ORIGINAL ARTICLE

Irritable Bowel Syndrome, Particularly the Constipation-Predominant Form, Involves an Increase in *Methanobrevibacter smithii*, Which Is Associated with Higher Methane Production

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Background/Aims: Because *Methanobrevibacter smithii* produces methane, delaying gut transit, we evaluated *M. smithii* loads in irritable bowel syndrome (IBS) patients and healthy controls (HC). **Methods:** Quantitative real-time polymerase chain reaction for *M. smithii* was performed on the feces of 47 IBS patients (Rome III) and 30 HC. On the lactulose hydrogen breath test (LHBT, done for 25 IBS patients), a fasting methane result ≥10 ppm using 10 g of lactulose defined methane-producers. **Results:** Of 47, 20 had constipation (IBS-C), 20 had diarrhea (IBS-D) and seven were not sub-typed. The *M. smithii* copy number was higher among IBS patients than HC (Log_{10}5.4, interquartile range [IQR; 3.2 to 6.3] vs 1.9 [0.0 to 3.4], p<0.001), particularly among IBS-C compared to IBS-D patients (Log_{10}6.1 [5.5 to 6.6] vs 3.4 [0.6 to 5.7], p=0.001); the copy number negatively correlated with the stool frequency (R=-0.420, p=0.003). The *M. smithii* copy number was higher among methane-producers than nonproducers (Log_{10}6.4, IQR [5.7 to 7.4] vs 4.1 [1.8 to 5.8], p=0.001). Using a receiver operating characteristic curve, the best cutoff for *M. smithii* among methane producers was Log_{10}6.0 (sensitivity, 64%; specificity, 86%; area under curve [AUC], 0.896). The AUC for breath methane correlated with the *M. smithii* copy number among methane producers (r=0.74, p=0.008). Abdominal bloating was more common among methane producers (n=9/11 [82%] vs 5/14 [36%], p=0.021). **Conclusions:** Patients with IBS, particularly IBS-C, had higher copy numbers of *M. smithii* than HC. On LHBT, breath methane levels correlated with *M. smithii* loads. **(Gut Liver 2016;10:932-938)**

Key Words: Methanogenic flora; Real-time polymerase chain reaction; Lactulose hydrogen breath test; Gut transit

INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder of unknown aetiology.[1,2] Several hypotheses have been proposed in pathogenesis of IBS including altered gut microbiota, visceral hypersensitivity, dysmotility, gastrointestinal infection and infestation, dysregulation of brain-gut axis, psychological and genetic factors.[2-4] Therapeutic manipulation of gut flora using antibiotic or probiotic is known to improve IBS symptoms.[5,6] Role of gut microbiota and its alterations is being explored in the pathogenesis of this enigmatic disorder.[7]

About 30% to 62% of individuals have methane producing bacteria in their gut.[8] These methanogens are important anaerobic archaea of colonic flora and produce methane by utilizing hydrogen and carbon dioxide.[8,9] Several *in vitro* and *in vivo* studies showed that methane inhibits gastrointestinal motility and therefore, its level may inversely correlate with stool frequency.[10,11] In addition, area under curve (AUC) for breath methane correlated with severity of constipation among patients with constipation-predominant IBS (IBS-C).[11] Moreover, treatments with antibiotics targeting methanogens have been shown to improve gut transit and constipation.[12-14]

Culture and molecular studies showed that *Methanobrevibacter smithii* is the dominant methanogen in the colon and its level varies from undetectable to 10^{10}/g dry weight of stool.[15,16] Recently, a study reported that breath methane can be measured only when the level of *M. smithii* exceeds 4.2×10^5 copies per gram of wet stool samples.[16] However, this study had limitation due to small sample size.[15] In addition, there is scanty data on relationship between *M. smithii* and other symptoms of IBS such as abdominal bloating, pain and discomfort. In an earlier

case-control study, patients with IBS were less often methane producers than healthy controls (HC); the author suggested that as synthesis of methane from hydrogen reduces the volume of gas, lack of methanogens among patients with IBS might explain occurrence of abdominal bloating among them.[17] No study, however, tested this hypothesis.

