Can a Glucose-Based Hydrogen and Methane Breath Test Detect Bacterial Overgrowth in the Jejunum?

Short Title: Breath Test for Jejunal Bacteria

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ABSTRACT (246 words)

OBJECTIVES: Direct diagnosis of small intestinal bacterial overgrowth (SIBO) requires the collection and culture of microbes from the jejunal lumen, and a finding of over 10^5 viable bacteria per ml. More often, SIBO is diagnosed indirectly, with a non-invasive test for the hydrogen and methane produced by microbial fermentation when a bolus of ingested glucose reaches the jejunum. Our objective is to determine how well this breath test detects the overgrowth of jejunal bacteria.

METHODS: 18 patients with symptoms consistent with SIBO received a glucose breath test. On a later day, the jejunal lumen was sampled via aspiration during enteroscopy. Jejunal aspirates were cultured on aerobic and anaerobic media. DNA was extracted from the same samples and analyzed by quantitative pan-bacterial PCR amplification of 16S ribosomal rRNA genes. PCR provided a culture-independent bacterial cell count, as well as a method to determine microbial viability.

RESULTS: Combined bacterial colony counts ranged from $6x10^3$ to $8x10^6$ CFU/ml, while microbial viability varied from 0.3% to near 100%. Bacterial colonies, DNA-based cell counts, and the anaerobe/aerobe ratio showed no significant correlation with glucose-stimulated levels of exhaled hydrogen or methane. Unexpectedly, patients with lower viability of jejunal bacteria produced significantly higher signals in the hydrogen-methane breath test, with a P-value of 0.014.

CONCLUSIONS: Glucose-stimulated hydrogen or methane production was not correlated with increased bacterial levels in the jejunum. Although a positive breath test result may not indicate SIBO, our results suggest that it reflects some type of jejunal dysfunction or dysbiosis.

INTRODUCTION

Small intestinal bacterial overgrowth (SIBO) is a chronic condition in which the upper jejunum is found to have a higher than normal level of bacteria, traditionally greater than 10^5 CFU (colony forming units) per milliliter (1, 2, 3). Aspirates from a normal jejunal lumen generally contain 10^3 to 10^4 CFU/ml of bacteria, in comparison with the 10^{11} to 10^{12} CFU/ml present in the colon (2). Although there have been varying opinions regarding the type and numbers of bacteria that define SIBO (4), there is a wide consensus that an excess of bacteria in the jejunum can impair its normal physiological function. Patients diagnosed with SIBO often experience gas, abdominal bloating and variable stool frequency. The condition can be associated with nutrient malabsorption by the small intestine, leading to food intolerance and nutritional deficiency (3). Gut-targeted treatment with the poorly-absorbed antibiotic rifaximin can temporarily alleviate the symptoms of SIBO-positive patients (5), although it is unclear how much of the therapeutic effect involves small bowel bacteria or decreases in the colonic microbiota. In cases where gut surgery has compromised function of the iliocaecal valve, SIBO is thought to arise because of a retrograde flow of microbes from the colon. However, in the majority of cases, SIBO is not associated with gastrointestinal surgery, and it is unclear how it originates (3). In non-surgical SIBO, analysis by 16S rRNA gene metagenomics has indicated that the greatly enlarged bacterial flora retains a normal jejunal species profile. In such idiopathic SIBO, there appears to be no major influx of colonic microbes into the jejunum (6).

SIBO diagnosis using small bowel aspirates is considered a “gold standard” method, but it requires invasive enteroscopy and time-consuming microbial culture. For this reason, non-invasive hydrogen and methane breath tests have become the most frequently used test for clinical diagnosis of SIBO (7, 8). Hydrogen and methane in exhaled breath are of purely microbial origin, and cannot be produced by cells of the human body. Many species of eubacteria in the lower gut produce hydrogen gas (H_2) as a by-product of anaerobic metabolism. Microbial hydrogen and methane are readily transferred to the

blood through vessels of the gut wall. Once they reach the lungs, these gases can be detected in the exhaled breath (7, 8). In the colon, methane gas (CH_4) is generated by archaebacteria under extremely anaerobic conditions (9, 10) that are generally only found in the dense microbial populations of the colonic lumen, where the oxygen pressure is well below 1 mm Hg O_2 (11), in contrast to the 40 to 58 mm Hg O_2 found in the intestinal wall (12). In the classic lactulose breath test, patients ingest a solution of lactulose, a sugar that cannot be metabolized or absorbed by the small intestine. Once ingested lactulose reaches the colon, it is metabolized by bacteria, which then produce hydrogen through anaerobic fermentation. With some individuals, the breath hydrogen time course reveals two peaks. The first is thought to be produced when lactulose encounters an unusually large population of bacteria in the distal small intestine (3), and the second hydrogen peak when the ingested lactulose finally reaches the colon (8).

