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ORIGINAL ARTICLE

Hydrogen Sulfide and Methane on Breath Test Correlate with Human Small Intestinal Hydrogen Sulfide Producers and Methanogens

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Abstract

Background Evidence indicates that 3 gas breath testing (BT) correlates with stool microbial populations. Breath methane (CH_4) levels correlate with stool *Methanobrevibacter smithii* levels and constipation, while hydrogen sulfide (H_2S) levels correlate with stool H_2S producers and diarrhea. However, their relationships to small bowel microbes are unknown.

Aims To assess relationships between small bowel microbes and breath gases.

Methods REIMAGINE study subjects completed a fasting single-sample BT for CH_4 and H_2S. During esophagogastroduodenoscopy without colon preparation, duodenal aspirates were obtained using double-lumen sterile aspiration catheters. Microbial DNAs underwent shotgun sequencing (NovaSeq6000).

Results Duodenal bacterial profiles differed significantly in subjects with breath $H_2S \geq 1.5$ ppm vs. those with < 1.5 ppm, with 2.08-$\log_2$fold greater prevalence of phylum Thermodesulfobacteriota. Higher breath H_2S levels correlated with greater duodenal prevalences of H_2S producers, including *Proteus mirabilis* ($P = 0.002$), *Desulfosarcina widdelii* ($P = 0.027$), and *Desulfobulbus oligotrophicus* ($P = 0.041$); co-occurrence of all 3 species correlated with ~ 50% higher breath H_2S levels ($P = 0.0001$). Duodenal archaeal profiles differed significantly in subjects with intestinal methanogen overgrowth (IMO, $CH_4 \geq 10$ ppm), with 2.94-$\log_2$fold greater prevalence of family Methanobacteriaceae vs. non-IMO subjects. Higher breath CH_4 correlated with greater prevalences of methanogens including *M. smithii* ($P = 0.02$), *Halarchaeum* sp. CBA1220 ($P = 0.003$), *Desulfurococcus mucosus* ($P = 0.046$), and *Halobaculum rubrum* ($P = 0.049$). IMO was more common in subjects with co-occurrence of all 4 species ($P = 0.04$). In IMO-positive subjects, CH_4 levels correlated with greater constipation severity ($P = 0.019$); *P. mirabilis* ($P = 0.021$) and *D. oligotrophicus* ($P = 0.003$) correlated with looser stool in IMO-negative subjects. *M. smithii* prevalence correlated with known hydrogen-producing syntrophs, e.g., *Christensenella minuta* ($P < 0.001$).

Conclusion This study demonstrates that duodenal prevalences of H_2S-producing bacteria and methanogenic archaea contribute to H_2S and CH_4 levels, respectively, on BT.

Keywords Breath testing · Duodenum · Microbiome · Methane · Hydrogen sulfide · *Methanobrevibacter smithii* · *Proteus mirabilis*

Introduction

Small intestinal bacterial overgrowth (SIBO) has been recognized as a clinical condition for half a century [1]. Early studies identified SIBO by culturing small bowel aspirates [2, 3]. Subsequently, breath testing (BT) became an established indirect technique for diagnosing SIBO based on levels of hydrogen (H_2) on the breath [4]. Originally, BT only measured H_2, but later testing also incorporated measurement of methane (CH_4). This allowed researchers to elucidate the importance of CH_4, which is produced in the gut

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by methanogenic archaea[5], in delayed gastrointestinal (GI) transit and constipation, including constipation-predominant irritable bowel syndrome (IBS-C) [6–9]; increased intestinal colonization with methanogens is now known as intestinal methanogen overgrowth (IMO) [10, 11]. Neither H_2 nor CH_4 are produced by human cells, so measuring these gases provides exclusive indirect markers of gut microbial composition. Adopting BT for the diagnosis of SIBO has facilitated an ever-increasing understanding of the role of SIBO in conditions such as irritable bowel syndrome (IBS) [12], celiac disease [13], and others.

Recently, a third gas, hydrogen sulfide (H_2S) has been introduced to BT [14]. H_2S is a gasotransmitter that plays important roles in inflammation and mucosal repair in the GI tract [15] and has been linked to diarrhea-predominant irritable bowel syndrome (IBS-D) [16, 17]. In humans, H_2S is mainly produced by gut bacteria, including sulfate reducers which have been linked to colorectal cancer, ulcerative colitis [18], and persistent diarrhea [19].

An ongoing criticism of BT is the lack of studies comparing BT to the small bowel microbiome. The advent of whole-genome sequencing has facilitated research in this area. A recent study using lactulose demonstrated that a rise in H_2 of ≥ 20 ppm by 90 min on BT was the best marker for SIBO as defined by culture and sequencing, suggesting an increased H_2-producing capacity in the small bowel [20] and confirming the validity of diagnosing SIBO using H_2 on BT. Another whole-genome sequencing study confirmed that breath CH_4 levels correlated with constipation and stool levels of *Methanobrevibacter smithii*, the predominant methanogen in humans, and also showed that breath H_2S levels correlated with stool levels of H_2S producers [11]. Although there is good correlation between breath gases and stool levels of CH_4 and H_2S producers, we hypothesized that breath CH_4 and H_2S levels would also be reflective of, or influenced by, small bowel microbial populations.

In this study, we analyze and compare CH_4 and H_2S levels on fasting single-sample BT to the results of shotgun sequencing analysis of duodenal microbiome composition.