Accordingly, we undertook a study with the following aims: (1) to determine the copy number of *M. smithii* among different subgroups of patients with IBS and HC using quantitative real-time polymerase chain reaction (qPCR); (2) to determine the copy number of *M. smithii* among methane producers and nonproducers; (3) to determine the cutoff level of *M. smithii* among methane producers using receiver operating characteristic (ROC) curve; (4) to correlate the *M. smithii* load and AUC for breath methane among methane producers; and (5) to study the relationship between different symptoms of IBS and methane production.

MATERIALS AND METHODS

1. Patients and controls

Patients with IBS diagnosed using Rome III criteria[18] attending the gastroenterology outpatient clinic of a multilevel teaching hospital in northern India were recruited. Patients were classified into three subtypes using Rome III criteria: IBS-C, diarrhea-predominant (IBS-D) and unsubtyped (IBS-U). Patients having organic or metabolic disorders, receiving antibiotics, probiotics or prokinetics, laxatives, drugs altering gastrointestinal motility within previous 4 weeks and those undergoing intestinal surgery in the past were excluded. All the patients were subjected to investigations such as stool microscopy and occult blood testing, hemogram, thyroid function tests and proctosigmoidoscopy. Age and gender matched healthy subjects were included as controls. All HC were apparently healthy and did not have functional gastrointestinal disorders according to Rome III criteria on structured interview. Written informed consent was obtained from each recruited subject. The Institutional Ethics Committee approved the study protocol (PGI/BE/712/2012).

2. Clinical evaluation

Each subject was asked to fill up a validated Hindi version of Rome III[19] questionnaire to record the demographic and clinical symptoms including abdominal bloating or distension, pain/discomfort, passage of mucus, urgency, straining, feeling of

incomplete evacuation, altered bowel habits or stool form, constipation or diarrhoea and type of stool (using Bristol stool chart with pictures as well as descriptors). According to Rome III criteria, type I and II stools were considered to denote constipation, type VI and beyond as diarrhea, and forms changing in days as irregular stool forms. Patient's dietary habit was also recorded in the standard proforma. Patients taking no food of animal origin except milk were classified as vegetarian and those taking foods of animal origin as nonvegetarians.

3. Sample collection and extraction of DNA

Three consecutive fresh stool samples were collected from each study participant. Fresh morning stool samples were collected and transported to the laboratory within 1 to 2 hours. Stool sample from each subject was homogenized and divided into three aliquots. All stool samples were stored at -80°C immediately; subsequently, DNA was extracted using the QIAamp Qiagen mini stool kit (QIAGEN, Hilden, Germany) following manufacturer's instructions with some modifications. Concentration of DNA was quantified by NanoDrop ND2000 Spectrophotometer (NanoDrop Products, Wilmington, DE, USA).

4. Culture condition of *M. smithii* (American Type Culture Collection-35061, positive controls for qPCR)

M. smithii (American Type Culture Collection [ATCC]-35061) was cultured in Wilkins-Chalgren media and incubated under anaerobic condition at 37°C for 48 hours. This ATCC strain was used as positive controls for qPCR and standard graph was generated. Genomic DNA from ATCC bacterial strain was extracted using heat extraction method and quantified by NanoDrop spectrophotometer.[20] Standard graph was used to calculate the copy number of 16S rRNA gene for *M. smithii* in stool samples. Standard graph was generated via serial dilution of control DNA (100 ng) to 8 folds (corresponding approximately 10^1 to 10^8 16S rRNA copies/μL). Real-time PCR was performed to estimate the number of copies of 16S rRNA gene of each of serially diluted DNA. Standard graph was used to calculate the efficacy of qPCR using the Rotor gene software (Corbett Research, Sydney, Australia).

