0.06	0.15 ± 0.04	0.03 ± 0.14
OTU657	6.57 ± 0.35	4.22 ± 1.13	7.22 ± 0.81	5.81 ± 2.88
OTU749	9.37 ± 0.22	7.58 ± 0.72	9.36 ± 0.51	9.52 ± 1.82
OTU754	0.70 ± 0.06	0.27 ± 0.21	0.77 ± 0.15	0.27 ± 0.53
OTU770	0.51 ± 0.05	0.97 ± 0.17	0.57 ± 0.12	0.75 ± 0.42
OTU853	1.01 ± 0.04	1.15 ± 0.11	1.00 ± 0.08	1.76 ± 0.29
OTU919	4.23 ± 0.09	4.06 ± 0.30	4.51 ± 0.21	4.46 ± 0.76
OTU940	7.78 ± 0.18**	5.57 ± 0.58	7.80 ± 0.41*	6.01 ± 1.46
OTU955	12.37 ± 0.23**	9.15 ± 0.75	11.52 ± 0.53	9.37 ± 1.90
OTU968	0.96 ± 0.04	1.18 ± 0.12	0.86 ± 0.09	1.18 ± 0.31
OTU990	11.32 ± 0.28	13.18 ± 0.90	11.65 ± 0.64	15.63 ± 2.27
Others	7.10 ± 0.22**	10.30 ± 0.73	7.31 ± 0.51**	8.12 ± 1.84

adjusted mean ± S.E. OTU: operational taxonomic units
* **: There was significant difference compare to CH_4 only (*: $p<0.05$, **: $p<0.01$).

produced CH_4 only was significantly older than subjects produced none and H_2 only (Table 1a), subjects in CH_4 positive was also older than those in CH_4 negative (Table 1b) and subjects produced high CH_4 was also older than those in low CH_4 (Table 2). These result also showed that the methane production of subjects in those groups increased with age. Although further investigations are needed, geographical difference was suggested be the cause of such result[21].

The first methanogen which has been well-characterized in human colon belongs to the genus *Methanobrevibacter*, and was identified as *M.smithii*[14]. Other methanogens such as species belonging to *Archaea*, including two genera *Methanogenium* and *Methanosarcina*, were also found[31]. In this study, DNA of *Archaea* appeared in high CH_4 (Table 3), which shows that the microorganisms produced methane in subjects produced high CH_4 was *Archaea*, as also reported in previous studies.

Although no bacteria were suggested to produce methane in human colon except for a domain *Archaea*, there were significant differences in bacterial proportions between CH_4 positive and CH_4 negative in this study (Table 4). Thus, these bacteria were suggested

to influence methane production indirectly due to following reasons. Typically, patterns of balance between breath hydrogen and breath methane tend to be either high hydrogen and low methane concentrations, or high methane and low hydrogen concentrations[32]. It has been suggested that methane is produced in human intestine predominantly by a hydrogen-utilizing bacteria and thus adequate assessment of bacterial carbohydrate fermentation would require parallel measurement of breath hydrogen and methane[33]. In this study, CH_4 only consisted of subjects with high breath methane and low breath hydrogen concentrations, and H_2 only consisted of subjects with high breath hydrogen and low breath methane concentrations. There were significant differences of bacterial components between subjects in CH_4 only and H_2 only; OTU317 (*Prevotella*) of CH_4 only was significantly higher than that of H_2 only, and OTU940 (*C.* subcluster XIVa, Enterobacteriales) of CH_4 only was significantly lower than that of H_2 only (Table 5). In CH_4 only, OTU317 was suggested to cause enhanced methane production. In contrast, OTU940 was suggested to decrease methane production in H_2 only. Additionally, OTU317 of both negative was significantly lower than CH_4 only, and OTU940 was significantly higher than CH_4 only. As both negative was low hydrogen and low methane group, we hypothesized that different materials competed against methane, such as hydrogen sulfide and equol[17, 34], which were produced in subjects of both negative. Therefore, we suggest a possibility that OTU940 increased some materials competing against methane such as hydrogen sulfide, equol, and acetate in environment of high concentration of hydrogen. In contrast, OTU 317 seemed to increase methane production. Although more investigation is required, OTU 317 might have something enhancing methane production such as enzymes, or might decrease microorganisms competing against methanogens.

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