Breath tests based on the ingestion of glucose are, in principle, more sensitive and specific for jejunal bacteria. Their rationale is that the jejunum normally absorbs nearly all of the glucose bolus before it can reach the distal ileum or colon. In the setting of excessive jejunal bacteria, glucose ingestion should produce only a single, early peak of hydrogen and/or methane. Magnitude of the peak rise above baseline is, in principle, positively correlated with the number of bacteria in the jejunum. In clinical practice, a peak rise above baseline greater than 20 parts per million hydrogen or 10 parts per million methane has been defined as a SIBO-positive breath test (4). Many patients produce little or no methane, a situation that appears associated with an absence of methanogenic microbes in the colon (9). Other patients produce significant amounts of methane but little or no hydrogen. In these cases, it is suspected that hydrogen is initially generated, but then consumed by the biosynthesis of methane (9, 10).

Positive hydrogen and methane breath test results have been found associated with a wide range of small bowel disorders (3, 4, 13) and are frequently used in the diagnosis of SIBO. However, it remains

unclear how reliably breath tests reflect the level of bacteria in the jejunum (14, 15). There is also room for improvement in the direct analysis of jejunal microbes. A persistent source of uncertainty with tests on aspirates of the jejunal lumen is that many species of gut bacteria could be under-counted because they do not grow readily in standard bacterial culture (1, 4, 16). In addition, facultative anaerobes could be over-counted by methods that use both aerobic and anaerobic media (17). To address these concerns, we have supplemented microbial culture by pan-bacterial quantitative PCR of purified DNA (6). This method can rapidly detect all bacterial cells without the need for culture, and decreases species bias.

METHODS.

Research Subjects. Our study included 4 male and 14 female patients, aged 26 to 79. Informed consent was obtained and the protocol was approved by the Institutional Review Board of Texas Tech University at El Paso, following principles of the Declaration of Helsinki. This report analyzes the glucose breath test results of the same patients characterized previously for their jejunal microbiota (6). Each patient had common SIBO-associated symptoms, such as bloating, abdominal discomfort, gas, or irregular bowel habits. None of the patients showed evidence of acute gastroenteritis, diabetes, inflammatory bowel disease, malignancy, small bowel obstruction or small bowel fistulas. None had undergone gastrointestinal surgery, including type 1 or type 2 Billroth procedures, small bowel resections, colonic resections, ileostomy or bariatric surgery.

Glucose-based breath test. In preparation for the breath test, subjects received no antibiotics for the preceding 4 weeks, and had not eaten tofu, beans, or large protein meals the night before the test. Subjects did not smoke or sleep during the measurement period. The mouth was cleaned by tooth brushing and mouthwash just before the test. Hydrogen and methane measurements were made on-site, using a QuinTron Breath Tracker SC Digital Microlyzer with carbon dioxide calibration (QuinTron

Instrument Company, Milwaukee, Wisconsin). Gas levels were first measured in two independent breath samples, and the results averaged to obtain hydrogen and methane baselines. Then, 90 grams of glucose dissolved in 100 ml water was ingested by each patient and breath samples were measured every 20 minutes for 180 minutes (8). All gas peak maxima appeared between 40 and 100 minutes of glucose ingestion. Net hydrogen and methane were determined by subtracting the pre-ingestion baseline from the maximum peak value. Clinically positive breath test results were defined as: net hydrogen greater than 20 ppm or net methane greater than 10 ppm at any time during the post-glucose sampling period (4). Before it leaves the gut, hydrogen is avidly consumed in the major methanogenic pathway, which has the stoichiometry $4\ H_2 + CO_2 \Rightarrow CH_4 + 2\ H_2O$ (9, 10). To correct for this methanogenesis-associated decrease in the hydrogen signal, we added the net breath hydrogen level to 2 times the net breath methane level $\{H_2 + 2x\ CH_4\}$. Our differential weighting of the two gases was based on the 2-times heavier weighting of net methane in the current definition of a clinically positive breath test (4).