5. qPCR

qPCR was performed in Corbett Research 6000 Q-PCR instrument (Rotor gene 6000 software). The primers used to amplify the *M. smithii* and universal bacteria are listed in Table 1.[21,22]

Table 1. Primer Sequences for Target Bacterium

Target bacterium	Primer sequence (5′ to 3′)	Annealing temperature, °C	Amplicon size, bp	Reference
Methanobrevibacter smithii	FP: CCGGGTATCTAATCCGGTTC	62	123	Dridi *et al.*[21]
	RP: CTCCCAGGGTAGAGGTGAAA			
Universal bacteria	FP: TCCTACGGGAGGCAGCAGTG	65	446	Lyra *et al.*[22]
	RP: GGACTACCAGGGTATCTAATCCTGT			

Each PCR was carried out in a final volume of 25 μL, comprising of 12.5 μL of Power SYBR® Green PCR master mixture (Applied Biosystems, Carlsbad, CA, USA), 0.25 μL of 10 pmole of forward and reverse primers and 100 ng of DNA. Each set of reaction was run with positive and negative controls. qPCR was run in duplicate for each sample. The following thermal cycling parameters were used for amplification of DNA: reaction cycle at 95°C for 10 minutes to activate the AmpliTaq Gold® DNA polymerase (Applied Biosystems, Carlsbad, CA, USA) followed by 40 cycles of initial denaturation at 95°C for 15 seconds, 30 seconds of annealing at optimal temperature (Table 1), elongation at 72°C for 30 seconds. The quality of qPCR product was confirmed by melting curve analysis with increasing temperature from 60°C to 95°C (at regular increment of 0.5 degree for 5 seconds) to confirm that the fluorescence signal was due to desired amplicon and not from primer dimer or nonspecific PCR products. The qPCR amplified amplicon was further confirmed by applying on 2% agarose gel electrophoresis along with 100 base pair ladder.

6. Breath test

Lactulose hydrogen breath test (LHBT) was performed according to a standard protocol.[23] All study subjects undergoing LHBT were advised to avoid complex carbohydrate rich diets one day before the test. Smoking and physical exercise were not allowed 2 hours before and during the test. Patients washed their mouth with antiseptic mouthwash to avoid any erroneous results due to action of oral bacteria on the test sugar. Breath sample was collected to determine the basal breath methane level (ppm) after a 12 hours of fasting using breath gas analyzer (Breathtracker SC, Digital Microlyzer; Quintron, Milwaukee, WI, USA). An average of 3 readings was taken for fasting breath methane level. Thereafter, patients ingested 15 mL solution containing 10 g lactulose and level of breath methane was estimated every 15 minutes for a duration of 240 minutes.[24] Rise in methane level was calculated by subtracting the average basal methane value from the highest value obtained. Fasting breath methane level ≥10 ppm or increase by ≥10 ppm above basal after 10-g lactulose ingestion was diagnostic criteria of methane-producer.[25] Total production of methane gas was calculated by AUC.[24] The person performing the breath test was blinded to the result of fecal microbiota analysis.

7. Statistical analysis

SPSS version 15.0 (SPSS Inc., Chicago, IL, USA) and Graph Prism version 5.0 (Graph Pad Software Inc., La Jolla, CA, USA) were used for statistical analysis. The ratio of copy number of 16S rRNA of *M. smithii* to total 16S rRNA copy number of universal bacteria was calculated to find out relative difference. The value of relative difference was transformed into $\log_{10}$ and presented as median and interquartile range (IQR). Categorical and continuous data were analyzed using chi-square and Mann-Whitney U tests, respectively. Kruskal-Wallis test was used for comparisons of more than two variables. Bonferroni correction was applied for multiple comparisons. Spearman rank correlation was applied to determine the correlation between copy number of *M. smithii* and AUC for breath methane. ROC curve was used to determine the best cutoff level of *M. smithii* to identify methane producers. The p-values <0.05 were considered as significant.

