The Links Between Human Breath Methane, Dietary Fibre Digestion, and the Gut Microbiota

A thesis presented in partial fulfilment of the requirements
for the degree of

Doctor of Philosophy
in
Nutrition Science

at Massey University Manawatu,
New Zealand.

Laura Marie Payling

2022

Abstract

The concentration of methane that is exhaled in human breath has been associated with the composition and fibre-fermenting function of the human colonic microbiota. The current research aimed to investigate whether composition and fibre fermentation function of the colonic microbiota differ in individuals who are low breath methane emitters (LE) or high breath methane emitters (HE).

Healthy adult individuals (18) were recruited and breath tested. Unexpectedly, they showed positive correlations between breath hydrogen and methane. Then, the highest and lowest breath methane emitters provided faecal samples used for shotgun metagenomic sequencing and as faecal inocula for *in vitro* colonic fermentations with dietary fibres (β-glucan and lignocellulose). Individuals who were LE reported higher dietary vitamin E, fibre, and fat intakes and a *Bacteroides*-driven microbiota composition compared to HE individuals who reported a greater starch intake and a *Prevotella*-driven microbiota composition.

The faecal microbiota from individuals who were HE had a greater abundance of taxa from the *Methanobrevibacter* genus and more methane gas production during *in vitro* colonic fibre fermentation compared to the microbiota of individuals who were LE; however, the results were variable within the HE group. There was a greater rate and extent of dietary fibre fermentation in LE compared to HE individuals during *in vitro* colonic fermentation, which aligned with the greater fibre intakes of LE individuals. Furthermore, the faecal microbiota of LE individuals showed increased beneficial organic acid

production and a greater abundance of functional pathways related to amino acid metabolism compared to HE during *in vitro* colonic fermentation.

These results did not align with published research on human breath methane and the gut microbiota. However, there was consensus with emerging hypotheses suggesting that there are important pathways involved in hydrogen sulphide production and hydrogen utilisation that are largely unexplored. Further investigations in these areas could help redefine our understanding of fibre fermentation by the human colonic microbiota.

Acknowledgements

My first thanks go to my chief supervisor Professor Warren McNabb. Warren had an important role in guiding the planning and development of my project, as well as interpreting data and decision-making. Warren was a brilliant mentor and defiantly supported me during my time at Massey. Dr Karl Fraser helped with metabolomic data analysis, aligning my experiments into a thesis, and overall moral support. Professor Nicole Roy and Dr Simon Loveday helped by intricately reviewing drafts of Chapters and the final thesis with their excellent attention to detail. Dr Dragana Gagic helped with microbiota data analysis and the planning and reviewing of Chapter 4. Dr Ian Sims helped with understanding, measuring and interpreting the chemical composition of carbohydrate substrates that were used as fermentation substrates.

Thank you to the Tertiary Education Commission for funding my PhD project through the Riddet Institute Centre of Research Excellence and Massey University, and to AgResearch Ltd. for providing state-of-the-art laboratories. The Riddet Institute and AgResearch Ltd. also provided many development opportunities for which I am grateful, including presentation skills and statistics workshops, leading change workshops, and strategy meetings. The opportunities at the Riddet Institute were largely orchestrated by John Henley-King, Dr Sarah Golding, and the Riddet Institute Student Society, thank you!

Thank you to my colleagues who helped in different areas of my project. Dr Stefan Muetzel, Dr Peter Janssen, German Molano, Paul Maclean, Dr Wayne Young, Dr Jane Mullaney and Dr Dulantha Ulluwishewa are some from

AgResearch Ltd. who must be mentioned for their help in ideation, experimental design, and sample analysis.

Huge thanks go to Dr Stefan Hill and Dr Laura Raymond of Scion, who hosted me at their laboratory in Rotorua and kindly taught me how to work with nuclear magnetic resonance spectroscopy.

My appreciation also goes to Kate Morland of Studio Rubix for coaching me to become a Registered Nutritionist alongside my PhD. Learning and practising nutrition and dietetics kept me grounded and constantly challenged me to relate my research to the real world.

Thank you to Rita Ann Carey. Rita provided support, friendship, and a peaceful place for me to finish writing.

Finally, thanks go to my parents, my friends from England, and my partner William for their unwavering confidence in me, love, and support. Thanks for helping me find my path when I got lost several times along the way.

Contents

List of Figures

List of Tables

List of Abbreviations

ANOVA	Analysis of variance
BMI	Body mass index
CAZymes	Carbohydrate-active enzymes
CFU	Colony forming units
GC	Gas chromatograph
GIT	Gastrointestinal tract
HE	High breath methane emitters
LE	Low breath methane emitters
NMR	Nuclear magnetic resonance spectroscopy
NSP	Non-starch polysaccharides
PCA	Principal component analysis
PCoA	Principle coordinates analysis
PERMANOVA	Permutational multivariate analysis of variance
RS	Resistant starches
SparCC	Sparse correlations for compositional data
SRB	Sulphate-reducing bacteria
TSP	3-(trimethylsilyl)-propionate acid-d4

List of Supplementary Materials

Supplementary File 1	Serves of common food groups by the Australian and New Zealand Guides for Healthy Eating
Supplementary File 2	Spearman's correlations between diet and metadata
Supplementary File 3	Boxplots of alpha-diversity indices of samples from high and low breath methane emitters
Supplementary File 4	PERMANOVA of beta-diversity with diet and metadata
Supplementary File 5	PERMANOVA of microbial composition (feature level) and diet
Supplementary File 6	Spearman's correlations between microbiota composition and diet
Supplementary File 7	Spearman's correlations of KEGG pathways
Supplementary File 8	Spearman's correlations of KEGG pathways and phyla relative abundance
Supplementary File 9	Scatterplot of the relative abundance of Euryarchaeota phylum and methane metabolism genes
Supplementary File 10	Mann Whitney U test of breath methane and microbiota genera and species
Supplementary File 11	Spearman's correlations of microbiota genera
Supplementary File 12	Mann Whitney U test of breath methane and KEGG pathways
Supplementary File 13	Scatterplot of data used for headspace gas volume calibrations
Supplementary File 14	Tables of alpha-diversity and PCoA of beta diversity of *in vitro* fermentation samples
Supplementary File 15	Kruskal-Wallis test of family and genera relative abundance over time
Supplementary File 16	Boxplots of the relative abundance of families and genera over time

Supplementary File 17	Kruskal-Wallis test of genera by substrate
Supplementary File 18	Boxplots of the relative abundance of microbial genera and species by substrate at 48 h colonic fermentation
Supplementary File 19	Kruskal-Wallis test of KEGG pathways and fermentation time point
Supplementary File 20	Boxplots of the relative abundance of KEGG pathways according to time point and substrate
Supplementary File 21	Kruskal-Wallis test of KEGG pathways by substrate
Supplementary File 22	Dot plots of the median organic matter concentration from *in vitro* fermentation samples
Supplementary File 23	Boxplots of alpha-diversity of *in vitro* colonic fermentation samples
Supplementary File 24	Mann Whitney U test of microbial abundance by breath methane at 0 h of colonic fermentation
Supplementary File 25	Boxplots of the relative abundance of microbial phyla and genera in colonic fermentation samples at 0 h
Supplementary File 26	Mann Whitney U test of KEGG pathways by colonic fermentation time point
Supplementary File 27	Spearman's correlations of microbial genera and potential functions
Supplementary File 28	Images of spectra from solution-state nuclear magnetic resonance and gas chromatography
Supplementary File 29	ANOVA of aqueous metabolites significant by time point
Supplementary File 30	Boxplots of metabolite concentrations according to time point and substrate

Publications

Payling, L., Fraser, K., Loveday, S.M., Sims, I., Roy, N., and McNabb, W. (2020). The effects of carbohydrate structure on the composition and functionality of the human gut microbiota. Trends in Food Science and Technology 97, 233–248. https://doi.org/10.1016/j.tifs.2020.01.009.

Payling, L., Roy, N.C., Fraser, K., Loveday, S.M., Sims, I.M., Hill, S. J., Raymond, L. G., and McNabb, W. C. (2021). A protocol combining breath testing and ex vivo fermentations to study the human gut microbiome. STAR Protocols 100227. https://doi.org/10.1016/j.xpro.2020.100227.

Payling, L., Roy, N.C., Fraser, K., Loveday, S.M., Sims, I.M., Gagic, D. and McNabb, W. C. In preparation for submission to The American Journal of Gastroenterology. The relationship between breath methane, breath hydrogen, the colonic microbiota, and diet (Data from Chapter 4).

Payling, L., Roy, N. C., Hill, S. J., Raymond, L. G., Fraser, K., Gagic, D., Loveday, S. M., Sims, I. M. and McNabb, W. C. In preparation for submission to The ISME Journal. The links between human breath methane, microbiota composition, and fibre fermentation in an *in vitro* colonic model (Data from Chapters 5 and 6).

Conference Abstracts and Presentations

Payling *et al.* 2018. How does fibre structure affect microbiota functionality? AgResearch Microbial Communities Symposium, oral presentation.

Payling *et al.* 2019. A study to investigate human breath methane and dietary fibre fermentation by the colonic microbiota. Focus on Fibre (Otago), oral presentation.

Payling *et al.* 2019. Dietary fibre from the microbiota's perspective. Food Structures, Digestion and Health (FSDH) Conference, poster presentation.

Payling *et al.* 2021. The production of hydrogen sulphide by the human gut microbiota. Riddet Institute Colloquium, oral presentation.

Payling *et al.* 2022. The production of hydrogen sulphide by the human gut microbiota. HVN Foodomics conference, poster presentation.

Training and Other Activities

Expert Sensory Panellist, Massey FEAST, 2018.

Writing and Producing a Science Story Competition, 2nd Place, Bazzacam Ltd., 2018.

Mass Cytometry Course, Malaghan Institute of Medical Research, 2018.

Intern, Studio Rubix Nutrition and Dietetics, 2018 – 2019.

Change Leader, AgResearch Organisational Transformation Initiative, 2019.

Business Enterprise and Innovation, Massey University, 2019.

Conference session chairperson, FSDH conference, 2019.

President, The Riddet Institute Student Society, 2019 – 2020.

Metagenomics Summer School, Genomics Aotearoa and University of Auckland, 2020.

Student Health and Safety Video Competition, Winner, Massey University, 2020.

Presentation Skills, Influence Consulting 2020 and 2021.

Student Communicator Award, Riddet Institute Colloquium, 2021.

Statistics Workshop for Scientists, Massey University, 2021.

mixOmics R Essentials for Biological Data Integration, University of Melbourne, 2021.

Associate Registered Nutritionist, Studio Rubix Nutrition and Dietetics, 2020 – 2022.

Chapter 1

General Introduction

Diet and nutrition are linked to non-communicable diseases that are responsible for more than 70 % of worldwide deaths (World Health Organisation, 2003, 2021). Non-communicable diseases are related to environmental and lifestyle factors that include the quality and quantity of food consumed and the interaction of food components with the gastrointestinal tract (GIT) microbiota (World Health Organisation, 2003), including the microbiota of the small intestine and the colon.

The microbiota describes the microorganisms that inhabit the human GIT and contain approximately 150 times more genes than the human genome (Qin *et al.*, 2010). This collection of microbial genomes (metagenomes) has a remarkable influence on the metabolism, immune system, and health status of the host. The colon harbours the densest population of microorganisms in the human body (the colonic microbiota) and has been an important focus for health and nutrition research in the last decade (Shanahan, 2012)

Several factors can disrupt the dynamics between the colonic microbiota and the host. These include antibiotic usage, a high-fat diet, alcohol, hygiene, immunodeficiency, hyper immunity, and lifestyle (Das and Nair, 2019). In addition, microbiota dysfunction has been linked with GIT disorders, non-communicable diseases, and allergenic disorders (Bull and Plummer, 2014).

However, the exact composition of a healthy microbiota is unknown. Many attempts have been made to describe a healthy microbiota, including its core composition, taxonomic groupings, variability over time, development with age, and function. It has been one of the most exciting research areas in biomedicine in recent years, yet studies fail to reach a consensus on what characterises a healthy colonic microbiota.

This lack of consensus is partly due to the wide range of methods used to study the microbiota. A team of experts have produced standard methods for handling and processing faecal samples for metagenomic analysis (Costea *et al.*, 2017). If adopted, these standards may improve the repeatability, comparability, and reproducibility of human microbiota research. However, using faecal samples as a proxy for the colonic microbiota is commonplace and adds further potential error to understanding the colonic microbiota.

Additionally, the inherent complexity and variability of the colonic microbiota are challenging. Variation in the colonic microbiota has been linked with age, environment, health, genetics, socioeconomic status, geography, pregnancy, diet, exercise, antibiotic usage, and surgery (National Academies of Sciences Engineering and Medicine, 2018). Of these, diet is a key influencer that is modifiable and practical. David *et al.* (2014) showed that changes in colonic microbiota composition were evident at the species level after one day of changing between a plant-based diet and an animal-based diet. At the family level, changes were observed one to four weeks after a dietary change (Bonder *et al.*, 2016), whilst variation at the genus level appeared to be controlled by long-term habitual diet (Wu *et al.*, 2011).

Different taxa are affected in different individuals when the microbiota changes rapidly with diet (Wu *et al.*, 2011). Therefore, dietary interventions such as probiotics and prebiotics often show mixed efficacy. Several studies have found that individuals with long-term high dietary fibre intakes have a microbiota that is more responsive to modulation than those with low dietary fibre intakes (Eid *et al.*, 2015; Brahma *et al.*, 2017; Healey *et al.*, 2018). There is great individual variability in response to food, and part of that can be explained and predicted

by microbiota composition. For example, individual blood glucose responses to meals have been predicted using physiological and microbiota data (Vanamala, Knight and Spector, 2015; Zeevi *et al*., 2015).

Research challenges must be overcome to understand what constitutes a healthy colonic microbiota. Only then can microbiota dysfunction begin to be addressed. That is part of the goal of the Riddet Institute's Centre of Research Excellence Research Platform 2.2, investigating interactions between food structure, digestion, and the role of the microbiota in GIT function.

References

1. Bonder, M., Tigchelaar, E., Cai, X., Trynka, G., Cenit, M., Hrdlickova, B., Zhong, H., Vatanen, T., Gevers, D., Wijmenga, C., Wange, Y., Zhernakova, A. (2016). The influence of a short-term gluten-free diet on the human gut microbiome. Genome Medicine, 8, 45.
2. Brahma, S., Martínez, I., Walter, J., Clarke, J., Gonzalez, T., Menon, R., and Rose, D.J. (2017). Impact of dietary pattern of the fecal donor on *in vitro* fermentation properties of whole grains and brans. Journal of Functional Foods 29, 281–289. https://doi.org/10.1016/j.jff.2016.12.042.
3. Bull, M.J., and Plummer, N.T. (2014). Part 1: The Human Gut Microbiome in Health and Disease. Integrative Medicine 13, 17–22. https://doi.org/10.1053/j.astro.2014.03.032.
4. Costea, P. I., Zeller, G., Sunagawa, S., Pelletier, E., Alberti, A., Levenez, F., Tramontano, M., Driessen, M., Hercog, R., Jung, F.-E., Kultima, J. R., Hayward, M. R., Coelho, L. P., Allen-Vercoe, E., Bertrand, L., Blaut, M., Brown, J. R. M., Carton, T., Cools-Portier, S., Bork, P. (2017). Towards standards for human fecal sample processing in metagenomic studies. Nature Biotechnology, 35(11), 1069–1076. https://doi.org/10.1038/nbt.3960.

5. Das, B., and Nair, G.B. (2019). Homeostasis and dysbiosis of the gut microbiome in health and disease. Journal of Biosciences 44, 1–8. https://doi.org/10.1007/s12038-019-9926-y.

6. David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A. V., Devlin, A.S., Varma, Y., Fischbach, M.A., *et al.* (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature 505, 559–563. https://doi.org/10.1038/nature12820.

7. Eid, N., Osmanova, H., Natchez, C., Walton, G., Costabile, A., Gibson, G., Rowland, I., and Spencer, J.P.E. (2015). Impact of palm date consumption on microbiota growth and large intestinal health: A randomised, controlled, cross-over, human intervention study. British Journal of Nutrition 114, 1226–1236. https://doi.org/10.1017/S0007114515002780.

8. Healey, G., Murphy, R., Butts, C., Brough, L., Whelan, K., and Coad, J. (2018). Habitual dietary fibre intake influences gut microbiota response to an inulin-type fructan prebiotic: a randomised, double-blind, placebo-controlled, cross-over, human intervention study. British Journal of Nutrition 119, 176–189. https://doi.org/10.1017/s0007114517003440.

9. National Academies of Sciences Engineering and Medicine (2018). Environmental Chemicals, the Human Microbiome, and Health Risk (Washington: The National Academies Press).

10. Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D. R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Zoetendal, E. (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464(7285), 59–65. https://doi.org/10.1038/nature08821.

11. Shanahan, F. (2012). The Colonic Microbiota and Colonic Disease. Current Gastroenterology Reports 14, 446–452. https://doi.org/10.1007/s11894-012-0281-5.

12. Vanamala, J.K.P., Knight, R., and Spector, T.D. (2015). Can Your Microbiome Tell You What to Eat? Cell Metabolism 22, 960–961. https://doi.org/10.1016/j.cmet.2015.11.009.

13. World Health Organisation (2003). Diet, nutrition and the prevention of chronic diseases. 102. https://doi.org/ISBN 92 4 120916 X ISSN 0512-3054 (NLM classification: QU 145).

14. World Health Organisation (2021). Noncommunicable diseases. Fact sheet (online). https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases.

15. Wu, G. D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y. Y., Keilbaugh, S. A., Bewtra, M., Knights, D., Walters, W. A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F. D., and Lewis, J. D. (2011). Linking long-term dietary patterns with gut microbial enterotypes. Science 334(6052), 105–108. https://doi.org/10.1126/science.1208344.

16. Zeevi, D., Korem, T., Zmora, N., Israeli, D., Rothschild, D., Weinberger, A., Ben-Yacov, O., Lador, D., Avnit-Sagi, T., Lotan-Pompan, M., Suez, J., Mahdi, J. A., Matot, E., Malka, G., Kosower, N., Rein, M., Zilberman-Schapira, G., Dohnalová, L., Pevsner-Fischer, M., Segal, E. (2015). Personalized Nutrition by Prediction of Glycemic Responses. Cell 163(5), 1079–1095. https://doi.org/10.1016/j.cell.2015.11.001.

Chapter 2
Literature Review

The Role of Diet and the Colonic Microbiota in Human Health

Parts of this Chapter were published in Payling, L., Fraser, K., Loveday, S.M., Sims, I., Roy, N., and McNabb, W. (2020). The effects of carbohydrate structure on the composition and functionality of the human gut microbiota. Trends in Food Science and Technology 97, 233–248. https://doi.org/10.1016/j.tifs.2020.01.009.

2.1 Abstract

The characteristics of a healthy human colonic microbiota may include diversity, keystone species abundance, and a functional enrichment for fibre fermentation and organic acid production. However, more research is needed to understand the precise links between factors and mechanisms of action.

Clinical investigations of the colonic microbiota have used breath testing to identify individuals with dysbiosis, using breath methane and hydrogen as markers. Research has suggested that individuals who are HE have a higher abundance of taxa from the Firmicutes phylum than individuals who are LE. The microbiota from HE individuals also showed a greater extent of insoluble fibre fermentation due to a keystone species called *Ruminococcus champanellensis*, which was absent in the microbiota from LE individuals. Further research must validate findings linking breath methane with microbiota composition and functionality. These steps are important toward the goal of rationally modulating the microbiota for optimal health.

2.2 Introduction

This literature review discusses and critically assesses published research on the role of diet in modulating the colonic microbiota of healthy humans. It addresses studies' limitations and highlights knowledge gaps for further investigation.

2.3 What is a Healthy Microbiota?

The colonic microbiota, a community of approximately 40 trillion microorganisms, is key in the relationship between diet and human health (Sender *et al.*, 2016). The colon harbours the densest population of microorganisms in the human body, at 10^{11} microbial cells per gram of digesta (Sender *et al.*, 2016) (Figure 1).

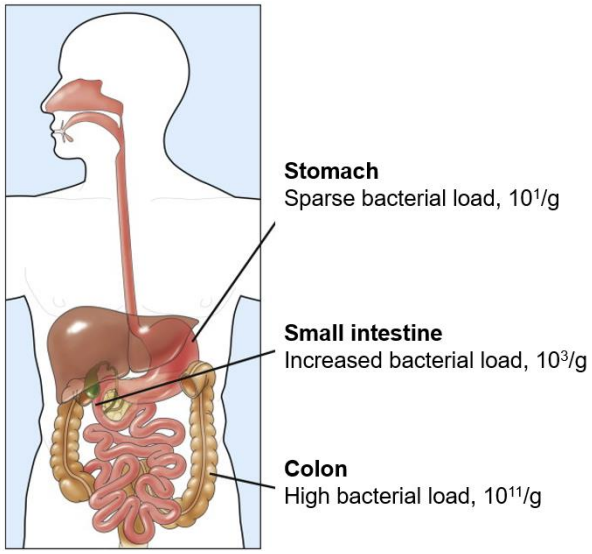


Figure 1 The density of microbial cells at different sites within the gastrointestinal tract. Figure adapted from Ohland and Jobin (2015) with permission.

The colonic microbiota ferments a range of dietary and endogenous materials for metabolism, including carbohydrates, proteins, and lipids. Protein fermentation uses dietary and endogenous proteins from host enzymes, mucin, and sloughed-off intestinal epithelial cells. Protein fermentation is greater at

higher pH; therefore, it is more prevalent in the distal colon than the proximal colon (Macfarlane *et al.*, 1992; Rowland *et al.*, 2018a). However, most microorganisms in the colon preferentially ferment carbohydrates, so adding fermentable carbohydrates, even at higher pH, reduces protein fermentation by approximately 60 % (Smith and Macfarlane, 1996).

The colonic microbiota can also degrade triglycerides and phospholipids into polar head groups and free lipids, although it is thought that the microbiota cannot utilise free lipids. However, free lipids affect microbiota composition through antimicrobial properties (Oliphant and Allen-Vercoe, 2019). Research on the role of the colonic microbiota in lipid metabolism is in its early stages compared to research on carbohydrate fermentation.

Most dietary proteins and lipids are digested in the upper GIT of humans, but many complex polysaccharides, including non-starch polysaccharides (NSP) and resistant starches (RS), are not. Humans lack the enzymatic repertoire to digest complex polysaccharides, i.e., dietary fibre. Instead, dietary fibre passes through the stomach and small intestine intact, and in the colon, it is mostly hydrolysed to carbohydrate fractions and fermented to metabolic end products. 10 to 60 g of dietary fibre reaches the colon daily, depending on the diet (Egert *et al.*, 2006; Power *et al.*, 2014). The resulting metabolites modify the functionality of the microbiota and the host (Egert *et al.*, 2006).

A healthy colonic microbiota ferments fibre, provides metabolites that are an energy source to mucosal cells and alters host metabolism, bile acid metabolism, vitamin synthesis, barrier function, inflammatory response modulation, immune development and regulation, enteric nervous system

development and function, and protection against pathogen colonisation (Kho and Lal, 2018).

Several cohort studies of healthy individuals found that the dominant bacterial phyla in the colon were Firmicutes, Bacteroidetes, and Actinobacteria, with Proteobacteria and Verrucomicrobia in lower numbers (Eckburg, 2005; Flint, Scott, Louis, *et al.*, 2012; Qin *et al.*, 2010; Tap *et al.*, 2009; Walker *et al.*, 2011a). Note, there is a recent publication (October 2021) of new nomenclature which renames many phyla (Oren & Garrity, 2021). Examples include Firmicutes, now Bacillota, and Bacteroidetes now Bacteroidota. Considering much of this thesis was constructed before the change, and the scientific literature is aligned with the original nomenclature, the original nomenclature is used throughout this thesis.

At the genus level, relative abundance is commonly dominated by *Bacteroides, Prevotella* (both from the Bacteroidetes phylum), or *Ruminococcus* (from the Firmicutes phylum). These patterns have been called enterotypes and are thought to be stable in an individual throughout adulthood. They are mostly determined by long-term carbohydrate and protein intakes (Cho and Blaser, 2012; Rinninella *et al.*, 2019). However, the stratification of colonic microbiota composition into enterotypes may be oversimplifying the complexity of the microbiota composition. Whilst enterotype categorisation helps understand complex microbial communities, dominant taxa may be better described on a continuum, such as *Bacteroides*- or *Prevotella*-driven (Jeffery *et al.*, 2012).

2.3.1 Diversity

The alpha-diversity of the colonic microbiota includes richness (number of species present) and evenness (distribution of species abundances). High

diversity is usually considered optimal for a healthy microbiota, much like a rainforest or reef (Turnbaugh *et al.*, 2009). Biodiversity provides functional redundancy, which diminishes the risk to human health when a species is lost due to environmental changes (Roy *et al.*, 2014). Furthermore, diversity is important for host homeostasis, considering that host-microbiota relationships govern nutrition, immunity, and metabolism.

Larsen and Claassen (2018) used a network theory model to show that increased colonic microbial diversity led to increased efficiency and resiliency of the community. When the colonic ecosystem was more diverse, fewer species interactions were required, equating to lower energy usage and increased efficiency. Furthermore, high diversity resulted in greater taxonomic stability and functional redundancy, reducing the impact of species loss (Larsen and Claassen, 2018).

Diet diversity, particularly dietary fibres and RS, promotes microbiota diversity. The degradation and fermentation of these substrates require a variety of carbohydrate-binding molecules and enzymes for hydrolysis. Diverse species meet these requirements. Sonnenburg *et al.* (2016) used a mouse model to show that reducing the amount of dietary fibre reaching the colon resulted in a loss of species and diversity. The taxa with low abundance were not passed on to the next generation of mice, but diversity was largely restored if the dietary fibre was increased in the new generation. However, after four generations of feeding low-fibre diets, it was impossible to reverse the loss of diversity with dietary intervention (Sonnenburg *et al.*, 2016). This finding highlights the crucial role of dietary colonic substrates in microbiota diversity. A similar correlation between diet and microbiota diversity is well evidenced in humans.

Individuals from Africa tend to eat plant-based foods that provide a high quantity and diversity of fermentable polysaccharides and thus have higher microbiota diversity than individuals from Western countries who eat more animal products and refined carbohydrates (Senghor *et al.*, 2018).

Other than bacteria, diversity also includes archaea (mainly *Methanobrevibacter smithii*), eukaryotes (mainly yeasts) and viruses (mainly phage), which have been under-represented in many microbiota datasets thus far (Lozupone *et al.*, 2012). Approximately 25 species of archaea have been found in the human GIT, and many of these are methanogenic (Figure 2), including those found in dental plaques (Nkamga *et al.*, 2017). *M. smithii* is most often isolated from the colon, with a prevalence of up to 98 %. In total, methanogens account for approximately 10 % of the organisms in the GIT.

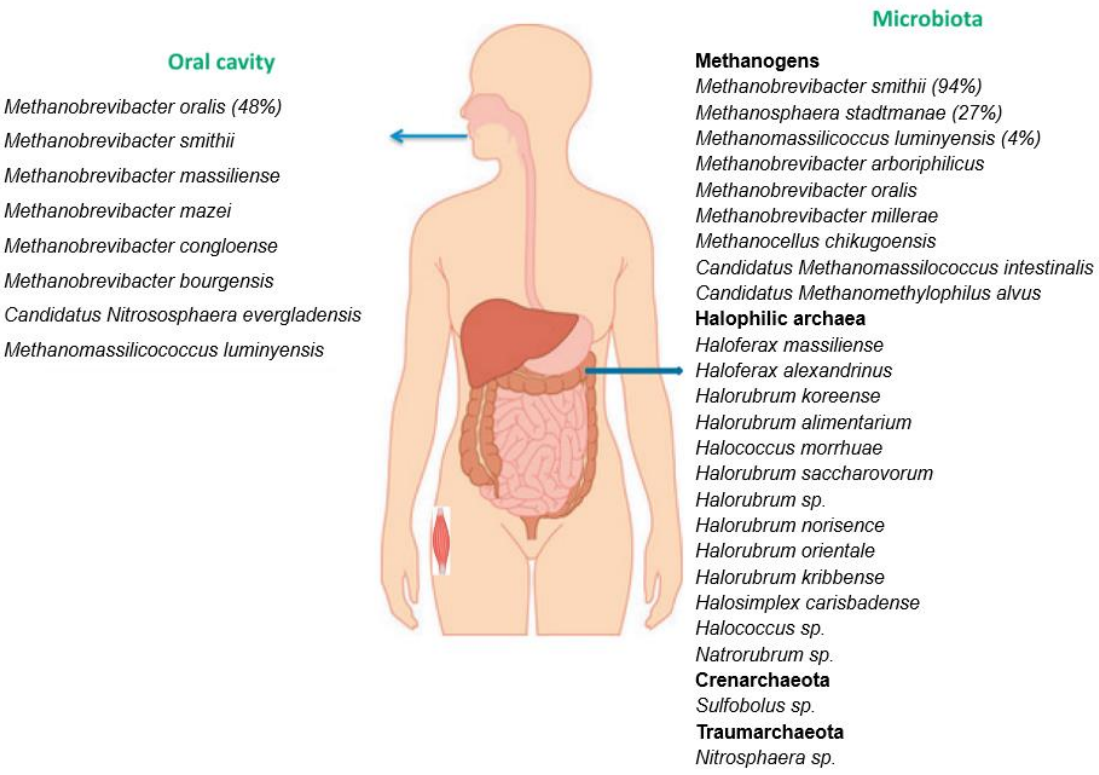


Figure 2 The species of archaea in the human gastrointestinal tract and mouth discovered using culture and metagenomic sequencing approaches.
Figure adapted from Nkamga *et al.* (2017) with permission.

2.3.2 Stability

A stable microbiota is a community that maintains similar average species abundances over time (Henson and Phalak, 2017). Stability is related to human health, as healthy individuals have a microbiota where 60 to 80 % of species are stable from one to five years (David *et al.*, 2015; Faith *et al.*, 2013).

Stability is affected by antibiotic usage, illness, and overseas travel (David *et al.*, 2015; Jalanka-Tuovinen *et al.*, 2011). The original composition can be reinstated after minor perturbations, such as short-term overseas travel. However, greater insults like infection can result in many taxa being replaced by genetically and functionally similar species (David *et al.*, 2015). The unstable fraction of the microbiota is composed of competing species that inhabit overlapping niches, and species replacement can occur within days (David *et al.*, 2015). Regardless of these fluctuations, the inter-individual differences in colonic microbiota composition remain greater than intra-individual variation over time (Gilbert *et al.*, 2018; Huttenhower *et al.*, 2012).

The concept of colonic microbiota stability is poorly understood. It has been argued that the cooperative metabolism of the microbiota fosters stability, but established ecological principles describe cooperation driving instability due to coupling, positive feedback loops, and dependency (Coyte *et al.*, 2015).

2.3.3 Functionality

While there is debate over optimal composition and stability, much research focuses on microbiota functionality. Microbiota functionality can be characterised by gene family combinations, metabolic modules, and regulatory pathways.

Specific core functions of the colonic microbiota include glycosaminoglycan biodegradation, organic acid production, lipopolysaccharide enrichment, and vitamin and amino acid production (Lloyd-Price *et al.*, 2016). These core functions often remain consistent in healthy individuals, even when species abundance changes (Lloyd-Price *et al.*, 2016) (Figure 3).

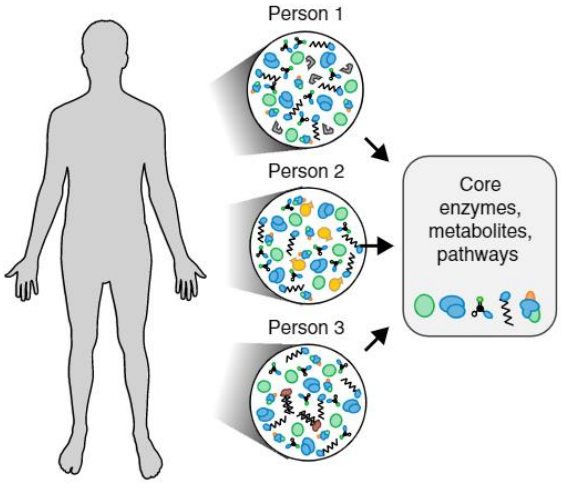


Figure 3 The core functions of the microbiota (enzymes, metabolites, and pathways) in faecal samples among individuals with differing microbiota composition.
Figure from Lloyd-Price *et al.* (2016) with permission.

Microbial species may exert beneficial or harmful effects depending on their functionality. For example, *Bacteroides fragilis* can support health through beneficial interactions with the immune system and protects against colitis in mouse models (Mazmanian *et al.*, 2008). However, it can also cause sepsis in immunocompromised hosts (Cerf-Bensussan and Gaboriau-Routhiau, 2010).

Cantarel, Lombard and Henrissat (2012) hypothesised that microbiota functionality is determined by substrate availability. They found that colonic community functionality was consistent in 148 individuals with similarities in the colonic metagenome, which was rich in gene families encoding enzymes for fibre fermentation (Cantarel *et al.*, 2012).

2.3.4 Metabolites

Fibre fermentation produces many metabolites that play a key role in GIT health. In a healthy individual, most polysaccharides that enter the colon are hydrolysed into their sugar components and fermented to produce organic acids (mainly acetate, butyrate and propionate, with lower concentrations of lactate, succinate and formate) and gases, including carbon dioxide, hydrogen, and methane (Ríos-Covián *et al.*, 2016).

2.3.4.1 Organic Acids

Normal colonic organic acid concentrations range from 1 to 3 mM, with lesser but varying concentrations of the pyruvate metabolites acetyl CoA, succinate, and lactate, which can undergo further metabolic processing and cross-feeding (Fernández-Veledo and Vendrell, 2019; Oliphant and Allen-Vercoe, 2019). The microbiota composition of an individual determines the ratios of different organic acids. For example, studies of three individual microbiota samples showed that butyrate made up approximately 60 % of the net organic acid production in two individuals, while the third showed higher propionate and valerate production, with butyrate constituting only 27 % of the net organic acids (Bourriaud *et al.*, 2005).

Organic acids are widely considered beneficial because they positively affect the host and the microbiota through local and systemic actions (Table 1). Briefly, butyrate promotes colonic cell health, partly through its role as an energy source for enterocytes (Roediger, 1982). It also reduces oxidative stress, promotes apoptosis (Hamer *et al.*, 2009; Xu *et al.*, 2017), and has beneficial roles in pathogen inhibition, insulin sensitivity, inflammation, motility, barrier function and pain sensation (Gao *et al.*, 2009; Jung *et al.*, 2015; Peng *et al.*,

2009; Soret *et al.*, 2010; Tedelind *et al.*, 2007; Vanhoutvin *et al.*, 2009; C. Zhang *et al.*, 2010; Zhou *et al.*, 2006). Acetate aids in body weight regulation, inflammation, pathogen inhibition, and GIT function (Frost *et al.*, 2014; Fukuda *et al.*, 2011; Ishizaka *et al.*, 1993; Mortensen and Nielsen, 1990; Tedelind *et al.*, 2007). Propionate aids blood glucose homeostasis, inflammation, and body weight regulation (Tedelind *et al.*, 2007; Todesco *et al.*, 1991; Xiong *et al.*, 2004). However, propionate can also cross the blood-brain barrier and might promote neurological inflammation in certain disease states (Shultz *et al.*, 2008). Most of these studies were conducted in animal models and/or human cell lines, and further human studies are needed to confirm the health effects of organic acids.

Table 1 A summary of literature showing the effects of organic acids on the host.

Metabolite	Effect on host	Effect description	Study type	Reference
Total organic acids	Pathogen inhibition	Reduced *Salmonella* invasion due to reduced colonic pH	*In vitro* (human intestinal epithelial cells)	Durant *et al.*, 2008
	Lipid metabolism	Molecular switch from lipid synthesis to lipid utilisation	Animal model (mice)	den Besten *et al.*, 2015; Lin *et al.*, 2012
		Reduced bodyweight and increased insulin sensitivity	Animal model (mice)	den Besten *et al.*, 2015; Lin *et al.*, 2012
	Gastrointestinal function	Increased ileal motor events*, increased ileal motility	Healthy humans	Kamath, Phillips, and Zinsmeister, 1988
Acetate	Pathogen inhibition	Protected against pathogenic *E. coli*	Animal model (mice)	Fukuda *et al.*, 2011
	Bodyweight regulation	Suppressed appetite	Animal model (mice)	Frost *et al.*, 2014
	Inflammation	Reduced TNFα secretion (anti-inflammatory)	*In vitro* (human neutrophil cell line)	(Tedelind, Westberg, Kjerrulf, and Vidal, 2007
	Carcinogenesis	Reduced NF-κB from colon cancer cells (anti-inflammatory)	*In vitro* (human colon adenocarcinoma cell line)	Tedelind *et al.*, 2007
		Increased antibody production from lymphocytes, increased natural killer cell (stimulate cell-mediated immunity) activity	Healthy humans and cancer patients	Ishizaka, Kikuchi, and Tsujii, 1993
	Gastrointestinal function	Dilated colonic arteries and increased colonic blood flow	*In vitro* (dissected human colonic arteries)	Mortensen and Nielsen, 1990
Butyrate	Pathogen inhibition	Increased mucin secretion, which increased adherence of probiotic strains and reduced adherence of pathogenic *E. coli*	*In vitro* (colorectal cell line)	Jung, Park, Jeon, and Han, 2015
	Gut barrier function	Increased intestinal epithelial integrity by regulating assembly of tight junctions	*In vitro* (Caco-2 cell line)	Peng, Li, Green, Holzman, and Lin, 2009

	Insulin sensitivity and bodyweight regulation	Increased insulin sensitivity and reduced adiposity by maintaining fasting blood glucose, fasting insulin, and body fat on a high-fat diet	Animal model (mice)	Gao *et al.*, 2009
	Inflammation	Increased peptide YY and glucagon-like peptide 1 expression, reduced food intake, increased satiety	Animal model (rats)	Zhou *et al.*, 2006
		Reduced TNFα secretion (anti-inflammatory)	*In vitro* (human neutrophil cell line)	Tedelind *et al.*, 2007; Y. Zhang *et al.*, 2010
	Carcinogenesis	Reduced IL-6 expression (anti-inflammatory)	*In vitro* (mouse colon organ cultures)	Tedelind *et al.*, 2007
		Reduced NF-κB from colon cancer cells (anti-inflammatory)	*In vitro* (human colon adenocarcinoma cell line)	Tedelind *et al.*, 2007
	Intestinal comfort	Reduced intestinal pain and discomfort	Healthy humans	Vanhoutvin *et al.*, 2009
	Gastrointestinal function	Increased colonic motility	Animal model (rats)	Soret *et al.*, 2010
	Promote colonic cell health	Provided energy for colonic epithelial cells for cell renewal and health	*In vitro* (rat colonocytes)	Roediger, 1982
		Reduced colonic oxidative stress	Healthy humans	Hamer *et al.*, 2009
		Promoted colonic cell apoptosis	*In vitro* (HeLa human cell line)	Xu *et al.*, 2017
Propionate	Inflammation	Reduced TNFα secretion from challenged neutrophils (anti-inflammatory)	*In vitro* (human neutrophil cell line)	Tedelind *et al.*, 2007
		Reduced cytokine in inflammatory signalling pathway	*In vitro* (human colon adenocarcinoma cell line)	Tedelind *et al.*, 2007
	Carcinogenesis	Reduced NF-κB from colon cancer cells Anti-inflammatory	*In vitro* (mouse colon organ cultures)	Tedelind *et al.*, 2007
		Reduced IL-6 expression		

Blood glucose and microbiota modulation	Reduced blood glucose response and increased faecal Bifidobacteria	Healthy humans	Todesco, Venketshwer, Bosello, and Jenkins, 1991
Bodyweight regulation	Increased leptin levels	Animal model (mice)	Xiong *et al.*, 2004
Neurological depreciation	Neurological inflammation, neurological impairment	Animal model (rats)	Shultz *et al.*, 2008

*Ileal motor events were visually identified as prolonged propagated contractions and discrete clustered contractions from motility tracings.

Many fermentation metabolites are substrates for metabolic cross-feeding, in which the end-products from one organism become the fuel for another. For example, organic substrates such as proteins, lipids or carbohydrates/fibre are fermented, and the substrate is oxidised, producing ATP, NADH, pyruvate and hydrogen. Pyruvate, the end-product of glycolysis, is an intermediate that is further metabolised depending on the type of fermentation. Pyruvate fermentation can form several alcohols, including ethanol, propanol, and 2,3-butanediol. High concentrations of these endogenous alcohols, produced by taxa of the Proteobacteria phylum, can contribute to pathogenesis, including non-alcoholic fatty liver disease (Oliphant and Allen-Vercoe, 2019).

