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REVIEW

Hydrogen cross-feeders of the human gastrointestinal tract

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ABSTRACT

Hydrogen plays a key role in many microbial metabolic pathways in the human gastrointestinal tract (GIT) that have an impact on human nutrition, health and wellbeing. Hydrogen is produced by many members of the GIT microbiota, and may be subsequently utilized by cross-feeding microbes for growth and in the production of larger molecules. Hydrogenotrophic microbes fall into three functional groups: sulfate-reducing bacteria, methanogenic archaea and acetogenic bacteria, which can convert hydrogen into hydrogen sulfide, methane and acetate, respectively. Despite different energy yields per molecule of hydrogen used between the functional groups, all three can coexist in the human GIT. The factors affecting the numerical balance of hydrogenotrophs in the GIT remain unconfirmed. There is increasing evidence linking both hydrogen sulfide and methane to GIT diseases such as irritable bowel syndrome, and strategies for the mitigation of such health problems through targeting of hydrogenotrophs constitute an important field for further investigation.

Introduction

Microbes in the human gastrointestinal tract (GIT) form complex communities that are often dependent upon one another for the provision of nutrients. The nutrients required by each microbial strain vary widely, as do the metabolites released. As such, the microbial population in the GIT, termed the microbiota, maintains a web of interactions in which the products of one strain may be further utilized by another, in addition to impacting upon the host. Such exchanging of metabolites between microbes is referred to as cross-feeding. Much research has been undertaken into the mechanics and characteristics of these relationships, particularly with regard to bacterial cross-feeding on the products of carbohydrate fermentation and short-chain fatty acid (SCFA) production.[1–7] However, an important yet understudied product of bacterial metabolism involved in SCFA-producing metabolic pathways is hydrogen, which is also targeted by cross-feeding microbes (Figure 1) and has a range of implications for the nutrition and health of the host.

Hydrogen is a common metabolite found within the human GIT, produced through widely-used microbial carbohydrate breakdown pathways. Two common examples of hydrogen-producing metabolic pathways are the Embden-Meyerhof-Parnas pathway (commonly called glycolysis), converting glucose to pyruvate, and the formation of acetate from pyruvate via acetyl-CoA.[8,9] The hydrogen produced in these reactions acts as an electron sink, allowing for the disposal of reducing power.[10] Electron sink products are a necessary part of bacterial metabolism, which allow for the disposal of free electrons produced during substrate catabolism.

Hydrogen is a major and efficient electron sink product in the human GIT, alongside other fermentation products such as ethanol, lactate and succinate.[11] However, the quantity of hydrogen produced in the GIT is dependent upon the population structure of the microbiota in each individual. The two major phyla of the GIT microbiota are the Firmicutes and the Bacteroidetes, which together form over 85% of the total bacterial population in adults.[12,13] The relative proportions of these phyla have been shown to vary between individuals[14,15] and according to diet.[16] Of the Firmicutes and Bacteroidetes, culture-based studies suggest that free hydrogen is mainly produced by the former (for a review, see ref. [9]). The co-culture
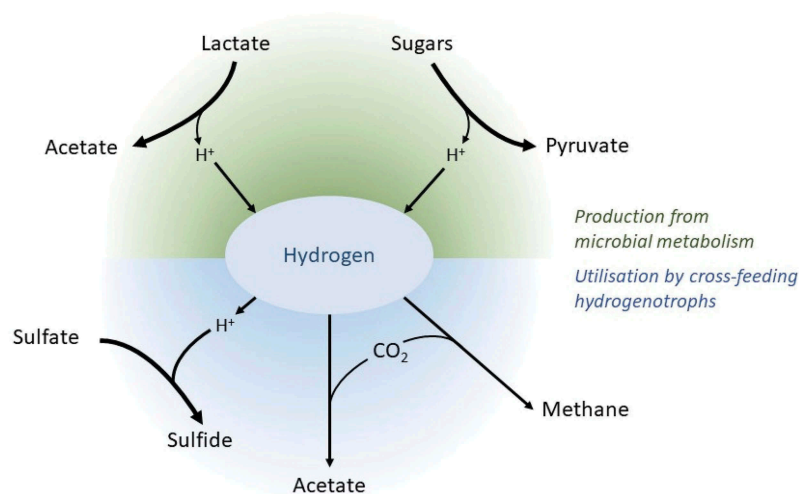
Figure 1. Selected sources of hydrogen production in the GIT and microbial cross-feeding pathways by which it is removed.

growth of a *Bacteroides* strain (Bacteroidetes) with a *Ruminococcus* strain (Firmicutes) on cellulose resulted in decreased cumulative hydrogen production compared to *Ruminococcus* monoculture, despite increased overall cellulose degradation.[17] Importantly, both strains were able to persist together in this experiment, as is the case in the human GIT. A recent genome survey of 343 sequenced genomes from the Human Microbiome Project found that more than 70% contained hydrogenase-encoding sequences, the majority of which were members of either the Firmicutes or Bacteroidetes phyla.[18] This implies that hydrogen cycling within these phyla may be more important than previously thought, but it is unclear how such cycling contributes to the gastrointestinal hydrogen concentration. Nonetheless, the concentration of hydrogen in the GIT will depend in part upon the balance between those bacteria that produce hydrogen during fermentation and those that do not, as shown by the culture-based study.

The hydrogen concentration in the GIT affects both the microbiota and the host. High hydrogen concentrations can impair the metabolism of both hydrogen-producing and non-producing bacteria. A high hydrogen partial pressure inhibits the regeneration of the coenzyme NAD^+ from NADH, slowing the rate of substrate catabolism and thus hindering bacterial growth.[19,20] The partial pressure of hydrogen also determines the thermodynamic favorability of SCFA production. The production of acetate and butyrate results in a greater release of free hydrogen than does propionate formation, thus the latter is more thermodynamically favorable at high ambient hydrogen concentrations.[21]

As well as affecting the metabolism of microbes, hydrogen can have a detrimental effect on the human host, with proposed roles in various GIT disorders, which will be discussed later in this review, and include Irritable Bowel Syndrome (IBS), inflammatory bowel disease and obesity. Hydrogen is removed from the GIT in a number of ways, mediated by both the host and the microbiota. Some hydrogen is expelled directly from the GIT or absorbed into the bloodstream.[11] The remaining hydrogen can be converted into other metabolites by hydrogenotrophic members of the microbiota, in sufficient quantities to have an effect on the host. Major gaps exist in our understanding of the mechanisms behind the inter-individual differences observed in hydrogenotroph colonization and metabolism. Without this knowledge, it is not possible to make strong inference about the role of hydrogenotrophs in disease, nor to propose remedial strategies based on the control of the microbiota. This review will present the current understanding of hydrogenotrophic microbes, their occurrence and prevalence in the microbiota, and their associated health impacts.