Methods

Subject Recruitment

Samples for this study were obtained through the REIMAGINE study [21]. Consecutive patients aged 18–85 years undergoing standard-of-care esophagogastroduodenoscopy (EGD) or antegrade double-balloon enteroscopy (DBE) without colonoscopy were asked to participate in the REIMAGINE study. Consenting participants provided fasting serum samples and completed a medical history questionnaire, after which samples of duodenal luminal fluid were obtained during endoscopy. In 2024, the REIMAGINE study was amended to include acquiring a single fasting breath sample on the morning of endoscopy. Cedars-Sinai Institutional Review Board approved the study protocol (Pro00035192) and all participants provided informed written consent prior to participation.

Breath Testing

On the day of endoscopy, a single fasting exhaled breath sample was collected in sealed multilayer foil breath bags to measure carbon dioxide (CO_2), CH_4, and H_2S levels by gas chromatography (Gemelli Biotech, Raleigh, NC). It was not possible to perform full 2-h lactulose breath tests in these subjects prior to the scheduled standard-of-care endoscopy, and as changes in breath hydrogen (H_2) levels cannot be determined from single-sample breath tests, H_2 levels were not considered in this study. Previous studies suggest single fasting breath samples are sufficient to assess CH_4 [22] and H_2S production [11]. A positive CH_4 breath test for intestinal methanogen overgrowth (IMO) was defined as ≥ 10 parts per million (ppm) [22]. There is currently no established consensus or guideline regarding a threshold for H_2S levels.

Small Intestinal Sample Collection and Processing

Aspirates were obtained with a dual-lumen protected catheter (Hobbs Medical, Inc.) via sterile aspiration. Up to 2 mL of luminal fluid were collected from the second portion of the duodenum [21]. Care was taken not to add fluid into the duodenum prior to aspiration. Aspirated samples were immediately transferred on ice to the laboratory, where sterile 1 × Dithiothreitol (DTT) (EMD Millipore Corp., Darmstadt, Germany) was added (1:1 ratio). Samples were processed as described previously and microbial pellets were stored at − 80 °C [21].

DNA Isolation

DNAs were isolated from duodenal aspirate microbial pellets using MagAttract PowerMicrobiome DNA KF kits (Qiagen) on a KingFisher Flex System (Thermo Fisher Scientific) as described previously [21], using 1xDTT aliquots as negative controls. DNA purity and concentration were determined using a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

Library Preparation and Shotgun Sequencing

Libraries for whole-genome (shotgun) sequencing were prepared using Illumina-DNA Prep kit and IDT for Illumina-DNA/RNA UD Indexes (Illumina), as described previously [23]. Library qualities were analyzed on an Agilent 2100

Bioanalyzer System (Agilent Technologies, Santa Clara, CA). Sequencing was performed on a NovaSeq platform (Illumina), using NovaSeq 6000 S2 Reagent Kits v1.5 (300 cycles) and analyzed using CLC Genomics Workbench 22.0.2/20.0.3 and Microbial Module (Qiagen). Reads were demultiplexed and then trimmed using the Trim Reads Tool (Qiagen) and following parameters: quality limit of 0.05, maximum number of ambiguities of 2, and a trim adapter list containing the sequences of Illumina adapters. For co-occurrence analysis, host reads were removed using the *Homo sapiens* Genome Reference Consortium Human Build 38 as the genomic index. After trimming and two-level de-hosting, reads were mapped to the PlusPFP database (10/9/2023 release) with Dragen Metagenomics Pipeline (version 3.5.13) and the built-in kraken2 tool (version 2.0.8) using default parameters. Microbial metabolic pathways were built as previously described [23] and identified using the MetaCyc Pathway Database (2022-05).

For microbiome comparisons, a deeper de-hosting step was performed using k-mer-based read classification using Kraken 2 v. 2.1.3. As a *Homo sapiens* reference, a collection of 408 unmasked genome assemblies was compiled from two haploid human genome assemblies (GRCh38.p14 released by the Genome Reference Consortium, NCBI RefSeq assembly GCF_000001405.40, NCBI BioProject PRJNA168, and T2T-CHM13v2.0 released by the Telomere-to-Telomere (T2T) Consortium, NCBI RefSeq assembly GCF_009914755.1, NCBI BioProject PRJNA807723) and a collection of 406 haploid human pangenome assemblies released by the Human Pangenome Reference Consortium (NCBI BioProject PRJNA730822). During this step a confidence of 0.0 and a minimum hit group of 2 were used. After this step, unaligned reads were mapped to the PlusPFP (version 9/4/2024) using kraken2, and a confidence score of 0.5 and a minimum hit group of 2 were used. The PlusPFP database (version 9/4/2024) database also contained a human DNA sequence to retrieve any leftovers not aligned in the previous step.

Statistical Analysis

Continuous variables were compared using *t*-test or Mann–Whitney *U* test for 2 groups. Comparisons between three or more groups were performed by one-way ANOVA or Kruskal–Wallis. Correlations were analyzed by Spearman rank correlation coefficients. Statistical analysis was performed using SPSS 24.0 (SPSS, Chicago, IL), Graphs were constructed using GraphPad Prism 9.2.0 (GraphPad Software, La Jolla, CA). Significance was set at $P < 0.05$. Venn diagrams were constructed using Displayr (New South Wales, Australia).