Pyruvate can be converted to acetyl-CoA by pyruvate formate-lyase, liberating formate. This pathway is present in many species from the *Bacteroides* genus. However, formate can be oxidised by formate hydrogen-lyase, producing hydrogen and carbon dioxide. Formate oxidation is a biomarker for inflammatory-associated dysbiosis (Hughes *et al.*, 2017; Vivijs *et al.*, 2015). Acetogens, typically taxa from the Firmicutes phylum (e.g., *Blautia hydrogenotrophica*), can also use formate to produce acetate via the Wood-Ljungdahl pathway (Laverde Gomez *et al.*, 2019). Acetate can be converted to

butyrate by different butyrate-producing species, often from the Firmicutes phylum, e.g., *Eubacterium rectale, Faecalibacterium prausnitzii, Roseburia* spp.*, Eubacterium hallii, Coprococcus* spp.*,* and *Anaerostipes* spp. (Ríos-Covián *et al*., 2016). Additionally, some genera from the Bacteroidetes phylum, such as *Odoribacter*, *Alistipes* and *Butyricimonas,* can produce butyrate, but these genera usually occur in lower abundance than butyrate producers from the Firmicutes phylum (Vital *et al*., 2014).

Alternatively, pyruvate can be converted to lactate, whilst NADH and hydrogen are converted to NAD^+. Taxa of the Firmicutes phylum (e.g., *Coprococcus catus* and *Megasphaera elsdenii)* can utilise lactate to produce propionate or butyrate (Louis and Flint, 2017). Lactate is a preferred substrate for species from the *Desulfovibrio* genus during sulphate reduction to hydrogen sulphide, where lactate is oxidised to acetate (Marquet *et al*., 2009). The conversion of lactate to butyrate is pH-sensitive, and *in vitro* colonic fermentations showed that lactate utilisation is highest around pH 5.9 to 6.4. At a pH lower than 5.2, the conversion of lactate to butyrate is limited, and lactate accumulates (Belenguer *et al*., 2007).

Pyruvate can be converted to succinate through carboxylation and reduction reactions (Cao *et al*., 2013). In the colonic microbiota, most hexose and pentose sugars are fermented through the succinate pathway by taxa from the Bacteroidetes phylum and the Negativicutes class of the Firmicutes phylum (Fernández-Veledo and Vendrell, 2019). Taxa from the Bacteroidetes phylum and some taxa from the Firmicutes phylum *(*e.g.*, Phascolarctobacterium succinatutens)* can subsequently use succinate to produce propionate (Figure 4).

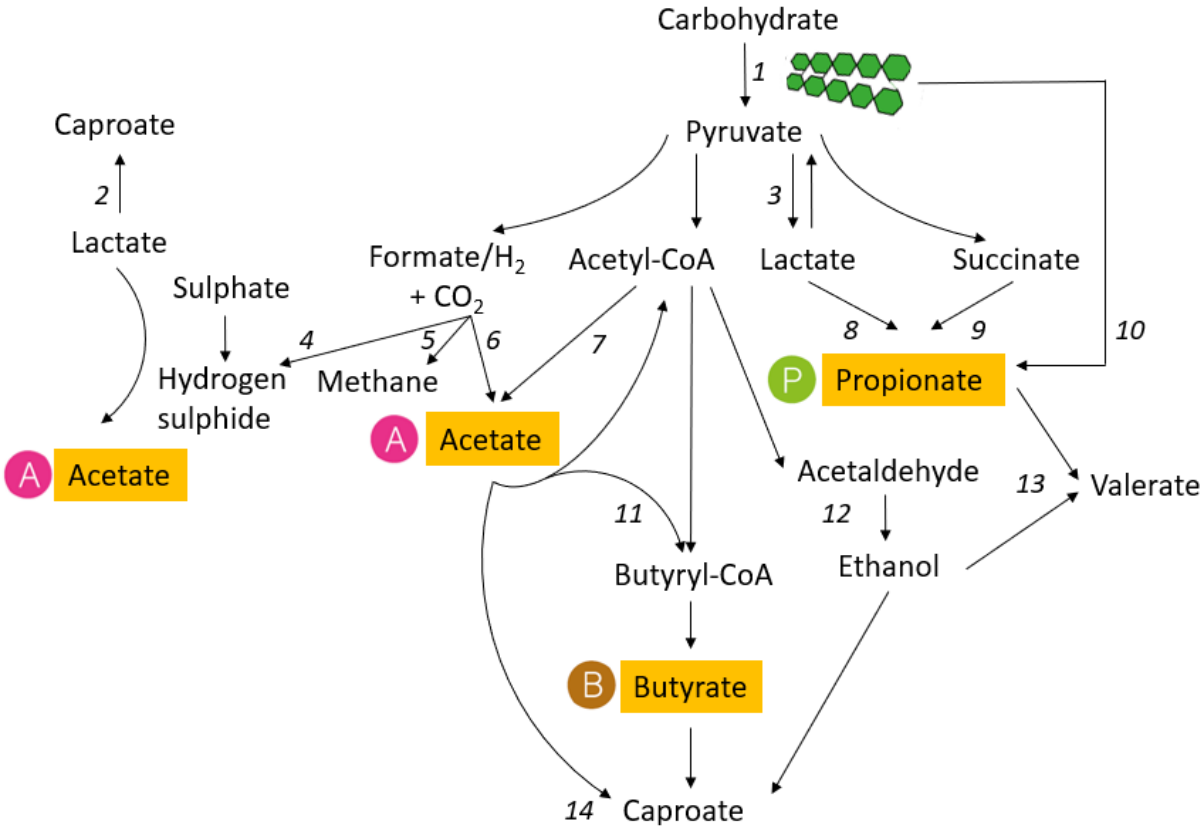


Figure 4 Common metabolic cross-feeding pathways used by commensal colonic bacteria.

Adapted from Payling *et al.*, 2020 with permission.

1 Carbohydrate fermenters, e.g., Bacteroidetes, Firmicutes, Actinobacteria
2 Alternative caproate production pathways; *Megasphaera elsdenii* and *Ruminococcaceae spp.*
3 Lactate converted to pyruvate as a precursor for butyrate synthesis: some Firmicutes (clostridial cluster XIVa)
4 Sulphate reducers, e.g., *Desulfovibrio spp.*
5 Methanogens, e.g., *Methanobrevibacter smithii* (archaea)
6 The Wood-Ljungdahl pathway; Firmicutes, e.g., *Blautia hydrogenotrophica*
7 Oxidative decarboxylation of pyruvate; many enteric bacteria
8 Acrylate pathway, lesser propionate production pathway; Firmicutes, e.g., *Coprococcus catus* and *Megasphaera elsdenii*
9 Main propionate production pathways; Bacteroidetes and some Firmicutes, e.g., *Phascolarctobacterium succinatutens*
10 Propanediol pathway from fucose and rhamnose; *Proteobacteria* and *Ruminococcus* spp.
11 Firmicutes, e.g., *Eubacterium rectale*, *Faecalibacterium prausnitzii*, *Roseburia spp.*, *Eubacterium hallii, Coprococcus spp.* and *Anaerostipes spp.*
12 Ethanol production pathway; *Bacteroides, Clostridium, Eubacterium, Blautia, Coprococcus, Dorea, Lachnoclostridium, Roseburia, Lactobacillus, Ruminoclostridium, Ruminococcus, Streptococcus* and *Escherichia* genera.
13 Valerate production coupling ethanol to propionate; *Clostridium* genus
14 Reverse β oxidation of acetate and butyrate with ethanol as the electron donor

2.3.4.2 Gases

Each type of fermentation results in different hydrogen concentrations. Mixed fermentation producing formate, ethanol and acetate can yield four moles of hydrogen per mole of glucose (Ntaikou, 2021). The same level of hydrogen production can be achieved when acetate is the sole organic acid metabolite. However, when butyrate is the sole organic acid metabolite, the potential hydrogen production is reduced to two moles per mole of glucose (Ntaikou, 2021).

Hydrogen is a fermentation intermediate, and its utilisation is essential to allow the continuation of substrate oxidation for energy production. Hydrogen cross-feeding in the human colon occurs between different species and is a syntrophic metabolism from which both species benefit (Seth and Taga, 2014). The removal of hydrogen allows complete oxidation of organic substrates and, therefore, a higher energy yield from anaerobic fermentation. Hydrogen is an electron sink product, so it needs to be depleted to maintain electron flow and continue substrate fermentation in an energetically favourable manner. Hydrogen utilisation is considered a keystone function as it is integral to the function of a healthy microbiota. Without hydrogen utilisation, gas accumulation in the colon is five to ten times above normal levels, and anaerobic fermentation cannot continue (Rowland *et al.*, 2018a).

Three functional groups of microorganisms (sulphate reducers, methanogens, and acetogens) benefit from using hydrogen as a substrate. To generate energy, these groups produce either hydrogen sulphide, methane, or acetate (Smith *et al.*, 2018) (Figure 4). Each is favourable under different conditions, but all three groups can co-exist in the colon.

Hydrogen sulphide producers, called sulphate reducing bacteria (SRB), reduce sulphate, using it as the electron acceptor to produce hydrogen sulphide. This reaction is known as dissimilatory sulphate reduction, and the most common SRB in the human colon are of the *Desulfovibrio* genus. Sulphate is derived from sulphated mucins (glycoproteins that line the epithelium from the stomach to the rectum (Prasanna, 2016; Robbe *et al.*, 2003)), dietary sources such as cysteine or methionine, or sulphated dietary carbohydrates such as carrageenans and fucoidans. Sulphate and hydrogen sulphide can be involved in a sulphur cycle, in which hydrogen sulphide can be methoxylated to methanethiol and dimethyl sulphate. This reaction may be part of a detoxification process. Methanethiol can also be converted to hydrogen sulphide, oxidised to sulphate, and then sulphate used by SRB (Oliphant and Allen-Vercoe, 2019). Hydrogen sulphide can be used in assimilatory sulphate reduction to produce cysteine, whereby electrons from NADPH are used to reduce sulphite to sulphide by facultative anaerobes. The pathway is commonly found in bacteria and yeast and has recently been proposed as a possible mechanism to modulate hydrogen sulphide concentrations in the colonic lumen to improve symptoms of inflammatory bowel disease (Kushkevych *et al.*, 2020).

Methanogens can use hydrogen or formate, reducing carbon dioxide into methane (hydrogenotrophs). The most common species in the human colon is the archaea *M. smithii*. The activity of *M. smithii* is inhibited by low pH, hence it is usually found in the distal colon (Crespo-Piazuelo *et al.*, 2018). Unlike most microorganisms, methanogens have little ability to ferment carbohydrates and instead rely on hydrogen for energy (Nkamga *et al.*, 2017). Whilst most

methanogens in the human colon are hydrogenotrophs, there can also be methylotrophic methanogens present, which use methylated compounds, including methanol, to convert the methyl group into methane (Gaci *et al.*, 2014)

Acetate producers, called acetogens, convert carbon dioxide and hydrogen to acetate without further gas production. Many enteric bacteria can perform reductive acetogenesis by the Wood-Ljungdahl pathway, and acetogens can metabolise many substrates, including mono- and di-saccharides. Furthermore, methanol is a substrate for acetogenesis, where the methyl group is transferred to the Wood-Ljungdahl pathway to reduce carbon dioxide to acetate (Kremp and Müller, 2021). Species of the genus *Eubacterium* utilise this pathway (Oliphant and Allen-Vercoe, 2019).

There are several sources of colonic methanol for methanogenesis and acetogenesis. Fruits, vegetables, and alcoholic beverages are the main sources of exogenous methanol, and biotin synthesis is the main pathway responsible for microbiota-produced methanol. It is a widespread function of the colonic microbiota (Dorokhov *et al.*, 2015).

Most competition for colonic hydrogen occurs between SRB and methanogens. SRB have a greater affinity for hydrogen than methanogens, and their reduction of sulphate is thermodynamically favourable compared to methanogenesis and acetogenesis. Hence, sulphate reduction dominates when sulphate is available (G. Gibson *et al.*, 1993).

Acetogenesis can occur alongside either of these pathways but is less energetically favourable than sulphate reduction or methanogenesis.

Acetogenesis is a minor pathway for hydrogen disposal, although it may be favoured in acidic conditions (Gibson *et al.*, 1993).

Some research has linked the three types of hydrogen disposal to the three different enterotypes, *Bacteroides*, *Prevotella*, or *Ruminococcus* genus dominant, demonstrating how hydrogen utilisation is linked with microbiota composition (Arumugam *et al.*, 2011).

Individuals with a high abundance of faecal methanogens (10^7 to 10^{10} methanogenic organisms/g dry weight faeces) usually test positive on a methane breath test (>1-2 ppm methane) (De Lacy Costello *et al.*, 2013). This observation is the case for about one-third of the Western population (De Lacy Costello *et al.*, 2013; Levitt *et al.*, 2006). In addition, an *in vitro* study with faeces from methane breath-tested individuals reported that methanogen abundance also correlated with functional differences in fibre fermentation (Robert and Bernalier-Donadille, 2003). These findings will be discussed in Sections 2.4.4.2 Keystone Species and 2.5.1.1 Human Studies.

Some research shows that colonic methanogenesis is common in bowel cancer and irritable bowel syndrome, but others found it uncommon in inflammatory bowel disease (Ghavami *et al.*, 2018; Pimentel *et al.*, 2003; Sahakian *et al.*, 2010). It is thought that methane is associated with constipation, so it is correlated with disorders characterised by constipation and not diarrhoea (Pimentel *et al.*, 2003). A series of animal and human experiments confirmed that methane gas slows small intestine transit and motility (Pimentel *et al.*, 2006). However, most methane research has been done on individuals with suspected or diagnosed GIT disorders, so further research is needed to confirm the role of methane in a healthy GIT. Human research analysing faecal DNA

found that the abundance of *M. smithii* was markedly lower in inflammatory bowel disease patients compared to healthy controls. The researchers suggested that *M. smithii* could be a marker of a healthy GIT (Ghavami *et al.*, 2018).

Hydrogen sulphide in high concentrations (micromolar to millimolar) is more certainly detrimental to human health (Attene-Ramos *et al.*, 2006; Christl *et al.*, 1996). However, the microbiota is not the only source of hydrogen sulphide in humans. The enzymes responsible for hydrogen sulphide production are ubiquitous in the human body, including in epithelial cells (Paul and Snyder, 2015). Hydrogen sulphide is an important gasotransmitter in the human body, regulating vascular tone and blood pressure and having cytoprotective roles. It affects almost all cellular processes in the body (Paul and Snyder, 2015; Tomasova *et al.*, 2016).

When colonic concentrations of hydrogen sulphide are low (nanomolar to low micromolar), the contribution of microbially-produced hydrogen sulphide is minimal compared to the production of hydrogen sulphide from intestinal epithelial cells (Figure 5, left). These cells utilise hydrogen sulphide as an energy source via a sulphide-oxidation unit. However, when the microbiota produces larger amounts of hydrogen sulphide, the sulphide-oxidation unit is downregulated, limiting the use of hydrogen sulphide for ATP generation. In addition, hydrogen sulphide accumulation reduces the integrity of the protective mucus layer and triggers an inflammatory response, as seen in Crohn's disease patients (Figure 5, right). Similarly, dysfunctional hydrogen sulphide pathways are noted in colorectal carcinogenesis (Blachier *et al.*, 2019).

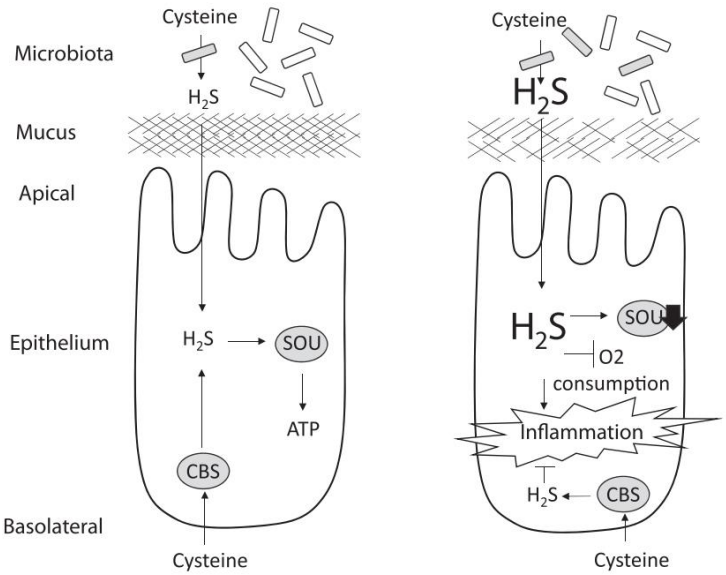


Figure 5 The production and utilisation of hydrogen sulphide (H_2S) by the colonic microbiota and epithelial cells in a healthy and diseased state.
Left (healthy): Hydrogen sulphide is generated by epithelial cells via cystathionine β-synthase (CBS) and processed by a sulphide oxidation unit (SOU) to produce ATP. Right (dysfunctional), excessive hydrogen sulphide production from the microbiota downregulates SOU, degenerates the protective colonic mucus layer and triggers an inflammatory response, as seen in Chron's disease. Figure from Blachier *et al.* (2019) with permission.

Acetate is proposed to be the most beneficial pathway of hydrogen disposal for human health. The host can absorb and use acetate as an energy source, increasing beneficial butyrate production through a mutualistic metabolism with *Ruminococcus intestinalis* (Smith *et al.*, 2018).

2.3.5 Summary

The current description of a healthy microbiota is a microbiota found in a healthy individual, yet its composition in healthy individuals is variable. Research indicates that it should be diverse in richness and enriched for common functions, including fibre fermentation, organic acid production, and vitamin and amino acid synthesis, and not excessive in methane or hydrogen

sulphide production. Further studies of microbiota composition and functionality in healthy individuals can help to test these observations.

2.4 Feeding a Healthy Microbiota

The nutrient-specific factors that differ between health and disease states include intakes of digestible carbohydrates, lipids, proteins, and fibre. However, the importance of these nutrients in developing nutrition-related diseases is controversial (Duvigneaud *et al.*, 2007).

A consistent finding is that overweight and obese individuals have higher carbohydrate and lower fibre intakes than healthy-weight individuals (Davis *et al.*, 2006; Slattery *et al.*, 1992). The protective mechanisms of dietary fibre are a low energy value that reduces energy density and physiochemical effects that increase satiety (Duvigneaud *et al.*, 2007).

The benefits of dietary fibre for human health have been noted since the 1970s when Trowell and Burkitt defined the dietary fibre hypothesis stating that diets low in fibre increased the risk of coronary heart disease, obesity, diabetes, dental caries, vascular disorders, and numerous colonic diseases (Cummings and Engineer, 2017; Trowell and Burkitt, 1981). These observations came years after the industrial revolution, which brought machine milling and food refinement to the Western world, correlated with increased non-communicable diseases and laxative prescriptions (Cummings and Engineer, 2017). In addition, the increased palatability and convenience of processed foods drove a reduction in the consumption of whole foods, thereby changing the provision of nutrients to the microbiota and reducing the production of microbiota-derived metabolites, which are beneficial to human health. Humans have adapted to the host-gut microbiota symbiosis over millennia, which takes

advantage of the diverse fermentative capabilities of microorganisms. There is evidence that the dominant taxa of the human gut microbiota adapted to this niche at least 15 million years ago in ancient hominids (Moeller et al., 2016), including an enlarged colon to increase retention time and retain microbes (Ley *et al.*, 2008). This improved the digestibility and nutritional value of plant foods for evolving hominids, who did not possess the enzymes required to degrade complex plant polysaccharides.

The colonic microbiota is key in the relationship between dietary fibre and human health. Research has shown that 10 - 60 g of dietary fibre reaches the colon daily depending on intake (recommended daily intake is 25-30 g/day for adults (National Health and Medical Research Council *et al.*, 2006)), and as a result, most microorganisms in the colon preferentially ferment carbohydrates (Egert *et al.*, 2006; Power *et al.*, 2014). Fibre fermentation results in hydrolysed carbohydrate fractions and metabolic end-products, which modify the state and functionality of the microbiota and the host. However, fermentation characteristics are affected by the amount and structure of the substrate (Fåk *et al.*, 2015; Hamaker and Tuncil, 2014).

2.4.1 Fibre Structure

The structure of dietary fibre has been extensively reviewed by carbohydrate chemists and food technologists (Boland *et al.*, 2014; Sinnott, 2007). The general structure can be considered at three main levels: macrostructure, mesostructure and molecular structure. Dietary fibre macrostructures are derived from plant food components, including RS, cellulose, and some hemicellulose (Flint, Scott, Duncan, *et al.*, 2012). Macrostructures are larger than approximately 0.2 mm and are visible to the naked eye. For example,

fragments of the epidermis, bran layers, vascular tissue, and other identifiable plant tissues. Mesostructures, often called microstructures, are structural features in the micrometre size range visible under a light microscope, such as starch granules and plant cells. Molecular structures, also called nanostructures, describe how atoms and repeating units are arranged in polymer chains (Zhang, 2014).

2.4.1.1 Macrostructure

Macrostructures relate to physical properties, of which fibre solubility has been commonly used to describe dietary fibres. The enzymic-gravimetric method AOAC 991.43 determines fibre dissolution in water (AOAC, 1994). This method is a proxy for solubility in the GIT, but the physiological conditions of the GIT cause fibres to behave differently *in vivo* compared to *in vitro* (Choct, 2015; Food and Agriculture Organisation of the United Nations and World Health Organisation, 1998). A more suitable classification of dietary fibre focuses on viscosity, water-binding properties, the binding of ions, organic molecules and microorganisms, and fermentation properties, such as the rate, site, and products of fermentation (McCleary and Prosky, 2000). However, there is a lack of standardised methods for these measures.

Gidley and Yakubov (2019) described an approach for characterising the physicochemical properties of dietary fibres according to their biological functions in the GIT. This method involved two-dimensional mapping based on molecule/particle size and local density (Figure 6). However, different methods are required for various fibre types, and the techniques are not widely adopted or reported in the literature (Gidley and Yakubov, 2019).

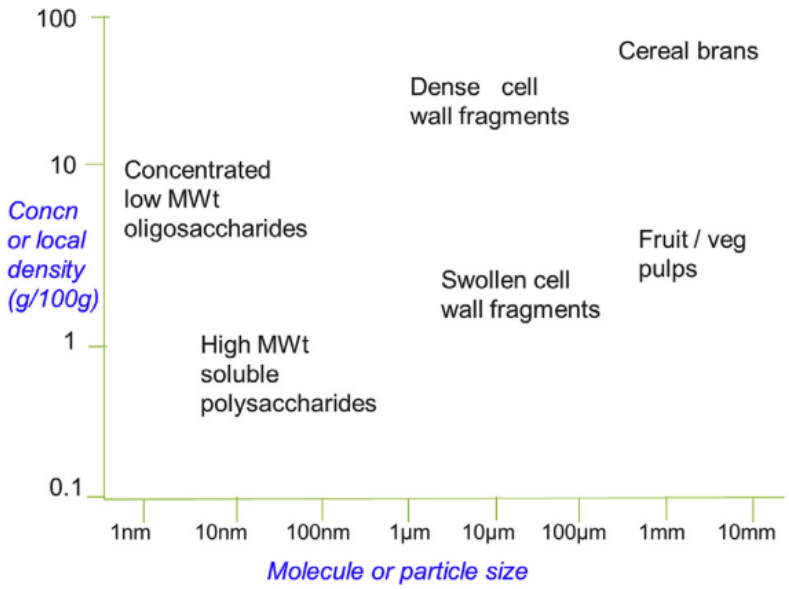


Figure 6 Two-dimensional mapping of the physiochemical properties of fibres according to their molecular/particle size and concentration/local density.
MWt: molecular weight. Figure from Gidley and Yakubov (2019) with permission.

2.4.1.2 Mesostructure

Mesostructures refer to the three-dimensional arrangement of polymers into micrometre-sized structures. For example, cellulose is a polymer common in the human diet and arranged in mesostructures. Individual polymers of cellulose are assembled into microfibrils through hydrogen bonding (Flint, Scott, Duncan, *et al.*, 2012). The microfibril structure is semi-crystalline, containing both amorphous and crystalline phases that vary in orderliness. In the crystalline regions, polymers are tightly packed in a repeating homogeneous pattern, and in the amorphous regions, the polymers are randomly distributed, resulting in less mechanical strength than in the crystalline regions (Zhang, 2014).

The ratio of crystalline to amorphous regions depends upon the cellulose source and extraction or processing methods used. For example, vegetable sources of cellulose can have crystallinities of 34 % to 57 % (Szymańska-Chargot *et al.*,

2017), but processed celluloses where the amorphous regions have been dissolved can be more than 70 % crystalline (Aguayo *et al.*, 2018).

RS is another example of mesostructural arrangement. Type I RS is physically inaccessible starch granules locked inside whole grains or beans that are destroyed during processing and cooking (Sun, 2017). Type II RS describes starch in its native form, where the polymers are a double helix inside starch granules, giving a semi-crystalline structure that is mostly insoluble (Sun, 2017). Type III RS is formed when starch is gelatinised and cooled or stored, allowing lesser branched polymers to be retrograded, i.e., re-crystallised into double helices with hydrogen bonds (Faisant *et al.*, 1993).

2.4.1.3 Molecular Structure

At the molecular level, many factors affect the structure, including branching, degree of polymerisation, molecular weight, glycosidic linkages, sugar composition, and esterification.

Macrostructure, mesostructure and molecular structure add complexity and variability to the substrates that the colonic microbiota receives, and this complexity is one of the main drivers of microbiota diversity. Each species' genome encodes for a set of enzymes, so many different species are needed for a wide range of enzymatic functions.

2.4.2 Food Processing

Most food is processed before eating, including milling, drying, pre-cooking, canning, and cooking. These processes impact food structures beyond their native properties. The main effects of food processing are the breakdown of structures at all scales and the increased solubility of dietary fibres (Table 2).

These characteristics likely reduce the concentration of insoluble carbohydrates reaching the colon.

Table 2 The effects of food processing on carbohydrate composition and structure. Table from Payling *et al.*, 2020, with permission.

Process	Food	Results	Structural change	Reference
Milling	Wheat	Damaged starch granules, increased water absorption, and increased susceptibility to gelatinisation.	Reduced crystallinity and double helix content.	(Eliasson, 2004)
Extrusion	Wheat products	Increased solubility.	-	(Björck *et al.*, 1984; Ralet *et al.*, 1990)
	Corn meal	Increased hydration capacity.	-	
	Oatmeal	Increased total NSP and increased hydration capacity.	-	(Camire and Flint, 1991)
	Potato peel	Increased ratio of soluble to insoluble NSP.	-	
Steaming and flaking	Cereals	Low-level starch gelatinisation.	Starch gelatinisation.	(Hagander *et al.*, 1987)
Cooling, packaging, and storage	Starchy foods	Amylose retrogrades over the short term during cooling. Amylopectin retrogrades over the long term during storage.	Starch retrogradation. Formation of Type III RS. Highly crystalline and insoluble.	(Eliasson, 2016)
Canning	Green beans	Decreased uronic acid-containing polymers.	-	(Margareta *et al.*, 1994)
	Green peas	Increased mid-range MW polymers.	-	
	Swede	Decreased insoluble fibre.	-	(Nyman *et al.*, 1987)
	Carrots, peas, beans, Brussel sprouts	No change.	-	
Freezing	Vegetables	Bursts plant cells and increases accessibility of digestive enzymes.	Destruction of plant cell wall.	(McDougall *et al.*, 2002)
	Swede	Increased fibre solubility.	-	(Nyman *et al.*, 1987)
Blanching	Carrot	Loss of 25% of total low MW CHO, increased ratio of soluble to insoluble NSP.	-	(Nyman *et al.*, 1987)
	Swede	Loss of 30% of total low MW CHO.	-	
	Peas, green beans, Brussel sprouts	Loss of 12% of low MW CHO.	-	

Boiling	Green beans	Loss of neutral sugars decreased galactose-containing polymers. Increased mid-range MW polymers.	-	(Margareta *et al.*, 1994)
	Green peas	Loss of ~22% low MW CHO.	-	
	Peas, beans, sprouts	Loss of 50% low MW CHO.	-	(Nyman *et al.*, 1987)
	Swede	Loss of 45% low MW CHO.	-	
	Carrot		-	
Baking	Flour	Increased RS by 0.5-1%.	Increased RS formation.	(McDougall *et al.*, 2002)
	Corn meal	No change.	-	
	Oatmeal	No change.	-	(Camire and Flint, 1991)
	Potato peel	Increased total NSP.	-	
Microwave cooking	Green beans	Loss of high MW fraction.	-	(Margareta *et al.*, 1994)
	Green peas	Increased mid-range MW polymers.	-	
Re-heating	Retrograded starch (Type III RS)	Crystallinity of amylopectin is lost at 70 °C and above. Crystallinity of amylose remains up to 145 °C.	At typical re-heating temperatures, amylopectin becomes soluble and amorphous, but amylose remains crystalline and insoluble.	(Food and Agriculture Organisation of the United Nations and World Health Organisation, 1998)

NSP; Non-starch polysaccharide
RS; Resistant starch
MW; Molecular weight
CHO; Carbohydrate

In addition, food processing can generate type III RS that provides a substrate for microbial fermentation, but the structure has limited diversity. The diversity of fermentation substrates is a key driver of microbial alpha-diversity, so diets rich in highly refined carbohydrates can be detrimental. A better understanding of the interaction between food structures and the microbiota may help promote diets which maintain and enhance a healthy microbiota.

2.4.3 Digestion and Fermentation

Crucial digestive processes occur in the mouth, stomach, and small intestine. A combination of host and microbial activities determines the amount and structure of substrate reaching the colon for fermentation.

2.4.3.1 The Mouth

In the mouth, both physical (mastication) and chemical (salts, mucins, and enzymes) digestion occur (Hillman *et al.*, 2017; Sarkar, Ye and Singh, 2017) (Figure 7). These processes contribute to 40 % of starch breakdown before the small intestine, although other nutrients are minimally impacted (Goodman, 2010).

The oral microbiota is a mix of transient and commensal populations, which can form biofilms on the surfaces of the mouth (Avila, Ojcius and Yilmaz, 2009). The bacteria utilise nutrients delivered in the saliva, such as peptides and partially dissolved carbohydrates (Avila, Ojcius and Yilmaz, 2009). There is no evidence to suggest that the oral microbiota significantly impacts fibre structures due to a short retention time and high availability of soluble nutrients (Avila, Ojcius and Yilmaz, 2009). After swallowing, the food bolus passes down the oesophagus into the stomach. However, there is little information on the oesophageal microbiota (Hillman *et al.*, 2017).

2.4.3.2 The Stomach

In the stomach, there is mechanical, acidic, and enzymatic digestion. Mechanical digestion reduces particle size, whilst acidic and enzymatic digestion further degrades food structures via the acids and proteolytic enzymes in gastric juices (Boland, Golding and Singh, 2014; Hillman *et al.*, 2017).

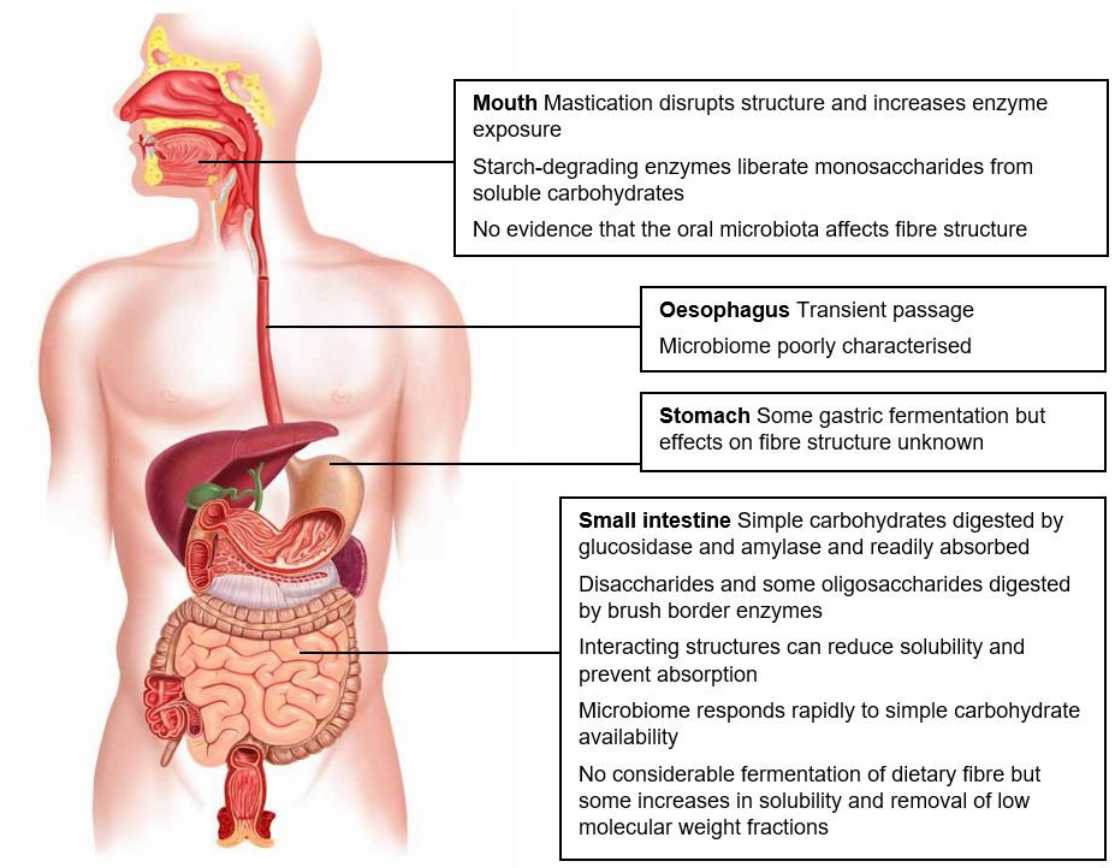


Figure 7 The processes of digestion and fermentation along the human gastrointestinal tract. Graphic credit: Leonello Calvetti, Science Photo Library. Figure credit from Payling *et al.* (2020) with permission.

The short retention time and the acidic pH of three to five mean the stomach microbiota consists of only approximately 10^1 microorganisms/g digesta (Ohland and Jobin, 2015). The low pH and short retention time mean that most bacteria are transient and have low metabolic activity; however, a more alkaline pH in the mucosa encourages more permanent active populations (Hillman *et al.*, 2017).

2.4.3.3 The Small Intestine

Most digestion and absorption occur in the small intestine (Hillman *et al.*, 2017). The small intestine is divided into three segments: duodenum, jejunum, and ileum, which are seven metres long in total (Kraus, 1993). Further chemical

digestion and some absorption occur in the duodenum, whilst significant absorption occurs in the jejunum and ileum (Goodman, 2010; Boland, Golding and Singh, 2014).

The density of the small intestinal microbiota (10^4 to 10^5 colony forming units (CFU)/mL) is higher than that of the stomach but lower than the colonic microbiota (10^7 to 10^8 CFU/mL). High bile acid concentrations in the small intestine limit microbial abundance (Kastl *et al.*, 2020). The small intestinal microbiota is composed mainly of fast-growing facultative anaerobes such as *Lactobacilli* and *Streptococci*, which ferment simple carbohydrates (Roy *et al.*, 2014). The production of acetate, lactate, and butyrate (and sometimes formate) has been observed; however, butyrate production is lower than in the colon (Zoetendal *et al.*, 2012). Furthermore, the small intestine has a gradient of antimicrobials from high at the proximal end to low at the distal end, allowing for a greater abundance of microorganisms in the distal regions of the small intestine (Donaldson *et al.*, 2016).

The small intestine *in vitro* models inoculated with porcine ileal digesta found an average of 30 % organic matter fermentability in the ileum, with higher acetate production than colonic fermentation (Montoya, de Haas and Moughan, 2018; Hoogeveen *et al.*, 2020). However, similar work found that acetate production was less than half of the concentration from colonic fermentation (Montoya, Blatchford and Moughan, 2021). Little is known about the small intestinal microbiota due to poor sampling accessibility in healthy individuals (Aidy, van den Bogert and Kleerebezem, 2015).

High percentages of dietary RS, cellulose and lignin have been recovered at the terminal ileum (>80 %), suggesting that the small intestinal microbiota does

not ferment these (Holloway, Tasman-Jones and Lee, 1978; Faisant *et al.*, 1995, 1995). However, these fibres may still be subject to structural change. For example, RS granules recovered from the ileum had subtle changes in ultrastructure and crystallinity, reducing the melting temperature and increasing the porosity and surface area (Faisant *et al.*, 1995). In addition, gastric and small intestinal digestion simulations with dietary fibre from kiwifruit found a decrease in the yield and molecular weight of insoluble fibre after digestion (Carnachan *et al.*, 2012). These structural alterations have consequences for colonic fermentation.

Other than digestion-resistant fibres, readily digestible substrates may pass through the small intestine undigested. For example, studies have recovered 10 to 25 % of starch at the ileum, which was water-soluble and easily digested under small intestinal *in vitro* conditions (Faisant *et al.*, 1993; Faisant *et al.*, 1995; Noah *et al.*, 1998).

2.4.3.4 The Colon

Digestion in the colon is completely microbial. Substrates not digested or absorbed in the upper GIT are fermented, influencing the composition and function of the colonic microbiota.

The main substrates fermented are starch, NSP and protein. Lipids are found in colonic contents but are derived from a mix of undigested dietary fats, endogenous secretions, and bacterial metabolites. It has been estimated that 50 to 75 % of faecal lipid are of bacterial origin (Timmis, 2010; Chen *et al.*, 1998). In humans, only a small proportion of dietary lipid reaches the colon, five to eight grams daily. In addition, the microbiota can metabolise dietary

triglycerides into free fatty acids and glycerol using lipases, but this is not a widespread function compared to carbohydrate fermentation (Gérard, 2020).

Most of the fermentable dietary substrate reaching the colon of individuals consuming Western diets is composed of NSP and protein, approximately 12 g of each reaching the colon daily (Cummings and Englyst, 1987). Fibre arrives mostly as insoluble complex particles derived from plant polysaccharides or RS (Flint, Duncan and Louis, 2017). The structures arrive in various states of solubility, chain length, or even complexed with other molecules, which affect the extent of fermentation (Flint *et al.*, 2008). Readily-digested substrates such as starch and glucose are 90 to 100 % fermentable compared to NSP, which is approximately 70 % fermentable (Faisant *et al.*, 1995; Noah *et al.*, 1998).

Following a retention time of 20 to 56 hours in the colon (Southwell *et al.*, 2009), a few recalcitrant structures remain, mostly lignin and crystalline cellulose. These fractions are expelled in the faeces and constitute approximately 25 % of the organic solids (Rose *et al.*, 2015). Colonic fermentation is highly efficient, and only small fractions of undigested material are left. Most faecal weight (25 to 54 % of organic solids) is bacterial biomass in healthy individuals. The faeces also contain some protein/nitrogen (2 to 25 % organic solids) and lipids (2 to 15 % organic solids) (Rose *et al.*, 2015). Approximately 35 % of the faecal protein originates from endogenous sources (Starck, Wolfe and Moughan, 2018).

The colonic microbiota has 10^{10} to 10^{12} bacteria/g digesta (Hillman *et al.*, 2017), making it one of the densest microbial environments known to ecology (Senghor *et al.*, 2018). Most bacteria preferentially ferment carbohydrates producing organic acids, gases, and microbial biomass. Functionally,

carbohydrate fermenters can be classified as specialists or generalists. Those with genes for over 200 carbohydrate-active enzymes (CAZymes), such as taxa from the Bacteroidetes phylum, are considered generalists as their broad genetic potential allows them to access structurally diverse substrates and adapt quickly to changing nutrient availability (Briggs, Grondin and Brumer, 2021). Generalists account for most of the saccharolytic bacteria in the colon. Specialists, such as taxa from the Firmicutes phylum, have lower numbers of CAZymes and hence a smaller genome (Flint, Scott, Duncan, *et al.*, 2012; Cockburn and Koropatkin, 2016).

Bacterial abundance changes along the length of the colon as the environment changes. Along its 1.5 m length, it is anaerobic with a neutral or slightly acidic pH (Bornhorst and Paul Singh, 2014). In the ascending/right/proximal colon, there is a high concentration of carbohydrates and water compared to the descending/left/distal colon (Figure 8).

Rapidly fermentable carbohydrates are fermented proximally, leaving a lower concentration of carbohydrates in the distal colon, primarily insoluble. Therefore, the proximal colon has a higher fermentative capacity and hydrogen concentrations, resulting in a pH of approximately 5.6. The pH increases to 6.6 in the distal colon (Van De Wiele *et al.*, 2007). The water concentration also decreases along the length of the colon due to reabsorption (Bornhorst and Paul Singh, 2014).

The proximal colon usually has a higher abundance of taxa from the Firmicutes phylum, which are more acid-tolerant than the taxa from the *Bacteroides* genus, usually found in higher abundance in the distal colon.