The hydrogenotrophic functional groups of the human gastrointestinal tract

Hydrogenotrophic microbes in the GIT largely fall into three functional groups, determined principally by the products of their hydrogen metabolism:

methanogens, which produce methane; sulfate-reducing bacteria (SRB), which convert free sulfate to sulfide compounds; and acetogens, which produce acetate. Each utilize hydrogen to varying degrees under certain conditions and all have been shown to coexist within the human GIT.[22–24] Microbiome data shows that although functional genes from all three hydrogenotrophic groups are consistently found throughout the colon, they form a numerically minor part of the microbiota.[18,24–26] Arumugam et al. suggested that the three enterotypes into which they could separate individuals in their microbiome sequencing work may be influenced by the dominant form of hydrogenotrophy occurring in each enterotype.[26] However, confirming this hypothesis was prevented by the low abundance of genetic material from the hydrogenotrophic functional groups obtained in samples and deserves further investigation.

The role of hydrogenotrophic microbes in reducing the partial pressure of hydrogen in the GIT reduces the associated inhibitory effect on carbohydrate metabolism.[21,27,28] The relationship between hydrogen producers and consumers therefore constitutes a mutualism, beneficial to both.

Methanogens

The Archaeon *Methanobrevibacter smithii* is the most abundant and, in some cases, the sole methanogen found within the human GIT,[13,24,29–32] although *Methanosphaera stadtmanae* is also commonly observed.[30,33] *M. smithii* converts CO_2 and hydrogen into methane (CH_4)[8], in the following reaction (Figure 2):

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$
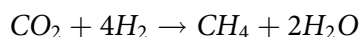
M. smithii is also able to utilize formate (HCO_2^-) in the same manner, because the similar reduction potentials of formate and hydrogen make interconversion between the two manageable.[34] Expression of genes involved in formate utilization by *M. smithii* has been shown to be upregulated in the presence of formate-producing bacteria.[35] Samuel et al. also found evidence to support minor levels of ethanol and methanol uptake by this archaeon as lesser energy sources, as well as acetate uptake, for use in an incomplete tricarboxylic acid cycle.[35] They

suggest that these latter pathways are responsible for biomass accumulation.

Methanogens are limited by low pH, even in the range found within the proximal colon, which can be as low as pH 5.5.[36,37] They are also limited by their inability to degrade sugars[35,38] and are therefore obligate cross-feeders, dependent upon the products of carbohydrate degraders for survival.

Sulfate-reducing bacteria

The SRB show greater taxonomic diversity in the microbiota than the methanogens and are able to utilize a wider range of substrates for growth. Most commonly it is the *Desulfovibrio* genus that is the dominant SRB clade in the colon,[24,36] members of which are able to reduce sulfate compounds to hydrogen sulfide (H_2S), whilst simultaneously oxidizing lactate to acetate. *Desulfovibrio piger* has an obligate requirement for sulfate in order to oxidize lactate,[39] and interestingly was able to reduce sulfate effectively at pH 5.5 – substantially more acidic conditions than the neutral pH supportive of GIT methanogens. Other SRB species are able to metabolize substrates such as the SCFAs acetate, butyrate and propionate, as well as ethanol and pyruvate,[40] although these genera are usually absent or found at low counts in the GIT.[24,41–44] A standard stoichiometric equation for sulfate reduction is as follows[19]:

$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O$$

or

$$2CH_3CHOHCOO^- + SO_4^{2-}$$
$$\rightarrow 2CH_3COO^- + 2HCO_3^- + H_2S$$
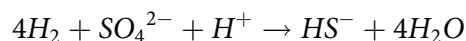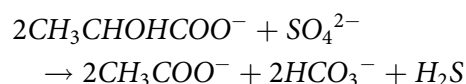
$$(2\,Lactate + Sulfate$$
$$\rightarrow 2\,Acetate + 2\,Bicarbonate$$
$$+ Hydrogen\ Sulfide)$$

with the inclusion of the lactate to acetate conversion (Figure 3).[45–47] Both lactate oxidation and sulfate reduction are energy yielding pathways for SRB and use of intermediates of the lactate oxidation pathway as carbon sources for growth can be expected to alter the stoichiometry of these reactions.[45]