Comparisons and statistical analyses were performed with CLC Genomics Workbench v.25.0.1 and CLC Microbial Genomics Module v.25.0.1 (Qiagen). The number of reads (after de-hosting steps) assigned to bacterial and archaeal taxa were compared, after removing taxa represented by singletons and doubletons per group. Despite similar library sizes between groups, fold changes were calculated from the GLM, which corrects for differences in library size between the samples and the effects of confounding factors. An unbiased analysis was performed to compare differences in relative abundance (RA) between groups at the species level. A negative binomial GLM model was used to obtain maximum likelihood estimates for the FC of a feature between groups, and the Wald test was used for determination of significance. False discovery rate was performed to correct the P value. FDR P value < 0.05 was considered strongly significant for all bacterial analysis, while a FDR P value < 0.1 was considered a trend towards being significant. Considering the very low diversity and abundance in the small bowel, a FDR P value < 0.1 was considered significant for the archaeome studies. Microbial richness and evenness were evaluated using the Shannon index, and Bray–Curtis was used to evaluate duodenal microbial beta-diversity. Partial Least Squares Discriminant Analysis (PLS-DA) studies were conducted using the MetaboAnalyst, version 6.0 (https://www.metaboanalyst.ca/home.xhtml). Data were $\log_{10}$ transformed and Pareto scaling was applied.

Results

Subject Demographics

Duodenal aspirates from 110 subjects (females: 64.4%, age: 56.4 ± 15.8 years, BMI: 26.3 ± 6.5 kg/m^2) were analyzed. BT data were collected for all 110 subjects; however, H_2S levels were not recorded for one subject. Of the 110 subjects, 22 (20%) had breath CH_4 levels ≥ 10 ppm and were considered positive for intestinal methanogen overgrowth (IMO). H_2S levels on BT (from 109 subjects) ranged from 0.31 to 3.54 ppm. The top 10 indications for endoscopy in study subjects are shown in Supplemental Table 1.

Duodenal microbial profiles were analyzed in all subjects via whole-genome (shotgun) sequencing of duodenal aspirates. On average, 174,029,497 reads (SD = ± 110,620,821) were generated per sample. 75 to 96% of the initial reads mapped to human DNA, and approximately 44% (SD = ± 18.52%) of the unmapped reads mapped to the PlusPFP database. Of these, 99% (SD = ± 0.08%, range: 17 to 100%) mapped to Bacteria. The remaining reads mapped to Eukaryotes, Fungi, Viruses, and Archaea, in this order. The average number of classified reads was not statistically different between groups ($P = 0.23$).

H_2S producers in the Duodenum Correlated with H_2S on Breath Test

The duodenal bacterial profile of subjects with ≥ 1.5-ppm H_2S on BT appeared to be different from subjects with < 1.5 ppm H_2S on BT, regardless of concurrent IMO (Supplemental Fig. 1), with increased bacterial alpha-diversity ($P = 0.003$, Supplemental Fig. 2). In addition, the relative abundance (RA) of phylum Thermodesulfobacteriota, which comprises the majority of sulfate-reducing bacteria (SRB), was significantly increased in the duodenum of subjects with ≥ 1.5 ppm H_2S on BT compared to subjects with < 1.5 ppm H_2S (log_2 fold change (log_2FC): 2.08, FDR P value = 6.61E−4). A Partial Least Squares Discriminant Analysis (PLS-DA) of this phylum revealed that the majority of species leading to the separation of the groups in a principal component plot were *Desulfovibrio desulfuricans*, *D. fairfieldensis*, and other unidentified *Desulfovibrio* species (Supplemental Fig. 3). The RA of these and other species from phylum Thermodesulfobacteriota were also significantly increased in subjects with ≥ 1.5 ppm H_2S on BT compared to subjects with < 1.5 ppm (FDR P value < 0.05, Supplemental Table 2).

Duodenal RA of known H_2S-producing species [24–26] exhibited correlations with breath H_2S levels, including five species from phylum Thermodesulfobacteriota, *Desulfovibrio desulfuricans* ($R = 0.23$, $P = 0.018$), *Desulfobulbus oligotrophicus* ($R = 0.22$, $P = 0.025$), *Desulfobulbus oralis* ($R = 0.20$, $P = 0.033$), *Desulfosarcina widdelii* ($R = 0.24$, $P = 0.011$), and *Desulfuromonas* sp. DDH964 ($R = 0.23$, $P = 0.017$) and one from phylum Chrysiogenota, *Desulfurispirillum indicum* ($R = 0.24$, $P = 0.011$). *Desulfoluna limicola* trended toward significance ($R = 0.17$, $P = 0.067$). The duodenal RA of another well-known H_2S producer, *Proteus mirabilis* (phylum Proteobacteria), also correlated with breath H_2S levels ($R = 0.32$, $P = 0.001$) (Supplemental Fig. 4, Table 1).

We next examined associations between the presence and co-occurrence of the eight specific H_2S producers described above and breath H_2S levels. H_2S levels were significantly increased in subjects with any three or more of these H_2S producers in their duodenal microbiome ($P = 0.0239$) (Fig. 1). Individually, there were no differences in breath H_2S levels in subjects with or without duodenal *Desulfurispirillum indicum* ($P = 0.085$), *Desulfuromonas* sp. DDH964 ($P = 0.143$), *Desulfobulbus oralis* ($P = 0.346$), *Desulfoluna limicola* ($P = 0.109$), or *Desulfovibrio desulfuricans* ($P = 0.179$) (Supplemental Fig. 5). However, subjects with *Proteus mirabilis* ($P = 0.002$), *Desulfosarcina widdelii* ($P = 0.027$) or *Desulfobulbus oligotrophicus* ($P = 0.041$) had

