Metabolism of Dietary Fiber by Human Gut Microbes: Interspecies Interactions and the Influence of Molecular Hydrogen

by

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Dedication

For my family

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Abstract

The human gut microbiota is a diverse and abundant community of microbes that colonize the gut. These microbes and their human hosts co-evolved in the context of each other, resulting in a close functional integration of the microbiota into human physiology. Indeed, the gut microbiota has been likened to a “forgotten organ” that plays a wide variety of roles in human health and disease. Despite extensive research characterizing connections between the gut microbiota and human health, development of microbiota-targeted therapies has been hindered by high levels of inter-individual variation coupled with a poor understanding of the interspecies interactions and environmental factors that shape the composition and function of the microbiota itself. The work reported in this thesis addresses this deficiency with respect to a gut microbiota function of particular interest: production of the anti-inflammatory and anticarcinogenic microbial metabolite butyrate.

In the second chapter, I characterize the response of the gut microbiota to consumption of resistant starch from potato (RSP), a dietary supplement that has been shown to fuel microbial butyrate production in approximately 60% of individuals. Using data from a large human cohort, I find that while the resistant starch degraders *Bifidobacterium adolescentis* (Ba) and *Ruminococcus bromii* (Rb) can both respond to RSP supplementation, the presence of Ba suppresses Rb response. Furthermore, an Rb response to RSP is associated with growth of the butyrogen *Eubacterium rectale* (Er),

while a Ba response is instead weakly associated with growth of the butyrogen *Faecalibacterium prausnitzii* (Fp). *In vitro* experiments show this is likely due to greater fitness of Er at the high substrate concetrations produced by Rb growing on RSP compared to adaptation of Fp for low substrate concentration characteristic of RSP degradation by Ba. In the third chapter, I characterize the role of intestinal H_2 as a modulator of fermentation in the gut microbiota. *In vitro* experiments demonstrate that high levels of H_2 shift butyrogen metabolism in a predictable fashion that can include stimulation of butyrate production. I also find that hydrogenotrophic gut methanogens can decrease butyrate production by efficiently consuming H_2, accounting for some of the inter-individual variation in butyrate production observed during RSP supplementation. In the fourth chapter, I build off these findings by exploring the potential of a variety of dietary supplements to increase intestinal H_2. I also use data collected in this research to explore the feasibility of using fasting breath H_2 as an indicator of recent fiber consumption.

Taken together, this work identifies previously unrecognized factors influencing butyrate production by the human gut microbiota, as well as shedding light on the specifics of RSP degradation by the microbiota. The findings highlight H_2 concentration in particular as a factor differentiating individual gut microbiomes with relevance beyond the specific case of RSP fermentation. Accounting for the effect of intestinal H_2 will improve understanding of inter-individual differences in gut fermentation, in particular in response to microbiota-targeted supplements. Future work that explores targeted modulation of H_2 concentrations could stimulate butyrate production or otherwise manipulate gut microbial metabolism for the benefit of human health.

Chapter 1: Introduction

1.1 Background: Microbiota-targeted therapy, prebiotics, and variable results

The human gut microbiota is a community of microbes that colonizes the human gastrointestinal tract. Although it was first described over 120 years ago [1], only in the past twenty years have advances in DNA sequencing technology revealed the full complexity and diversity of this remarkable ecosystem [2]. The revolution in our ability to characterize the gut microbiota brought with it a recognition of the deep integration of its structure and activity with the physiology of its human host [3]. Humans have co-evolved together with their gut microbes, which they acquire from their families and environments and pass down vertically through generations, each influenced by selective pressures from the other [4]. The resulting network of metabolic and immunological interactions between host and microbe has led to the gut microbiota being described as a "forgotten organ" [5].

As an ever-increasing number of aspects of human health and disease are linked to the gut microbiota, interest has grown in the development of microbiota-targeted therapies—targeted perturbations of the microbiota intended to improve or maintain health [6]. One feature of the microbiome that is a particularly promising candidate for manipulation by microbiota-targeted therapies is production of short chain fatty acids (SCFAs) [7]. Representing the most abundant metabolic products of the gut microbiota, SCFAs (along with the gases H_2, CO_2, and sometimes CH_4) are produced by intestinal

microbes fermenting carbohydrates to obtain energy and carbon for growth [8]. The primary SCFAs are acetic, propionic, and butyric acid, usually referred to as their conjugate bases acetate, propionate, and butyrate [9]. In an archetypical example of host-microbiota co-evolution, the SCFAs are absorbed by the large intestine and exert myriad influences on human physiology [7]. Butyrate is of particular interest due to its beneficial role in numerous aspects of human health coupled with the fact that it is not produced by any human enzymes and therefore must be supplied entirely by gut microbes [10, 11].

Perhaps the simplest way to modulate SCFA production by the microbiota is to supply gut microbes with substrate that specifically fuels the production of the desired SCFA, usually butyrate [11]. Substrates that are utilized by the microbiota in order to produce a health benefit are known as prebiotics, and dietary supplementation with prebiotics represents one of the major approaches to microbiota-targeted therapies [12]. While many prebiotic interventions have attempted to stimulate butyrate production by the gut microbiota, often obtaining positive results, a high level of inter-individual variability in response to prebiotics has historically kept such trials from unqualified success [13–15]. Even when a significant average increase in fecal butyrate is observed across the study cohort, variable responses to supplementation are often the more salient feature of the data [16–19].

The research reported in this thesis was intended to explain this variability by characterizing the underlying factors distinguishing individual responses to prebiotic supplementation. Once these factors are understood, interventions can be tailored to account for them or only given to those with a high chance of responding appropriately. In this way, an understanding of the gut microbiome richer in mechanistic detail can

support the development of promising new approaches to the improvement and maintenance of health.

1.2 Research program

The overarching goal of my research program was to gain a better mechanistic understanding of the interspecies interactions and features of the gut environment that mediate the response of the gut microbiota to prebiotic supplementation. This goal grew out of the variable responses to RSP supplementation observed in a pilot study that attempted to stimulate butyrate production in healthy individuals, resulting in a specific focus on RSP as a prebiotic and butyrate production as a response [16]. The central guiding principle that informed my research strategy was to elucidate mechanisms whenever possible, even at the cost of ignoring the complexities that always exist in the human gut microbiota. This is the reason that a relatively small number of well-characterized species with representative strains available for *in vitro* research were chosen as the focus of the research in Chapters 2 and 3.

The research in Chapter 2 addresses the overarching goal most directly by investigating the interactions between the two starch degraders known to be most important in RSP degradation, *Ruminococcus bromii* (Rb) and *Bifidobacterium adolescentis* (Ba) [16, 20–23], and the two butyrogens known to be most responsive to RSP supplementation, *Eubacterium rectale* (Er) and *Faecalibacterium prausnitzii* (Fp) [16, 17, 19, 24]. The initial analyses were performed on human data, followed by *in vitro* experiments with representative strains to test whether the strains themselves were responsible for the observed effects and to attempt to elucidate the mechanism.

By contrast, the research in Chapter 3 began with a hypothesis formulated on the basis of the existing literature describing the influence of H_2 on butyrogenic fermentation. H_2 concentrations within the range of likely gut physiological conditions modulated the fermentation end products of bacteria closely related to gut butyrogens *in vitro*, leading to the simple expectation that it plays a similar role in the gut. This hypothesis was first tested extensively in increasingly complex cultures *in vitro*, again using representative strains of the butyrogens known to be most prominent in the human gut. H_2-mediated interspecies interactions between butyrogens and hydrogenotrophic methanogens were predicted and validated *in vitro*. Only afterwards were predictions for the human cohort checked against the results of the RSP supplementation studies, allowing the human data to serve as a confirmation of the relevance *in vitro* work rather than a source of hypotheses to be tested *in vitro*.

Chapter 4 diverged from the mechanistic approach of Chapters 2 and 3 in order to explore ways to apply the findings on the role of H_2 in the gut microbiota. In this way, it serves as practical follow-up to reconnect Chapter 3 more closely back to the overarching goal of the research program as a whole.

Chapter 2: Competitive and Cooperative Interactions Characterize the Gut Microbiota Response to a Resistant Starch Supplement that Increases Butyrate

2.1 Abstract

Butyrate is crucial for maintaining barrier functions of the colonic epithelium, yet mammals are dependent on bacteria in the gut for its production. Dietary fiber is the primary fuel for metabolism of gut microbes and its paucity in many human diets led us to test the efficacy of a readily available fiber supplement—resistant starch from potatoes (RSP)—to enhance butyrate production in a large human cohort. RSP supplementation stimulated fermentation and butyrate production overall, but its success varied considerably among individuals. We found that two bacterial taxa known to degrade RSP engaged in one-sided competition in response to supplementation. Associations between abundance of RSP degraders and RSP-responsive butyrogens emerged during supplementation. *In vitro* cultures identified quantity of cross-fed substrate as a mechanisms underlying specific interactions between RSP degraders and butyrogens that influenced invervention success. The combined results set the stage for individualized manipulation of butyrate production as a promising microbiota-targeted therapeutic.

2.2 Introduction

Increasing understanding of the many connections between the human gut microbiota and host physiology has led to significant interest in microbiota-targeted

therapy—rational manipulation of the gut microbiota in order treat disease or maintain health. One promising approach to targeted microbiota manipulation is dietary supplementation with prebiotics. First conceptualized in 1995 [25], a prebiotic has most recently been defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" [12]. In simple terms, prebiotics are food sources for beneficial microbes. The strategy underlying prebiotic supplementation is to provide a substrate that specifically supports the growth and/or metabolism of gut microbes whose abundance or metabolism is expected to positively impact host health. An important requirement of prebiotics is that they be resistant to digestion and absorption by the host so that they reach the target microbes in the colon intact [26].

Since they provide a substrate for microbial metabolism, prebiotics are particularly suitable for use in interventions intended to increase production of a beneficial metabolite. Of all the metabolites produced by the gut microbiome, perhaps the most intense interest has centered on butyrate, which has been described as "the most interesting bacterial fermentation product in the human colon" due to its many associations with beneficial aspects of host health [7, 11]. Butyrate is the preferred energy source of the colonocytes that compose the colonic epithelium [27]. More interestingly, butyrate acts as a histone deacetylase (HDAC) inhibitor, a function that can exert broad control over host cell gene expression with potentially wide-ranging effects [7, 10]. Its abundance in the human colon is negatively associated with colorectal cancer occurrence [28], and rodent studies have suggested it plays a causal role in cancer prevention [29–31]. It also acts as an anti-inflammatory signal that has been shown to improve symptoms of ulcerative colitis in both humans and rodent models through inhibition of NF-κB activation in lamina propria

macrophages ([32–34]. One study in a murine model suggested it could even have antidepressant-like effects [35].

While a number of different prebiotics have been shown to stimulate butyrate production by the human gut microbiota, resistant starch (RS) has emerged as a particularly promising candidate [36, 37]. As early as 1983, it was recognized that a portion of dietary starch transits the small intestine without being digested by host amylases and arrives in the cecum intact [38]. The mechanism of this resistance varies and is used to classify resistant starches. RS1 is physically protected by plant components, RS2 is in the form of tightly-packed granules, RS3 is crystallized by cooking and cooling, and RS4 is chemically modified to prevent digestion [8]. RS is a particularly interesting as a candidate prebiotic because its fermentation by gut microbes produces higher levels of butyrate than fermentation of non-starch polysaccharide (NSP) prebiotics [39–41].

While virtually all dietary starches are resistant to some extent, the resistance of starches in typical American foodstuffs is generally low [42]. For this reason, supplementation with purified (or synthesized, in the case of RS4) RS is the most common prebiotic approach. RS2 consisting of raw potato starch (resistant starch from potato, RSP) or high-amylose maize starch (resistant starch from maize, RSM) is commonly investigated as a candidate prebiotic in human trials [13]. An early RSP feeding study in humans showed the largest increase in fecal butyrate over other common fermentation products [43], and stable isotope analysis of mice consuming RSP showed butyrate to be the predominant product of its fermentation by the gut microbiota [44].

Despite these promising signs, human studies investigating RS as a prebiotic aimed at stimulating butyrate production by gut microbe have been plagued by high levels of inter-individual variability [14, 15]. Even when RS supplementation increases fecal butyrate concentration on average, a significant minority of individuals often show unchanged or decreased fecal butyrate [16–19]. Some of this variation is likely due to the fact that the microbiota of ~20% of individuals does not effectively ferment RS, resulting in its recovery intact in the feces [43, 45].

Results from RS supplementation trials coupled with *in vitro* functional and genomic studies of human gut microbes have consistently identified a relatively small number of microbial species intimately involved RS degradation and fermentation to butyrate in the human gut. Hydrolysis of the starch polysaccharides is largely attributable to *Ruminococcus bromii* (Rb) and species of bifidobacteria, most prominently *Bifidobacterium adolescentis* (Ba) [16, 20–22]. While both of these species thrive on RS as a carbon source, neither produces butyrate, leading to the conclusion that butyrogens utilize the products of these starch degraders in a process known as cross-feeding, which has been demonstrated *in vitro* [46, 47]. Based on responsiveness to RS supplementation in human studies, *Eubacterium rectale* (Er) and, to a lesser extent, *Faecalibacterium prausnitzii* (Fp) have been identified as the butyrogens most involved in RS-mediated stimulation of butyrate production [16, 17, 19, 24].

While significant progress has been made towards understanding RS degradation and fermentation in the human gut microbiota, the factors accounting for inter-individual variation remain unclear, hampering efforts to improve the success of RS supplementation or identify the individuals most likely to benefit. In this study, we attempt

to characterize the microbial ecology underlying microbiota responses to RSP supplementation in a large human cohort. We use a focused approach that takes advantage of prior findings to investigate specifically the species known to be most relevant: Rb, Ba, Er, and Fp. Using 16S rRNA sequencing, we identified amplicon sequence variants corresponding to these species and analyzed the patterns of their abundance to identify interspecies interactions such as competition and cross-feeding. We found that the presence of Ba strictly excludes Rb from responding to RSP supplementation, and that Rb is preferentially associated with Er, while Ba preferentially associates with Fp to a lesser extent. *In vitro* experiments confirmed these preferential degrader-butyrogen associations and found that they are likely mediated by the concentration of glucose resulting from starch degradation.

2.3 Materials & Methods

2.3.1 Human cohort

Results from a portion of this study's human cohort were previously reported by Baxter et al. (2019) [17]. Participants were recruited through Authentic Research Sections of the University of Michigan BIO173 introductory biology course. Subjects were excluded based on self-reported inflammatory bowel syndrome, inflammatory bowel disease, colorectal cancer, and consumption of antibiotics in the past 6 months.

2.3.2 Study design and sample collection

The study took place during a number of separate semesters over the course of 3 years, from the winter semester of 2016 to the winter semester of 2019. While all

supplements consumed consisted of either resistant starch from potato (RSP) or resistant starch from maize (RSM), they varied in source, total dose, and frequency. The supplements consumed were Bob's Red Mill potato starch (Bob's Red Mill Natural Foods, Milwaukie, OR) consumed as a 20 g dose twice daily, resistant potato starch from LODAAT Pharmaceuticals (Oak Brook, IL) consumed as an 18 g or 20 g dose twice daily, or HiMaize 260 consumed as a 20 g dose twice daily.

In each semester, the study followed a 3-week course. During the first week, fecal and breath samples were collected before consumption of RS. During the second week, RS consumption began at a half dose and increased to the full dose. During the third week, RS consumption continued at the full dose while fecal and breath samples were collected.

2.3.3 Human sample analysis – HPLC and 16S rRNA sequencing

Fecal sample collection, preparation, and quantification of short-chain fatty acid concentration by high-performance liquid chromatography (HPLC) was performed as previously described in Baxter et al. (2019) [17]. 16S rRNA gene sequencing and sequence data processing to obtain amplicon sequence variant (ASV) relative abundances were also performed as described in Baxter et al. (2019) without sequence rarefaction [17].

2.3.4 Microbial strains and culture

Faecalibacterium prausnitizii A2-165 (DSM 17677) was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). *Ruminococcus bromii*

VPI 6883 (ATCC 27255) was obtained from the American Type Culture Collection (ATCC). *Eubacterium rectale* A1-86 (DSM 17629), was obtained from collaborators. *Bifidobacterium adolescentis* 269-1 was isolated as described in Chapter 3 (3.3.2).

The standard growth medium was a modified version of YCFA medium (Lopez-Siles, 2011) with the addition of glucose or fructose (0.5 mg/mL), cysteine (0.1 mg/mL), thiamine (5 µg/mL) and riboflavin (5 µg/mL). To prepare RSP media, 15 g of resistant potato starch (Bob's Red Mill, Milwaukee, OR) was washed twice in distilled water and suspended in 30 mL 70% ethanol. The RSP suspension was dispensed into a serum bottle under an atmosphere of 20% CO_2 and 80% N_2 and sealed with a butyl rubber stopper and aluminum crimp. 50 µL of RSP suspension was added to 5 mL modified YCFA without glucose or fructose to create culture tubes containing the RSP media used in most experiments. Instead of adding RS via a concentrated starch suspension in ethanol, the Ba-Rb competitions on RSP and RSM used starches washed and incubated >24 hrs in 70% alcohol before the alcohol being decanted and the starch being dried in a sterile fashion. Media was then added to the dried starch in the drying vessel to produce the desired concentration of RSP or RSM.

2.3.5 CFU enumeration

Modified YCFA medium was mixed with pre-autoclaved Bacto™ Agar (6 g/L), poured into Nunc® 100 x 25 mm petri plates and left overnight in the anaerobic chamber to deoxygenate. 100 µL of cultures to be enumerated were plated, incubated until colonies developed, and then counted.

2.3.6 Thin-layer chromatography (TLC)

As standards, 2 mL of 2.5 mM glucose and 2 mL of 5 mM seven maltooligosaccharides (G2-G7) were spotted silica gel-60 aluminum TLC plates that had been heat treated at 100 ˚C for 20 minutes and cooled. Samples were then spotted, and the TLC assay was run using the FBW solvent system containing formic acid / n-butanol / distilled water (6 : 4 : 1, v / v / v) for 15 minutes and developed using Orcinol-H_2SO_4-ethanol reagent.

2.4 Results

To assess RSP degradation by the human gut microbiota, a longitudinal study design was used in which fecal samples were collected from human subjects before and during the consumption of powdered RSP. Up to three baseline fecal samples were collected from each subject over the course of one week before supplementation. During the next week, RSP supplement consumption was increased stepwise until it reached 18-20g of resistant starch daily. Up to three further fecal samples were then collected from each subject over the course of a week of full-dose RSP consumption.

As expected, summed relative abundance of the five RSP degraders previously identified by Baxter et al. (2019) [17] increased dramatically in response to RSP supplementation (Fig. 2.1A). This was accompanied by a significant, although less dramatic, increase in fecal SCFA concentration, indicating that supplement consumption stimulated fermentation in the gut microbiota (Fig. 2.1B). This increase was driven primarily by acetate (Supplemental Fig. 2.1A) and secondarily by butyrate (Supplemental Fig. 2.1B), which both increased significantly, while propionate concentration actually

decreased on average (Supplemental Fig. 2.1C). RSP degrader abundance was correlated with fecal SCFA concentration during RSP supplementation (Fig. 2.1C) but not at baseline (Supplemental Fig. 2.1D). Analyzed individually, only fecal acetate concentration was found to positively correlate with RSP degrader abundance during supplementation, with propionate negatively correlated and butyrate showing no correlation (Supplemental Fig. 1.1E-G).

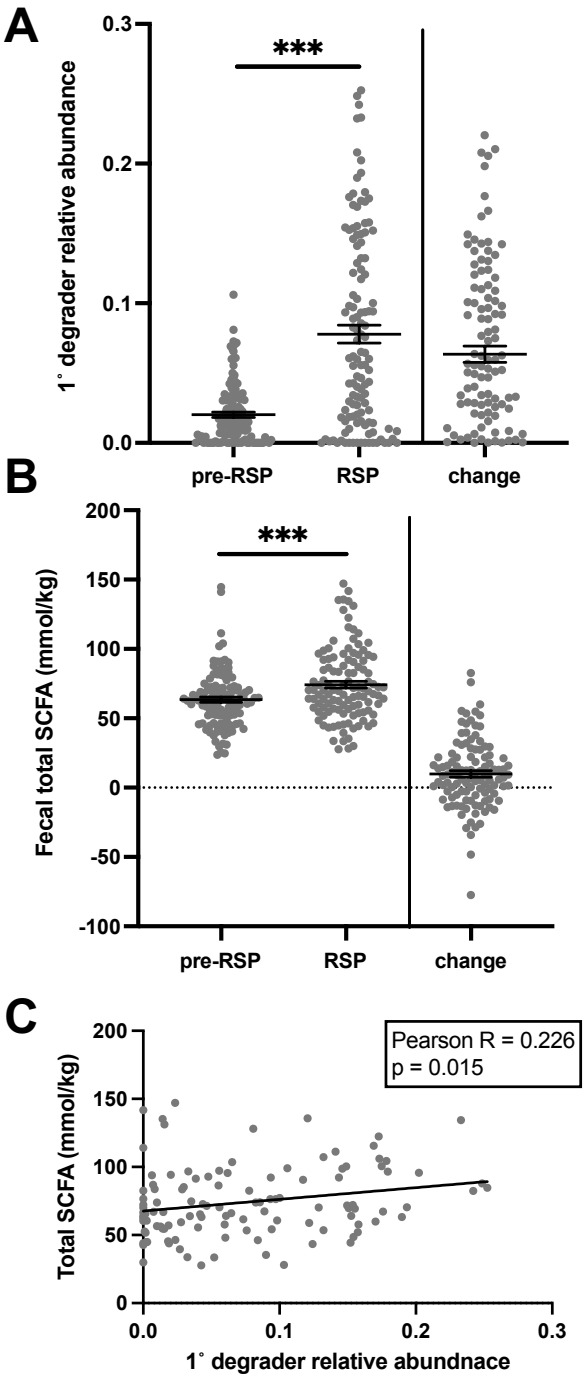


Figure 2.1. Resistant starch from potato (RSP) supplementation triggers blooms of primary starch degraders and increases SCFA production in the human gut.
Summed relative abundances of five previously identified RSP degraders (A) and total fecal SCFA (sum of acetate, butyrate, and propionate) (B) before and during RSP supplementation. Each point represents the average of 1-3 fecal samples from a single individual collected the week before supplementation or during the second week of supplementation. Changes represent the difference of during and before averages for each individual. Error bars indicate SEM. Statistical significance calculated using paired t-tests (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$). Pearson correlation of summed relative abundances of RSP degraders versus total fecal SCFA during RSP supplementation (C).

Among the five previously identified RSP degraders, ASV12 and ASV31 (corresponding to Ba and Rb) were by far the most prevalent and abundant overall. Amplicon sequence variants (ASVs) corresponding to the other previously identified RSP degraders were detected at a relatively much lower abundance and prevalence overall. Therefore, ASV12 and ASV31 responses to RSP supplementation were examined and compared in greater detail. While both ASVs increased in average relative abundance during supplementation, ASV12 exhibited both a larger and a more consistent response than ASV31 (Fig. 2.2A). Interestingly, the opposite pattern was observed in a smaller cohort that consumed a resistant starch from maize (RSM) supplement, with ASV31 exhibiting a larger and more consistent response (Fig. 2.2A).

When individuals were classified by detection of ASV12 and ASV31 in baseline samples, a unidirectional suppression of ASV31 response to RSP by ASV12 became apparent. With only two exceptions, ASV31 never increased in relative abundance in response to RSP when ASV12 was detected before supplementation. By contrast, it often showed a robust response when ASV12 was absent at baseline (Fig. 2.2B). In stark contrast, ASV12 consistently responded to RSP irrespective of the presence or absence of ASV31 before supplementation (Fig. 2.2C).

In vitro cultures of *B. adolescentis* 269-1 and *R. bromii* VPI 6883—strains sharing 16S V4 sequences with ASV12 and ASV31, showed more robust growth of Ba than Rb with RSP as the carbon source—recapitulating the dominance of ASV12 observed *in vivo* during RSP supplementation (Fig. 2.2D). Notably, Rb growth was more robust than that

of Ba with RSM as the carbon source, indicating that the Ba dominance is substrate-specific, again consistent with *in vivo* observations (Fig. 2.2D).