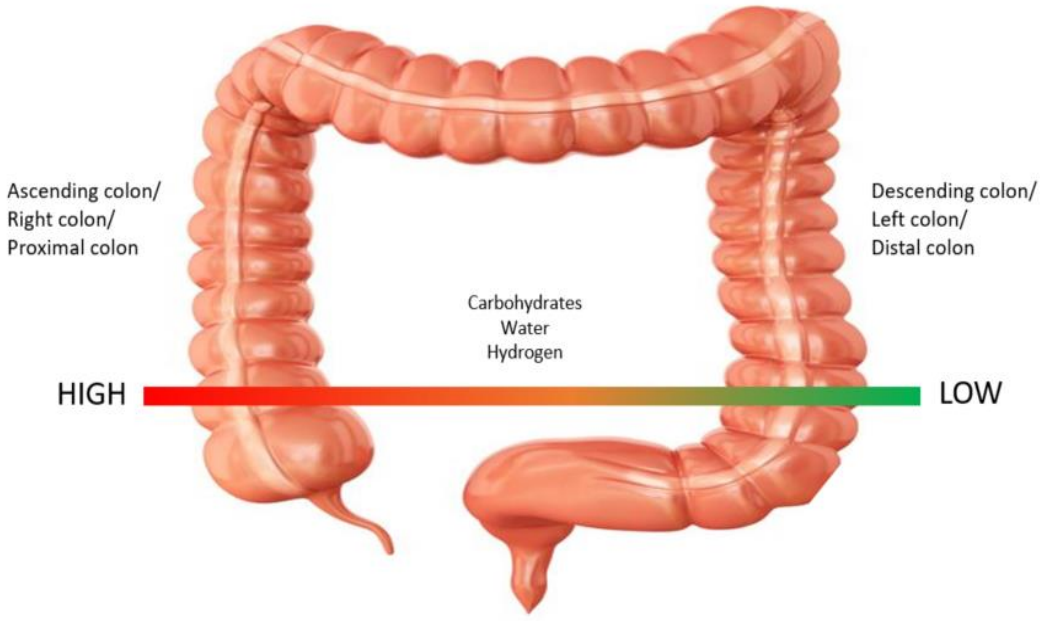


Figure 8 The concentrations of carbohydrates, water, and hydrogen, in the proximal colon compared to the distal colon.

2.4.4 Substrate-Microbiota Interactions

2.4.4.1 Individual Variation

Deciphering the complex interactions between the human colonic microbiota and dietary substrates is key to microbiota modulation for optimal health. However, the human microbiota shows high individual variability due to diet, leading to conflicting food research results (Leeming *et al.*, 2019).

Zeevi *et al.* (2015) found that postprandial blood glucose levels were highly variable in an 800-person cohort fed standardised meals. Part of the variability was associated with the relative abundance of taxa from the *Proteobacteria* genus, *Enterobacteriaceae* family, and Actinobacteria phylum in faecal samples. Pathways of the microbiota, including bacterial chemotaxis, flagellar assembly, ABC transporters, systems involved in bacterial quorum sensing and infection, and transport of the amino acids lysine, arginine, and glutamate, were also linked with postprandial blood glucose. Whilst some of the taxonomic

differences were linked with variation in diet and physiology, which may help to explain the association with postprandial blood glucose, several factors, including microbiota pathways, have not been explored or explained experimentally (Zeevi *et al.*, 2015).

Human studies looking at specific effects of increased dietary fibre found that some participants responded with an increase in host inflammatory markers whilst others showed a decrease. Increased inflammation was linked with lower microbial alpha-diversity and a greater relative abundance of taxa from the *Coprococcus*, *Ruminococcus*, *Oscillospira,* and *Anaerostipes* genera compared to those who experienced reduced inflammation, although there was high individual variability (Wastyk *et al.*, 2020). Conversely, the abundance of CAZymes consistently explained dietary fibre responses. As CAZymes increased, the abundance of several host disease-associated proteins decreased, particularly those linked to the inflammatory responses (Figure 9) (Wastyk *et al.*, 2020). These findings suggest that the fibre fermenting function of the microbiota might explain some individual responses to food and interactions with human health.

There is lower inter-person variability in microbiota functionality compared to composition, which is attributed to the high functional redundancy of the microbiota. Redundancy describes functions that several species can perform, so the function remains when one species is lost or in low abundance. High functional redundancy is thought to promote the resilience and stability of the human colonic microbiota (Tian *et al.*, 2020). However, some functions of the microbiota have low functional redundancy and can be lost when a certain species is missing or in low abundance.

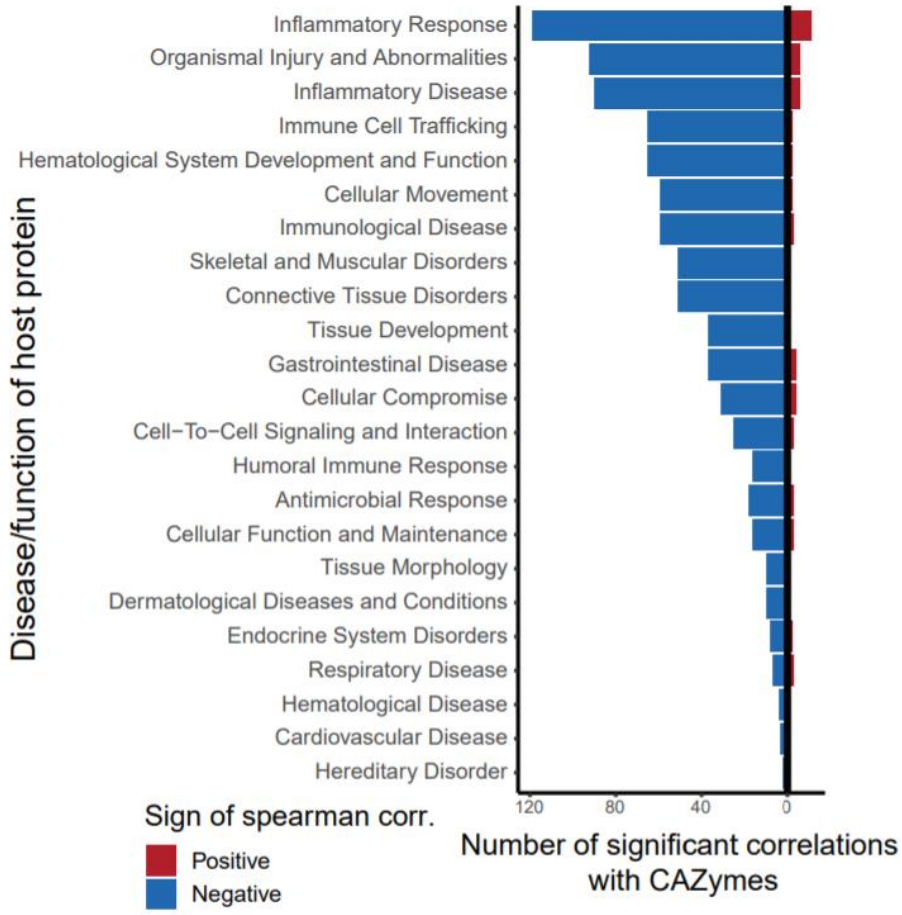


Figure 9 The number of significant correlations between carbohydrate-active enzymes (CAZymes) and host disease-associated proteins.
Negative correlations are in blue, and positive correlations are in red. Figure from Wastyk *et al.* (2020) with permission.

In a human study with 14 volunteers, most participants fermented at least 96 % of dietary RS after a three-week adaptation; however, only approximately 40 % of dietary RS was fermented in two individuals. These individuals had a lower abundance of faecal *Ruminococcus bromii* (<1 %) compared to the other 12 participants (4 to 7 % faecal *R. bromii*) (Walker *et al.*, 2011). Further research using faecal samples from one of the volunteers with low RS fermentation showed that *R. bromii* supplementation increased RS fermentation *in vitro* (Ze *et al.*, 2012). Furthermore, genomic analysis of *R. bromii* found a unique

repertoire of genes coding for starch degrading enzymes that form a multi-enzyme complex referred to as an 'amylosome' (Ze *et al.*, 2015). This finding suggests that *R. bromii* may have unique starch utilisation genes and could be a keystone species for RS fermentation.

2.4.4.2 Keystone Species

The concept of keystone species was first proposed by an ecologist named Robert Paine in 1966, as he showed that removing sea stars greatly impacted the ecology of shorelines in the USA. His definition describes keystone species as important for community structure and stability with a non-redundant function (Paine, 1966). Varying definitions have since been discussed and are outlined in a comprehensive review by Cottee, Jones and Whittaker (2012). Here, they reject a specific definition encompassing proportionality, where a keystone species must have a disproportionate influence on its community relative to its abundance or biomass, as this requires characterisation of the community and will result in most species being eligible for description as keystone species. Further, proportionality is difficult to quantify and defend. Instead, they favour a broader definition of "a species that is of demonstrable importance for ecosystem function", referring to a range of ecosystem processes that support functional resilience in the community (Cottee-Jones and Whittaker, 2012). This definition was adopted and refined to produce a definition of keystone taxa in microbial ecology; "highly connected taxa that individually or in a guild exert a considerable influence on microbiota structure and functioning irrespective of their abundance across space and time. These taxa have a unique and crucial role in microbial communities, and their removal

can cause a dramatic shift in microbiota structure and functioning" (Banerjee, Schlaeppi and van der Heijden, 2018).

Regarding fibre fermentation, keystone species often have small genomes and roles in the initial access and fermentation of substrate macrostructures (also known as a specialist degrader or primary degrader) (Ze *et al.*, 2013). There are known primary degraders in the Firmicutes phylum that have systems allowing adherence to fibre particles and substrate colonisation, including a unique organisation of extracellular enzymes such as cellulosomes and amylosomes. These features enable them to attack the material with limited surface area and robust hydrogen-bonding networks (Ze *et al.*, 2013; Flint, Duncan and Louis, 2017; Williams *et al.*, 2017). For example, *R. bromii* produces amylosomes that provide superior access to insoluble RS (Ze *et al.*, 2015). Furthermore, the hydrolysis products liberated by *R. bromii* can be fermented by *Bifidobacterium adolescentis*, *E. rectale* and *Prevotella* spp. in a cross-feeding process (Kovatcheva-Datchary *et al.*, 2009).

Many keystone species in fibre degradation have distinct substrate specificities (Albenberg and Wu, 2014) and rely on complex fibres as an energy source. They are not adapted to compete for soluble carbohydrates. For example, *R. bromii* can utilise starch, fructose, galactose, and maltose but does not grow effectively on glucose (Ze *et al.*, 2012; Kim *et al.*, 2017). Similarly, *R. champanellensis*, a keystone degrader of cellulose, can ferment microcrystalline cellulose but has limited utilisation of soluble sugars (only cellobiose) (Chassard *et al.*, 2012).

The presence of keystone species in faecal samples (as a proxy of the colon microbiota) has been linked with human health. A highly diverse microbiota has more keystone species and higher organic acid production than those with

lower diversity. Individuals from agrarian societies have higher organic acid production compared to individuals from Western societies, even when dietary fibre intakes are matched. The lower concentration of organic acids is likely due to a lack of keystone fibre degraders in the Western microbiota (De Filippo *et al.*, 2010; Wu *et al.*, 2016). Animal studies suggested that reduced dietary fibre intakes resulted in a loss of species and diversity, where taxa with low abundance were not passed on to the next generation (Sonnenburg *et al.*, 2016).

Fibre-fermenting keystone species are also a crucial component of microbiota recovery after antibiotics. Using data from more than 100 individuals, Chng *et al.* (2020) found 21 species that correlated with ecological recovery after perturbation. An initial group of species, including *Bacteroides uniformis* and *Bacteroides thetaiotaomicron,* were associated with epithelial colonisation and dietary fibre fermentation. Their functions supported the growth of a second group dependent on fibre hydrolysis products, including *B. adolescentis* and *Bacteroides caccae*. Cross-feeding helped repopulate the community and rebuild a diverse microbial web, including butyrate producers such as *F. prausnitzii* and *Roseburia* spp. that provide energy for colonocytes and positively correlate with human health (Chng *et al.*, 2020).

Keystone species of the colonic microbiota have also been correlated with human breath methane emissions. The faecal microbiota composition of HE individuals (>2 ppm) included several *Ruminococcus* spp., including *R. champanellensis,* which were able to degrade microcrystalline cellulose. Whilst the faecal microbiota of LE individuals (<2 ppm) was not able to degrade microcrystalline cellulose and did not contain these species (Robert and Bernalier-Donadille, 2003). Later, Chassard *et al.* (2010) found that a

proportion of individuals who were LE had a microbiota that could degrade cellulose, but communities were from the Bacteroidetes phyla instead of the Firmicutes phyla found in high methane breath emitters. Keystone species likely exist for many of the components of the plant cell wall, but they have not yet been identified (Wu *et al.*, 2016).

2.4.4.3 Cross-Feeding

Many effects of microorganisms are mediated through cross-feeding, in which the metabolites of one species become the fuel for another. In several colonic microbiota models and ecological theories, cross-feeding is a key driver of population diversity (Rainey *et al.*, 2000; Hoek and Merks, 2017; Goldschmidt, Regoes and Johnson, 2018).

Bacteroides cellulosilyticus and *B. caccae* are keystone species in the degradation of arabinogalactan proteoglycans which are plant glycans abundant in the human diet (Tan *et al.*, 2012; Cartmell *et al.*, 2018). Arabinogalactan proteoglycans typically have a type II arabinogalactan structure; a 1,3-β-D-galactan backbone requiring a surface endo-β-1,3-galactanase for cleavage (Tan *et al.*, 2012; Cartmell *et al.*, 2018). In a study of 15 species of the *Bacteroides* genus, only two species (*B. cellulosilyticus* and *B. caccae*) hydrolysed the 1,3-β-D-galactan backbone into oligosaccharides (Cartmell *et al.*, 2018). However, all strains could utilise oligosaccharides. Therefore 13 species of the *Bacteroides* genus relied on the other two to initiate degradation and provide hydrolysis products for cross-feeding (Cartmell *et al.*, 2018). The initial degradation of arabinogalactan is affected by the substrate's molecular structure. In contrast to type II, type I arabinogalactan was utilised by 14 species of the *Bacteroides* genus and only differs by the carbon with which

the glycosidic bond is made, giving a 1,4-β-D-linked galactose backbone (Luis *et al.*, 2018).

Whilst enzyme specificity is determined at the level of molecular structure. The substrate mesostructures often determine enzyme accessibility. One example is the organisation of microfibrils in cellulose. Highly crystalline regions are less accessible to enzymatic hydrolysis than amorphous regions because they are packed together in regular structures that promote insolubility and reduce susceptibility to enzymatic attack (McCleary and Prosky, 2000). In addition, amorphous regions contain randomly distributed polymers with less mechanical strength and resistance than crystalline regions (Zhang *et al.*, 2014; Payling *et al.*, 2020). This mesostructure means that access and colonisation of cellulose by colonic microorganisms require specialist genes that are not widespread in the microbiota. *R. champanellensis* has a key role (primary degrader) in cellulose fermentation and produces a variable ratio of succinate and acetate depending on the cellulose source (Chassard *et al.*, 2012) (Figure 10). This observation suggests a mesostructural effect as different cellulose sources have specific ratios of crystalline to amorphous regions. For example, microcrystalline cellulose is up to 53 % crystalline, whereas apple cellulose is approximately 25 % crystalline (Szymańska-Chargot, Cybulska and Zdunek, 2011). The ratios of succinate and acetate produced from cellulose fermentation affect cross-feeding. Succinate can be converted to propionate, and acetate can be converted to butyrate or used as a carbon source for methanogens (Hobson and Stewart, 1997) (Figure 10).

In addition, substrate hydrolysis products such as cellodextrins and cellobiose are liberated by primary degraders for substrate cross-feeding. Human colonic

commensals, including *Bifidobacterium breve* and *B. dentium,* have cellobiose phosphorylase activity, which permits the uptake and catabolism of cellodextrins and cellobiose (Pokusaeva, Fitzgerald and Van Sinderen, 2011; Vigsnaes *et al.*, 2013). However, these bacteria cannot use polysaccharides, so their growth relies on scavenging oligosaccharides released by primary degraders (Munoz *et al.*, 2020).

Finally, substrate macrostructure affects the interaction between substrates and the microbiota. In the plant cell wall, polysaccharides are matrixed with cellulose, a range of hemicelluloses (xylans, galactans, β-glucans, xyloglucans and glucomannans), pectin, and phenolic compounds (Chassard and Bernalier-Donadille, 2006). The matrix reduces the solubility of polysaccharides that are often soluble following extraction and isolation. It also increases the complexity of the substrate, requiring different enzymatic activities for degradation.

Purified celluloses such as carboxymethyl cellulose are minimally fermented in the human colon, yet whole-food cellulose in vegetables is extensively degraded, affecting macrostructures (Cummings, 1984). In addition, there is a limited understanding of how the complex cell wall affects the microbiota because experimental work commonly uses purified fibres (Williams *et al.*, 2017).

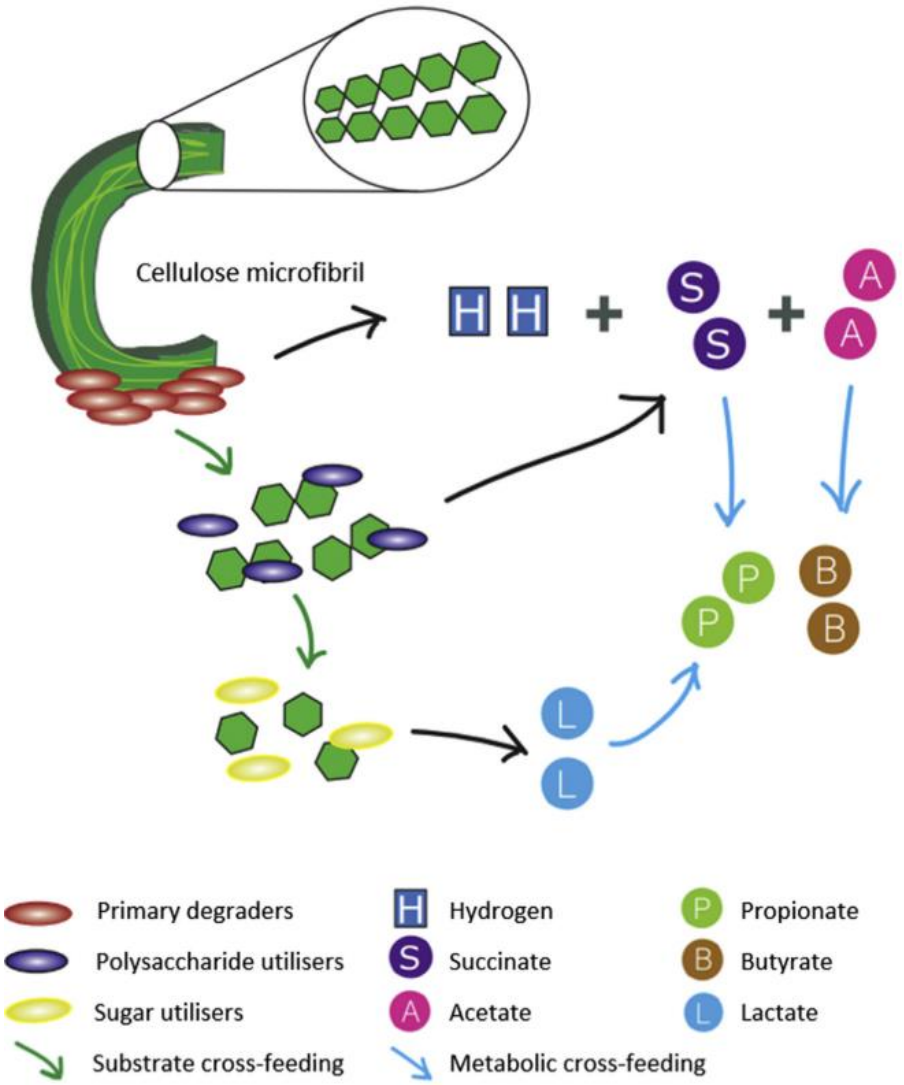


Figure 10 Substrate and metabolic cross-feeding of cellulose in the human colon. Figure from Payling *et al.* (2020) with permission.

2.4.5 Summary

Dietary fibre promotes healthy digestion and metabolism in humans and acts as a fermentable substrate for the colonic microbiota. The interactions between fibre and microbiota are affected by dietary fibre structures and the ecology and functionality of the microbiota. However, fibre structures are affected by food processing, preparation, and digestion, which adds complexity.

The variation in individual responses to diet also adds complexity but is partly explained by differences in microbiota composition and function. While

microbiota composition varies, functionality often remains consistent due to the high functional redundancy of genes. The exceptions are keystone species, where a few species have a crucial role in providing substrate to others in the community. Primary degraders initiate cross-feeding to provide diverse substrates for a complex microbial fermentation web. Therefore, the substrate structure and the cross-feeding webs of the colonic microbiota must be considered when attempting to modulate the colonic microbiota.

2.5 Studying a Healthy Microbiota

2.5.1 Methodology

The relationship between dietary fibre and the colonic microbiota is complex. Well-designed experiments can help decipher this relationship but rely on robust, reliable, and precise methodologies to provide detailed data relevant to the *in vivo* situation. Key methods are described and evaluated below.

2.5.1.1 Human Studies

Many human microbiota studies are epidemiological, so they consider the microbiota and host together but often cannot establish cause and effect. Nevertheless, microbiota composition has been correlated with more than 25 diseases or disorders, including hypertension, type I and II diabetes, various liver diseases, irritable bowel syndrome, inflammatory bowel disease, depression, coeliac disease, colon cancer, obesity, Alzheimer's disease, Parkinson's disease, autism and more (Kostic *et al.*, 2015; Tilg, Cani and Mayer, 2016; Halfvarson *et al.*, 2017; Vogt *et al.*, 2017; Yan *et al.*, 2017; Rinninella *et al.*, 2019).

Epidemiological studies have correlated the microbiota with dietary patterns, including Western, gluten-free, vegetarian, vegan, omnivore, and

Mediterranean. In addition, the microbiota has been correlated with dietary nutrients, including protein, fat, carbohydrates, fibre, and other dietary components such as probiotics and polyphenols (Singh *et al.*, 2017).

However, these studies do not show cause and effect, limiting their application. Some researchers argue that mouse studies using antibiotic-treated mice, germ-free mice, or mice with a human faecal microbiota transplant are needed to establish cause and effect (Shang, 2019). Others argue that the high success of causation in rodent studies (95 %) is inaccurate and that more stringent guidelines are needed to prevent false-positive results. False-positive results arise from bias at technical, experimental, analytical, interpretative and publication levels, but they are particularly prevalent in studies using human gut microbiota-associated rodents, as the model is not standardised. Experiments often use a small number of human donors but many mice, which artificially inflates the sample size and predisposes experiments to false-positive results (Walter *et al.*, 2020).

Furthermore, human dietary studies are often inaccurate due to their reliance on self-recorded food intakes. Approximately 50 % of food records show significant under-reporting (Macdiarmid and Blundell, 1997; Ravelli and Schoeller, 2020). Additional limitations exist for the reporting of carbohydrate foods, including underreporting due to participant perceptions of healthy eating, and the effects of processing, storage, and preparation, which affect chemical composition and nutritional value, but are difficult to capture. Researchers have attempted to improve accuracy by publishing guides and standardisation, but such guidelines have only recently been published for diet data in microbiota analyses. The recommendation was that multiple days of diet

data be collected before microbiota sampling, and three to five consecutive faecal samples should be taken to reduce intra-individual variation (Johnson *et al.*, 2020). However, these guidelines can be challenging due to limitations on time and resources.

In addition, these studies use faecal samples to represent the colonic microbiota due to the impractical and invasive nature of direct sampling. Faecal samples can be used to seed *in vitro* models under colon-like conditions representing populations of the distal and proximal colon (Flint, Scott, Duncan, *et al.*, 2012; Venema, 2015). However, spatial variation in colonic composition is lost when faecal samples are collected and homogenised. As a result, distinct differences between microbial communities of the colonic mucosa, digesta, and lumen exist, characterised by different populations: *Bacteroidaceae*, *Prevotellaceae,* and *Rikenellaceae* families in the digesta and *Lachnospiraceae* and *Ruminococcaceae* families in the inter-fold regions of the lumen (Figure 11).

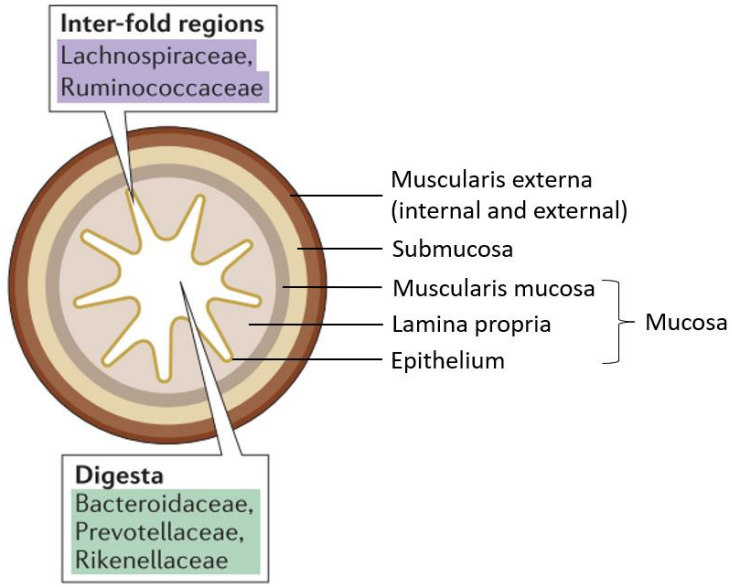


Figure 11 The spatial distribution of bacteria through a cross-section of the human distal colon. Figure from Donaldson *et al.* (2016) with permission.

In faecal samples, patches of 1 cm can vary in composition (Donaldson, Lee and Mazmanian, 2016). Taxa of the *Enterobacteriaceae* family are at the interface of faeces and mucus, low in abundance or absent in faeces. Species of the *Bifidobacterium* genus are found mostly in the faeces and absent in the mucus, whereas *E. rectale*, *F. prausnitzii*, *Bacteroides* spp., *Eubacterium cylindroides*, *Clostridium histolyticum* and *Clostridium lituseburense* were found in the highest concentrations in the faeces and lowest concentrations in the mucus (Swidsinski *et al.*, 2008). This variation is due to aggregates of interacting organisms, the heterogeneity of plant fibres, or mucosa physiology, which differ along the length of the colon (Donaldson, Lee and Mazmanian, 2016).

Furthermore, the collection, processing, and analysis of human faecal samples are complex, with potential bias and error at every step. Sample handling and storage affect microbiota composition, including temperature fluctuations and freeze-thaw cycles. In the American Gut Project, blooms of *Escherichia coli* were found in samples due to sample transit times and lack of preservation (Mcdonald *et al.*, 2018) (Figure 12).

The International Human Microbiome Standards group described specific protocols according to transit time. For example, samples than can be handled in the laboratory within 24 hours should be deposited in a sterile plastic bag and sealed in a container with an anaerobic generator sachet. Samples that will remain outside the laboratory for more than 24 hours should be frozen and shipped on dry ice or collected in a stabilisation solution (International Human Microbiome Standards, 2015).

The method of DNA extraction can also affect results. Different DNA extraction protocols can affect the ratios of taxa from the Bacteroidetes and Firmicutes

phyla, the most dominant organisms in the colon. In addition, some protocols can recover 100 times more DNA than others (Wesolowska-Andersen *et al.*, 2014; Costea *et al.*, 2017; Nature Research Custom Media and Zymo Research, 2018). This variability is due to differences in the bacterial cell wall structure, with Gram-positive bacteria having a thicker cell wall that is harder to lyse than Gram-negative bacteria. Most Firmicutes spp. are gram-positive, while most Bacteroidetes spp. are gram-negative. Some solutions include analysing a known mock community and studying the bias that occurs with different methods. Mock communities can also be used to study the variation due to extraction processes, sample batching, and different laboratories. (Nature Research Custom Media and Zymo Research, 2018).

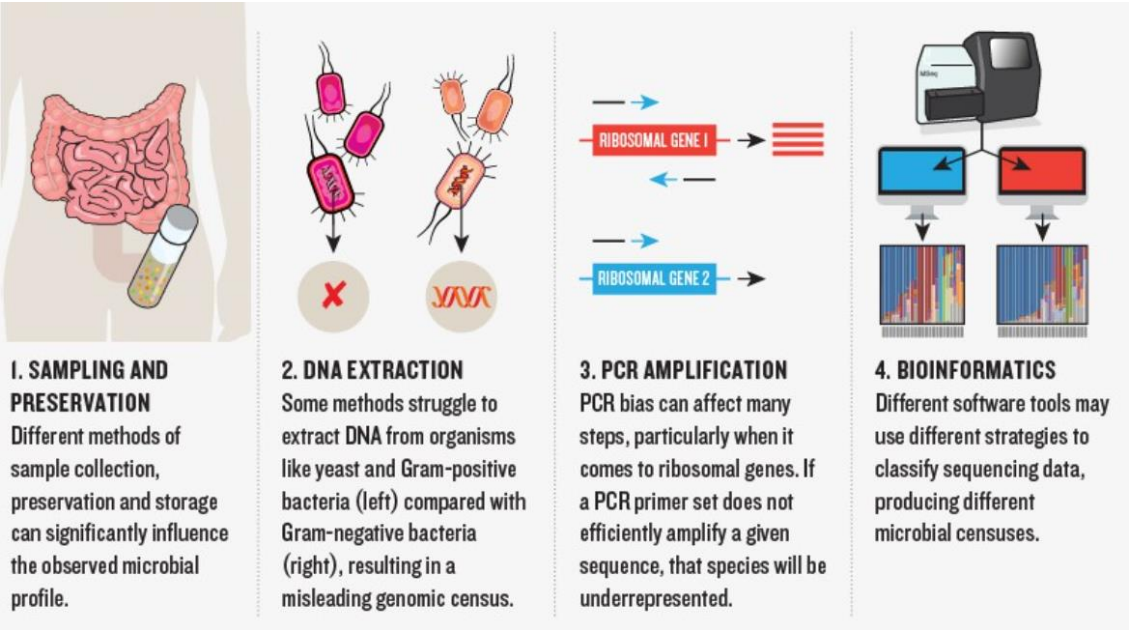


Figure 12 Four main steps where error and bias may be introduced in microbiota research. Figure credit from Nature Research Custom Media and Zymo Research (2018) with permission.

PCR amplification, used for 16S rRNA analyses, also introduces bias if sequences are not amplified uniformly. Often, GC-rich and long repetitive sequences are amplified poorly. Long repetitive sequences or GC-rich regions

promote the formation of secondary structures in the DNA, which block the action of DNA polymerase, resulting in incomplete extension of primers and low amplification. Several reagents and PCR cycling protocols have been developed to improve amplification, but in some cases, the challenge remains (Orpana, Ho and Stenman, 2012). Shotgun metagenomic sequencing is an alternative method which removes PCR bias as there is no amplification step. In shotgun sequencing, the entire metagenome is analysed as opposed to the 16S rRNA gene, allowing genera in lower abundance and annotated species to be identified (Durazzi *et al.*, 2021).

Finally, a comparison of 11 different bioinformatic tools found that microbial abundance differed by up to three times depending on the software used. This variation is partly due to taxonomic misclassification, which can be ameliorated using abundance filtering and ensemble approaches (taking the consensus of several tools). For shotgun data, combining tools that use different classification strategies (alignment, k-mer analysis, or assembly) can optimise the ensemble approach (McIntyre *et al.*, 2017).

Faecal samples and diet records are the most common information collected during human microbiota studies. Additionally, some studies have used breath testing to facilitate microbiota research. The participant provides a breath sample in a bag, which is measured by gas chromatography to quantify hydrogen and methane concentrations. Breath hydrogen has been used to diagnose sugar malabsorption in the small intestine. About one-third of individuals will excrete lower concentrations of breath hydrogen but higher concentrations of methane due to their microbiota's ability to convert hydrogen

to methane (Levitt *et al.*, 2006; De Lacy Costello, Ledochowski and Ratcliffe, 2013).

Some research has used breath testing to characterise individuals into HE and LE groups and found functional differences in the microbiota (Robert and Bernalier-Donadille, 2003). Breath gas emissions of 32 individuals over 15 days showed variation in hydrogen emission, but methane emission was stable. Breath hydrogen is high in the early morning, decreases and progressively increases throughout the day, and is mostly influenced by carbohydrate ingestion (le Marchand *et al.*, 1992). The study concluded that four days of measurement are required to accurately characterise breath hydrogen, whereas only one day is needed for methane (le Marchand *et al.*, 1992). In 15 individuals who were followed for 18 to 36 months, breath methane was consistent for 11 participants (73 %). Two participants showed reduced breath methane, and two showed increased breath methane. One of the four participants had received antibiotics which may explain the change, but for the other three, the cause of the change was unknown (Strocchi *et al.*, 1994). Although breath testing does not provide precise information on microbial communities, it has proven to be a practical, affordable, and non-invasive indicator of methanogen abundance and may be linked to microbiota functionality.

2.5.1.2 *In Vitro* Studies

In vitro models are commonly used for human colonic microbiota studies. The setup can vary from simple batch cultures to complex continuous models that run for several weeks.

Batch culture is a static model that is a crude simplification of the colonic microbiota. It can only run for a limited time, as substrate availability and the

concentration of end-products become a hindrance to bacterial metabolism (Edwards *et al.*, 1996). On the other hand, the setup is simpler than a continuous system, less resource-intensive, easily accessible, and repeatable. It is the most common setup used to understand the microbiota's metabolism of substrates and to generate mechanistic hypotheses for future work (Pham and Mohajeri, 2018).

Continuous systems have the advantage that substrate can be continuously fed, end products removed by dialysis, and they can be dynamic with peristaltic mixing and nutrient absorption. One example is the TNO *in vitro* model of the colon (TIM-2), which has been validated for its compositional and metabolic likeness to the colon of adult sudden death victims (Macfarlane, Gibson and Cummings, 1992; Venema, 2015). However, due to their complexity and cost, these models are not widely available (Verhoeckx *et al.*, 2015).

All *in vitro* colonic fermentation models are subject to the bias of faecal sampling inputs and cannot consider the interaction between the colonic microbiota and host cells (Donaldson, Lee and Mazmanian, 2016). However, the accessibility of *in vitro* systems allows experimental control and easy sample collection. While there is a published standard method for *in vitro* digestion studies (Brodkorb *et al.*, 2019), there is no comparable standardisation for *in vitro* colonic fermentations. This standardisation would improve the replicability and reliability of the method.

2.5.1.3 'Omic' Methodologies

The application of molecular-based techniques for studying the human colonic microbiota using faecal samples was transformative in the field. Tools such as 16S, metagenomics, metatranscriptomics, metaproteomics and metabolomics

have enabled deeper insights into diet-microbiota-host relationships (Guirro *et al.*, 2018).

Genomics was the first of the 'omics' disciplines to be developed, including analysing the genomes of microorganisms in the human colon. Of the next-generation sequencing methods, 16S rRNA sequencing was used because the gene is present in almost all bacteria, has a conserved function, and is large enough for informatics (1500 base pairs). It involves amplification of portions of the hypervariable regions (V1-V9) of the 16S rRNA gene (Janda and Abbott, 2007). An advancement on 16S rRNA sequencing was shotgun metagenomic sequencing, where the DNA is isolated from the microbiota (often using a commercial DNA extraction kit), and the DNA reads of whole genomes are sequenced, *de novo* assembled and/or aligned to reference genomes using a database (Sirangelo, 2018). It is differentiated from 16S rRNA sequencing by analysing all DNA in a sample instead of marker genes. This difference means the data from shotgun sequencing can be used for additional analyses such as functional profiling. It also detects some archaea, viruses, virophages, eukaryotes, and bacteria. Limitations are that it cannot provide information on the expression level of genes or proteins and cannot distinguish between active, dormant, or dead organisms (Jovel *et al.*, 2016).

Metatranscriptomics informs on gene transcription, which has greater functionality than metagenomics. The mRNA is isolated from the community, sequenced, and matched to a database to provide information on the active gene's function. Limitations are the difficulty in obtaining enough quality RNA and effectively separating the mRNA from other RNAs. In addition, the

databases for metatranscriptomics are not as comprehensive as the databases for metagenomics, which can limit data interpretation (Sirangelo, 2018).

Metaproteomics allows a further level of functional characterisation, which considers that not all mRNA transcripts are translated into proteins. However, it is estimated that there are 63 million proteins in human faecal samples, so the protein environment is complex, and standardised procedures and databases are still developing. Limitations include efficient extraction of proteins, removal of chemicals used in the sample extraction process, removal of host cells, enrichment of microbial cells, and increasing sample purity through the pre-fractionation of proteins and peptides (Petriz and Franco, 2017).

Metabolomics uses mass spectrometry or nuclear magnetic resonance (NMR) spectroscopy to provide information on the metabolites in a sample (Rombouts *et al.*, 2017). Like metatranscriptomics and metaproteomics, the metabolomics reference databases are limited and not as advanced as those for metagenomics (Sirangelo, 2018). The Human Metabolome Database 5.0 contains reference information for 218,000 metabolites, and the more specific Human Fecal Metabolome Database contains approximately 6000 metabolites (Karu *et al.*, 2018; Wishart *et al.*, 2021). In comparison, genetic databases for the human gut microbiota contain genomes that encode more than 170 million protein sequences (Almeida *et al.*, 2021).

Metabolomics can measure metabolites that are intracellular, extracellular, or found in the headspace, either of endogenous or microbial origin (Villas-Boas, 2016). Metabolites produced by the colonic microbiota include vitamins, amino acids, proteins, peptides, polysaccharide-peptidoglycans, substrate hydrolysis

products, bacteriocins, organic acids, and quorum sensing and virulence factors (Lockett *et al.*, 2016). These metabolites constitute up to 92 % of the faecal metabolome. The most prominent in faeces are organic acids, with lipids, bile acids, fatty acids, amino acids, sugars, amines, alcohols, phenolic compounds, and vitamins occurring in lower concentrations (Gall *et al.*, 2018; Zhgun and Ilyina, 2020). A challenge is that many metabolites, except organic acids, are produced at low concentrations. Exclusion processes can isolate other metabolites from organic acids, including size-based filtering to separate small bioactive from large macromolecules. Secondary metabolites and peptides can be isolated using denaturing treatments such as proteases and heat, and then the sample is fractioned with solid-phase extraction or high-performance liquid chromatography. However, large volumes of supernatant may be required to provide a sufficient concentration of the targeted metabolites (Ó Cuív *et al.*, 2016). The main considerations for metabolomics include the extraction of metabolites from the sample, separation or fractionation by chromatography, ionisation of the molecules of interest, detection of mass signals, and finally, analyte identification (Čuperlović-Culf *et al.*, 2010).

Integrating these fields, commonly referred to as a multi-omic approach, is a holistic way to analyse the microbial composition, function, and metabolism of the microbiota (Figure 13). Challenges include the integration of metadata, data from different platforms, and the management and bioinformatics of large datasets (Abram, 2015; Beale, Karpe and Ahmed, 2016; Sirangelo, 2018). Nevertheless, some studies have integrated multi-omic data to highlight key functionalities linked with key genes and species in networks (Roume *et al.*,

2015). This approach is a progressive step in identifying functionally important community members of the human colonic microbiota.

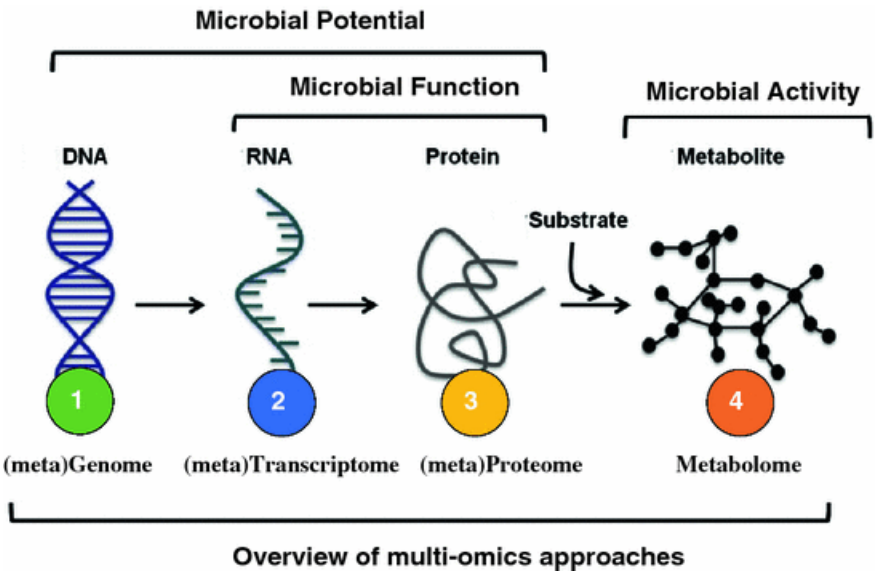


Figure 13 A multi-omic approach used to gain a deep insight into the composition and functionality of the human gastrointestinal tract microbiota.
Figure originally from Beale *et al.* (2016) and adapted from Abram (2015) with permission.

2.5.2 Summary

Abundant literature shows correlations between the human colonic microbiota and various diseases and dietary factors. A greater understanding of the role of the colonic microbiota in health will increasingly come from cause-and-effect studies. However, more research is needed to define appropriate models. *In vitro* fermentation models have a role in developing mechanistic hypotheses to be tested in further animal or human studies. In all forms of study, combining omic methodologies has advanced the understanding of the complex networks and interactions of the human colonic microbiota.