Sulfate may be derived in the GIT from a number of dietary sources, particularly high-
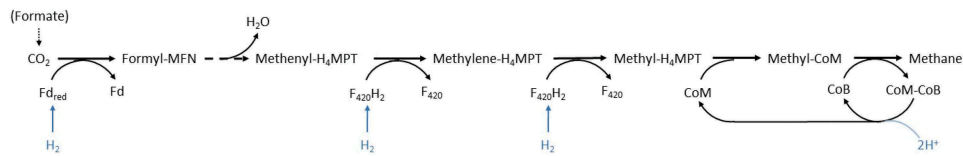
Figure 2. Simplified diagram for hydrogenotrophic methanogenesis. The dashed arrow represents multiple reactions and intermediates. Formate may be a precursor, but is converted to CO_2 for methanogenesis. Formyl-MFN = formyl-methanofuran; H_4 MPT = tetrahydromethanopterin; Fd = ferredoxin; Fd_{red} = reduced ferredoxin; F_{420} = coenzyme F_{420}; CoM/CoB = coenzyme M/B. See refs. [35] and [125] for full pathway descriptions.
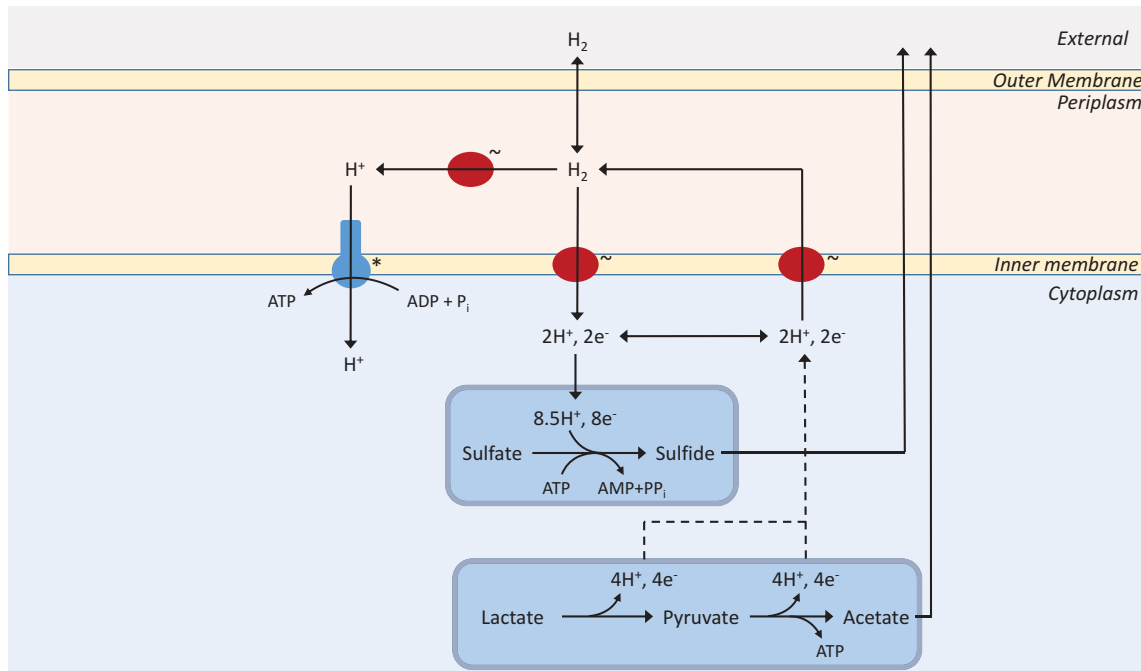

Figure 3. Simplified diagram for the simultaneous oxidation of lactate and reduction of sulfide, based on data for the prominent SRB *Desulfovibrio vulgaris* Hildenborough. Both lactate oxidation and sulfate reduction occur in the cell cytoplasm. The protons and electrons produced during lactate oxidation are acted upon by a number of hydrogenase enzymes, with interconversion between H_2, and $2H^+ + 2e^-$. A proton gradient is maintained across the cytoplasmic membrane, enabling the synthesis of ATP by membrane-bound ATPase. * = ATP synthase; ~ = hydrogenase enzyme. See refs. [34, 45, 46] and [126] for full pathway descriptions.

protein foods such as animal products, which contain the sulfur amino acids cysteine, methionine and taurine, as well as the inorganic sulfate present in *Brassica* vegetables.[48] Sulfate may also be generated from the breakdown of endogenous sulfur-containing mucins.[49,50] However, SRB are unable to degrade these structures, so rely on other members of the microbiota to release free sulfate during their metabolism of mucins.[10] Willis et al. investigated the cross-feeding actions of the SRB *Desulfovibrio desulfuricans* on free sulfate produced by *Bacteroides fragilis* during mucin breakdown.[49] Both *D. desulfuricans* cell counts and the sulfide produced were increased in co-culture compared to a SRB monoculture, clearly displaying a cross-feeding relationship. The

sulfate-reduction pathway of SRB is dependent on the availability of sulfate, and thus is often dependent on cross-feeding for this substrate.

Although the metabolism of hydrogen by SRB puts them in competition with other hydrogenotrophic microbes, the broader metabolic capabilities of the SRB allow them to compete with other functional groups of the microbiota, metabolizing more complex substrates. *D. piger* has been shown to cross-feed on lactate produced by *Bifidobacterium adolescentis*,[39] and to effectively compete for this substrate against the butyrate-producers *Eubacterium hallii* and *Anaerostipes caccae*. Butyrate production was decreased in all co-cultures containing the SRB, as were cell counts for *E. hallii* and *A. caccae*, due to competition for

lactate. By contrast, *D. piger* cell counts were unaffected by the presence or absence of either or both of the butyrate producers, potentially due to more effective lactate scavenging by the SRB. The authors hypothesized that the hydrogen produced by the other bacteria in the co-cultures may have given a further advantage to *D. piger*, which is supported by the increased final sulfide concentrations in the co-cultures. However, this is impossible to determine explicitly, since only initial and final cell counts were measured in this experiment, leaving no way to ascertain growth rates or the time taken to reach steady state. Moreover, both the butyrate-producing species studied here have been shown to metabolize sugars as well as lactate,[7] a dynamic that was not analyzed in this study. Nevertheless, the behavior exhibited by *D. piger* shows more complex cross-feeding interactions than those of the methanogens.

Acetogens

Acetate is produced via a number of microbial metabolic pathways, however in the context of hydrogen cross-feeding, we consider only the acetate formed via reductive acetogenesis (Figure 4)[19]:

$$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$$
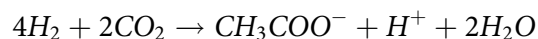
Reductive acetogenesis uses the Wood-Ljungdahl metabolic pathway. Another common and pertinent term found in the literature is "homoacetogenesis", but the definition is not uniform across the field of study. Homoacetogenesis has been defined as the formation of acetate as the sole reduced end product in the metabolism of a substrate,[51] but discordantly as the "production of more than 2 mol of acetate per mol of sugar consumed".[52] We avoid the use of the term homoacetogenesis and, in the remainder of this review, our use of the term acetogen is restricted to bacteria producing acetate via the Wood-Ljungdahl pathway. This also excludes the incomplete oxidizers of SCFAs among the SRB, which, as previously noted, are acetate producers when feeding on organic molecules such as lactate, but do not perform reductive acetogenesis.