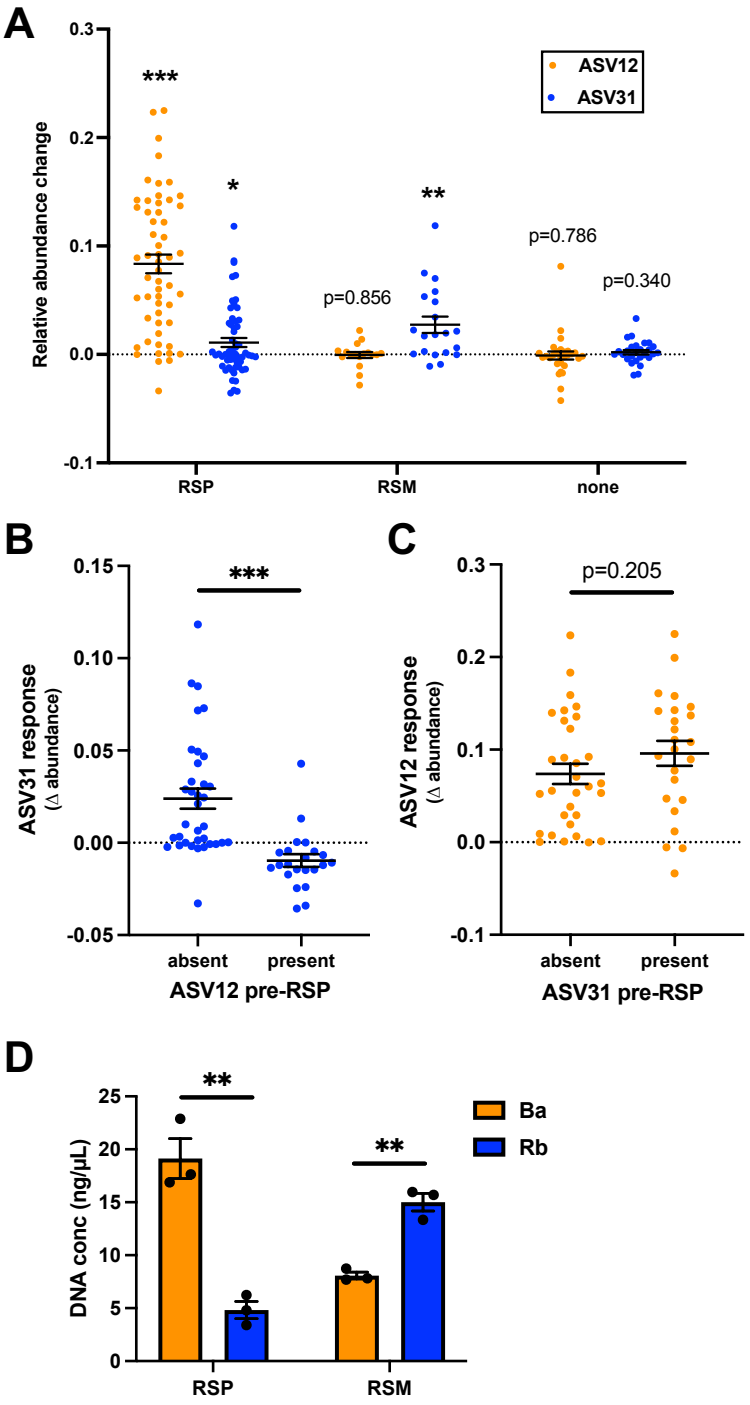


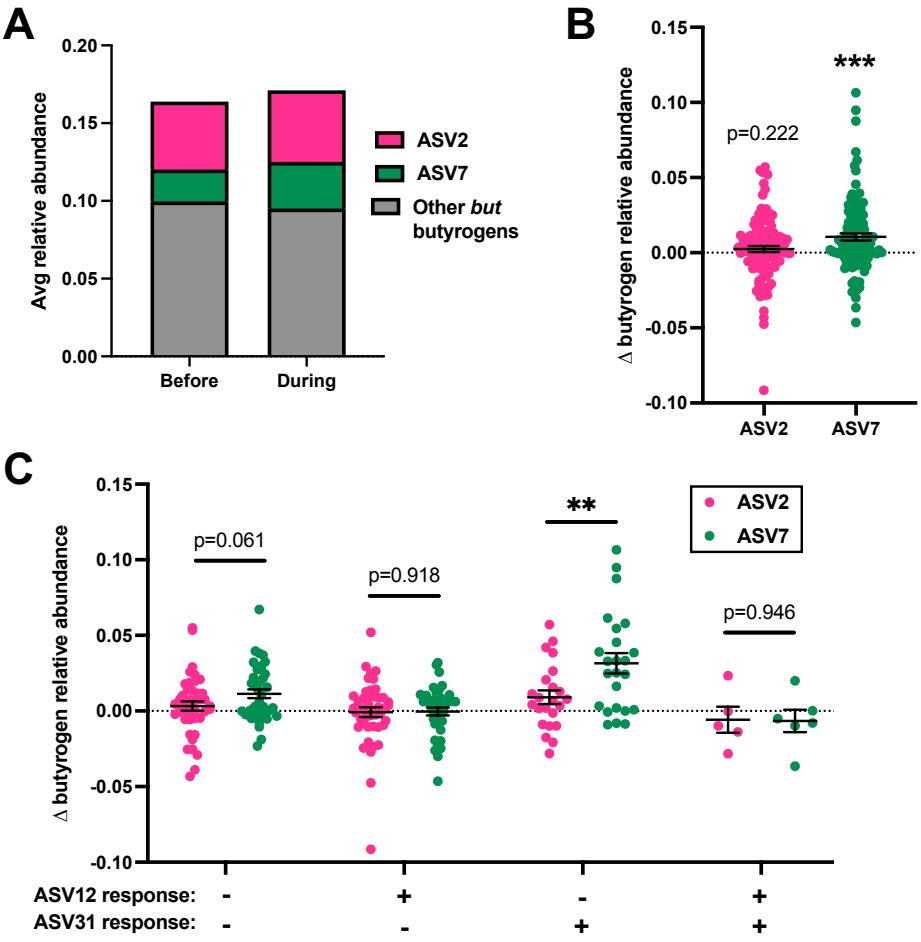
Figure 2.2. Starch degrader ASV12 (*B. adolescentis*) outcompetes ASV31 (*R. bromii*) during RSP supplementation.
Change in relative abundance of ASV12 and ASV31 from the week before supplementation to the second week of supplementation (A). RSP, resistant starch from maize (RSM), and non-supplemented controls are shown. Change during RSP supplementation in relative abundance of ASV12 (B) and ASV31 (C), based

on presence or absence of ASV12 or ASV31 in any samples taken before supplementation. Points in A-C represent the average of 1-3 fecal samples from a single individual. Concentration of *B. adolescentis* and *R. bromii* DNA recovered from 48-hour co-cultures on RSP or RSM substrate (D). Error bars indicate SEM. Statistical significance calculated using paired t-tests (A-C) or two-sample t-tests (D) (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

After investigating degradation of RSP supplement, the downstream response of secondary degraders to the supplement was examined. Since butyrate production is of particular interest to human health, butyrogenic secondary degraders were selected for in-depth analysis. In particular, ASV2 and ASV7 represented highly abundant butyrogens with representative strains (*F. prausnitzii* A2-165 and *E. rectale* A1-86, respectively) available for *in vitro* validation of patterns observed in the human cohort. Taken together, ASV2 and ASV7 accounted for ~40% of total relative abundance of butyrogens using the *but* pathway at baseline and ~45% during supplementation (Fig. 2.3A). While ASV7 significantly increased in average relative abundance in response to RSP supplementation, ASV2 did not (Fig. 2.3B).

Interestingly, classifying individuals by their RSP degrader response to supplementation revealed an association between degrader and butryogen responses. In individuals that exhibited an increase in ASV12 average relative abundance during supplementation (+12), neither ASV2 nor ASV7 significantly increased in relative abundance (Fig. 2.3C). ASV7 increased in average relative abundance in individuals either lacking ASV12 or in whom ASV12 did not increase in response to supplementation (-12). The ASV7 response was especially prominent when ASV31 was observed to increase in relative abundance (-12/+31) (Fig. 2.3C). This association of ASV7 and ASV31 was also apparent in a moderate and highly significant Pearson correlation of their average relative abundances during RSP supplementation (Fig. 2.3D). Relative

abundances of ASV2 and ASV12 were also found to be weakly correlated during supplementation. Notably, no correlations were observed for the converse pairings of degraders and butyrogens (ASV7~ASV12 and ASV2~ASV31) or at baseline (Fig. 2.3D). Despite these observed patterns, statistical analysis did not bear out a categorical association between ASV2 and ASV12 responders or ASV7 and ASV31 responders, in part due to insufficient sample sizes (Supplemental Fig. 2.2A).



D

Degrader-butyrogen interaction	During RSP supplement		Before RSP supplement (baseline)	
	Pearson R	p	Pearson R	p
ASV12 – ASV2	0.289	**0.036***	0.112	0.452
ASV12 – ASV7	0.159	0.285	-0.037	0.818
ASV31 – ASV2	0.092	0.524	0.105	0.46
ASV31 – ASV7	0.438	**0.001****	0.086	0.534

Figure 2.3. Responses of the butyrogens ASV31 (*R. bromii*) and ASV7 (*E. rectale*) to RSP supplementation are associated with starch degrader responses.
Average relative abundances of ASV2, ASV7, and all other putative butyryl-CoA:acetyl:CoA transferase (but) butyrogens before and during RSP supplementation (A). Change in relative abundances of ASV2 and ASV7 in response to RSP supplementation (B). Average change in ASV2 and ASV7 relative abundance from the week before RSP supplementation to the second week of supplementation in individuals classified by the pattern of primary degrader response to RSP supplementation (C). A positive response (+) indicates an ASV increased in average relative abundance or became detectable during supplementation, while a lack of response (-) indicates an ASV decreased in abundance during supplementation or was not detected in any samples from the individual. Error bars indicate SEM. Statistical significance calculated using paired t-tests (B) or Student's t-tests (D) (* = p<0.05; ** = p<0.01; *** = p<0.001). Pearson correlations of relative abundances of the primary degraders ASV12 and ASV31 with the butyrogens ASV2 and ASV7 (D). Bold indicates statistically significant correlations.

On the whole, increased butyrate during RSP supplementation appeared to be primarily associated with increased ASV7 relative abundance (+7) and an absence of increased ASV12 relative abundance (-12) (Fig. 2.3E, Supplemental Fig. 2.2B-H).

To further investigate the observed associations between RSP degrader and butyrogen responses to RSP supplementation, *in vitro* experiments were performed using the four representative strains previously described. In tri-cultures grown on RSP inoculated with one of the RSP degraders (either Ba or Rb) and both butyrogens, competition between the butyrogens depended on the degrader present. When Ba was the RSP degrader present, Fp outcompeted Er. By contrast, when Rb was the RSP degrader present, Er outcompeted Fp (Fig. 2.4A). These results suggested that RSP degraders played a causative role in the associations observed between degrader and butyrogen responses in the human cohort, in particular between ASV7 and ASV31. Interestingly, butyrate production also appeared to depend on degrader-butyrogen pairings *in vitro*. With RSP as the carbon source, more butyrate was produced by Ba+Fp co-culture than by Ba+Er co-culture. Intriguingly, butyrate production showed an opposite pattern with Rb as the RSP degrader: more butyrate was produced by Rb+Er than by Rb+Fp (Fig. 2.4B). This dependence of butyrate production on degrader-butyrogen pairings was not clearly observed in the human cohort, where fecal butyrate change during supplementation appeared to be negatively associated with ASV12 response and positively associated with ASV7 response, independent of degrader-butyrogen pairings (Supplemental Fig. 2.2B-H).

Finally, we demonstrated that greater availability of free glucose and maltooligosaccharides resulting from RSP degradation by Rb versus Ba likely accounted for the observed degrader-butyrogens associations *in vitro* and, by extension, *in vivo*. Rb is known to produce free glucose and maltooligosaccharides as it degrades RSP, while this production of accessible carbohydrates during growth of RSP has not been reported for Ba [47]. These results were validated using HPLC (Supplemental Fig. 2.3A-B) and TLC (Supplemental Fig. 2.3C). Hypothesizing that this difference in degradation byproducts accounted for dependence of Fp versus Er competitive fitness on the RSP degrader present, we grew co-cultures of Fp and Er for five days in medium supplemented with either 2 mg (high) or 0.2 mg (low) glucose each day. Er outcompeted Fp on high glucose while Fp outcompeted Er on low glucose (Fig. 2.4C). These competition results mirrored those obtained from cultures grown on RSP with Rb and Ba as degraders, respectively (Fig. 2.4A).

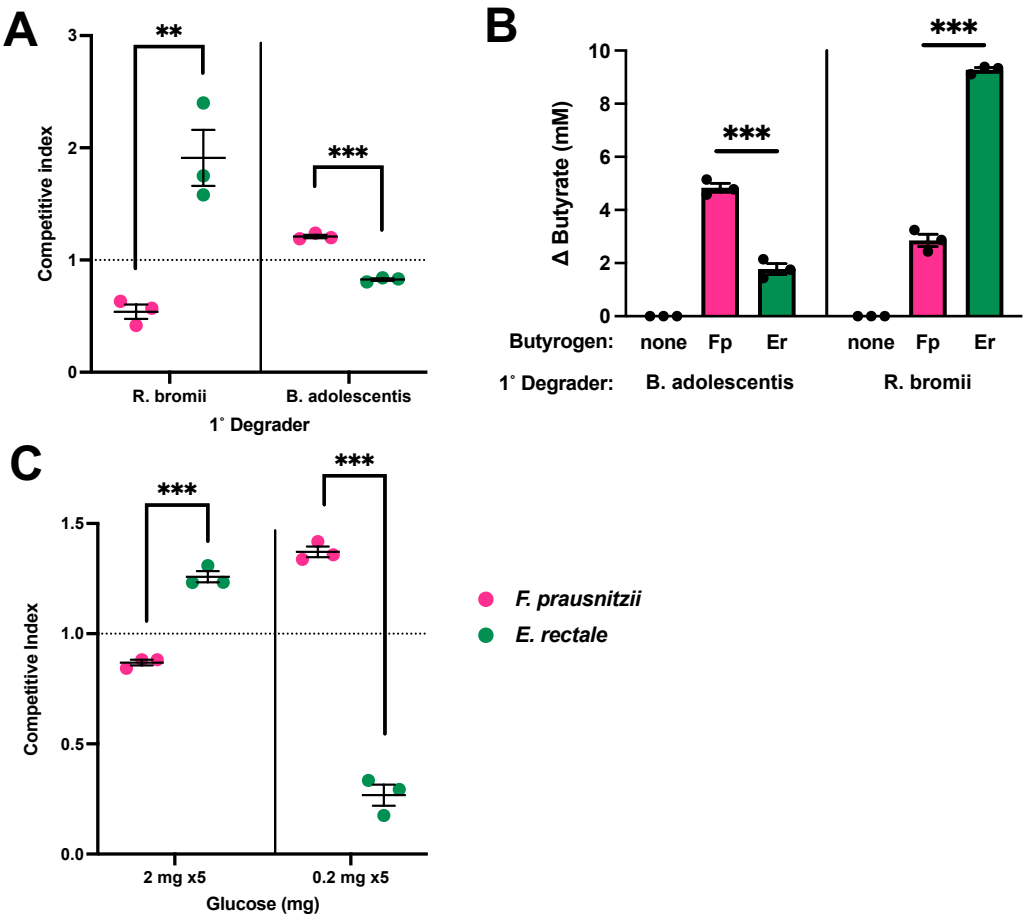


Figure 2.4. RSP degradation by B. adoloscentis (Ba) and low glucose concentrations favor growth of F. prausnitzii (Fp) over E. rectale (Er).
Competitive index of Fp and Er on RSP substrate in tri-cultures of Fp, Er, and either Rb or Ba (A). Butyrate produced on RSP substrate by Ba and *R. bromii* (Rb) and by co-cultures of either Ba or Rb and either Fp or Er (B). Competitive index of Fp and Er in co-cultures supplemented with either 2 mg or 0.2 mg of glucose each day for five days (C). Competitive indices were calculated as the ratio of the relative abundance of a strain in the inoculum (input) to its relative abundance in the endpoint sample (output). Error bars indicate SEM. Statistical significance calculated using two-sample t-tests (* = p<0.05; ** = p<0.01; *** = p<0.001).

2.5 Discussion

The *in vivo* and *in vitro* work described in this chapter focused on the interactions between four species in the gut microbiota: the RSP degraders Rb and Ba (identified with ASV12 and ASV31, respectively) and the butyrogens Fp and Er (identified with ASV2 and ASV7, respectively). These specific ASVs were selected for in-depth analysis on the basis of *in vivo* reports consistently identifying them as responsive to RS supplementation [13,

19, 24], their adherence to RS granules in an *ex vivo* system [21], and labeling with ^{13}C-containing RSP in an *in vitro* colon model [22]. Results from two previous analyses of earlier subsets of the human cohort analyzed in this study were also informative in selecting the appropriate ASVs [16, 17]. While this targeted approach largely avoided the pitfalls of repeated hypothesis testing and inter-individual variability, in addition to facilitating *in vitro* follow-ups of observations, it likely excluded from analysis some microbial strains that play relevant roles in RSP degradation and fermentation. One clear indication of this is the significant average increase observed in fecal butyrate in individuals with no response of ASV12 or ASV31 to RSP supplementation (Supplemental Fig. 2.2G). It is likely that RSP hydrolysis in these individuals was due to other degraders such as the *Clostridium chartatabidum* identified in an earlier analysis of a subset of the present cohort [17]. Notably, no average increase in butyrate was observed in individuals lacking an ASV2 or ASV7 response to RSP, suggesting that these two ASVs substantially accounted for the overall butyrate increase (Supplemental Fig. 2.2H). Future work applying an unbiased screening approach to this dataset could be useful to identify additional taxa responsive to RSP.

The most novel finding in this study was that glucose concentration mediates competition between Er and Fp (Fig. 2.4C). This result not only explains the observed association of Er responses with Rb responses rather than Bf responses (Fig. 2.3C-D), but also sheds light on the different ecological niches inhabited by these two prominent human gut butyrogens.

The association between Rb and Er in RS degradation has been well-known since a landmark study identified Rb as a “keystone species” that makes saccharides derived

from RS available to a variety of gut microbes lacking the specialization necessary to degrade refractory starch granules [47]. In this study, Rb was found to produce free glucose and maltose during growth on RS that fueled growth of Er in co-culture on RS. Subsequent studies have confirmed the role of Rb as a prolific cross-feeder, likely due to its lack of a glucose transporter to take up the free glucose it generates [48]. It has been found to also support the growth of *Ruminococcus gnavus* and *Bacteroides thetaiotaomicron* in co-culture on RS [49, 50]. One study dramatically visualized its donation of substrate to the environment as a glucose “halo” that surrounded Rb spotted onto solid media containing starch [50].

Cross-feeding is essential to the growth of Er on RS, as it cannot effectively grow on RS substrate alone [20, 47]. Curiously, Er possesses carbohydrate binding modules (CBMs) that tightly bind RSM despite not being able to utilize that substrate [51]. This observation combined with findings that Er transporters specialize in the uptake of glucose and maltooligosaccharides [52], suggest that it may have co-evolved with Rb to take advantage of cross-feeding products. In this light, our findings of Er-Rb association in preference to Er-Ba association in RSP response (Fig. 2.3C-D) and of butyrate production and growth of Er exceeding that of Fp in co-culture with Rb on RSP (Fig. 2.4A-B) are unsurprising.

More puzzling is the apparent preferential association of Ba with Fp over Er hinted at *in vivo* (Fig. 2.3D and clearly demonstrated in growth on RSP *in vitro* (Fig. 2.4A-B). Cross-feeding from Ba growing on RS to butyrogens has also been observed, but it appears to be mediated by lactate and possibly acetate [46, 53]. Fp is not a lactate-utilizing butyrogen, so this route of cross-feeding is unlikely to be at play [54].

Oligosaccharide-mediated crossfeeding has been proposed [46], but oligosaccharides have not been found to be an abundant product of RS degradation by Ba [47], including in this study (Supplemental Fig. 2.3C). Given all this, how is it that Fp outcompetes Er on RSP when Ba is the starch degrader?

The observation that Fp is far better adapted than Er to growth in low glucose conditions provides a plausible answer (Fig. 2.4C). In contrast to Er, which thrives in the abundant glucose and oligosaccharides released by Rb, Fp specializes in survival when available substrate is relatively scarce, such as when Ba degrades RS without releasing free glucose or oligosaccharides. Both butyrogens benefit by cross-feeding from RS degraders, but are adapted to different availability of cross-fed substrate. From an ecological perspective, they occupy different niches along a spectrum of substrate availability. While it is well-known in the context of prebiotic supplementation that the quantity of available substrate can influence competition between gut microbes respond [15, 55], to our knowledge this is the first description of the quantity of cross-fed substrate mediating which microbes most benefit from cross-feeding.

Another striking result was the one-sided competition between Ba and Rb for RSP substrate *in vivo*. The presence of Ba before RSP supplementation was found to essentially preclude any Rb response to RSP, while Ba responses were completely unaffected by the presence or absence of Rb (Fig. 2.2B-C). This clear dominance of Ba over Rb on RSP substrate has been noted in prior studies [56–58]. The most recent of these was a small study conducted in Japan as a partial replication of our earlier report on a subset of the cohort described in the present work [17, 58]. Despite only having ten participants, this study found an identical pattern of Rb never responding to RSP

supplementation in the presence of Ba, but often responding when Ba is absent [58]. This replication across continents is a very encouraging sign for the robustness and applicability of this finding.

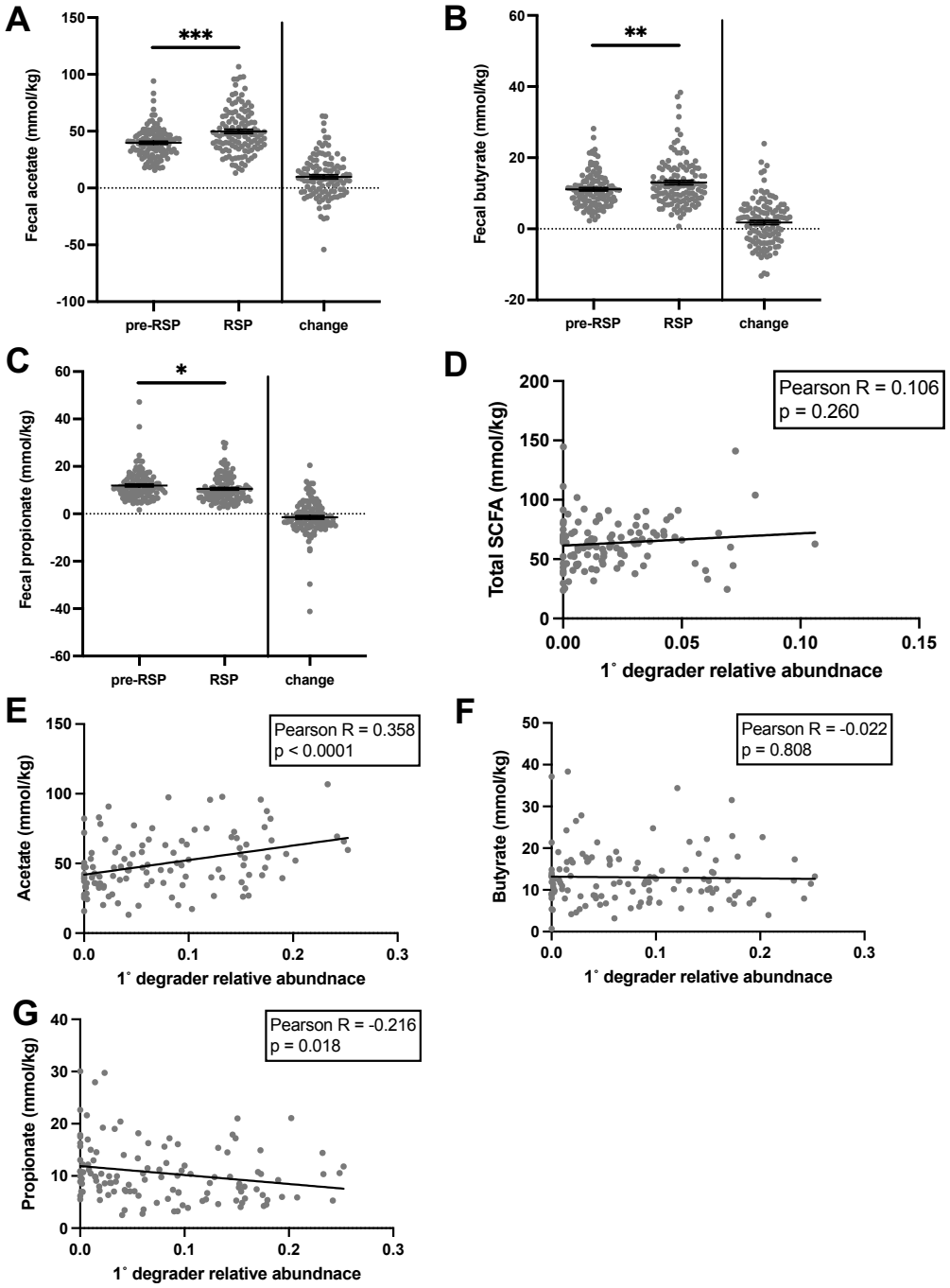
The mechanism underlying this lopsided competition is not clear. Functional and genomic studies show that both Rb and Ba are well-adapted to bind and degrade RSP [48, 59–62]. Both possess extracellular proteins with a CBM74 domain, which appears to be specialized to bind RSP over RS from other sources [20, 51, 63]. The advantage of Ba may be connected to its RSP-binding pili, a feature that Rb notably lacks [61, 64].

Taken as a whole, our findings suggest that individuals harboring Ba in their gut microbiota are relatively poor candidates for prebiotic interventions with RSP aimed at stimulating butyrate production. Ba will prevent a Rb response, in turn reducing the likelihood of a cross-feeding mediated Er response and higher butyrate production. Prebiotic trials of RSP should either select individuals lacking Ba or analyze results from Ba-positive and Ba-negative individuals separately.

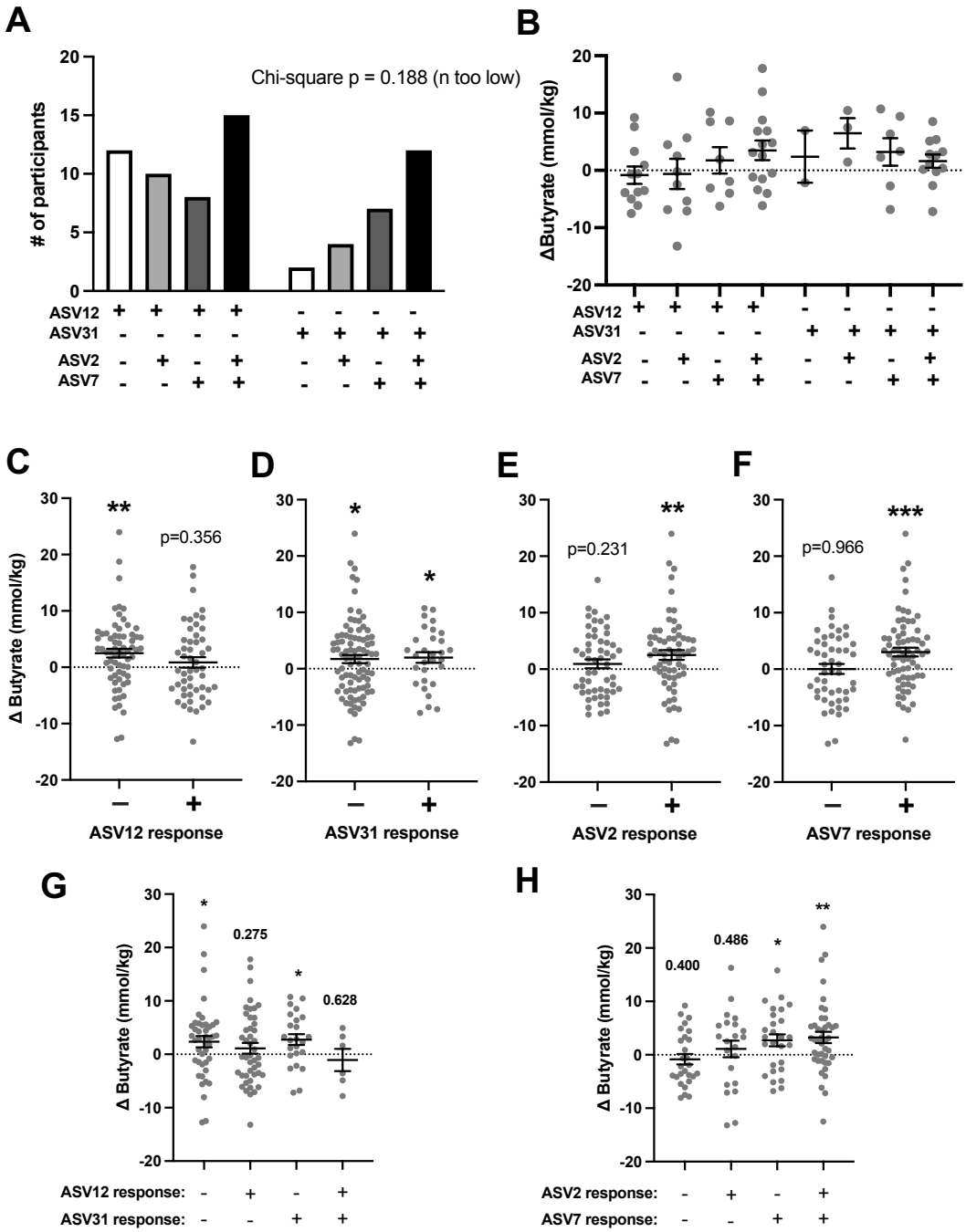
2.6 Acknowledgements

Nanditha Ravishankar played a significant role in the research presented in this chapter. In the course of her master's research in the Schmidt Lab, she discovered the preferential *R. bromii-E. rectale* and *B. adolescentis-F. prausnitzii* associations in co-culture on RSP, generated the data presented in Fig. 2.4A-B and Supplemental Fig. 2.3, and provided the materials and methods used to generate that data. When this chapter is submitted for publication, she will be a co-first author due to her intellectual and experimental contributions to the project.