Collection of jejunal aspirates. As previously (6), jejunal aspirates were collected directly after an overnight fast. The oral cavity was first cleaned extensively by tooth brushing and the use of germicidal mouthwash. An Olympus SIF-Q180 Enteroscope was employed to access the jejunum. The air column was capped during passage, and opened once the probe reached 20 cm beyond the ligament of Treitz. At this point, 50 ml of sterile deionized water was injected into the jejunum through the instrument. After 1 minute, 40 ml of this water lavage solution was retrieved by aspiration through the instrument and stored on ice in a sterile container. The samples were divided into equal halves and analyzed separately for bacterial counts and DNA composition. To estimate dilution of the jejunal fluid, aspirate supernatant, obtained after centrifugation for 10 minutes at 10,000 x g, was measured with a Fisher Traceable® precision electrical conductivity meter. The conductivity reference standard was a solution

containing 142 mM NaCl, 4.5 mM KCl and 0.5 mM $CaCl_2$, modeled on the major electrolytes of jejunal fluid (18). During fasting, luminal contents are primarily physiological fluids secreted by the jejunum, and have an electrolyte composition that shows minimal variation between individuals (18). Internal compartment volumes available to the lavage solution can vary substantially (19). Electrical conductivity allowed us to determine lavage dilution factors, and calculate microbial abundances per ml of luminal fluid. Lavage produced a greater total microbial yield from a wider area of the jejunum than the traditional small volume aspirates of undiluted intestinal juice. This higher yield decreased possible effects of microbial contamination from higher levels of the gut.

Microbial culture. Colony counts were obtained as described previously (6, 17). Serial dilutions of jejunal aspirate samples were plated within 3 hours of aspiration. Aerobic cultures were plated on trypticase soy agar with 5% sheep blood or on Levine eosin methylene blue agar (Becton Dickinson, Franklin Lakes, New Jersey), and incubated at 37°C for 24 hours. Anaerobic cultures were plated on CDC anaerobic blood agar, KV blood agar (kanamycin-vancomycin), or phenylethyl alcohol blood agar (Thermo Scientific, Lenexa, KS), and incubated in Gas Pak jars at 37°C for 48 hours. Summation of bacterial counts from all 5 culture conditions, followed by normalization to undiluted luminal fluid, provided total bacterial CFUs per ml.

DNA extraction and bacteria-specific QPCR analysis. Microbes and cellular debris from half of the jejunal aspirate were pelleted by centrifugation for 10 minutes at 10,000 x g and the pellet stored frozen at -80°C. DNA extraction and purification from the frozen pellet employed the Power Soil Microbial DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA), which mechanically disrupted bacteria by grinding with garnet beads. Yield of bacterial DNA was estimated by quantitative PCR (QPCR) carried out in triplicate using an Applied Biosystems Step One Plus thermal cycler with ABI Amplitaq Gold®

and SYBR Green detection. QPCR amplification of the V1-V3 region of the 16S rRNA gene with pan-bacterial DNA oligonucleotide primers 27ForM-20mer AGAGTTTGATCMTGGCTCAG and 533RevK-19mer TTACCGCGGCKGCTGGCAC was performed at a stringent annealing temperature of 59°C. C_t values were converted to microbial DNA concentration and *E. coli* genome equivalents using a standard dilution series of *E. coli* K12 DNA as described previously (6).

Statistical Analysis. R Consortium software, version 3.2.5 obtained from www.r-project.org, was used for statistical analysis of the data. Data from breath test-positive and test-negative groups was compared using the Mann-Whitney U-test. Diagnostic threshold-independent correlation of quantitative breath test results with bacterial load was determined using Spearman's correlation coefficient, a conservative non-parametric test for fit to an increasing monotonic function. The Spearman rank-order statistic is not affected by linear or log transformation of the data. P-values represented 2-tailed tests of the null hypothesis of no correlation.

RESULTS.

Jejunal aspirate and glucose breath test results are summarized in **Table 1**. Patients 1 through 10 had SIBO, based on a bacterial titer over 10^5 CFU/ml. Regarding baseline measurements of breath samples collected just prior to glucose ingestion, hydrogen was detected in 16 patients, while methane was detected in only 7. Ingested glucose generally produced increases in both hydrogen and methane, reaching peak values within 40 to 80 minutes after ingestion. Net hydrogen increase ranged up to 177 ppm (Table 1, column 7). Methane levels and their percent increases were generally lower, with a maximum rise of 18.5 ppm in net methane (Table 1, column 10).

