

Methanogenesis associated with altered microbial production of short-chain fatty acids and human-host metabolizable energy

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Abstract

Methanogens are methane-producing, hydrogen-oxidizing (i.e. hydrogenotrophic) archaea. Numerous studies have associated methanogens with obesity, but these results have been inconsistent. One link to metabolism may be methanogens' hydrogen-oxidizing ability, thus reducing hydrogen partial pressure and thermodynamically enhancing fermentation of sugars to short-chain fatty acids (SCFAs) that the host can absorb. Because research linking methanogenesis to human metabolism is limited, our goal with this exploratory analysis was to investigate relationships between methanogens and other hydrogenotrophs, along with the association of methanogens with human metabolizable energy (ME). Using results from a randomized crossover feeding study including a western diet and a high-fiber diet, well-characterized human participants, and continuous methane measurements, we analyzed hydrogenotroph abundance and activity, fecal and serum SCFAs, and host ME between high and low methane producers. We detected methanogens in about one-half of participants. We found no evidence that methanogens' consumption of hydrogen to produce methane affected other hydrogenotrophs. High methane producers had greater serum propionate and greater gene and transcript abundance of a key enzyme of the hydrogen-consuming, propionate-producing succinate pathway. High methane producers also had greater ME than low producers on the high-fiber diet. A network analysis revealed positive relationships between the methane-production rate and bacteria capable of degrading fiber and fermenting fiber-degradation products, thus forming a trophic chain to extract additional energy from undigested substrates. Our results show that methanogenesis in a microbial consortium was linked to host ME through enhanced microbial production, and subsequent host absorption, of SCFAs.

Keywords: hydrogenotrophs; methane; methanogens; human metabolism; short-chain fatty acids; metabolizable energy

Introduction

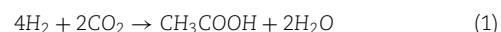
The human body hosts ~38 trillion bacterial cells, most of which reside in the colon [1]. Besides bacteria, the intestinal microbiota also includes archaea, protists, and viruses [2]. One of the important functions of the intestinal microbiota is fermentation [3]. Unmetabolized macronutrients entering the colon become substrates for bacterial hydrolysis and fermentation, which generates simple products, such as H₂, CO₂, and short-chain fatty acids (SCFAs) [4].

Although SCFAs have received much attention due to their impact on host health [5], H₂ remains understudied despite its importance to the microbiome. High H₂ partial pressure inhibits fermenting bacteria from regenerating NAD⁺ from NADH, which decreases the fermentation of complex substrates and impairs microbial growth [6, 7]. H₂ partial pressure also influences the thermodynamics of SCFA production: Low partial pressure favors

acetate and butyrate production, while high partial pressure favors propionate production [8].

The H₂ produced by fermentation can be oxidized by microorganisms known as hydrogenotrophs in well-known microbial processes that produce acetate, sulfide, or methane [9]. The three common groups of hydrogenotrophic microorganisms in the human colon are homoacetogenic bacteria, sulfate-reducing bacteria (SRB), and methanogenic archaea. These three groups can use H₂ as an electron donor to produce energy [9]. The hydrogenotrophic groups, their substrates and products, and the fate of their products are summarized below (Fig. 1).

Homoacetogenic bacteria, which include species in the genera *Blautia*, *Clostridium*, and *Ruminococcus*, oxidize H₂ and reduce CO₂ to make acetate using the Wood-Ljungdahl pathway [10]. The stoichiometry for homoacetogenesis is:



Received: 27 January 2025. Revised: 24 April 2025. Accepted: 20 May 2025

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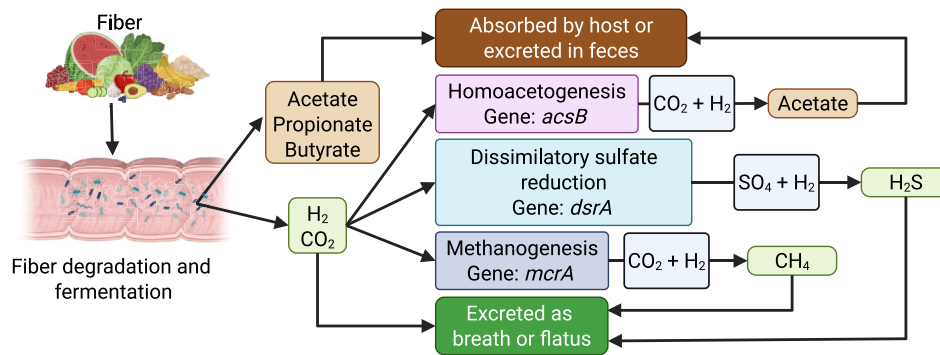
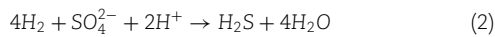


Figure 1. Hydrogenotrophic processes in the human colon when the macronutrient input is carbohydrate. Fiber that reaches the colon is hydrolyzed and fermented by the microbiome to produce SCFAs, H_2 , and CO_2 . Most SCFAs are absorbed by the host or excreted in feces. Although some H_2 is released through the breath or flatus, most is metabolized in the colon by homoacetogenesis, dissimilatory sulfate reduction, or methanogenesis. Acetate produced through hydrogenotrophy is absorbed by the host or excreted in feces. CH_4 and some hydrogen sulfide produced through hydrogenotrophy are released through the breath or flatus. Figure created using biorender.

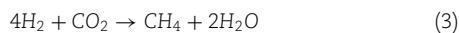
Homoacetogens carrying out reaction (1) are autotrophs, which means that their carbon source is inorganic carbon, or CO_2 . However, most homoacetogens are not obligate hydrogenotrophs and can ferment organic substrates, such as glucose, as the electron donor and carbon source [11].

SRB in the genera *Desulfovibrio* and *Fusobacteria* oxidize H_2 and reduce sulfate to sulfide through dissimilatory sulfate reduction [12]:



Like homoacetogens, SRB can also ferment organic substrates, such as lactate and pyruvate [11].