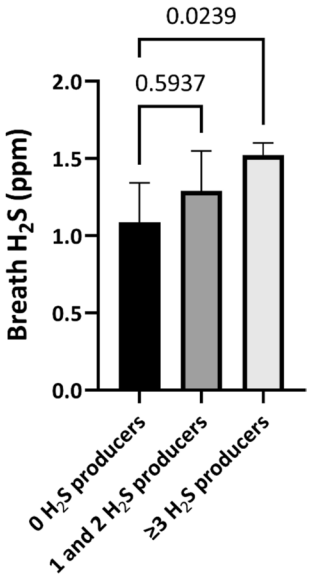

Fig. 1 Comparison of breath H_2S levels in subjects ($N = 109$) with zero to three or more of these H_2S producers in their small bowel (duodenal) microbiome: *Proteus mirabilis*, *Desulfovibrio desulfuricans*, *Desulfobulbus oligotrophicus*, *Desulfobulbus oralis*, *Desulfosarcina widdelii*, *Desulfurispirillum indicum*, *Desulfuromonas* sp. DDH964, and *Desulfoluna limicola*

Table 1 Prevalences of duodenal bacteria in the study population, and correlations between their relative abundances (RA) and breath H_2S levels (ppm)

	Bacterial species	Prevalence within the study population ($N = 109$)	Correlation between duodenal RA and breath H_2S levels	
			R	*P* value
1	*Desulfobulbus oligotrophicus*	51.38%	0.22	0.025
2	*Desulfobulbus oralis*	71.56%	0.20	0.033
3	*Desulfoluna limicola*	41.28%	0.17	0.067
4	*Desulfosarcina widdelii*	55.05%	0.24	0.011
5	*Desulfovibrio desulfuricans*	64.22%	0.23	0.018
6	*Desulfurispirillum indicum*	53.21%	0.24	0.011
7	*Desulfuromonas* sp. DDH964	56.88%	0.23	0.017
8	*Proteus mirabilis*	77.98%	0.32	0.001

higher breath H_2S levels than subjects without these species (Fig. 2). Subjects who had all three of these H_2S producers in their duodenal microbiome had ~ 50% higher breath H_2S levels than subjects with none of these species ($P = 0.0001$, Fig. 3).

Other species known to produce H_2S [24–26] were also evaluated, including *C. difficile, H. pylori, Fusobacterium* spp., *Shewanella* spp., *Citrobacter* spp., *Salmonella* spp., *Proteus* spp., and all species from the order Desulfovibrionales. The richness and evenness of these species were very significantly different between subjects with ≥ 1.5-ppm H_2S on BT compared to subjects with breath $H_2S < 1.5$ ppm (Shannon index $P = 0.0025$, Supplemental Fig. 6), and several H_2S bacterial producers were overrepresented in the duodenum of subjects with ≥ 1.5-ppm H_2S on BT (Supplemental Tables 3 & 4), including several *Proteus* species. For example, subjects with ≥ 1.5-ppm H_2S on BT had higher duodenal RA of *P. vulgaris* (log_2FC:2.24, FDR P value = 1.31E−3) and *P. mirabilis* when compared to subjects with breath $H_2S < 1.5$ ppm (log_2FC:1.17, FDR P value = 0.10, Supplemental Table 3).

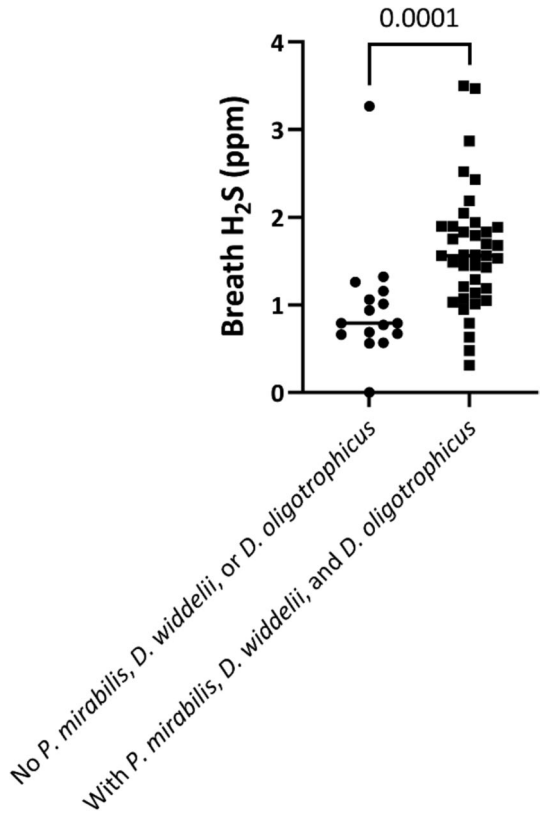

Fig. 3 Comparison of breath H_2S levels in subjects with *Proteus mirabilis*, *Desulfosarcina widdelii*, and *Desulfobulbus oligotrophicus* ($N = 39$) and those without ($N = 16$) in their duodenal microbiome

CH_4 Producers in the Duodenum Correlated with CH_4 on Breath Test

The duodenal archaeal profile of subjects with IMO appeared to differ from subjects without IMO (Supplemental Fig. 7) and trended toward clustering together on a principal component analysis of beta-diversity ($P = 0.08$, Supplemental Fig. 8). The RA of the entire Methanobacteriaceae family was 2.94-log_2 fold increased in the duodenum of IMO subjects when compared to non-IMO subjects (P value = 1.29E-3, FDR P value = 0.07).