2.6 Future Perspectives

The healthy colonic microbiota is not well defined. However, it is thought that it should be diverse in richness, enriched for fibre fermentation, organic acid production and vitamin and amino acid synthesis, yet not excessive in methane or hydrogen sulphide production. Further mechanistic studies of microbiota functionality in healthy individuals are needed to test this hypothesis.

The important role of fibre fermentation in the healthy microbiota relates to the structure of dietary fibres. The macrostructures, mesostructures, and molecular structures explain some interactions between dietary fibre and the microbiota. However, these structures are affected by food processing, preparation, and digestion, which add complexity.

The variation in individual responses to diet also adds complexity and is related to individual differences in colonic microbiota composition and the abundance of keystone species that have an important role in fibre fermentation and cross-feeding.

In vitro models are suitable tools for mechanistic testing and are well-suited to studying substrate metabolism by the colonic microbiota. However, further animal and/or human studies are required to establish cause and effect. Meanwhile, multi-omic approaches permit the study of the microbiota's metabolism in response to dietary substrates, which is critical to modulating the human colonic microbiota for optimal health using diet.

2.7 Thesis Aims

A key goal of microbiota research is to identify the characteristics of optimal and sub-optimal microbiota and to have tools to manipulate the former. It is known that diet is a driver of microbiota composition; however, the precise interactions between fermentable substrates and the microbiota that result in the production of metabolites are unclear. A better understanding of this area is required for precise microbiota modulation.

Human breath methane has been linked to differences in microbiota composition and functionality and may be an important variable in substrate-microbiota interactions. Previous studies have assessed HE compared to LE group differences by culture and single sample faecal sequencing, which provides a snapshot of composition and functionality. However, questions remain about how breath methane relates to the composition and functionality of a complex microbial community and the underlying mechanisms of this relationship. Therefore, fermentation models may help investigate the interactions between the colonic microbiota and fermentable substrates in individuals who are HE and LE.

Under this premise, the aims of the PhD project are as follows:

1. **Investigate differences in the composition and predictive function of the human faecal microbiota between HE and LE individuals.**

The research will validate the differences in microbiota composition and functionality noted in Kumpitsch *et al.* (2021) and Robert and Bernalier-Donadille (2003) using methane breath testing and faecal microbiota shotgun sequencing. In addition, this data will answer whether individuals who are HE

harbour more taxa from the Firmicutes phylum and individuals who are LE have more taxa from the Bacteroidetes phyla.

2. **Assess whether breath methane and faecal community composition link to differences in fibre fermentation in an *in vitro* model of colonic fermentation.**

The second part of the research will validate whether the composition and potential metabolic differences of the microbiota seen in faecal sequencing from a single time point are evident during fibre fermentation. Faecal samples will be used to inoculate an *in vitro* model of colonic fermentation, and dietary fibres will be added to emulate colonic fermentation. Samples will be taken in a time-course to assess changes in microbiota composition and fermentation and analysed using shotgun metagenomic and metabolomic approaches. Highly fermentable soluble fibre will be used as a positive control compared to insoluble fibre to investigate whether the microbiota of HE individuals has a greater rate and extent of insoluble fibre fermentation than LE individuals.

One caveat with using an *in vitro* model is that strategies must be employed to increase the repeatability and reliability of the data. This limitation will be accounted for using published methods and standard operating procedures where available.

In summary, the thesis aims to use a combination of human study and *in vitro* fermentation models to study the associations between breath methane and microbiota composition and fibre fermentation (Figure 14). First, this work will help to validate previous findings linking breath methane emissions with microbiota composition and predictive functionality. Secondly, it will assess

whether the findings from sequencing single faecal samples are reflected in analyses from a fermentation model.

The results from this research will help to establish whether human breath methane is linked to microbiota composition and fibre fermentation. Then, hypotheses for future studies can be generated regarding specific interactions between dietary substrates and the gut microbiota. This knowledge is important to understanding the microbiota as a complex system within individuals and developing strategies for successful microbiota modulation.

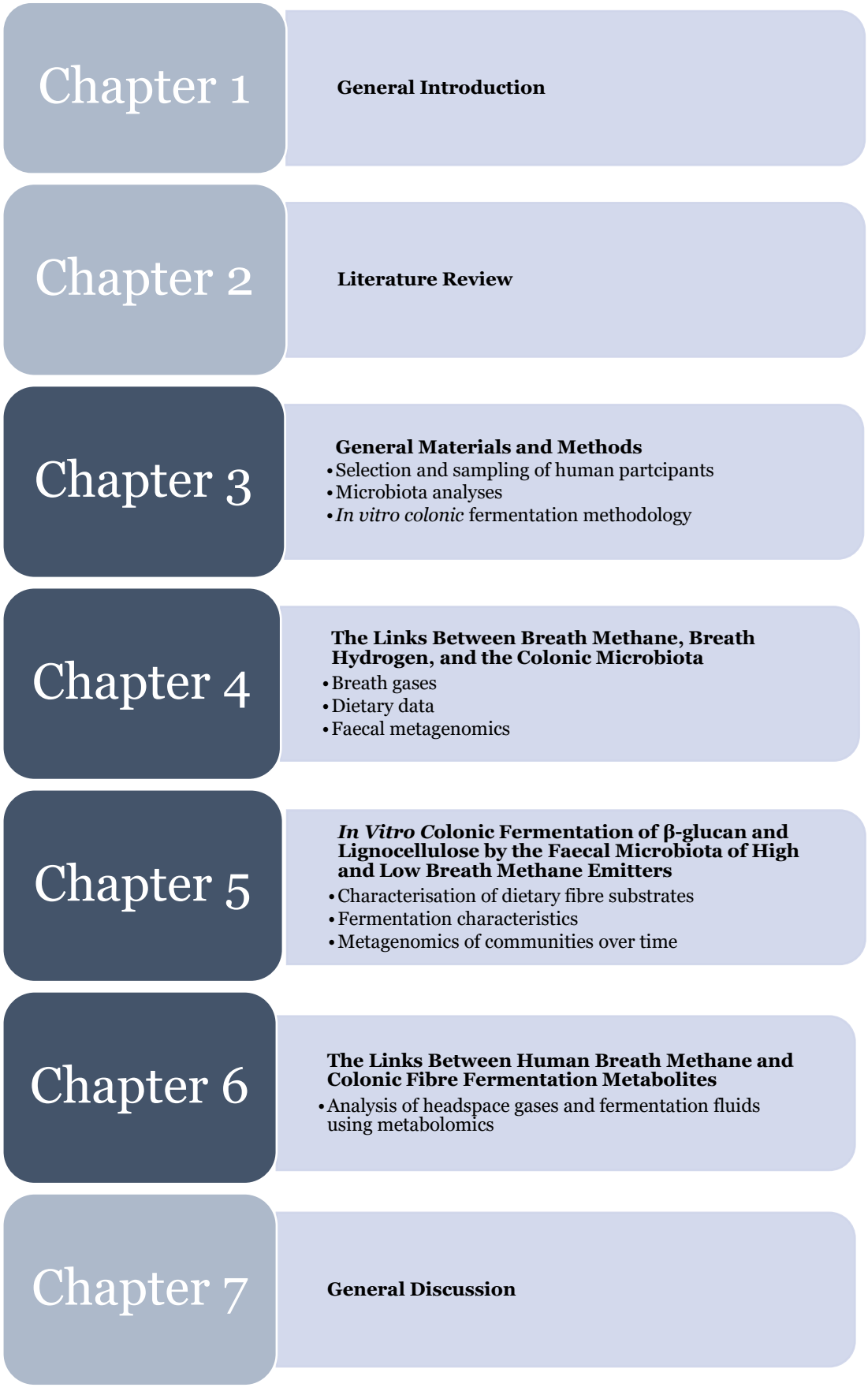


Figure 14 Different chapters of the thesis.

References

1. Abram, F. (2015). Systems-based approaches to unravel multi-species microbial community functioning. Computational and Structural Biotechnology Journal 13, 24–32. https://doi.org/10.1016/j.csbj.2014.11.009.
2. Aguayo, M., Fernández Pérez, A., Reyes, G., Oviedo, C., Gacitúa, W., Gonzalez, R., and Uyarte, O. (2018). Isolation and Characterization of Cellulose Nanocrystals from Rejected Fibers Originated in the Kraft Pulping Process. Polymers (Basel) 10, 1145. https://doi.org/10.3390/polym10101145.
3. Aidy, S., van den Bogert, B., and Kleerebezem, M. (2015). The small intestine microbiota, nutritional modulation and relevance for health. Current Opinion in Biotechnology 32, 14–20. https://doi.org/10.1016/j.copbio.2014.09.005.
4. Albenberg, L.G., and Wu, G.D. (2014). Diet and the Intestinal Microbiome: Associations, Functions, and Implications for Health and Disease. Gastroenterology 146, 1564–1572. https://doi.org/10.1053/j.gastro.2014.01.058.Diet.
5. Almeida, A., Nayfach, S., Boland, M., Strozzi, F., Beracochea, M., Shi, Z. J., Pollard, K. S., Sakharova, E., Parks, D. H., Hugenholtz, P., Segata, N., Kyrpides, N. C., and Finn, R. D. (2021). A unified catalog of 204,938 reference genomes from the human gut microbiome. Nature Biotechnology 39(1), 105–114. https://doi.org/10.1038/s41587-020-0603-3.
6. AOAC (1994). Soluble, Insoluble, and Total Dietary Fiber in Foods and Food Products. In AOAC International Approved Methods, (St. Paul, MN, USA: AOAC International), pp. 1–3.
7. Arumugam, M., Raes, J., Pelletier, E., le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J. M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Bork, P. (2011). Enterotypes of the human gut microbiome. Nature 473(7346), 174–180. https://doi.org/10.1038/nature09944.

8. Attene-Ramos, M.S., Wagner, E.D., Plewa, M.J., and Gaskins, H.R. (2006). Evidence that hydrogen sulfide is a genotoxic agent. Molecular Cancer Research 4, 9–14. https://doi.org/10.1158/1541-7786.MCR-05-0126.

9. Avila, M., Ojcius, D.M., and Yilmaz, Ö. (2009). The Oral Microbiota: Living with a Permanent Guest. DNA and Cell Biology 28, 405–411. https://doi.org/10.1089/dna.2009.0874.

10. Banerjee, S., Schlaeppi, K., and van der Heijden, M.G.A. (2018). Keystone taxa as drivers of microbiome structure and functioning. Nature Reviews Microbiology 16, 567–576. https://doi.org/10.1038/s41579-018-0024-1.

11. Beale, D.J., Karpe, A. V., and Ahmed, W. (2016). Beyond Metabolomics: A Review of Multi-Omics-Based Approaches. In Microbial Metabolomics, (Cham: Springer International Publishing), pp. 289–312.

12. Belenguer, A., Duncan, S.H., Holtrop, G., Anderson, S.E., Lobley, G.E., and Flint, H.J. (2007). Impact of pH on lactate formation and utilization by human fecal microbial communities. Applied and Environmental Microbiology 73, 6526–6533. https://doi.org/10.1128/AEM.00508-07.

13. Björck, I., Nyman, M.\, and Asp, N.-G. (1984). Extrusion-cooking and dietary fiber: Effects on dietary fiber content and on degradation in the rat intestinal tract. Cereal Chemistry 61, 174–179.

14. Blachier, F., Beaumont, M., and Kim, E. (2019). Cysteine-derived hydrogen sulfide and gut health: a matter of endogenous or bacterial origin. Current Opinion in Clinical Nutrition and Metabolic Care 22, 68–75. https://doi.org/10.1097.

15. Boland, M., Golding, M., and Singh, H. (2014). Understanding Food Structures in Natural and Processed Foods and their Behavior During Physiological Processing. In Food Structures, Digestion and Health, (Elsevier Inc.), pp. 3–81.

16. Bornhorst, G.M., and Paul Singh, R. (2014). Gastric Digestion In Vivo and *In vitro*: How the Structural Aspects of Food Influence the Digestion Process. Annual Review of Food Science and Technology 5, 111–132. https://doi.org/10.1146/annurev-food-030713-092346.

17. Bourriaud, C., Robins, R.J., Martin, L., Kozlowski, F., Tenailleau, E., Cherbut, C., and Michel, C. (2005). Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. Journal of Applied Microbiology 99, 201–212. https://doi.org/10.1111/j.1365-2672.2005.02605.x.

18. Briggs, J.A., Grondin, J.M., and Brumer, H. (2021). Communal living: glycan utilization by the human gut microbiota. Environmental Microbiology 23, 15–35. https://doi.org/10.1111/1462-2920.15317.

19. Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., le Feunteun, S., Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. Nature Protocols 14(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1.

20. Camire, M.E., and Flint, S.I. (1991). Thermal Processing Effects on Dietary Fiber Composition and Hydration Capacity in Corn Meal, Oatmeal, and Potato Peels. Cereal Chemistry 68, 645–647.

21. Cantarel, B.L., Lombard, V., and Henrissat, B. (2012). Complex carbohydrate utilization by the healthy human microbiome. PLoS ONE 7, 1–10. https://doi.org/10.1371/journal.pone.0028742.

22. Cao, Y., Zhang, R., Sun, C., Cheng, T., Liu, Y., and Xian, M. (2013). Fermentative succinate production: An emerging technology to replace the traditional petrochemical processes. BioMed Research International 2013, 1-12. https://doi.org/10.1155/2013/723412.

23. Carnachan, S.M., Bootten, T.J., Mishra, S., Monro, J.A., and Sims, I.M. (2012). Effects of simulated digestion *in vitro* on cell wall polysaccharides from kiwifruit (Actinidia spp.). Food Chemistry 133, 132–139. https://doi.org/10.1016/j.foodchem.2011.12.084.

24. Cartmell, A., Muñoz-Muñoz, J., Briggs, J. A., Ndeh, D. A., Lowe, E. C., Baslé, A., Terrapon, N., Stott, K., Heunis, T., Gray, J., Yu, L., Dupree, P., Fernandes, P. Z., Shah, S., Williams, S. J., Labourel, A., Trost, M., Henrissat, B., and Gilbert, H. J. (2018). A surface endogalactanase in Bacteroides

thetaiotaomicron confers keystone status for arabinogalactan degradation. Nature Microbiology 3(11), 1314–1326. https://doi.org/10.1038/s41564-018-0258-8.

25. Cerf-Bensussan, N., and Gaboriau-Routhiau, V. (2010). The immune system and the gut microbiota: friends or foes? Nature Reviews Immunology 10, 735–744. https://doi.org/10.1038/nri2850.

26. Chassard, C., and Bernalier-Donadille, A. (2006). H2 and acetate transfers during xylan fermentation between a butyrate-producing xylanolytic species and hydrogenotrophic microorganisms from the human gut. FEMS Microbiology Letters 254, 116–122. https://doi.org/10.1111/j.1574-6968.2005.00016.x.

27. Chassard, C., Delmas, E., Robert, C., Lawson, P.A., and Bernalier-Donadille, A. (2012). Ruminococcus champanellensis sp. nov., a cellulose-degrading bacterium from human gut microbiota. International Journal of Systematic and Evolutionary Microbiology 62, 138–143. https://doi.org/10.1099/ijs.0.027375-0.

28. Chen, H. L., Haack, V. S., Jacecky, C. W., Vollendorf, N. W., and Marlett, J. A. (1998). Mechanisms by which wheat bran and oat bran increase stool weight in humans. American Journal of Clinical Nutrition, 68, 711–719. https://academic.oup.com/ajcn/article/68/3/711/4648701.

29. Chng, K. R., Ghosh, T. S., Tan, Y. H., Nandi, T., Lee, I. R., Ng, A. H. Q., Li, C., Ravikrishnan, A., Lim, K. M., Lye, D., Barkham, T., Raman, K., Chen, S. L., Chai, L., Young, B., Gan, Y. H., and Nagarajan, N. (2020). Metagenome-wide association analysis identifies microbial determinants of post-antibiotic ecological recovery in the gut. Nature Ecology and Evolution 4(9), 1256–1267. https://doi.org/10.1038/s41559-020-1236-0.

30. Cho, I., and Blaser, M.J. (2012). Applications of Next-Generation Sequencing: The human microbiome: at the interface of health and disease. Nature Publishing Group 13, 260–270. https://doi.org/10.1038/nrg3182.

31. Choct, M. (2015). Fibre - Chemistry and Functions in Poultry Nutrition. Avicultura 28, 113–119.

32. Christl, S.U., Eisner, H.D., Dusel, G., Kasper, H., and Scheppach, W. (1996). Antagonistic Effects of Sulfide and Butyrate on Proliferation of Colonic Mucosa: A Potential Role for These Agents in the Pathogenesis of Ulcerative Colitis. Digestive Diseases and Sciences 41, 2477–2481. https://doi.org/10.1007/bf02100146.

33. Cockburn, D.W., and Koropatkin, N.M. (2016). Polysaccharide Degradation by the Intestinal Microbiota and Its Influence on Human Health and Disease. Journal of Molecular Biology 428, 3230–3252. https://doi.org/10.1016/j.jmb.2016.06.021.

34. Costea, P. I., Zeller, G., Sunagawa, S., Pelletier, E., Alberti, A., Levenez, F., Tramontano, M., Driessen, M., Hercog, R., Jung, F.-E., Kultima, J. R., Hayward, M. R., Coelho, L. P., Allen-Vercoe, E., Bertrand, L., Blaut, M., Brown, J. R. M., Carton, T., Cools-Portier, S., Bork, P. (2017). Towards standards for human fecal sample processing in metagenomic studies. Nature Biotechnology 35(11), 1069–1076. https://doi.org/10.1038/nbt.3960.

35. Cottee-Jones, H.E.W., and Whittaker, R.J. (2012). perspective: The keystone species concept: a critical appraisal. Frontiers of Biogeography 4, 117–127. https://doi.org/10.21425/F5FBG12533.

36. Coyte, K.Z., Schluter, J., and Foster, K.R. (2015). The ecology of the microbiome: Networks, competition, and stability. Science 350, 663–666. https://doi.org/10.1126/science.aad2602.

37. Crespo-Piazuelo, D., Estellé, J., Revilla, M., Criado-Mesas, L., Ramayo-Caldas, Y., Óvilo, C., Fernández, A.I., Ballester, M., and Folch, J.M. (2018). Characterization of bacterial microbiota compositions along the intestinal tract in pigs and their interactions and functions. Scientific Reports 8, 12727. https://doi.org/10.1038/s41598-018-30932-6.

38. Cummings, J.H. (1984). Cellulose and the human gut. Gut 25, 805–810.

39. Cummings, J.H., and Engineer, A. (2017). Denis Burkitt and the origins of the dietary fibre hypothesis. Nutrition Research Reviews 1–15. https://doi.org/10.1017/S0954422417000117.

40. Cummings, J.H., and Englyst, H.N. (1987). Fermentation in the human large intestine and the available substrates. The American Journal of Clinical Nutrition 45, 1243–1255.

41. Čuperlović-Culf, M., Barnett, D.A., Culf, A.S., and Chute, I. (2010). Cell culture metabolomics: applications and future directions. Drug Discovery Today 15, 610–621. https://doi.org/10.1016/j.drudis.2010.06.012.

42. Curtis, M.M., Hu, Z., Klimko, C., Narayanan, S., Deberardinis, R., and Sperandio, V. (2014). The Gut Commensal Bacteroides thetaiotaomicron Exacerbates Enteric Infection through Modification of the Metabolic Landscape. Cell Host and Microbe 16, 759–769. https://doi.org/10.1016/j.chom.2014.11.005.

43. David, L.A., Materna, A.C., Friedman, J., Campos-Baptista, M.I., Blackburn, M.C., Perrotta, A., Erdman, S.E., and Alm, E.J. (2015). Host lifestyle affects human microbiota on daily timescales. Genome Biology 15, 1–15. https://doi.org/10.1186/gb-2014-15-7-r89.

44. Davis, J.N., Hodges, V.A., and Gillham, B. (2006). Normal-Weight Adults Consume More Fiber and Fruit than Their Age- and Height-Matched Overweight/Obese Counterparts. Journal of the American Dietetic Association 106, 833–840. https://doi.org/10.1016/j.jada.2006.03.013.

45. Donaldson, G.P., Lee, S.M., and Mazmanian, S.K. (2016). Gut biogeography of the bacterial microbiota. Nature Reviews Microbiology 14, 20–32. https://doi.org/10.1038/nrmicro3552.

46. Dorokhov, Y.L., Shindyapina, A. v, Sheshukova, E. v, and Komarova, T. v (2015). Metabolic Methanol: Molecular Pathways and Physiological Roles. Physiological Reviews 95, 603–644. https://doi.org/10.1152/physrev.00034.2014.-Methanol.

47. Durazzi, F., Sala, C., Castellani, G., Manfreda, G., Remondini, D., and De Cesare, A. (2021). Comparison between 16S rRNA and shotgun sequencing data for the taxonomic characterization of the gut microbiota. Scientific Reports 11, 1–10. https://doi.org/10.1038/s41598-021-82726-y.

48. Duvigneaud, N., Wijndaele, K., Matton, L., Philippaerts, R., Lefevre, J., Thomis, M., Delecluse, C., and Duquet, W. (2007). Dietary factors

associated with obesity indicators and level of sports participation in Flemish adults: a cross-sectional study. Nutrition Journal 6(1), 26. https://doi.org/10.1186/1475-2891-6-26.

49. Eckburg, P.B. (2005). Diversity of the Human Intestinal Microbial Flora. Science 308, 1635–1638. https://doi.org/10.1126/science.1110591.

50. Edwards, C.A., Gibson, G., Champ, M., Jensen, B.-B., Mathers, J.C., Nagengast, F., Rumney, C., and Quehl, A. (1996). *In vitro* Method for Quantification of the Fermentation of Starch by Human Faecal Bacteria. Journal of the Science of Food and Agriculture 71, 209–217. https://doi.org/10.1002.

51. Egert, M., De Graaf, A.A., Smidt, H., De Vos, W.M., and Venema, K. (2006). Beyond diversity: Functional microbiomics of the human colon. Trends in Microbiology 14, 86–91. https://doi.org/10.1016/j.tim.2005.12.007.

52. Eliasson, A.-C. (2004). Starch in Food. Structure, function and applications (Cambridge: CRC Press).

53. Eliasson, A.-C. (2016). Chapter 10 Starch: Physicochemical and Functional Aspects. In Carbohydrates in Food, (6000 Broken Sound Parkway NW, Suite 300, Boca Raton, FL 33487-2742: CRC Press Taylor and Francis Group), pp. 479–578.

54. Englyst, H.N., and Cummings, J.H. (1985). Digestion of the polysaccharides of some cereal foods in the human small intestine. The American Journal of Clinical Nutrition 42, 778–787. https://doi.org/10.1093/ajcn/42.5.778.

55. Englyst, H. N., Trowell, H., Southgate, D. A. T., and Cummings, H. H. (1987). Dietary fiber and resistant starch. American Journal of Clinical Nutrition, 46, 873–874. https://academic.oup.com/ajcn/article/46/6/873/4716076

56. Faisant, N., Champ, M., Colonna, P., Buleon, A., Molis, C., Langkilde, A.M., Schweizer, T., Flourie, B., and Galmiche, J.P. (1993). Structural features of resistant starch at the end of the human small intestine. European Journal of Clinical Nutrition 47, 285–296.

57. Faisant, N., Buléon, A., Colonna, P., Molis, C., Lartigue, S., Galmiche, J.P., and Champ, M. (1995). Digestion of raw banana starch in the small intestine

of healthy humans: structural features of resistant starch. British Journal of Nutrition 73, 111. https://doi.org/10.1079/BJN19950013.

58. Faith, J.J., Guruge, J.L., Charbonneau, M., Subramanian, S., Seedorf, H., Goodman, A.L., Clemente, J.C., Knight, R., Andrew, C., Leibel, R.L., *et al.* (2013). The long-term stability of the human gut microbiota. Science 341, 1–22. https://doi.org/10.1126/science.1237439.

59. Fåk, F., Jakobsdottir, G., Kulcinskaja, E., Marungruang, N., Matziouridou, C., Nilsson, U., Stålbrand, H., and Nyman, M. (2015). The physico-chemical properties of dietary fibre determine metabolic responses, short-chain fatty acid profiles and gut microbiota composition in rats fed low- and high-fat diets. PLoS ONE 10, 1–16. https://doi.org/10.1371/journal.pone.0127252.

60. Fernández-Veledo, S., and Vendrell, J. (2019). Gut microbiota-derived succinate: Friend or foe in human metabolic diseases? Reviews in Endocrine and Metabolic Disorders 20, 439–447. https://doi.org/10.1007/s11154-019-09513-z.

61. De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S., Collini, S., Pieraccini, G., and Lionetti, P. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. Proceedings of the National Academy of Sciences 107, 14691–14696. https://doi.org/10.1073/pnas.1005963107.

62. Fisher, C.K., and Mehta, P. (2014). Identifying keystone species in the human gut microbiome from metagenomic timeseries using sparse linear regression. PLoS ONE 9, 1–10. https://doi.org/10.1371/journal.pone.0102451.

63. Flint, H.J., Bayer, E.A., Rincon, M.T., Lamed, R., and White, B.A. (2008). Polysaccharide utilization by gut bacteria: Potential for new insights from genomic analysis. Nature Reviews Microbiology 6, 121–131. https://doi.org/10.1038/nrmicro1817.

64. Flint, H.J., Scott, K.P., Louis, P., and Duncan, S.H. (2012a). The role of the gut microbiota in nutrition and health. Nature Reviews Gastroenterology and Hepatology 9, 577–589. https://doi.org/10.1038/nrgastro.2012.156.

65. Flint, H.J., Scott, K.P., Duncan, S.H., Louis, P., and Forano, E. (2012b). Microbial degradation of complex carbohydrates in the gut. Gut Microbes 3. https://doi.org/10.4161/gmic.19897.

66. Flint, H.J., Duncan, S.H., and Louis, P. (2017). The impact of nutrition on intestinal bacterial communities. Current Opinion in Microbiology 38, 59–65. https://doi.org/10.1016/j.mib.2017.04.005.

67. Food and Agriculture Organisation of the United Nations, and World Health Organisation (1998). Carbohydrates in human nutrition. FAO Food and Nutrition Paper 66.

68. Friend, D.W., Cunningham, H.M., and Nicholson, J.W.G. (1963). The production of organic acids in the pig. Division of Animal and Poultry Science 43, 156–168.

69. Frost, G., Sleeth, M. L., Sahuri-Arisoylu, M., Lizarbe, B., Cerdan, S., Brody, L., Anastasovska, J., Ghourab, S., Hankir, M., Zhang, S., Carling, D., Swann, J. R., Gibson, G., Viardot, A., Morrison, D., Louise Thomas, E., and Bell, J. D. (2014). The short-chain fatty acid acetate reduces appetite via a central homeostatic mechanism. Nature Communications 5(1), 1–11. https://doi.org/10.1038/ncomms4611.

70. Fu, G., Yue, P., Hu, Y., Li, N., Shi, Z., and Peng, F. (2018). Fractionation of DMSO-Extracted and NaOH-Extracted Hemicelluloses by Gradient Ethanol Precipitation from Neosinocalamus affinis. International Journal of Polymer Science 2018, 1–8. https://doi.org/10.1155/2018/9587042.

71. Fukuda, S., Toh, H., Hase, K., Oshima, K., Nakanishi, Y., Yoshimura, K., Tobe, T., Clarke, J. M., Topping, D. L., Suzuki, T., Taylor, T. D., Itoh, K., Kikuchi, J., Morita, H., Hattori, M., and Ohno, H. (2011). Bifidobacteria can protect from enteropathogenic infection through production of acetate. Nature, 469(7331), 543–547. https://doi.org/10.1038/nature09646.

72. Gaci, N., Borrel, G., Tottey, W., O'Toole, P.W., and Brugère, J.F. (2014). Archaea and the human gut: New beginning of an old story. World Journal of Gastroenterology 20, 16062–16078. https://doi.org/10.3748/wjg.v20.i43.16062.

73. Gall, G. le, Guttula, K., Kellingray, L., Tett, A.J., Hoopen, R. ten, Kemsley, K.E., Savva, G.M., Ibrahim, A., and Narbad, A. (2018). Metabolite quantification of faecal extracts from colorectal cancer patients and healthy controls. Oncotarget 9, 33278–33289. https://doi.org/10.18632/oncotarget.26022.

74. Gao, Z., Yin, J., Zhang, J., Ward, R.E., Martin, R.J., Lefevre, M., Cefalu, W.T., and Ye, J. (2009). Butyrate Improves Insulin Sensitivity and Increases Energy Expenditure in Mice. Diabetes 58, 1509–1517. https://doi.org/10.2337/db08-1637.

75. Garrett, W. S., Gallini, C. A., Yatsunenko, T., Michaud, M., Delaney, M. L., Punit, S., Karlsson, M., Bry, L., Jonathan, N., Gordon, J. I., Onderdonk, A. B., and Glimcher, L. H. (2011). Induce Spontaneous and Maternally Transmitted Colitis. Cell Host Microbe, 8(3), 292–300. https://doi.org/10.1016/j.chom.2010.08.004.

76. Gérard, P. (2020). The crosstalk between the gut microbiota and lipids. OCL - Oilseeds and Fats, Crops and Lipids 27. https://doi.org/10.1051/ocl/2020070.

77. Ghavami, S.B., Rostami, E., Sephay, A.A., Shahrokh, S., Balaii, H., Aghdaei, H.A., and Zali, M.R. (2018). Alterations of the human gut Methanobrevibacter smithii as a biomarker for inflammatory bowel diseases. Microbial Pathogenesis 117, 285–289. https://doi.org/10.1016/j.micpath.2018.01.029.

78. Gibson, G., Macfarlane, G.T., and Cummings, J. (1993). Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut 34, 437–439.

79. Gidley, M.J., and Yakubov, G.E. (2019). Functional categorisation of dietary fibre in foods: Beyond 'soluble' vs 'insoluble.' Trends in Food Science and Technology 86, 563–568. https://doi.org/10.1016/j.tifs.2018.12.006.

80. Gilbert, J.A., Blaser, M.J., Caporaso, J.G., Jansson, J.K., Lynch, S. V., and Knight, R. (2018). Current understanding of the human microbiome. Nature Medicine 24, 392–400. https://doi.org/10.1038/nm.4517.

81. Goldschmidt, F., Regoes, R.R., and Johnson, D.R. (2018). Metabolite toxicity slows local diversity loss during expansion of a microbial cross-feeding community. ISME Journal 12, 136–144. https://doi.org/10.1038/ismej.2017.147.

82. Goodman, B.E. (2010). Insights into digestion and absorption of major nutrients in humans. Advances in Physiology Education 34, 44–53. https://doi.org/10.1152/advan.00094.2009.

83. Granfeldt, Y., Hagander, B., and Björck, I. (1995). Metabolic responses to starch in oat and wheat products. On the importance of food structure, incomplete gelatinization or presence of viscous dietary fibre. European Journal of Clinical Nutrition 43, 189–199.

84. Guirro, M., Costa, A., Gual-Grau, A., Mayneris-Perxachs, J., Torrell, H., Herrero, P., Canela, N., and Arola, L. (2018). Multi-omics approach to elucidate the gut microbiota activity: Metaproteomics and metagenomics connection. Electrophoresis 39, 1692–1701. https://doi.org/10.1002/elps.201700476.

85. Hagander, B., Björck, I., Asp, N.G., Efendić, S., Holm, J., Nilsson-Ehle, P., Lundquist, I., and Scherstén, B. (1987). Rye products in the diabetic diet. Postprandial glucose and hormonal responses in non-insulin-dependent diabetic patients as compared to starch availability *in vitro* and experiments in rats. Diabetes Research and Clinical Practice 3, 85–96. https://doi.org/10.1016/S0168-8227(87)80012-8.

86. Hajishengallis, G., Darveau, R.P., and Curtis, M.A. (2012). The keystone-pathogen hypothesis. Nature Reviews Microbiology 10, 717–725. https://doi.org/10.1038/nrmicro2873.

87. Halfvarson, J., Brislawn, C. J., Lamendella, R., Vázquez-Baeza, Y., Walters, W. A., Bramer, L. M., D'Amato, M., Bonfiglio, F., McDonald, D., Gonzalez, A., McClure, E. E., Dunklebarger, M. F., Knight, R., and Jansson, J. K. (2017). Dynamics of the human gut microbiome in inflammatory bowel disease. Nature Microbiology 2(5), 17004. https://doi.org/10.1038/nmicrobiol.2017.4.

88. Hamaker, B.R., and Tuncil, Y.E. (2014). A perspective on the complexity of dietary fiber structures and their potential effect on the gut microbiota. Journal of Molecular Biology 426, 3838–3850. https://doi.org/10.1016/j.jmb.2014.07.028.

89. Hamer, H.M., Jonkers, D.M.A.E., Bast, A., Vanhoutvin, S.A.L.W., Fischer, M.A.J.G., Kodde, A., Troost, F.J., Venema, K., and Brummer, R.-J.M. (2009). Butyrate modulates oxidative stress in the colonic mucosa of healthy humans. Clinical Nutrition 28, 88–93. https://doi.org/10.1016/j.clnu.2008.11.002.

90. Hartman, A.L., Lough, D.M., Barupal, D.K., Fiehn, O., Fishbein, T., Zasloff, M., and Eisen, J.A. (2009). Human gut microbiome adopts an alternative state following small bowel transplantation. Proceedings of the National Academy of Sciences of the United States of America 106, 17187–17192. https://doi.org/10.1073/pnas.0904847106.

91. Helle, S., Cameron, D., Lam, J., White, B., and Duff, S. (2003). Effect of inhibitory compounds found in biomass hydrolysates on growth and xylose fermentation by a genetically engineered strain of S. cerevisiae. Enzyme and Microbial Technology 33, 786–792. https://doi.org/10.1016/S0141-0229(03)00214-X.

92. Henson, M., and Phalak, P. (2017). By-product Cross Feeding and Community Stability in an In Silico Biofilm Model of the Gut Microbiome. Processes 5, 13. https://doi.org/10.3390/pr5010013.

93. Hillman, E., Lu, H., Yao, T., and Nakatsu, C. (2017). Microbial Ecology along the Gastrointestinal Tract. Microbes and Environments 32, 300–313. https://doi.org/10.1264/jsme2.ME17017.

94. Hobson, P.N., and Stewart, C.S. (1997). The Rumen Microbial Ecosystem (Dordrecht: Springer).

95. Hoek, M.J.A. van, and Merks, R.M.H. (2017). Emergence of microbial diversity due to cross-feeding interactions in a spatial model of gut microbial metabolism. BMC Systems Biology 11, 1–18. https://doi.org/10.1186/s12918-017-0430-4.

96. Holloway, W.D., Tasman-Jones, C., and Lee, S.P. (1978). Digestion of certain fractions of dietary fiber in humans. American Journal of Clinical Nutrition 31, 927–930. https://doi.org/10.1093/ajcn/31.6.927.

97. Holloway, W.D., Tasman-Jones, C., and Bell, E. (1980). The hemicellulose component of dietary fiber. The American Journal of Clinical Nutrition 33, 260–263. https://doi.org/10.1093/ajcn/33.2.260.

98. Hoogeveen, A.M.E., Moughan, P.J., De Haas, E.S., Blatchford, P., McNabb, W.C., and Montoya, C.A. (2020). Ileal and hindgut fermentation in the growing pig fed a human-type diet. British Journal of Nutrition 124, 567–576. https://doi.org/10.1017/S0007114520001385.

99. Hughes, E.R., Winter, M.G., Duerkop, B.A., Spiga, L., Furtado de Carvalho, T., Zhu, W., Gillis, C.C., Büttner, L., Smoot, M.P., Behrendt, C.L., *et al.* (2017). Microbial Respiration and Formate Oxidation as Metabolic Signatures of Inflammation-Associated Dysbiosis. Cell Host and Microbe 21, 208–219. https://doi.org/10.1016/j.chom.2017.01.005.

100. Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J.H., Chinwalla, A.T., Creasy, H.H., Earl, A.M., Fitzgerald, M.G., Fulton, R.S., *et al.* (2012). Structure, function and diversity of the healthy human microbiome. Nature 486, 207–214. https://doi.org/10.1038/nature11234.

101. International Human Microbiome Standards (2015). Standard Operating Procedures (online). http://www.human-microbiome.org/index.php#SOPS.

102. Ishizaka, S., Kikuchi, E., and Tsujii, T. (1993). Effects of acetate on human immune system. Immunopharmacology and Immunotoxicology 15, 151–162. https://doi.org/10.3109/08923979309025991.

103. Jalanka-Tuovinen, J., Salonen, A., Nikkilä, J., Immonen, O., Kekkonen, R., Lahti, L., Palva, A., and de Vos, W.M. (2011). Intestinal microbiota in healthy adults: Temporal analysis reveals individual and common core and relation to intestinal symptoms. PLoS ONE 6. https://doi.org/10.1371/journal.pone.0023035.

104. Janda, J.M., and Abbott, S.L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and

pitfalls. Journal of Clinical Microbiology 45, 2761–2764. https://doi.org/10.1128/JCM.01228-07.

105. Jeffery, I. B., Claesson, M. J., O'Toole, P. W., and Shanahan, F. (2012). Categorization of the gut microbiota: enterotypes or gradients? Nature Reviews Microbiology, 10(9), 591–592. https://doi.org/10.1038/nrmicro2859.

106. Johnson, A.J., Zheng, J.J., Kang, J.W., Saboe, A., Knights, D., and Zivkovic, A.M. (2020). A Guide to Diet-Microbiome Study Design. Frontiers in Nutrition 7, 1–16. https://doi.org/10.3389/fnut.2020.00079.

107. Jovel, J., Patterson, J., Wang, W., Hotte, N., O'Keefe, S., Mitchel, T., Perry, T., Kao, D., Mason, A.L., Madsen, K.L., *et al.* (2016). Characterization of the gut microbiome using 16S or shotgun metagenomics. Frontiers in Microbiology 7, 1–17. https://doi.org/10.3389/fmicb.2016.00459.

108. Jung, T.H., Park, J.H., Jeon, W.M., and Han, K.S. (2015). Butyrate modulates bacterial adherence on LS174T human colorectal cells by stimulating mucin secretion and MAPK signalling pathway. Nutrition Research and Practice 9, 343–349. https://doi.org/10.4162/nrp.2015.9.4.343.

109. Kaoutari, A. El, Armougom, F., Gordon, J.I., Raoult, D., and Henrissat, B. (2013). The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. Nature Reviews Microbiology 11, 497–504. https://doi.org/10.1038/nrmicro3050.

110. Karu, N., Deng, L., Slae, M., Guo, A.C., Sajed, T., Huynh, H., Wine, E., and Wishart, D.S. (2018). A review on human fecal metabolomics: Methods, applications and the human fecal metabolome database. Analytica Chimica Acta 1030, 1–24. https://doi.org/10.1016/j.aca.2018.05.031.

111. Kastl, A.J., Terry, N.A., Wu, G.D., and Albenberg, L.G. (2020). The Structure and Function of the Human Small Intestinal Microbiota: Current Understanding and Future Directions. Cellular and Molecular Gastroenterology and Hepatology 9, 33–45. https://doi.org/10.1016/j.jcmgh.2019.07.006.

112. Kho, Z.Y., and Lal, S.K. (2018). The human gut microbiome - A potential controller of wellness and disease. Frontiers in Microbiology 9, 1–23. https://doi.org/10.3389/fmicb.2018.01835.

113. Kim, C.C., Kelly, W.J., Patchett, M.L., Tannock, G.W., Jordens, Z., Stoklosinski, H.M., Taylor, J.W., Sims, I.M., Bell, T.J., and Rosendale, D.I. (2017). Monoglobus pectinilyticus gen. Nov., sp. nov., a pectinolytic bacterium isolated from human faeces. International Journal of Systematic and Evolutionary Microbiology 67, 4992–4998. https://doi.org/10.1099/ijsem.0.002395.

114. Kostic, A.D., Gevers, D., Knip, M., Xavier, R.J., La, H., Oikarinen, S., Harmsen, H.J.M., Goffau, M.C. De, Welling, G., Alahuhta, K., *et al.* (2015). The Dynamics of the Human Infant Gut Microbiome in Development and in Progression toward Type 1 Resource The Dynamics of the Human Infant Gut Microbiome in Development and in Progression toward Type 1 Diabetes. Cell Host and Microbe 17(2), 260–273. https://doi.org/10.1016/j.chom.2015.01.001.

115. Kovatcheva-Datchary, P., Egert, M., Maathuis, A., Rajilić-Stojanović, M., De Graaf, A.A., Smidt, H., De Vos, W.M., and Venema, K. (2009). Linking phylogenetic identities of bacteria to starch fermentation in an *in vitro* model of the large intestine by RNA-based stable isotope probing. Environmental Microbiology 11, 914–926. https://doi.org/10.1111/j.1462-2920.2008.01815.x.

116. Kraus, D. (1993). Concepts in modern biology (New Jersey: Globe Fearon Co).

117. Kremp, F., and Müller, V. (2021). Methanol and methyl group conversion in acetogenic bacteria: Biochemistry, physiology and application. FEMS Microbiology Reviews 45. https://doi.org/10.1093/femsre/fuaa040.