Analysis of the aspirates revealed over a 200-fold range in the amount of bacterial DNA (BGE/ml) between individuals (**Figure 1a**), and over a 1,000-fold range in live colony counts (CFUs/ml) (**Figure**

1b). We were able to estimate the fraction of viable, colony forming bacteria in the jejunal aspirates of each patient by dividing the number of colony forming units (CFUs) by the number of bacterial genome equivalents (BGEs) of DNA, which represents a culture-independent cell count. This viability index ranged over 300-fold between individual patients (**Figure 1c**), suggesting major differences in bacterial health, growth and senescence. In our earlier report of jejunal species composition (6), differences in bacterial load and viability did not appear significantly related to phylum or genus composition of the jejunal microbiota. Fortunately, the large range in bacterial density within the jejunum offered an excellent opportunity to evaluate the breath test as an indirect method for quantifying jejunal bacteria. With this wide range, variation in breath test and bacterial measurement was less likely to obscure significant correlations. Our first round of analysis used clinical criteria (4) to divide the patients into groups that were positive or negative for the breath test. The Mann-Whitney U-test indicated that there was no significant difference in bacterial load between these two groups, whether measured by means of bacterial DNA (BGE/ml) (**Figure 1a**) or by combined colony counts (CFU/ml) (**Figure 1b**). Microbial viability revealed the greatest difference between the breath test positive and negative groups, but at P-value = 0.124, this still fell short of significance.

The data was also evaluated by plotting glucose-stimulated hydrogen and hydrogen-methane against bacterial abundance (**Figure 2**). This use of raw gas production data avoided the possible introduction of bias by the selection of a clinical breath test diagnostic threshold. Because of the wide range in bacterial load and breath test signals, data were plotted on a log scale for both axes. Again, the load of bacterial DNA showed no significant correlation with the levels of net hydrogen or methane in the breath test (**Figure 2a,d**). When aspirated bacteria were measured by colony forming units, there was actually a weak negative correlation, which meant that higher hydrogen and methane production was associated with lower numbers of jejunal bacteria (**Figure 2c,d**). This result is the opposite of that expected from the rationale of the glucose breath test. For the 18 individuals in this study, this negative

correlation had a P-value of 0.22, which did not reach the threshold of significance. Although we expected that more anaerobic conditions throughout the gut would be associated with better hydrogen and methane production, we observed no significant relationship between breath test results and the ratio of anaerobes to aerobes in our aspirate cultures (Table 1, column 4). The Spearman correlation with the ratio of anaerobes to aerobes was clearly not significant for either hydrogen (P-value = 0.567), or for hydrogen-methane (P-value = 0.408).

However, when we compared breath test results with the bacterial viability for each subject, we observed significant correlation of decreased microbial viability with increasing breath hydrogen (R = 0.496; P-value = 0.036) (**Figure 3a**). An even higher Spearman correlation (R = - 0.569) was obtained for the combined hydrogen-methane result, with a P-value of 0.014 (**Figure 3b**). An important question regarding low viability is whether the large fraction of non-viable bacterial DNA detected by quantitative PCR represented dead, dying or dormant cells. It should be noted that DNA measured by quantitative bacteria-specific PCR can, in principle, survive long after cells have lysed and broken open. In our study, samples used for DNA extraction had been pelleted with moderate force centrifugation, at 10,000 x g for 10 minutes. Free DNA released from lysed cells would not be pelleted under these conditions, but the pellet should be enriched for DNA confined within bacterial cells. If the cells were dead but their cell walls intact, they might still confine the DNA, but damage to the membrane would likely make the bacteria porous to smaller molecules, including DNAase I and other hydrolytic enzymes secreted by the pancreas. Within the jejunum, DNA trapped in porous cells should be broken down to fragments too small to detect by QPCR. A final possibility is that many of these DNA-containing cells are not dead, but dormant. Under many circumstances, bacteria can enter a quiescent state where they remain alive, but do not form colonies in standard culture (16).

DISCUSSION.