The predominant methanogen in the human colon is *Methanobrevibacter smithii*, although *Methanosphaera stadtmanae* and *Methanomassiliicoccus* spp. are sometimes detected [13]. Methanogens can be categorized into three groups: (i) hydrogenotrophic methanogens that reduce CO_2 (or formate); (ii) acetoclastic methanogens that ferment acetate to CH_4 and CO_2 ; and (iii) methylotrophic methanogens that oxidize and reduce methanol to CH_4 and CO_2 [14]. All methanogens found in human intestines so far are hydrogenotrophic [15]:



Unlike homoacetogens and SRB, hydrogenotrophic methanogens do not have alternative metabolic pathways: They rely solely on H_2 and CO_2 for their metabolism, and detected CH_4 is always produced via a H_2 -consuming process.

CH_4 also is unique among the hydrogenotrophs because it is only made by archaea and is not metabolized by the human body [16]. In contrast, H_2S is produced from other microbial sulfur metabolisms [17] and metabolized by mitochondria in host colonocytes [18]. Likewise, acetate is produced through fermentation by many bacteria [19], and it is a common substrate for any respiring bacteria [20].

Some evidence supports that methanogens have an impact on host metabolism in mouse models [11]. For example, mice inoculated with methanogens showed increased weight and adiposity despite consuming the same amount of food as controls [21, 22]. However, the role of methanogens in human metabolism is complicated and controversial. Studies have found conflicting correlations between methanogens and obesity/leanness [23] and anorexia [24].

Here, we investigated relationships between methanogens and the other hydrogenotrophs, along with the impact of methanogens on human-host metabolism, in samples from a tightly controlled randomized crossover feeding study with well characterized human participants [25]. Briefly, the researchers evaluated the microbial contribution to human-host energy balance using two distinctly different diets: the Western Diet (WD) and the Microbiome-enhancer Diet (MBD). The WD was comprised of foods that were low in fiber and resistant starch, small in particle size (such as peanut butter vs. whole nuts on the MBD), and included processed foods. The MBD, in contrast, was designed to deliver more microbial substrates to the colon by being less absorbable by the host. The MBD contained more whole foods, fiber, resistant starch, and was limited in processed foods. They found that diet altered microbiome's structure, and that the microbiome's metabolic activity contributed to host metabolizable energy (ME).

The results from the controlled randomized crossover feeding study [25] are particularly well-suited for a deeper investigation into methanogens and their role in the microbiome and host metabolism. That study strictly accounted for energy input (diet), energy expenditure, and energy output (urine, feces, gas). Additionally, they continuously measured CH_4 production during the inpatient portion of the study using a first-in-human method within a whole room calorimeter [26]. Combining the study's CH_4 measurements with its multi-omic data allows us to detect relationship among the methanogens, the other hydrogenotrophs, and the human host.

Because the hydrogenotrophs compete for H_2 , we hypothesized that high methanogenic activity would lead to a lowering of homoacetogenic and sulfate-reduction activities. These changes in the microbial community would then be associated with factors related to the human host's metabolism: e.g. fecal SCFA output, serum SCFA concentration, and host ME. We found that, rather than directly affecting host metabolism, methanogenesis may be a biomarker for a microbiome with enhanced ability for energy extraction.

Subjects and methods

Overview of clinical study

Details of the clinical study (NCT02939703) from which the data and samples for this work were derived were previously published [25, 26]. Briefly, the clinical study was approved by

the AdventHealth Institutional Review Board and conducted at the AdventHealth Translational Research Institute in Orlando, Florida. After signing informed consent and evaluating eligibility, 17 participants (nine men and eight women) were enrolled and completed the study, which was a randomized crossover-controlled feeding study with the WD as a control and the MBD as an intervention. This design minimized the impact of confounders as each participant served as their own control. The study period took place over 61 days. Each participant's caloric requirement was determined during the baseline period (days 1–9), and meals were prepared uniquely for each participant to maintain energy balance. Participants consumed those meals outpatient for 11 days then inpatient for the next 11 days with a >14-day washout between diet periods. During the 11-day inpatient stay for each diet, each participant's energy expenditure was measured in whole room calorimeter for 6 days and fecal samples were collected.

CH₄ measurements

CH₄ release was measured continuously during each 6-day period that the participant was in the whole-room calorimeter. CH₄ concentration was measured with an off-axis integrated-cavity output spectroscopy (OA-ICOS). A detailed description and validation of the method can be found elsewhere [27].

Colonic transit time

Colonic transit time (CTT) was measured while participants were in the whole-room calorimeter. Participants ingested a SmartPill (Medtronic) that sent data to a sensor worn by the participants recording temperature, pressure, and pH [26].

Host metabolizable energy

Briefly, host ME, the energy from the diet available to the host [28], was computed as the total energy intake minus the energy lost in the feces. Calculation details are previously published [25].

Fecal and serum short chain fatty acids

Fecal and fasting serum SCFAs were quantified by targeted metabolomics (Metabolon, Inc., Mooresville, NC). Fecal SCFAs were normalized as previously published [25].

Quantification of key hydrogenotroph genes

The abundances of the homoacetogens, SRB, and methanogens were measured using the quantitative polymerase chain reaction (qPCR) for genes that identify each hydrogenotroph: *acsB* for homoacetogens, *dsrA* for SRB, and *mcrA* for methanogens. DNA samples used for the qPCR assays of key hydrogenotroph genes were the same as those used for qPCR of 16S rRNA genes as previously published [25]. Primers, thermocycler settings, standards, and references for each gene are summarized in [Supplementary Table 1](#). All qPCR assays were performed on a Thermofisher Applied Biosystems Quant Studio 3. Standards for each gene were custom IDT gBlocks gene fragments of the target genes from a representative microbe of each hydrogenotroph group. 7-point calibration curves for each assay were generated in triplicate using gene copy numbers ranging from 10¹ to 10⁸ of their respective gene standards.

The qPCR values were transformed from log to exponential values, normalized to daily fecal output, giving us daily fecal copy number (gene copy numbers/day). Because each hydrogenotroph contains one gene copy per cell of their respective gene [29–31], qPCR measurements of gene copy number provided an accurate estimate of hydrogenotroph cell numbers/day in the feces.