RESULTS

1. Demographic and clinical parameters of patients with IBS and HC

The demographic and clinical parameters of patients with IBS and HC are summarized in Table 2. Patients with IBS (n=47) and HC (n=30) were comparable in age (34-year [19 to 68] vs 35-year [21 to 69], p=0.925) and gender (39/47 [83%] vs 22/30 [73%] male, p=0.309). Twenty patients had IBS-C, 20 IBS-D and remaining seven could not be sub-typed using Rome III criteria.

2. Quantitative determination of *M. smithii* and its association with clinical symptoms

M. smithii was detected in 19 of 20 (95%) patients with IBS-C, 15 of 20 (75%) with IBS-D and 7 of 7 (100%) with IBS-U. Number of copies of *M. smithii* was higher among patients with IBS than HC ($\log_{10}$5.4, IQR [3.2 to 6.3] vs 1.9 [0.0 to 3.4], p<0.001) (Fig. 1A). Number of copies of *M. smithii* was higher among patients with IBS-C than HC and IBS-D ($\log_{10}$6.1 [5.5 to 6.6] vs 1.9 [0.0 to 3.4] and $\log_{10}$3.4 [0.6 to 5.7]; p<0.001 and p=0.004, respectively) (Fig. 1B). Copy number of *M. smithii* inversely correlated with stool frequency among patients with IBS (r=-0.420, p=0.003) (Fig. 1C). In addition, number of copies of *M. smithii* was lower among IBS patients passing more than 3 stools per day than those without (n=25/47, $\log_{10}$3.6, IQR [2.4 to 5.6] vs 22/47, $\log_{10}$5.9, IQR [4.9 to 6.6]; p=0.002). *M. smithii* load was higher in patients with Bristol stool type 1 to 2 than those with stool type 3 to 5 and type 6 to 7 ($\log_{10}$5.6 [5.0 to 6.5] vs $\log_{10}$3.4 [0.6 to 5.9] and $\log_{10}$3.7 [2.4 to 5.5]; p<0.03 and p<0.02, respectively).

3. Relationship between *M. smithii* load and breath methane on LHBT

LHBT was performed among (25/47) patients with IBS (12 patients with IBS-C and 13 with IBS-D). Of 25 patients who underwent LHBT, IBS-C patients were more often methane-producer than IBS-D (8/12 [67%] vs 3/13 [23%], p=0.047). Number of copies of 16S rRNA of *M. smithii* was higher among methane-producers than non-producers ($\log_{10}$6.4 [5.7 to 7.4] vs 4.1 [1.8 to 5.8], p=0.001) (Fig. 2A).

Table 2. Demographics and Clinical Symptoms in Patients with IBS and HC

| Parameter	IBS (n=47)	HC (n=30)	p-value
Age, yr	34 (19–68)	35 (21–69)	0.925*
Male sex	39 (83)	22 (73)	0.309
Predominant bowel habits (Rome III criteria)			
IBS-C	20 (43)	-	-
IBS-D	20 (43)	-	-
IBS-U	7 (14)	-	-
Clinical symptoms			
Visible abdominal distension	34 (72)	2 (7)	<0.001
Abdominal bloating/feeling of abdominal distension >1/4 of days	22 (47)	0	<0.001
Abdominal pain	35 (74)	1 (3)	<0.001
Abdominal discomfort	47 (100)	2 (7)	<0.001
Relief of pain/discomfort with bowel movement	46 (98)	2 (7)	<0.001
More frequent stool at onset of pain	13 (28)	0	0.001
Loose stool at onset of pain	14 (30)	0	0.001
Urgency	17 (36)	0	<0.001
Passage of mucus	35 (74)	1 (3)	<0.001
Passage of mucus >1/4 of defecation	14 (28)	0	0.001
Feeling of incomplete evacuation	45 (96)	3 (10)	<0.001
Feeling of incomplete evacuation >1/4 of defecation	29 (62)	0	<0.001
Straining during defecation	27 (57)	1 (3)	<0.001
Irregular stool form	33 (70)	1 (3)	<0.001
Irregular stool frequency	39 (83)	1 (3)	<0.001
Diet			
Vegetarian	14 (30)	12 (40)	NS
Nonvegetarian	33 (70)	18 (60)	NS