118. Kushkevych, I., Cejnar, J., Treml, J., Dordević, D., Kollar, P., and Vítězová, M. (2020). Recent Advances in Metabolic Pathways of Sulfate Reduction in Intestinal Bacteria. Cells 9, 1–16. https://doi.org/10.3390/cells9030698.

119. De Lacy Costello, B.P.J., Ledochowski, M., and Ratcliffe, N.M. (2013). The importance of methane breath testing: a review. Journal of Breath Research 7, 24001. https://doi.org/10.1088/1752-7155/7/2/024001.

120. Lamichhane, S., Sen, P., Alves, M.A., Ribeiro, H.C., Raunioniemi, P., Hyötyläinen, T., and Orešič, M. (2021). Linking gut microbiome and lipid metabolism: Moving beyond associations. Metabolites 11, 1–15. https://doi.org/10.3390/metabo11010055.

121. Larsen, O.F.A., and Claassen, E. (2018). The mechanistic link between health and gut microbiota diversity. Scientific Reports 8, 6–10. https://doi.org/10.1038/s41598-018-20141-6.

122. Laverde Gomez, J.A., Mukhopadhya, I., Duncan, S.H., Louis, P., Shaw, S., Collie-Duguid, E., Crost, E., Juge, N., and Flint, H.J. (2019). Formate cross-feeding and cooperative metabolic interactions revealed by transcriptomics in co-cultures of acetogenic and amylolytic human colonic bacteria. Environmental Microbiology 21, 259–271. https://doi.org/10.1111/1462-2920.14454.

123. Lawley, B., Sims, I.M., and Tannock, G.W. (2013). Whole-transcriptome shotgun sequencing (RNA-seq) screen reveals upregulation of cellobiose and motility operons of lactobacillus ruminis l5 during growth on tetrasaccharides derived from barley β-Glucan. Applied and Environmental Microbiology 79, 5661–5669. https://doi.org/10.1128/AEM.01887-13.

124. Leeming, E.R., Johnson, A.J., Spector, T.D., and Roy, C.I.L. (2019). Effect of diet on the gut microbiota: Rethinking intervention duration. Nutrients 11, 1–28. https://doi.org/10.3390/nu11122862.

125. Levitt, M.D., Furne, J.K., Kuskowski, M., and Ruddy, J. (2006). Stability of Human Methanogenic Flora Over 35 Years and a Review of Insights Obtained From Breath Methane Measurements. Clinical Gastroenterology and Hepatology 4, 123–129. https://doi.org/10.1016/j.cgh.2005.11.006.

126. Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R., *et al.* (2008). Evolution of Mammals and Their Gut Microbes. Science 320, 1647–1651. https://doi.org/10.1126/science.1155725.

127. Lloyd-Price, J., Abu-Ali, G., and Huttenhower, C. (2016). The healthy human microbiome. Genome Medicine 8, 1–11. https://doi.org/10.1186/s13073-016-0307-y.

128. Lockett, T.J., Bird, A.R., Christophersen, C., Clarke, J.M., Conlon, M.A., and Topping, D.L. (2016). Microbes, Metabolites and Health. In Microbial Metabolomics: Applications in Clinical, Environmental, and Industrial Microbiology, D.J. Beale, K.A. Kouremenos, and E.A. Palombo, eds. (Cham: Springer International Publishing), pp. 13–48.

129. Louis, P., and Flint, H.J. (2017). Formation of propionate and butyrate by the human colonic microbiota. Environmental Microbiology 19, 29–41. https://doi.org/10.1111/1462-2920.13589.

130. Lozupone, C.A., Stombaugh, J.I., Gordon, J.I., Jansson, J.K., and Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. Nature 489, 220–230. https://doi.org/10.1038/nature11550.

131. Luis, A.S., Briggs, J., Zhang, X., Farnell, B., Ndeh, D., Labourel, A., Baslé, A., Cartmell, A., Terrapon, N., Stott, K., *et al.* (2018). Dietary pectic glycans are degraded by coordinated enzyme pathways in human colonic Bacteroides. Nature Microbiology 3, 210–219. https://doi.org/10.1038/s41564-017-0079-1.

132. Macdiarmid, J.I., and Blundell, J.E. (1997). Dietary under-reporting: What people say about recording their food intake. European Journal of Clinical Nutrition 51, 199–200. https://doi.org/10.1038/sj.ejcn.1600380.

133. Macfarlane, G.T., Gibson, G.R., and Cummings, J.H. (1992). Comparison of fermentation reactions in different regions of the human colon. Journal of Applied Bacteriology 72, 57–64. https://doi.org/10.1111/j.1365-2672.1992.tb04882.x.

134. Maldonado-Contreras, A., Goldfarb, K.C., Godoy-Vitorino, F., Karaoz, U., Contreras, M., Blaser, M.J., Brodie, E.L., and Dominguez-Bello, M.G. (2011). Structure of the human gastric bacterial community in relation to Helicobacter pylori status. ISME Journal 5, 574–579. https://doi.org/10.1038/ismej.2010.149.

135. le Marchand, L., Wilkens, L.R., Harwood, P., and Cooney, R. v. (1992). Use of Breath Hydrogen and Methane as Markers of Colonic Fermentation in Epidemiological Studies: Circadian Patterns of Excretion. Environmental Health Perspectives 98, 199–202. https://doi.org/10.1289/ehp.9298199.

136. Margareta, E., Nyman, G.L., Svanberg, S.J.M., and Asp, N.L. (1994). Molecular weight distribution and viscosity of water-soluble dietary fibre isolated from green beans, brussels sprouts and green peas following different types of processing. Journal of the Science of Food and Agriculture 66, 83–91. https://doi.org/10.1002/jsfa.2740660113.

137. Marquet, P., Duncan, S.H., Chassard, C., Bernalier-Donadille, A., and Flint, H.J. (2009). Lactate has the potential to promote hydrogen sulphide formation in the human colon. FEMS Microbiology Letters 299, 128–134. https://doi.org/10.1111/j.1574-6968.2009.01750.x.

138. Mazmanian, S.K., Round, J.L., and Kasper, D.L. (2008). A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 453, 620–625. https://doi.org/10.1038/nature07008.

139. McCleary, B. V, and Prosky, L. (2000). Advanced Dietary Fibre Technology (Oxford, UK: Blackwell Science Ltd).

140. McDonald, D., Hyde, E., Debelius, J. W., Morton, J. T., Gonzalez, A., Ackermann, G., Aksenov, A. A., Behsaz, B., Brennan, C., Chen, Y., DeRight Goldasich, L., Dorrestein, P. C., Dunn, R. R., Fahimipour, A. K., Gaffney, J., Gilbert, J. A., Gogul, G., Green, J. L., Hugenholtz, P., Gunderson, B. (2018). American Gut: an Open Platform for Citizen Science Microbiome Research. MSystems 3(3), 1–28. https://doi.org/10.1128/mSystems.00031-18.

141. McDougall, G.J., Morrison, I.M., Stewart, D., and Hillman, J.R. (2002). Plant Cell Walls as Dietary Fibre: Range, Structure, Processing and Function. Journal of the Science of Food and Agriculture 70, 133–150. https://doi.org/10.1002/(sici)1097-0010(199602)70:2<133:aid-jsfa495>3.3.co;2-w.

142. McIntyre, A. B. R., Ounit, R., Afshinnekoo, E., Prill, R. J., Hénaff, E., Alexander, N., Minot, S. S., Danko, D., Foox, J., Ahsanuddin, S., Tighe, S., Hasan, N. A., Subramanian, P., Moffat, K., Levy, S., Lonardi, S., Greenfield,

N., Colwell, R. R., Rosen, G. L., and Mason, C. E. (2017). Comprehensive benchmarking and ensemble approaches for metagenomic classifiers. Genome Biology 18(1), 1–19. https://doi.org/10.1186/s13059-017-1299-7.

143. Michalak, L., La Rosa, S.L., Leivers, S., Lindstad, L.J., Røhr, Å.K., Aachmann, F.L., and Westereng, B. (2020). A pair of esterases from a commensal gut bacterium remove acetylations from all positions on complex β-mannans. Proceedings of the National Academy of Sciences of the United States of America 117, 7122–7130. https://doi.org/10.1073/pnas.1915376117.

144. Moeller, A. H., Caro-Quintero, A., Mjungu, D., Georgiev, A. V., Lonsdorf, E. V., Muller, M. N., Pusey, A. E., Peeters, M., Hahn, B. H., & Ochman, H. (2016). Cospeciation of gut microbiota with hominids. Science (New York, N.Y.), 353(6297), 380–382. https://doi.org/10.1126/science.aaf3951.

145. Mok, C., and Dick, J.W. (1991). Moisture Adsorption of Damaged Wheat Starch. Cereal Chemistry 68, 405–409.

146. Montoya, C.A., de Haas, E.S., and Moughan, P.J. (2018). Development of an in vivo and *in vitro* ileal fermentation method in a growing pig model. Journal of Nutrition 148, 298–305. https://doi.org/10.1093/jn/nxx038.

147. Montoya, C.A., Blatchford, P., and Moughan, P.J. (2021). *In vitro* ileal and caecal fermentation of fibre substrates in the growing pig given a human-type diet. British Journal of Nutrition 125, 998–1006. https://doi.org/10.1017/S0007114520003542.

148. Morrison, W.R., and Tester, R.F. (1994). Properties of damaged starch granules. IV. Composition of ball-milled wheat starches and of fractions obtained on hydration. Journal of Cereal Science 20, 69–77. https://doi.org/10.1006/jcrs.1994.1046.

149. Mortensen, F. V, and Nielsen, H. (1990). Short chain fatty acids dilate isolated colonic resistance arteries. Gut 31, 1391–1394.

150. Munoz, J., James, K., Bottacini, F., and Van Sinderen, D. (2020). Biochemical analysis of cross-feeding behaviour between two common gut commensals when cultivated on plant-derived arabinogalactan. Microbial Biotechnology 13, 1733–1747. https://doi.org/10.1111/1751-7915.13577.

151. National Health and Medical Research Council, Australian Government Department of Health and Ageing, and New Zealand Ministry of Health (2006). Nutrient Reference Values for Australia and New Zealand (Canberra: National Health and Medical Research Council).

152. Nature Research Custom Media, and Zymo Research (2018). Setting standards for reproducibility in gut microbiome research (online). https://www.nature.com/articles/d42473-018-00136-7.

153. Nkamga, V.D., Henrissat, B., and Drancourt, M. (2017). Archaea: Essential inhabitants of the human digestive microbiota. Human Microbiome Journal 3, 1–8. https://doi.org/10.1016/j.humic.2016.11.005.

154. Noah, L., Guillon, F., Bouchet, B., Buléon, a, Molis, C., Gratas, M., and Champ, M. (1998). Digestion of carbohydrate from white beans (Phaseolus vulgaris L.) in healthy humans. The Journal of Nutrition 128, 977–985. https://doi.org/10.1093/jn/128.6.977.

155. Ntaikou, I. (2021). Microbial production of hydrogen. In Sustainable Fuel Technologies Handbook, S. Dutta, and C. Mustansar Hussain, eds. (Massachusetts: Academic Press), pp. 315–377.

156. Nyman, M., Palsson, K.-E., and Asp, N.-G. (1987). Effects of Processing on Dietary Fiber in Vegetables. Lebensmittel-Wissenschaft und -Technologie 20, 29–36.

157. Ó Cuív, P., Burman, S., Pottenger, S., and Morrison, M. (2016). Exploring the Bioactive Landscape of the Gut Microbiota to Identify Metabolites Underpinning Human Health. In Microbial Metabolomics: Applications in Clinical, Environmental, and Industrial Microbiology, D.J. Beale, K.A. Kouremenos, and E.A. Palombo, eds. (Cham: Springer International Publishing), pp. 49–82.

158. Ohland, C.L., and Jobin, C. (2015). Microbial Activities and Intestinal Homeostasis: A Delicate Balance Between Health and Disease. Cellular and Molecular Gastroenterology and Hepatology 1, 28–40. https://doi.org/10.1016/j.jcmgh.2014.11.004.

159. Ohwaki, K., Hungate, R.E., Lotter, L., Hofmann, I.R.R., Owaki, K., Hungate, R.E., Lotter, L., Hofmann, R.R., and Maloiy, G. (1974). Stomach

fermentation in East African Colobus monkeys in their natural state. Applied Microbiology 27, 713–723. https://doi.org/10.1007/s00125-013-2912-2.

160. Oliphant, K., and Allen-Vercoe, E. (2019). Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. Microbiome 7. https://doi.org/10.1186/s40168-019-0704-8.

161. Oren, A., & Garrity, G. M. (2021). Valid publication of the names of forty-two phyla of prokaryotes. International Journal of Systematic and Evolutionary Microbiology 71(10). https://doi.org/10.1099/ijsem.0.005056.

162. Orpana, A.K., Ho, T.H., and Stenman, J. (2012). Multiple heat pulses during PCR extension enabling amplification of GC-rich sequences and reducing amplification bias. Analytical Chemistry 84, 2081–2087. https://doi.org/10.1021/ac300040j.

163. Paine, R.T. (1966). Food Web Complexity and Species Diversity. The American Naturalist 100, 65–75.

164. Paul, B.D., and Snyder, S.H. (2015). H2S: A Novel Gasotransmitter that Signals by Sulfhydration. Trends in Biochemical Sciences 40, 687–700. https://doi.org/10.1016/j.tibs.2015.08.007.

165. Payling, L., Fraser, K., Loveday, S.M., Sims, I., Roy, N., and McNabb, W. (2020). The effects of carbohydrate structure on the composition and functionality of the human gut microbiota. Trends in Food Science and Technology 97, 233–248. https://doi.org/10.1016/j.tifs.2020.01.009.

166. Peng, L., Li, Z., Green, R.S., Holzman, I.R., and Lin, J. (2009). Butyrate Enhances the Intestinal Barrier by Facilitating Tight Junction Assembly via Activation of AMP-Activated Protein Kinase. Cell 139, 1619–1625. https://doi.org/10.3945/jn.109.104638.1619.

167. Petriz, B.A., and Franco, O.L. (2017). Metaproteomics as a Complementary Approach to Gut Microbiota in Health and Disease. Frontiers in Chemistry 5(4). https://doi.org/10.3389/fchem.2017.00004.

168. Pham, V.T., and Mohajeri, M.H. (2018). The application of *in vitro* human intestinal models on the screening and development of pre-and probiotics. Beneficial Microbes 9, 725–742. https://doi.org/10.3920/BM2017.0164.

169. Pimentel, M., Mayer, A.G., Park, S., Chow, E.J., Hasan, A., and Kong, Y. (2003). Methane production during lactulose breath test is associated with gastrointestinal disease presentation. Digestive Diseases and Sciences 48, 86–92. https://doi.org/10.1023/A:1021738515885.

170. Pokusaeva, K., Fitzgerald, G.F., and Van Sinderen, D. (2011). Carbohydrate metabolism in Bifidobacteria. Genes and Nutrition 6, 285–306. https://doi.org/10.1007/s12263-010-0206-6.

171. Power, S.E., O'Toole, P.W., Stanton, C., Ross, R.P., and Fitzgerald, G.F. (2014). Intestinal microbiota, diet and health. British Journal of Nutrition 111, 387–402. https://doi.org/10.1017/S0007114513002560.

172. Prasanna, L. C. (2016). Analysis of the Distribution of Mucins in Adult Human Gastric Mucosa and Its Functional Significance. Journal of Clinical and Diagnostic Research, 10(2), 01–04. https://doi.org/10.7860/JCDR/2016/12323.7162.

173. Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., *et al.* (2010). A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464, 59–65. https://doi.org/10.1038/nature08821.

174. Rainey, P.B., Buckling, A., Kassen, R., and Travisano, M. (2000). The emergence and maintenance of diversity: Insights from experimental bacterial populations. Trends in Ecology and Evolution 15, 243–247. https://doi.org/10.1016/S0169-5347(00)01871-1.

175. Ralet, M.C., Thibault, J.F., and Delia Valle, G. (1990). Influence of extrusion-cooking on the structure and properties of wheat bran. Journal of Cereal Science 11, 249–259.

176. Ravelli, M.N., and Schoeller, D.A. (2020). Traditional Self-Reported Dietary Instruments Are Prone to Inaccuracies and New Approaches Are

Needed. Frontiers in Nutrition 7, 1–6. https://doi.org/10.3389/fnut.2020.00090.

177. Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G.A.D., Gasbarrini, A., Mele, M.C., Rinninella, E., Raoul, P., Cintoni, M., *et al.* (2019). What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms 7(1), 14. https://doi.org/10.3390/microorganisms7010014.

178. Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de los Reyes-Gavilán, C.G., and Salazar, N. (2016). Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. Frontiers in Microbiology 7, 1–9. https://doi.org/10.3389/fmicb.2016.00185.

179. Robbe, C., Capon, C., Maes, E., Rousset, M., Zweibaum, A., Zanetta, J. P., and Michalski, J. C. (2003). Evidence of regio-specific glycosylation in human intestinal mucins: Presence of an acidic gradient along the intestinal tract. Journal of Biological Chemistry, 278(47), 46337–46348. https://doi.org/10.1074/jbc.M302529200.

180. Robert, C., and Bernalier-Donadille, A. (2003). The cellulolytic microflora of the human colon: Evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects. FEMS Microbiology Ecology 46, 81–89. https://doi.org/10.1016/S0168-6496(03)00207-1.

181. Roediger, W.E.W. (1982). Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterology 83, 424–249. https://doi.org/10.1016/S0016-5085(82)80339-9.

182. Rombouts, C., Hemeryck, L.Y., Van Hecke, T., De Smet, S., De Vos, W.H., and Vanhaecke, L. (2017). Untargeted metabolomics of colonic digests reveals kynurenine pathway metabolites, dityrosine and 3-dehydroxycarnitine as red versus white meat discriminating metabolites. Scientific Reports 7, 1–13. https://doi.org/10.1038/srep42514.

183. Rose, C., Parker, A., Jefferson, B., and Cartmell, E. (2015). The characterization of feces and urine: A review of the literature to inform advanced treatment technology. Critical Reviews in Environmental Science

and Technology 45, 1827–1879. https://doi.org/10.1080/10643389.2014.1000761.

184. Roume, H., Heintz-Buschart, A., Muller, E.E.L., May, P., Satagopam, V.P., Laczny, C.C., Narayanasamy, S., Lebrun, L.A., Hoopmann, M.R., Schupp, J.M., *et al.* (2015). Comparative integrated omics: identification of key functionalities in microbial community-wide metabolic networks. Biofilms and Microbiomes 1, 15007. https://doi.org/10.1038/npjbiofilms.2015.7.

185. Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., and Tuohy, K. (2018). Gut microbiota functions: metabolism of nutrients and other food components. European Journal of Nutrition 57, 1–24. https://doi.org/10.1007/s00394-017-1445-8.

186. Roy, N.C., Bassett, S.A., Young, W., Thum, C., and McNabb, W.C. (2014). Chapter 18 The Importance of Microbiota and Host Interactions Throughout Life. In Food structures, digestion and health. (Amsterdam: Elsevier Inc.), pp. 489-511.

187. Sahakian, A.B., Jee, S.R., and Pimentel, M. (2010). Methane and the gastrointestinal tract. Digestive Diseases and Sciences 55, 2135–2143. https://doi.org/10.1007/s10620-009-1012-0.

188. Sarkar, A., Ye, A., and Singh, H. (2017). Oral processing of emulsion systems from a colloidal perspective. Food and Function 8, 511–521. https://doi.org/10.1039/C6FO01171C.

189. Schoeler, M., and Caesar, R. (2019). Dietary lipids, gut microbiota and lipid metabolism. Reviews in Endocrine and Metabolic Disorders 20, 461–472. https://doi.org/10.1007/s11154-019-09512-0.

190. Seeberger, PH. (2017). Chapter 2 Monosaccharide Diversity. In Essentials of Glycobiology (online). https://www.ncbi.nlm.nih.gov/books/NBK453086/.

191. Sender, R., Fuchs, S., and Milo, R. (2016). Revised estimates for the number of human and bacteria cells in the body. PLOS Biology 14, 1–14. https://doi.org/10.1371/journal.pbio.1002533.

192. Senghor, B., Sokhna, C., Ruimy, R., and Lagier, J.C. (2018). Gut microbiota diversity according to dietary habits and geographical provenance. Human Microbiome Journal 7–8, 1–9. https://doi.org/10.1016/j.humic.2018.01.001.

193. Seth, E.C., and Taga, M.E. (2014). Nutrient cross-feeding in the microbial world. Frontiers in Microbiology 5, 1–6. https://doi.org/10.3389/fmicb.2014.00350.

194. Shang, Q. (2019). From correlation to causation: The missing point in the study of functional foods and gut microbiota. Journal of Functional Foods 61, 103466. https://doi.org/10.1016/j.jff.2019.103466.

195. Shetty, S.A., Hugenholtz, F., Lahti, L., Smidt, H., and de Vos, W.M. (2017). Intestinal microbiome landscaping: Insight in community assemblage and implications for microbial modulation strategies. FEMS Microbiology Reviews 41, 182–199. https://doi.org/10.1093/femsre/fuw045.

196. Shultz, S.R., MacFabe, D.F., Martin, S., Jackson, J., Taylor, R., Boon, F., Ossenkopp, K.-P., and Cain, D.P. (2008). Intracerebroventricular injections of the enteric bacterial metabolic product propionic acid impair cognition and sensorimotor ability in the Long–Evans rat: Further development of a rodent model of autism. Behavioural Brain Research 200, 33–41. https://doi.org/10.1016/j.bbr.2008.12.023.

197. Singh, R.K., Chang, H.W., Yan, D., Lee, K.M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T.H., *et al.* (2017). Influence of diet on the gut microbiome and implications for human health. Journal of Translational Medicine 15, 1–17. https://doi.org/10.1186/s12967-017-1175-y.

198. Sinnott, M. (2007). Carbohydrate Chemistry and Biochemistry: Structure and Mechanism (Cambridge: Royal Society of Chemistry).

199. Sirangelo, T.M. (2018). Human gut microbiome analysis and multi-omics approach. International Journal of Pharma Medicine and Biological Sciences 7, 52–57. https://doi.org/10.18178/ijpmbs.7.3.52-57.

200. Slattery, M., Mcdonald, A., Bild, D.E., Caan, B.J., Hilner, J.E., Jacobs, D.R.Jr., and Liu, K. (1992). Associations of body fat and its distribution with dietary intake, physical activity, alcohol, and smoking in blacks and whites. The American Journal of Clinical Nutrition 943–949. https://doi.org/10.1093/ajcn/55.5.943.

201. Smith, E.A., and Macfarlane, G.T. (1996). Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. Journal of Applied Bacteriology 81, 288–302. https://doi.org/10.1111/j.1365-2672.1996.tb04331.x.

202. Smith, N.W., McNabb, W.C., Roy, N.C., Altermann, E.H., and Shorten, P.R. (2018). Hydrogen cross-feeders of the human gastrointestinal tract. Gut Microbes 10(3), 270-288. https://doi.org/10.1080/19490976.2018.1546522.

203. Sonnenburg, E.D., Smits, S.A., Tikhonov, M., Higginbottom, S.K., Wingreen, N.S., and Sonnenburg, J.L. (2016). Diet-induced extinction in the gut microbiota compounds over generations. Nature 529, 212–215. https://doi.org/10.1038/nature16504.Diet-induced.

204. Soret, R., Chevalier, J., De Coppet, P., Poupeau, G., Derkinderen, P., Segain, J.P., and Neunlist, M. (2010). Short-Chain Fatty Acids Regulate the Enteric Neurons and Control Gastrointestinal Motility in Rats. Gastroenterology 138, 1772-1782. https://doi.org/10.1053/j.gastro.2010.01.053.

205. Southwell, B.R., Clarke, M.C.C., Sutcliffe, J., and Hutson, J.M. (2009). Colonic transit studies: Normal values for adults and children with comparison of radiological and scintigraphic methods. Pediatric Surgery International 25, 559–572. https://doi.org/10.1007/s00383-009-2387-x.

206. Starck, C.S., Wolfe, R.R., and Moughan, P.J. (2018). Endogenous Amino Acid Losses from the Gastrointestinal Tract of the Adult Human - A Quantitative Model. Journal of Nutrition 148, 1871–1881. https://doi.org/10.1093/jn/nxy162.

207. Strocchi, A., Ellis, C.J., Furne, J.K., and Levitt, M.D. (1994). Study of constancy of hydrogen-consuming flora of human colon. Digestive Diseases and Sciences 39, 494–497. https://doi.org/10.1007/BF02088333.

208. Sun, Q. (2017). Starch Nanoparticles. In Starch in Food (Second Edition) (Sawston: Woodhead publishing), pp. 691-745.

209. Swidsinski, A., Loening–Baucke, V., Verstraelen, H., Osowska, S., and Doerffel, Y. (2008). Biostructure of Fecal Microbiota in Healthy Subjects and Patients with Chronic Idiopathic Diarrhea. 135, 568-579. https://doi.org/10.1053/j.gastro.2008.04.017.

210. Szymańska-Chargot, M., Cybulska, J., and Zdunek, A. (2011). Sensing the structural differences in cellulose from apple and bacterial cell wall materials by Raman and FT-IR Spectroscopy. Sensors 11, 5543–5560. https://doi.org/10.3390/s110605543.

211. Szymańska-Chargot, M., Chylińska, M., Gdula, K., Kozioł, A., and Zdunek, A. (2017). Isolation and Characterization of Cellulose from Different Fruit and Vegetable Pomaces. Polymers 9, 495. https://doi.org/10.3390/polym9100495.

212. Tan, L., Showalter, A.M., Egelund, J., Hernandez-Sanchez, A., Doblin, M.S., and Bacic, A. (2012). Arabinogalactan-proteins and the research challenges for these enigmatic plant cell surface proteoglycans. Frontiers in Plant Science 3, 1–10. https://doi.org/10.3389/fpls.2012.00140.

213. Tap, J., Mondot, S., Levenez, F., Pelletier, E., Caron, C., Furet, J.P., Ugarte, E., Muñoz-Tamayo, R., Paslier, D.L.E., Nalin, R., *et al.* (2009). Towards the human intestinal microbiota phylogenetic core. Environmental Microbiology 11, 2574–2584. https://doi.org/10.1111/j.1462-2920.2009.01982.x.

214. Tedelind, S., Westberg, F., Kjerrulf, M., and Vidal, A. (2007). Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: A study with relevance to inflammatory bowel disease. World Journal of Gastroenterology 13, 2826–2832. https://doi.org/10.3748/wjg.v13.i20.2826.

215. Tian, L., Wang, X.W., Wu, A.K., Fan, Y., Friedman, J., Dahlin, A., Waldor, M.K., Weinstock, G.M., Weiss, S.T., and Liu, Y.Y. (2020). Deciphering functional redundancy in the human microbiome. Nature Communications 11, 1–11. https://doi.org/10.1038/s41467-020-19940-1.

216. Tilg, H., Cani, P.D., and Mayer, E.A. (2016). Gut microbiome and liver diseases. Recent Advances in Basic Science 65, 2035–2044. https://doi.org/10.1136/gutjnl-2016-312729.

217. Timmis, K. N. (2010). Gastrointestinal Tract: Fat Metabolism in the Colon. In Handbook of hydrocarbon and lipid microbiology. (Berling: Springer), pp. 3111-3118.

218. Todesco, T., Venketshwer, R., Bosello, O., and Jenkins, D. (1991). Propionate Lowers Blood Glucose and alters lipid metabolism. American Journal of Clinical Nutrition 54, 560–565.

219. Tomasova, L., Konopelski, P., and Ufnal, M. (2016). Gut bacteria and hydrogen sulfide: The new old players in circulatory system homeostasis. Molecules 21, 1–18. https://doi.org/10.3390/molecules21111558.

220. Trosvik, P., and de Muinck, E.J. (2015). Ecology of bacteria in the human gastrointestinal tract--identification of keystone and foundation taxa. Microbiome 3, 44. https://doi.org/10.1186/s40168-015-0107-4.

221. Trowell, H., and Burkitt, D.P. (1981). Western Diseases: Their Emergence and Prevalence (Cambridge, MA: Harvard University Press), pp. 1-456.

222. Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., *et al.* (2009). A core gut microbiome in obese and lean twins. Nature 457, 480–484. https://doi.org/10.1038/nature07540.

223. Vanhoutvin, S.A.L.W., Troost, F.J., Kilkens, T.O.C., Lindsey, P.J., Hamer, H.M., Jonkers, D.M.A.E., Venema, K., and Brummer, R.J.M. (2009). The effects of butyrate enemas on visceral perception in healthy volunteers. Neurogastroenterology and Motility 21, 952–958. https://doi.org/10.1111/j.1365-2982.2009.01324.x.

224. Venema, K. (2015). The TNO *In vitro* Model of the Colon (TIM-2). In The Impact of Food Bioactives on Health: *In vitro* and Ex Vivo Models, K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, and H. Wichers, eds. (Cham: Springer International Publishing), pp. 293–304.

225. Verhoeckx, K., Cotter, P., López-Expósito, I. Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., and Wichers, H. (2015). The Impact of Food Bioactives on Health (Cham: Springer International Publishing).

226. Vigsnaes, L. K., Nakai, H., Hemmingsen, L., Andersen, J. M., Lahtinen, S. J., Rasmussen, L. E., Hachem, M. A., Petersen, B. O., Duus, J., Meyer, A. S., Licht, T. R., and Svensson, B. (2013). In vitro growth of four individual human gut bacteria on oligosaccharides produced by chemoenzymatic synthesis. Food and Function 4(5), 784–793. https://doi.org/10.1039/c3fo30357h.

227. Villas-Boas, S.G. (2016). Introduction to Microbial Metabolomics. In Microbial Metabolomics: Applications in Clinical, Environmental, and Industrial Microbiology, D.J. Beale, K.A. Kouremenos, and E.A. Palombo, eds. (Cham: Springer International Publishing), pp. 1–12.

228. Vital, M., Howe, A.C., and Tiedje, J.M. (2014). Revealing the Bacterial Butyrate Synthesis Pathways by Analyzing (Meta)genomic Data. MBio 5, 1–11. https://doi.org/10.1128/mbio.00889-14.

229. Vivijs, B., Haberbeck, L.U., Mfortaw Mbong, V.B., Bernaerts, K., Geeraerd, A.H., Aertsen, A., and Michiels, C.W. (2015). Formate hydrogen lyase mediates stationary-phase deacidification and increases survival during sugar fermentation in acetoin-producing enterobacteria. Frontiers in Microbiology 6, 1–11. https://doi.org/10.3389/fmicb.2015.00150.

230. Vogt, N. M., Kerby, R. L., Dill-mcfarland, K. A., Harding, S. J., Merluzzi, A. P., Johnson, S. C., Carlsson, C. M., Asthana, S., Zetterberg, H., Blennow, K., Bendlin, B. B., and Rey, F. E. (2017). Gut microbiome alterations in Alzheimer's disease. Scientific Reports September, 1–11. https://doi.org/10.1038/s41598-017-13601-y.

231. Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., Brown, D., Stares, M. D., Scott, P., Bergerat, A., Louis, P., McIntosh, F., Johnstone, A. M., Lobley, G. E., Parkhill, J., and Flint, H. J. (2011). Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME Journal 5(2), 220–230. https://doi.org/10.1038/ismej.2010.118.

232. Walter, J., Armet, A.M., Finlay, B.B., and Shanahan, F. (2020). Perspective Establishing or Exaggerating Causality for the Gut Microbiome: Lessons from Human Microbiota-Associated Rodents. Cell 180, 221–232. https://doi.org/10.1016/j.cell.2019.12.025.

233. Wastyk, H.C., Fragiadakis, G.K., Perelman, D., Dahan, D., Merrill, B.D., Yu, F.B., Topf, M., Gonzalez, C.G., Robinson, J.L., Elias, J.E., *et al.* (2020). Gut Microbiota-Targeted Diets Modulate Human Immune Status. BioRxiv 2020.09.30.321448.

234. Wesolowska-Andersen, A., Bahl, M., Carvalho, V., Kristiansen, K., Sicheritz-Pontén, T., Gupta, R., and Licht, T. (2014). Choice of bacterial DNA extraction method from fecal material influences community structure as evaluated by metagenomic analysis. Microbiome 2, 19. https://doi.org/10.1186/2049-2618-2-19.

235. Van De Wiele, T., Boon, N., Possemiers, S., Jacobs, H., and Verstraete, W. (2007). Inulin-type fructans of longer degree of polymerization exert more pronounced *in vitro* prebiotic effects. Journal of Applied Microbiology 102, 452–460. https://doi.org/10.1111/j.1365-2672.2006.03084.x.

236. Williams, B.A., Grant, L.J., Gidley, M.J., and Mikkelsen, D. (2017). Gut fermentation of dietary fibres: Physico-chemistry of plant cell walls and implications for health. International Journal of Molecular Sciences 18. https://doi.org/10.3390/ijms18102203.

237. Wishart, D. S., Guo, A., Oler, E., Wang, F., Anjum, A., Peters, H., Dizon, R., Sayeeda, Z., Tian, S., Lee, B. L., Berjanskii, M., Mah, R., Yamamoto, M., Jovel, J., Torres-Calzada, C., Hiebert-Giesbrecht, M., Lui, V. W., Varshavi, D., Varshavi, D., Gautam, V. (2021). HMDB 5.0: the Human Metabolome Database for 2022. Nucleic Acids Research 1–10.

https://academic.oup.com/nar/advance-article/doi/10.1093/nar/gkab1062/6431815.

238. Witt, T., and Stokes, J.R. (2015). Physics of food structure breakdown and bolus formation during oral processing of hard and soft solids. Current Opinion in Food Science 3, 110–117. https://doi.org/10.1016/j.cofs.2015.06.011.

239. Wu, G. D., Compher, C., Chen, E. Z., Smith, S. A., Shah, R. D., Bittinger, K., Chehoud, C., Albenberg, L. G., Nessel, L., Gilroy, E., Star, J., Weljie, A. M., Flint, H. J., Metz, D. C., Bennett, M. J., Li, H., Bushman, F. D., and Lewis, J. D. (2016). Comparative metabolomics in vegans and omnivores reveal constraints on diet-dependent gut microbiota metabolite production. Gut 65(1), 63–72. https://doi.org/10.1136/gutjnl-2014-308209.

240. Wu, S., Rhee, K. J., Albesiano, E., Rabizadeh, S., Wu, X., Yen, H. R., Huso, D. L., Brancati, F. L., Wick, E., McAllister, F., Housseau, F., Pardoll, D. M., and Sears, C. L. (2009). A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. Nature Medicine 15(9), 1016–1022. https://doi.org/10.1038/nm.2015.

241. Xiong, Y., Miyamoto, N., Shibata, K., Valasek, M.A., Motoike, T., Kedzierski, R.M., and Yanagisawa, M. (2004). Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proceedings of the National Academy of Sciences 101, 1045–1050. https://doi.org/10.1073/pnas.2637002100.

242. Xu, S., Zhao, J.-Y., Zhao, S.-M., Xu, W., Huang, L., and Liu, C.-X. (2017). Butyrate induces apoptosis by activating PDC and inhibiting complex I through SIRT3 inactivation. Signal Transduction and Targeted Therapy 2, 16035. https://doi.org/10.1038/sigtrans.2016.35.

243. Yan, Q., Gu, Y., Li, X., Yang, W., Jia, L., Chen, C., Han, X., Huang, Y., Zhao, L., Li, P., Fang, Z., Zhou, J., Guan, X., Ding, Y., Wang, S., Khan, M., Xin, Y., Li, S., and Ma, Y. (2017). Alterations of the Gut Microbiome in Hypertension. Frontiers in Cellular and Infection Microbiology 7(August), 1–9. https://doi.org/10.3389/fcimb.2017.00381.

244. Ze, X., Duncan, S.H., Louis, P., and Flint, H.J. (2012). Ruminococcus bromii is a keystone species for the degradation of resistant starch in the human colon. ISME Journal 6, 1535–1543. https://doi.org/10.1038/ismej.2012.4.

245. Ze, X., Le Mougen, F., Duncan, S.H., Louis, P., and Flint, H.J. (2013). Some are more equal than others: The role of "keystone" species in the degradation of recalcitrant substrates. Gut Microbes 4. https://doi.org/10.4161/gmic.23998.

246. Ze, X., ben David, Y., Laverde-Gomez, J. A., Dassa, B., Sheridan, P. O., Duncan, S. H., Louis, P., Henrissat, B., Juge, N., Koropatkin, N. M., Bayer, E. A., and Flint, H. J. (2015). Unique Organization of Extracellular Amylases into Amylosomes in the Resistant Starch-Utilizing Human Colonic Firmicutes Bacterium Ruminococcus bromii. American Society for Microbiology 6(5), e01058-15. https://doi.org/10.1128/mbio.01058-15.

247. Zeevi, D., Korem, T., Zmora, N., Israeli, D., Rothschild, D., Weinberger, A., Ben-Yacov, O., Lador, D., Avnit-Sagi, T., Lotan-Pompan, M., Suez, J., Mahdi, J. A., Matot, E., Malka, G., Kosower, N., Rein, M., Zilberman-Schapira, G., Dohnalová, L., Pevsner-Fischer, M., Segal, E. (2015). Personalized Nutrition by Prediction of Glycemic Responses. Cell 163(5), 1079–1095. https://doi.org/10.1016/j.cell.2015.11.001.

248. Zhang, X. (2014). Fundamentals of Fiber Science (Pennsylvania: DEStech Publications Inc.).

249. Zhang, C., Zhang, M., Wang, S., Han, R., Cao, Y., Hua, W., Mao, Y., Zhang, X., Pang, X., Wei, C., Zhao, G., Chen, Y., and Zhao, L. (2010). Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. ISME Journal 4(2), 232–241. https://doi.org/10.1038/ismej.2009.112.

250. Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. (2014). PEAR: A fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30, 614–620. https://doi.org/10.1093/bioinformatics/btt593.

251. Zhgun, E.S., and Ilyina, E.N. (2020). Fecal Metabolites as Non-Invasive Biomarkers of Gut Diseases. Acta Naturae 12, 4–14. https://doi.org/10.32607/actanaturae.10954.

252. Zhou, J., Hegsted, M., McCutcheon, K.L., Keenan, M.J., Xi, A., Raggio, A.M., and Martin, R.J. (2006). Peptide YY and proglucagon mRNA expression patterns and regulation in the gut. Obesity 14, 683–689. https://doi.org/10.1038/oby.2006.77.

253. Zoetendal, E.G., Raes, J., Van Den Bogert, B., Arumugam, M., Booijink, C.C., Troost, F.J., Bork, P., Wels, M., De Vos, W.M., and Kleerebezem, M. (2012). The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. ISME Journal 6, 1415–1426. https://doi.org/10.1038/ismej.2011.212.

Chapter 3
General Materials and Methods

Parts of this Chapter were published in Payling, L., Roy, N. C., Fraser, K., Loveday, S. M., Sims, I. M., Stefan, J., Laura, G., and McNabb, W. C. (2021). A protocol combining breath testing and ex vivo fermentations to study the human gut microbiome. STAR Protocols 2, 100227. https://doi.org/10.1016/j.xpro.2020.100227.

3.1 Abstract

The methods described herein investigated the links between human breath methane and the composition and fibre fermentation of the colonic microbiota from healthy human adults.

The study was observational using healthy adult human volunteers who were breath tested and grouped according to their breath methane concentration.

Two dietary fibres with different compositions and structures (oat β-glucan and potato lignocellulose) were added to a colonic batch fermentation model with faecal inoculum from the breath-tested participants. Samples of headspace gas, fermentation supernatant, and pellets were collected over 48 h. Several analytical approaches were used to measure the microbial composition and fibre fermentation.

3.2 Introduction

This Chapter describes the methods that apply to multiple Research Chapters, including participant recruitment, breath testing, metadata collection, faecal sample collection, *in vitro* colonic fermentation experiments, and shotgun metagenomics. At the beginning of the study, all potential participants were screened and breath tested, and metadata were collected. Then, *in vitro* colonic fermentation experiments were run consecutively, using the faecal inoculum

from one participant at a time (Figure 15). Any additional methods used in the study that are specific to only one Chapter are described in that Chapter (Figure 16).