DNA sequencing and sequence processing

We used DNA sequences and taxonomic data that were previously published [25]. However, we generated annotated genes abundances for this manuscript. To generate the gene abundance data, DNA sequences were quality controlled with FastQC (version 0.12.0) [32]. Adapters were trimmed using TrimGalore (version 0.6.5) [33]. DNA sequences were then aligned to Hg38 (GRCh38.p14) using bowtie2 (version 2.4.4) [34]. Aligned sequences were removed, and the remaining reads were paired and annotated using HUMAnN3 (version 3.8) [35] using standard parameters. A small pseudo-count (equal to half of the lowest non-zero count) was added to any zeros and gene abundances were centered log-ratio transformed as suggested for the analysis of compositional data [36, 37].

RNA extraction, library preparation, and sequencing

For RNA sequencing, aliquots were taken from the same fecal samples used for DNA sequencing. Fecal-sample processing, RNA extraction, library prep, and mRNA sequencing were performed at the University of North Carolina at Chapel Hill Microbiome Core (Chapel Hill, NC, USA), which is supported by the following grants: Gastrointestinal Biology and Disease (CGIBD P30 DK034987) and the UNC Nutrition Obesity Research Center (NORC P30 DK056350). RNA was extracted using the Qiagen RNeasy PowerMicrobiome Kit (Cat No./ID: 26000–50). RNA depletion was performed using QIAseq FastSelect –5S/16S/23S Kit (Cat No./ID: 335925), and library prep was performed using QIAseq Stranded Total RNA Lib Kit (Cat No./ID: 180745). RNA samples were sequenced using the HiSeq 4000 PE150 platform (Illumina). To avoid batching effects, fecal samples were randomized prior to nucleic acid extraction and all samples were sequenced at the same time.

RNA-sequence processing

RNA-sequencing outputs were quality controlled with FastQC (version 0.12.0) [32]. Adapters were trimmed using TrimGalore (version 0.6.5) [33]. RNA sequences were aligned against Hg38 (GRCh38.p14) using STAR (version 2.7.11a) [38]. Aligned sequences were removed, and the remaining reads were paired and annotated using HUMAnN3 (version 3.8) [35] using standard parameters. A small pseudo-count (equal to half of the lowest non-zero count) was added to any zeros and transcript abundances were centered log-ratio transformed as suggested for the analysis of compositional data [36, 37].

Wilcoxon signed rank test

The Wilcoxon signed rank test was used to compare the median log₁₀(daily fecal copy number/day) for hydrogenotroph marker genes *mcrA* (methanogens), *acsB* (homoacetogens), and *dsrA* (SRB). Samples in which a gene was undetected were given a gene copy number of 1 and then log transformed.

Categorizing high and low methane producers

Because methane production rates followed a bimodal distribution ([Supplementary Figure 1](#)), study participants were categorized as either a high CH₄ producer or a low CH₄ producer for subsequent statistical testing. Methane production rates were log₁₀ transformed and tested for multimodality by the Excess Mass Test (Excess mass = 0.22, P-value < 2.2e – 16) using the *modetest* function from the R package “multimode” (version 1.5) [39]. Using the *locmodes* function in the same package, the threshold for high CH₄ production was found to be 37 mL CH₄/day. Thus, participants

were evaluated on a per sample basis and categorized as a high methane producer (>37 mL CH_4/day) or low methane producer (<37 mL CH_4/day) irrespective of diet.

Linear mixed model

We used a linear mixed model (LMM) to test the relationship between high and low CH_4 producers, our binary categorical primary independent variable of interest, and outcome variables. We accounted for the effects of diet, diet period, diet sequence, and colonic transit time (CTT) by including them as covariates in our LMMs. We included participant ID as a random factor to account for the non-independence of the samples. An interaction term between CH_4 producers and diet was included in the model for host ME. Details about dependent and independent variables, covariates, and random term used in our LMMs can be found in [Supplement Table 2](#). LMMs were run using the `lmer` command from the R package “lmerTEST” (version 3.1–3) [40].

The residuals of each LMM were evaluated for a normal distribution by using `qqnorm` and `shapiro.test` commands, both of which were from the R package “stats” (version 4.2.2) [41]. The Benjamini-Hochberg method was used to correct for multiple comparisons and adjusted P -values ≤ 0.10 were considered significant. The same analyses were completed with CH_4 as a continuous variable. The results of the continuous-variable analyses are in the [Supplementary Data](#).

Network analysis

Microbiome network analysis was performed using the Cytoscape (version 3.10.2) [42] plugin Conet (version 1.1.1) [43], an ensemble co-occurrence analysis tool. Conet was run with default settings using Pearson, Spearman, Mutual Information, Bray–Curtis dissimilarity, and Kullback–Leibler dissimilarity to detect associations between microorganisms. The Benjamini-Hochberg method was used to correct for multiple comparisons, and adjusted P -values ≤ 0.05 were considered significant.

Results and discussion

Homoacetogens and SRB were present in all participants, but not all participants had methanogens

We investigated the abundance of methanogens, homoacetogens, and SRB among the trial participants. We performed qPCR targeting genes that encode enzymes in the hydrogenotrophic pathways for each group: *mcrA* for methanogens, *acsB* for homoacetogens, and *dsrA* for SRB.