Data are presented as median (range) or number (%).
IBS, irritable bowel syndrome; HC, healthy controls; IBS-C, constipation-predominant IBS; IBS-D, diarrhea-predominant IBS; IBS-U, unsubtyped IBS; NS, not significant.
*Nonparametric independent sample test. A chi-square test was used for all other categorical data. $p < 0.05$ was considered significant.
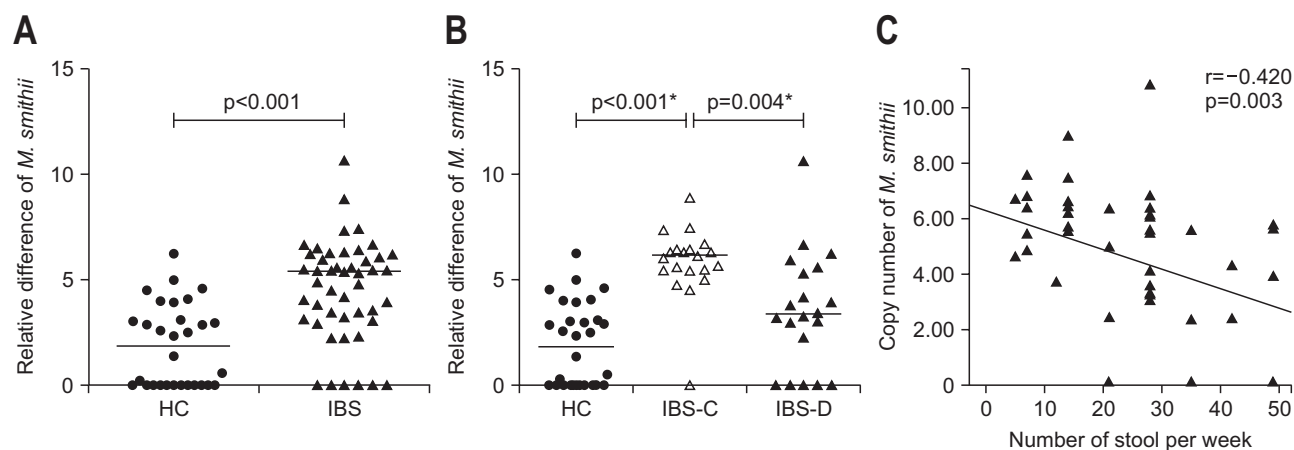
Fig. 1. (A) Copy numbers of *Methanobrevibacter smithii* among patients with irritable bowel syndrome (IBS) and healthy controls (HC). (B) Copy numbers of *M. smithii* among patients with constipation-predominant IBS (IBS-C), diarrhea-predominant IBS (IBS-D), and HC. (C) Correlation between copy numbers of *M. smithii* and number of stools per week. $p < 0.05$ was considered as significant. *Bonferroni corrected p-value.
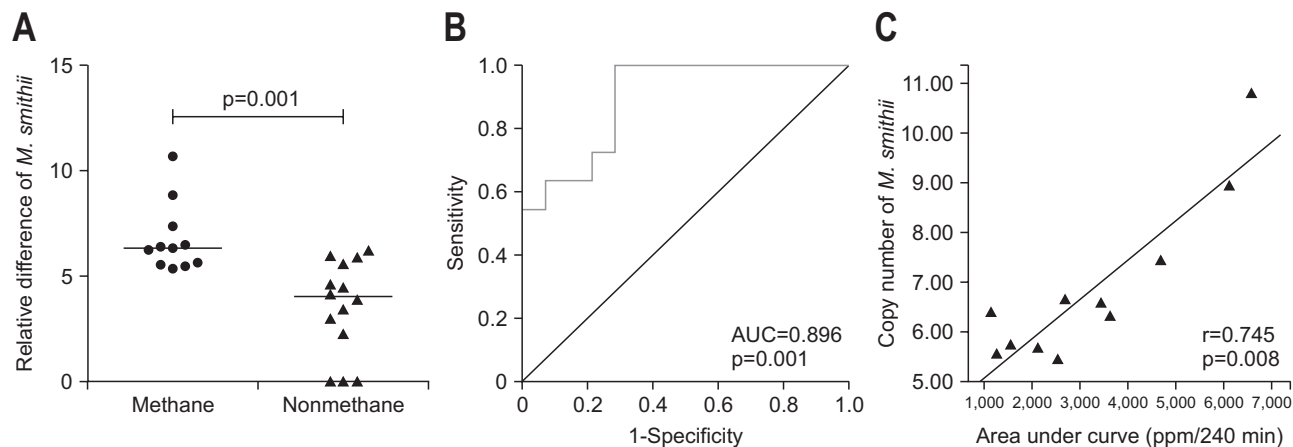
Fig. 2. (A) Copy numbers of *Methanobrevibacter smithii* among methane producers and nonproducers. (B) Receiver operating characteristic curve showing the area under the curve (AUC). (C) Correlation between copy numbers of *M. smithii* and the AUC. p<0.05 was considered as significant.