Genome sequenced acetogens possess the genes necessary for the metabolism of a greater range of substrates than either SRB or methanogens.[9,53] The GIT acetogen *Blautia hydrogenotrophica* (previously *Ruminococcus hydrogenotrophicus*, but since reclassified[52,54]) has been shown to metabolize a range of mono- and disaccharides, as well as hydrogen and CO_2, required for reductive acetogenesis.[52] Work culturing the hydrogen producer *Ruminococcus albus* with a GIT acetogen displayed acetate levels in co-culture of around three times that recorded for *R. albus* alone.[55] Hydrogen was near undetectable after 5 days of co-culture, due to its conversion to acetate via the Wood-Ljungdahl pathway.

Acetogens have a positive impact on the human body in terms of energy harvest, since acetate can be absorbed by the host for use as an energy source.[56] Acetate produced via the Wood-Ljungdahl pathway has been shown to account for up to 33% of acetate produced by fecal cultures.[57] Moreover, acetogens may provide benefits to other bacterial groups. An *in vitro* cross-feeding mutualism has been observed between *Roseburia intestinalis* and *B. hydrogenotrophica* when the pair were grown on xylan.[58] *R. intestinalis* converted xylan to butyrate, releasing hydrogen for acetogenesis. Also observed was cross-feeding by *R. intestinalis* on the acetate produced by the acetogen, resulting in higher
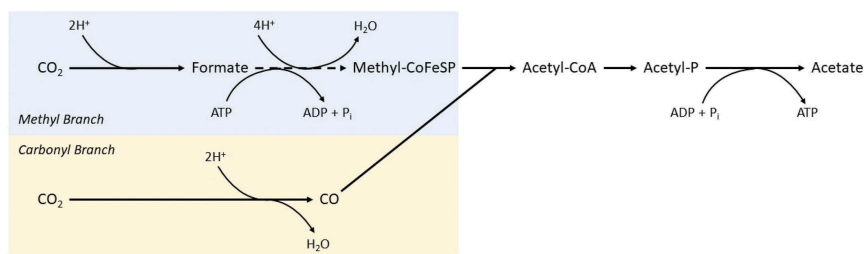

Figure 4. Simplified diagram of the Wood-Ljungdahl pathway. The dotted arrow indicates multiple reactions with a number of intermediates, leading to the formation of Methyl-CoFeSP (Co/Fe-containing corrinoid iron-sulphur protein), which is removed during the conversion to Acetyl-CoA. See refs. [11] and [127] for full pathway descriptions.

butyrate production by the co-culture than the monoculture.

Competition between the hydrogenotrophic functional groups

The competition between the hydrogenotrophic functional groups can be investigated by considering the biochemistry of the reactions performed by each group. Sulfate reduction is thermodynamically the most favorable of the three, and has a Gibbs free energy change of -152.2 kJ mol^{-1}, compared to -130 kJ mol^{-1} for methanogenesis and -95 kJ mol^{-1} for acetogenesis.[10,19,59] Moreover, of the three functional groups, SRB have the greatest affinity for hydrogen (lowest half-saturation constant, K_S,[60] significantly lower than methanogens). Methanogens have a far lower hydrogen concentration threshold than acetogens for growth on this substrate (a minimum of 116 ± 20 ppm for *M. smithii*, compared to a minimum $1,100 \pm 200$ ppm for acetogens, although this number varies widely between strains[61]), which together demonstrate the hierarchical nature of hydrogen usage capability between the three functional groups. SRB should be favored in an environment with abundant substrate, however a lack of sufficient sulfate has been shown to negate their advantage.[42] Between methanogens and acetogens, the methanogenic population should reduce the concentration of hydrogen to such an extent as to be unavailable for use in the Wood-Ljungdahl pathway.[59] However, as shown by observational studies, low pH can improve selection for SRB and acetogens due to its inhibitory effect on methanogens.[36]

The domination of mixed fecal cultures by SRB was clearly demonstrated more than 30 years ago. Gibson et al. found significant methane production by fecal cultures sampled from individuals with a positive methane breath test.[62] However, when these cultures were mixed with non-methanogenic fecal cultures, methane production was almost completely inhibited, with high sulfide production recorded instead. Methane production was recoverable only when sulfate reduction was chemically inhibited. Acetate production was not measured, but could be expected to have remained relatively consistent between cultures, as reductive acetogenesis should not be favorable in competition with sulfate-reduction and methanogenesis. This result is in contrast with that of a later experiment that showed, when following a very similar protocol, that methanogenesis dominated cultures grown from a mixture of methanogenic and non-methanogenic fecal samples.[63] As stated by the authors of the second publication, the two results are difficult to reconcile. They hypothesized that the thermodynamic advantages of SRB may not be correct for all species in the GIT, however there is also the possibility that pH played a role. pH was not recorded in the work of Gibson et al.,[62] whereas it remained at pH 6.8 or higher in the experiments of Strocchi et al.[63] It is therefore possible that pH was lower in the earlier experimentation, which would have been more favorable to the SRB.

Limited data exists on the direct co-culture combination of the hydrogenotrophic functional groups. However, some inference can be made from studies in gnotobiotic mice. Samuel et al. colonized mice with *Bacteroides thetaiotaomicron* and then either the methanogen *M. smithii* or the SRB *D. piger*.[64] *B. thetaiotaomicron* produced mainly acetate when combined with *M. smithii*, and this association rapidly reached much higher intestinal population densities than the SRB combination, as well as increasing host adiposity compared with both the SRB combination and mono-association with *B. thetaiotaomicron*. Significant gene expression changes were detected in *B. thetaiotaomicron* when in combination with *M. smithii*, including upregulation of the expression of genes involved in the production of the enzyme pyruvate-formate lyase. This points to increased production of formate, which can be utilized by *M. smithii*. This was combined with a shift to more acetate production in the methanogenic association, with reduced propionate and butyrate. The cause of this change was thought to be the observed reduction in the cecal NADH/NAD$^+$ ratio, which indicated that the metabolism of *M. smithii* created a more favorable environment for the breakdown of sugars by *B. thetaiotaomicron*. By contrast, only minimal gene expression changes were detected in the SRB association. Unfortunately, no association with both the methanogen and the SRB was undertaken to ascertain direct competition dynamics, so we cannot determine whether the effect of *M. smithii* upon *B. thetaiotaomicron* metabolism would persist in the presence of *D. piger*, or which of the hydrogenotrophs

would dominate the GIT environment in this case. From a biochemistry perspective, the extinction of *M. smithii* due to competitive exclusion would be expected. However, the more successful association observed between the methanogen and *B. thetaiotaomicron* casts doubt upon this hypothesis.