We detected methanogens in only nine participants for both diets and one participant for only the MBD ([Fig. 2](#)). Consequently, the average $\log_{10}$ (gene copy number/day) was 5.3 ± 4.8 for methanogens. In contrast, we detected homoacetogens and SRB in all 17 participants for both diets. On average, homoacetogens were more abundant than SRB: $\log_{10}$ (gene copy number/day) of 10.2 ± 0.46 for homoacetogens versus 8.8 ± 0.86 for SRB, but homoacetogens and SRB were more abundant than methanogens. Detecting methanogens in only some participants is consistent with past results showing that the abundance of *M. smithii* in the human gut was either high or very low [44, 45]. The presence or absence of methanogens can have important implications for hydrogenotrophs in a microbiome. In mixed-culture experiments, SRB became the dominant hydrogenotroph when methanogens were inhibited [11]. In co-culture experiments, *Bacteroides thetaiotaomicron* expressed more glycoside hydrolases, enzymes that break down polysaccharides, and degraded and fermented

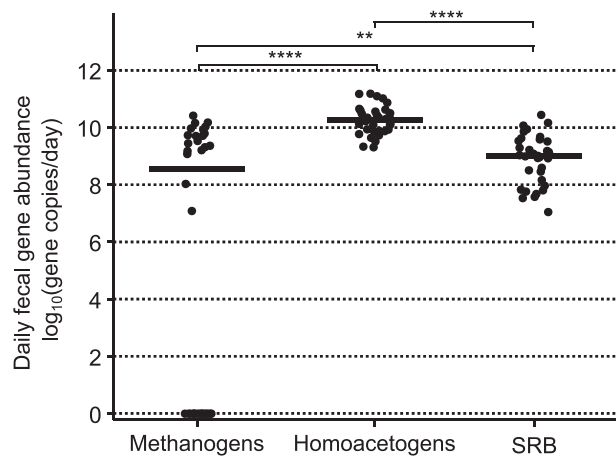


Figure 2. Abundances of hydrogenotrophs in daily fecal copy numbers [$\log_{10}$ (gene copies/day)] measured by qPCR. Daily fecal copy numbers are gene copy numbers measured in feces and normalized to daily fecal output. Homoacetogens and SRB were detected in all participants in both diets. Methanogens were only detected in nine participants in both diets and one participant in the MBD. Homoacetogens were more abundant than SRB and methanogens. SRB were more abundant than methanogens. Abundance medians are marked by horizontal black bars. Comparison tests between abundance medians were performed with Wilcoxon signed rank test. *: Adj. P -value < 0.10 , **: Adj. P -value < 0.05 , ***: Adj. P -value < 0.01 , ****: Adj. P -value < 0.0001 . NS is non-significant with an adjusted P -value > 0.10 . (BH adjustment, $N = 17$).

more glycans when the methanogen *M. smithii* was present, compared to when the SRB *Desulfovibrio piger* was present [22].

Homoacetogens and SRB did not show patterns of competition with methanogens

The absence of methanogens in a portion of the participants might lead to differences in competition for H_2 among the hydrogenotrophs. We hypothesized that, in the absence of methanogens, homoacetogens or SRB (or both) would become more abundant and active. To test the hypothesis that homoacetogens or SRB (or both) would become more abundant and active, we compared mean methanogen, SRB, and homoacetogen daily fecal copy numbers and gene and transcript abundances of key genes in each hydrogenotrophic pathway between high and low CH_4 producers. We used the key genes and their transcripts instead of all the genes in the pathways, because many of the genes in the hydrogenotrophic homoacetogenic pathway and dissimilatory sulfate reduction pathway are also used in other types of metabolisms, such as the one-carbon pathway and assimilatory sulfate reduction pathway, respectively [12, 22, 46].

Compared to low CH_4 producers, high CH_4 producers had larger methanogen daily copy numbers in the feces and abundance of *mcrA* genes and transcripts, ([Fig. 3A](#)). In contrast, homoacetogen daily copy numbers, *acsB* genes, and *acsB* transcripts were not significantly different between the two groups ([Fig. 3B](#)). Although SRB copy numbers were higher in high CH_4 producers, *dsrA* gene and transcript abundances were not significantly different between the two groups ([Fig. 3C](#)).

Our results suggest that competition for H_2 was not a factor limiting the growth and accumulation of SRB and homoacetogens. One explanation is that SRB and homoacetogens have diverse metabolisms and do not rely solely on H_2 as an electron donor [46, 47]. For example, SRB can utilize lactate for sulfate respiration [17] or for fermentation in the absence of sulfate [48, 49]. Homoacetogens can also ferment sugars instead of oxidizing H_2 during