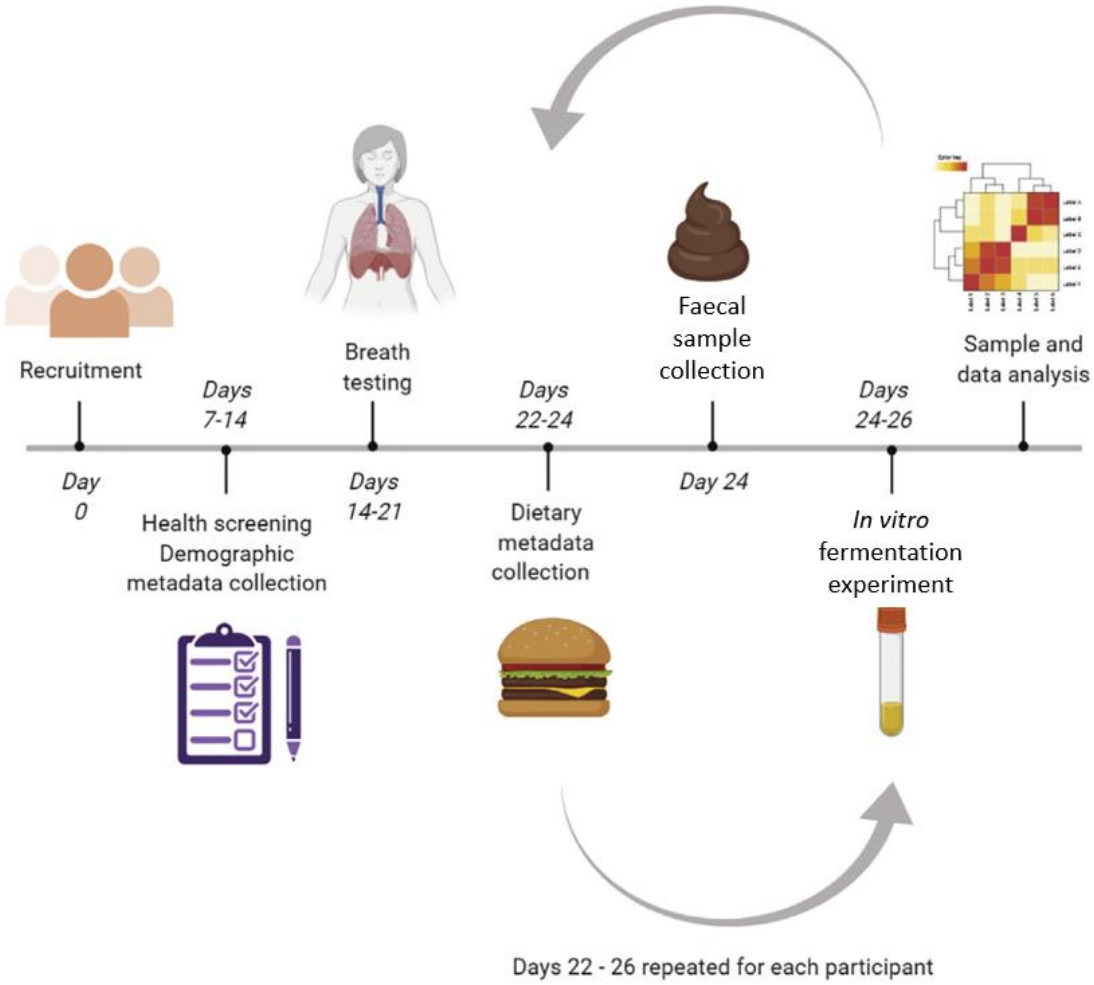


Figure 15 Diagram showing the study design, including participant recruitment and *in vitro* fermentations.
Figure from Payling *et al.* (2021) with permission.

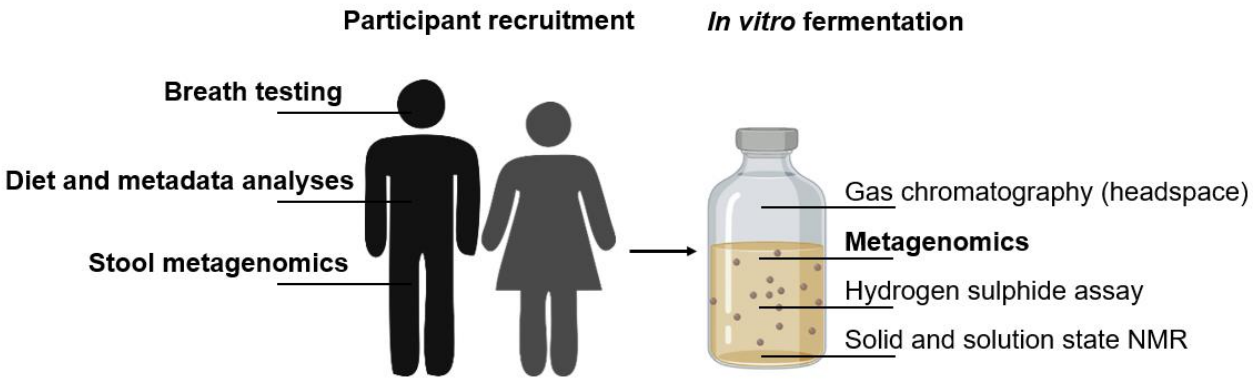


Figure 16 The main methods used in the thesis.
Those described in the current Chapter are highlighted in bold. The remaining methods are described in forthcoming research Chapters.

3.3 Ethics Approval

The study was reviewed and approved by the Massey University Human Ethics Committee (Southern A, Application 19/03, 08-04-19) and is registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) (ACTRN12619001721190, 06-12-19).

3.4 Participant Recruitment

A recruitment email was sent to the staff and student email lists at several Research Institutions and Universities in Palmerston North, New Zealand, on 02-12-19. Potential participants were sent a study information sheet, followed by a health screening questionnaire designed to select only healthy participants. The health screening was based on the inclusion and exclusion criteria below.

3.4.1 Inclusion Criteria

- Agree to complete a health screening questionnaire
- Medically healthy (according to the exclusion criteria listed below)
- Body Mass Index (BMI) of 18.5 to 30
- Agree to provide a three-day food and hydration diary

3.4.2 Exclusion Criteria

- Any current health concerns
- Previously diagnosed with norovirus, hepatitis A or GIT parasites
- Currently experiencing any flu-like symptoms (fever, headache, dehydration, weight loss, lethargy)
- Noticed blood in faeces in the last three months
- Previously diagnosed with *Salmonella*, *Shigella*, *Yersinia*, *Campylobacter*, *Aeromonas*, *Candida*, *Escherichia coli, Klebsiella, Cryptosporidium* or *Entamoeba histolytica*
- A member of the household has had diarrhoea, vomiting, stomach pain or upset stomach in the last three months
- Experienced GIT symptoms in the last three months (loss of appetite, nausea, vomiting or diarrhoea)
- Taken medicines in the last two weeks, excluding contraception
- Used antibiotics or laxatives in the last three months
- A frequent stool type of 1 or 7, according to the Bristol Stool Chart (extreme constipation or diarrhoea, respectively)
- Defaecate less than once per day on average
- A history of GIT disorder (irritable bowel syndrome, inflammatory bowel disease, colon cancer, colitis, diverticulitis)

Ten healthy human adult volunteers were recruited and breath-tested for methane and hydrogen concentrations. Recruitment was completed on 30-10-20. Participant metadata on demographics, health, and diet were collected. In

addition, participants provided a faecal sample used to inoculate an *in vitro* model of colonic fermentation.

Due to the multivariate nature of the study, a power analysis was challenging, and so the scientific literature was reviewed to assess the level of replication in similar experiments. Published studies using faecal inocula for *in vitro* colonic fermentation used one to ten participants (Sanz, Gibson and Rastall, 2005; Walker *et al.*, 2005; Leitch *et al.*, 2007; Parkar *et al.*, 2012; Sulek *et al.*, 2014; Duncan *et al.*, 2016; Ho *et al.*, 2018). Most commonly, three to four participants were used. Five participants per group (10 participants in total) exceeded the number often used in literature but maintained the study at a manageable scale. Literature suggested that approximately 35 % of the population are HE, so it was predicted that 14 participants would need to be recruited to identify five HE (Weaver *et al.*, 1986; Robert and Bernalier-Donadille, 2003). In practice, 31 individuals were screened to find five HE that matched the health inclusion and exclusion criterion. Participants signed a form declaring informed consent to participate during enrolment.

3.5 Breath Testing

Participants fasted for 12 hours (overnight fast) and then provided a sample of alveolar air using an AlveoSampler kit (QuinTron, Milwaukee, USA) (Rezaie *et al.*, 2015; Gottlieb *et al.*, 2017). The participant breathed into a mouthpiece attached to a 400 mL discard bag which collected 'dead space' air comprising air from the airway passages at the end of the previous inhalation. Once the discard bag was full, a syringe attached to the mouthpiece was opened to collect 30 mL of alveolar air within the same breath. Within one hour of sample collection, the alveolar air was transferred from the collection syringe into a

sample holding bag (QuinTron, Milwaukee, USA), which provided seven days of sample stability. The same day, samples were flown from Palmerston North to Auckland in the holding bags to analyse methane, hydrogen, and carbon dioxide on a Quintron Breathtracker (QuinTron, Milwaukee, USA). The concentration of carbon dioxide was used to check for atmospheric air contamination.

Participants emitting ≥5 ppm methane were considered HE, and participants emitting <5 ppm were considered LE. The 5-ppm cut-off for a spot methane breath test was validated against the published expert consensus method by Rezaie *et al.* (2015) and in a retrospective audit of more than 100 clinical patients (Harvie, Tuck and Schultz, 2019).

3.6 Metadata Collection

Participants self-recorded a three-day food and hydration diary before faecal sample collection. The diary was based on the validated resource guide for dietary assessment by the Food and Agriculture Organisation of the United Nations (FAO, 2018). The information collected included details of meals, snacks, and drinks, including a description of the food or drink, its mass, weight or volume, preparation method, brand, and details of dietary supplements. In addition, participants were asked to record whether this was representative of their typical diet over the last three months and, if the answer was no, to describe how these three days differed.

Demographic and health metadata, including age, sex, height, body weight, BMI, and frequency and quality of bowel movements, were collected as part of the health screening questionnaire during participant recruitment. In addition,

the quality of bowel movements was recorded using the Bristol Stool Chart (Lewis and Heaton, 1997).

3.7 Faecal Sample Collection

Faecal sample collection followed the International Human Microbiota Standards Protocol 'IHMS_SOP 02 V2' (Dore *et al.*, 2015). Participants were provided with a faecal sample collection kit containing a Fisherbrand Commode Specimen Collection System (Fisher Scientific, Waltham, USA) lined with a plastic bag, an anaerobic sachet (Anaerocult P, Merck KGaA, Darmstadt, Germany), 3 mL water for sachet activation, gloves, an ice pack, a plastic bag, a cool box, and kit usage instructions (Figure 17). The participant provided the sample whilst at home inside a plastic bag lining the Specimen Collection System. The plastic bag was folded closed but not sealed, and the anaerobic sachet was placed on top. The sachet was activated by adding water according to the manufacturer's instructions, and the Specimen Collection System container was sealed to minimise oxygen contamination. The sample was placed in the cool box with the ice pack and collected or delivered to the research facility within one hour of defecation. At the research facility, the sample was immediately transferred to an anaerobic workstation (Whitley A85 Workstation, Don Whitley Scientific, Bingley, UK) containing 80 % nitrogen, 15 % carbon dioxide and 5 % hydrogen.

The sample was weighed and inspected inside the chamber, and the defecation time, delivery time, sample weight, and Bristol Stool Scale rating were recorded.

Figure 17 The Fisherbrand Commode Specimen Collection System used for safe and efficient collection of faecal samples (left) and the faecal collection kit provided to participants (right). Image (left) from Fisher Scientific with permission.

3.8 *In Vitro* Colonic Fermentation Experiments

On arrival of the faecal samples to the anaerobic workstation, five aliquots of 0.5 g faeces were partitioned into 2 mL cryovials and immediately frozen at -80 °C for later DNA extraction. From the remaining sample, 96 g were transferred into a sterile sample collection pottle, and 300 mL of sterile pre-reduced sodium phosphate buffer (0.2 M, pH 6.5) were added. The pottle was sealed with a magnetic stirrer bar and mixed on a magnetic stirrer plate in anaerobic conditions for five minutes to produce an inoculum of 32 % w/v. Next, the inoculum was strained through a double layer of cheesecloth with 16 threads per cm in both directions within the anaerobic workstation. This method of homogenisation helps to ensure that the inoculum contains microorganisms attached to residual dietary fibres (Williams *et al.*, 2005).

Fermentation experiments were run with one participant at a time, each containing different treatments. Treatments 1 and 2 were positive blanks containing oat β-glucan or potato lignocellulose but no faecal inoculum (positive blanks). Treatment 3 was a negative blank containing the faecal inoculum but no dietary fibre. Treatments 4 and 5 contained the faecal inoculum and oat β-glucan or potato lignocellulose (Figure 18). Lignocellulose was selected due to its high cellulose concentration, so that the cellulose utilising ability of gut microbiotas from HE and LE individuals could be assessed and compared to that reported in Robert and Bernalier-Donadille (2003) and Chassard et al. (2010).

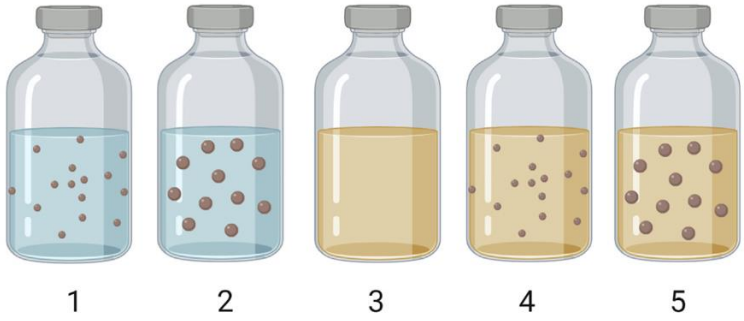


Figure 18 The setup of *in vitro* fermentation bottles.
Treatment 1 (positive blank 1): 10 mL anaerobic sodium phosphate buffer and 100 mg oat β-glucan. Treatment 2 (positive blank 2): 10 mL anaerobic sodium phosphate buffer and 100 mg potato lignocellulose. Treatment 3 (negative blank): 5 mL faecal inoculum and 5 mL anaerobic sodium phosphate buffer. Treatment 4: 5 mL faecal inocula, 5 mL anaerobic sodium phosphate buffer and 100 mg oat β-glucan. Treatment 5: 5 mL faecal inocula, 5 mL anaerobic sodium phosphate buffer and 100 mg potato lignocellulose. Figure from Payling *et al.* (2021) with permission.

Oat β-glucan (BCAN®, Garuda International, Inc., Exeter, USA) is a readily fermentable fibre and potential prebiotic (Daou and Zhang, 2012), containing at least 70 % β-glucan (Garuda International Inc., 2018). Potato lignocellulose is a poorly fermentable fibre that has been extracted using a standard method of grating in the presence of sulphite, sieving through a 100 μm mesh, washing,

freezing, and freeze-drying to extract starch and water-soluble compounds resulting in a composition of 60 to 80 % cellulose, 10 to 20 % lignin, and 10 to 20 % hemicellulose (IsoLife, Wageningen, The Netherlands).

Substrates were weighed (100 mg) into sterile 50 mL serum bottles (treatments 1, 2, 4 and 5), with one set of duplicate bottles for each time point (six time points). The bottles were transferred to the anaerobic workstation the day before the fermentation to ensure deoxygenation. Five millilitres of faecal inoculum (or 5 mL sodium phosphate buffer for positive blanks) was added to each 50 mL serum bottle containing 100 mg substrate or negative blanks (no substrate) and an additional 5 mL sodium phosphate buffer. Each serum bottle contained 10 mL of fluid in total (final concentration 16 % w/v inocula for treatments 3, 4, and 5), with 40 mL of headspace composed of 80 % nitrogen, 15 % carbon dioxide, and 5 % hydrogen. The bottles were sealed with a bung and crimp cap and then transferred to a shaking incubator at 37 °C and 50 rpm.

At the following time points (hours of fermentation), one set of bottles (duplicate) was harvested; 0, 3, 6, 10, 24 and 48 hours. Bottles for harvesting were removed from the incubator and transferred to a static water bath at 37 °C, where gas pressure was measured with a probe. Headspace gas (2 mL) was extracted using a syringe and transferred into a 5.9 mL vial (Exetainer, Labco, Lampeter, UK) for GC analysis of hydrogen and methane. Aliquots of 0.1 mL fermentation fluid were taken from treatments 3 to 5 and mixed with 1 mL zinc acetate solution (2 %) for sulphide determination. All samples were frozen at -80 °C.

Samples were plunged into an ice water bath for 20 minutes to slow bacterial activity (Feng *et al.*, 2018; Warren *et al.*, 2018). Next, samples were transferred

to 15 mL centrifuge tubes, the pH measured, and then centrifuged at 6705 x *g* for 15 minutes at 4°C and again at 24,000 x *g* for 10 minutes at 4 °C (Titgemeyer *et al.*, 1991). Finally, the final supernatant was removed and aliquoted into 2 mL fractions for solution-state NMR.

The pellet was homogenised with a spatula and split into two aliquots for DNA extraction and solid-state NMR. Replicates of the entire fermentation were kept from the start and end of fermentation to analyse the changes in dry matter and organic matter. All samples were stored at -80 °C.

3.9 Shotgun Metagenomics

Frozen faecal aliquots were thawed at 4 °C. DNA was extracted using the Macherey-Nagel NucleoSpin Soil kit following the manufacturer's instructions with the addition of a bead-beating step for four minutes (Mini-Beadbeater 96, BioSpec, Bartlesville, USA) (Young *et al.*, 2020). Extracted DNA was quality control checked by visualisation on agarose gels and quantifying DNA purity (OD260/OD280, OD260/OD230) on a NanoPhotometer® spectrophotometer (Implen, Westlake Village, USA). DNA concentration was measured using a Qubit® dsDNA Assay Kit (Qubit® 2.0 Fluorometer, Life Technologies, Carlsbad, USA). Samples with OD values between 1.8 to 2.0 and DNA concentrations superior to 1 µg were used to construct libraries. All the DNA extracts passed the quality control verification.

DNA samples were shipped to Annoroad Gene Technology, China, where sequencing was performed as a service. Sequencing libraries were prepared using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, USA), following the manufacturer's protocol, and index codes were added to attribute sequences to each sample. Briefly, the DNA samples

were fragmented by sonication to a size of 300 base pairs, and then DNA fragments were end-polished, A-tailed, and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. Finally, PCR products were purified (AMPure XP system, Beckman Coulter Diagnostics, Brea, USA), and libraries were analysed for size distribution on an Agilent 2100 Bioanalyzer and quantified using real-time PCR. Samples were sequenced via shotgun metagenomics on an Illumina NovaSeq 6000. There were 24-30 million paired-end reads per run (read length 2 × 150 base pairs) and an output of approximately 4 gigabase pairs per sample.

Data processing was conducted by bioinformatician Paul Maclean, AgResearch Ltd., New Zealand. There were 7.6 M raw reads, which were paired using the default setting of PEAR (Zhang et al. 2014) to provide 3.7 M paired reads. Pairs underwent adapter and low-quality region trimming using the default settings of Trimmomatic (Bolger et al., 2014), removing 0.04% of the data to provide 3.7 M clean-read pairs. Reads matching the host genome were removed by the 'Decontamination using Kmers' method. Specifically, the function "bbduk.sh" from the BBMAP package version 38.22 was used to filter and discard any reads matching a reference k-mer from the human genome (GRCh38.p13). Then a DIAMOND BLASTX search was run to align unassembled reads to the NCBI non-redundant database of annotated protein sequences, producing 3.3 M assigned read pairs (89% of the data were assigned). The sequences were binned according to taxonomic and functional classifications using MEGAN (Bağcı, Patz and Huson, 2021). The student (Laura Payling) was provided with abundance tables and conducted all further analyses including filtering, sum scaling, diversity and relative abundance analyses.

The relevant forthcoming chapters describe additional analysis methods used in the thesis. The data from the application of these methods may advance the mechanistic understanding of dietary fibre fermentation in the human microbiota.

References

1. Bağcı, C., Patz, S., and Huson, D.H. (2021). DIAMOND+MEGAN: Fast and Easy Taxonomic and Functional Analysis of Short and Long Microbiome Sequences. Current Protocols 1, 1–29. https://doi.org/10.1002/cpz1.59.
2. Bolger A. M., Lohse M., Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data (2014). Bioinformatics 1, 30(15), 2114-20. doi: 10.1093/bioinformatics/btu170.
3. Daou, C., and Zhang, H. (2012). Oat Beta-Glucan: Its Role in Health Promotion and Prevention of Diseases. Comprehensive Reviews in Food Science and Food Safety 11, 355–365. https://doi.org/10.1111/j.1541-4337.2012.00189.x.
4. Dore, J., Ehrlich, S.D., Levenez, F., Pelletier, E., Alberti, A., Bertrand, L., Bork, P., Costea, P.I., Sunagawa, S., Guarner, F., *et al.* (2015). IHMS_SOP 02 V1: Standard operating procedure for faecal samples self-collection, laboratory analysis handled within 4 hours. https://brd.nci.nih.gov/brd/sop/download-pdf/2101.
5. Duncan, S.H., Russell, W.R., Quartieri, A., Rossi, M., Parkhill, J., Walker, A.W., and Flint, H.J. (2016). Wheat bran promotes enrichment within the human colonic microbiota of butyrate-producing bacteria that release ferulic acid. Environmental Microbiology 18, 2214–2225. https://doi.org/10.1111/1462-2920.13158.
6. FAO (2018). Dietary Assessment: A resource guide to method selection and application in low resource settings (Rome: Food and Agriculture Organization of the United Nations), pp. 1-172.
7. Feng, G., Flanagan, B.M., Mikkelsen, D., Williams, B.A., Yu, W., Gilbert, R.G., and Gidley, M.J. (2018). Mechanisms of utilisation of arabinoxylans

by a porcine faecal inoculum: competition and co-operation. Scientific Reports 8. https://doi.org/10.1038/s41598-018-22818-4.

8. Garuda International Inc. (2018). B-CAN® Soluble Oat Fiber (70% oat beta-glucan) (online). https://garudaint.com/product.php?id=82.
9. Gottlieb, K., Le, C., Wacher, V., Sliman, J., Cruz, C., Porter, T., and Carter, S. (2017). 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. Gastroenterology Report 5(3), 193-199. https://doi.org/10.1093/gastro/gow048.
10. Harvie, R.M., Tuck, C.J., and Schultz, M. (2019). Evaluation of lactulose, lactose, and fructose breath testing in clinical practice: A focus on methane. JGH Open 4, 198–205. https://doi.org/10.1002/jgh3.12240.
11. Ho, A.L., Kosik, O., Lovegrove, A., Charalampopoulos, D., and Rastall, R.A. (2018). *In vitro* fermentability of xylo-oligosaccharide and xylo-polysaccharide fractions with different molecular weights by human faecal bacteria. Carbohydrate Polymers 179, 50–58. https://doi.org/10.1016/j.carbpol.2017.08.077.
12. Leitch, E.C.M.W., Walker, A.W., Duncan, S.H., Holtrop, G., and Flint, H.J. (2007). Selective colonization of insoluble substrates by human faecal bacteria. Environmental Microbiology 9, 667–679. https://doi.org/10.1111/j.1462-2920.2006.01186.x.
13. Lewis, S.J., and Heaton, K.W. (1997). Stool form scale as a useful guide to intestinal transit time. Scandinavian Journal of Gastroenterology 32, 920–924. https://doi.org/10.3109/00365529709011203.
14. Payling, L., Roy, N. C., Fraser, K., Loveday, S. M., Sims, I. M., Stefan, J., Laura, G., and Warren, C. (2021). A protocol combining breath testing and ex vivo fermentations to study the human gut microbiome. STAR Protocols 2, 100227. https://doi.org/10.1016/j.xpro.2020.100227.
15. Parkar, S.G., Rosendale, D., Paturi, G., Herath, T.D., Stoklosinski, H., Phipps, J.E., Hedderley, D., and Ansell, J. (2012). *In vitro* Utilization of Gold and Green Kiwifruit Oligosaccharides by Human Gut Microbial

Populations. Plant Foods for Human Nutrition 67, 200–207. https://doi.org/10.1007/s11130-012-0293-1.

16. Rezaie, A., Chang, B., Chua, K.S., Lin, E.A., and Pimentel, M. (2015). Accurate Identification of Excessive Methane Gas Producers by a Single Fasting Measurement of Exhaled Methane: A Large-scale Database Analysis (Conference proceedings). American Journal of Gastroenterology 1787, S759–S760.
17. Robert, C., and Bernalier-Donadille, A. (2003). The cellulolytic microflora of the human colon: Evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects. FEMS Microbiology Ecology 46, 81–89. https://doi.org/10.1016/S0168-6496(03)00207-1.
18. Sanz, M.L., Gibson, G.R., and Rastall, R.A. (2005). Influence of disaccharide structure on prebiotic selectivity *in vitro*. Journal of Agricultural and Food Chemistry 53, 5192–5199. https://doi.org/10.1021/jf050276w.
19. Sulek, K., Vigsnaes, L.K., Schmidt, L.R., Holck, J., Frandsen, H.L., Smedsgaard, J., Skov, T.H., Meyer, A.S., and Licht, T.R. (2014). A combined metabolomic and phylogenetic study reveals putatively prebiotic effects of high molecular weight arabino-oligosaccharides when assessed by invitro fermentation in bacterial communities derived from humans. Anaerobe 28, 68–77. https://doi.org/10.1016/j.anaerobe.2014.05.007.
20. Titgemeyer, E.C., Bourquin, L.D., Fahey, G.C., and Garleb, K.A. (1991). Fermentability of various fiber sources by human fecal bacteria *in vitro*. American Journal of Clinical Nutrition 53, 1418–1424. https://doi.org/10.1093/ajcn/53.6.1418.
21. Walker, A.W., Duncan, S.H., Mcwilliam leitch, E.C., Child, M.W., and Flint, H.J. (2005). pH and Peptide Supply Can Radically Alter Bacterial Populations and Short-Chain Fatty Acid Ratios within Microbial Communities from the Human Colon pH and Peptide Supply Can Radically Alter Bacterial Populations and Short-Chain Fatty Acid Ratios within Mi. Applied Environmental Microbiology 71, 3692–3700. https://doi.org/10.1128/AEM.71.7.3692.

22. Warren, F.J., Fukuma, N.M., Mikkelsen, D., Flanagan, B.M., Williams, B.A., Lisle, A.T., Ó Cuív, P., Morrison, M., and Gidley, M.J. (2018). Food Starch Structure Impacts Gut Microbiome Composition. MSphere 3(3), e00086-18. https://doi.org/10.1128/mSphere.00086-18.

23. Weaver, G.A., Krause, J.A., Miller, T.L., and Wolin, M.J. (1986). Incidence of methanogenic bacteria in a sigmoidoscopy population: An association of methanogenic bacteria and diverticulosis. Gut 27, 698–704. https://doi.org/10.1136/gut.27.6.698.

24. Williams, B.A., Bosch, M.W., Boer, H., Verstegen, M.W.A., and Tamminga, S. (2005). An *in vitro* batch culture method to assess potential fermentability of feed ingredients for monogastric diets. Animal Feed Science and Technology 123-124, 445–462. https://doi.org/10.1016/j.anifeedsci.2005.04.031.

25. Young, W., Arojju, S.K., McNeill, M.R., Rettedal, E., Gathercole, J., Bell, N., and Payne, P. (2020). Feeding Bugs to Bugs: Edible Insects Modify the Human Gut Microbiome in an *in vitro* Fermentation Model. Frontiers in Microbiology 11. https://doi.org/10.3389/fmicb.2020.01763.

26. Zhang J., Kobert K., Flouri T., Stamatakis A. (2014). PEAR: a fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics, 1, 30(5), 614-20. doi: 10.1093/bioinformatics/btt593.

Chapter 4

The Links Between Breath Methane, Breath Hydrogen, and the Colonic Microbiota

This Chapter is being drafted into a publication by Payling, L., Roy, N.C., Fraser, K., Loveday, S.M., Sims, I.M., Gagic, D. and McNabb, W. C. The relationship between breath methane, breath hydrogen, the colonic microbiota, and diet. The American Journal of Gastroenterology.

4.1 Abstract

Healthy adults who are HE or LE have a different faecal microbiota in terms of composition and fibre fermentation. However, few studies investigated this using next-generation sequencing methods. This study aimed to investigate the relationship of breath methane with colonic microbiota composition and predicted function, including links with breath hydrogen.

Eighteen healthy individuals were tested for breath methane, and the five highest and five lowest breath methane emitters were selected to provide faecal samples for DNA shotgun metagenomic sequencing. Unexpectedly, a positive correlation between breath methane and breath hydrogen was observed.

Individuals that were LE had a *Bacteroides*-driven microbiota, enriched with several other genera, including *Blautia, Slackia* and *Clostridia*, and more pathways related to bile acid biosynthesis and oxidative phosphorylation than individuals that were HE. In comparison, higher breath methane was positively associated with a *Prevotella*-driven microbiota, co-occurring with *Eggerthella, Akkermansia* and *Desulfovibrio* genera, and more pathways related to xenobiotic metabolism, amino acid metabolism, and fatty acid degradation.

The study confirmed differences in the microbiota composition of individuals who were HE and LE at the genus and species levels, which had different driving genera. Dietary factors, including vitamin E, fibre, starch, and fat, contributed to these differences.

4.2 Introduction

Diet diversity, particularly dietary fibres and RS, promotes alpha-diversity of the colonic microbiota in humans. The microbiota ferments dietary fibres as an energy source, and the different levels of structure in the substrates drive diversity in microbial composition because different species are needed for a wide range of enzymatic functions. During fermentation, the main gases produced are carbon dioxide, hydrogen, and methane (Ríos-Covián *et al.*, 2016; Rowland *et al.*, 2018). The only major sources of methane and hydrogen in human breath are colonic methanogenesis and fermentation, respectively (Ostojic, 2017; Kumpitsch *et al.*, 2020).

These gases pass from the colonic lumen into the bloodstream and diffuse into the lungs, where they are exhaled. Approximately 20 % of colonic methane is exhaled in breath, whilst the rest is excreted in flatus (Bond, Engel and Levitt, 1971). Whilst the excretion pattern is consistent between individuals, it does vary according to colonic gas accumulation, with a greater proportion of gas excreted in breath compared to flatus when colonic hydrogen accumulation is low (Hammer, 1993). Nevertheless, individual differences in breath gas concentrations reflect differences in colonic production.

About one-third of individuals excrete a lower concentration of breath hydrogen but a higher concentration of methane due to the ability of the colonic microbiota for methanogenesis (Levitt *et al.*, 2006; De Lacy Costello, Ledochowski and Ratcliffe, 2013). Hydrogen utilisation by colonic methanogens reduces the partial pressure of hydrogen and allows increased polysaccharide fermentation, producing beneficial organic acids (Rezaie *et al.*, 2017). Alternatively, hydrogen partial pressure is reduced by acetogenesis

and/or sulphate reduction (Gibson, Macfarlane and Cummings, 1993; Smith *et al.*, 2018).

Breath testing has proven to be a practical, affordable, and non-invasive method to detect methanogenic activity, though it does not provide information on microbial communities.

Research has attempted to better understand the mechanisms of methane production in humans by studying the composition and function of colonic microbiota of individuals who are HE and LE. *In vitro*, the faecal microbiota of HE individuals has a greater capacity for fibre fermentation than LE individuals. Keystone species, including *R. champanellensis,* are mediators of this response and known fibre fermenters and hydrogen producers (Robert and Bernalier-Donadille, 2003).

These data were confirmed in a study that sequenced the faecal microbiota of 100 adults who were breath-tested (Kumpitsch *et al.*, 2020). They found that individuals who were HE had a faecal microbiota specialised for fibre degradation, including taxa from the *Ruminococcaceae* family. In addition, individuals who were HE had a 1000-fold greater abundance of faecal *M. smithii* compared to LE and lower reported vitamin B12 and fat intakes (Kumpitsch *et al.*, 2020). However, the study did not report breath hydrogen.

4.3 Aim and Hypotheses

This study aimed to investigate the links between breath hydrogen and methane and the composition and potential functionality of the colonic microbiota in individuals who were HE or LE. This knowledge could contribute to a better understanding of colonic microbiota ecology, which will support the development of microbiota modulation strategies for better health outcomes.

The hypotheses were as follows:

1. Individuals who are HE have a lower breath hydrogen concentration than individuals who are LE.
2. Individuals who are HE have a higher abundance of taxa from the Firmicutes phylum, while individuals who are LE have more taxa from the Bacteroidetes phylum.
3. The microbiota of HE individuals has a higher abundance of genes relating to methane metabolism, whereas the microbiota of LE individuals has a higher abundance of genes relating to sulphate reduction.

4.4 Methods

Eighteen participants were recruited, breath-tested, and asked to provide diet data and metadata (Figure 19) as outlined in Chapter 3, General Materials and Methods. The five highest and five lowest breath methane emitters were selected to provide faecal samples for shotgun metagenomic sequencing. The sequencing methodology was also described in Chapter 3.

The five-ppm breath methane cut-off was the expert consensus method for determining HE or LE (Gottlieb *et al.*, 2017). Individuals who were HE or LE served as two contrasting groups for the analysis.

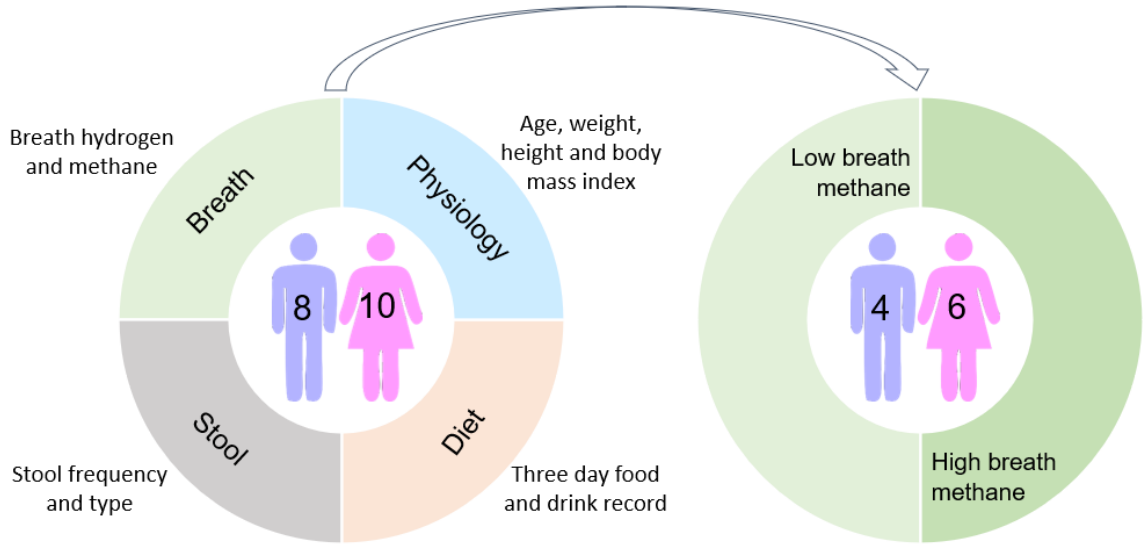


Figure 19 The number of males and females recruited in the participant cohort and the metadata that were collected.
Participant sex is indicated by human silhouette colour, blue representing male and pink representing female.

4.4.1 Diet and Metadata

Metadata were coded and entered into a digital database. Personal data were managed strictly to comply with privacy and confidentiality guidelines (Massey University, 2017).

Food and hydration records were checked for completeness, and participants were approached to complete any records where further information was required and available. Compositional data for food and drink were generated using Foodworks 10 (Xyris, Australia) by inputting the mass of foods consumed and calculating serves based on the New Zealand and Australian Food Composition Databases (Xyris Food Groups).

4.4.2 Data Analyses

The breath data met the key assumptions of a regression using the least squares method, including residuals with a mean of zero, homoscedasticity of the residuals, and residuals that were not inter-correlated.

Dietary data were analysed by macronutrient and micronutrient composition and dietary patterns, which were based on serves of common food groups as denoted by the Australian and New Zealand Guides for Healthy Eating (Supplementary File 1). Dietary data were adjusted to an energy basis (per 100 calories) due to associations of energy intake with other dietary information and breath gases. A Mann-Whitney U Test was used to assess differences in nutrient intake and food group serves between groups of participants that were HE and LE.

Alpha- and beta-diversity metrics, including the Shannon-Wiener Index, Simpson's Index, the number of taxonomic units (richness), and Bray-Curtis dissimilarity, were calculated from the faecal microbiota sequence data using the packages Phyloseq and Vegan in R (McMurdie and Holmes, 2013; Oksanen *et al.*, 2019). In addition, Bray-Curtis dissimilarities were applied to Principal Coordinates Analysis (PCoA) to compare beta-diversity between samples from HE and LE individuals in MicrobiomeAnalyst (Chong *et al.*, 2020).

The identified taxonomic units that did not align to k-mers of the reference human genome were filtered to remove those with more than 90 % zero counts or those that appeared in only one sample. Similarly, predicted genes were filtered to remove low variance genes (10 % interquartile range) and those that occurred in less than three participants (Figure 20). The KEGG output was arranged hierarchically, with Level 1 representing the broadest level of function, e.g., metabolism and cellular processes, Level 2 represented the next level of function, which was more specific, e.g., carbohydrate metabolism, amino acid metabolism, and Level 3 represented detailed pathways such as glycolysis,

glycan degradation. Level 2 and 3 data were used for analysis as these were the most relevant to the hypotheses.

Permutational multivariate analysis of variance (PERMANOVA) (package Vegan in R, Oksanen *et al.*, 2019) was used to explore the relationships between diet, metadata, beta-diversity, microbiota composition, and KEGG pathways. Spearman's correlations were conducted for dietary data, metadata, and microbiota data to investigate correlations within and between datasets. The probability values (p) were adjusted for multiple comparisons (p-adj) using the Benjamini-Hochberg Method (Benjamini and Hochberg, 1995). Trends were considered where p-adj >0.05, but trends in unadjusted probability (p) values were evident (p <0.10). PERMANOVA analyses were conducted on the entire dataset combining samples from individuals who were LE and HE to maximise the detection power of the study, whilst specific differences between the two groups were tested using univariate analyses. The Mann-Whitney U Test was used to assess whether microbiota composition or potential function differed according to metadata categories or the breath methane groupings. PERMANOVA, Spearman's correlation, and Mann-Whitney U tests are non-parametric and robust to violations of homogeneity of variance and normality.

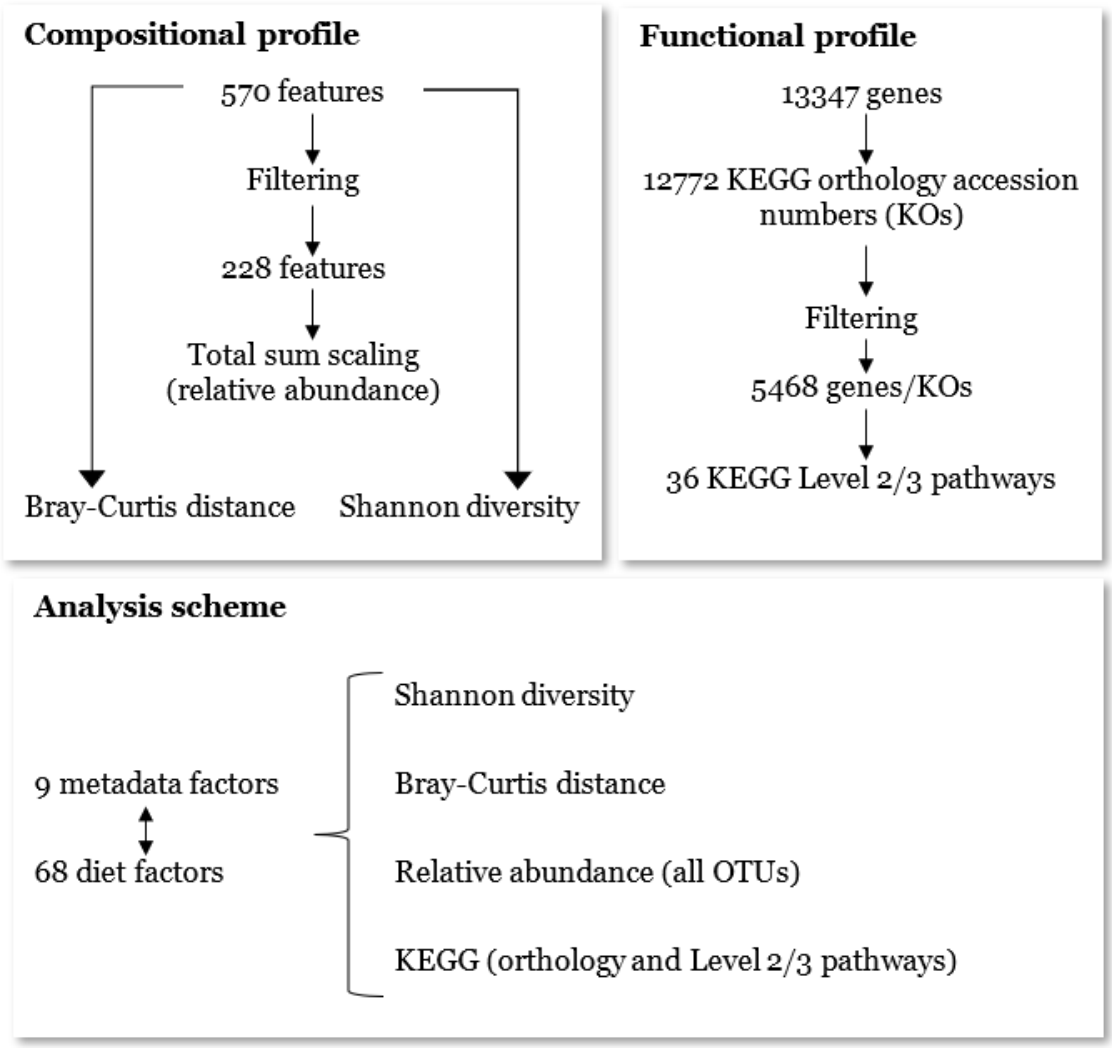


Figure 20 The analysis scheme used for shotgun metagenomic sequencing.