2.7 Supplemental Figures

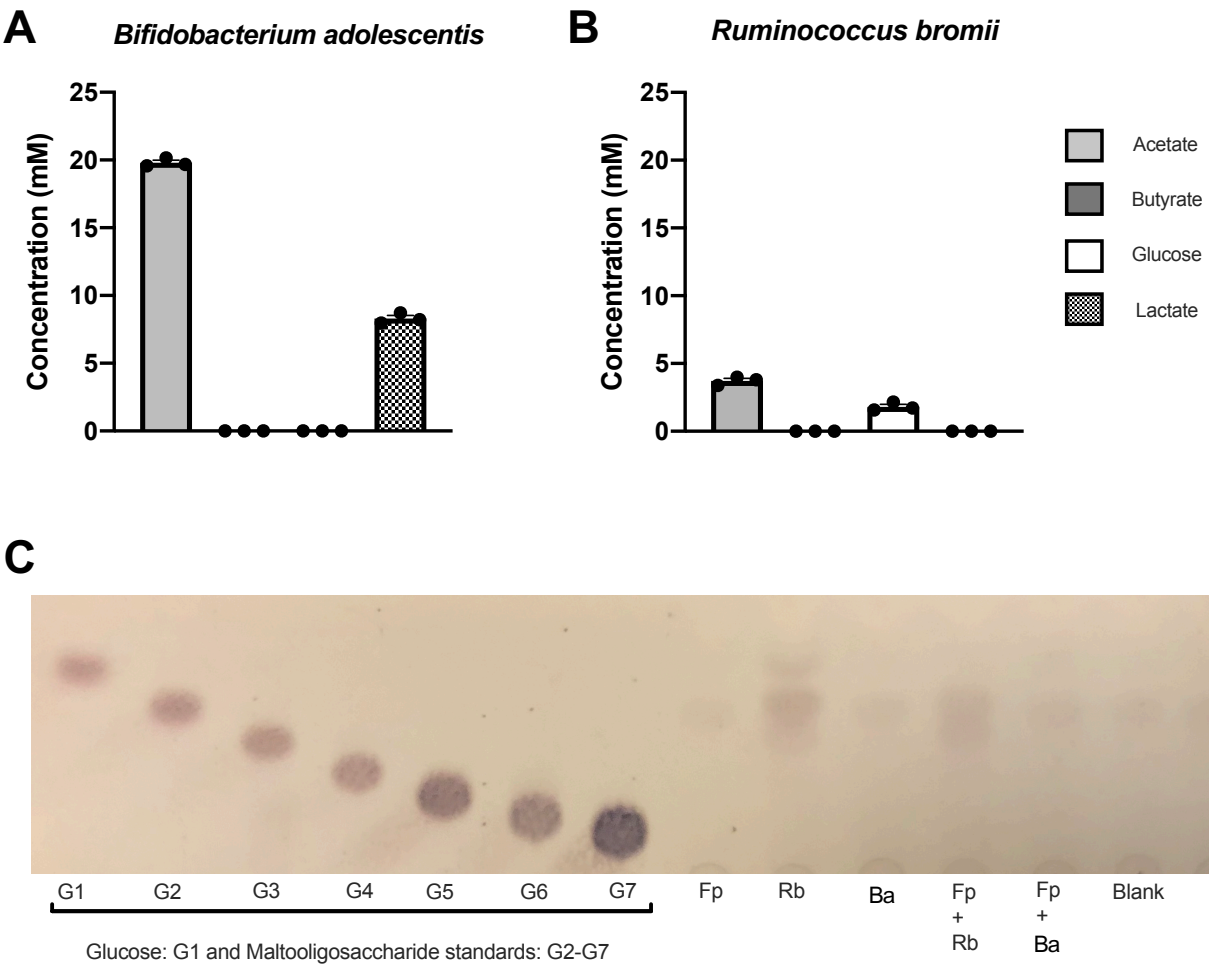


Supplemental Figure 2.1. Responses of individual SCFAs to RSP supplementation.
(A-C) Fecal acetate (A), butyrate (B), and propionate (C) before and during RSP supplementation. Each point represents the average of 1-3 fecal samples from a single individual collected the week before supplementation or during the second week of supplementation. Changes represent the difference of during and before averages for each individual. Error bars indicate SEM. Statistical significance calculated using paired t-tests (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$). (D-G) Pearson correlation of summed relative abundances of RSP degraders versus total fecal SCFA before RSP supplementation (D) and versus acetate (E), butyrate (F), and propionate (G) during RSP supplementation.



Supplemental Figure 2.2. Butyrate responses to RSP supplementation in individuals with varying patterns of RSP degrader and butryogen response.

Number of individuals with each pattern of RSP and butyrogen response. "+" means the indicated ASV increased in relative abundance from before to during RSP supplementation; "-" means the indicated ASV either did not increase in relative abundance from before to during supplementation or was never detected in the individual. (B-H) Butyrate response of individuals with each pattern of RSP and/or butyrogen response. Each point represents one individual. Error bars indicate SEM. Statistical significance calculated with paired t-tests comparing average fecal butyrate before and during supplementation (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).



Supplemental Figure 2.3. Products of RS degrader growth on RSP.
SCFA profile of *B. adolescentis* (A) and *R. bromii* (B) grown in YCFA medium containing 0.5% RPS. Samples were harvested at 48h. (C) TLC analysis of glucose and maltooligosachharides released from RPS by the action of *B. adolescentis* (Ba) or *R. bromii* (Rb) and their respective co-cultures with butyrate producer *F. prausnitzii* (Fp+Rb and Fp+Bf) in modified YCFA medium (without acetate) containing 0.5% RPS after 48 h.

Chapter 3: H_2 Generated by Fermentation in the Human Gut Microbiome Influences Metabolism and Competitive Fitness of Gut Butyrate Producers

A version of this chapter was published as:

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3.1 Abstract

Hydrogen gas (H_2) is a common product of carbohydrate fermentation in the human gut microbiome and its accumulation can modulate fermentation. Concentrations of colonic H_2 vary between individuals, raising the possibility that H_2 concentration may be an important factor differentiating individual microbiomes and their metabolites. Butyrate-producing bacteria (butyrogens) in the human gut usually produce some combination of butyrate, lactate, formate, acetate and H_2 in branched fermentation pathways to manage reducing power generated during the oxidation of glucose to acetate and carbon dioxide. We predicted that a high concentration of intestinal H_2 would favor the production of butyrate, lactate and formate by the butyrogens at the expense of acetate, H_2, and CO_2. Regulation of butyrate production in the human gut is of particular interest due to its role as a mediator of colonic health through anti-inflammatory and anti-carcinogenic properties.

For butyrogens that contained a hydrogenase, growth under a high H_2 atmosphere or in the presence of the hydrogenase inhibitor CO stimulated production of organic

fermentation products that accommodate reducing power generated during glycolysis, specifically butyrate, lactate and formate. Also as expected, production of fermentation products in cultures of *Faecalibacterium prausnitzii* strain A2-165, which does not contain a hydrogenase, was unaffected by H_2 or CO. In a synthetic gut microbial community, addition of the H_2-consuming human gut methanogen *Methanobrevibacter smithii* decreased butyrate production alongside H_2 concentration. Consistent with this observation, *M. smithii* metabolic activity in a large human cohort was associated with decreased fecal butyrate, but only during consumption of a resistant starch dietary supplement, suggesting the effect may be most prominent when H_2 production in the gut is especially high. Addition of *M. smithii* to the synthetic communities also facilitated the growth of *E. rectale*, resulting in decreased relative competitive fitness of *F. prausnitzii.*

H_2 is a regulator of fermentation in the human gut microbiome. In particular, high H_2 concentration stimulates production of the anti-inflammatory metabolite butyrate. By consuming H_2, gut methanogenesis can decrease butyrate production. These shifts in butyrate production may also impact the competitive fitness of butyrate producers in the gut microbiome.

3.2 Introduction

Hydrogen gas (H_2) is a common product of bacterial metabolism in anoxic environments, when electron acceptors for anaerobic respiration are limited. H_2 is commonly produced when fermentative microbes use protons as electron acceptors to dispose of reducing power, reducing them to H_2 via hydrogenases [65–67]. Thermodynamic principles render H_2 production less favorable when H_2 concentrations

are high, impacting the metabolism of H_2-producing microbes [68–70]. Hydrogenase genes occur in phylogenetically diverse microbes including 71% of the reference genomes in the Human Microbiome Project Gastrointestinal Tract database, suggesting that H_2 concentration may be a major factor influencing fermentation in the human gut microbiota [71].

H_2 produced during bacterial fermentation in the large intestine can be consumed by other microbes, escape in flatus or diffuse into the blood stream where it is subsequently released into the lungs and exhaled. The summation of these processes results in a concentration of H_2 in intestinal gas ranging from undetectable to over 40% v/v (Supplemental Fig. 3.1) [72, 73]. Diet is a major determinant of H_2 production in the human colon. In particular, fermentable, microbiota-accessible carbohydrates (MACs) [74] largely drive H_2 production [75, 76]. Despite the ubiquity of H_2 in the environment of the large intestine, concrete information is lacking about how the concentration of hydrogen regulates fermentation of specific gut microbes. An effect of H_2 concetration on human gut butyrogens has been predicted [77], and would be of particular significance because of the anti-inflammatory and anticarcinogenic effects of butyrate [10, 28, 31, 34].

The fermentation scheme of typical human gut butyrogens is depicted in Figure 3.1A. Carbohydrate substrates (most simply represented by glucose) are first processed via glycolysis. Per glucose, the reactions of glycolysis form two pyruvate and phosphorylate two ADP to form ATP by substrate-level phosphorylation (SLP). Importantly, glucose oxidation to pyruvate reduces two moles of the cofactor NAD^+ to NADH [78]. The necessity of regenerating NAD^+ from this NADH to maintain redox

balance represents both a central constraint on possible fermentations and an opportunity to conserve additional energy [66, 79].

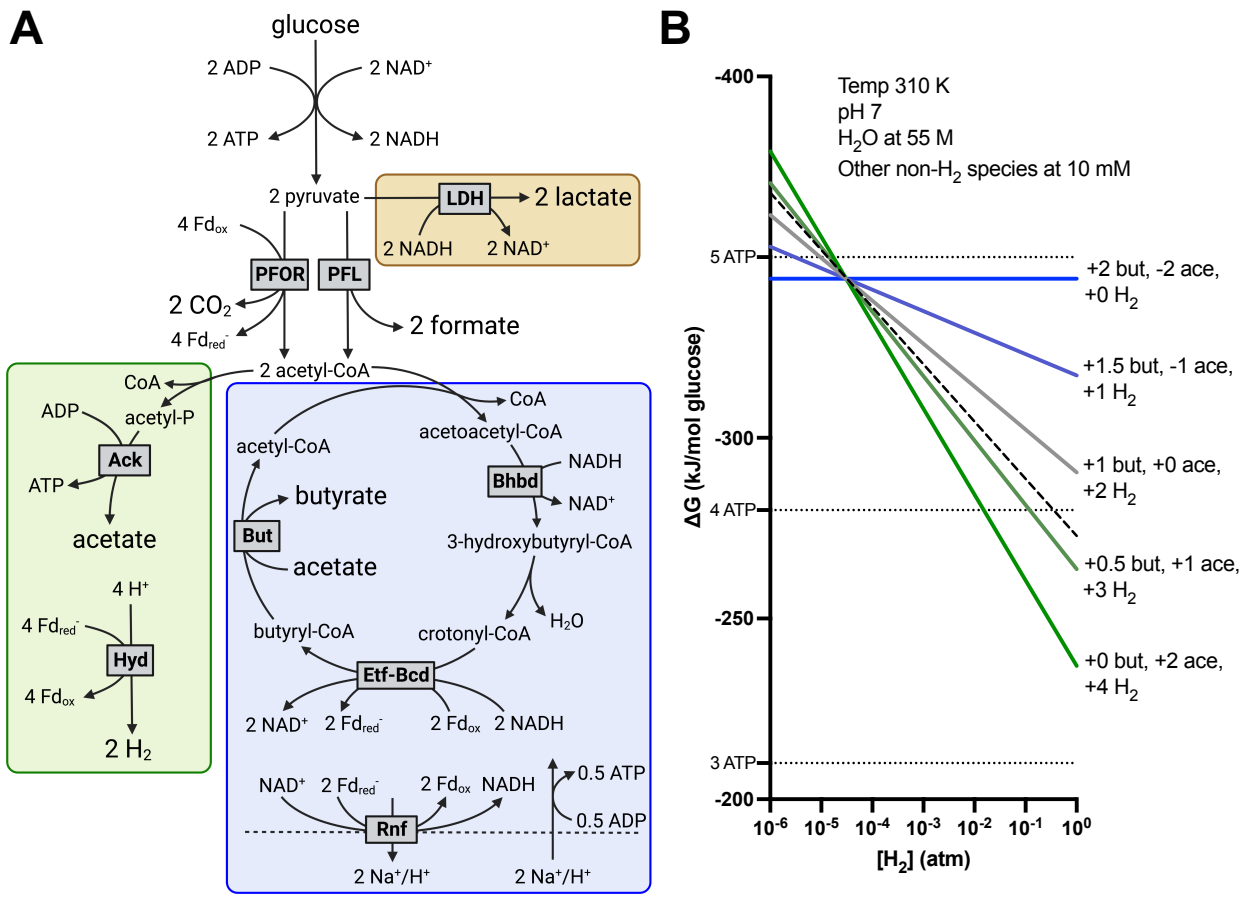


Figure 3.1. Stoichiometry and thermodynamics of fermentation in human gut butyrogens. (A) Generic fermentation pathways in human gut butyrogens that can yield combinations of H_2, CO_2, formate, lactate, acetate, and butyrate (adapted from Louis and Flint, 2017 [78]). Fd, ferredoxin; LDH, lactate dehydrogenase; PFOR, pyruvate:ferredoxin oxidoreductase; PFL, pyruvate formate-lyase; Ack, acetate kinase; Hyd, ferredoxin hydrogenase; Bhbd, hydroxybutyryl-CoA dehydrogenase; Etf-Bcd, butyryl-CoA dehydrogenase electron-transferring flavoprotein complex; But, butyryl-CoA:acetyl CoA transferase; Rnf, Rnf complex. The division of acetyl-CoA between acetate production (green box) and butyrate production (blue box) is variable; an equal division is shown as a representative case with simple stoichiometry. The division of pyruvate between the PFOR and PFL routes of acetyl-CoA formation is also variable. Stoichiometry is balanced from glucose to either lactate (orange box) or to butyrate and acetate, via either PFOR or PFL. (B) Illustration of the effect of $[H_2]$ on ΔG of glucose fermentation to butyrate and acetate. A range of possible fermentation balances are shown, with the net molar production or consumption of butyrate (but), acetate (ace), and H_2 per glucose indicated. Dotted horizontal lines indicate theoretical ΔG thresholds for formation of a given number of ATP, assuming ΔG of ADP phosphorylation as +70 kJ/mol [79]. Dashed line indicates the least butyrate formation likely to be feasible for human gut butyrogens. Representative physiological conditions were used as indicated.

Following glycolysis, pyruvate may be reduced to lacate by lactate dehydrogenase (LDH). This pathway fully reoxidizes the NADH produced in glycolysis, but produces no additional ATP [80]. More commonly, pyruvate is converted into acetyl-CoA and either CO_2 or formate. Production of acetyl-CoA and CO_2 is catalyzed by pyruvate:ferredoxin

oxidoreductase (PFOR) and is coupled with the reduction of ferredoxin (Fd), a small iron-sulfur protein with a lower reduction potential than NAD^+ [79, 81, 82]. By contrast, pyruvate cleavage into acetyl-CoA and formate is catalyzed by pyruvate formate-lyase (PFL) and does not generate any additional reduced species [81, 83]. Formate may subsequently be used in anabolic pathways or simply secreted as a fermentation product [83]. *In vivo*, both the PFOR and PFL pathways of acetyl-CoA formation can be active simultaneously [84].

As with pyruvate, acetyl-CoA in human gut butyrogens can proceed down either of two branched pathways culminating in acetate or butyrate production [66, 77, 78]. In acetate production, the acetyl group is transferred from CoA to phosphate to form acetyl phosphate (acetyl-P). In a reaction catalyzed by acetate kinase (Ack), this phosphate is then transferred to ADP to generate ATP via SLP, releasing acetate. This pathway conserves energy as ATP but does not contribute to reoxidation of NADH or reduced ferredoxin (Fd_{red}^-) [66, 77, 85]. Butyrate production, by contrast, is an important sink for reducing power. In this pathway, two acetyl-CoA are combined to form acetoacetyl-CoA, which is then reduced to 3-hydroxybutyryl-CoA by hydroxybutyryl-CoA dehydrogenase (Bhbd) in a reaction that reoxidizes one NADH cofactor to NAD^+ [86]. The next reaction forms crontonyl-CoA, which is then further reduced to butyryl-CoA by the butyryl-CoA dehydrogenase electron-transferring flavoprotein complex (Bcd-Etf). This electron-bifurcating complex couples crotonyl-CoA reduction to the endergonic reduction of Fd_{ox} by NADH in an overall thermodynamically feasible reaction [79, 87]. The resulting Fd_{red}^-, in addition to that formed by PFOR, is reoxidized by ferredoxin hydrogenase through the reduction of protons to H_2 [78, 88]. Sufficient flux through the butyrate production pathway

results in an overabundance of Fd_{red}^- and a shortfall of NADH. Butyrogens can conserve additional energy in this case through anaerobic respiration using the Rnf complex, which couples Fd_{red}^- oxidation and NAD^+ reduction (ferredoxin:NAD^+ oxidoreductase) to cation transport across the membrane, synthesizing ATP by a chemiosmotic mechanism [79, 89]. Final release of butyrate is by exchange with free acetate catalyzed by butyryl-CoA:acetyl CoA transferase (But), which results in net acetate consumption when more acetyl-CoA flows to butyrate production than to acetate production [78, 85, 90].

A salient feature of the branched metabolism of gut butyrogens described above is that increasing butyrate production reduces the refductant available for H_2 formation. Conversely, increasing acetate production necessarily entails the formation of more H_2 in order to regenerate NAD^+ [66, 78]. As a result, thermodynamic equilibrium increasingly favors butyrate over acetate production as ambient H_2 concentrations rise (illustrated in Fig. 1B) [70, 77, 91]. This has led to the expectation that higher gut H_2 concentration favors butyrate over acetate production [70, 77]. While this result or similar results demonstrating the same principle have been observed in species related to human gut butyrogens [92–94], to the best of our knowledge the effect of ambient H_2 concentration on fermentation by the predominant human gut butyrogens *Roseburia intestinalis*, *Eubacterium rectale*, and *Faecalibacterium prausnitzii* [77, 95] has not been directly investigated, with the exception of one study that found no effect of autogenous H_2 on cultures of *R. intestinalis* [96].

H_2 concentration in bacterial culture can be reduced by stirring [94, 97], sparging [92], or co-culture with hydrogen-consuming microbes (hydrogenotrophs) [68, 93, 98, 99]. Under these circumstances, hydrogenases can generate more H2 and oxidized end

products (e.g. acetate), with correspondingly less production of reduced organic end products (e.g. ethanol, butyrate) [92–94, 97, 99]. Three guilds of hydrogenotrophs colonize the human gut: methanogens, sulfate reducers, and reductive acetogens [100–104]. These hydrogenotrophs actively consume H_2 and may therefore play an important role in regulating the H_2 concentrations to which human gut butyrogens are exposed.

In this study, we investigate the effect of H_2 concentration on the profile of fermentation end products of *R. intestinalis*, *E. rectale*, *F. prausnitzii*. We find evidence that physiologically relevant variations in H_2 concentration influence the favored routes of reductant disposal in H_2-producing human gut butyrogens, resulting in shifts in the production of fermentation products. Specifically, exposure to high H_2 concentrations increases production of butyrate, lactate, and formate at the expense of acetate and, presumably, CO_2. These metabolic shifts appear to impact the competitive fitness of certain butyrogens. We propose a model where the profile of fermentation products from these taxa, and metabolically similar fermenters in the human colon, is modulated by local colonic H_2 concentration. This, in turn, is a balance of production by fermenters and elimination by hydrogenotrophs. Finally, we report observations from a large human cohort consuming resistant potato starch as a MAC expected to stimulate fermentation and H_2 production. Consistent with our model, we found that hydrogenotrophic gut methanogenesis was associated with decreased fecal butyrate during supplement consumption.

3.3 Methods

3.3.1 Human study

3.3.1.1 Cohort

Results from a portion of this study's human cohort were previously reported by Baxter et al. (2019) [17] Participants were recruited through Authentic Research Sections of the University of Michigan BIO173 introductory biology course. Subjects were excluded based on self-reported inflammatory bowel syndrome, inflammatory bowel disease, colorectal cancer, and consumption of antibiotics in the past 6 months.

3.3.1.2 Study design and sample collection

The study took place during a number of separate semesters over the course of 3 years, from the winter semester of 2016 to the winter semester of 2019. While all supplements consumed consisted of resistant starch from potato (RSP), they varied in source, total dose, and frequency. The supplements consumed were Bob's Red Mill potato starch (Bob's Red Mill Natural Foods, Milwaukie, OR) consumed as a 20g dose twice daily, a 20 g dose mixed with 2.5 g psyllium twice daily, a 40 g dose once daily, or a 40 g dose twice daily; or resistant potato starch from LODAAT Pharmaceuticals (Oak Brook, IL) consumed as a 20 g dose once daily.

In each semester, the study followed a 3-week course. During the first week, fecal and breath samples were collected before consumption of RSP. During the second week, RSP consumption began at a half dose and increased to the full dose. During the third week, RSP consumption continued at the full dose while fecal and breath samples were collected.

3.3.1.3 Human sample analysis

Fecal sample collection, preparation, and quantification of short-chain fatty acid concentration by high-performance liquid chromatography (HPLC) was performed as previously described in Baxter et al. (2019) [17]. Breath samples consisted of 30 mL of end-expiratory breath collected in a 30 mL gastight syringe. Immediately after collection, samples were injected into a QuinTron BreathTracker SC analyzer (QuinTron Instrument Company Inc., Milwaukee, WI, Cat#QTLNRBTGCSC) for analysis. Concentrations of H_2, methane, and carbon dioxide gas were measured, and hydrogen and methane measurements were normalized based on an assumed nominal concentration of 3.5% carbon dioxide. The BreathTracker analyzer was calibrated daily using a standard calibration gas containing 150 ppm H_2, 75 ppm methane, and 6% carbon dioxide (QuinTron, Cat#QT07500-G).

3.3.1.4 Human samples and methanogenesis classification

Concentrations of fecal metabolites obtained from HPLC (described above) were normalized to the wet weight of fecal material. Concentrations of CH_4 and H_2 in breath samples were quantified as described above. For each fecal metabolite and breath gas, samples with values lying more than three interquartile ranges below the lower quartile or above the upper quartile were excluded from analysis according to the method of Tukey's Fences [105].

Subjects were classified as methanogenic or non-methanogenic, with separate classifications for the periods before and during supplement consumption. Methanogenic subjects were defined as those with over 4 ppm methane in at least one breath sample. This cutoff was based on a study of responses to consumption of lactulose (a fiber

inaccessible to human enzymes but rapidly degraded by the gut microbiota) which suggested that a baseline threshold of 4 ppm above background is best predictive of increased breath methane [106]. We used this threshold because our intent was to identify subjects where methanogenesis was not just present, but a significant component of the gut ecosystem. In the Winter 2016 and Winter 2019 semesters, an elevated baseline concentration of 1 ppm methane was observed across most samples. Since this was likely due to instrument calibration rather than biological activity, this elevated baseline was subtracted before classifying individuals as methanogenic or non-methanogenic. The average concentration of each fecal metabolite and breath gas before and during starch supplement consumption was then calculated for each subject. The average concentration of fecal metabolites and breath gases in methanogenic and non-methanogenic subjects was then compared using two-tailed Student's t-tests in GraphPad Prism 9.

3.3.2 Microbial strains and culture

Methanobrevibacter smithii F1 (DSM 2374), *Faecalibacterium prausnitizii* A2-165 (DSM 17677), and *Roseburia intestinalis* L1-82 (DSM 14610) were obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). *Ruminococcus bromii* VPI 6883 (ATCC 27255) was obtained from the American Type Culture Collection (ATCC). *Eubacterium rectale* A1-86 (DSM 17629), *Bacteroides thetaiotaomicron* VPI 5482 (DSM 2079), *Bacteroides vulgatus* Eggerth and Gagnon (ATCC 8482), and *Prevotella copri* CB7 (DSM 18205) were obtained from collaborators.

Also included in the synthetic community were the strains *Bifidobacterium adolescentis* 269-1 and *Anaerostipes caccae* 127-8-5, which are isolates from fecal samples obtained in the course of the human cohort study. *B. adolescentis* 269-1 was obtained from a fecal sample serially diluted and plated on Bifidus Selective Medium agar (BSM Agar, Sigma-Aldrich) including the BSM supplement according to the manufacturer's instructions. Plates were incubated at 37˚C in an anoxic atmosphere of 5% carbon dioxide, between 1.5-3.5% H_2, and balance N_2 in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI). *Bifidobacterium* colonies were identified as having a pink center and light brown edge, and were restreaked on BSM agar

A. caccae 127-8-5 was obtained from a fecal sample stored at -80˚C in an OMNIgene-Gut collection kit tube (DNA Genotek, Ottawa, Ontario, Cat#OMR-200). The fecal sample was serially diluted and plated on SABU agar, a medium containing 2 g/L taurocholate to stimulate spore germination (full list of medium components in Supplementary Table 3.1). Plates were incubated at 37˚C in the anaerobic chamber described above, and colonies that grew were picked.