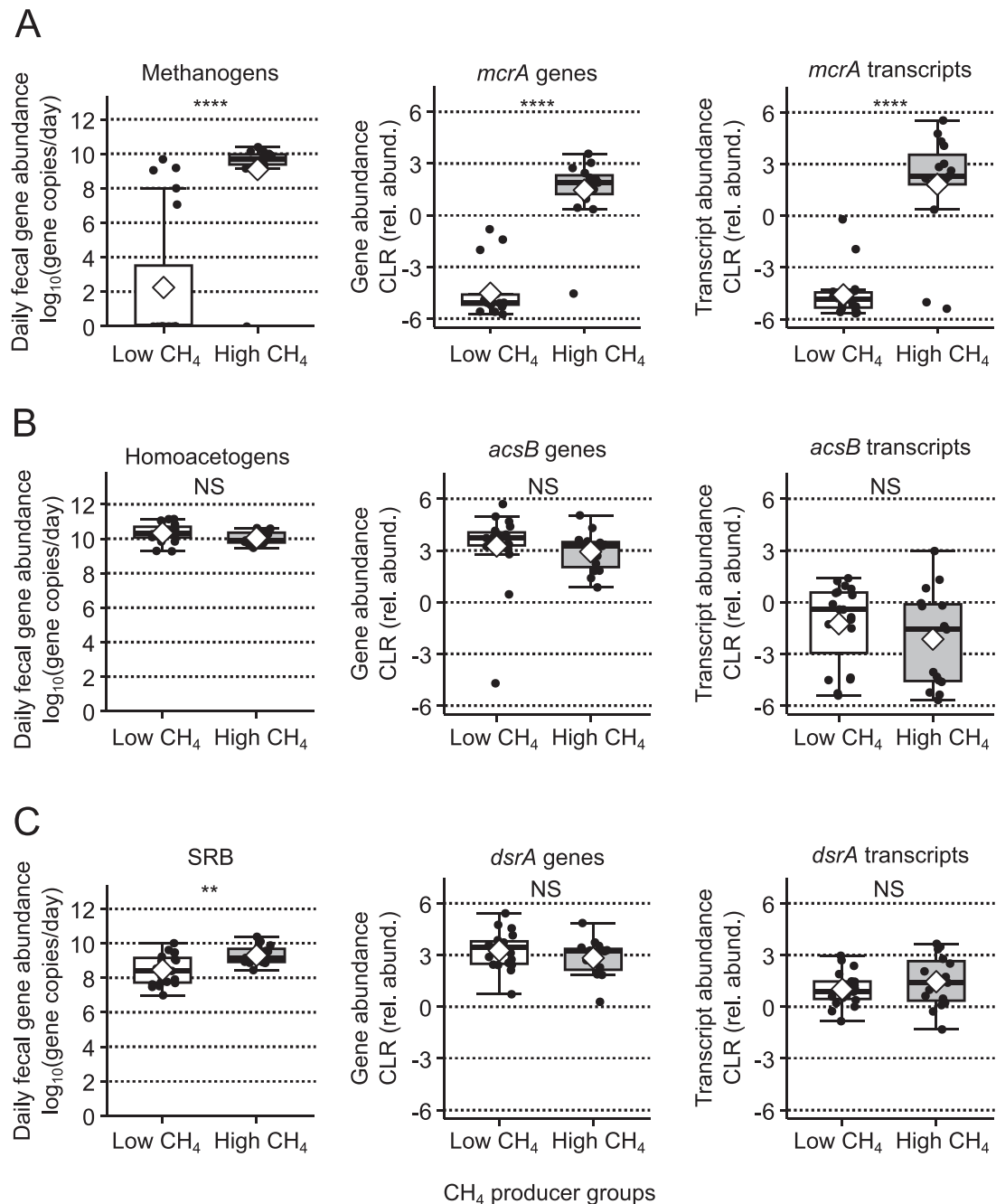


Figure 3. Comparisons of mean hydrogenotroph daily fecal copy numbers, gene and transcript abundance between CH₄ groups. (A) Methanogen daily fecal copy numbers, *mcrA* genes, and *mcrA* transcripts were significantly higher in high CH₄ producers than low CH₄ producers. (B) Homoacetogen daily fecal copy numbers, *acsB* genes, and *acsB* transcripts were not significantly different between CH₄ groups. (C) SRB daily fecal copy numbers were significantly higher in high CH₄ producers than low CH₄ producers, but *dsrA* genes, and *dsrA* transcripts were not significantly different between the CH₄ groups. CLR is the centered log-ratio transformation. Group means are marked by white diamonds. Group medians are marked by horizontal black lines. All means comparisons were made by linear mixed model. *: Adj. P-value <0.10, **: Adj. P-value <0.05, ***: Adj. P-value <0.01, ****: Adj. P-value <0.0001. NS is non-significant with an adjusted P-value >0.10. (BH adjustment, N = 17).

hydrogenotrophic homoacetogenesis [50]. Although homoacetogens can grow mixotrophically, using hydrogenotrophy and fermentation simultaneously for energy generation, an experiment evaluating the mixotrophy of *Blautia coccooides*, a homoacetogen found in the human gut, revealed that hydrogenotrophy was inhibited by glucose [51]. The high abundance of *acsB* genes relative to the low abundance of *acsB* transcripts suggests that homoacetogens may not have been relying on H₂ for electrons. Another explanation is that the electron flow to CH₄ was too

small to have had a major impact on the other hydrogenotrophs' metabolic function. Indeed, electron flow to CH₄ was ~1% of the electron-equivalent intake to the large intestine [52].

High CH₄ producers had higher serum propionate concentrations than low CH₄ producers

Although hydrogenotrophs did not appear to compete for H₂ and the flow of electrons into CH₄ was small, CH₄ production still may

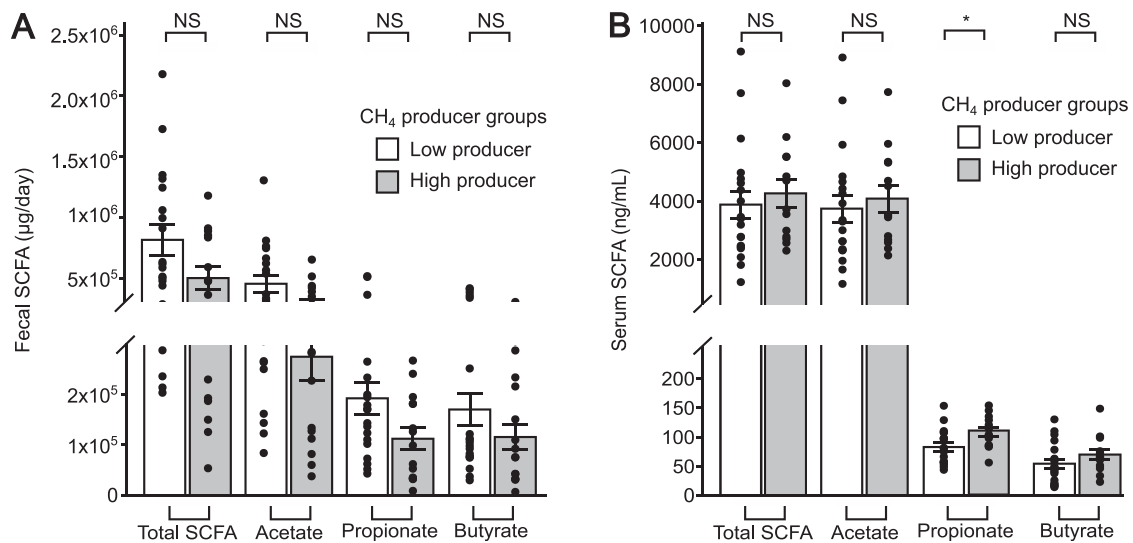


Figure 4. Comparisons of mean fecal SCFA output and serum SCFA concentration between CH₄ groups. (A) Total fecal SCFA, fecal propionate and fecal butyrate were not significantly different between CH₄ groups. (B) Serum propionate concentrations were significantly higher in high CH₄ producers than low CH₄ producers, but total SCFA, serum acetate, and serum butyrate were not significantly different between CH₄ groups. All means comparisons were made by linear mixed model. *: Adj. P-value <0.10, **: Adj. P-value <0.05, ***: Adj. P-value <0.01, ****: Adj. P-value <0.001. NS is non-significant with an adjusted P-value >0.10. (BH adjustment, N = 17).

have had an impact on the H₂ concentration in the large intestine. Because fermentation is inhibited by high H₂ partial pressure [53], additional H₂ consumption by methanogens may still have relieved a thermodynamic inhibition on fermentation and led to greater fecal SCFA outputs and serum SCFA concentrations. Fecal SCFA were not significantly different between groups, although the means were consistently lower for the high CH₄ producers (Fig. 4A). In contrast, serum propionate concentrations were higher in high CH₄ producers than low CH₄ producers (Fig. 4B).