The predominant methanogen, *M. smithii*, was present in the duodenum of 63.64% of subjects and *M. smithii* RA correlated with breath CH_4 levels ($R = 0.221$, $P = 0.02$). RA of eight other archaeal species [27–30] also correlated with breath CH_4 (Table 2, Supplemental Fig. 9). Three of these, *Halarchaeum* sp. CBA1220, *Desulfurococcus mucosus*, and *Halobaculum rubrum*, co-occurred with *M.*

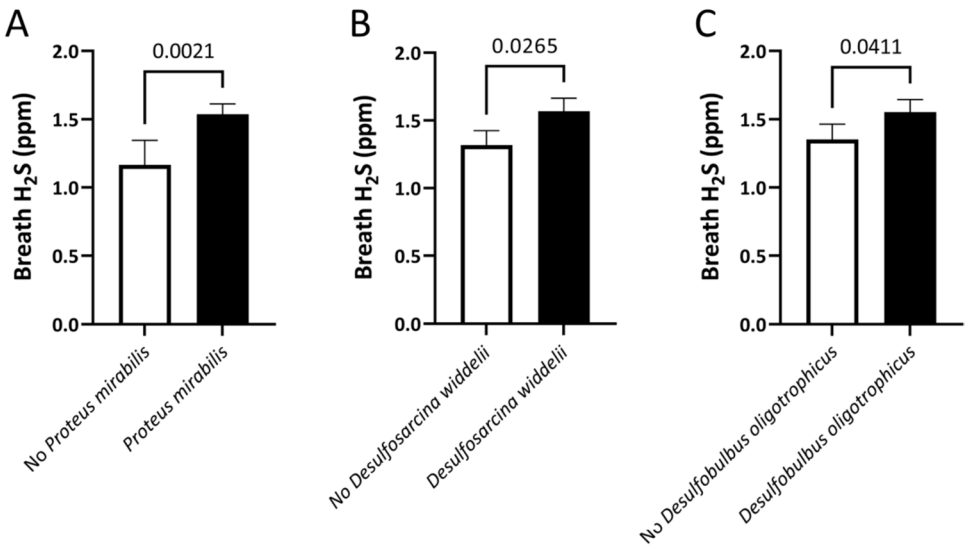

Fig. 2 Comparison of breath H_2S levels in subjects with ($N = 85$) or without ($N = 24$) *Proteus mirabilis* (**A**), with ($N = 60$) or without ($N = 49$) *Desulfosarcina widdelii* (**B**), and with (N = 56) or without (N = 53) *Desulfobulbus oligotrophicus* (**C**) in their duodenal microbiome

Table 2 Prevalences of duodenal archaea in the study population and correlations between their relative abundances (RA) and breath CH_4 levels (ppm) and duodenal *Methanobrevibacter smithii* RA

	Archaeal species	Prevalence within the study population ($N=110$)	Correlation between duodenal RA and breath CH_4 levels		Correlation between duodenal RA and duodenal RA of *M. smithii*	
			R	*P* value	*R*	*P* value
1	*Methanothermococcus okinawensis*	40%	0.319	0.001	0.282	0.003
2	*Halomicroarcula* sp. SHR3	55.45%	0.291	0.002	0.194	0.042
3	*Halarchaeum* sp. CBA1220	63.64%	0.277	0.003	0.190	0.362
4	*Thermococcus argininiproducens*	29.09%	0.226	0.017	0.277	0.003
5	*Methanobrevibacter smithii*	63.64%	0.221	0.02	N/A	N/A
6	*Candidatus* Nitrosotenuis cloacae	36.36%	0.209	0.028	0.379	< 0.0001
7	*Halobacterium litoreum*	57.27%	0.209	0.028	0.312	0.025
8	*Desulfurococcus mucosus*	27.27%	0.19	0.046	0.077	0.713
9	*Halobaculum rubrum*	61.82%	0.188	0.049	0.079	0.707

smithii in the duodenum (Supplemental Fig. 10), but there were no direct correlations between the RAs of any of these species. Breath CH_4 levels increased significantly with co-occurrence of two ($P=0.018$), three ($P=0.027$), or four ($P=0.008$) of these methanogens, as compared to none (Fig. 4A). A positive BT for IMO ($CH_4 \geq 10$ ppm) was also more common when all four of these species co-occurred ($P=0.04$) (Fig. 4B). Furthermore, breath CH_4 levels in IMO-positive subjects correlated with constipation severity ($R=0.577$, $P=0.019$).

CH_4 Producers Correlated with Known H_2-Producing Bacterial Syntrophs in the Duodenum

The bacterial families Christensenellaceae and Ruminococcaceae include known H_2-producing syntrophs for methanogens [31–34]. Consistent with this, positive correlations were found between RAs of bacterial species from these families and the archaeal species that correlated with breath CH_4. *M. smithii* RA correlated with RAs of *Christensenella minuta* ($R=0.313$, $P<0.001$), *Ruminococcus gnavus* ($R=0.433$, $P<0.0001$), *Ruminococcus lactaris*