The taxonomic identity of the 269-1 and 127-8-5 isolates was determined using Sanger sequencing of the 16S rRNA gene. 16S rRNA was amplified using primers designated 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3‘) and sequenced from the 8F primer. These sequencing results have been deposited in Zenodo (https://doi.org/10.5281/zenodo.6643453).

All microbial strains used in this study are available upon request made to the lead contact.

All microbial strains were preserved in frozen stocks at -80˚C with either 5% DMSO or 20% glycerol as a cryopreservative. To begin cultivation, for all strains except *M. smithii* F1, a small amount of material was scraped from the frozen stocks and added to 5-10 mL of SAB4 base medium (components in Supplementary Table 3.1) supplemented with either 4 g/L D-glucose or 2 g/L each of D-glucose and D-fructose in the Coy anaerobic chamber described above. These cultures were incubated at 37˚C and passaged as necessary (no more than four passages, most commonly one or two) to produce mid- or late-exponential phase cultures used to inoculate experimental cultures. For *M. smithii* F1, frozen stocks were thawed in the anaerobic chamber and transferred using a 1 mL syringe fitted with a 23 gauge needle into a Balch tube sealed with a butyl rubber stopper and aluminum crimp (Chemglass Life Sciences, Vineland, NJ, Cat#CLS-4209) containing 5 mL SAB4 base medium under a headspace of 80% H_2 + 20% CO_2 mixed gas at 20 psig. These primary cultures were incubated at 37˚C on an orbital shaker at 150 rpm and passaged anaerobically as necessary to produce mid- or late-exponential phase cultures used to inoculate experimental cultures.

3.3.3 Monoculture experiments

Cultures were grown at 37˚ in 10 mL (H_2 headspace experiments) or 5 mL (CO headspace experiments) SAB4 base medium (Supplementary Table 3.1) supplemented with 4 g/L D-glucose and 2.31 g sodium bicarbonate in Balch tubes sealed with butyl rubber stoppers and aluminum crimps (as described in Experimental Model and Subject Details). For shaking cultures, the Balch tubes were placed on their side in an orbital shaker at 150 rpm.

All Balch tubes were prepared with a headspace of 80% N_2 + 20% CO_2 mixed gas at atmospheric pressure. In experiments involving the addition of H_2 to the headspace, all cultures were prepared with a headspace at 3 atm gauge pressure containing the indicated partial pressure of H_2 and the balance N_2. Gases were added using a custom gas manifold, and a pressure gauge was used to adjust regulators to supply the correct pressure (SSI Technologies Inc., Janesville, WI, Cat#MG-30-A-9V-R). Ultra-high purity grades of N_2 and H_2 were used. In experiments involving the addition of carbon monoxide (CO), 2.2 mL of either pure CO or N_2 was added using a syringe fitted with a stopcock and needle. All gases used in this study were purchased from Cryogenic Gases Inc., a division of Metro Welding Supply Corp. (Detroit, MI).

Growth curves were obtained by making regular measurements of the OD_{600} in the culture tubes using a Spec-20 spectrophotometer (Thermo Spectronic Model 333183). Before each series of measurements, the spectrophotometer was zeroed using a Balch tube containing uninoculated medium from the same batch used in the experiment.

Monoculture experiments under H_2 were performed twice for *E. rectale* and *F. prausnitzii* and three times for *R. intestinalis*. One experiment each for *E. rectale* and *R. intestinalis* included conditions with ppH_2 of 2 and 3 atm in addition to 0 and 1 atm. All conditions in all experiments under H_2 had three to five biological replicates. Monoculture experiments with CO were performed once for each butyrogen with four (*R. intestinalis*, *F. prausnitzii*) or five (*E. rectale*) biological replicates.

3.3.4 Synthetic community experiments

Cultures of synthetic community members (excluding *M. smthii* F1) were grown from stock in SAB4 base medium supplemented with 2 g/L each of D-glucose and D-fructose in the anaerobic chamber described above (and passaged so as to obtain mid- or late-exponential phase cultures of all the microbes simultaneously (as described above in Experimental Model and Subject Details). Once this was achieved, equal cell numbers of each synthetic community member (estimated using OD_{600} measurements) were combined to create an inoculation mix, which was used to inoculate Balch tubes for the experimental cultures, which were subsequently sealed. These Balch tubes contained 10 mL of the SAB4 base medium supplemented with D-glucose and D-fructose described above. Since they were inoculated and sealed in the anaerobic chamber, their initial headspace matched that of the anerobic chamber (5% carbon dioxide, between 1.5-3.5% H_2, balance N_2). Each experimental culture was grown for 24 hours, then passaged at a 1:100 dilution into another Balch tube for two subsequent 24-hour cultures. For shaking cultures, the Balch tubes were placed on their side in an orbital shaker at 150 rpm.

Cultures of *M. smithii* F1 were grown from stock in Balch tubes and passaged so as to obtain mid- or late-exponential phase cultures at the same time as the other synthetic community members. *M. smithii* cells were added to the appropriate experimental cultures as an inoculum separate from the inoculation mix described above. Additional inocula of *M. smithii* were added at each passage of the synthetic community from pure cultures of *M. smithii* were that maintained in Balch tubes during the course of the experiment. The number of *M. smithii* cells added in each inoculum was estimated using OD_{600} and kept consistent.

The synthetic community experiment was performed twice with five biological replicates for each condition each time. Shaking cultures were only included in the second experiment.

3.3.4.1 Synthetic community relative abundance quantification

1 mL samples of synthetic community cultures were centrifuged for two minutes at 11,000g. Genomic DNA was extracted from the pellet using a DNeasy PowerLyzer Microbial Kit (Qiagen, Cat#12255-50) according to the manufacturer's instructions. The V4 region of the 16S rRNA gene was amplified and sequenced on the Illumina MiSeq platform using a 2x250-bp paired-end kit as described in Kozich et al. (2013) [107].

The resulting 16S amplicon data was analyzed using mothur v1.39.5 [108]. The mothur script and logfile have been deposited in Zenodo (https://doi.org/10.5281/zenodo.6621661). In summary, paired-end reads were merged into contigs, screened for sequencing errors, and aligned to the SILVA v132 reference database [107]. Aligned sequences were pre-clustered at 1 difference, screened for chimeras, and classified using the SILVA v132 reference database. Sequences identified as mitochondria, chloroplasts, or Eukaryotes were removed. Sequences were then clustered into 99% OTUs, which reproduced the 9 community members (plus *M. smithii*) known to be present in the cultures, and a shared file was exported. Microsoft Excel (Microsoft Corporation, Redmond, WA) was used to calculate relative abundances from the shared file, and the results were imported into GraphPad Prism 9 (GraphPad Software, San Diego, CA), where statistical analyses were carried out as described in the figure legends.

3.3.5 Aqueous fermentation product quantification

Samples of 1 mL bacterial culture were centrifuged for two minutes at 11,000g and the supernatant passed through a 0.22 µm MultiScreen$_{HTS}$ GV 0.22 µm filter plate (Millipore Sigma, Burlington, MA). Similar to the procedure described by Baxter et al. (2019) [17], filtrates were transferred into 100-µl inserts inside 1.5-ml screw cap vials in preparation for analysis by HPLC. Quantification of SCFAs was performed using a Shimadzu HPLC system (Shimadzu Scientific Instruments, Columbia, MD) that included an LC-10AD vp pump A, LC-10AD vp pump B, DGU-14A degasser, CBM-20A communictaions bus module, SIL-20AC HT autosampler, CTO-10AS vp column oven, RID-10A RID detector, and an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA). We used a mobile phase of 0.01 N H_2SO_4 at a total flow rate of 0.6 ml per min with the column oven temperature at 50˚C. The sample injection volume was 10 µl, and each sample eluted for 40 min. The concentrations were calculated using standard curves generated for each product from a cocktail of short-chain organic acid standards at concentrations of 40, 20, 10, 5, 2.5, 1, 0.5, 0.25, and 0.1 mM. These standards were run before and after each batch of samples, and standard curves were generated using averaged values. The baseline of the chromatographs was manually corrected to ensure consistency between samples and standards. Samples were analyzed in a randomized order.

3.3.6 Gaseous fermentation product quantification

Gas samples were removed from the headspace of cultures using syringes fitted with stopcocks. Methane content was measured using a Shimadzu GC-2014A greenhouse gas analyzer gas chromatograph (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with a flame ionization detector (FID) fed by ultra-high purity H_2 and zero-grade air. Ultra-high purity N_2 was used as the carrier gas. Sample separation was performed with a 1.0 M Hayesep T 80/100 mesh column, and 4.0 M Hayesep D 80/100 mesh column, and a 0.7 M Shimalite Q 100/180 mesh column. Before each series of measurements, accuracy was checked using a 500 ppm methane standard (Argus-Hazco, Byron Center, MI, Cat#GD40-007-A-221S).

H_2 content was measured using a Peak Performer 1 gas chromatograph (Cat#910-105) with a reducing compound photometer (RCP) detector and post-column diluter (Peak Laboratories, Mountain View, CA) calibrated using a 10 ppm H_2 standard (GASCO 105L-H2N-10, Cal Gas Direct Incorporate, Huntington Beach, CA). Ultra-high purity N_2 was used as the carrier gas. When necessary, samples were diluted in room air using syringes fitted with stopcocks before measurement to reduce the H_2 concentration below 100 ppm, which was the upper detection limit.

3.3.7 Total protein quantification

Total protein content was used as an indicator of bacterial biomass. 1 mL samples of microbial cultures at endpoint were centrifuged for 2 minutes at 11,000g. The supernatant fraction was stored at -80˚C for later analysis. The pellet was resuspended in 1.5 mL distilled H_2O and sonicated to lyse cells. Sonication was perfomed on ice using a Branson Digital Sonifier 450 equippd with a 102C converter and microtip, which was

placed directly in the bacterial suspension. 35% amplitude was used for a 3-minute cycle of 1 second on followed by 14 seconds off (total 12 seconds sonication time). Protein concentrations in the resulting lysate and the saved supernatant fraction were determined using a Pierce Coomassie Plus Bradford assay reagent (Thermo Scientific, Cat#23238) with bovine serum albumin (BSA) standards per the manufacturer's instructions. The results from the lysate and supernatant were added together to obtain the total protein yield of the culture.

3.3.8 Microbial culture fermentation products

In monocultures of *R. intestinalis*, *E. rectale*, and *F. prausnitzii*, depending on the moles of butyrate formed per glucose fermented, acetate can be either a net product (<1 mol butyrate per mol glucose) or net substrate (>1 mol butyrate per mol glucose) of fermentation. Even when it is a net substrate, however, some acetyl-CoA still flows to acetate production and ATP formation by acetate kinase. In order to not obscure this nuance by reporting the net consumption of acetate that was observed in some cultures, results for fermentation products were expressed as the percent of total carbon consumed that was used in the formation of each product, rather than simple carbon recovery. This metric was calculated by first reasoning that since all three of these butyrogens produce butyrate by consuming acetate via the butyryl-CoA:acetate CoA enzyme, each mole of butyrate produced represeted a mole of acetate (abundantly available in the SAB4 medium) consumed [90]. Therefore, a molar value of acetate that was theoretically consumed was set equivalent to moles of produced butyrate. Total fermented carbon was then calculated by adding the moles of carbon in the consumed glucose to the moles of

carbon in theoretically consumed acetate. Total acetate produced was then calculated by adding the moles of theoretically consumed acetate to the change in acetate measured in the culture at endpoint versus blank medium, which varied from consumption to production depending on strain and condition. Since neither butyrate, formate, nor lactate were present in the medium or expected to be consumed during microbial metabolism, their total quantity produced was simply taken to be their endpoint concentration. Percent total fermented carbon in each substrate was then calculated as the moles of carbon in the produced substrate divided by the total moles of fermented carbon.

In the synthetic communities, the presence of diverse potential metabolic pathways rendered the above approach impractical. Instead, results for each fermentation product were expressed as change in the product in moles (an increase in all but one culture where acetate decreased) divided by the total moles of substrate (glucose and fructose) consumed in the culture.

All statistical analyses of metabolite data obtained from microbial cultures were performed using GraphPad Prism 9 and are described in the figure legends.

3.4 Results

To test the hypothesis that H_2 modulates the production of fermentation products by human gut butyrogens, we studied pure cultures of strains representing abundant butyrogens in the human gastrointestinal tract. *Eubacterium rectale* A1-86 and *Roseburia intestinalis* L1-82 were selected to represent the generalized metabolic pathways of butyrogens that could be affected by H_2 (Fig. 3.1). *Faecalibacterium prausnitzii* A2-165

was chosen as a representative of butyrogens that lack a hydrogenase and are therefore unlikely to be affected by H_2.

Replicate cultures of these three butyrogens were grown under a headspace of either H_2 or N_2 and shaken continuously to equilibrate headspace gases with the culture medium. As predicted, the presence of a H_2 headspace shifted the profile of fermentation away from acetate towards more reduced organic acids (e.g. lactate and butyrate) for both H_2-producing butyrogens (Figs. 3.2A, 3.2C). The same pattern of fermentation products was recapitulated in the presence of carbon monoxide (CO; Figs. 3.2B, 3.2D), a potent inhibitor of ferredoxin hydrogenase [109]. The profiles of reduced organic acids differed between the hydrogen-producing butyrogens. In cultures of *R. intestinalis*, reducing power was diverted to butyrate and formate. Lactate production increased very significantly, but remained only a trace product in both conditions (<0.5% substrate carbon). By constrast, cultures of *E. rectale*, saw reducing power diverted primarily to lactate, with a smaller diversion to formate and no change in butyrate. Since formate production reduces intracellular Fd_{red}^- versus the alternative production of CO_2, it clearly represents a diversion reducing power away from H_2 production via ferredoxin hydrogenase, as does butyrate production (Fig. 3.1A). Increasing the partial pressure of H_2 in the headspace up to 3 atm led to larger shifts in a rough dose-response pattern (Supplemental Fig. 3.2). Unlike *E. rectale* and *R. intestinalis*, *F. prausnitzii* lacks hydrogenase activity [110]. As expected, its fermentation products were unaffected by the presence of H_2 or CO in the headspace (Fig. 3.2E-F).

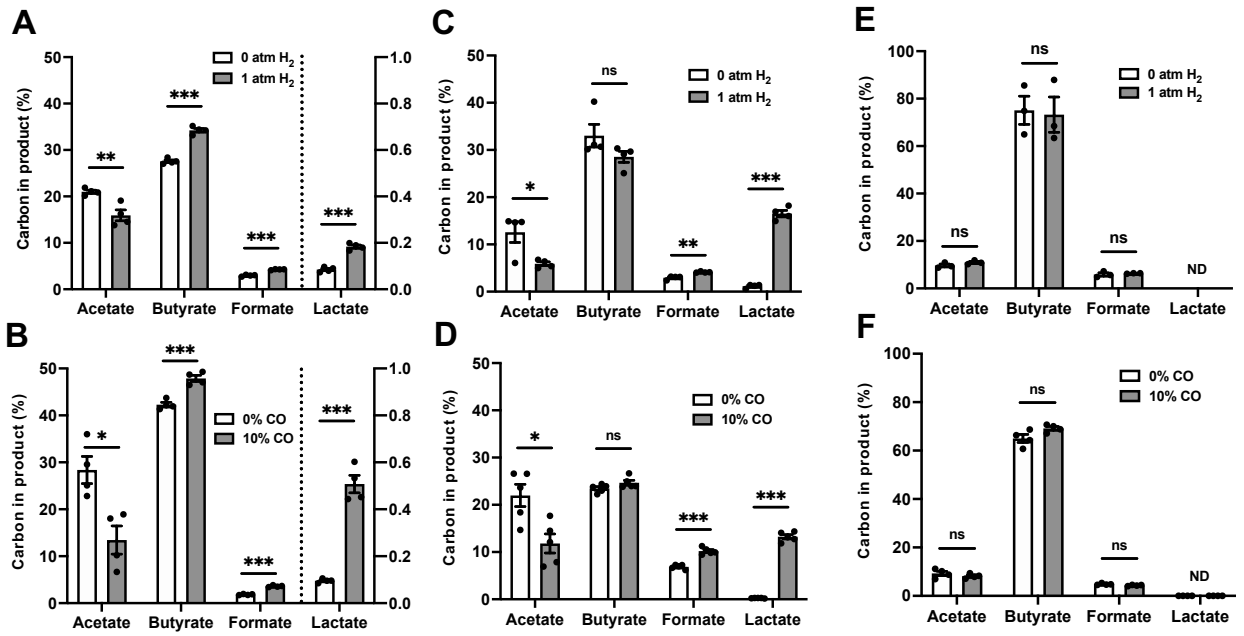


Figure 3.2. Variation in fermentation products in cultures of human gut butyrogens grown under different atmospheres.
Endpoint fermentation products in cultures of *R. intestinalis* (note lactate is shown on smaller scale on right axis) (A-B), *E. rectale* (C-D), and *F.* prausnitzii (E-F) grown in shaken cultures with H_2, N_2, or CO—a potent inhibitor of hydrogenases. Error bars indicate SEM. Statistical significance calculated using two-sided Student's two-sample t-tests (* = p<0.05; ** = p<0.01; *** = p<0.001).

To assess whether H_2 has an impact on butyrate production in more complex microbial communities, we assembled a synthetic community of microbes isolated from the human gut. This mixture consisted of representative strains of common butyrate producers (*F. prausnitzii*, *E. rectale*, *R. intestinalis*, and *Anaerostipes caccae*), two common resistant starch degraders (*Bifidobacterium adolescentis* and *Ruminococcus bromii*), and several members of the abundant gut phylum Bacteroidetes (*Bacteroides vulgatus*, *Bacteroides thetaiotaomicron*, and *Prevotella copri*). We compared butyrate production by this community from equimolar quantities of glucose and fructose in the presence or absence of *M. smithii*, the predominant H_2-consuming methanogen in the human gut [111, 112].

The relative abundances of the constituent microbes, as well as production of CH_4, H_2, and fermentation products, were monitored over the course of three consecutive subcultures of the synthetic community. The addition of *M. smithii* resulted in the production of methane (Fig. 3.3A) and decreased the concentration of H_2 and production of butyrate as predicted (Fig. 3.3B-C). The corresponding increase in acetate, which was observed in monocultures, was only observed in the second subculture of the synthetic community (Supplemental Fig. 3.3D). The expected shift in acetate production by butyrogens may have been masked by the copious production of acetate by other members of the synthetic community, such as the two *Bacteroides* species. Lacate and formate were also produced, along with the characteristic *Bacteroides* fermentation products propionate and succinate (Supplemental Fig. 3.3E-H). Lactate production was reduced by the addition of *M. smithii*. Since *M. smithii* consumes all available formate, its impact on formate production could not be determined.

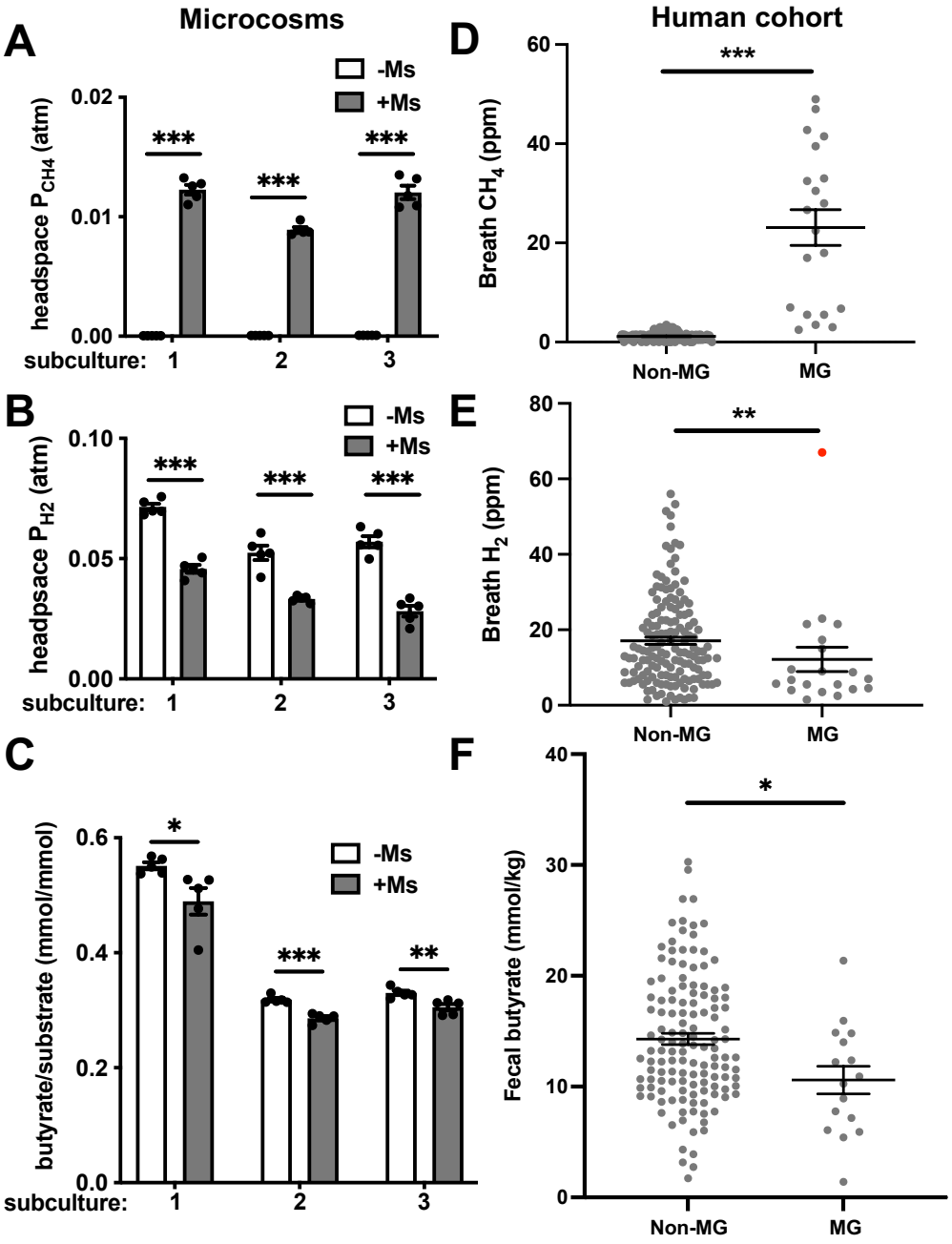


Figure 3.3. Influence of methanogenesis on butyrate production in a synthetic gut community and the human gut microbiota.

Methane (A), H_2 (B), and butyrate (C) production by a 9-species synthetic human gut community grown with (shaded bars) and without (open bars) the addition of *M. smithii*. Butyrate was measured in three successive subcultures, with a new inoculum of *M. smithii* added at each passage. In a human cohort consuming a resistant potato starch supplement, breath and fecal samples were used to determine weekly average breath CH_4 (D), breath H_2 (E), and fecal butyrate (F) in individuals with and without active gut methanogenesis, defined as at least one breath methane measurement greater than 4 ppm CH_4 in the measurement period (MG) or no breath measurements over 4 ppm in the same period (non-MG). Error bars indicate SEM. Statistical significance calculated using two-sided Student's two-sample t-tests (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$). One breath H_2 measurement shown in the methanogenic group with a value over 60 ppm (data point shown in red) was excluded from statistical analysis.

Removal of *M. smithii* from the synthetic community increased H_2 levels and favored the growth of *F. prausnitzii* (Fig. 4). The increase in *F. prausnitzii* suggests that accumulation of H_2 in the absence of a methanogen forced butyrogens with ferredoxin hydrogenase to shift their fermentation towards less energetically favorable pathways. Consistent with this explanation is the decreased abundance of *E. rectale* (Fig. 3.4), which had a slower growth rate and lower yield under higher H_2 in monoculture (Supplemental Table 3.2). In the absence of a methanogen, *R. intestinalis* exhibited a similar, but less obvious, decrease in abundance (Fig. 3.4), although its growth rate and yield were not significantly impacted by high H_2 in monoculture (Supplemental Table 3.2).

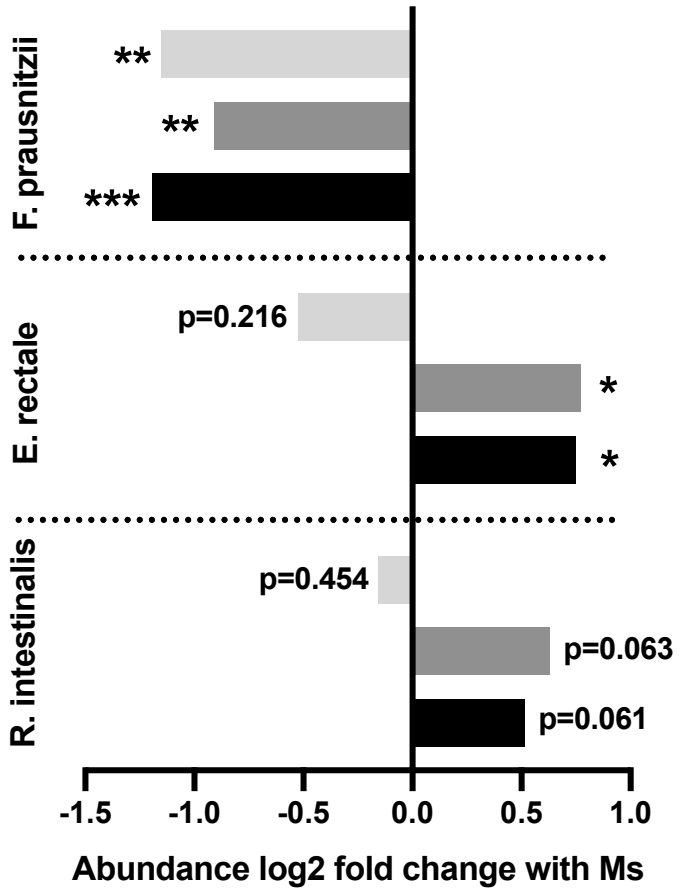


Figure 3.4. Influence of methanogenesis on competitive fitness of butyrogens in a mock gut community.
Fold change in relative abundance of *F. prausnitzii*, *E. rectale*, and *R. intestinalis* in a 9-species synthetic human gut community with the addition of *M. smithii* compared to the same community without *M. smithii*. Relative abundance was quantified at the end of three successive 24-hour subcultures (first subculture in light gray, second in dark gray, third in black), with a new inoculum of *M. smithii* added to the appropriate cultures at each passage. For each species, there were five replicate cultures in each condition. Statistical significance calculated from relative abundance values using two-sided Student's two-sample t-tests (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

Incubation of the synthetic community cultures with vigorous shaking completely abrogated the effect of *M. smithii* on butyrate production and the relative abundances of butyrate producers (Supplemental Fig. 3.3C, 3.3I). This may be because local accumulation of dissolved H_2 is prevented when H_2 in the culture medium is rapidly equilibrated with the headspace, preventing H_2 consumption by *M. smithii* from making a difference by reducing this accumulation. Shaking entirely prevented net production of

lactate, which also suggests decreased exposure of the butyrogens to high H_2 (Supplemental Fig. 3).