Acetogen cross-feeding in the mouse GIT has also been demonstrated between *B. thetaiotaomicron* and *B. hydrogenotrophica*, a combination that increased the efficiency of fermentation by *B. thetaiotaomicron*.[65] Similarly to the *M. smithii* bi-associations of Samuel et al.,[64] the presence of the acetogen reduced the NADH/NAD$^+$ ratio in fecal samples, implying that the regeneration of NAD$^+$ is facilitated by the reductive acetogenesis carried out by *B. hydrogenotrophica*. There is, as yet, no evidence to support the reduction of the NADH/NAD$^+$ ratio by an SRB in the mouse GIT. This may be due to the scarcity of studies of this nature, but may also imply that SRB are of little benefit in the facilitation of more efficient substrate breakdown by carbohydrate degraders.

It is important to note that the three functional groups will not always be in competition, since hydrogen is only essential to the methanogens. A mutualistic relationship has been observed between the SRB *Desulfovibrio vulgaris* and the methanogen *Methanococcus maripaludis in vitro*.[66] The SRB is able to convert lactate to acetate, CO_2 and hydrogen, whilst the methanogen utilizes the released CO_2 and hydrogen for methane production. This cross-feeding reduces the partial pressure of hydrogen, creating a more favorable metabolic environment for the SRB. In this particular combination, *D. vulgaris* acts as a net hydrogen producer, indicating that competition between the functional groups is environmentally dependent. Sulfate was not present in the medium in sufficient quantity for metabolism by *D. vulgaris*, thus preventing competitive interaction.

More recently, Rey et al. examined the behavior of *D. piger* using both mouse models and an *in vitro* study.[67] The SRB was introduced to gnotobiotic mice that had been associated with a consortia of eight non-hydrogenotrophic human GIT bacteria. The authors hypothesized that the observed enhanced growth of *D. piger* was due to both hydrogen production and the degradation of sulfur-containing host mucins by other members of the consortia,

providing free sulfate and hydrogen for *D. piger*. This relationship was confirmed with *in vitro* co-cultures of *D. piger* and the mucin degrader *B. thetaiotaomicron*. In co-culture with the wild type *B. thetaiotaomicron* in medium supplemented with chondroitin sulfate, the SRB showed good growth, and high levels of hydrogen sulfide were produced. However, co-cultures including an engineered *B. thetaiotaomicron* strain unable to synthesize a certain sulfatase enzyme, resulted in reduced overall culture growth and sulfide production. Neither bacterium could release free sulfate from the chondroitin sulfate compound. Applying this to mucin in the GIT environment, *D. piger* grew better in mice associated with the wild type *B. thetaiotaomicron* than those harboring the sulfatase mutant. This provides compelling evidence for cross-feeding by *D. piger* on sulfate released by the mucin degradation of *B. thetaiotaomicron in vivo*. Moreover, additional sulfate supplemented to the diets of these mice showed little effect on *D. piger* growth, implying that it obtains sufficient sulfate from host secretions in the presence of the mucin degrader. We would expect that in the bi-association including the *B. thetaiotaomicron* sulfatase mutant, supplementary sulfate would enhance the growth of *D. piger* only, but this scenario does not appear to have been investigated.

The lack of any published data regarding the direct *in vivo* or *in vitro* competition between all three hydrogenotrophic functional groups means that it is impossible to conclude which is likely to dominate in the GIT. Moreover, the relatively inaccessible nature of the human GIT means that direct measurement of hydrogen flux between members of the microbiota is extremely difficult. One recently posed strategy for tracking hydrogen *in vitro* and *in vivo* is the use of stable isotope probing coupled with Raman spectroscopy to analyze the flux of labelled hydrogen (for a review, see ref. [68]) This technique has to date only been explored in a few experiments, but has the potential to be an important tool in microbiota experimentation. Promise is also being shown by telemetric capsules, which are able to measure luminal gas concentrations throughout the GIT in real time.[69] We expect to see this technique applied in the near future for the study of hydrogenotrophy and methane production.

The current lack of experimental data on hydrogenotroph competition encourages the construction of an *in vivo* or culture-based model for such a combination. Alternatively, mathematical modelling could be used to make predictions of coculture dynamics based on monoculture data, but any such approach would benefit from the construction of the experimental system for verification of model results. Although these data are currently unavailable, there exists a wealth of literature concerning the hydrogenotrophic population of human fecal samples, upon which many etiological hypotheses have been based.

The balance between hydrogenotrophic functional groups in the gastrointestinal tract

There have been a vast number of studies, particularly over the past 30 years, which have attempted to ascertain the balance between the three hydrogenotrophic functional groups in the human microbiota. The majority of these studies have relied upon analysis of fecal samples or fecal cultures, coupled with breath-testing for hydrogen and methane. A number of such studies suggested that methanogens and SRB were mutually exclusive, or nearly so, in the GIT due to competition for hydrogen, the only mutual substrate of the two. Gibson et al. studied two groups of 20 individuals from the UK and South Africa to determine the methanogenic and sulfate-reducing potential of each.[41] Significant differences in the dominant functional group were found between the two locations, with most of the rural South Africans being predominantly methanogenic, whereas the UK cohort had higher levels of SRB in their feces. In both sample groups, individuals who were methane producers rarely had SRB detected in their feces, and those with high concentrations of SRB did not produce methane. However, enumeration of bacteria was performed solely *via* culture-based methods in this work, and methane status was determined by breath testing, a technique that has been shown to return negative results in individuals who carry methanogens in low concentrations,[70] and therefore is not conclusive in determining the presence of methanogens in the GIT.[9,30,71]

In a later assessment of 30 South African volunteers, cultured samples from methane-positive individuals were shown to have lower rates of both sulfate-reduction and acetogenesis than those from methane-negative individuals.[36] Further work by the same authors with a larger cohort of volunteers allowed for separation of individuals into three distinct classes: methanogenic, with no SRB detected in feces; methanogenic with low numbers of SRB detected in feces; and non-methanogenic with high SRB counts in feces.[72] Of the 87 individuals tested in this later study, only nine fell into the second class, providing further evidence for the mostly mutually exclusive nature of SRB and methanogens.