Given the widespread use of hydrogen and methane breath tests for SIBO diagnosis (3, 8), we were surprised that our results revealed no positive correlation of test results with the density of jejunal bacteria. Over the past two decades, several studies have questioned whether breath tests provide a reliable diagnosis for SIBO (14, 15, 20). These studies have generally concerned lactulose breath tests, and used culture to measure bacteria in jejunal aspirates. Various explanations have been proposed for the poor agreement between microbial culture of aspirates and breath test signals. One is the difficulty of detecting double peaks in the lactulose test (20), which may contribute to poor reproducibility of this test (21). For aspirate analysis, a serious issue is that culture methods tend to greatly favor the growth of certain species, and these often vary in abundance between individuals. We have addressed the first concern by concentrating on the glucose breath test, which is considered more specific for jejunal bacteria. In principle, ingested glucose does not reach the colon because it is almost fully absorbed by the small intestine. We have addressed the inherent variability of microbial culture by employing a PCR-based method that selectively quantifies all bacterial DNA, and is not dependent on culture (6). In the past, another matter of concern with aspirate analysis was the possibility that microbes retrieved from the jejunum consisted almost entirely of oral bacteria acquired as contaminants during passage of the enteroscopy probe through the mouth (4). Our characterization of bacterial species recovered from the jejunal lumen (6) suggests that this is not a significant problem when the mouth is properly cleansed. Most of our jejunal aspirates contained high levels of the genera *Escherichia*, *Citrobacter* and *Klebsiella*, which are effectively absent from the mouth. The genera *Selenomonas, Capnocytophaga, Kingella* and *Tannerella* are abundant in oral microbiota, but were found only at very low levels in our jejunal samples (6, 22).

Microbial methane production is especially dependent on highly anaerobic conditions, to an even greater extent than hydrogen production (9, 10). Such conditions are only found within dense microbial masses of the colonic lumen, where facultative anaerobes consume nearly all available gaseous oxygen

(11). In contrast, the jejunum is well supplied with oxygen-carrying blood vessels, and its sparse microbiota lacks the capacity to create a highly anaerobic environment. Microbial species composition of the jejunum by metagenomic analysis indicates that it is devoid of the extreme anaerobes that are normally abundant in the colon (6, 23, 24). Even in individuals with SIBO-level microbiota, the jejunum has an oxygenated environment with a 10^4 to 10^6-fold lower density of bacteria than the colon. It is therefore not an optimal site for the production of detectable amounts of hydrogen and methane. Even our highest DNA-based count of 2.9×10^7 bacteria per ml, estimates under a milligram of bacteria in 200 ml fluid volume for the entire small bowel (19). Even in an optimized bioreactor (25), this would not be enough bacteria to produce the hydrogen generally detectable in the glucose breath test.

What do we make of the great range in viability of jejunal bacteria, and its significant inverse correlation with breath test signal? Microbial viability in the jejunum could be affected by a combination of many features of the luminal environment, including acidity, digestive enzymes, bile salts, immunological responses, inflammation or anti-microbial peptides. Our earlier study did not find an obvious relationship between viability and species composition of the jejunal microbiota (6), but this could still play a role. Microbial viability and breath test signals may both be secondary indicators of more basic underlying physiological disturbances in the luminal environment.

In summary, our results do not support the use of a positive glucose breath test to diagnose bacterial overgrowth in the small intestine. However, our results suggest that the breath test is sensitive to the viability of jejunal microbiota. The origin of this new relationship is unknown, and further work is necessary to determine why the breath test provides a clinically useful indicator for suspected jejunal dysfunction and dysbiosis. Currently, the collection and analysis of jejunal aspirates still remains the most direct and reliable means of diagnosing SIBO. Coupled with PCR for quantifying bacteria and high throughput DNA sequencing for microbial identification, this traditional method offers a powerful approach to understanding many disorders affecting the jejunum.

STUDY HIGHLIGHTS

WHAT IS CURRENT KNOWLEDGE

- Hydrogen and methane breath tests are used for diagnosis of small intestinal bacterial overgrowth.
- Their diagnostic accuracy is not well documented.

WHAT IS NEW HERE

- Bacterial PCR is a promising culture-independent method for analysis of jejunal fluid.
- Positive glucose breath tests are not correlated with increased jejunal bacteria.
- However, positive breath tests are associated with a decreased fraction of living jejunal bacteria.

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TABLE and FIGURE LEGENDS.