Table 3. Relationships between Different Symptoms of Irritable Bowel Syndrome among Methane Producers and Methane Nonproducers

Symptom	Methane producer (n=11)	Methane nonproducer (n=14)	p-value
Abdominal bloating	9 (82)	5 (36)	0.021
Abdominal pain	9 (82)	11 (79)	NS
Relief of pain or discomfort with bowel movement	11 (100)	14 (100)	NS
Urgency	2 (18)	7 (50)	NS
Passage of mucus per rectum	7 (64)	9 (64)	NS
Feeling of incomplete evacuation >1/4 of defecation	9 (82)	8 (57)	NS
Straining	7 (64)	5 (36)	NS
Irregular stool form	7 (64)	12 (86)	NS
Irregular stool frequency	8 (73)	12 (86)	NS
More frequent stool at onset of pain	1 (9)	9 (64)	0.005
Loose stool at onset of pain	1 (9)	8 (57)	0.013

Data are presented as number (%).
NS, not significant (p>0.05).

4. Cutoff level of *M. smithii* among methane producers on LHBT

Using ROC curve, an optimal cutoff of *M. smithii* among methane producers on LHBT was $\log_{10}6.0$ (area under ROC curve, 0.896 [95% CI, 0.77 to 1.00; p=0.001]), which had sensitivity and specificity of 64% and 86%, respectively (Fig. 2B).

5. Correlation between *M. smithii* load and AUC for breath methane

AUC for breath methane correlated positively with number of copies of *M. smithii* among methane producers (Spearman correlation coefficient, r=0.745; p=0.008) (Fig. 2C).

6. Relationship between different symptoms of IBS and methane production

Abdominal bloating was more common among methane producers than nonproducers (n=9/11 [82%] vs 5/14 [36%], p=0.021). Methane producers more often had Bristol stool type 1 to 2; in contrast, the methane nonproducers had stool type 6 to 7 (n=8/12 [66%] vs 10/13 [77%], p=0.047). Other symptoms like abdominal pain, relief of pain or discomfort with bowel movement, urgency, passage of mucus with stool, feeling of incomplete evacuation, straining, irregular stool form and frequency were comparable among methane producers and nonproducers (Table 3).

DISCUSSION

The present study shows that (1) number of copies *M. smithii* was higher among patients with IBS, particularly IBS-C than IBS-D and HC; (2) *M. smithii* load inversely correlated with stool frequency among patients with IBS; (3) number of copies of *M. smithii* was less among patients with IBS having more than 3 stool per day than those without; (4) number of copies of *M. smithii* was higher among methane producers than nonproducers; (5) threshold value of *M. smithii* among LHBT positive methane producers was $\log_{10}6.0$; (6) AUC for breath methane

correlated positively with load of *M. smithii* among methane producers; and (7) methane producers more often had abdominal bloating than nonproducers.