At a similar time, Macfarlane et al. found that the intestinal contents of sudden death victims followed the same pattern, harboring predominately either a methanogenic or a sulfate-reducing community.[73] Although only two individuals were examined, the more complete examination of the microbiota permitted in sudden death victims agreed with the classing of volunteers proposed by Gibson et al.[72]

However, there exist a number of counter-examples to this mutual exclusivity. The majority of the volunteer cohort tested by Pochart et al. were shown to harbor both methanogens and SRB.[74] Following a similar protocol to Gibson et al.,[41] the volunteers were split into methane-excreting and non-excreting groups by means of breath-testing before fecal analysis was performed. All of the methane-excreting individuals harbored both methanogens and SRB, as did most of the non-excretors. There were those individuals who had SRB but no methanogens in their samples, so the cohort could be divided in the manner of Gibson et al.,[72] but with very different proportions of the cohort in each class. The methanogen-free group was in the minority in this study. The sample size of 19 was small, but this group later assayed another cohort of 19, and found no significant difference in SRB counts between individuals who excreted methane and those who did not.[22] El Oufir et al. found that, although SRB cell counts differed significantly between methane-positive and negative individuals in their study cohort, and negatively correlated with methanogen counts, every individual tested had detectable SRB

in their feces, regardless of methane excretor status.[75] Suarez et al.[76] found that methane and sulfide were both present in the flatus of their study subjects, giving further evidence against mutual exclusivity.

Such contrasting results, from such a large number of separate studies, indicate that there is potentially a complex pattern to hydrogenotroph colonization that has not yet been clearly identified. We cannot discount the possibility that the varied conclusions from different investigations is influenced by the different geographic and ethnic backgrounds of the individuals studied, as observed by Gibson et al.[41] More recent comparative analysis of microbiome data from a number of countries indeed found significantly higher similarity in the microbiota composition of individuals from the same country than from different countries.[77] In recent times, due to new evidence and the contradictory nature of previous data, the hypothesis of mutual exclusion has been discounted. No significant relationship between the presence or abundance of methanogens and SRB was found by Hansen et al. when sequencing fecal samples from 68 sets of twins.[33] However, they did find a significant relationship between the presence of methanogens and the abundance of several clusters within the Firmicutes phylum, which may be explained by the high hydrogen production of strains in these clusters, suggesting a further possible variable affecting hydrogenotroph colonization.

This was not the first instance of positive associations between specific hydrogenotrophs and other bacterial functional groups studied. In examining the earlier hypothesis that high methanogen counts were related to increased counts of cellulolytic bacteria,[78] Chassard et al. sampled a larger group of volunteers and observed a positive relationship between the two.[28] Although increased cellulolytic activity was not found on all cellulose sources, it was observed that methane-negative fecal samples tended to contain a greater proportion of Gram-negative bacteria, whereas methane positive samples were associated with more Gram-positive bacteria. This may be reflective of the aforementioned greater hydrogen production by the Gram-positive Firmicutes, resulting in higher concentrations of available substrate for methanogens.[17] However, bacterial isolation was performed in samples taken from only 7 of the 49 volunteers originally sampled (3 methane negative and 4 methane positive). Therefore, while there is evidence for some link between methanogen concentrations and the presence of the cellulolytic strains targeted by these authors, drawing conclusions about a dominant cellulolytic phylum associated with methanogenesis requires a larger study. There was also no investigation on the effect of these cellulolytic bacteria on the other hydrogenotrophic functional groups. Correlations between functional groups observed in studies such as that of Hansen et al.[33] ought to provoke more culture-based studies of the kind performed by Chassard et al.,[28] to better understand the mechanism driving the positive association.

Separate to the relationship between SRB and methanogen colonization, there may be an observable relationship between these functional groups and the acetogens. A negative correlation has been documented between acetogens and methanogens, implying that perhaps there was some competition between these two functional groups, not necessarily involving SRB.[22] Similar relationships between acetogen and methanogen abundance has been found elsewhere.[23,59,79,80] Recent microbiome data analysis of fecal samples from 106 Japanese individuals was unable to detect *M. smithii* in 92% of individuals. This constituted significantly lower methanogen colonization than was found in all eleven other countries considered in this study, and was coupled with an increased abundance of members of the genus *Blautia*.[77] Sulfate-reducing bacteria, by contrast, did not vary greatly between countries. Bernalier et al. found that though SRB counts did not vary widely between the individuals tested in their experiment, mean acetogen counts were significantly lower in individuals with high methanogen counts.[59] Moreover, when methanogens were chemically inhibited in fecal cultures from methane-producing individuals, total acetate production by the culture was threefold greater than that of the original fecal culture. This implies that while methanogens competed effectively for hydrogen, acetogens were always present in these samples, and converted hydrogen to acetate in meaningful concentrations only in the absence of competition for hydrogen. This is consistent with

the thermodynamic and threshold hydrogen concentration restrictions presented earlier.

Aside from the direct competition between functional groups, there may also be non-microbial factors that strongly influence the relative abundance of each group. El Oufir et al. found that treatment of volunteers with a costive increased methanogen counts and decreased SRB counts in feces, with the converse true during treatment with a laxative.[75] Their findings point to a link between GIT transit time and the predominant mechanism of hydrogen consumption. The observed slow growth rates of methanogens support the hypothesis that reduced transit time negatively affects their population size.[81]

In considering the colonization of the colon by hydrogenotrophs, we must also consider spatial variables, as environmental conditions are not uniform throughout the GIT. An *in vitro* 3-stage model of the human colon was inoculated with a fecal slurry containing both methanogenic and sulfate-reducing microbes to analyze their *in situ* competition and the effect of endogenous mucins on this interaction.[42] The three vessels were of different volumes, arranged in sequence and maintained at fixed pH levels. Methane production was consistently greater in the latter two stages, representative of the transverse and distal colon, where the pH was higher (pH 6.5 and 7). However, once the continuous infusion of sulfated mucins to the model was initiated, methanogenesis was strongly inhibited. Sulfide production increased dramatically upon addition of mucins due to the increased availability of sulfate, most notably in the latter two vessels, where methanogenesis had been highest previously. Therefore, the differing availability of sulfated mucins in different parts of the colon will affect the dominance of SRB.