Fecal and serum SCFAs are difficult to interpret on their own. Fecal SCFAs are the result of microbial production and cross feeding, and host absorption. Once absorbed by colonocytes, butyrate is used as an energy source and propionate is used for intestinal gluconeogenesis [54]. Any remaining SCFAs then pass through the liver and into systemic circulation [20, 55] and are measured as serum SCFAs. Acetate is the most abundant serum SCFA, while propionate and butyrate are measured in concentrations considerably smaller than acetate [20, 55]. Isotope studies have shown that serum acetate comes from microbial and endogenous sources, but propionate and butyrate are mostly almost entirely microbially produced in the intestine [20, 55]. Therefore, the opposing relationships of fecal SCFA and serum propionate with CH₄ production suggest a faster rate of SCFA uptake in participants that produced more methane.

High CH₄ producers had a higher abundance of genes and transcripts for a propionate-producing pathway

Given the positive association between serum propionate and CH₄-production rate, we investigated if propionate production could have been altered by methanogenesis. We evaluated the relationship between the CH₄-production rate and the abundance of key microbial genes and transcripts for microbial pathways for propionate production. The common propionate-producing pathways, the succinate and acrylate pathways [54], consume H₂ [55]. The succinate pathway has two variants, one using methylmalonyl-CoA decarboxylase (*mmd*) and the other using methylmalonyl-CoA carboxyltransferase (MMCT). The acrylate

pathway is characterized by the acryloyl-CoA reductase (*acr*) [54, 55]. The abundances of the *mmd* genes and transcripts were higher in high CH₄ producers (Fig. 5A), MMCT genes and transcripts were not different between groups (Fig. 5B), and *acr* gene abundance, but not transcript abundance, was higher in high CH₄ producers (Fig. 5C).

Miceli et al. [56] showed that methanogenic microbial communities, while maintaining CH₄ production rates, increased propionate production in response to increased carbohydrate availability for fermentation. We calculated the average amount of H₂ consumed by methanogenesis and propionate production in participants with detectable methanogen fecal copy numbers. The calculations can be found in the Supplementary Materials. We found that methanogenesis consumed, on average, ~0.12 e⁻ eq/day of H₂, whereas we estimated that propionate production could have consumed as much as 716 e⁻ eq/day of H₂, assuming all propionate was produced via the succinate pathway. Our calculations support the notion that electron flow to propionate production was substantially greater than to methanogenesis.

Host ME was higher for high CH₄ producers, but only for the MBD

Absorption of microbially generated SCFA has been estimated to contribute up to 10% of human daily caloric uptake [57], and our results indicate that high CH₄ production may be an indicator or biomarker of increased SCFA absorption by the host. Thus, we compared host ME between high and low CH₄ producers. Because diet had a strong effect on host ME, we also evaluated if diet and CH₄-producer group had a combined effect on host ME. High CH₄ producers had a significantly higher host ME than low producers on the MBD, but not on the WD (Fig. 6).

Because methanogens in the human colon comprise only ~1.2% of the microbiome [58] and rely solely on H₂ plus CO₂ (or formate) for energy [59, 60], they neither produce nor consume SCFAs. In principle, methanogens could have contributed to increased host ME by consuming H₂ and thermodynamically accelerating fermentation to SCFAs. As we showed above, the