4.5 Results

4.5.1 Breath Hydrogen and Methane

The 18 recruited participants included ten females and eight males, aged 24 to 42, of which 39 % were HE (Table 3). The median breath methane and hydrogen concentrations were 2 and 4 ppm for the LE group and 7 and 14 ppm for the HE group, respectively (Table 4). The HE group had greater breath methane and hydrogen concentrations compared to the LE group ($p < 0.01$) (Table 4).

Table 3 Metadata of recruited participants.

Participant	Height, cm	Weight, kg	Body Mass Index	Sex	Age	Faecal frequency	Faecal quality	Breath hydrogen, ppm	Breath methane, ppm
1	168	67	24	Female	42	1	3	1	1
2	191	95	26	Male	25	2	3	0	1
3	165	62	23	Female	27	1	3	4	1
4	177	66	21	Male	26	1.5	4	4	1
5	175	71	23	Female	28	1	3.5	1	2
6	176	80	26	Male	27	1.5	4.5	55	18
7	170	67	23	Female	30	0	3	14	7
8	157	58	24	Female	25	1	3	9	6
9	160	65	25	Male	34	2	4	66	26
10	155	45	19	Female	31	1	4	30	13
11	177	65	21	Male	38	2	3.5	3	2
12	177	78	25	Female	30	2.5	3.5	8	3
13	180	61	19	Female	36	1	3.5	6	2
14	183	66	20	Female	27	2.5	3	8	3
15	188	70	20	Male	39	1	3	6	4
16	189	85	24	Male	25	1	3.5	11	3
17	170	54.5	19	Female	32	1	4	14	6
18	175	93	30	Male	28	2	3.5	10	5

Faecal frequency, number of times per day. Faecal quality according to the Bristol Stool Chart (Type 1 constipated, Type 3-4 normal, Type 7 diarrhoea). Participants one to ten are the sub-group that were selected for faecal sampling. Blue data are low breath methane emitters, and red data are high breath methane emitters.

Table 4 The median breath hydrogen and breath methane concentrations in groups of high and low breath methane emitters, and statistics according to a Mann Whitney-U test.

Median	LE	HE	95 % Confidence Interval for the Difference	p-adj
Breath methane, ppm	2	7	(4, 16)	0.001
Breath hydrogen, ppm	4	14	(6, 51)	0.001

p-adj is the p-value adjusted for ties. The HE group included 7 participants, and the LE group included 11 participants.

There was a positive relationship (R^2 >0.90, p <0.001) between breath hydrogen and methane (Figure 21). The slope coefficient was 2.68, with a standard error of 0.13, and lower and upper 95 % confidence intervals of 2.41 and 2.95, respectively.

Breath methane and hydrogen concentrations were negatively correlated with dietary sugar intake but positively correlated with starch intake (r > 0.50, p-adj <0.05). Breath hydrogen concentration was negatively correlated with total fat, saturated fat, monounsaturated fat, and polyunsaturated fatty acid docosapentaenoic acid intake (r ≥0.40, p-adj <0.05) (Supplementary File 2).

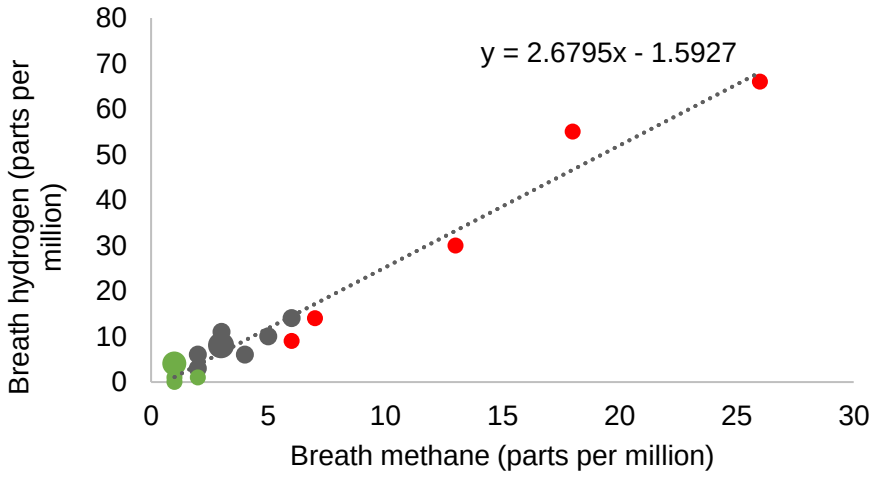


Figure 21 Breath hydrogen and methane from 18 participants.
Red and green data points represent the five highest and five lowest breath methane emitting participants, respectively, selected for faecal sampling. Enlarged data points indicate two points that are overlayed.

4.5.2 Overview of Microbial Composition and Potential Function

The sub-group of participants selected for faecal sampling based on breath testing data consisted of six females and four males aged 25 to 42.

Sequencing of faecal samples provided an average of 7.6 M clean reads (7.4 to 8.0 M) and the average number of assigned microbial reads was 3.3 M (3.1 to 3.4 M).

4.5.2.1 Alpha- and Beta-Diversity

Alpha-diversity describes the level of community variation within a sample; commonly used indices are the number of taxonomic units (richness), the Shannon-Wiener Index (richness and evenness), and the Simpson Index (richness and evenness) (Roswell, Dushoff and Winfree, 2021). The number of observed taxonomic units in samples ranged from 121 to 169. The Shannon-Wiener Index ranged from 3.0 to 3.6, and the Simpson Index ranged from 0.89 to 0.95. The indices did not vary according to breath methane ($p > 0.05$) (Supplementary File 3).

Beta-diversity is a measure of dissimilarity between the communities of different samples (Lahti, Sudarshan and Ernst, 2021) and is commonly measured using the Bray-Curtis index. Variation in the Bray-Curtis index was explained by dietary vitamin E and vitamin A (41 % and 35 %, respectively, p-adj <0.05) (Supplementary File 4) and tended to be explained by breath methane grouping (16 %, $p < 0.10$) (Supplementary File 4, Figure 22).

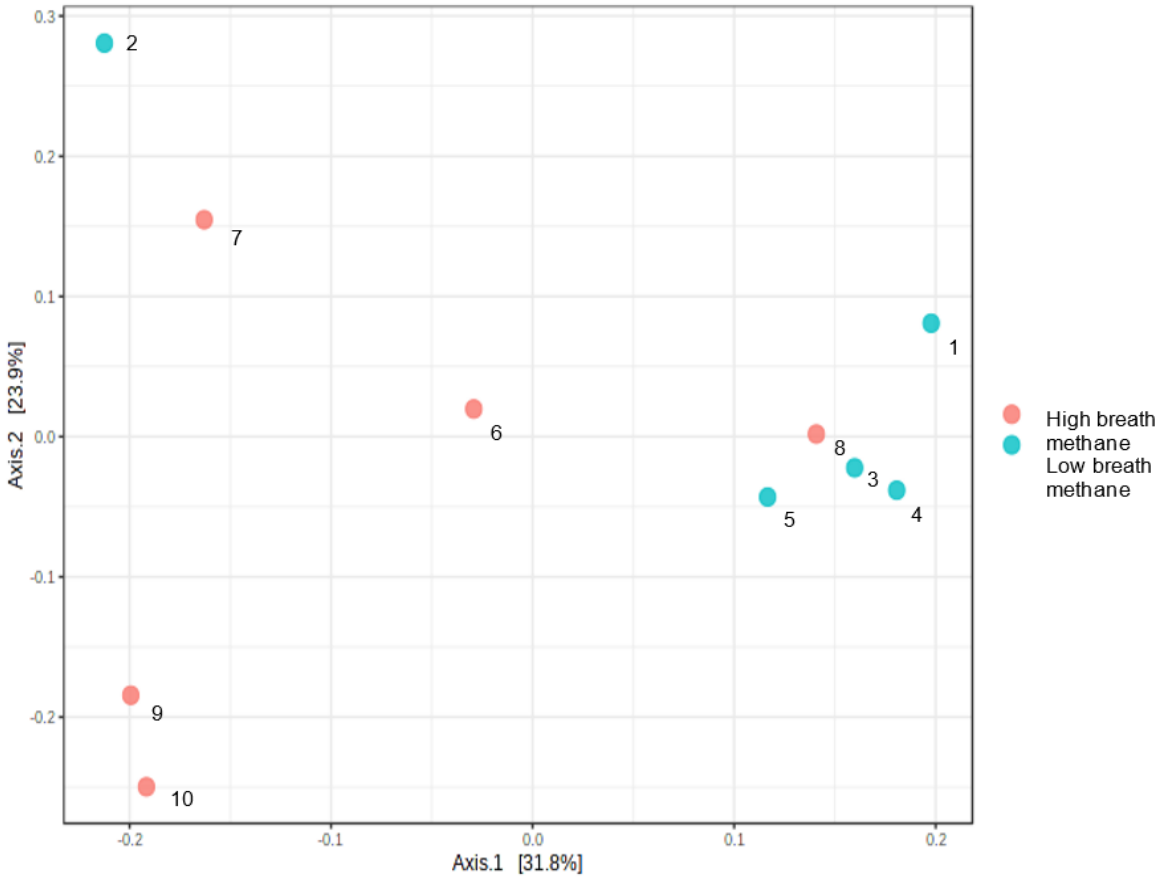


Figure 22 Principal coordinates analysis of faecal microbiota beta-diversity from ten participants using the Bray-Curtis index.
Data points are coloured by high (pink) and low (blue) participant breath methane and labelled by participant ID.

4.5.2.2 Relative Bacterial Abundance and Dietary Factors

LE individuals had higher dietary intakes of energy, protein, fat, fibre, sugar, and vitamin E compared to HE individuals (p-value < 0.05); however, their starch intakes were similar (p-value >0.10) (Table 5). When adjusted to an energy basis, there were no differences between the two groups for the intakes of macronutrients or vitamin E, except sugar which was highest in HE individuals (p-value <0.05) (Table 5).

Table 5 The average daily macronutrient and vitamin E intakes of high and low breath methane emitters according to a Mann-Whitney U Test.

	Low breath methane emitters	High breath methane emitters	P-value
Energy, kcal	2588.21	1929.17	0.004
Protein, g	112.02	76.85	0.019
Fat, g	107.01	62.43	0.003
Fibre, g	32.37	19.75	0.037
Starch, g	136.24	158.37	0.786
Sugar, g	90.98	52.18	0.009
Vitamin E, mg	18.68	7.00	0.014
Protein, g/100 kcal	4.07	3.91	0.786
Fat, g/100 kcal	3.90	3.69	0.205
Fibre, g/100 kcal	1.21	1.16	0.786
Starch, g/100 kcal	6.05	7.96	0.085
Sugar, g/100 kcal	2.82	3.95	0.019
Vitamin E, mg/100 kcal	0.56	0.41	0.085

Dietary fibre and vitamin E explained 10 % and 25 % of the variance in the microbiota composition, respectively (p-adj <0.05) (Supplementary File 5). vitamin E positively correlated with taxa from the *Bacteroidaceae* family, negatively correlated with taxa from the *Acidaminococcaceae* family (r >0.80, p-adj <0.05), and tended to have a negative association with taxa from the *Desulfovibrionaceae* family (r ≥0.80, p <0.05, p-adj >0.05) (Supplementary File 6). Dietary fibre tended to have a positive association with *Faecalibacterium* and *Lachnospira* genera (r ≥0.70, p <0.05, p-adj >0.05) (Supplementary File 6).

4.5.2.3 Potential Microbial Function

At KEGG Level 2/3, the most abundant genes were related to cellular and signalling processes, genetic information processing, carbohydrate metabolism, and amino acid metabolism, regardless of HE or LE status (Figure 23).

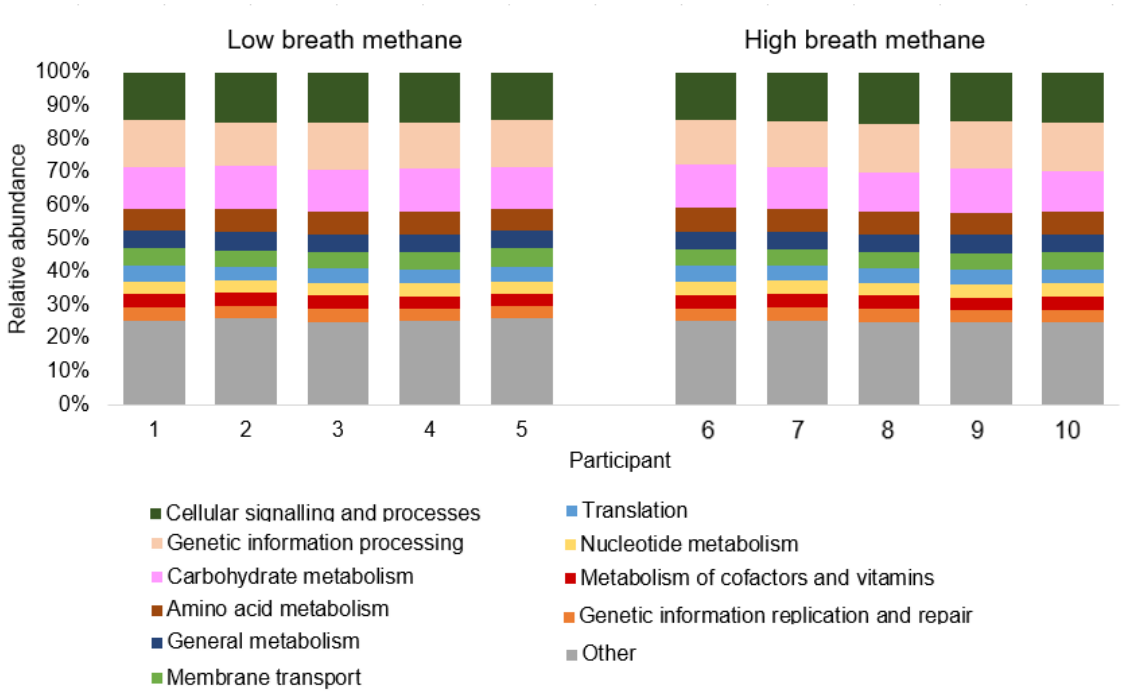


Figure 23 The relative abundance of the top ten predicted functions of the faecal microbiota at KEGG Level 2/3.

From top to bottom, colours represent dark green cellular signalling and processes, peach genetic information processing, pink carbohydrate metabolism, brown amino acid metabolism, dark blue general metabolism, green membrane transport, light blue translation, yellow nucleotide metabolism, red metabolism of cofactors and vitamins, orange genetic information replication and repairs, grey all other pathways combined.

There were trends for methane metabolism to positively correlate with genes involved in genetic information replication and repair, infectious disease, terpenoids and polyketides metabolism, transcription, quorum sensing and biofilm formation, amino acid metabolism, and nucleotide metabolism ($r > 0.50$, $p < 0.10$, p-adj > 0.05) (Supplementary File 7). Conversely, methane

metabolism tended to be negatively correlated with signal transduction and sulphur metabolism (r >0.50, p <0.10, p-adj >0.05) (Supplementary File 7).

4.5.2.4 Microbiota abundance and potential function

There were 28 Spearman's correlations between the relative abundance of phyla and Level 2/3 pathways, most of which were positive (Supplementary File 8). There was a positive correlation between the Euryarchaeota phylum (*M. smithii*) and genes involved in methane metabolism (r >0.90, p-adj <0.05), although this was driven by one participant (Supplementary File 9). The relative abundance of the Euryarchaeota phylum was also positively correlated with pathways relating to infectious disease and the metabolism of terpenoids and polyketides (r >0.70, p-adj <0.05) (Supplementary File 8).

The relative abundance of the Firmicutes phylum correlated positively with functions related to fatty acid biosynthesis, genetic information folding and sorting, and synthesis of secondary metabolites (r >0.60, p-adj <0.05) and had a negative trend with methane metabolism (r >0.60, p <0.05, p-adj >0.05). The relative abundance of the Bacteroidetes phylum was positively associated with genes involved in glycan metabolism and bile acid synthesis (r >0.70, p-adj <0.05) (Supplementary File 8).

4.5.3 Breath Methane and Microbial Composition

Microbial composition at the phylum level was not associated with breath methane concentration (p-adj>0.10). Neither was the composition at the family level linked with breath methane concentration (p-adj>0.10); however, composition at the genus level was linked with breath methane concentration. The most abundant genera in faecal samples were *Bacteroides,*

Faecalibacterium, Bifidobacterium, Alistipes, Roseburia, Ruminococcus, and *Prevotella*, regardless of LE or HE status (Figure 24).

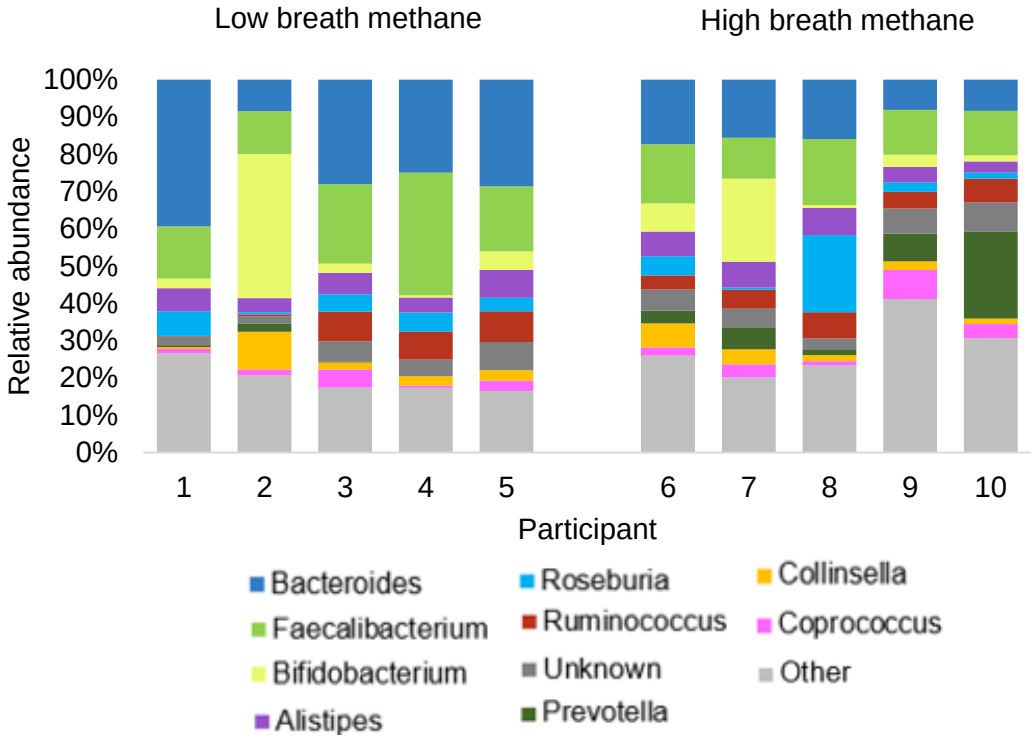


Figure 24 The relative abundance of top 10 taxa in faecal samples from high and low breath methane emitters at the genus level.
From top to bottom, blue *Bacteroides*, light green *Faecalibacterium*, light yellow *Bifidobacterium*, purple *Alistipes*, light blue *Roseburia*, red *Ruminococcus*, grey unidentified at the genus level, dark green *Prevotella*, yellow/orange *Collinsella*, pink *Coprococcus*, light grey all other genera.

Regardless of HE or LE status, there were trends for breath methane to be positively associated with *Eggerthella* (r >0.60, p <0.05, p-adj >0.05) (Figure 25) and for HE samples to have a greater relative abundance of that genus (p <0.10, p-adj >0.05) (Supplementary File 10). Breath methane was numerically positively associated with *Dialister, Methanobrevibacter, Megasphaera, Acidaminococcus, Prevotella,* and *Akkermansia* genera (r ≥0.40, p >0.10, p-adj >0.05) (Figure 25). However, there was no difference in the relative abundance of *M. smithii* between the two groups (p >0.10, p-adj >0.05), as the

methanogen was not detected in any samples from the LE group and only two samples from the HE group (Figure 26). The *Methanobrevibacter* genus correlated positively with *Megasphaera* and *Acidaminococcus* genera (r >0.90, p-adj <0.05) (Supplementary File 11). The *Prevotella* genus tended to correlate positively with *Akkermansia* and *Desulfovibrio* genera (r >0.80, p <0.01, p-adj >0.05), but negatively with the *Bacteroides* genus (r >0.50, p <0.10, p-adj >0.05) (Supplementary File 11).

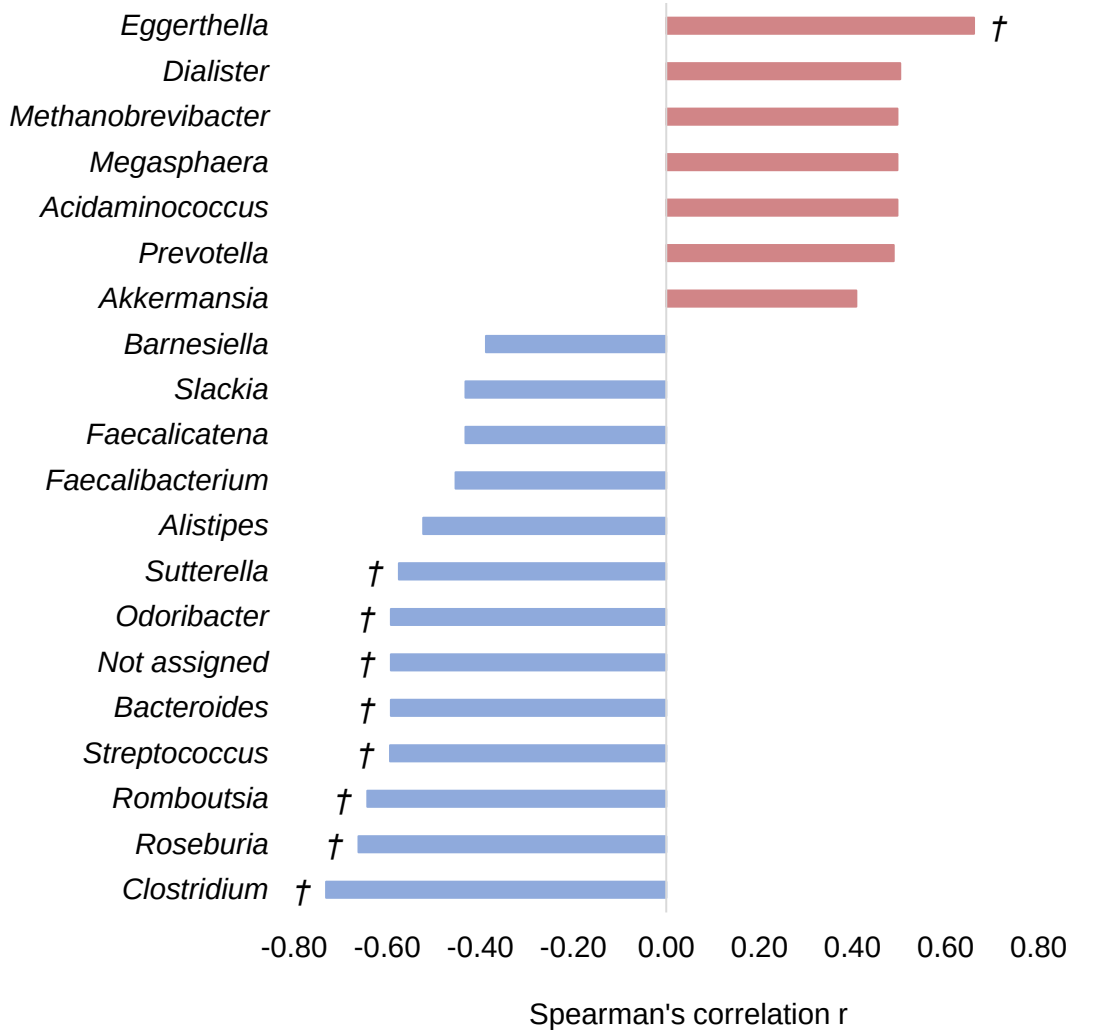


Figure 25 The Spearman correlation coefficient of the top 20 genera that correlated with breath methane grouping.

† Trend, p <0.10, p-adj >0.05. Genera with red bars were positively correlated with breath methane, and genera with blue bars were negatively correlated with breath methane.

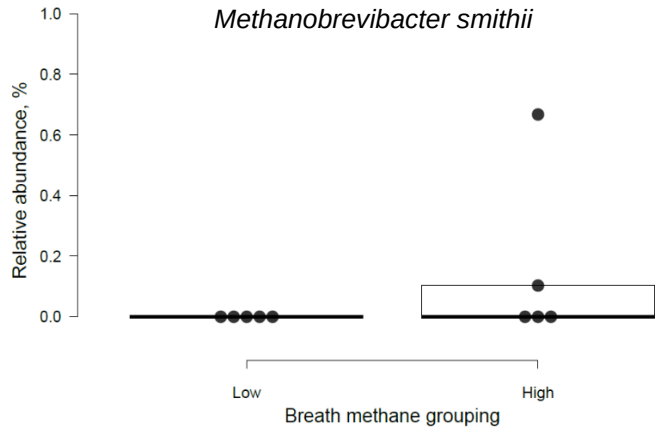


Figure 26 The relative abundance of *Methanobrevibacter smithii* in faecal samples from low and high-breath methane emitters.

Breath methane tended to be negatively correlated with *Clostridium, Roseburia, Romboutsia, Streptococcus, Bacteroides*, unassigned genera, *Odoribacter*, and *Sutterella* genera (r >0.50, p <0.10, p-adj >0.05) and numerically negatively associated with *Alistipes*, *Faecalibacterium, Faecalicatena, Slackia* and *Barnesiella* (r ≥0.40, p >0.10, p-adj >0.05) (Figure 25). Many of these genera from the Firmicutes (*Clostridium, Roseburia, Romboutsia, Streptococcus)* and Bacteroidetes (*Bacteroides* and *Odoribacter)* phyla tended to have higher relative abundance in samples from the LE group compared to the HE group (p-adj <0.10) (Supplementary File 10). The *Bacteroides* genus tended to correlate positively with *Blautia* and *Slackia* genera (r >0.60, p <0.10, p-adj >0.05) (Supplementary File 11).

There were no differences in the relative abundance of keystone species *B. thetaiotaomicron, R. bromii, R. champanellensis* and *Enterococcus faecalis* in samples from HE or LE individuals (p-adj <0.05) (Supplementary File 10).

4.5.4 Breath Methane and Potential Microbial Function

There were trends for amino acid metabolism pathways to be positively correlated with breath methane and oxidative phosphorylation to be negatively correlated with breath methane ($r > 0.50$, $p < 0.10$, p-adj > 0.05) (Figure 27).

In addition, the relative abundance of genes involved in bile acid biosynthesis (sterol and secondary bile acids) tended to be higher in the LE compared to the HE group ($p < 0.10$, p-adj > 0.05), and those involved in fatty acid degradation and xenobiotics metabolism tended to be higher in the HE compared to the LE group ($p < 0.10$, p-adj > 0.05) (Supplementary File 12). Furthermore, there appeared to be greater variation in the relative abundance of genes involved in methane metabolism pathways in the HE group compared to the LE group (Figure 28).

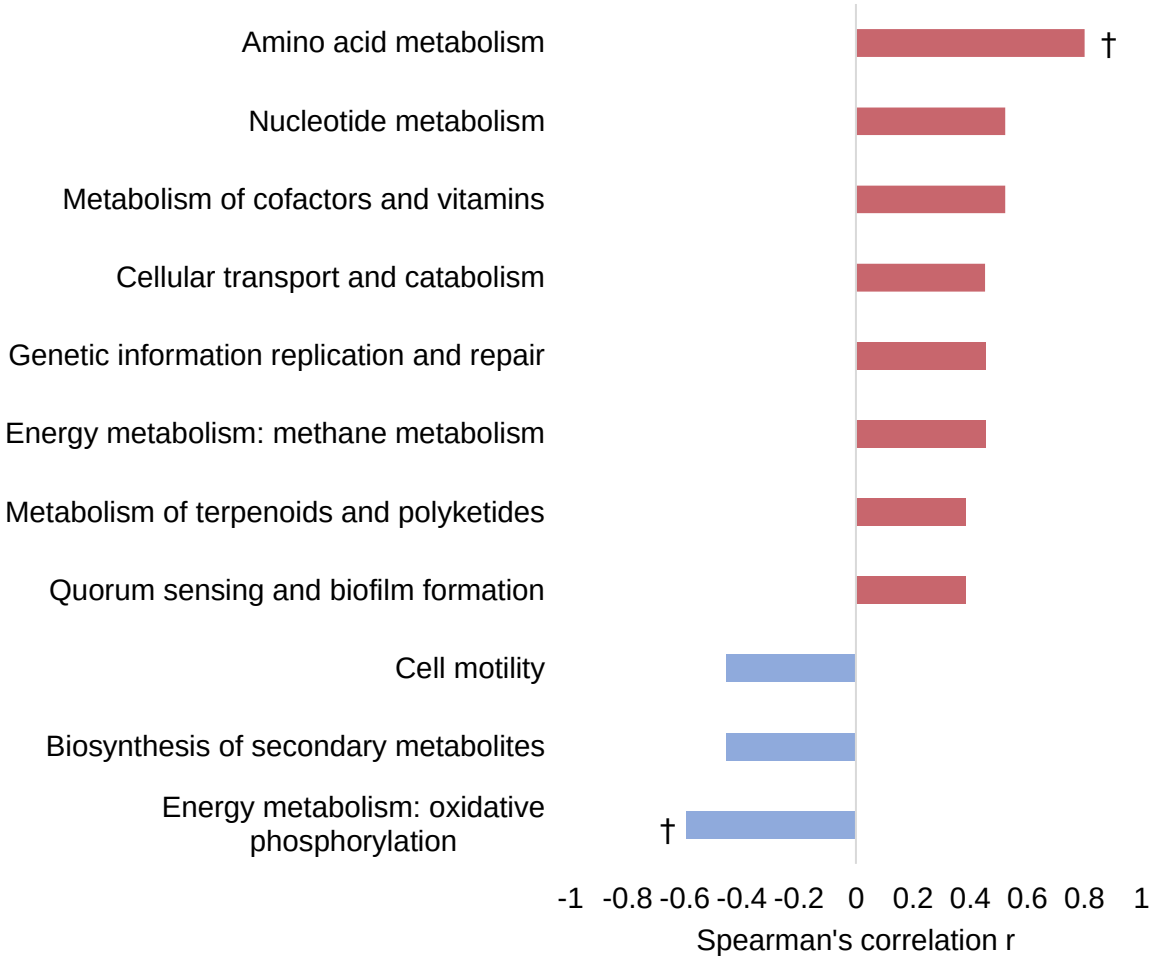


Figure 27 The Spearman correlation coefficient of the top 10 KEGG pathways correlated with breath methane.

† Trend, p <0.10 and p-adj >0.05. Pathways with red bars positively correlated with breath methane, and pathways with blue bars negatively correlated with breath methane.

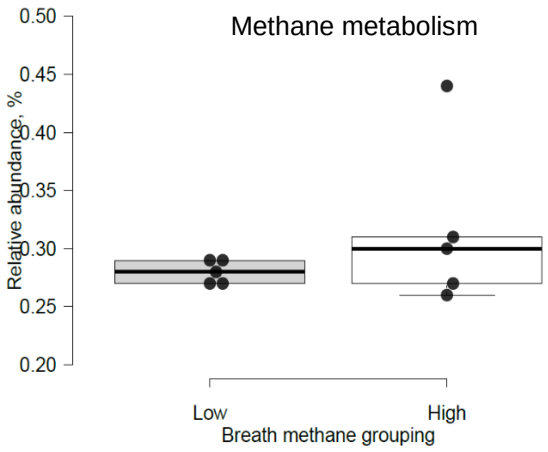


Figure 28 The relative abundance of methane metabolism genes in faecal samples from low and high-breath methane emitters.

4.6 Discussion

This study quantified the breath methane and hydrogen concentrations of 18 healthy adults, of which 39 % were HE. The average age of the participants was 31 years old and mostly females of European descent. A review of 19 studies reported an average of 38 % HE in adults and children, which aligns with the current study (Polag and Keppler, 2019).

The current study was sufficiently powered to reveal differences in breath gas concentrations between LE and HE groups with a sample size of ten participants. In addition, the differentiation between groups was aided by selecting the highest and lowest methane breath emitters from a wider pool of participants.

4.6.1 Microbial Diversity

Based on the alpha-diversity index, community richness and evenness were similar across breath methane groups, although Kumpitsch *et al.* (2020) found that this index was higher for HE than LE individuals. However, the reliability of the current data and that of Kumpitsch *et al.* (2020) is limited because only one sample was sequenced per participant. Diversity indices are more reliable with increased sub-sampling (sequencing different regions of one sample) because the consistency of capturing low-abundant species is greater (Wilhm, 1970; Roswell, Dushoff and Winfree, 2021). This approach is costly and out of scope for many studies, but it will become achievable as sequencing costs reduce.

The beta-diversity analyses identified some compositional variance between breath methane groups; however, these groupings were heterogenous. Corroborating this, a study with 30 participants found that samples did not

cluster according to breath methane in PCoA (Kumpitsch *et al.*, 2020). Therefore, breath methane concentration is linked to microbiota composition, but the compositional 'fingerprint' is determined by additional factors not captured in these studies.

4.6.2 Dietary Intakes

Breath hydrogen concentration was negatively correlated with dietary fat intake in agreement with other studies (Hoffmann *et al.*, 2013; Kumpitsch *et al.*, 2020). In the rumen, it is thought that dietary fat reduces hydrogen accumulation via fatty acid biohydrogenation, reducing fermentable organic matter intake, reducing fibre fermentation, and inhibiting methanogen activity (Alvarez-Hess *et al.*, 2019). However, this seems unlikely in the current study as individuals who were LE had a higher dietary fibre intake than individuals who were HE. Alternatively, human studies have suggested that high dietary lipid intake causes colonic gas retention, and colonic gas accumulation causes greater gas excretion in the flatus, lowering breath gas concentrations (Hammer, 1993; Serra *et al.*, 2002).

The higher dietary fibre intake in individuals who were LE coincided with a greater relative abundance of taxa from the *Bacteroides* genus compared to HE individuals. Conversely, previous studies showed that HE individuals had greater dietary fibre intake and a higher relative abundance of fibre-degrading taxa from the *Ruminococcaceae* family compared to LE (Robert and Bernalier-Donadille, 2003; Kumpitsch *et al.*, 2020). Many species of the *Bacteroides* genus possess large repertoires of CAZymes for fibre degradation (Flint *et al.*, 2012), so it is possible that species of the *Bacteroides* genus were associated with fibre fermentation in the current study compared to species from the

Ruminococcaceae family in other research (Robert and Bernalier-Donadille, 2003; Kumpitsch *et al.*, 2020).

Dietary starch intake correlated positively with breath gases, much like other studies which found positive associations between the *Methanobrevibacter* genus and long-term carbohydrate consumption in humans (Hoffmann *et al.*, 2013). Hoffman *et al.* (2013) hypothesised that the *Candida* fungus degraded starch into simple sugars, and then taxa from *Prevotella* and *Ruminococcus* genera fermented the sugars producing hydrogen for methane synthesis by taxa from the *Methanobrevibacter* genus. In the current study, the sequencing methodology was not designed to detect fungi. Moreover, there were no correlations between the relative abundance of *Prevotella*, *Ruminococcus,* and *Methanobrevibacter* genera.

Vitamin E intake explained 25 % of the variance in microbiota composition. Individuals who were LE tended to have a higher vitamin E intake and a higher relative abundance of taxa from the *Bacteroides* genus. Indeed, vitamin E and *Bacteroides* were strongly positively correlated. Vitamin E is an antioxidant, and several mouse studies and one human study have found that increased dietary vitamin E intake results in an increased relative abundance of the Bacteroidetes phylum and reduced intestinal inflammation (Mandal *et al.*, 2016; Pierre *et al.*, 2018; Choi *et al.*, 2020; Yang *et al.*, 2020). Conversely, several studies have reported that vitamin E was associated with the opposite effect; increased taxa from the Firmicutes phylum and reduced taxa from the Bacteroidetes phylum (Li, Krause and Somerset, 2017; Tang *et al.*, 2017; Williams *et al.*, 2017). However, the interpretation of dietary data are limited by the accuracy of the databases used to generate nutritional information from

food intake data. The databases used were based on food analysis data from 2017, and therefore may not be accurate for food composition in 2022. Furthermore, dietary fibre is a broad term encompassing several types of indigestible carbohydrates. Whilst relationships between dietary fibre and the gut microbiota have been observed in the current study and in other scientific literature, there is huge diversity among dietary fibres and the ways in which they affect nutritional physiology and the gut microbiota. A commonly used approach is to categorise fibres as soluble or insoluble according to the substrates behaviour *in vitro*. This lacks specificity to the *in vivo* and nutritional effects of dietary fibre. An improvement of the dietary fibre nutritional grouping would be to consider different dietary fibres based on their biological functionality in the gastrointestinal tract. This approach has been described by Gidley and Yakubov (2019); however, analysing the different dietary fibres consumed by an individual is unrealistic. In practice, food composition databases would have to adopt the approach and analyse different foods and ingredients.

4.6.3 Breath Methane, Breath Hydrogen, and Faecal Microbiota Composition and Potential Function in High and Low Breath Methane Emitters

4.6.3.1 Hypothesis One: Individuals who are high breath methane emitters have a lower breath hydrogen concentration than individuals who are low breath methane emitters

In contrast to the stated hypothesis, the breath data showed a positive correlation between hydrogen and methane concentrations. This finding disagrees with several publications where HE individuals were proposed to

have a lower concentration of breath hydrogen due to the microbial conversion of 4 mol of hydrogen into 1 mol of methane (Cloarec *et al.*, 1990; Levitt *et al.*, 2006; De Lacy Costello, Ledochowski and Ratcliffe, 2013; Harvie, Tuck and Schultz, 2019).

However, methanogenesis is a sink for hydrogen, thereby reducing the partial pressure of hydrogen and potentially favouring additional hydrogen production (Bauchop and Mountfort, 1981; Rezaie *et al.*, 2017b). Furthermore, potent hydrogen-producers, such as taxa from the *Ruminococcus* and *Enterococcus* genera, are often positively associated with methanogenic archaea due to interspecies hydrogen transfer. Robert and Bernalier-Donadille (2003) found a greater prevalence of hydrogen-producing, cellulose-degrading *Ruminococcus* and *Enterococcus* spp. in samples from HE compared to LE individuals. Specifically, these species were *E. faecalis* (NCBI: txid1351), *R. champanellensis* (NCBI: txid213810) and unclassified *Ruminococcus* spp. (NCBI: txid213814) (Robert and Bernalier-Donadille, 2003; Schoch, 2020). However, the relative abundance of *Ruminococcus* and *Enterococcus* genera did not differ between breath methane groups in the current study. *E. faecalis* and many *Ruminococcus spp*. were identified but present in a few samples, and *Ruminococcus champanellensis* were undetected.

There were trends for genera of high hydrogen-producing species (e.g., *Clostridium*, *Odoribacter* and *Streptococcus*) to be more abundant in samples from LE individuals (McKay, Holbrook and Eastwood, 1982; Göker *et al.*, 2011; Ostojic, 2017), even though this group had a lower breath hydrogen concentration. Therefore, there was no evidence to suggest that individuals with

high breath hydrogen had an increased colonic abundance of high hydrogen-producing microorganisms.

4.6.3.2 Hypothesis Two: Individuals who are high breath methane emitters have a higher abundance of taxa from the Firmicutes phylum, while individuals who are low breath methane emitters have more taxa from the Bacteroidetes phylum

In contrast to the stated hypothesis, there was no consistent relationship between breath methane concentration and the relative abundance of taxa from Firmicutes and Bacteroidetes phyla. However, the breath methane groupings explained 16 to 17 % of the beta-diversity variation.

The data suggested that the faecal microbiota of HE and LE individuals differed. Specifically, the faecal microbiota of LE individuals was *Bacteroides*-driven, co-occurring with *Clostridia, Sutterella* and *Blautia*, among others. Similarly, Arumugam *et al.* (2011) showed that the *Bacteroides*-driven enterotype was positively associated with *Clostridium*. Kumpitsch *et al.* (2020) found that samples from LE individuals had a greater abundance of *Bacteroides*, *Sutterella* and *Blautia* genera compared to samples from HE individuals. Arumugam *et al.* (2011) and Hoffmann *et al.* (2013) reported a negative association between *Bacteroides* and *Methanobrevibacter* genera, which was not observed in the current study, even though the *Bacteroides* genus was more abundant in samples from LE compared HE individuals.