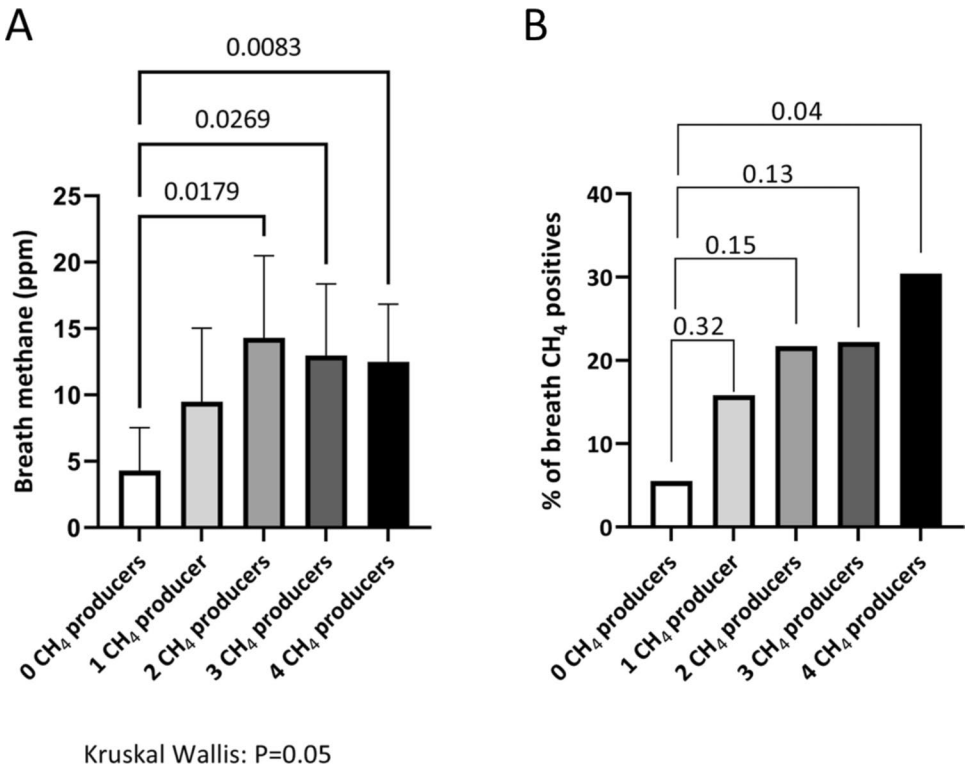

Fig. 4 **A** Comparison of breath methane levels in subjects with zero to four of these methane producers in their duodenum: *M. smithii*, *Halarchaeum* sp. CBA1220, *D. mucosus*, and *H. rubrum*. **B** Comparison of the percentage of IMO-positive subjects ($CH_4 \geq 10$ ppm) when zero to four of these methane producers were present

($R=0.329$, $P<0.001$), *Ruminococcus albus* ($R=0.349$, $P<0.001$), *Ruminococcus bicirculans* ($R=0.244$, $P=0.01$), *Ruminococcus bovis* ($R=0.335$, $P<0.001$), *Ruminococcus champanellensis* ($R=0.286$, $P=0.003$), and *Ruminococcus gauvreauii* ($R=0.346$, $P<0.001$) (Supplemental Table 5). Other methanogens that correlated with breath CH_4 but not with *M. smithii* RA (Table 2) also correlated with RAs of Christensenellaceae and Ruminococcaceae species (Supplemental Table 5).

M. smithii also correlated positively with another H_2-producing bacterium, *Dorea formicigenerans* ($R=0.463$, $P<0.0001$), but correlated negatively with *Enterococcus faecalis* ($R=-0.212$, $P=0.027$) and *Enterococcus faecium* ($R=-0.231$, $P=0.015$).

Pathway Analysis Supported the Presence of H_2S Production and Methanogenesis in the Small Bowel

Microbial metabolic pathways were generated for 105 study subjects. An average of 64.9% of cleaned reads mapped to a microbial genome, of which 0.09% mapped to archaeal genomes (range 0.001–1.9%). *Proteus mirabilis* RA correlated with assimilatory sulfate reduction pathways I ($R=0.280$, $P=0.004$), II ($R=0.226$, $P=0.020$), and III ($R=0.324$, $P=0.001$), and with the assimilatory sulfite reductase enzyme (NADPH) ($R=0.297$, $P=0.002$) found in assimilatory sulfate reduction pathways I and III, in which sulfite is converted to H_2S. *P. mirabilis* also correlated with the dissimilatory sulfate reduction I (to hydrogen sulfide) pathway ($R=0.286$, $P=0.003$), in which sulfate is reduced to H_2S. *D. oligotrophicus* RA correlated with assimilatory sulfate reduction pathways I ($R=0.262$, $P=0.007$), II ($R=0.209$, $P=0.033$), III ($R=0.296$, $P=0.002$), and IV ($R=0.234$, $P=0.016$), and the assimilatory sulfite reductase enzyme (NADPH) ($R=0.287$, $P=0.003$). *P. mirabilis* ($R=0.211$, $P=0.031$) and *D. oligotrophicus* ($R=0.309$, $P=0.001$) also correlated with the sulfate assimilation and cysteine biosynthesis superpathway.

M. smithii RA correlated with the 'methanogenesis from H_2 and CO_2' pathway ($R=0.197$, $P=0.044$) and importantly, also correlated with the archaeal coenzyme F420 biosynthesis I pathway ($R=0.256$, $P=0.008$). Coenzyme F420 is involved in key steps during methanogenesis [35]. *Halarchaeum* sp. CBA1220 RA also correlated with the 'methanogenesis from H_2 and CO_2' pathway ($R=0.230$, $P=0.018$).