To explore whether the influence of *M. smithii* on butyrate production is relevant in the human gut, we collected breath samples from a human cohort both before and during consumption of resistant starch from potatoes (RSP). Resistant starch is not degraded by human amylases and reaches the gut microbiota undigested, where it can serve as a substrate for fermentation. We previously reported that RSP supplementation in a portion of this cohort increased fecal butyrate overall [17]. In this study, measurement of breath methane was used to assess gut methanogenesis both before and during RSP consumption. During RSP consumption, active gut methanogenesis was associated with lower levels of breath H_2 (Fig. 3.3E) and lower fecal butyrate concentration (Fig. 3.3F) compared to individuals lacking gut methanogenesis. These findings are consistent with the results from *in vitro* cultures and suggest that the H_2 produced from RSP breakdown in the gut may play an important role in the stimulation of butyrate production by the microbiota. Indeed, individuals with gut methanogenesis did not follow overall trend of increased fecal butyrate during RSP consumption (Supplemental Fig. 3.4D). Removal of H_2 by hydrogenotrophs such as *M. smithii* appears to shift butyrogen metabolism *in vivo* as well as *in vitro*. Interestingly, gut methanogenesis in the same individuals before RSP consumption was not associated with decreased breath H_2 or fecal butyrate (Supplemental Fig. 3.4A-C).

3.5 Discussion

The *in vitro* results reported in this study revealed shifts in fermentation products of human gut butyrogens grown under a headspace containing 1 atm partial pressure of H_2. While this quantity of H_2 is not found in intestinal gas, the H_2 concentration relevant for microbial physiology is not that of the gas above a microbial culture, but rather that of the H_2 dissolved in the aqueous phase where microbes dwell [101, 113]. Studies in bioreactors have shown that H_2-producing microbial communities experience dissolved H_2 concentrations many times greater than equilibrium with the headspace, with reports indictating 3- to 100-fold overconcentrations in various conditions and bioreactor designs [113–116]. Since H_2 in intestinal gas (analogous to bioreactor headspace gas) ranges from <1% to >40% (v/v) with a median of approximately 15% (Supplemental Fig. 3.1) [72, 73], it is likely that dissolved H_2 in the human colon ranges above and below that produced by equilibration with 1 atm H_2. Therefore, the metabolic shifts induced in human gut butyrogens by the 1 atm H_2 headspace used in our *in vitro* cultures could also occur *in vivo*. Observations from a human cohort were consistent with this hypothesis (Fig. 3.3D-F).

Much of the H_2 produced in the human gut is consumed *in situ* by hydrogenotrophic microbes [100, 101]. Accordingly, in this study we investigated the consumption of H_2 by gut methanogens, reasoning that active methanogenesis must at some level lead to a reduction in dissolved H_2. The fact that nearly all CH_4 in the human gut is produced by the single culturable species, *Methanobrevibacter smithii* [112, 117], allowed us to use a simple, *in vitro* synthetic gut community to model the influence of methanogenesis on fermentation in the human gut.

Previous studies of the role of gut methanogenesis have often focused on aspects of human health and sometimes produce conflicting results [104, 118]. Explaining these inconsistencies, and distinguishing between correlation and causation, is difficult without mechanistically-founded expectations about the effect of H_2 removal on the gut microbiota [118]. In the current study, we hoped to obtain more interpretable results by first studying pure cultures of important gut microbes to test theoretical expectations (Fig. 3.2). Establishing the effects of H_2 concentration in this system allowed us to develop predictions for highly simplified synthetic gut communities in which H_2 was modulated by *M. smithii* (as in the gut) rather than by direct experimental manipulation of the headspace gas (Fig. 3.3A-C). Finding a methanogen-mediated decrease in butyrate production in this system in turn allowed us to understand the observation of lower fecal butyrate in methanogenic individuals (previously reported by Abell et al. (2009) [119] in a small cohort of eight individuals) as consistent with the predicted effect of gut methanogens rather than simply an intriguing association (Fig. 3.3D-F). Notably, the increase in lactate production observed in *E. rectale* under high H_2 likely also drives increased fecal butyrate given that lactate in the human colon appears to be rapidly fermented to SCFAs including butyrate [120–122]. Certain human gut butyrogens, notably *Anaerostipes caccae* and *Eubacterium hallii* appear to specialize in this route of butyrate production when lactate is available, while *R. intestinalis*, *E. rectale*, and *F. prausnitzii* have not been observed to significantly utilize lactate as a substrate [123, 124]. Our synthetic gut community included *A. caccae*, and so provided an *in vitro* model of the process.

A previous study also reported decreased butyate and increased acetate production by human gut butyrogens in the prevalent but low-abundance genus

Christensenella in *in vitro* co-culture with *M. smithii* [125]. The reported shift in fermentation was similar to our findings in *R. intestinalis*, indicating that the effects of H_2 concentration and methanogenesis we describe are common to other human gut butyrogens beyond the strains we investigated. Another study failed to find any influence of co-culture with *M. smithii* on *R. intestinalis* fermentation, and found that co-culture with the hydrogenotrophic acetogen *Blautia hydrogenotrophica* actually increased butyrate production [126]. However, these results were driven by acetate availability, as acetate was not provided in the culture medium and net acetate consumption is required for production of high levels of butyrate (Fig. 3.1). This likely does not reflect the environment of the human colon, where acetate is abundant [9]. The most direct evidence to date of *M. smithii* modulating fermentation *in vivo* does not involve a butyrogen, but rather the commonly-studied *Bacteroides thetaiotaomicron*. A study using gnotobiotic mice showed that *M. smithii* modulates *B. theta* fermentation products *in vivo*, increasing acetate and formate production at the expense of propionate, which the authors interpreted as due to consumption of H_2 and/or formate by *M. smithii* [127].

In the present study, simple *in vitro* experiments with single species allowed a more specific description of the influence of H_2 removal on human gut fermentation beyond the commonly repeated broad description of it as facilitating, enhancing, or improving the efficiency of human gut fermentation on the whole [104, 111, 128–131]. The principle that H_2 removal facilitates H_2-producing fermentation in the human gut is well-founded and accounts for the decrease in competitive fitness of the hydrogenogenic butyrogens *E. rectale* and (marginally) *R. intestinalis* in the synthetic community experiments reported here (Fig. 3.4), as well as the impairment of *E. rectale* growth rate and yield under very

high H_2 (Supplemental Table 3.2). However, this perspective obscures the fact that H_2 accumulation does not simply shut down fermentation in the human gut, as it does in other well-studied systems such as sewage digesters. There, endergonic oxidation of butyrate and propionate to acetate requires an intimate syntrophic association between fermenters and methanogens [68, 91]. Our findings show that unlike these obligate syntrophs, the predominant human gut butyrogens *E. rectale* and *R. intestinalis* [77, 95] can cope with elevated H_2 by disposing of reducing equivalents via butyrate and lactate instead. They do suffer some loss of metabolic efficiency, especially in the case of *E. rectale* which forgoes roughly half of the its ATP formation per glucose with its dramatic shift from butyrate and acetate production to lactate fermentation. However, they continue to grow using “backup” metabolic strategies. They are therefore examples of “facultative syntrophs” [69] for whom H_2 accumulation results in a fermentation shift rather than a fermentation arrest. Counterintuitively, high H_2 concentration actually *stimulates* production of the fermentation products butyrate and lactate in these organisms. As predicted by estimates of the Gibbs free energy of a range of fermentation balances (Fig. 3.1B), exposure to increasing concentrations of H_2 shifted fermentation products in a roughly dose-response fashion, showing that the shift is progressive and not governed by a fixed H_2 threshold (Supplemental Fig. 3,2).

The reduced fecal butyrate we report in methanogenic individuals only appears during consumption of an RSP supplement (Fig. 3.3F) and is not observed in the same individuals before supplement consumption (Supplemental Fig. 3.4A-C). The most likely explanation of this result is that RSP consumption is necessary in most individuals to stimulate sufficient production of H_2 in the colon to change the thermodynamic situation

if not efficiently removed. This possibility is supported by higher average H_2 during versus before RSP consumption ($p=0.005$). Another explanation, not mutually exclusive with the first, is based on the biogeography of methanogens in the human colon. A number of reports indicate that methanogens are more abundant in the distal colon and rectum rather than the proximal colon [103, 132–134]. As a refractive substrate, RSP may reach the distal colon in higher quantity than most other substrates in the diet before supplementation. Therefore, RSP fermentation could be more influenced by methanogens than fermentation of substrates that are mostly degraded before reaching the distal colon. Other guilds of human gut hydrogenotrophs–the sulfate reducers and reductive acetogens–may play a greater role in modulating fermentation of these substates. Further work should seek to include these guilds of hydrogenotrophs in our understanding of the role of H_2 in modulating fermentation in the gut microbiome.

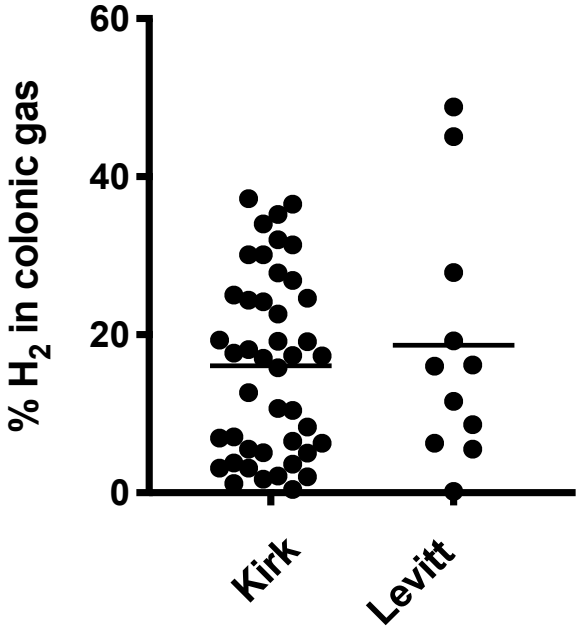
A final point of consideration is the negative association between active gut methanogenesis and successful stimulation of butyrate by RSP supplementation. While RSP supplementation generally increased fecal butyrate [17], methanogenic individuals showed no increase in fecal butyrate on average (Supplemental Fig. 3.4D). Although this is a correlative finding, this study provides a theoretical basis for a causal role of methanogenesis in decreasing butyrate production via efficient H_2 removal. Given the myriad positive effects of butyrate on colon health [10], consideration should be given to reducing methanogenesis (and perhaps hydrogenotrophy in general) during supplement interventions intended to stimulate butyrate production. An alternative approach would be to administer H_2 to stimulate butyrate production directly. A large body of research has studied H_2 administration for its apparent antioxidant and anticarcinogenic effects, often

via the consumption of water supersaturated with H_2 [135–137]. Our findings in this study raise the possibility that these treatments may also stimulate butyrate production in the gut microbiota, especially in combination with supplement interventions.

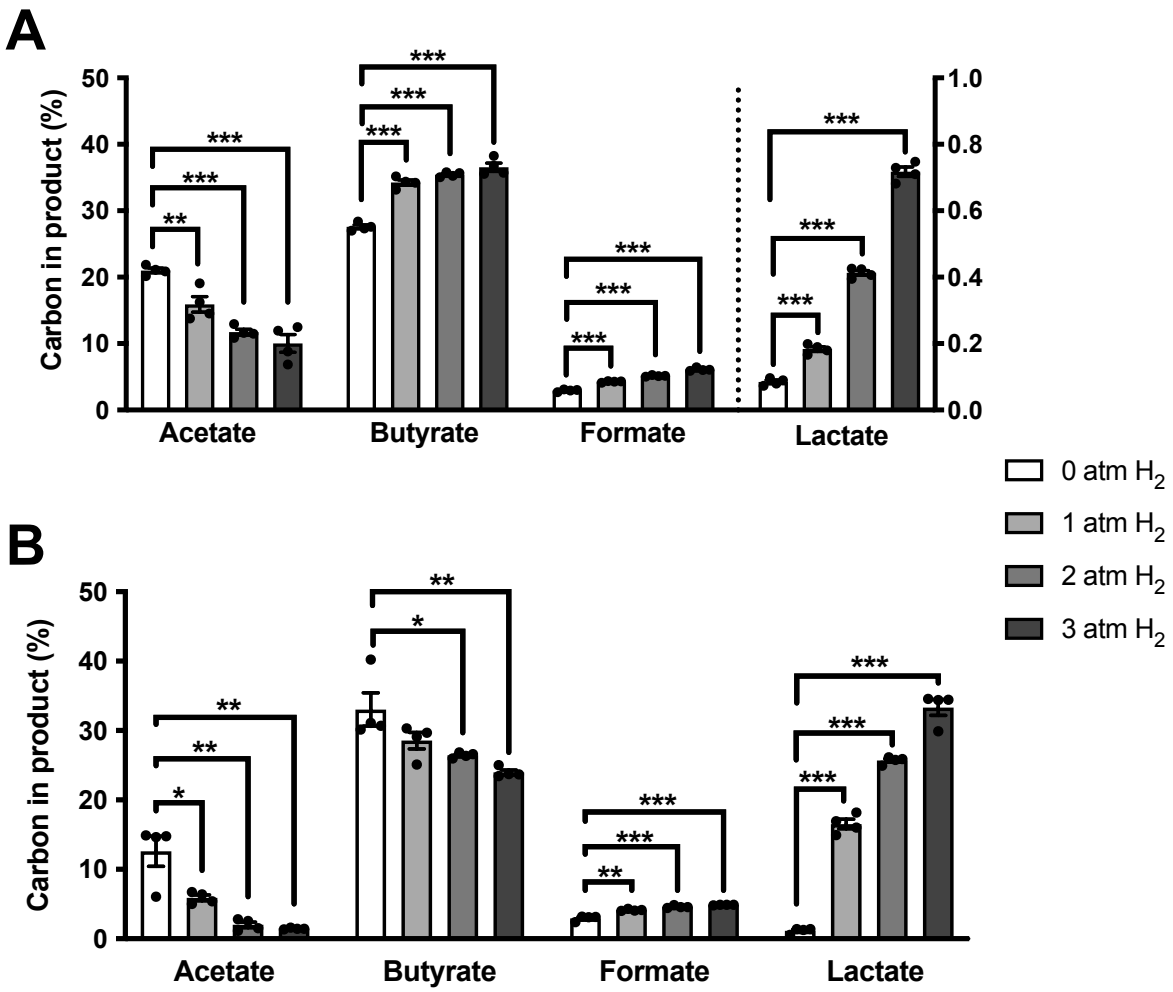
3.6 Conclusions

H_2 has often been proposed as an regulator of metabolic processes in the human gut microbiota [104], but concrete informating is lacking on its specific role in the complex gut ecosystem. In this study, we examined the effect of H_2 concentration on one prominent aspect of the human gut microbiota: production of the anti-inflammatory and anti-carcinogenic bacterial metabolite butyrate. Using *in vitro* approaches, we were able to observe the effect of H_2 on three prominent human gut butyrogens: *R. intestinalis*, *E. rectale*, and *F. prausnitzii*. We found that high H_2 concentration upregulated butyrate production by *R. intestinalis*, but not in *E. rectale*, which instead upregulated lactate production. *F. prausnitzii* was unaffected by H_2. We further found that H_2 consumption by the predominant gut methanogen *M. smithii* was sufficient to alter butyrate production by the H_2-regulated butyrogens. Findings from a large human cohort supported a model in which gut H_2 concentration, which is a balance between H_2 production by fermenting bacteria and H_2 consumption by methanogens, influences the total butyrate production by the gut microbiota.

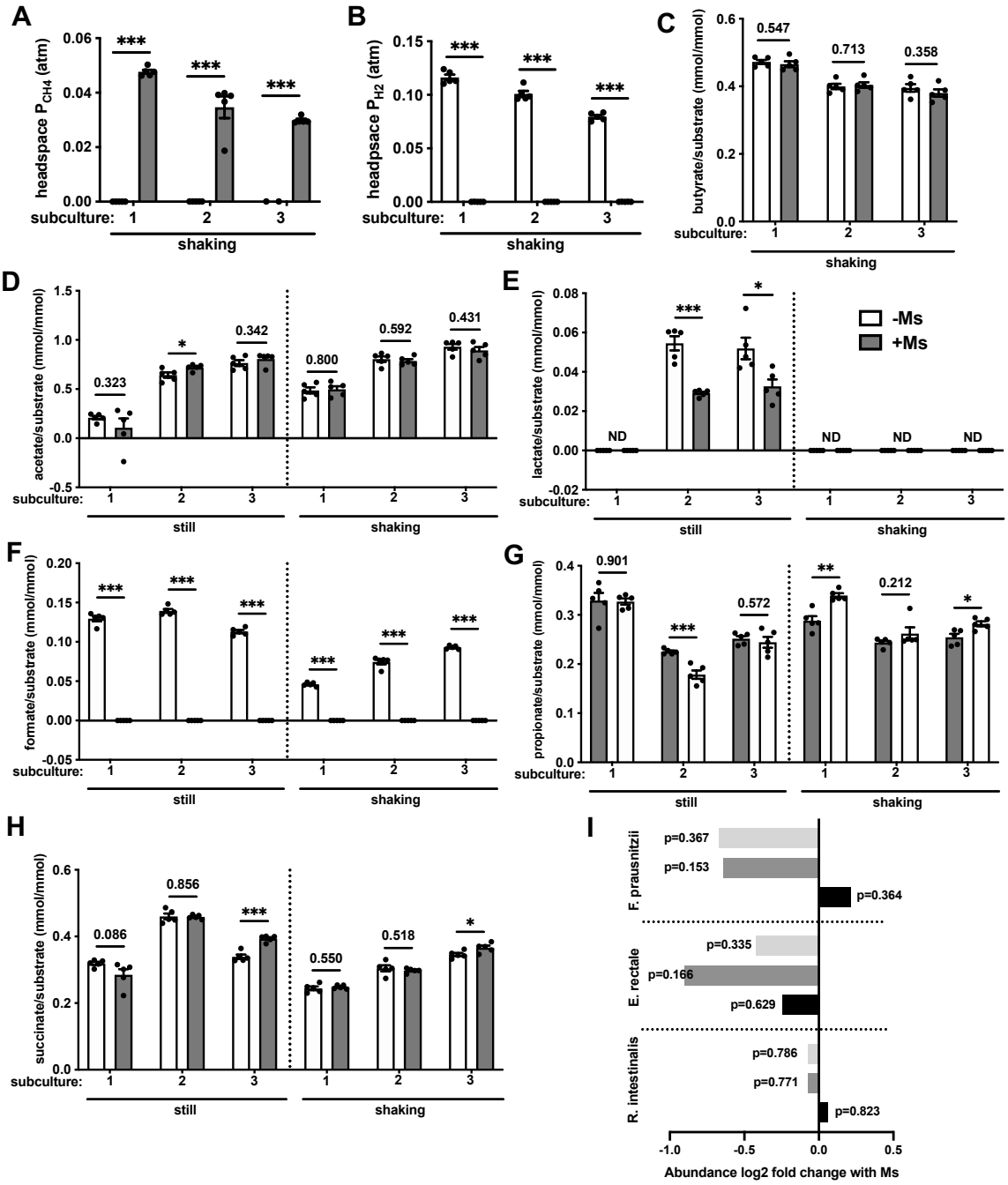
3.7 Supplemental Figures



Supplemental Figure 3.1. H_2 measured in human intestinal gas (v/v) by two different methodologies. Kirk (1949) [72] collected total flatus produced during a 10-hour period, while Levitt (1971) [73] washed out and collected the total gas content of the large and small intestines by infusion of argon into the jejunum.

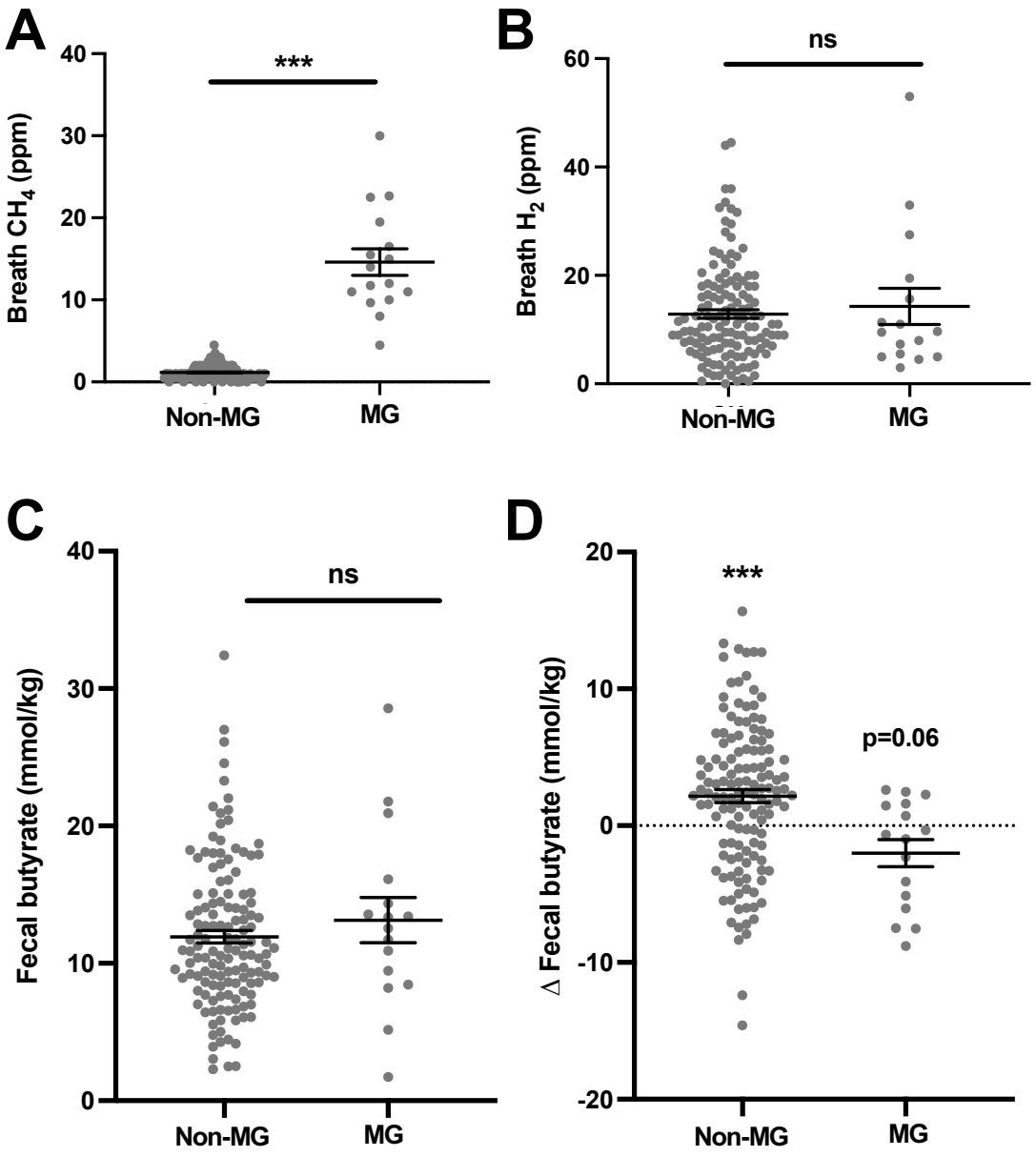


Supplemental Figure 3.2. Dose response of fermentation products of human gut butyrogens to headspace H_2.
Endpoint fermentation products in cultures of *R. intestinalis* (A) and *E. rectale* (B), grown in shaken cultures with increasing partial pressure of H_2 in the culture headspace. Total headspace pressure was 3 atm in all conditions, with N_2 used as the balance gas. Error bars indicate SEM. Statistical significance calculated using two-sided Student's two-sample t-tests (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).



Supplemental Figure 3.3. Influence of methanogenesis on fermentation and growth of a synthetic gut community with variable agitation.

Production of methane (A), H_2 (B), butyrate (C), acetate (D), lactate (E), formate (G), and succinate (H) by a 9-species synthetic human gut community grown with (shaded bars) and without (open bars) the addition of *M. smithii*. Cultures incubated on an orbital shaker at 150 rpm; production in still cultures is also shown for all products except butyrate. Note that formate is consumed by *M* smithii, accounting for its absence from +Ms cultures. Products were measured in three successive subcultures, with a new inoculum of *M. smithii* added at each passage. Error bars indicate SEM. Statistical significance calculated using two-sided Student's two-sample t-tests (* = p<0.05; ** = p<0.01; *** = p<0.001). Fold change in relative abundance of *F. prausnitzii*, *E. rectale*, and *R. intestinalis* in shaking synthetic community cultures (I). Statistical significance calculated from relative abundance values using two-sided Student's two-sample t-tests.