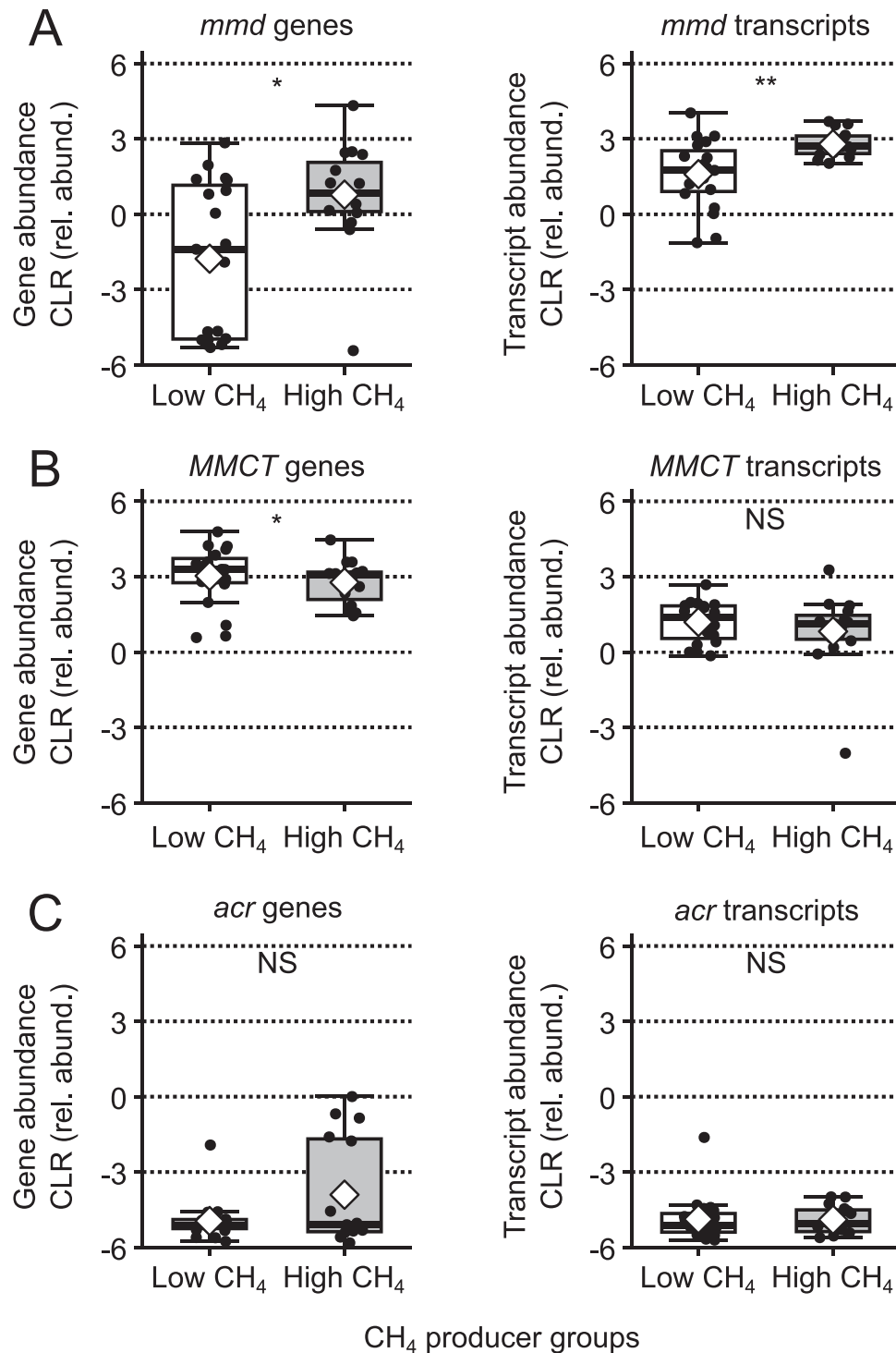


Figure 5. Comparison of gene and transcript abundance for the propionate-producing succinate (*mmd* and *MMCT*) and acrylate (*acr*) pathways between CH₄ groups. (A) *Mmd* (methylmalonyl-CoA decarboxylase) gene and transcript abundance were significantly higher in high CH₄ producers than low CH₄ producers. (B) *MMCT* (methylmalonyl-CoA carboxyltransferase) gene and transcript abundance were not significantly different between high and low CH₄ producers. (C) *Acr* (acryloyl-CoA reductase) genes were significantly higher in high CH₄ producers than low CH₄ producers, but transcripts were not significantly different between high and low CH₄ producers. CLR is centered log-ratio transformation. Group means are marked by a white diamond. Group medians are marked by horizontal black line. All means comparisons were made by linear mixed model. *: Adj. P-value <0.10, **: Adj. P-value <0.05, ***: Adj. P-value <0.01, ****: Adj. P-value <0.0001. NS is non-significant with an adjusted P-value >0.10. (BH adjustment, N = 17).

electron flow to CH₄ was small relative to the potential electron flow to propionate. A more compelling explanation is that methanogens were a key component of a microbial community that enabled greater host uptake of energy from the large intestine in a fiber-rich, whole food diet.

Fiber-degrading bacteria and propionate-producing bacteria co-occur with *M. Smithii*

Considering the positive association between host ME and CH₄-production rate in the MBD, along with methanogens' inability

Table 1. Microorganisms positively associated with CH₄ production rate. Bacteria and archaea significantly and positively associated with CH₄ production. Asaccharolytic bacteria lack the ability to degrade sugars and utilize other substrates, such as amino acids, or cross-feed on bacterial metabolites such as lactate. All relationships were statistically significant after correcting for multiple comparisons (adj. P-value <0.05, BH adjustment, N = 17).

Fiber Degraders	Fermenters	Asaccharolytic	Unknown
<i>Bacteroides caccae</i>	<i>Alistipes shahii</i>	<i>Desulfovibrio piger</i>	<i>Eubacterium</i> sp. CAG:251
<i>Bifidobacterium adolescentis</i>	<i>Collinsella stercoris</i>	<i>Methanobrevibacter smithii</i>	<i>Oscillibacter</i> sp. 57_20
<i>Bifidobacterium pseudocatenulatum</i>	<i>Holdemanella biformis</i>	<i>Phascolarctobacterium succinatutens</i>	<i>Slackia isoflavoniconvertens</i>
<i>Coprococcus catus</i>	<i>Odoribacter splanchnicus</i>		
<i>Coprococcus eutactus</i>			
<i>Eubacterium eligens</i>			
<i>Eubacterium halli</i>			
<i>Lachnospira pectinoschiza</i>			
<i>Parabacteroides merdae</i>			
<i>Prevotella copri</i>			
<i>Roseburia faecis</i>			

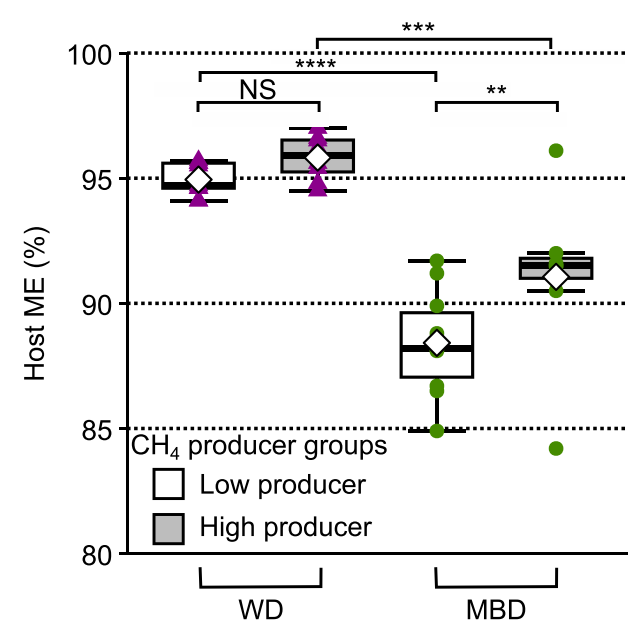


Figure 6. Host ME in high and low CH₄ producers by diet. Host ME was significantly higher on the WD than the MBD for high CH₄ producers and low CH₄ producers. In the WD, host ME was not significantly different between high and low CH₄ producers. However, in the MBD, high CH₄ producers had significantly higher host ME than low CH₄ producers. WD is the western diet. MBD is the microbiome enhancer diet. Group means are marked by a white diamond. Group medians are marked by horizontal black lines. All means comparisons and variable relationships were made by linear mixed model. *: Adj. P-value <0.10, **: Adj. P-value <0.05, ***: Adj. P-value <0.01, ****: Adj. P-value <0.0001. NS is non-significant with an adjusted P-value >0.10. (BH adjustment, N = 17).

to degrade fiber or ferment sugars, we looked for co-occurring bacteria that made the methanogenic microbial community better equipped to degrade and ferment organic substrates. We used CoNet [42], a network co-occurrence inference tool, to find bacteria strongly associated with CH₄ production. After correction for multiple comparisons, 22 bacteria were positively correlated with CH₄ production (Table 1).