Table 1. Bacterial Culture, Bacterial DNA and Breath Test Results

	Jejunal Aspirates				Glucose Breath Test Results							
	1	2	3	4	5	6	7	8	9	10	11	12
patient	Colonies	Bacterial DNA	Viability	Anaerobe	H_2 Base	H_2 Peak	Net H_2	CH_4 Base	CH_4 Peak	Net CH_4	Clinical	$H_2 + 2xCH_4$
	CFU / ml	BGE / ml	CFU / BGE	Ratio	ppm	ppm	ppm	ppm	ppm	ppm	Pos-neg	Net ppm
1	7.9×10^6	3.2×10^6	2.469	1.85	3	2	-1	16.5	17	0.5	neg	0
2	5.2×10^6	5.5×10^6	0.945	8.35	0	0	0	0	0	0	neg	0
3	1.5×10^6	4.7×10^6	0.319	18.23	1	4	3	0	0	0	neg	3
4	7.9×10^5	1.3×10^7	0.061	0.68	7	18	11	30	70	40	Positive	91
5	4.7×10^5	2.9×10^7	0.016	0.32	11.5	65	53.5	14	19	5	Positive	63.5
6	4.2×10^5	2.4×10^7	0.018	1.21	1.5	3	1.5	72.5	91	18.5	Positive	38.5
7	3.1×10^5	1.3×10^7	0.024	2.69	13	27	14	0	0	0	neg	14
8	2.0×10^5	2.3×10^6	0.087	16.54	1	3	2	1	7	6	neg	14
9	1.6×10^5	3.1×10^7	0.005	11.66	2	16	14	0	0	0	neg	14
10	1.1×10^5	7.9×10^6	0.014	2.37	19.5	42	22.5	0	5	5	Positive	32.5
11	6.6×10^4	1.0×10^6	0.066	7.13	1.5	5	3.5	0	0	0	neg	3.5
12	4.2×10^4	1.5×10^5	0.280	11.99	0	2	2	0	0	0	neg	2
13	3.1×10^4	5.2×10^6	0.006	3.69	1.5	9	7.5	0	0	0	neg	7.5
14	2.8×10^4	1.3×10^6	0.022	8.01	3.5	3	-0.5	49.5	52	2.5	neg	4.5
15	2.2×10^4	8.5×10^6	0.003	36.04	30	199	169	0	9	9	Positive	188
16	2.2×10^4	3.2×10^6	0.007	15.13	2	3	1	49	66	17	Positive	35
17	9.9×10^3	2.8×10^5	0.035	6.04	42	219	177	0	10	10	Positive	197
18	5.7×10^3	7.3×10^5	0.008	3.67	2	11	9	0	0	0	neg	9

Table 1. Bacterial culture, bacterial DNA and breath test results. Primary data for 18 subjects ranked by colony counts. Column 1: Yield of total combined aerobic and anaerobic viable colonies per ml of undiluted jejunal fluid. Column 2: Bacterial DNA, in genome equivalents (intact bacterial cell count) per ml. Column 3: Viability ratio of combined colonies per bacterial genome (bacterial cell). Column 4: Ratio of live colony number under anaerobic conditions to those growing aerobically. Column 5: Hydrogen baseline. Column 6: Hydrogen maximum value. Column 7: Net increase in breath hydrogen (column 6 minus column 5). Column 8: Methane baseline. Column 9: Methane maximum value. Column 10: Net increase breath methane. Column 11: Clinical breath test outcome, defined as ≥ 20 ppm H_2 or ≥ 10 ppm CH_4. (reference 4). Column 12: Combined

breath hydrogen and methane measure, which is the sum of net hydrogen and 2 times the net methane. This is based on the clinical test criterion used in column 11, in which methane carries twice the weight of hydrogen (reference 4).