Supplemental Figure 3.4. Influence of methanogenesis on breath H_2 and fecal butyrate before consumption of an RSP supplement.
In a human cohort before consumption of an RSP supplement, breath and fecal samples were used to determine weekly average breath CH_4 (A), breath H_2 (B), and fecal butyrate (C) in individuals with and without active gut methanogenesis. Fecal butyrate measurements taken during subsequent consumption of RSP were used to calculate the change in fecal butyrate associated with RSP supplementation (D). Error bars indicate SEM. Statistical significance calculated using two-sided Student's two-sample t-tests in A-C and one-sided t-tests for difference from a mean of zero in D (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

SAB4 base medium

Component	Quantity per L medium
Bacto tryptone	10 g
Bacto yeast extract	2.5 g
Sodium acetate	2.7 g
MOPS	20.9 g
L-cysteine	1 g
K_2HPO_4	0.5 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	0.8 g
KCl	50 mg
$CaCl_2.2H_2O$	30 mg
NaCl	1.5 g
NH_4Cl	1 g
EDTA	5 mg
$NiCl_2.6H_2O$	0.7 mg
$FeSO_4.7H_2O$	1 mg
$MnSO_4.H_2O$	5 mg
$ZnSO_4.7H_2O$	1.8 mg
$CuSO_4.5H_2O$	0.1 mg
$CoCl_2.6H_2O$	1.8 mg
$Na_2MoO_4.2H_2O$	0.1 mg
$Na_2SeO_3.5H_2O$	0.1 mg
$Na_2WO_4.2H_2O$	0.2 mg
Biotin	40 µg
Folic acid	40 µg
Pyridoxine HCl	0.2 mg
Thiamine HCl	0.1 mg
Riboflavin	0.1 mg
Nicotinic acid	0.1 mg
D-Pantothenic acid hemi-	0.1 mg
p-Aminobenzoic acid (PA	0.1 mg
Lipoic acid (thioctic acid)	0.1 mg
Vitamin B12	2 µg
2-merchaptoethanesulfo	0.2 mg
Resazurin	1 mg
Hemin	10 mg
Valeric acid	101 mg
Isovaleric acid	102 mg
Isobutyric acid	88 mg

SABU agar

Component	Quantity per L medium
Bacto tryptone	10 g
Bacto yeast extract	2.5 g
Sodium acetate	2.75 g
Sodium bicarbonate	3.75 g
Cysteine HCl	4 g
Fructose	2 g
Soluble starch	2 g
Cellobiose	2 g
Sodium taurocholate	1 g
K_2HPO_4	0.5 g
KH_2PO_4	0.5 g
$MgSO_4.7H_2O$	0.8 g
KCl	50 mg
$CaCl_2.2H_2O$	30 mg
NaCl	1.5 g
NH_4Cl	1 g
EDTA	5 mg
$NiCl_2.6H_2O$	0.7 mg
$FeSO_4.7H_2O$	1 mg
$MnSO_4.H_2O$	5 mg
$ZnSO_4.7H_2O$	1.8 mg
$CuSO_4.5H_2O$	0.1 mg
$CoCl_2.6H_2O$	1.8 mg
$Na_2MoO_4.2H_2O$	0.1 mg
$Na_2SeO_3.5H_2O$	0.1 mg
$Na_2WO_4.2H_2O$	0.2 mg
Biotin	40 µg
Folic acid	40 µg
Pyridoxine HCl	0.2 mg
Thiamine HCl	0.1 mg
Riboflavin	0.1 mg
Nicotinic acid	0.1 mg
D-Pantothenic acid hemi-	0.1 mg
p-Aminobenzoic acid (PA	0.1 mg
Lipoic acid (thioctic acid)	0.1 mg
Vitamin B12	2 µg
Resazurin	1 mg
Hemin	10 mg
Valeric acid	10.2 mg
Isovaleric acid	10.2 mg
Isobutyric acid	10.7 mg

Supplemental Table 3.1. Components of SAB4 base medium.
Media was prepared on a magnetic stir plate and sterilized by autoclaving or passage through a 0.22 µm filter.

Supplementary Table 1		3 atm N2	3 atm H2
Generation time (min)	*F. prausnitzii*	237.0 (11.7)	240.2 (11.5)
	E. rectale	**66.8 (5.9)*** **	**93.9 (6.4)*** **
	R. intestinalis	72.1 (9.6)	78.8 (8.3)
Protein biomass yield (mg)	*F. prausnitzii*	0.544 (0.063)	0.538 (0.041)
	E. rectale	**0.225 (0.016)***	**0.204 (0.003)***
	R. intestinalis	0.247 (0.001)	0.259 (0.015)

Supplemental Table 3.2. Influence of H_2 on growth rate and yield of human gut butyrogens.
Generation time in exponential phase and yield (quantified by total protein) of the common human gut butyrogens *F. prausnitzii*, *E. rectale*, and *R. intestinalis* grown under a headspace containing 3 atm of either N2 or H2. Asterisks and bold indicate a significant difference under H2 versus N2 (Student's t-test, $p<0.05$). Standard deviation is shown in parentheses. Four replicate cultures of
F. prausnitzii and *E. rectale* were present in each condition; five replicate cultures of R. intestinalis were present in each condition.

Chapter 4: The Effect of Dietary Fiber and Candidate Prebiotic Supplements on Fasting Breath H_2

4.1 Abstract

Fermentation of carbohydrates in the human gut microbiota produces hydrogen gas (H_2) that is excreted in expired breath. In light of findings suggesting that high intestinal H_2 concentration shifts gut fermentation towards butyrate, H_2 production by prebiotic supplements may be considered an end in itself. We examined the effect of daily dietary fiber intake and six commercially available candidate prebiotics on fasting breath H_2 in large human cohorts. Inulin was found to produce the largest increase in fasting breath H_2, while resistant starch from potato (RSP) caused a smaller increase. This suggests the possibility of using inulin as a “co-prebiotic” to maximize butyrate production from RSP fermentation via high intestinal H_2.

4.2 Introduction

The H_2 and CH_4 breath tests, now almost always combined into a single test, were pioneered around 1970 [138–140]. The breath test is based on two facts. First, bacterial fermentation in the colon produces H_2 while archaeal methanogenesis produces CH_4. Microbial activity represent the only source of these gases in the human body, as they are not produced by any human metabolism [141]. Second, gases present at higher concentrations in the intestinal lumen than in the host tissue diffuse into the bloodstream and are excreted into the atmosphere (where the concentration is lower still) during gas

exchange in the lungs [142]. Taken together, these facts mean that simply measuring the concentration of H_2 and CH_4 in expired breath gives real-time information about microbial fermentation and methanogenesis in the gut.

Since its invention, the breath test has found common use in clinical gastroenterology [143, 144] to measure orocecal transit time [145, 146], diagnose small intestinal bacterial overgrowth (SIBO) [147], and to determine when a swallowed carbohydrate is not digested and absorbed before reaching the colon, a condition known to gastroenterologists as malabsorption [148, 149]. Beyond its clinical applications, breath testing has been used in academic study of the human gut microbiome for its ability to provide information about the activity of the gut microbes [118, 150–152].

It has long been noted that consumption of foods high in dietary fiber can lead to elevated breath H_2 the next day [153–155]. For this reason, carbohydrate-free diets are recommended for 15 hours before a clinical breath test, ideally with no high-fiber foods consumed in the whole day before the test, in order to obtain the uncontaminated, easily interpretable results [153]. In the research reported here, we asked the question of whether this effect could be approached from the other direction. That is, can the "contaminating" breath H_2 detected long after fiber consumption give an indication of the amount of fiber in the diet? In addition, can fasting breath H_2 measured the day after consuming a supplement give an indication of that supplement's prebiotic content?

There is much precedent to using the H_2 breath test to explore food properties instead of human physiology or microbial activity. It is often used to explore digestibility of a refined carbohydrate product and less frequently to determine the resistant carbohydrate portion of whole foods or meals [156, 157]. The novelty of the present study

is twofold. First, we investigate fasting breath H_2 as an indicator of dietary fiber intake in general, not of any particular food product. Second, when evaluating prebiotic supplements, we explore the use of a single sample of fasting breath H_2 rather than a lengthy and labor-intensive time course running nine or more hours from supplement consumption [156, 157]. Obtaining usable results with this protocol would allow evaluation of candidate prebiotics in a far larger number of individuals, an important consideration given the infamous inter-individual variability of the gut microbiome.

The final motivation for the prebiotic studies reported here was the finding reported in Chapter 3 of this thesis that an elevated H_2 concentration shifts the fermentation profile of human gut butyrogens towards butyrate [152]. As discussed in Chapter 2 of this thesis, RS is a promising prebiotic to target butyrate production. However, it is also a relatively poor substrate for H_2 production in the colon, resulting in the excretion of only a fraction as much H_2 as inulin per gram [158]. In light of this, we hypothesize that a prebiotic that fuels abundant H_2 production could be co-administered with RSP to boost its butyrogenic potential. We hope that the studies reported here will help select an optimal prebiotic for this this novel application.

4.3 Methods

4.3.1 Human cohort

Participants were recruited through the University of Michigan BIO173 introductory biology course.

4.3.2 Dietary logs

Dietary logs were recorded using the MyFitnessPal app (MyFitnessPal, Inc). Dietary logs were completed for all food and drink the day before collecting a fasting breath sample for three consecutive days both before and after the *ad hoc* dietary fiber increase.

4.3.3 Supplements

The supplements used were Bob's Red Mill potato starch (Bob's Red Mill Natural Foods, Milwaukie, OR), Prebiotic Fiber Supplement pouches containing NUTRAFLORA FB P-95 (GoBiotix), Fiber One Oats & Chocolate bars (BJ's Wholesale Club, Inc), Fiberful granola bars of the rolled oats & chocolate chips flavor (Trader Joe's), Supergut resistant starch bars (Supergut), and Chex Mix snack bars of the birthday cake flavor (General Mills).

4.3.4 Sample collection and measurement

To collect breath samples, participants inhaled normally, held their breath for five seconds, then exhaled normally through a plastic drinking straw fully inserted into a round-bottom glass tube. The straw was then withdrawn and the tube immediately sealed using a screw cap fitted with a rubber septum.

Samples were extracted from the sealed tubes using an AlveoVac extraction system connected to a QuinTron BreathTracker SC analyzer used to measure breath gas concentrations (both from QuinTron Instrument Company Inc., Milwaukee, WI). Breath samples with a CO_2 correction reported as "Too High" or greater than 4% CO_2 were excluded from analysis. For each and breath gas, samples with values lying more than three interquartile ranges below the lower quartile or above the upper quartile were excluded from analysis according to the method of Tukey's Fences [105].

4.3.5 Data analysis and statistics

Data were collated, organized, and analyzed using R. Final statistical analyses were carried out using Graphpad Prism 9 (GraphPad Software, San Diego, CA).

4.4 Results

In order to assess the effect of everyday dietary fiber on fasting breath H_2, 422 participants created daily dietary log using the MyFitnessPal app, then collected fasting breath samples the next morning. This procedure was repeated three times. The participants then repeated the procedure three additional times while making *ad hoc* dietary adjustments to attempt to increase their dietary fiber intake. Pearson correlation tests between breath H_2 and CH_4 concentrations and prior day dietary components were then performed, with each diet log–breath sample pair treated as a separate data point and no distinction between samples taken before and after the *ad hoc* dietary adjustment (Fig. 4.1A, Supplemental Fig. 4.1A). After correcting for multiple comparisons, the only significant correlate of fasting breath H_2 was dietary fiber ($R = 0.093$, $p<0.05$) (Fig. 4.1A). Unexpectedly, fasting breath CH_4 was found to have a significant inverse correlation with vitamin C ($R = -0.084$, $p<0.05$) (Supplemental Fig. 4.1A).

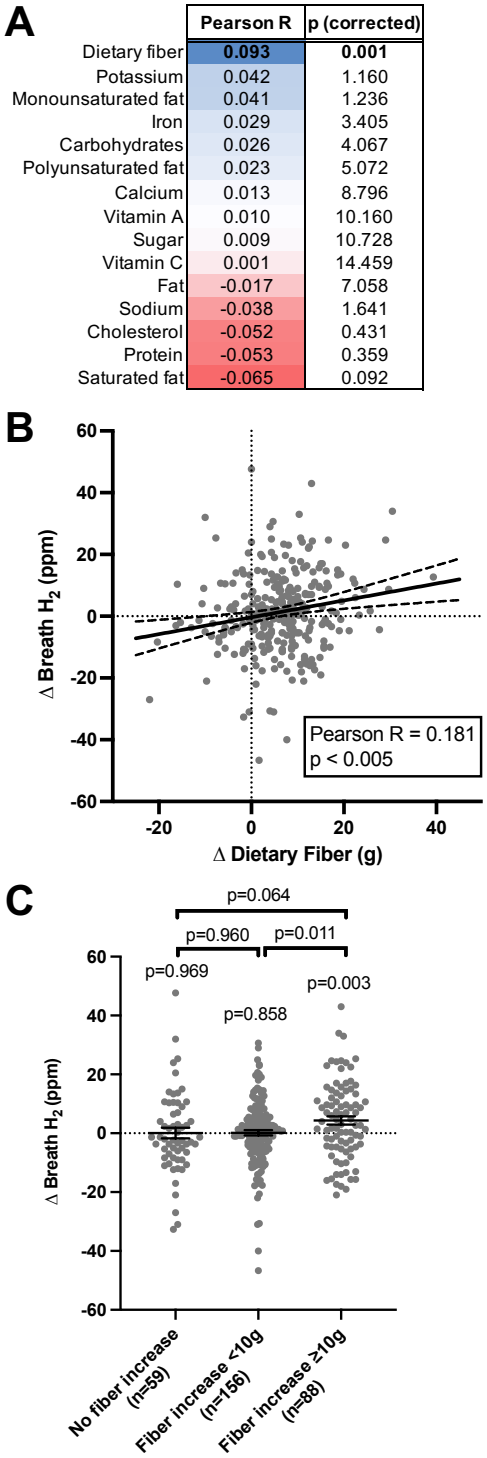


Figure 4.1. Dietary fiber is associated with fasting breath H2 during self-directed diet and ad hoc fiber increase.
(A) Pearson correlations of prior day dietary components with fasting breath H2. P-values were corrected for multiple comparisons using the Bonferroni method. (B) Ad hoc increase in average dietary fiber consumption versus change in average next day fasting breath H2. Linear regression shown with 95% CI. (C) Changes in average next day fasting breath H2 for different classes of *ad hoc* dietary fiber increase. P-values shown over a single condition represent paired t-tests of H2 change, while comparisons between columns represent Student's two-sample t-tests. Error bars show SEM.

The impact of the *ad hoc* dietary fiber increase on fasting breath H_2 was determined by first calculating for each individual the change in average dietary fiber from the three days before the adjustment to the three days after. These changes were then compared to the changes in average fasting breath H_2 over the same period using a Pearson correlation test. A relatively weak but significant correlation was found between the increase (or decrease) in dietary fiber intake and the increase in fasting breath H_2 ($R = 0.181$, $p<0.005$) (Fig. 4.1B). No such correlation existed between dietary fiber and fasting breath CH_4 (Supplemental Fig. 4.1B). Individuals were then classified into three groups based on their dietary adjustment: those that did not record a successful increase in average dietary fiber intake, those that increased intake by <10 g, and those that increased intake by ≥10 g. Average fasting breath H_2 only increased significantly in the group that added ≥10 g average daily dietary fiber to their diet (Fig. 4.1C). Somewhat surprisingly, there was no trend, however slight, towards increased fasting breath H_2 in the group that added <10 g of daily dietary fiber.

Following this initial investigation, subsequent studies using large human cohorts were carried out to quantify the effect of prebiotic fiber supplements on next-day fasting breath H_2. In each of these studies, participants were instructed to collect two fasting breath samples in the morning to establish baseline breath H_2 and CH_4 concentrations. Participants then consumed a supplement at a time not more than 12 hours before the intended breath sample, slept, and collected a fasting breath sample in the morning. This procedure was repeated once more, not necessarily on the following day. In each study,

roughly half the participants consumed no supplement before the latter two breath samples in order to serve as controls.

Supplement studies were conducted with resistant starch from potato (RSP), GoBiotix Prebiotic Fiber Supplement pouches (GBX), Fiber One Oats & Chocolate bars (F1), Trader Joe's Fiberful granola bars (rolled oats & chocolate chips flavor) (FF), Supergut bars (SG), and Chex Mix snack bars (birthday cake flavor) (CM). For many of the supplement bars, only one half was consumed (indicated as "0.5"). The amount of fiber in the supplements varied from none (CM) to 9 g (RSP, F1). Types of fiber included resistant starch (RSP, Supergut, likely smaller amounts in other bars not specifically added), inulin (F1 in the form of chicory root extract), oat β-glucans (Supergut), and fructooligosaccharides (GBX, FF). The major dietary components of these supplements as reported on their labels at the doses they were consumed are shown in Fig. 4.2A.

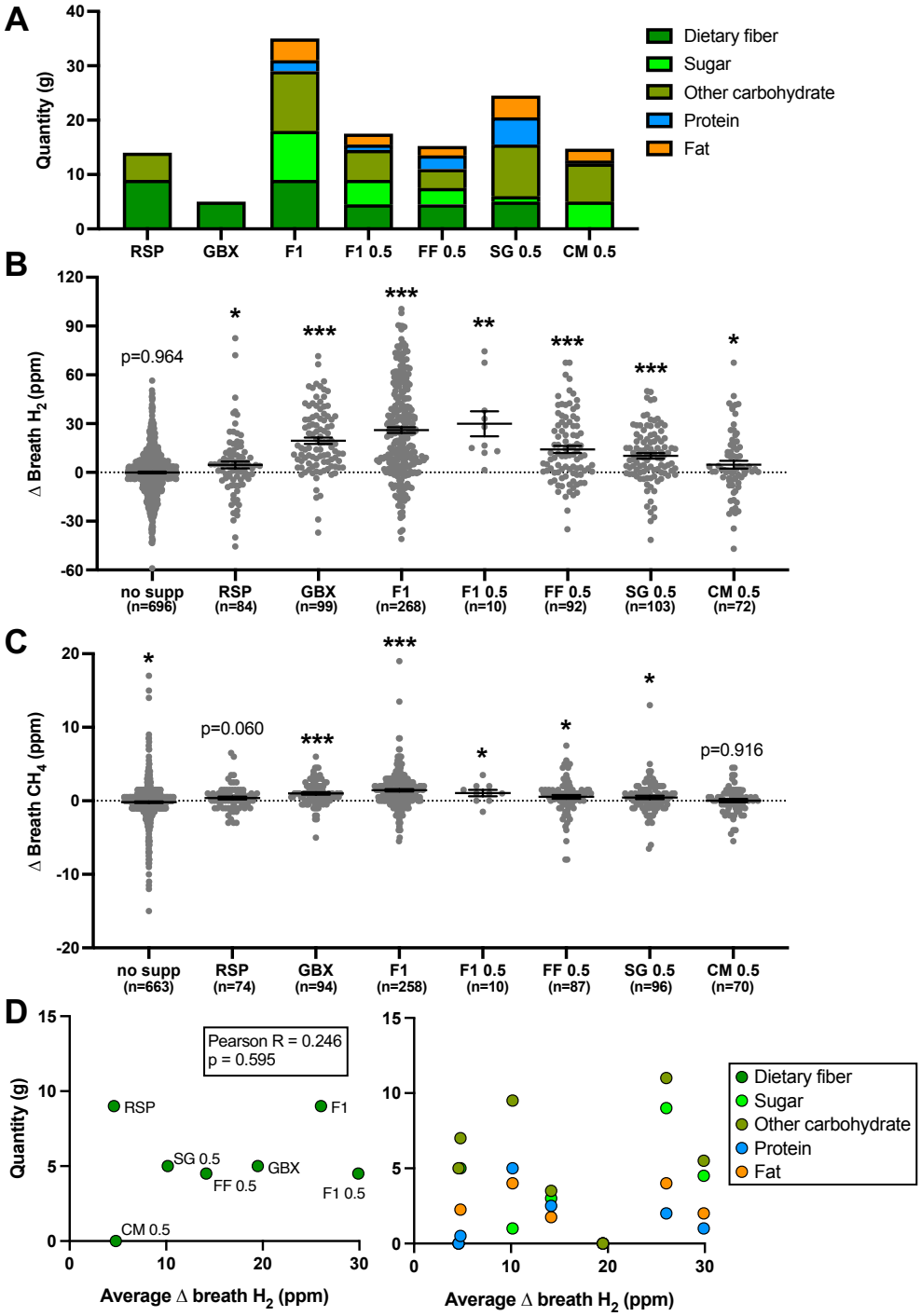


Figure 4.2. Effect of dietary fiber supplementation on next day fasting breath H2 and CH4.
(A) Major dietary components of supplements consumed by a human cohort: purified resistant starch from potato (RSP), GoBiotix fructooligosaccharide powder (GBX), FiberOne bar (F1), ½ FiberOne bar (F1 0.5), ½ Fiberful bar (FF 0.5), ½ SuperGut bar (SG 0.5), and ½ Chex Mix bar (CM 0.5). (B-C) Change relative to baseline of average fasting breath H_2 or CH_4 following prior day consumption of indicated supplement. Each point represents one individual. P-vales represent paired t-tests of fasting breath gases at baseline and following supplementation (* = p<0.05; ** = p<0.01; *** = p<0.001). (D) Dietary components of supplements versus average change relative to baseline of fasting breath H_2 following prior day supplement consumption. Dietary fiber is shown alone of left panel with labeled supplements for clarity. All other components are shown on right panel.

All of the supplements resulted in a significant average increase in next-day fasting breath H_2, although the increases seen with RSP and CM 0.5 were small (Fig. 4.2B). The largest average fasting breath H_2 increase was seen with F1, with GBX also producing a large increase. Both F1 and GBX consumption more than doubled next-day fasting breath H_2 (Supplemental Fig. 4.2A). Surprisingly, F1 0.5 caused just as large an increase in fasting breath H_2 as F1, although confidence in this result is limited by the relatively small number of participants consuming it (n=10) (Fig 4.2B). Interestingly, the effect of the supplements on fasting breath H_2 did not appear to correlate with either their fiber content or any other of their dietary components (Fig. 4.2D). F1 and GBX consumption also led to the largest increases in average fasting breath CH_4 (Fig. 4.2C). These increases were significant but relatively much smaller than those observed with H_2, even in relative terms (Supplemental Fig. 4.2B). Fasting CH_4 increases observed with other supplements were of marginal significance and very small effect size.

Conventional time courses of breath gases were not obtained, as participants only took one breath sample after consumption of each supplement dose. However, recording the times of supplement consumption and breath sampling allowed crude average time courses to be constructed using breath samples from multiple individuals taken at varying times after supplement consumption (Fig. 4.3A-B). Due to the nature of the experimental design and participant instructions, most breath samples were taken between 8-12 hours post supplementation (hps), limiting the length of the time courses that could be constructed. Times were rounded to the nearest hour and only hours with five or more

samples were included in the time courses displayed. Average H_2 and CH_4 concentrations at 8 and 12 hps were compared using two-sample Student's t-tests, but these tests were not always informative and consistent statistical analysis of the results was difficult in general.

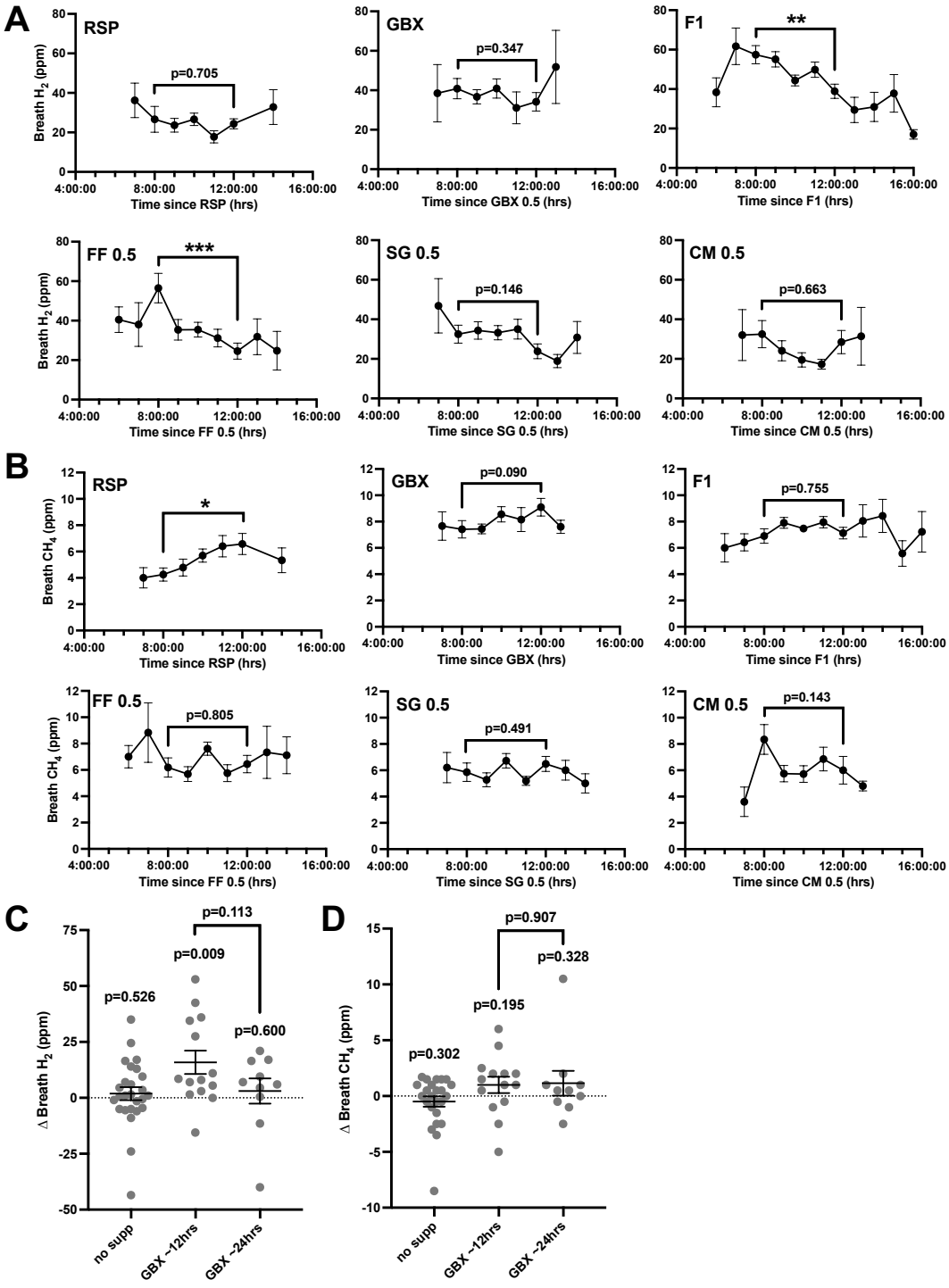


Figure 4.3. Time courses of fasting breath gases following supplement consumption.
(A-B) Fasting breath H_2 (A) or CH_4 (B) for each supplement measured the specified time after supplement consumption. Samples were rounded to and averaged together at the nearest hour. Only hours with >4 measurements are shown. Error bars indicate SEM. P-values were obtained from two sample Student's t-tests comparing average breath gases at 8 hrs to 12 hrs (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$). (C-D) Fasting breath H_2 (C) or CH_4 (D) measured approximately 12 or 24 hrs after consumption of GBX. Each point represents one individual. P-values shown over a single condition represent paired t-tests of H_2 change, while comparisons between columns represent Student's two-sample t-tests. Error bars indicate SEM.

Of the fasting H_2 time courses, only F1 showed the clearest decline in breath H_2 over time, which lasted from 7 to 13 hps (Fig. 4.3A). FF 0.5 also showed a decline over the same time frame, while SG 0.5, CM 0.5, and RSP may have shown declines, but were difficult to discern. GBX showed no sign of decline. No consistent trends of H_2 increase over the time frame were apparent for any of the supplements (Fig. 4.3A). CH_4 time courses showed no trends in general, including for F1, which had displayed the largest increase in fasting breath CH_4 (Fig. 4.3B). One very notable exception was RSP, which displayed a very smooth curve of increasing CH_4 from 7-12 hps. Variability of the data in this time course was also particularly low compared to other supplements and even the fasting H_2 measured in the same samples.