Bacteria positively associated with CH₄ production were either fiber degraders—(*Bacteroides caccae* [61], *Bifidobacterium adolescentis* [62], *Bifidobacterium pseudocatenulatum* [63], *Coprococcus catus* [64], *C. eutactus* [65], *Eubacterium eligens* [61], *E. halli* [66], *Lachnospira pectinoschiza* [62], *Parabacteroides merdae* [64], *Prevotella copri* [65], and

Rosburia faecis [67])—or a diverse group of fermenters—(*Alistipes shahii* [67], *Collinsella stercoris* [68], *Holdemanella biformis* [69], and *Odoribacter splanchnicus* [70]). Five of the 22 positively associated bacteria produce propionate via the succinate pathway (*B. caccae* [71], *P. copri* [54], *C. eutactus* [72], *O. splanchnicus* [71], and *Phascolarctobacterium succinatutens* [73]), which aligns with the results on propionate-producing pathways (Fig. 5).

We hypothesize that a network consortium of fiber degraders, fermenters, propionate producers, and methanogens provided the host with more SCFA, and other substrates, and contributed to higher host ME (Fig. 6). Indeed, co-culture experiments show that in the presence of methanogens bacterial metabolism is steered toward degradation of polysaccharides [22, 62, 64], growth is uninhibited by H₂ or fermentation products [66], and SCFA production increases [65, 67, 71].

In the proposed microbial consortium, fiber degraders break down polysaccharides into sugars; fermenters consume the sugars to release acetate, butyrate, propionate, and H₂; and methanogens and propionate producers consume the H₂ and keep fermentation thermodynamically favorable. Thus, the methanogenic microbial communities can utilize more complex substrates than non-methanogenic microbial communities. Methanogenic microbial communities also can release more substrates, in addition to SCFAs, such as other carboxylates and monosaccharides in the intestinal lumen for the host to absorb, increasing host ME. SCFA transporters in colonocytes can also transport other carboxylates such as lactate and pyruvate [72], which the colonocytes can use for biomass or energy production. Colonocytes also have monosaccharide transporters and are thus able to absorb sugars released from polysaccharide degradation [74]. Furthermore, the expression of SCFA and monosaccharide transporters in colonocytes is increased by their respective substrates [75, 76] which would increase absorption of those substrates. This explains how the methanogenic communities can potentially produce more SCFAs and other substrates for the host to absorb and contribute to previously observed differences in energy extraction when the microorganisms are properly fed, such as in the MBD [25].

Conclusion and future directions

Methanogens and methane production were present in only about one-half of the study participants. Although methanogen fecal copy numbers and *mcrA* genes and transcripts were higher in the

high CH₄ producers, SRB and homoacetogen fecal copy numbers, genes, and transcripts had no relationship with CH₄ production. Consequently, we saw no evidence that the methanogens' uptake of H₂ to produce methane affected the other hydrogenotrophs. This probably occurred because SRB and homoacetogens, unlike methanogens, do not rely on H₂ oxidation for energy generation. Methanogenesis was associated with higher host ME on the MBD and high serum propionate on both diets, but not with fecal SCFAs, suggesting that methanogens were linked to enhanced SCFA production and uptake. We also found that bacteria positively associated with CH₄ are well-suited to degrade and ferment fiber, as well as consuming H₂ through propionate production.

Taken together, our results add important missing mechanistic insights into the relationship between methanogens and energy extraction. Methanogens appear to be part of a microbial consortium capable of enhanced energy extraction and absorption with the MBD (a fiber-rich, whole-food diet). This microbial consortium might contribute to previously observed variation in host ME on the MBD. However, future studies with larger sample sizes are needed to confirm our results.

Future research should focus on investigating whether methane or methanogens affect production and absorption of SCFAs and other substrates in the colon. As we show, the impact that methanogens have on host metabolism may not be in removing H₂, but rather by influencing other physiological parameters, such as SCFA production and possibly SCFA absorption. Alternatively, methanogens might simply constitute a detectable signal for enhanced energy extraction. Understanding the interactions of methanogens with the human host and other microorganisms in the human large intestine could lead to dietary or other means to modulate methanogen activity in ways that improve the host's metabolic health. Evaluating the presence and activity of methanogens that accumulate on the intestinal epithelium would provide important information that cannot be obtained solely from fecal samples. Additionally, future experiments that involve in vitro microbial communities of homoacetogens, SRB, and methanogens would allow for direct measurements of all hydrogenotrophic activities for a wide range of conditions. This would provide a higher resolution picture of the structural and functional relationships among the hydrogenotrophs.

Nucleic acid sequences

DNA and RNA sequencing data from this study can be found in the BioProject database under the accession codes PRJNA913183 and PRJNA947193.

Author contributions

BD and RK-B designed the study analysis. BD performed bioinformatic and statistical analyses. SRS, RK-B, and BER designed the parent clinical study and obtained funding. KDC and EAC supervised the clinical trial. EAC supervised methane measurements. FY helped with statistical analyses. TLD helped with QA/QC and critical feedback on the data. BD wrote the first draft of the manuscript with edits and input from all authors. All authors critically reviewed the manuscript, provided feedback, and agreed with the content.

Supplementary material

Supplementary material is available at *The ISME Journal* online.

Conflicts of interest

The authors declare there are no conflicts of interest.

Funding

This project was funded by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health (Award Number RO1DK105829). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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