CH_4 Producers Correlate with the Presence of H_2S Producers in the Duodenum

Analyses revealed a trend toward correlation between H_2S and CH_4 levels on BT ($R=0.181$, $P=0.059$). Further, RAs of some of the duodenal H_2S producers identified in this study also correlated with breath CH_4 levels (*P. mirabilis* [$R=0.191$, $P=0.047$], *D. oligotrophicus* [$R=0.207$, $P=0.031$], and *D. widdelii* [$R=0.228$, $P=0.017$]), and with RAs of some of the CH_4 producers identified: *P. mirabilis* correlated with *M. smithii* ($R=0.190$, $P=0.047$), *M. okinawensis* ($R=0.192$, $P=0.045$), and *Halomicroarcula* sp. SHR3 ($R=0.218$, $P=0.022$); *D. oligotrophicus* correlated with *Halarchaeum* sp. CBA1220 ($R=0.351$, $P<0.0001$), *Candidatus* Nitrosotenuis cloacae ($R=0.313$, $P<0.0001$), and *Halobaculum rubrum* ($R=0.277$, $P=0.004$); and *D. widdelii* correlated with *M. smithii* ($R=0.254$, $P=0.008$), *Halomicroarcula* sp. SHR3 ($R=0.316$, $P=0.001$), *Halarchaeum* sp. CBA1220 ($R=0.200$, $P=0.037$), and *Candidatus* Nitrosotenuis cloacae ($R=0.201$, $P=0.036$). In IMO-negative subjects, *P. mirabilis* ($R=0.281$, $P=0.021$) and *D. oligotrophicus* ($R=0.353$, $P=0.003$) RAs correlated with looser stool.

Discussion

Here, we demonstrate that CH_4 levels on breath testing (BT) exhibit correlations with levels of methanogens in the small intestine. Importantly, the predominant methanogen *M. smithii* and other methanogens are present in the small intestine and appear to play additive roles in contributing to breath CH_4 levels, in addition to contributions from colonic methanogens. We also show that H_2S levels on BT exhibit correlations with levels of H_2S-producing bacteria in the small intestine, including specifically *P. mirabilis*, *D. oligotrophicus*, and *D. widdelii*. These data support the concept that breath gas profiles are influenced, at least in part, by small bowel microbial composition.

Over the last half century, SIBO and BT have been increasingly understood. Despite the long history of BT as an indirect technique for assessing SIBO, and more recently IMO, the methodology to validate BT was not available until recently. An important question was whether BT findings were related to the levels of microorganisms producing these gases. The lack of data supported criticisms of BT, such as suggestions that BT, and particularly lactulose BT, is merely a marker of transit [36, 37]. Moreover, the measurement of breath H_2S, and the understanding that overgrowth of H_2S producers may contribute to patient symptoms [14], is still evolving. Here, we show that breath CH_4 and H_2S levels are in fact influenced by small bowel microbial composition. Enhanced understanding of different types of microbial overgrowth and potential competition for gas utilization will improve how we interpret breath testing.

Methane is an important gas in breath testing. Substantial evidence now suggests that CH_4 produced in the gut alters gut neuromuscular function [6] and is associated with constipation [7]. Further, constipation severity is proportional to

breath CH_4 levels [38] and stool *M. smithii* levels. In a randomized-controlled study, reducing CH_4 with a combination of antibiotics improved constipation [8, 39]. Recently, stool microbiome analysis in IBS-C subjects found direct correlations between breath CH_4, constipation, and *M. smithii* levels [11]. Moreover, a recent consensus now recognizes CH_4 as a contributor to constipation in IBS-C [40]. However, studies of CH_4 on BT to date, including microbiome studies, have utilized stool. Here, we confirm that, as previously shown [41], *M. smithii* and other methanogens also colonize the small intestine and that the duodenal archaeal profile of subjects with IMO is significantly different from that in non-IMO subjects, with an almost threefold increase in the relative abundance (RA) of the entire Methanobacteriaceae family in the duodenum of IMO subjects. Further, we demonstrate relationships between breath CH_4 levels and the duodenal RAs of *M. smithii* and other archaea including *Halarchaeum* sp. CBA1220, *Desulfurococcus mucosus*, and *Halobaculum rubrum*. Lastly, the RAs of both *M. smithii* and *Halarchaeum* sp. CBA1220 correlated with the archaeal pathway for 'methanogenesis from H_2 and CO_2.' These findings add to stool data on methanogens, and demonstrate that methanogens are found in both the small and the large intestine.