In order to obtain more rigorous and interpretable temporal data, a small study with GBX was carried out in which participants consumed the supplements either 12 or 24 hrs before collecting breath samples. There were no restrictions on other foods consumed after the supplement as long as the breath sample was taken after a fast. A significant increase in average fasting breath H_2 was only observed at 12 hps, while H_2 at 24 hps and CH_4 at both time points were not significantly elevated (Fig. 4.3C-D).

A final and unexpected result was a moderately strong rank correlation between fasting breath H_2 and CH_4 during consumption of all supplements and at baseline in most cases as well (Fig. 4.4A). This consistent statistical relationship between the only two variables measured in this study confounded attempts to explore other relationships between them. Differences in the strength of the correlation appeared intriguing. For example, GBX and FF 0.5, the two fructooligosaccharide-containing supplements, were

also the only two supplements to show clearly stronger rank correlations after supplement consumption compared to baseline. It is tempting to interpret these patterns, but the highly derived nature of the result precludes proper statistical analysis. In addition, large differences in correlation strength between baseline measurements of the different supplements is a cause for concern, as these should all be the same. Subsampling from the very large control group showed that this sort of variability in rank correlation was to be expected as a result of chance at the sample sizes present in the supplement groups. Nonetheless, fasting breath H_2 versus CH_4 at baseline and after prior-day supplementation are shown for the most "interesting" supplements—RSP, GBX, and FF 0.5 (Fig. 4.4B-D). If nothing else, these plots provide visualizations of the consistent H_2-CH_4 rank correlation.

A

H_2 vs. CH_4 Spearman R	Baseline	Supp
No supp	**0.237**	**0.252**
RSP	**0.42**	**0.271**
GBX	0.187	**0.245**
F1	**0.254**	**0.286**
FF 0.5	0.183	**0.388**
SG 0.5	**0.26**	**0.304**
CM 0.5	**0.474**	**0.414**

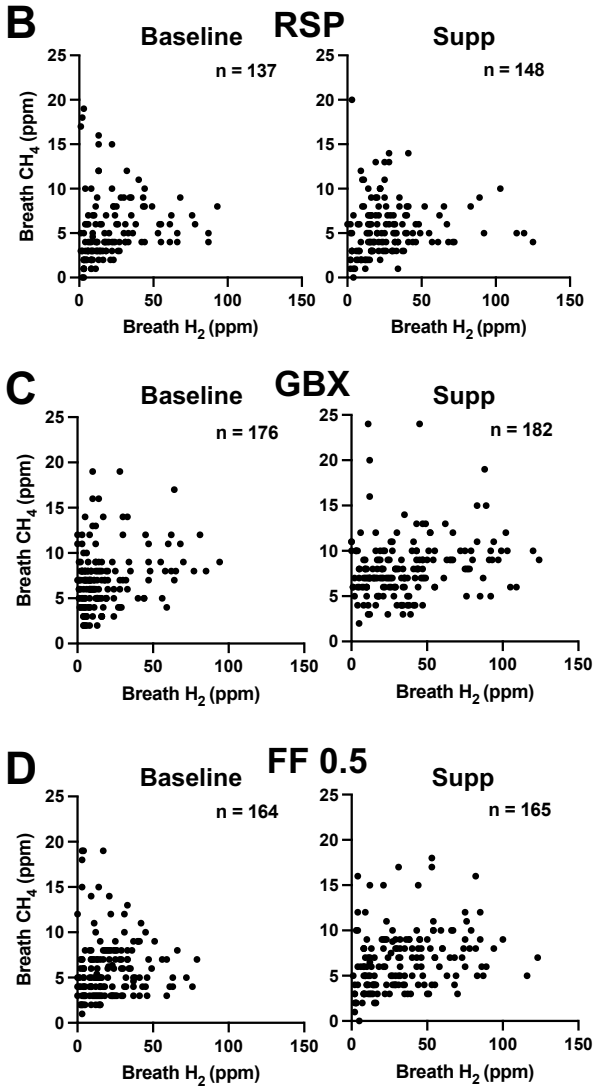


Figure 4.4. Dietary fiber supplements differentially alter the relationship of fasting breath H_2 and CH_4.

(A) Spearman correlations of fasting breath H_2 with fasting breath CH_4 at baseline and following prior- day consumption of each supplement. Bolded values indicate statistical significance ($p<0.05$, Bonferroni correction). (B-D) Fasting breath H_2 vs. CH_4 at baseline and following prior-day consumption of RSP (B), GBX (C), and FF 0.5 (D). Each point represents one individual.

4.5 Discussion

The results obtained in this study confirm that RSP is a relatively poor prebiotic for stimulating H_2 production compared to inulin (F1) or fructooligosaccharies (GBX, FF), in line with past findings [100, 158]. The dominance of Ba in RSP degradation described in Chapter 2 of this thesis and in recent literature may account for this[58], as Ba metabolism—in contrast to many other carbohydrate fermenters of the gut microbiome—does not produce any H_2 [53]. This observation leads toward the strategy of optimizing butyrate yield from RSP through co-administration with a highly hydrogenogenic prebiotic intended to generate high colonic H_2 and thereby shift fermentation towards butyrate [152]. Of the prebiotic candidates investigated, inulin in the form of F1 bars appears to be the most promising candidate for this application based on exceptionally large increase in fasting breath H_2 that followed its consumption (Fig. 4.2B). In addition, time course data indicate that the H_2 production it stimulates peaks around 8 hps, in which case a simple thrice-daily dosing schedule would be suitable to keep intestinal H_2 high at all times (Fig. 4.3A).

Additional study is needed to ensure that an RSP-inulin combination prebiotic would not result in uncomfortable bloating due to excessive gas production, a known risk of inulin supplementation [159]. These symptoms often appear when daily intake of inulin supplement is over 10 g [160, 161]. This limit would prevent the consumption of more than one F1 bar per day (Fig. 3.2A). Fortunately, the fact that halving the F1 dose did not reduce the next-day breath H_2 whatsoever suggests that a full bar is not necessary to

achieve high levels of intestinal H_2 (Fig. 4.3A). Future research investigating F1 tolerance could also determine the minimum dose that saturates fasting breath H_2.

One of the goals of this study was to assess fasting breath H_2 as an indicator of dietary fiber intake. While we did find a clear positive and significant relationship between dietary fiber and fasting breath H_2, the variability in this relationship appears far too high to use fasting breath H_2 as an indicator of dietary fiber intake on an individual level (Fig. 4.1A-C). The observation that *ad hoc* dietary fiber increases below 10 additional g/day were ineffective in increasing fasting breath H_2 was surprising, especially given that most of the prebiotic supplements were highly effective despite none of their doses exceeding 9 g (Fig. 4.1C, Fig. 4.2A-B). One potential reason for this discrepancy is that nutritional dietary fiber includes non-fermentable substrates such as cellulose and lignin that are not accessible to either the host or the gut microbiota [162]. This finding emphasizes the potential benefits of refined prebiotics in addition to ordinary dietary fiber in stimulating the gut microbiota.

Although the synthesized average fasting breath H_2 time courses generated in this work were of poor resolution with high variability, they were generally in accord with previously described patterns of H_2 excretion in breath over the course of the day (Fig. 4.3A) [163, 164]. Breath H_2 is usually found to be relatively high in the morning before eating. It then decreases steadily until the early or late afternoon, at which point it begins to rise, apparently staying elevated until the next morning [163, 164]. This appears to represent delayed and somewhat overlapping responses to a small breakfast and lunch followed by a large dinner. Breath CH_4 has also been noted in the literature to be relatively constant throughout the day and even unaffected by fasting, which greatly decreases

breath H_2 [118, 163]. This is generally consistent with the flat CH_4 time courses we observed, with the notable exception of RSP (Fig. 4.3B).

On the whole, the concentrations of fasting breath H_2 that we measured in this study were high compared to those reported in the literature. The average baseline fasting breath H_2 among participants in all prebiotic groups was consistently 18-20 ppm, with a substantial tail reaching over 50 ppm (Supplemental Fig. 4.2A). In the literature, average fasting breath H_2 in healthy individuals is consistently reported at 5-6 ppm, with one study reporting <1% of individuals over 30 ppm [150, 155, 165, 166]. This discrepancy is likely due to most of our participants fasting for between 8-12 hrs instead of the >12 hrs widely used as best practice [150, 155, 165, 166]. Future studies using our protocol should seek to correct this deficiency if feasible to bring results into better conversation with existing literature on fasting breath H_2.

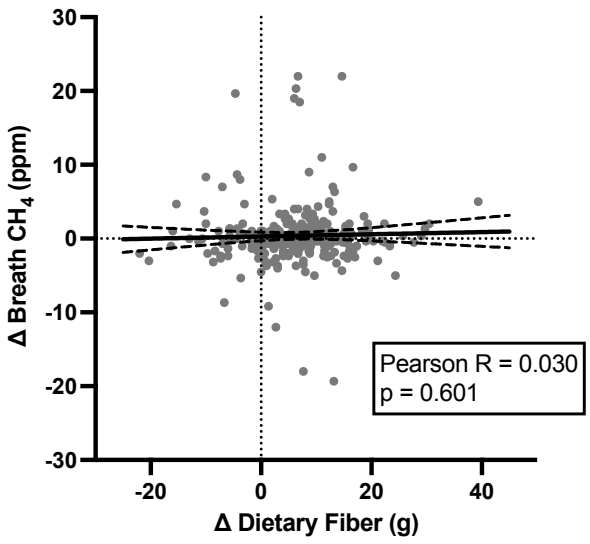
In sum, the results reported here demonstrate a clear positive relationship between dietary fiber intake and fasting breath H_2 (albeit too noisy to apply as a measure of fiber intake), confirm reports of anomalously low H_2 excretion from RSP prebiotics, and identify inulin in the form of F1 bars as a potential “co-prebiotic” targeted at shifting RSP fermentation towards butyrate by producing high concentrations of intestinal H_2. Future trials should seek to better elucidate the time course of H_2 production from F1 in order to design an optimal dosing schedule, as well as test the tolerability of RSP/inulin co-prebiotics at various doses. These are the next steps along the path to creating a more effective prebiotic supplement targeted at stimulating butyrate production by the gut microbiota.

4.6 Supplemental Figures

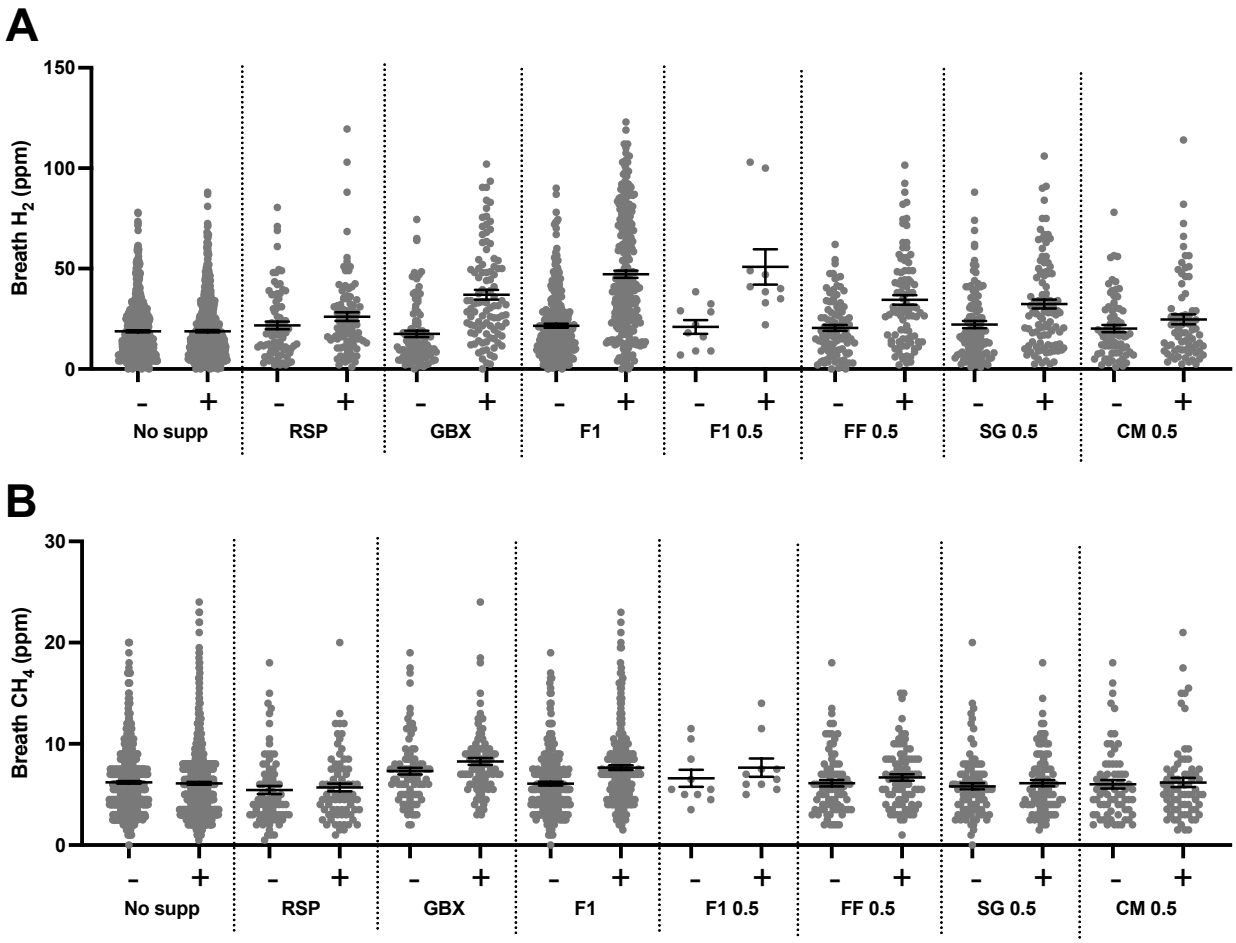
A

	Pearson R	p (corrected)
Carbohydrates	0.042	1.275
Sugar	0.022	5.507
Fat	0.006	11.966
Protein	0.001	14.447
Dietary fiber	-0.002	14.211
Iron	-0.007	11.820
Sodium	-0.015	8.141
Calcium	-0.021	6.105
Saturated fat	-0.029	3.503
Cholesterol	-0.039	1.676
Polyunsaturated fat	-0.046	0.917
Vitamin A	-0.052	0.571
Monounsaturated fat	-0.053	0.433
Potassium	-0.058	0.255
Vitamin C	**-0.084**	**0.011**

B



Supplemental Figure 4.1. Diet component correlates of fasting breath CH_4.
Pearson correlations of prior day dietary components with fasting breath CH_4. P-values were corrected for multiple comparisons using the Bonferroni method. (B) *Ad hoc* increase in average dietary fiber consumption versus change in average next day fasting breath H_2. Linear regression shown with 95% CI.



Supplemental Figure 4.2. Next day fasting breath concentrations of H_2 and CH_4 following consumption of a fiber supplement.
Average concentration of fasting breath H_2 (A) and CH_4 (B) at baseline (-) and following prior day consumption of indicated supplement (+). Each point represents one individual. Statistics are the same as indicated on Fig. 4.2B-C.

Chapter 5: Conclusions

The research described in this thesis made two major contributions to the understanding of fiber degradation and fermentation by the human gut microbiota, in addition to a number of less significant findings.

First and foremost, the role of H_2 in modulating fermentation by major gut butyrogens was thoroughly characterized using *in vitro* approaches and validated by checking the main prediction against observations from a large human cohort. By demonstrating the H_2 acts primarily to shift fermentation towards more reduced end products, the findings in this chapter corrected a common misapprehension in the literature that high concentrations of H_2 inhibit gut fermentation [104, 111, 128–131]. The reality, as least as far as butyrate production is concerned, appears to be the opposite. Characterizing the influence of H_2 on fermentation also led to the prediction that hydrogenotrophs, whose main metabolic activity consists of consuming H_2, would decrease butyrate production. In the case of the methanogen *M. smithii* this prediction was borne out *in vivo*.

These findings are made more relevant by the fact that intestinal H_2 varies over such a wide range between individuals, from undetectable to over 40% v/v [72, 73, 167]. Since H_2 is an easily quantifiable feature that so dramatically distinguishes individual

microbiomes, understanding its influence on the microbiota is particularly valuable in predicting inter-individual variability.

The second important contribution is the finding that the RSP-responsive butyrogens *E. rectale* (Er) and *F. prausnitzii* (Fp) occupy adjacent niches distinguished by the availability of accessible carbohydrate substrate. As a result, their competition to cross-feed from RSP degraders is mediated by whether the degrader releases large amounts of accessible saccharides, as in the case of *R. bromii* (Rb), or very few, as in the case of *B. adolescentis* (Ba). To the best of our knowledge, this is the first time that competition between two gut microbes has been found to be mediated by availability rather than the identity of a cross-fed substrate. Future investigations of cross-feeding should take this possibility into account when exploring cross-feeding specificity.

From a broader perspective, adaptation for high or low substrate is to be expected in a highly pulsed-substrate environment like the gut where active fermentation occurs in distinct waves between meals, as is apparent from daily patterns of breath H_2 excretion [163, 164, 168]. Notably, CH_4 excretion stays relatively constant even as H_2 excretion varies widely, suggesting that methanogens are not adapted to take advantage of high substrate availability as H_2-producing fermenters are, but are also relatively unaffected by low substrate availability [118, 163]. The colon is an environment of alternating feast and famine, and new insights may be gained by asking which of its residents are adapted to enjoy the feast like Er and which prefer to weather the famine like Fp.

The final notable aspect of the research reported here is how the findings from all three chapters can be marshalled together to provide guidance in maximizing butyrate response to RSP supplementation. Namely, individuals lacking detectable Ba and breath

methane should be targeted as the most likely to respond favorably and intestinal H_2 should be boosted by administration of an inulin “co-prebiotic” to shift fermentation towards butyrate. Although the how successful such an approach would be remains to be seen, the ability to make rational adjustments and recommendations like these to improve microbiota-targeted interventions is indicative of real progress towards improving therapeutics based on mechanistic understanding of the gut microbiota.

References

1. Metchnikoff I. The Wilde Medal and Lecture of the Manchester Literary and Philosophical Society. Br Med J. 1901;1:1027–8.
2. Morgan XC, Huttenhower C. Chapter 12: Human Microbiome Analysis. PLoS Comput Biol. 2012;8:e1002808.
3. Marchesi JR, Adams DH, Fava F, Hermes GDA, Hirschfield GM, Hold G, et al. The gut microbiota and host health: a new clinical frontier. Gut. 2016;65:330–9.
4. Groussin M, Mazel F, Alm EJ. Co-evolution and Co-speciation of Host-Gut Bacteria Systems. Cell Host Microbe. 2020;28:12–22.
5. O'Hara AM, Shanahan F. The gut flora as a forgotten organ. EMBO Rep. 2006;7:688–93.
6. Lemon KP, Armitage GC, Relman DA, Fischbach MA. Microbiota-Targeted Therapies: An Ecological Perspective. Sci Transl Med. 2012;4.
7. Koh A, De Vadder F, Kovatcheva-Datchary P, Backhed F. From Dietary Fiber to Host Physiology: Short-Chain Fatty Acids as Key Bacterial Metabolites. Cell. 2016;165:1332–45.
8. Topping DL, Clifton PM. Short-Chain Fatty Acids and Human Colonic Function: Roles of Resistant Starch and Nonstarch Polysaccharides. Physiol Rev. 2001;81:1031–64.
9. Cummings JH, Pomare EW, Branch WJ, Naylor CP, Macfarlane GT. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut. 1987;28:1221–7.
10. Hamer HM, Jonkers D, Venema K, Vanhourvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. Aliment Pharmacol Ther. 2008;27:104–19.
11. Macfarlane GT, Macfarlane S. Fermentation in the Human Large Intestine. J Clin Gastroenterol. 2011;45:S120–7.
12. Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol. 2017;14:491–502.
13. Bendiks ZA, Knudsen KEB, Keenan MJ, Marco ML. Conserved and variable responses of the gut microbiome to resistant starch type 2. Nutrition Research. 2020;77:12–28.
14. DeMartino P, Cockburn DW. Resistant starch: impact on the gut microbiome and health. Curr Opin Biotechnol. 2020;61:66–71.
15. Dobranowski PA, Stintzi A. Resistant starch, microbiome, and precision modulation. Gut Microbes. 2021;13.
16. Venkataraman A, Sieber JR, Schmidt AW, Waldron C, Theis KR, Schmidt TM. Variable responses of human microbiomes to dietary supplementation with resistant starch. Microbiome. 2016;4:33.

17. Baxter NT, Schmidt AW, Venkataraman A, Kim KS, Waldron C, Schmidt TM. Dynamics of Human Gut Microbiota and Short-Chain Fatty Acids in Response to Dietary Interventions with Three Fermentable Fibers. mBio. 2019;10.
18. Hald S, Schioldan AG, Moore ME, Dige A, Lærke HN, Agnholt J, et al. Effects of Arabinoxylan and Resistant Starch on Intestinal Microbiota and Short-Chain Fatty Acids in Subjects with Metabolic Syndrome: A Randomised Crossover Study. PLoS One. 2016;11:e0159223.
19. Maier T V., Lucio M, Lee LH, VerBerkmoes NC, Brislawn CJ, Bernhardt J, et al. Impact of Dietary Resistant Starch on the Human Gut Microbiome, Metaproteome, and Metabolome. mBio. 2017;8.
20. Cerqueira FM, Photenhauer AL, Pollet RM, Brown HA, Koropatkin NM. Starch Digestion by Gut Bacteria: Crowdsourcing for Carbs. Trends Microbiol. 2020;28:95–108.
21. Leitch ECM, Walker AW, Duncan SH, Holtrop G, Flint HJ. Selective colonization of insoluble substrates by human faecal bacteria. Environ Microbiol. 2007;9:667–79.
22. Kovatcheva-Datchary P, Egert M, Maathuis A, Rajilić-Stojanović M, de Graaf AA, Smidt H, et al. Linking phylogenetic identities of bacteria to starch fermentation in an *in vitro* model of the large intestine by RNA-based stable isotope probing. Environ Microbiol. 2009;11:914–26.
23. Walker AW, Ince J, Duncan SH, Webster LM, Holtrop G, Ze X, et al. Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME J. 2011;5:220–30.
24. Vital M, Howe A, Bergeron N, Krauss RM, Jansson JK, Tiedje JM. Metagenomic Insights into the Degradation of Resistant Starch by Human Gut Microbiota. Appl Environ Microbiol. 2018;84.
25. Gibson GR, Roberfroid MB. Dietary Modulation of the Human Colonic Microbiota: Introducing the Concept of Prebiotics. J Nutr. 1995;125:1401–12.
26. Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, et al. Prebiotic effects: metabolic and health benefits. British Journal of Nutrition. 2010;104:S1–63.
27. Roediger WEW. Utilization of Nutrients by Isolated Epithelial Cells of the Rat Colon. Gastroenterology. 1982;83:424–9.
28. Weaver GA, Krause JA, Miller TL, Wolin MJ. Short chain fatty acid distributions of enema samples from a sigmoidoscopy population: an association of high acetate and low butyrate ratios with adenomatous polyps and colon cancer. Gut. 1988;29:1539–43.
29. McIntyre A, Gibson PR, Young GP. Butyrate production from dietary fibre and protection against large bowel cancer in a rat model. Gut. 1993;34:386–91.
30. Wong CSM, Sengupta S, Tjandra JJ, Gibson PR. The Influence of Specific Luminal Factors on the Colonic Epithelium: High-Dose Butyrate and Physical Changes Suppress Early Carcinogenic Events in Rats. Dis Colon Rectum. 2005;48:549–59.
31. D'Argenio G, Cosenza V, Cave MD, Iovino P, Valle ND, Lombardi G, et al. Butyrate enemas in experimental colitis and protection against large bowel cancer in a rat model. Gastroenterology. 1996;110:1727–34.

32. Scheppach W, Sommer H, Kirchner T, Paganelli G-M, Bartram P, Christl S, et al. Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. Gastroenterology. 1992;103:51–6.
33. Lührs H, Gerke T, Müller JG, Melcher R, Schauber J, Boxberger F, et al. Butyrate Inhibits NF-κB Activation in Lamina Propria Macrophages of Patients with Ulcerative Colitis. Scand J Gastroenterol. 2009;37:458–66.
34. Lee C, Kim BG, Kim JH, Chun J, Im JP, Kim JS. Sodium butyrate inhibits the NF-kappa B signaling pathway and histone deacetylation, and attenuates experimental colitis in an IL-10 independent manner. Int Immunopharmacol. 2017;51:47–56.
35. Sun J, Wang F, Hong G, Pang M, Xu H, Li H, et al. Antidepressant-like effects of sodium butyrate and its possible mechanisms of action in mice exposed to chronic unpredictable mild stress. Neurosci Lett. 2016;618:159–66.
36. Brouns F, Kettlitz B, Arrigoni E. Resistant starch and “the butyrate revolution.” Trends Food Sci Technol. 2002;13:251–61.
37. Bird A, Conlon M, Christophersen C, Topping D. Resistant starch, large bowel fermentation and a broader perspective of prebiotics and probiotics. Benef Microbes. 2010;1:423–31.
38. Stephen AM, Haddad AC, Phillips SF. Passage of carbohydrate into the colon. Gastroenterology. 1983;85:589–95.
39. Abell GCJ, Cooke CM, Bennett CN, Conlon MA, McOrist AL. Phylotypes related to *Ruminococcus bromii* are abundant in the large bowel of humans and increase in response to a diet high in resistant starch. FEMS Microbiol Ecol. 2008;66:505–15.
40. McOrist AL, Miller RB, Bird AR, Keogh JB, Noakes M, Topping DL, et al. Fecal Butyrate Levels Vary Widely among Individuals but Are Usually Increased by a Diet High in Resistant Starch1,2. J Nutr. 2011;141:883–9.
41. Weaver G, Krause J, Miller T, Wolin M. Cornstarch fermentation by the colonic microbial community yields more butyrate than does cabbage fiber fermentation; cornstarch fermentation rates correlate negatively with methanogenesis. Am J Clin Nutr. 1992;55:70–7.
42. Murphy MM, Douglass JS, Birkett A. Resistant Starch Intakes in the United States. J Am Diet Assoc. 2008;108:67–78.
43. Cummings JH, Beatty ER, Kingman SM, Bingham SA, Englyst HN. Digestion and physiological properties of resistant starch in the human large bowel. British Journal of Nutrition. 1996;75:733–47.
44. Herrmann E, Young W, Reichert-Grimm V, Weis S, Riedel C, Rosendale D, et al. In Vivo Assessment of Resistant Starch Degradation by the Caecal Microbiota of Mice Using RNA-Based Stable Isotope Probing—A Proof-of-Principle Study. Nutrients. 2018;10:179.
45. Christl SU, Katzenmaier U, Hylla S, Kasper H, Scheppach W. In Vitro Fermentation of High-Amylose Cornstarch by a Mixed Population of Colonic Bacteria. Journal of Parenteral and Enteral Nutrition. 1997;21:290–5.
46. Belenguer A, Duncan SH, Calder AG, Holtrop G, Louis P, Lobley GE, et al. Two Routes of Metabolic Cross-Feeding between *Bifidobacterium adolescentis* and Butyrate-Producing Anaerobes from the Human Gut. Appl Environ Microbiol. 2006;72:3593–9.