In contrast, a recent study of mice inoculated with *M. smithii* found that methanogen counts were highest in the small intestine, in disagreement with previous thinking.[82] To add to the lack of consensus in the observed data, a biopsy study examining the population of hydrogenotrophs present in the colonic mucosa at various sites in 25 healthy individuals concluded that, although gene copies from each of the three hydrogenotrophic functional groups were present in every volunteer tested, there was no obvious

pattern of spatial colonization.[24] A further contrast with previous results was the finding that SRB gene copies were higher than those of acetogenic bacteria in the proximal colon, with the opposite found in the distal colon. Methanogenic genes varied most widely in quantity between sites and individuals, from undetectable to 10^8 copies/g, with no clear pattern.

The contrasting results on the balance of hydrogenotrophs obtained in different experiments require better explanation. Although there is now irrefutable evidence to show that all three functional groups coexist in the human GIT, competition for hydrogen is likely still a driving force in determining the relative population sizes of each group.[71] Also, if an individual appears to harbor predominantly one hydrogenotroph at one sampling, this does not necessarily imply that the situation will remain so, as dominant functional groups have been shown to change over time.[83] The great influence of diet on hydrogenotroph population also cannot be ignored in this area of research. Changes in dietary sulfate, for example, have been shown to transiently alter both methanogen and SRB population sizes in the GIT,[84] an aspect that is not always included in the discussion of hydrogenotroph colonization. Despite a reasonably long history of analysis, we still have an insufficient understanding of the underlying causes for both the spatial distribution and inter-individual differences of hydrogenotrophic colonization of the human GIT. Without this knowledge, deriving the distribution of hydrogenotrophs most conducive to optimal nutrition and health remains a challenge.

The health implications of hydrogen in the gastrointestinal tract

Hydrogen itself has been shown to have both beneficial and detrimental health impacts. The build-up of hydrogen gas is a source of GIT discomfort in both adults and infants.[17,85] However, hydrogen gas can also be used as a therapeutic agent, particularly when administered in drinking water. Hydrogen-dissolved water has been shown to reduce oxidative stress via the removal of reactive oxygen species, with knock-on effects such as reducing the symptoms of obesity, metabolic

syndrome and Parkinson's disease (for reviews, see refs. [86] and [87]). Furthermore, hydrogen is a stable and safe molecule that can easily be transferred among cells, making it an attractive treatment.[86] The fact that patients with Parkinson's disease have a lower abundance of hydrogen-producing bacteria in their microbiota suggests that the hydrogen produced in the GIT may have an impact on the etiology of this degenerative disease.[87,88] The removal of hydrogen by hydrogenotrophs would then also contribute to the balance of hydrogen, which must be considered alongside the health impacts of the products of these cross-feeders.

Health impacts of hydrogen cross-feeding on the host

The products of hydrogen cross-feeding have been implicated in a variety of disease states, justifying the study of hydrogenotrophic microbes from a medical perspective. Lactate or hydrogen build-up in the GIT has been linked to colic symptoms in a recent study of 40 infants.[85] Although it is difficult to obtain solid information from their infant cohort study, mainly due to the difficulty in diagnosing colic and the variation in symptoms between individuals, the accompanying *in vitro* studies provide data on SRB in co-culture with hydrogen producers. Large volumes of hydrogen were produced by the prominent infant microbiota bacteria *Veillonella ratti* in monoculture, which the authors suggest contributes to discomfort. However, co-cultures of this hydrogen producer with the hydrogen-utilizing SRB *D. piger* resulted in greatly reduced hydrogen concentrations. Any therapeutic reduction in hydrogen levels must be weighed up against the increased hydrogen sulfide concentrations caused by the SRB. Whether a similar effect, without the production of hydrogen sulfide, could be achieved by methanogens has not been investigated. Methanogens have been isolated from both the gastric juice of newborns,[89] and from infant fecal samples.[30,70] It is thought that colonization of the infant by *M. smithii* originates from the mother, potentially through breast-feeding. However, analysis of breast-milk from 20 women found archaeal sequences in only 8,[90] implying that inoculation from other sources may be responsible. The study of hydrogenotrophs, and cross-feeding in general, in the

infant GIT is quite new. It is well-established that the infant microbiota is quite different to that of an adult and shows significant interindividual variation (for a review, see ref. [91]), but current data on the relationship between SRB and methanogens in this environment, and any health impacts of this relationship, is not available.

Hydrogen sulfide and/or the SRB that produce it have been found at higher concentrations in patients suffering from IBS,[92,93] ulcerative colitis[80,94,95] (although this could not be confirmed in all studies[96]) and colorectal cancer (CRC).[97,98] Hydrogen sulfide is also implicated in increased risk of DNA damage[99,100] and increased risk of developing CRC.[101] However, there also exist potentially health-promoting effects of hydrogen sulfide, such as maintaining mucus layer integrity,[102] and a potential cardioprotective role (for a review, see ref. [103]) It should also be noted that hydrogen sulfide is not exclusively produced by SRB. Human cells also produce hydrogen sulfide, predominantly as a signaling molecule, which is kept below toxic concentrations *via* oxidation at the mitochondria (for a review, see ref. [104]) This oxidation contributes electrons to the electron transport chain, therefore both human and bacterially produced sulfide may also be an energy source for the host.[105] Furthermore, microbial fermentation of sulfur-containing amino acids, such as cysteine, can also lead to hydrogen sulfide formation, and may, in some individuals, be a more significant source of sulfide than sulfate reduction.[106]

Methane has links to health issues also, although the literature is sparser on this topic in comparison to hydrogen sulfide. Higher methane production has been associated with constipation, including IBS-related constipation (IBS-C),[107–110] and increased GIT transit time.[75] However, microbiome analysis has shown that the abundance of methanogens was lower in IBS individuals, particularly those with IBS-C.[111] These studies only show associations and not causality, and a consensus has not been reached on the role of methanogens in IBS (for reviews, see refs. [25,110] and [112]). However, infusion of methane into the small intestinal tracts of dogs has been shown to increase transit time by affecting muscular motility.[113] As previously discussed, methanogen counts have been shown to positively correlate with increased transit

time.[75] It is possible, therefore, that a positive feedback loop may exist between increased transit time and increased methanogen counts, but this requires experimental validation.