In contrast to CH_4, there are fewer studies on breath H_2S and human health. Studies suggest that H_2S levels are proportional to diarrhea severity [11, 42], but again, are based on stool. In a recent study, subjects with diarrhea-predominant IBS had higher breath H_2S levels than subjects with other IBS subtypes [11], confirming earlier findings by Banik et al. [16]. Further, breath H_2S levels correlated with increases in H_2S-producing bacteria in stool, including *Fusobacterium* and *Desulfovibrio* spp., and with microbial metabolomic markers of H_2S production [11]. A subsequent animal study confirmed forced colonization of rats with H_2S producers *Desulfovibrio piger* or *Fusobacterium varium* resulted in increased gut H_2S production and a diarrhea-like phenotype [42]. Here, we show that the duodenal microbial profiles of subjects with ≥ 1.5 ppm H_2S on BT are significantly different from those of subjects with breath $H_2S < 1.5$ ppm, exhibiting increased alpha-diversity and a more than twofold increase in the duodenal RA of the entire phylum Thermodesulfobacteriota, to which most sulfate-reducing bacteria (SRB) belong, and that the difference between groups was driven by *Desulfovibrio desulfuricans* and other *Desulfovibrio* species. We also demonstrate that breath H_2S levels correlate with the RAs of H_2S-producing bacteria in the duodenum, including phylum Thermodesulfobacteriota species *Desulfovibrio desulfuricans, D. oligotrophicus*, and *D. widdelii*, as well as *Desulfurispirillum indicum* (phylum Chrysiogenota) and *Proteus mirabilis* (phylum Proteobacteria). Interestingly, *P. mirabilis* is a well-known H_2S producer [43] that was considered years ago as a possible contributor to SIBO [44]. Subjects with duodenal *P. mirabilis* exhibit higher H_2S levels on BT, and duodenal *P. mirabilis* RA correlates with the dissimilatory sulfate reduction I pathway (in which sulfate is reduced to H_2S), the assimilatory sulfate reduction pathways, and the enzyme responsible for sulfite conversion to H_2S. Consistent with this, a previous study showed that *P. mirabilis* can use multiple substrates to produce H_2S, including cysteine, cystine, homocystine, mercaptoacetate, sodium sulfite, and sodium thiosulfate [45]. Lastly, subjects with higher breath H_2S levels (≥ 1.5 ppm) had higher duodenal *P. mirabilis* RAs than those with breath $H_2S < 1.5$ ppm. While *P. mirabilis* duodenal RAs may appear low, we previously showed that the duodenum contains 10^3–10^9 16S bacterial copies/mL [46]. Therefore, this *P. mirabilis* RA represents 10^2 16S copies/mL of aspirate. Elevated breath H_2S may also require separate terminology from SIBO (i.e., individuals with a positive hydrogen breath test). The concept of intestinal sulfide overproduction (ISO) and how it relates to SIBO and IMO will need to be assessed by breath testing in a large clinical cohort.

Interestingly, there appears to be an overlap in colonization of subjects with methanogens and H_2S producers and the manifestation of stool phenotypes. Looseness of stool appears to only occur in the presence of H_2S producers and absence of methanogens. Constipation is the dominant feature any time methanogens result in a CH_4 level ≥ 10 ppm (i.e., IMO). Below this point, H_2S producers correlate with looser stool. It is, however, important to note that in this study only the small bowel is being assessed. There is a likely possibility that colonic production of both CH_4 and H_2S further compound gas production and levels on breath test, as well as phenotypes.

It is notable to find methanogens in the small intestine as the small intestine is often recognized as a higher oxygen portion of the gut that should not allow for the growth of strict anaerobes such as methanogens. The small intestine is home to both aerotolerant microbes (aerobes and facultative aerobes) and anaerobes. Friedman et al. showed that oxygen levels in the duodenum are much greater than in the distal gut but also showed that radial oxygen levels vary from lumen to mucosa [47], presumably creating niche environments that can be colonized by different types of microbes.

This study has some limitations. Here, analyses were based on fasting breath samples and not a full BT profile after carbohydrate ingestion. However, fasting samples have been shown to be sufficient to assess CH_4 [22], since methanogenesis itself is not a fermentative process, and appear also be sufficient to assess H_2S production [11]. We also could not measure breath H_2 levels or assess for overgrowth of H_2 producers. Hydrogen is rapidly diffused or consumed after production. As such, fasting H_2 is typically very low and requires carbohydrate ingestion for its

detection. Here, only a fasting single-sample BT could be performed prior to the scheduled standard-of-care endoscopies. Another limitation is a lack of small bowel shotgun sequencing from healthy subjects. This is a challenge and would require consent for a healthy individual to undergo upper endoscopy. Additionally, this study utilized duodenal luminal samples. We have previously shown that the luminal microbial compositions in different small intestinal segments are highly similar [48] although other studies do not fully support this [49, 50]. Lastly, diet can affect microbial composition, fermentation, and gas production in the gut. While all patients in this study were similarly fasted prior to endoscopy, diet in the 24–48 h before the endoscopy procedure was not controlled for.

In conclusion, CH_4 and H_2S levels on breath testing exhibit correlations with levels of methanogenic archaea and H_2S-producing bacteria in the small intestine. This, combined with previous data (using full breath testing) indicating that H_2 correlates with small bowel Enterobacteriaceae levels [20], suggests that breath test results are also influenced by small intestinal microbiome composition, and that the sources of these gases also include the small intestine. These results have important consequences. First, they reinforce the importance of breath testing and its interpretation. Excessive methane and methanogens are associated with constipation and the absence of excessive CH_4 allows the determination that higher H_2S producers mean looser stool. More importantly, identifying the organisms responsible for these gases, their precise gut locations, and their consequences for human health will help develop a new generation of approaches for treating SIBO, IMO and sulfide overproduction.

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Data Availability It is publishing industry standard that 16S rRNA gene sequencing data be available on request. However, it is also understood that shotgun sequencing poses a risk for deidentification due to the presence of human genetics in the sequencing. In general, availability of shotgun data is not expected.

Declarations

Conflict of interest Competing Interests: M.P. is a consultant for Ferring Pharmaceuticals Inc., Salvo Health, Dieta Health, Cylinder Health Inc., and Vivante Health Inc. M.P. has received grant support from Bausch Health and Synthetic Biologics. R.M. has received grant support from Bausch Health. A.R. is a consultant/speaker for and has received grant support from Bausch Health. Cedars-Sinai has a licensing agreement with Gemelli Biotech and Hobbs Medical. A.R., M.P., and R.M. have equity in Gemelli Biotech and GoodLFE. M. P. has equity in Cylinder Health and Salvo Health. The remaining authors report no conflicts of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Review Board and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The REIMAGINE study protocol was approved by the Cedars-Sinai Institutional Review Board (Pro00035192), and all participants provided informed written consent prior to participation.

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