47. Ze X, Duncan SH, Louis P, Flint HJ. *Ruminococcus bromii* is a keystone species for the degradation of resistant starch in the human colon. ISME J. 2012;6:1535–43.
48. Mukhopadhya I, Moraïs S, Laverde-Gomez J, Sheridan PO, Walker AW, Kelly W, et al. Sporulation capability and amylosome conservation among diverse human colonic and rumen isolates of the keystone starch-degrader *Ruminococcus bromii*. Environ Microbiol. 2018;20:324–36.
49. Crost EH, Le Gall G, Laverde-Gomez JA, Mukhopadhya I, Flint HJ, Juge N. Mechanistic Insights Into the Cross-Feeding of *Ruminococcus gnavus* and *Ruminococcus bromii* on Host and Dietary Carbohydrates. Front Microbiol. 2018;9.
50. Rangarajan AA, Chia HE, Azaldegui CA, Olszewski MH, Pereira G V., Koropatkin NM, et al. *Ruminococcus bromii* enables the growth of proximal *Bacteroides thetaiotaomicron* by releasing glucose during starch degradation. Microbiology (N Y). 2022;168.
51. Cockburn DW, Suh C, Medina KP, Duvall RM, Wawrzak Z, Henrissat B, et al. Novel carbohydrate binding modules in the surface anchored α-amylase of *Eubacterium rectale* provide a molecular rationale for the range of starches used by this organism in the human gut. Mol Microbiol. 2018;107:249–64.
52. Cockburn DW, Orlovsky NI, Foley MH, Kwiatkowski KJ, Bahr CM, Maynard M, et al. Molecular details of a starch utilization pathway in the human gut symbiont *Eubacterium rectale*. Mol Microbiol. 2015;95:209–30.
53. De Vuyst L, Leroy F. Cross-feeding between bifidobacteria and butyrate-producing colon bacteria explains bifdobacterial competitiveness, butyrate production, and gas production. Int J Food Microbiol. 2011;149:73–80.
54. Duncan SH, Louis P, Flint HJ. Lactate-Utilizing Bacteria, Isolated from Human Feces, That Produce Butyrate as a Major Fermentation Product. Appl Environ Microbiol. 2004;70:5810–7.
55. Tachon S, Zhou J, Keenan M, Martin R, Marco ML. The intestinal microbiota in aged mice is modulated by dietary resistant starch and correlated with improvements in host responses. FEMS Microbiol Ecol. 2013;83:299–309.
56. Teichmann J, Cockburn DW. In vitro Fermentation Reveals Changes in Butyrate Production Dependent on Resistant Starch Source and Microbiome Composition. Front Microbiol. 2021;12.
57. Alfa MJ, Strang D, Tappia PS, Graham M, Van Domselaar G, Forbes JD, et al. A randomized trial to determine the impact of a digestion resistant starch composition on the gut microbiome in older and mid-age adults. Clinical Nutrition. 2018;37:797–807.
58. Nagara Y, Fujii D, Takada T, Sato-Yamazaki M, Odani T, Oishi K. Selective induction of human gut-associated acetogenic/butyrogenic microbiota based on specific microbial colonization of indigestible starch granules. ISME J. 2022;16:1502–11.
59. Wang X, Conway PL, Brown IL, Evans AJ. In Vitro Utilization of Amylopectin and High-Amylose Maize (Amylomaize) Starch Granules by Human Colonic Bacteria. Appl Environ Microbiol. 1999;65:4848–54.
60. Duranti S, Turroni F, Lugli GA, Milani C, Viappiani A, Mangifesta M, et al. Genomic Characterization and Transcriptional Studies of the Starch-Utilizing Strain Bifidobacterium adolescentis 22L. Appl Environ Microbiol. 2014;80:6080–90.

61. Milani C, Mangifesta M, Mancabelli L, Lugli GA, Mancino W, Viappiani A, et al. The Sortase-Dependent Fimbriome of the Genus *Bifidobacterium*: Extracellular Structures with Potential To Modulate Microbe-Host Dialogue. Appl Environ Microbiol. 2017;83.
62. Cerqueira FM, Photenhauer AL, Doden HL, Brown AN, Abdel-Hamid AM, Moraïs S, et al. Sas20 is a highly flexible starch-binding protein in the *Ruminococcus bromii* cell-surface amylosome. Journal of Biological Chemistry. 2022;298:101896.
63. Valk V, Lammerts van Bueren A, Kaaij RM, Dijkhuizen L. Carbohydrate-binding module 74 is a novel starch-binding domain associated with large and multidomain α-amylase enzymes. FEBS J. 2016;283:2354–68.
64. Photenhauer A. Personal communication. 2023.
65. Schwartz E, Fritsch J, Friedrich B. H_2-Metabolizing Prokaryotes. In: The Prokaryotes. Berlin, Heidelberg: Springer Berlin Heidelberg; 2013. p. 119–99.
66. Thauer RK, Jungermann K, Decker K. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev. 1977;41:100–80.
67. Gottschalk G. Bacterial Metabolism. New York, NY: Springer New York; 1986.
68. Stams AJM. Metabolic interactions between anaerobic bacteria in methanogenic environments. Antonie Van Leeuwenhoek. 1994;66:271–94.
69. Stams AJM, Plugge CM. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat Rev Microbiol. 2009;7:568–77.
70. Macfarlane S, Macfarlane GT. Regulation of short-chain fatty acid production. Proc Nutr Soc. 2003;62:67–72.
71. Wolf PG, Biswas A, Morales SE, Greening C, Gaskins HR. H_2 metabolism is widespread and diverse among human colonic microbes. Gut Microbes. 2016;7:235–45.
72. Kirk E. The Quantity and Composition of Human Colonic Flatus. Gastroenterology. 1949;12:782–94.
73. Levitt MD. Volume and Composition of Human Intestinal Gas Determined by Means of an Intestinal Washout Technic. New England Journal of Medicine. 1971;284:1394–8.
74. Sonnenburg ED, Sonnenburg JL. Starving our Microbial Self: The Deleterious Consequences of a Diet Deficient in Microbiota-Accessible Carbohydrates. Cell Metab. 2014;20:779–86.
75. Muir JG, Lu ZX, Young GP, Cameron-Smith D, Collier GR, O'Dea K. Resistant starch in the diet increases breath hydrogen and serum acetate in human subjects. Am J Clin Nutr. 1995;61:792–9.
76. Oku T, Nakamura S. Comparison of digestibility and breath hydrogen gas excretion of fructo-oligosaccharide, galactosyl-sucrose, and isomalto-oligosaccharide in healthy human subjects. Eur J Clin Nutr. 2003;57:1150–6.
77. Louis P, Flint HJ. Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine. FEMS Microbiol Lett. 2009;294:1–8.
78. Louis P, Flint HJ. Formation of propionate and butyrate by the human colonic microbiota. Environ Microbiol. 2017;19:29–41.
79. Buckel W, Thauer RK. Flavin-Based Electron Bifurcation, Ferredoxin, Flavodoxin, and Anaerobic Respiration With Protons (Ech) or NAD+ (Rnf) as Electron Acceptors: A Historical Review. Front Microbiol. 2018;9.

80. Hillman ET, Kozik AJ, Hooker CA, Burnett JL, Heo Y, Kiesel VA, et al. Comparative genomics of the genus Roseburia reveals divergent biosynthetic pathways that may influence colonic competition among species. Microb Genom. 2020;6.
81. Ragsdale SW. Pyruvate Ferredoxin Oxidoreductase and Its Radical Intermediate. Chem Rev. 2003;103:2333–46.
82. Charon M, Volbeda A, Chabrière E, Pieulle L, Hatchikian EC, Fontecilla–Camps J. Crystal structures of the key anaerobic enzyme pyruvate:ferredoxin oxidoreductase, free and in complex with pyruvate. Nat Struct Biol. 1999;6:182–90.
83. Thauer RK, Kirchniawy FH, Jungermann KA. Properties and Function of the Pyruvate-Formate-Lyase Reaction in Clostridiae. Eur J Biochem. 1972;27:282–90.
84. Dostal A, Lacroix C, Bircher L, Pham VT, Follador R, Zimmermann MB, et al. Iron Modulates Butyrate Production by a Child Gut Microbiota *In Vitro*. mBio. 2015;6.
85. Louis P, Duncan SH, McCrae SI, Millar J, Jackson MS, Flint HJ. Restricted Distribution of the Butyrate Kinase Pathway among Butyrate-Producing Bacteria from the Human Colon. J Bacteriol. 2004;186:2099–106.
86. Bennett GN, Rudolph FB. The central metabolic pathway from acetyl-CoA to butyryl-CoA in *Clostridium acetobutylicum*. FEMS Microbiol Rev. 1995;17:241–9.
87. Li F, Hinderberger J, Seedorf H, Zhang J, Buckel W, Thauer RK. Coupled ferredoxin and crotonyl coenzyme A (CoA) reduction with NADH catalyzed by the butyryl-CoA dehydrogenase/Etf complex from *Clostridium kluyveri*. J Bacteriol. 2008;190:843–50.
88. Chen J-S, Mortenson LE. Purification and properties of hydrogenase from *Clostridium pasteurianum* W5. Biochimica et Biophysica Acta (BBA) - Protein Structure. 1974;371:283–98.
89. Biegel E, Müller V. Bacterial Na^+-translocating ferredoxin:NAD^+ oxidoreductase. Proceedings of the National Academy of Sciences. 2010;107:18138–42.
90. Vital M, Howe AC, Tiedje JM, Moran MA. Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta)genomic Data. mBio. 2014;5:e00889-14.
91. Angenent LT, Karim K, Al-Dahhan MH, Wrenn BA, Domíguez-Espinosa R. Production of bioenergy and biochemicals from industrial and agricultural wastewater. Trends Biotechnol. 2004;22:477–85.
92. Van Andel JG, Zoutberg GR, Crabbendam PM, Breure AM. Glucose fermentation by *Clostridium butyricum* grown under a self generated gas atmosphere in chemostat culture. Appl Microbiol Biotechnol. 1985;23:21–6.
93. Pavlostathis SG, Miller TL, Wolin MJ. Cellulose fermentation by continuous cultures of *Ruminococcus albus* and *Methanobrevibacter smithii*. Appl Microbiol Biotechnol. 1990;33:109–16.
94. Lamed RJ, Lobos JH, Su TM. Effects of Stirring and Hydrogen on Fermentation Products of *Clostridium thermocellum*. Appl Environ Microbiol. 1988;54:1216–21.
95. Duncan SH, Belenguer A, Holtrop G, Johnstone AM, Flint HJ, Lobley GE. Reduced Dietary Intake of Carbohydrates by Obese Subjects Results in Decreased Concentrations of Butyrate and Butyrate-Producing Bacteria in Feces. Appl Environ Microbiol. 2007;73:1073–8.

96. D'hoe K, Vet S, Faust K, Moens F, Falony G, Gonze D, et al. Integrated culturing, modeling and transcriptomics uncovers complex interactions and emergent behavior in a three-species synthetic gut community. Elife. 2018;7.
97. Doremus MG, Linden JC, Moreira AR. Agitation and pressure effects on acetone-butanol fermentation. Biotechnol Bioeng. 1985;27:852–60.
98. Kafkewitz D, Iannotti EL, Wolin MJ, Bryant MP. An anaerobic chemostat that permits the collection and measurement of fermentation gases. Appl Microbiol. 1973;25:612–4.
99. Weimer PJ, Zeikus JG. Fermentation of cellulose and cellobiose by Clostridium thermocellum in the absence of *Methanobacterium thermoautotrophicum*. Appl Environ Microbiol. 1977;33:289–97.
100. Christl SU, Murgatroyd PR, Gibson GR, Cummings JH. Production, metabolism, and excretion of hydrogen in the large intestine. Gastroenterology. 1992;102:1269–77.
101. Strocchi A, Levitt MD. Factors affecting hydrogen production and consumption by human fecal flora. The critical roles of hydrogen tension and methanogenesis. J Clin Invest. 1992;89:1304–11.
102. Gibson GR, Macfarlane S, Macfarlane GT. Metabolic interactions involving sulphate-reducing and methanogenic bacteria in the human large intestine. FEMS Microbiol Ecol. 1993;12:117–25.
103. Nava GM, Carbonero F, Croix JA, Greenberg E, Gaskins HR. Abundance and diversity of mucosa-associated hydrogenotrophic microbes in the healthy human colon. ISME J. 2012;6:57–70.
104. Carbonero F, Benefiel AC, Gaskins HR. Contributions of the microbial hydrogen economy to colonic homeostasis. Nat Rev Gastroenterol Hepatol. 2012;9:504–18.
105. Tukey JW. Exploratory Data Analysis. Reading, MA: Addison-Wesley; 1977.
106. Gottlieb K, Le C, Wacher V, Sliman J, Cruz C, Porter T, et al. Selection of a cut-off for high- and low-methane producers using a spot-methane breath test: results from a large north American dataset of hydrogen, methane and carbon dioxide measurements in breath. Gastroenterol Rep (Oxf). 2017;5:193-9.
107. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. Appl Environ Microbiol. 2013;79:5112–20.
108. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. Appl Environ Microbiol. 2009;75:7537–41.
109. Dabrock B, Bahl H, Gottschalk G. Parameters Affecting Solvent Production by Clostridium pasteurianum. Appl Environ Microbiol. 1992;58:1233–9.
110. Duncan SH, Hold GL, Harmsen HJM, Stewart CS, Flint HJ. Growth requirements and fermentation products of *Fusobacterium prausnitzii*, and a proposal to reclassify it as *Faecalibacterium prausnitzii* gen. nov., comb. nov. Int J Syst Evol Microbiol. 2002;52:2141–6.
111. Samuel BS, Hansen EE, Manchester JK, Coutinho PM, Henrissat B, Fulton R, et al. Genomic and metabolic adaptations of *Methanobrevibacter smithii* to the human gut. Proceedings of the National Academy of Sciences. 2007;104:10643–8.

112. Scanlan PD, Shanahan F, Marchesi JR. Human methanogen diversity and incidence in healthy and diseased colonic groups using mcrA gene analysis. BMC Microbiol. 2008;8:79.
113. Kraemer JT, Bagley DM. Supersaturation of dissolved H_2 and CO_2 during fermentative hydrogen production with N2 sparging. Biotechnol Lett. 2006;28:1485–91.
114. Pauss A, Samson R, Guiot S, Beauchemin C. Continuous measurement of dissolved H_2 in an anaerobic reactor using a new hydrogen/air fuel cell detector. Biotechnol Bioeng. 1990;35:492–501.
115. Pauss A, Guiot SR. Hydrogen monitoring in anaerobic sludge bed reactors at various hydraulic regimes and loading rates. Water Environment Research. 1993;65:276–80.
116. Pauss A, Andre G, Perrier M, Guiot SR. Liquid-to-Gas Mass Transfer in Anaerobic Processes: Inevitable Transfer Limitations of Methane and Hydrogen in the Biomethanation Process. Appl Environ Microbiol. 1990;56:1636–44.
117. Abu-Ali GS, Mehta RS, Lloyd-Price J, Mallick H, Branck T, Ivey KL, et al. Metatranscriptome of human faecal microbial communities in a cohort of adult men. Nat Microbiol. 2018;3:356–66.
118. Levitt MD, Furne JK, Kuskowski M, Ruddy J. Stability of Human Methanogenic Flora Over 35 Years and a Review of Insights Obtained From Breath Methane Measurements. Clinical Gastroenterology and Hepatology. 2006;4:123–9.
119. Abell GCJ, Conlon MA, McOrist AL. Methanogenic archaea in adult human faecal samples are inversely related to butyrate concentration. Microb Ecol Health Dis. 2009;18:154–60.
120. Bourriaud C, Robins RJ, Martin L, Kozlowski F, Tenailleau E, Cherbut C, et al. Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. J Appl Microbiol. 2005;99:201–12.
121. Belenguer A, Duncan SH, Holtrop G, Anderson SE, Lobley GE, Flint HJ. Impact of pH on Lactate Formation and Utilization by Human Fecal Microbial Communities. Appl Environ Microbiol. 2007;73:6526–33.
122. Wang SP, Rubio LA, Duncan SH, Donachie GE, Holtrop G, Lo G, et al. Pivotal Roles for pH, Lactate, and Lactate-Utilizing Bacteria in the Stability of a Human Colonic Microbial Ecosystem. mSystems. 2020;5.
123. Duncan SH, Louis P, Flint HJ. Lactate-Utilizing Bacteria, Isolated from Human Feces, That Produce Butyrate as a Major Fermentation Product. Appl Environ Microbiol. 2004;70:5810–7.
124. Falony G, Verschaeren A, De Bruycker F, De Preter V, Verbeke K, Leroy F, et al. In Vitro Kinetics of Prebiotic Inulin-Type Fructan Fermentation by Butyrate-Producing Colon Bacteria: Implementation of Online Gas Chromatography for Quantitative Analysis of Carbon Dioxide and Hydrogen Gas Production. Appl Environ Microbiol. 2009.
125. Ruaud A, Esquivel-Elizondo S, de la Cuesta-Zuluaga J, Waters JL, Angenent LT, Youngblut ND, et al. Syntrophy via Interspecies H_2 Transfer between *Christensenella* and *Methanobrevibacter* Underlies Their Global Cooccurrence in the Human Gut. mBio. 2020;11:1022.

126. Chassard C, Bernalier Donadille A. H2 and acetate transfers during xylan fermentation between a butyrate-producing xylanolytic species and hydrogenotrophic microorganisms from the human gut. FEMS Microbiol Lett. 2006;254:116–22.
127. Samuel BS, Gordon JI. A humanized gnotobiotic mouse model of host–archaeal–bacterial mutualism. Proceedings of the National Academy of Sciences. 2006;103:10011–6.
128. DiBaise JK, Zhang H, Crowell MD, Krajmalnik-Brown R, Decker GA, Rittmann BE. Gut microbiota and its possible relationship with obesity. Mayo Clin Proc. 2008;83:460–9.
129. Nakamura N, Lin HC, McSweeney CS, Mackie RI, Gaskins HR. Mechanisms of Microbial Hydrogen Disposal in the Human Colon and Implications for Health and Disease. Annual Review of Food Science and Technology. 2010;1:363–95.
130. Smith NW, Shorten PR, Altermann EH, Roy NC, McNabb WC. Hydrogen cross-feeders of the human gastrointestinal tract. Gut Microbes. 2019;10:270–88.
131. Fischbach MA, Sonnenburg JL. Eating For Two: How Metabolism Establishes Interspecies Interactions in the Gut. Cell Host Microbe. 2011;10:336–47.
132. Flourie B, Etanchaud F, Florent C, Pellier P, Bouhnik Y, Rambaud JC. Comparative study of hydrogen and methane production in the human colon using caecal and faecal homogenates. Gut. 1990;31:684–5.
133. Pochart P, Lémann F, Flourié B, Pellier P, Goderel I, Rambaud J-C. Pyxigraphic sampling to enumerate methanogens and anaerobes in the right colon of healthy humans. Gastroenterology. 1993;105:1281–5.
134. Macfarlane GT, Gibson GR, Cummings JH. Comparison of fermentation reactions in different regions of the human colon. J Appl Bacteriol. 1992;72:57–64.
135. Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med. 2007;13:688–94.
136. Hu Y, Wang P, Han K. Hydrogen Attenuated Inflammation Response and Oxidative in Hypoxic Ischemic Encephalopathy via Nrf2 Mediated the Inhibition of NLRP3 and NF-κB. Neuroscience. 2022;485:23–36.
137. Yang Y, Zhu Y, Xi X. Anti-inflammatory and antitumor action of hydrogen via reactive oxygen species (Review). Oncol Lett. 2018. https://doi.org/10.3892/ol.2018.9023.
138. Levitt MD, Ingelfinger FJ. Hydrogen and Methane Production in Man. Ann N Y Acad Sci. 1968;150:75–81.
139. Levitt MD. Production and Excretion of Hydrogen Gas in Man. New England Journal of Medicine. 1969;281:122–7.
140. Bond JH, Engel RR, Levitt MD. Factors Influencing Pulmonary Methane Excretion in Man: An Indirect Method of Studying the In Situ Metabolism of the Methane-Producing Colonic Bacteria. Journal of Experimental Medicine. 1971;133:572–88.
141. Gasbarrini A, Corazza GR, Gasbarrini G, Montalto M, Di Stefano M, Basilisco G, et al. Methodology and indications of H_2-breath testing in gastrointestinal diseases: the Rome Consensus Conference. Aliment Pharmacol Ther. 2009;29 Suppl 1:1–49.
142. Calloway DH, Murphy EL. The Use of Expired Air to Measure Intestinal Gas Formation. Ann N Y Acad Sci. 1968;150 1 Gastrointesti:82–95.

143. Shin W. Medical applications of breath hydrogen measurements. Anal Bioanal Chem. 2014;406:3931–9.
144. Simrén M, Stotzer P-O. Use and abuse of hydrogen breath tests. Gut. 2006;55:297–303.
145. Bond JH, Levitt MD, Prentiss R. Investigation of small bowel transit time in man utilizing pulmonary hydrogen (H_2) measurements. J Lab Clin Med. 1975;85:546–55.
146. Hirakawa M, Iida M, Kohrogi N, Fujishima M. Hydrogen breath test assessment of orocecal transit time: comparison with barium meal study. Am J Gastroenterol. 1988;83:1361–3.
147. King CE, Toskes PP, Watkins JB. Breath Tests in the Diagnosis of Small Intestine Bacterial Overgrowth. CRC Crit Rev Clin Lab Sci. 1984;21:269–81.
148. Levitt MD, Donaldson RM. Use of respiratory hydrogen (H_2) excretion to detect carbohydrate malabsorption. J Lab Clin Med. 1970;75:937–45.
149. Rumessen JJ. Hydrogen and methane breath tests for evaluation of resistant carbohydrates. Eur J Clin Nutr. 1992;46 Suppl 2:S77-90.
150. Corazza GR, Strocchi A, Gasbarrini G. Fasting breath hydrogen in celiac disease. Gastroenterology. 1987;93:53–8.
151. Sloan TJ, Jalanka J, Major GAD, Krishnasamy S, Pritchard S, Abdelrazig S, et al. A low FODMAP diet is associated with changes in the microbiota and reduction in breath hydrogen but not colonic volume in healthy subjects. PLoS One. 2018;13:e0201410.
152. Campbell A, Gdanetz K, Schmidt AW, Schmidt TM. H_2 generated by fermentation in the human gut microbiome influences metabolism and competitive fitness of gut butyrate producers. Microbiome. 2023;11:133.
153. Kotler DP, Holt PR, Rosensweig NS. Modification of the breath hydrogen test: increased sensitivity for the detection of carbohydrate malabsorption. J Lab Clin Med. 1982;100:798–805.
154. Brummer RJ, Armbrecht U, Bosaeus I, Dotevall G, Stockbruegger RW. The hydrogen (H_2) breath test. Sampling methods and the influence of dietary fibre on fasting level. Scand J Gastroenterol. 1985;20:1007–13.
155. Perman JA, Modler S, Barr RG, Rosenthal P. Fasting breath hydrogen concentration: normal values and clinical application. Gastroenterology. 1984;87:1358–63.
156. Anderson IH, Levine AS, Levitt MD. Incomplete absorption of the carbohydrate in all-purpose wheat flour. N Engl J Med. 1981;304:891–2.
157. Levitt MD, Hirsh P, Fetzer CA, Sheahan M, Levine AS. H_2 excretion after ingestion of complex carbohydrates. Gastroenterology. 1987;92:383–9.
158. Brighenti F, Casiraghi MC, Pellegrini N, Riso P, Simonetti P, Testolin G. Comparison of lactulose and inulin as reference standard for the study of resistant starch fermentation using hydrogen breath test. Ital J Gastroenterol. 1995;27:122–8.
159. Teferra TF. Possible actions of inulin as prebiotic polysaccharide: A review. Food Front. 2021;2:407–16.
160. Pedersen A, Sandström B, Van Amelsvoort JMM. The effect of ingestion of inulin on blood lipids and gastrointestinal symptoms in healthy females. British Journal of Nutrition. 1997;78:215–22.

161. Davidson MH, Maki KC, Synecki C, Torri SA, Drennan KB. Effects of dietary inulin on serum lipids in men and women with hypercholesterolemia. Nutrition Research. 1998;18:503–17.
162. US Food & Drug Administration. Questions and Answers on Dietary Fiber. 2021.
163. Tadesse K, Smith D, Eastwod MA. Breath Hydrogen (H_2) and Methane (CH_4) Excretion Patterns in Normal Man and in Clinical Practice. Exp Physiol. 1980;65:85–97.
164. Kagaya M, Iwata M, Toda Y, Nakae Y, Kondo T. Circadian rhythm of breath hydrogen in young women. J Gastroenterol. 1998;33:472–6.
165. Kumar S, Misra A, Ghoshal UC. Patients With Irritable Bowel Syndrome Exhale More Hydrogen Than Healthy Subjects in Fasting State. J Neurogastroenterol Motil. 2010;16:299–305.
166. Justino SR, Gonçalves Dias MC, Maculevicius J, Batista de Morais M, Sing TC, Halpern A, et al. Fasting breath hydrogen concentration in short bowel syndrome patients with colon incontinuity before and after antibiotic therapy. Nutrition. 2004;20:187–91.
167. Suarez F, Furne J, Springfield J, Levitt M. Insights into human colonic physiology obtained from the study of flatus composition. Am J Physiol Gastrointest Liver Physiol. 1997;272:G1028–33.
168. Costello BPJ de L, Ewen RJ, Ratcliffe NM. A sensor system for monitoring the simple gases hydrogen, carbon monoxide, hydrogen sulfide, ammonia and ethanol in exhaled breath. J Breath Res. 2008;2:037011.