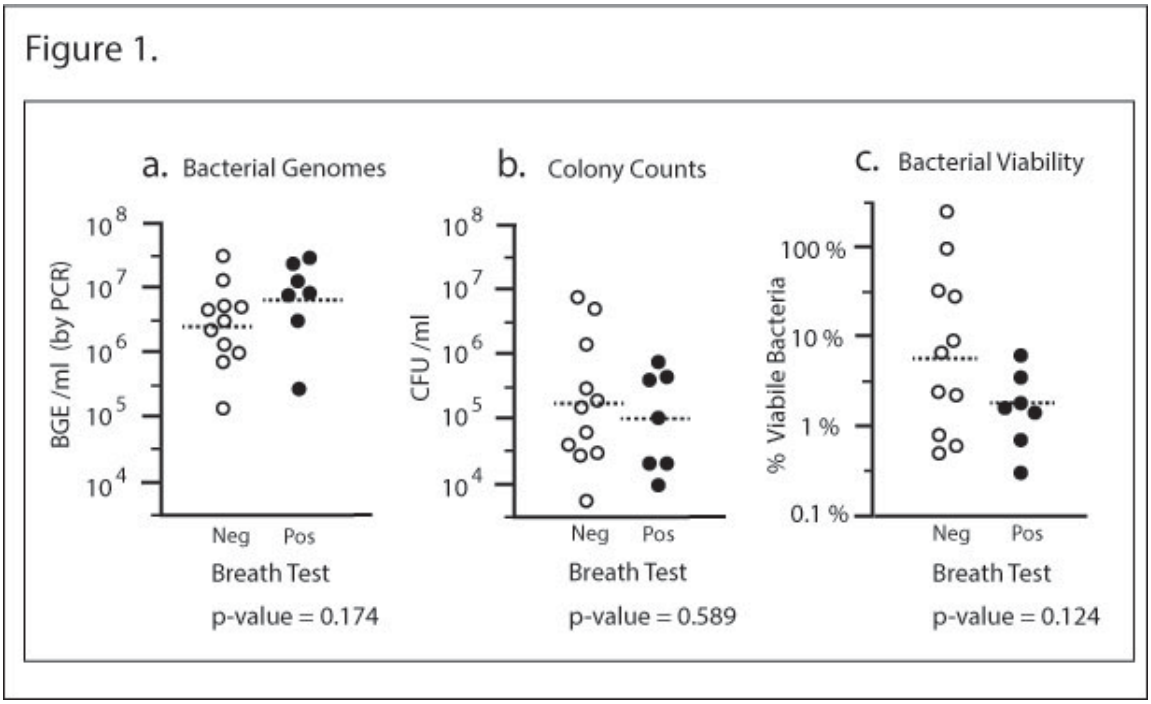

Figure 1. Comparison of jejunal bacteria for positive and negative breath tests. Interpretation of hydrogen and methane breath test data according to current clinical criteria of ≥ 20 ppm net H_2 or ≥ 10 ppm net CH_4 (reference 4; Table 1, column 11) identified 11 individuals who were test-negative (open dots) and 7 who were test-positive (black dots). Individual values for test-negative and test-positive groups were plotted against **a. Bacterial Genomes:** Bacterial genome equivalents (cell numbers) obtained by quantitiative PCR of isolated DNA. **b. Colony Counts:** Summation of aerobic and anaerobic colony counts. **c. Bacterial Viability:** Ratio of colony counts / genome equivalents in the aspirate, expressed as percent viability.