Human colon harbors diverse microbial species, which produces methane.[26] Ten percent of total anaerobes residing in human colon are methanogens and 80% to 100% of which is *M. smithii* by PCR.[26] There are limited data on *M. smithii* load among patients with IBS using qPCR. One study by Kim *et al*.[15] showed that *M. smithii* was predominant methanogen among patients with IBS-C, which is in accordance with our study. In contrast, another study showed that the number of copies of *M. smithii* was lower among patients with IBS than controls.[27] Therefore, more studies are necessary to determine whether higher level of *M. smithii* is associated with IBS-C. The present study provides further evidence of relationship between *M. smithii* and IBS-C.

We wish to emphasize that the load of *M. smithii* may be more important in pathogenesis of IBS, particularly IBS-C than mere detection of it.[15] Using qPCR, we found that optimal cutoff level of *M. smithii* in stool sample was $\log_{10}6$ to detect methane in breath on LHBT. In a previous study, the author used culture method and found that minimum level of methanogen must be 2×10^8 per gram of dry weight of stool to detect significant amount of methane in breath (>6 ppm).[28] *M. smithii* was identified using morphological, physiological, and immunological methods. Since, culture method can detect only live bacteria and *M. smithii*, as anaerobes, could die due to exposure of air.[28] qPCR is expected to be more efficient and sensitive tool for identification of colonic methanogens in stool samples.

Patients with IBS producing methane in breath on LHBT had abdominal bloating more often. These results were consistent with the previous study showing that methane production was associated with abdominal bloating, pain and flatulence[26] and contradicts the hypothesis suggested in another study claiming that methanogenesis might prevent abdominal bloating by reducing the volume of the gas while synthesis of methane from four atoms of hydrogen and one atom of cardon.[17] However, this was merely a hypothesis and abdominal bloating was not assessed in that study.[17] Our results reject this hypothesis. This is quite expected as abdominal bloating not only depends on volume of gas inside the lumen of the gut but its preferential retention within the small bowel, gut motility, visceral sensation and regularity and completeness of defecation.[26,29,30]

In this study, number of copies of *M. smithii* was higher among methane producer than nonproducer suggesting that *M. smithii* was responsible for producing methane on LHBT. This finding is consistent with a recent study showing that *M. smithii* load was higher among methane producers than nonproducers.[15] Evidence from the previous studies showed that higher level of breath methane production correlated with severity of constipation.[11] However, source of methane producing bacterium among patients with IBS was not clearly understood. Therefore, our study supports the previous hypothesis[15] that *M. smithii* could be considered as an important methanogen in stool sample responsible for breath methane production among patients with IBS. The number of copies of *M. smithii* inversely correlated with stool frequency among patients with IBS. Therefore, higher level of *M. smithii* could be considered as predisposing factor for the development of constipation.

Elimination of methanogenic flora using antibiotic treatment may contribute to therapeutic benefits at least in a subgroup of patients with IBS (IBS-C).[13] In a study of 111 Rome I IBS subjects, neomycin improved the constipation severity than placebo (44.0%±12.3% vs 5.0%±5.1%, p<0.05) among constipation-predominant methane producers.[13] In a case report, a patient with slow transit constipation associated with excess methane production improved after treatment with rifaximin, which reduced breath methane level, accelerated colonic transit and changed stool form and frequency.[12] Moreover, mixture of rifaximin and neomycin was more effective in relieving the methane production and constipation symptoms (87% and 85%) than neomycin (33% and 63%) or rifaximin alone (28% and 56%) among methane producing IBS patients.[31]

In conclusion, number of copies of *M. smithii* was higher among patients with IBS, particularly in IBS-C than HC and IBS-D. Moreover, number of copies of *M. smithii* was higher among methane producers than nonproducers. Best cutoff level of *M. smithii* among LHBT positive methane producers was $\log_{10}6.0$. AUC for breath methane had correlation with load of *M. smithii* among methane producers. Abdominal bloating was more common among methane producers than nonproducers.

CONFLICTS OF INTEREST

ACKNOWLEDGEMENTS

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