A separate area that has received much attention is the role of methanogenesis in obesity (for a review, see ref. [114]) Turnbaugh et al. found that genetically obese mice harbored higher levels of methanogens than their lean litter mates.[115] It was hypothesized that the consumption of hydrogen by methanogens improved the efficiency of carbohydrate metabolism by the microbiota, resulting in the observed increase in SCFA concentrations. Mice harboring high concentrations of methanogens therefore received a greater energy yield from their feed, which was confirmed by the lower residual energy found in their feces. This effect, due to the consumption of hydrogen in the GIT, may also result from the action of other hydrogenotrophs. Indeed, enzymes involved in reductive acetogenesis were significantly enriched in genetically obese mice. The most impressive result of this study was the fact that lean mice, when inoculated with the microbiota of an obese littermate, showed significant weight gain with no greater chow consumption. However, methanogens were one of many functional groups that varied between the lean and obese microbiota. Further work with mice inoculated with methanogens has implicated a high-fat diet as a cause for significant increases in both fecal methanogen counts and body weight, but does not provide evidence that methanogens are causative of obesity.[82] Contrastingly, two large trials involving qPCR analysis of feces from lean and obese humans showed depleted *M. smithii* counts in obese individuals, though did not pose direct mechanisms by which this species influences host adiposity.[15,31] The impact of methanogenesis on obesity remains far from clear.

Mitigation of the harmful effects of hydrogen cross-feeding

There have been several attempts to counter the negative health effects related to hydrogen metabolism in the GIT. The simplest interventions are dietary. Longer chain length prebiotics (dietary ingredients that can selectively enhance beneficial components of the indigenous GIT microbiota[116]) have been shown to produce less gases and at a reduced rate in *in vitro* fecal cultures compared to short chain prebiotics.[117] Moreover, fecal cultures grown with prebiotics such as resistant starch and fructooligosaccharides showed reduced hydrogen sulfide generation.[106] If methane reduction in humans becomes a desirable health outcome, then full advantage should also be taken of the results of studies conducted towards lessening the environmental impact of agriculture through reducing methane emissions from ruminants. Knapp et al. reviewed a number of methane-reducing techniques applied in the dairy industry, such as: increasing starch intake in the diet, which may shift bacterial fermentation in the rumen towards a higher propionate production, thus reducing the hydrogen available to methanogens; decreasing the passage time through the GIT, thus inhibiting methanogen population growth; and biological controls such as immunization, which directly target methanogens.[118] Supplementing the diets of methanogenic humans with sulfate has been shown to reduce methanogenesis in half of the individuals tested, although whether this effect would last longer than the 10 days of the trial was not investigated.[84] There have also been experiments in which acetogens have been used to reduce the formation of methane in the rumen or *in vitro*, but with limited success.[119,120] Whether such techniques will be necessary or effective in the human GIT is yet to be determined.

Methanogenesis and sulfate reduction both have links to negative health outcomes, making acetogenesis appear the more attractive manner in which to dispose of enteric hydrogen. The formation of acetate from free hydrogen constitutes a more energy efficient metabolic pathway for the host, as acetate may be absorbed into the bloodstream and used as an energy source.[56] Study of the kangaroo foregut, which bears similarity to the rumen, has shown that reductive acetogenesis can prevail over methanogenesis, despite its thermodynamic deficiency.[121] Methane production by the kangaroo microbiota in both *in situ* assays using stable-isotope probing and *in vitro* fermentations was minimal, while abundant incorporation of CO_2 into acetate was also shown. Several differences exist between the bovine and kangaroo

microbiota, but of note is that the acetogen *Blautia coccoides* was found in samples from all kangaroos tested, whereas only 40% of bovine samples contained this bacterium, and at a lower mean abundance. Acetogens can clearly perform the role of principal hydrogen consumer effectively, and have also been shown to be a viable replacement for methanogens in the lamb rumen,[122] however the mechanism by which methanogenesis is repressed in the kangaroo is unclear. Nor is any efficient mechanism for continued methanogen control in the rumen yet available.[123] Although this means that human nutrition cannot immediately benefit from animal research, cooperation between researchers on microbial hydrogen cross-feeding in the human and animal fields should be encouraged, as similar difficulties are faced by each. The motivation for study may differ between the two, with human health the focus of one and methane emission reduction often the goal of the other, but it can be expected that progress in one will aid progress in the other.

Future directions

Although many studies of bacterial cross-feeding leading to the production of SCFA have been published, few have considered the impact of hydrogen removal by hydrogenotrophic cross-feeders. It is unclear whether different results may have been obtained in these experiments if a hydrogenotrophic microbe had been included. In some cases, SCFA production may have increased, due to the mutually beneficial act of reducing the partial pressure of hydrogen performed by hydrogenotrophs. However, it is possible that the relationship would be more complex. It is conceivable, for example, that the inclusion of an SRB in an SCFA cross-feeding experiment may result in competitive cross-feeding for intermediates such as lactate, resulting in reduced SCFA yields, as observed by Marquet et al.[39] The versatile nature of many hydrogenotrophic strains makes prediction of the outcome of such cultures difficult. However, existing data from separate experiments could be combined into a predictive model, to give structured analytical backing to predictions.

Large-scale mathematical models of the human GIT microbiota that consider the removal of hydrogen via cross-feeding do exist. Kettle et al. present an example use of their microPop model in applying it to GIT fermentation.[124] The mathematical model considers the metabolites produced by a number of functional groups of bacteria, two of which are hydrogen-utilizing. However, this model has not been designed to investigate the products of hydrogen metabolism, as displayed by the lack of a sulfide-producing functional group. Such a model framework could easily be extended and adapted for the investigation of hydrogen cross-feeding in the GIT, once there exists a better understanding of how these microbes behave in the wider consortia.

The clear impacts of hydrogen cross-feeding on human health and nutrition should provoke further investigation of GIT hydrogenotrophs. The effect of both hydrogen and its subsequent metabolites on areas such as infant GIT discomfort, functional GIT disorders in adults, and energy yield from food, are not well understood. The first step in the greater understanding of these effects is to ascertain how the three functional groups behave in co-culture, and in the wider community of the microbiota.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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