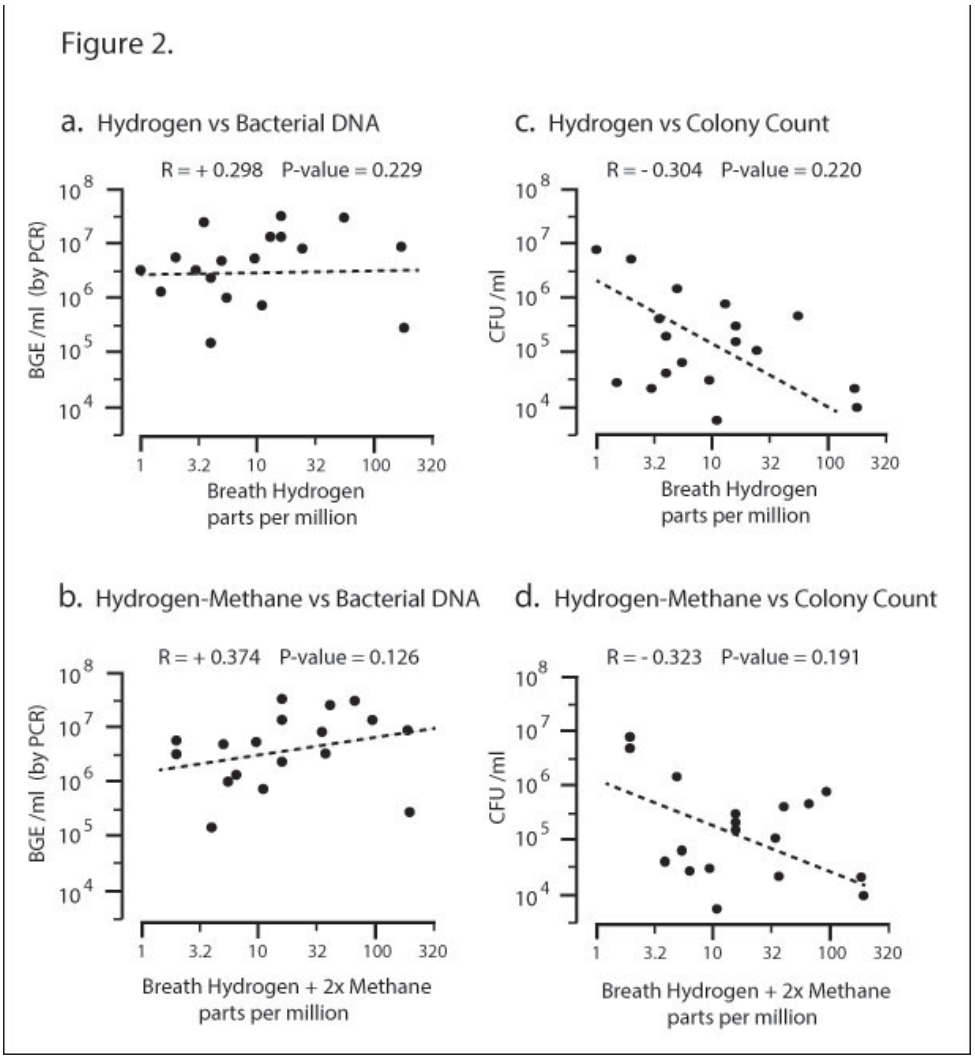

Figure 2. Gas production compared with bacterial load in the jejunum. Net breath test data from all patients is plotted against bacterial load, measured by **a, b:** bacterial genome number (DNA-based cell count), or **c, d:** total live colony count. **a:** Breath hydrogen vs Bacterial Genome Equivalents (BGE). **b:** Breath hydrogen-methane combined vs Bacterial Genome Equivalents (BGE/ml). **c:** Breath hydrogen vs Colony count (CFU/ml). **d:** Breath hydrogen-methane combined vs Colony count (CFU/ml). Spearman rank-order correlation and the P-value of the null hypothesis (no correlation) are presented above each graph. P-values ≤ 0.05 are considered significant. Each data axis is plotted on a $\log_{10}$ scale, with net breath gas values transformed by the addition of 2 ppm for compatibility to the log scale. This adjustment and log transformation do not alter the Spearman statistic.

Figure 3.

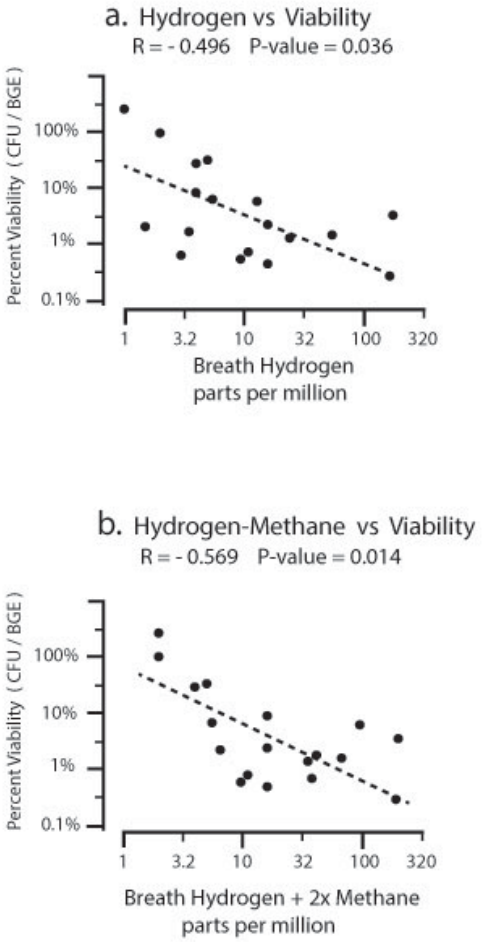

Figure 3. Gas production compared with bacterial viability in the jejunum. Net breath test data from all patients is plotted against bacterial viability. **a:** Breath hydrogen in parts per million vs Percent bacterial viability. **b:** Breath hydrogen-methane combined in parts per million vs Percent bacterial viability. Spearman correlation, P-values and graphs are obtained as in Figure 2. Viability ratio (Table 1, column 4) is graphed on a $\log_{10}$ scale as percent viability.