In the current study, high breath methane emission was associated with a *Prevotella*-driven microbiota, co-occurring with *Eggerthella, Akkermansia* and *Desulfovibrio* genera. Other studies corroborated the positive association between *Prevotella* and *Desulfovibrio* but found that *Akkermansia* was more

commonly positively associated with a third enterotype driven by *Ruminococcus* (Arumugam *et al.*, 2011).

At the species level, there were trends for the relative abundance of *B. caccae*, *Odoribacter splanchnicus* and *Roseburia inulinivorans* to be higher in samples from LE compared to HE individuals. These associations were not observed by Kumpitsch *et al.* (2020); however, they did observe an increase in the relative abundance of other *Bacteroides spp.* like *B. thetaiotaomicron* in samples from LE individuals. The current study found a positive correlation between *B. caccae* and *B. thetaiotaomicron*, with both species occurring in higher abundance in samples from LE individuals. *B. thetaiotaomicron* is a keystone species in fibre degradation in the human colon, specifically for arabinogalactan (Cartmell *et al.*, 2018), and it is known to cross-feed with *B. caccae*. This relationship can support the presence of butyrate-producing *Roseburia* spp.*,* although the butyrate producer *R. inulinivorans* was not implicated (Chng *et al.*, 2020). The data indicate the possibility of cross-feeding relationships between *Bacteroides* and *Roseburia spp.* in the faecal microbiota of LE individuals; however, culture-based or labelled-substrate studies would be needed to investigate further.

Scientific literature has reported a higher occurrence of keystone species in faecal samples from HE compared to LE individuals (Robert and Bernalier-Donadille, 2003; Kumpitsch *et al.*, 2020), but it was impossible to support or refute this finding in the current study. The keystone species *R. champanellensis* and the closely related *Ruminococcus* CAG:624 and CAG:379 (Ben David *et al.*, 2015) were not detected. Similarly, the detection of other keystone species, such as *R. bromii* and *B. thetaiotaomicron,* was inconsistent.

4.6.3.3 Hypothesis Three: The faecal microbiota of individuals who are high breath methane emitters have a higher abundance of genes relating to methane metabolism, whereas low breath methane emitters have a higher abundance of sulphate reduction genes

The abundance of methane and sulphate reduction pathways according to potential function did not differ between samples from HE and LE individuals. There was a positive correlation between the abundance of the Euryarchaeota phylum and methane metabolism genes and a trend for methane metabolism to be negatively associated with sulphur metabolism. However, the abundances of the *Methanobrevibacter* genus and methane metabolism genes were inconsistent in HE samples.

The relative abundance of genes involved in methane metabolism correlated negatively with those involved in sulphur metabolism, in agreement with Kumpitsch *et al.* (2020). These authors showed that samples from LE individuals had pathways enriched for sulphur metabolism, which may be due to direct competition for hydrogen between methanogens and taxa from the *Desulfovibrio* genus, as reported in several *in vitro* studies (Gibson, Cummings and Macfarlane, 1988; Gibson, Macfarlane and Cummings, 1993).

In contrast, taxa from the *Desulfovibrio* genus were positively associated with the *Prevotella*-driven microbiota found in samples from HE. This finding was unexpected, as the *Desulfovibrio* genus is considered the main sulphate-reducing bacteria in the human colon, unlikely to co-occur with methanogenesis (Gibson *et al.*, 1990; Smith *et al.*, 2018).

Surprisingly, there was no difference in the relative abundance of taxa from the *Methanobrevibacter* genus between samples from HE and LE individuals. The

relative abundances in samples from HE individuals were 0.25 % and 1.59 % in two samples but below detection in the remaining samples (80 %). Another study reported a highly variable relative abundance of *M. smithii*, averaging 2 % in samples from HE individuals and less than 0.2 % in samples from LE individuals (Kumpitsch *et al.,* 2020). Other studies reported that individuals emitting more than three to four ppm of breath methane had a high abundance of faecal methanogens by culture methods, approximating 0.03 to 7.5 % relative abundance (Stephen and Cummings, 1980; Weaver *et al.*, 1986; De Lacy Costello, Ledochowski and Ratcliffe, 2013; Kumpitsch *et al.*, 2020).

Studies using culture to quantify methanogens have not reported samples from HE with low faecal methanogen counts (Robert and Bernalier-Donadille, 2003). In addition, studies using quantitative PCR found that HE participants had at least 1.2 % of methanogens in faecal samples (Kim *et al.*, 2012). However, methodological differences, such as amplification bias with PCR and differences in breath testing procedures, reduce study comparability. Furthermore, recent studies have suggested that methanogens in dental plaques can contribute to breath methane emission, which may further confound results (Nkamga, Henrissat and Drancourt, 2017; Erdrich *et al.*, 2021).

Nevertheless, the current study found a positive correlation between the genes involved in methane metabolism and the relative abundance of the Euryarchaeota phylum, which agrees with Kumpitsch *et al.* (2020). However, one HE participant drove this relationship, which is not compelling evidence. In contrast to other studies, the genes involved in methane metabolism were negatively correlated with the abundance of taxa from the Firmicutes phylum,

but again, the effect was driven by one HE participant. In other studies, HE participants had a higher abundance of taxa from the Firmicutes phylum compared to LE participants (Chassard *et al.*, 2010; Kumpitsch *et al.*, 2020).

4.6.4 Strengths and Limitations

The current study is the first to report breath hydrogen and methane concentrations alongside the faecal microbiota composition in healthy individuals using community-based sequencing approaches. The measurement of breath hydrogen was missing in a recent study (Kumpitsch *et al.*, 2020) and previous studies used 16S rRNA and culture-dependent approaches (Robert and Bernalier-Donadille, 2003; Chassard *et al.*, 2010). The application of shotgun metagenomic sequencing in the current study enabled the characterisation of the complex faecal community, which provides more accurate and complete microbiota characterisation.

Limitations of the current study include the small number of participants and samples. Future studies could be better powered by including more participants, taking multiple samples from each participant, and sequencing sub-samples (several aliquots taken from one sample). A basic sample size calculation for the non-parametric Mann Whitney-U test based on *Methanobrevibacter* abundance in the current study, using an alpha of 0.05 and power of 0.80, suggested that 130 participants would be required to detect significant differences in *Methanobrevibacter* abundance between HE and LE groups. However, Johnson *et al.* (2020) recently described an approach to improve power with lower sample sizes, whereby the baseline faecal microbiota composition of participants is assessed during recruitment, which could be used to select HE participants with a high abundance of *M. smithii*.

Furthermore, the results showed differences between groups of participants who were HE and LE at the genera level, specifically *Bacteroides* and *Prevotella* relative abundance. Therefore, the ratio of these two genera may be a useful tool for power analyses to determine appropriate replication for studies concerning HE and LE individuals in future.

An additional limitation was the use of metagenomics to infer potential function of the gut microbiota. Whilst this is effective at highlighting the collection of genes within the metagenome that may be expressed, there is no measure of gene expression. Considering that gene expression is required for protein synthesis and ultimately to confer a physiological response, there can be a lack of congruency between metagenomic potential function and observed physiological response. For example, Shi et al. (2014) showed that metatranscriptomics, but not metagenomics, was effective at characterising the differences between high and low methane emitting sheep. This suggests that in sheep, methane emission is regulated more by gene expression than by microbiota community composition. Future studies assessing methane emission would benefit from conducting metatranscriptomics to build a more precise link between function and physiology.

4.7 Conclusion

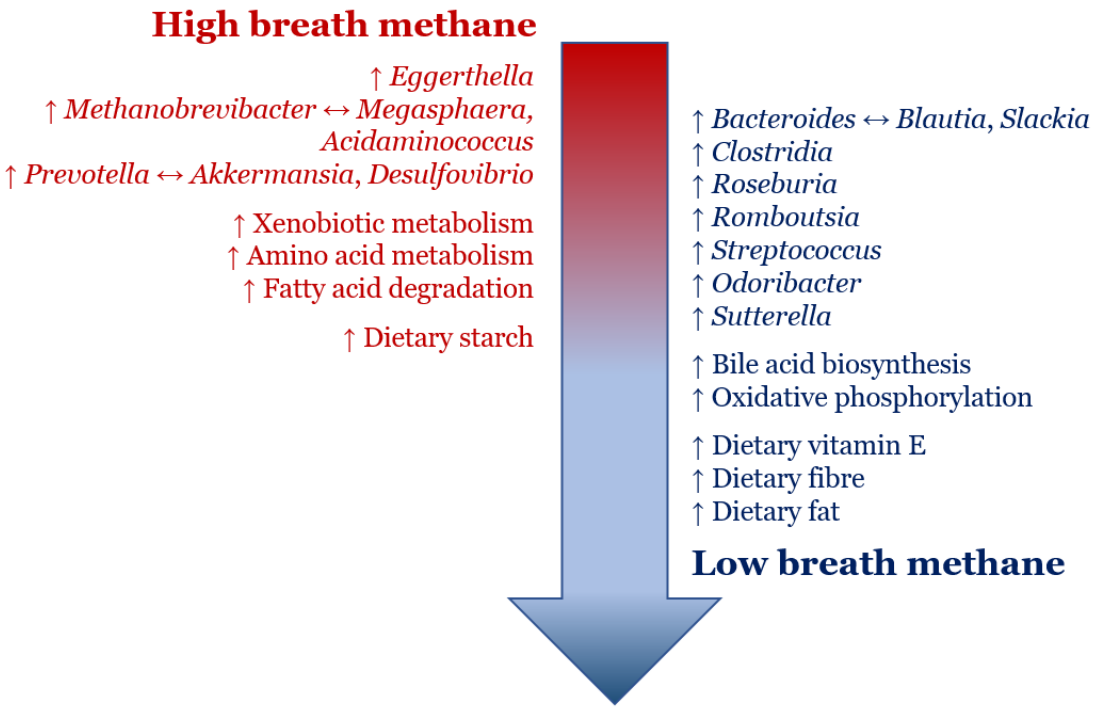


Figure 29 The key differences in reported dietary intakes and the relative abundance of genera and pathways according to participant breath methane.

Unexpectedly, a positive correlation between breath hydrogen and breath methane was found in healthy adults, contrasting with most published studies. This finding supports the hypothesis that healthy individuals who are HE harbour a colonic environment that promotes higher hydrogen production; however, it does not consider differences in hydrogen utilisation.

In contrast to the stated hypothesis, the faecal samples from HE individuals did not harbour more taxa from the Firmicutes phylum, and those from LE did not have more taxa from the Bacteroidetes phylum. However, there were differences in the faecal microbiota composition at the genus level. The faecal microbiota of the LE group was *Bacteroides*-driven, enriched with taxa from the *Clostridia, Roseburia, Romboutsia, Streptococcus,* and *Odoribacter* genera compared to the microbiota of the HE group. Furthermore, *Blautia* and *Slackia*

genera positively correlated with the *Bacteroides*-driven composition of the LE group, and *Sutterella* was negatively associated with breath methane. In comparison, the microbiota composition of the HE group had a greater abundance of *Eggerthella*, and breath methane was positively correlated with the abundance of *Methanobrevibacter*, *Prevotella* and *Akkermansia* (Figure 29).

The expected correlation between taxa from the *Methanobrevibacter* genus and genes involved in methane metabolism was observed but was limited by the heterogeneity of the HE group. Furthermore, there were no significant differences in methane or sulphur metabolism between samples from HE and LE individuals; however, samples from HE individuals had a greater abundance of amino acid metabolism pathways.

Overall, the findings contribute to understanding hydrogen and methane breath emissions and their relationship to the composition of the colonic microbiota in healthy individuals. However, findings remain limited to the microbiota composition and functionality at a time point. Further longitudinal analyses could provide a more dynamic view of the microbial communities in HE and LE individuals.

References

1. Alvarez-Hess, P.S., Williams, S.R.O., Jacobs, J.L., Hannah, M.C., Beauchemin, K.A., Eckard, R.J., Wales, W.J., Morris, G.L., and Moate, P.J. (2019). Effect of dietary fat supplementation on methane emissions from dairy cows fed wheat or corn. Journal of Dairy Science 102, 2714–2723. https://doi.org/10.3168/jds.2018-14721.
2. Arumugam, M., Raes, J., Pelletier, E., le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J. M., Bertalan, M.,

Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Bork, P. (2011). Enterotypes of the human gut microbiome. Nature 473(7346), 174–180. https://doi.org/10.1038/nature09944.

3. Bauchop, T., and Mountfort, D.O. (1981). Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. Applied and Environmental Microbiology 42, 1103–1110. https://doi.org/10.1128/aem.42.6.1103-1110.1981.
4. Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing Author (s): Yoav Benjamini and Yosef Hochberg Source: Journal of the Royal Statistical Society. 57(1), 289–300.
5. Bond, B.Y.J.H., Engel, R.R., and Levitt, M.D. (1971). Factors influencing pulmonary methane excretion in man. Journal of Experimental Medicine 133, 572–588.
6. Cartmell, A., Muñoz-Muñoz, J., Briggs, J. A., Ndeh, D. A., Lowe, E. C., Baslé, A., Terrapon, N., Stott, K., Heunis, T., Gray, J., Yu, L., Dupree, P., Fernandes, P. Z., Shah, S., Williams, S. J., Labourel, A., Trost, M., Henrissat, B., and Gilbert, H. J. (2018). A surface endogalactanase in Bacteroides thetaiotaomicron confers keystone status for arabinogalactan degradation. Nature Microbiology 3(11), 1314–1326. https://doi.org/10.1038/s41564-018-0258-8.
7. Chassard, C., Delmas, E., Robert, C., and Bernalier-Donadille, A. (2010). The cellulose-degrading microbial community of the human gut varies according to the presence or absence of methanogens. FEMS Microbiology Ecology 74, 205–213. https://doi.org/10.1111/j.1574-6941.2010.00941.x.
8. Chng, K. R., Ghosh, T. S., Tan, Y. H., Nandi, T., Lee, I. R., Ng, A. H. Q., Li, C., Ravikrishnan, A., Lim, K. M., Lye, D., Barkham, T., Raman, K., Chen, S. L., Chai, L., Young, B., Gan, Y. H., and Nagarajan, N. (2020). Metagenome-wide association analysis identifies microbial determinants of post-antibiotic ecological recovery in the gut. Nature

Ecology and Evolution 4(9), 1256–1267. https://doi.org/10.1038/s41559-020-1236-0.

9. Choi, Y., Lee, S., Kim, S., Lee, J., Ha, J., Oh, H., Lee, Y., Kim, Y., and Yoon, Y. (2020). Vitamin E (α-tocopherol) consumption influences gut microbiota composition. International Journal of Food Sciences and Nutrition 71, 221–225. https://doi.org/10.1080/09637486.2019.1639637.
10. Chong, J., Liu, P., Zhou, G., and Xia, J. (2020). Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nature Protocols, 15(3), 799–821. https://doi.org/10.1038/s41596-019-0264-1.
11. Cloarec, D., Bornet, F., Gouilloud, S., Barry, J.L., Salim, B., and Galmiche, J.P. (1990). Breath hydrogen response to lactulose in healthy subjects: Relationship to methane producing status. Gut 31, 300–304. https://doi.org/10.1136/gut.31.3.300.
12. ben David, Y., Dassa, B., Borovok, I., Lamed, R., Koropatkin, N. M., Martens, E. C., White, B. A., Bernalier-Donadille, A., Duncan, S. H., Flint, H. J., Bayer, E. A., and Moraïs, S. (2015). Ruminococcal cellulosome systems from rumen to human. Environmental Microbiology 17(9), 3407–3426. https://doi.org/10.1111/1462-2920.12868.
13. Erdrich, S., Tan, E.C.K., Hawrelak, J.A., Myers, S.P., and Harnett, J.E. (2021). Hydrogen–methane breath testing results influenced by oral hygiene. Scientific Reports 11, 1–11. https://doi.org/10.1038/s41598-020-79554-x.
14. Flint, H.J., Scott, K.P., Duncan, S.H., Louis, P., and Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. Gut Microbes 3. https://doi.org/10.4161/gmic.19897.
15. Gibson, G., Macfarlane, G.T., and Cummings, J. (1993). Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut 34, 437–439.

16. Gibson, G.R., Cummings, J.H., and Macfarlane, G.T. (1988). Competition for hydrogen between sulphate-reducing bacteria and methanogenic bacteria from the human large intestine. Journal of Applied Bacteriology 65, 241–247. https://doi.org/https://doi.org/10.1111/j.1365-2672.1988.tb01891.x.

17. Gibson, G.R., Cummings, J.H., Macfarlane, G.T., Allison, C., Segal, I., Vorster, H.H., and Walker, A.R. (1990). Alternative pathways for hydrogen disposal during fermentation in the human colon. Gut 31, 679–683. https://doi.org/10.1136/gut.31.6.679.

18. Göker, M., Gronow, S., Zeytun, A., Nolan, M., Lucas, S., Lapidus, A., Hammon, N., Deshpande, S., Cheng, J.F., Pitluck, S., *et al.* (2011). Complete genome sequence of odoribacter splanchnicus type strain (1651/6 T). Standards in Genomic Sciences 4, 200–209. https://doi.org/10.4056/sigs.1714269.

19. Gottlieb, K., Le, C., Wacher, V., Sliman, J., Cruz, C., Porter, T., and Carter, S. (2017). 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. Gastroenterology Report gow048. https://doi.org/10.1093/gastro/gow048.

20. Hammer, H.F. (1993). Colonic hydrogen absorption: Quantification of its effect on hydrogen accumulation caused by bacterial fermentation of carbohydrates. Gut 34, 818–822. https://doi.org/10.1136/gut.34.6.818.

21. Harvie, R.M., Tuck, C.J., and Schultz, M. (2019). Evaluation of lactulose, lactose, and fructose breath testing in clinical practice: A focus on methane. JGH Open 4, 198–205. https://doi.org/10.1002/jgh3.12240.

22. Hoffmann, C., Dollive, S., Grunberg, S., Chen, J., Li, H., Wu, G.D., Lewis, J.D., and Bushman, F.D. (2013). Archaea and Fungi of the Human Gut Microbiome: Correlations with Diet and Bacterial Residents. PLoS ONE 8. https://doi.org/10.1371/journal.pone.0066019.

23. Johnson, A.J., Zheng, J.J., Kang, J.W., Saboe, A., Knights, D., and Zivkovic, A.M. (2020). A Guide to Diet-Microbiome Study Design.

Frontiers in Nutrition 7, 1–16. https://doi.org/10.3389/fnut.2020.00079.

24. Kim, G., Deepinder, F., Morales, W., Hwang, L., Weitsman, S., Chang, C., Gunsalus, R., and Pimentel, M. (2012). Methanobrevibacter smithii is the predominant methanogen in patients with constipation-predominant IBS and methane on breath. Digestive Diseases and Sciences 57, 3213–3218. https://doi.org/10.1007/s10620-012-2197-1.

25. Kumpitsch, C., Fischmeister, F.P.S., Mahnert, A., Lackner, S., Wilding, M., Sturm, C., Holasek, S., Högenauer, C., Berg, I., Schöpf, V., *et al.* (2020). Methane emission of humans is explained by dietary habits, host genetics, local formate availability and a uniform archaeome. BioRxiv 1–45. https://doi.org/10.1101/2020.12.21.423794.

26. De Lacy Costello, B.P.J., Ledochowski, M., and Ratcliffe, N.M. (2013). The importance of methane breath testing: a review. Journal of Breath Research 7, 24001. https://doi.org/10.1088/1752-7155/7/2/024001.

27. Lahti, L., Sudarshan, S., and Ernst, F.G.M. (2021). Orchestrating Microbiome Analysis. Github (online) https://microbiome.github.io/OMA/.

28. Levitt, M.D., Furne, J.K., Kuskowski, M., and Ruddy, J. (2006). Stability of Human Methanogenic Flora Over 35 Years and a Review of Insights Obtained from Breath Methane Measurements. Clinical Gastroenterology and Hepatology 4, 123–129. https://doi.org/10.1016/j.cgh.2005.11.006.

29. Li, L., Krause, L., and Somerset, S. (2017). Associations between micronutrient intakes and gut microbiota in a group of adults with cystic fibrosis. Clinical Nutrition 36, 1097–1104. https://doi.org/10.1016/j.clnu.2016.06.029.

30. Mandal, S., Godfrey, K. M., McDonald, D., Treuren, W. v., Bjørnholt, J. v., Midtvedt, T., Moen, B., Rudi, K., Knight, R., Brantsæter, A. L., Peddada, S. D., and Eggesbø, M. (2016). Fat and vitamin intakes during pregnancy have stronger relations with a proinflammatory maternal

microbiota than does carbohydrate intake. Microbiome, 4. https://doi.org/10.1186/s40168-016-0200-3.

31. Massey University (2017). Code of Ethical Conduct for Research, Teaching and Evaluations Involving Human Participants (online). https://www.massey.ac.nz/massey/fms/PolicyGuide/Documents/c/code-of-ethical-conduct-for-research,-teaching-and-evaluations-involving-human-participants.pdf.

32. McDonald, J.H. (2014). Handbook of Biological Statistics (Baltimore, Maryland: Sparky House Publishing).

33. McKay, L.F., Holbrook, W.P., and Eastwood, M.A. (1982). Methane and hydrogen production by human intestinal anaerobic bacteria. Acta Pathologica Microbiologica Scandinavica Series B: Microbiology 90B, 257–260. https://doi.org/https://doi.org/10.1111/j.1699-0463.1982.tb00114.x.

34. McMurdie, P.J., and Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS ONE 8. https://doi.org/10.1371/journal.pone.0061217.

35. Nkamga, V.D., Henrissat, B., and Drancourt, M. (2017). Archaea: Essential inhabitants of the human digestive microbiota. Human Microbiome Journal 3, 1–8. https://doi.org/10.1016/j.humic.2016.11.005.

36. Oksanen, A. J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P. R., Hara, R. B. O., Simpson, G. L., Solymos, P., Stevens, M. H. H., and Szoecs, E. (2019). Vegan. Encyclopedia of Food and Agricultural Ethics 2395–2396. https://doi.org/10.1007/978-94-024-1179-9_301576.

37. Ostojic, S.M. (2017). Non-gut microbiota as a source of bioactive hydrogen. Postgraduate Medical Journal 93, 170. https://doi.org/10.1136/postgradmedj-2016-134411.

38. Pierre, J.F., Hinterleitner, R., Bouziat, R., Hubert, N.A., Leone, V., Miyoshi, J., Jabri, B., and Chang, E.B. (2018). Dietary antioxidant micronutrients alter mucosal inflammatory risk in a murine model of

genetic and microbial susceptibility. Journal of Nutritional Biochemistry 54, 95–104. https://doi.org/10.1016/j.jnutbio.2017.12.002.

39. Polag, D., and Keppler, F. (2019). Global methane emissions from the human body: Past, present and future. Atmospheric Environment 214, 116823. https://doi.org/10.1016/j.atmosenv.2019.116823.

40. Rezaie, A., Buresi, M., Lembo, A., Lin, H., McCallum, R., Rao, S., Schmulson, M., Valdovinos, M., Zakko, S., and Pimentel, M. (2017). Hydrogen and Methane-Based Breath Testing in Gastrointestinal Disorders: The North American Consensus. American Journal of Gastroenterology 112, 775–784. https://doi.org/10.1038/ajg.2017.46.

41. Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de los Reyes-Gavilán, C.G., and Salazar, N. (2016). Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. Frontiers in Microbiology 7, 1–9. https://doi.org/10.3389/fmicb.2016.00185.

42. Robert, C., and Bernalier-Donadille, A. (2003). The cellulolytic microflora of the human colon: Evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects. FEMS Microbiology Ecology 46, 81–89. https://doi.org/10.1016/S0168-6496(03)00207-1.

43. Roswell, M., Dushoff, J., and Winfree, R. (2021). A conceptual guide to measuring species diversity. Oikos 130, 321–338. https://doi.org/10.1111/oik.07202.

44. Rowland, I., Gibson, G., Heinken, A., Scott, K., Swann, J., Thiele, I., and Tuohy, K. (2018). Gut microbiota functions: metabolism of nutrients and other food components. European Journal of Nutrition 57. https://doi.org/10.1007/s00394-017-1445-8.

45. Schoch, C. Ciufo, S., Domrachev, M., Hotton, C. L., Kannan, S., Khovanskaya R., Leipe, D., Mcveigh, R., O'Neill, K., Robbertse, B., Sharma, S., Soussov, V., Sullivan, J. P., Sun, L., Turner, S., Karsch-Mizrachi, I. (2020). NCBI Taxonomy: a comprehensive update on curation, resources and tools. Database, 1-21, doi: 10.1093/database/baaa062.

46. Serra, J., Salvioli, B., Azpiroz, F., and Malagelada, J.R. (2002). Lipid-induced intestinal gas retention in irritable bowel syndrome. Gastroenterology 123, 700–706. https://doi.org/10.1053/gast.2002.35394.

47. Shi, W., Moon, C. D., Leahy, S. C., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., Seedorf, H., Kelly, W. J., Atua, R., Sang, C., Soni, P., Li, D., Pinares-Patiño, C. S., McEwan, J. C., Janssen, P. H., Chen, F., Visel, A., Rubin, E. M. (2014). Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. Genome research, 24(9), 1517–1525. https://doi.org/10.1101/gr.168245.113.

48. Smith, N.W., McNabb, W.C., Roy, N.C., Altermann, E.H., and Shorten, P.R. (2018). Hydrogen cross-feeders of the human gastrointestinal tract. Gut Microbes 1–19. https://doi.org/10.1080/19490976.2018.1546522.

49. Stephen, A.M., and Cummings, J.H. (1980). The microbial contribution to human faecal mass. The Journal of Medical Microbiology 13, 45–56.

50. Tang, M., Frank, D.N., Sherlock, L., Ir, D., Robertson, C.E., and Krebs, N.F. (2017). Effect of vitamin E with therapeutic iron supplementation. Journal of Pediatric Gastroenterology and Nutrition 63, 379–385. https://doi.org/10.1097/MPG.0000000000001154.Effect.

51. Weaver, G.A., Krause, J.A., Miller, T.L., and Wolin, M.J. (1986). Incidence of methanogenic bacteria in a sigmoidoscopy population: An association of methanogenic bacteria and diverticulosis. Gut 27, 698–704. https://doi.org/10.1136/gut.27.6.698.

52. Wilhm, T.L. (1970). Effect of Sample Size on Shannon ’s Formula. The Southwestern Naturalist 14, 441–445.

53. Williams, J. E., Carrothers, J. M., Lackey, K. A., Beatty, N. F., York, M. A., Brooker, S. L., Shafii, B., Price, W. J., Settles, M. L., McGuire, M. A., and McGuire, M. K. (2017). Human milk microbial community structure is relatively stable and related to variations in macronutrient and micronutrient intakes in healthy lactating women. Journal of Nutrition 147(9), 1739–1748. https://doi.org/10.3945/jn.117.248864

54. Yang, Q., Liang, Q., Balakrishnan, B., Belobrajdic, D.P., Feng, Q.J., and Zhang, W. (2020). Role of dietary nutrients in the modulation of gut microbiota: A narrative review. Nutrients 12. https://doi.org/10.3390/nu12020381

Chapter 5

In Vitro Colonic Fermentation of β-glucan and Lignocellulose by the Faecal Microbiota of High and Low Breath Methane Emitters

The data from this Chapter is being integrated with that of Chapter 6 for publication by Payling, L., Roy, N. C., Hill, S. J., Raymond, L. G., Fraser, K., Gagic, D., Loveday, S. M., Sims, I. M. and McNabb, W. C. The links between human breath methane, microbiota composition, and fibre fermentation in an *in vitro* colonic model. The ISME Journal.

5.1 Abstract

Adults that are HE and LE have different species in their colonic microbiota that may affect fibre fermentation. Therefore, the study aimed to culture *in vitro* microbial communities from individuals who were HE and LE and investigated their composition and fermentation capacity in response to dietary soluble (β-glucan) and insoluble (lignocellulose) fibres.

Faecal samples of five HE and five LE participants were used to inoculate an *in vitro* model of colonic fermentation supplemented with β-glucan and lignocellulose. At 0, 3, 6, 10, 24, and 48 h, fermentation samples were taken to measure the pH, gas volume, organic matter fermentability, microbial composition and microbial functional potential.

β-glucan was fermented faster than lignocellulose, indicated by increased gas volume and decreased pH from 0 to 24 h. By 48 h, both substrates had approximately 38 % organic matter fermentability and similar pH and gas volume values. In samples from LE participants, *Anaerobutyricum, Bacteroides, Blautia, Clostridium, Odoribacter, Phascolarctobacterium, Enterocloster*, *Sutterella* and *Streptococcus* genera (p-adj <0.05) had increased relative abundance compared to samples from HE participants. Yet samples from HE participants had increased relative abundance of *Akkermansia, Coprococcus, Dialister, Eggerthella, Flavonifractor,*

Parabacteriodes and *Prevotella* genera, and more pathways relating to methane and amino acid metabolism compared to samples from LE participants (p-adj <0.05).

The communities associated with fibre fermentation in the LE group were *Bacteroides*-driven, compared to communities from the HE group, which were *Prevotella*-driven.

5.2 Introduction

The human colonic microbiota derives energy from the fermentation of undigested dietary components, producing metabolites that can promote human health, such as organic acids and gases. Of these gases, hydrogen can reach concentrations of approximately 4 % in the colon (Kalantar-Zadeh *et al.*, 2018); however, high concentrations limit the oxidation of substrates and reduce the energy yield from fermentation. In a symbiotic action, hydrogen-utilising microorganisms, including methanogenic archaea, SRB, and acetogenic bacteria, utilise hydrogen.

Methanogens are the dominant hydrogen-utilisers of the colonic microbiota in approximately one-third of the population (Levitt *et al.*, 2006; De Lacy Costello, Ledochowski and Ratcliffe, 2013). These individuals are referred to HE. Several studies showed that individuals who are HE and LE have distinct faecal microbiota compositions. Specifically, HE individuals have a greater relative abundance of insoluble fibre-degrading keystone species, including several *Ruminococcus spp*. from the phylum Firmicutes, providing a greater cellulose fermentation capacity compared to LE individuals (Robert and Bernalier-Donadille, 2003; Chassard *et al.*, 2010; Kumpitsch *et al.*, 2020). The Firmicutes phylum contains known primary degraders, which are adept at accessing and

fermenting dietary fibre (Ze *et al.*, 2013; Flint, Duncan and Louis, 2017; Williams *et al.*, 2017). In addition, positive correlations between dietary fibre intakes, breath methane concentration, and *Methanobrevibacter* spp. abundance suggest a positive link between methanogens and fibre fermentation (Hoffmann *et al.*, 2013; Kumpitsch *et al.*, 2020).

Dietary fibre fermentation is important for maintaining colonic microbiota diversity, recovery after antibiotics, metabolite cross-feeding, and butyrate production (Sonnenburg *et al.*, 2016; Chng *et al.*, 2020). The potential link between breath methane emission and fibre fermentation warrants further investigation to improve understanding of the functional networks.

Most observational studies of dietary fibre interventions in adult humans have used culture-based methods or sequencing of faecal samples to give data on changes in microbiota composition and its potential function. Compared with human studies, established *in vitro* culture models of colonic fermentation may provide greater insights into the dynamics of fibre fermentation by the microbiota of individuals who are HE or LE.

5.3 Aim and Hypotheses

This study aimed to investigate the colonic fermentation of soluble and insoluble dietary fibres by the faecal microbiota of individuals who were HE or LE in a batch culture model of fermentation, described in Chapter 3.

The hypotheses were:

1. Colonic fermentations from individuals who are HE have a greater rate and extent of insoluble fibre fermentation compared to LE.

2. Colonic fermentations from individuals who are HE have a higher relative abundance of microorganisms from the Firmicutes phylum, while fermentations from individuals who are LE have more microorganisms from the Bacteroidetes phylum.
3. Colonic fermentations from individuals who are HE have a higher abundance of genes relating to methane metabolism, whereas fermentations from individuals who are LE have a higher abundance of genes relating to sulphate reduction.

5.4 Methods

The methods for *in vitro* colonic fermentation are described in Chapter 3. Below are details of additional analyses relevant to this Chapter.

5.4.1 Chemical Characterisation of Substrates

Substrates were chemically characterised by The Nutrition Laboratory at Massey University. Dry matter was determined by the AOAC 925.10 and 930.15 methods, ash was determined using the AOAC 942.05 method with the furnace at 550°C, and organic matter was determined by calculation (dry matter minus ash). Additionally, starch concentration was determined using the Megazyme kit for α-amylase according to method AOAC 996.11 and RS by the Megazyme assay K-RSTAR (AOAC 2002.02). β-glucan concentration was determined using the Megazyme assay K-BGLU (AACC Method 32-23.01, AOAC Method 995.16, AOAC Method 992.28, CODEX Method Type II, EBC Method 3.10.1, ICC Standard No. 166, and RACI Standard Method). Acid detergent lignin, cellulose, and insoluble hemicellulose were determined using the Fibertec method according to AOAC 973.18 and 2002.04.

In addition, the constituent sugar composition of lignocellulose was determined by high-performance anion-exchange chromatography by Dr Ian Sims at the Ferrier Research Institute (Victoria University of Wellington, New Zealand), a specialist in carbohydrate analysis. Duplicate samples of lignocellulose (3 to 4.5 mg) were weighed accurately into 7 mL Kimax glass tubes. Sulphuric acid (72 % w/w, 187 µL) was added to each sample, capped, and incubated at 30 °C for 2.5 h with intermittent mixing, following dilution to one molar acid by the addition of 2.06 mL water. Next, the samples were mixed and incubated at 100 °C for 3 h. Finally, the hydrolysates were cooled, diluted to 5 mL with water, and stored at 4 °C overnight. Prior to analysis, each sample was diluted further to give solutions of 20 to 25 µg/mL.

The diluted hydrolysates were analysed by high-performance anion-exchange chromatography (Dionex™ ICS-5000$^+$, Thermo Fisher Scientific Inc., Waltham, USA). A CarboPac PA-1 (4 x 250 mm; Dionex) column was equilibrated in 20 mM NaOH and eluted with simultaneous gradients of NaOH (20 mM from 0 to 25 min, then 20 to 100 mM from 25 to 30 min and held to 50 min) and sodium acetate (0 to 500 mM from 30 to 50 min) at a flow rate of one mL/min. The eluent was monitored by pulsed amperometric detection using the Dionex standard carbohydrate waveform. The sugars were identified from their elution times relative to standard sugar mixes and quantified from the response calibration curves of each sugar.

5.4.2 Organic Matter Fermentability

The fermentation pellets from 0 and 48 h of fermentation were analysed for dry matter and ash concentration, as described for substrates. Organic matter was calculated by dry matter concentration minus ash concentration. Organic

matter fermentability was calculated as the percentage reduction in organic matter at 48 h compared to 0 h of fermentation.

5.4.3 Total Gas Production and pH

The bottles were sampled at 0, 3, 6, 10, 24, and 48 h of fermentation. First, duplicate bottles were transferred from a 37 °C incubator to a 37 °C water bath. Next, the gas pressure in the headspace of each bottle was measured with a needle attached to a pressure probe through the bung of the fermentation bottle.

Calibration was conducted to convert gas pressure (kPa) into gas volume (mL). This calibration had seven points from 0 to 20 mL added gas, in triplicate, under conditions matching the experimental setup. The linear calibration curve (Supplementary File 13) was used to convert gas pressure readings (kPa) into gas volume (mL) as per the following equation:

$$\text{Gas production, mL} = (\text{gas pressure, kPa}/2.01) - 1.21.$$

During the collection of fermentation supernatants, the pH was measured with a pH meter (LAQUAtwin, Horiba, Kyoto, Japan).

5.4.4 Solid-State Nuclear Magnetic Resonance

Fermentation pellet samples from 0 and 48 h were freeze-dried and packed into four mm ZrO2 rotors. Rotors were spun at five kHz in a ^{13}C solid-state magic angle spinning NMR on a Bruker Avance III 200 MHz spectrometer fitted with a four mm Bruker SB multinuclear MAS probe (Bruker, Billerica, USA) under the guidance of Dr Stefan Hill (Scion, New Zealand), an expert in solid-state NMR of organic materials. A standard ^{13}C CP-MAS (cross polarisation-magic angle spinning) pulse sequence was used with a 1H preparation pulse of 4.25

μs, a 1 ms contact time, an acquisition time of 26.5 ms with high powered decoupling and a recycle delay of 1.5 s. A total of 5,120 transients were collected per sample. Hartmann-Hahn matching and referencing was carried out using glycine (176.5 ppm). All spectra were then Fourier transformed with a Gaussian line broadening of 25 Hz.

5.4.5 Shotgun Metagenomics

DNA was extracted from fermentation samples collected at 0, 6, 24, and 48 h sequenced (totalling 219 samples), and data were processed as described in Chapter 3. These time points were chosen to capture the progressive changes in microbiota composition whilst keeping the number of samples for sequencing within the project scope.

First, data were filtered to remove low abundant and low variance taxonomic units with less than four reads in 15 % of samples, and less than 10 % inter-quartile range variance, leaving 167 taxonomic units. Then, the data were total sum scaled to provide relative abundance values.

The analysis of KEGG pathways reflected that used for the metagenomic data of faecal samples (Chapter 4). The KEGG output was arranged hierarchically, and Level 2 and 3 data were used for analysis. Data were filtered and scaled, resulting in 29 pathways for analysis.

5.4.6 Data Analyses

The organic matter, pH and gas production data were checked for normality and had a non-parametric distribution. Therefore, descriptive statistics of the median values with the lower and upper quartiles (Q1 and Q3) denoting the variance were calculated.

NMR spectra were binned using AMIX software (Bruker, Billerica, USA) into regions of ten ppm, forming 20 different bins. Binned data were normalised to a constant sum, whereby the intensity of each bin was calculated as a fraction of the total spectrum. This method is a commonly used correction method in NMR, which allows the comparison of samples irrespective of variations in peak intensities (Emwas *et al.*, 2018). Data were then range scaled to achieve Gaussian (normal) distribution and evaluated using PCA in SIMCA 16 (Sartorius Stedim Data Analytics AB, Umeå, Sweden) and analysis of variance (ANOVA) or T-test in MetaboAnalyst 5.0 (Pang *et al.*, 2021). Spectral features were interpreted using published literature for ^{13}C chemical shifts, alongside the expertise of Dr Stefan Hill.

The microbiota analyses of the fermentation data were carried out as described for faecal data (Chapter 4). Time points were analysed independently to investigate the effect of substrate and participant breath methane without temporal autocorrelation (Coenen *et al.*, 2020).

Sparse Correlations for Compositional Data (SparCC) was used as a distance measure between taxa to visualise the network of microorganisms at each time point in MicrobiomeAnalyst (Chong *et al.*, 2020). SparCC correlations use log-ratio transformed data, a sparse network, and correlation iterations, which are well-suited methods for compositional data (Cosma-Grigorov *et al.*, 2020). Only relationships with a correlation coefficient greater than 0.40 and p-adj <0.05 are shown.

Heat tree analyses were used to depict differences in microbial taxa in the fermentation samples from LE and HE participants in MicrobiomeAnalyst (Chong *et al.*, 2020). Taxonomic classifications and median relative

abundances of taxa were used to build a hierarchical structure containing genera that were different between the two communities according to a non-parametric Wilcoxon Rank Sum test (p-adj <0.05) (Foster, Sharpton and Grünwald, 2017).

Where multiple comparisons were made, p-values were adjusted according to the Benjamini–Hochberg method (Benjamini and Hochberg, 1995) to reduce the likelihood of false-positive results (p-adj <0.05). Trends were considered where p-adj >0.05, but trends in unadjusted p-values were evident (p <0.10).

5.5 Results

5.5.1 Time, Substrate, and Participant Affected Fermentation

5.5.1.1 Substrate Characterisation

β-glucan and lignocellulose had similar moisture, ash, and organic matter concentrations (Table 6). β-glucan contained 72.3 % β-glucan, 3.9 % starch, and small amounts of RS, hemicellulose, cellulose, and lignin. Lignocellulose had 83.2 % w/w glucose, which comprised the substrate composition of 34.1 % RS and 49.2 % cellulose (estimated from total glucose minus starch and RS). Other sugars determined in the lignocellulose analysis were galactose (3.86 % of total sugars), galacturonic acid (1.02 % of total sugars), arabinose, fucose, and rhamnose (each <1 % total sugars).

Solid-state NMR showed that time was the main driver of compositional differences in the samples throughout the 48 h of fermentation (Figure 30). Those at 0 h had greater relative peak areas relating to carbohydrates, whereas samples at 48 h had greater peak areas relating to alkyl groups, aromatics,

phenolics, amides, and esters (Figure 31), which are usual constituents of fermentation metabolites. Participant variation explained some differences between samples, but substrate had little effect (Figure 30).

Table 6 The chemical composition of β-glucan and lignocellulose substrates used for fermentations.

Characteristic (g/100 g)	β-glucan	Lignocellulose
Moisture	2.7	3.0
Ash	1.8	0.7
Organic matter	95.5	96.3
β-glucan	72.3	<0.1
Starch	3.9	<0.1
Resistant starch	0.1	34.1
Hemicellulose	0.3	12.3
Cellulose	<0.1	49.2
Lignin	<0.1	0.8
Total	81	100

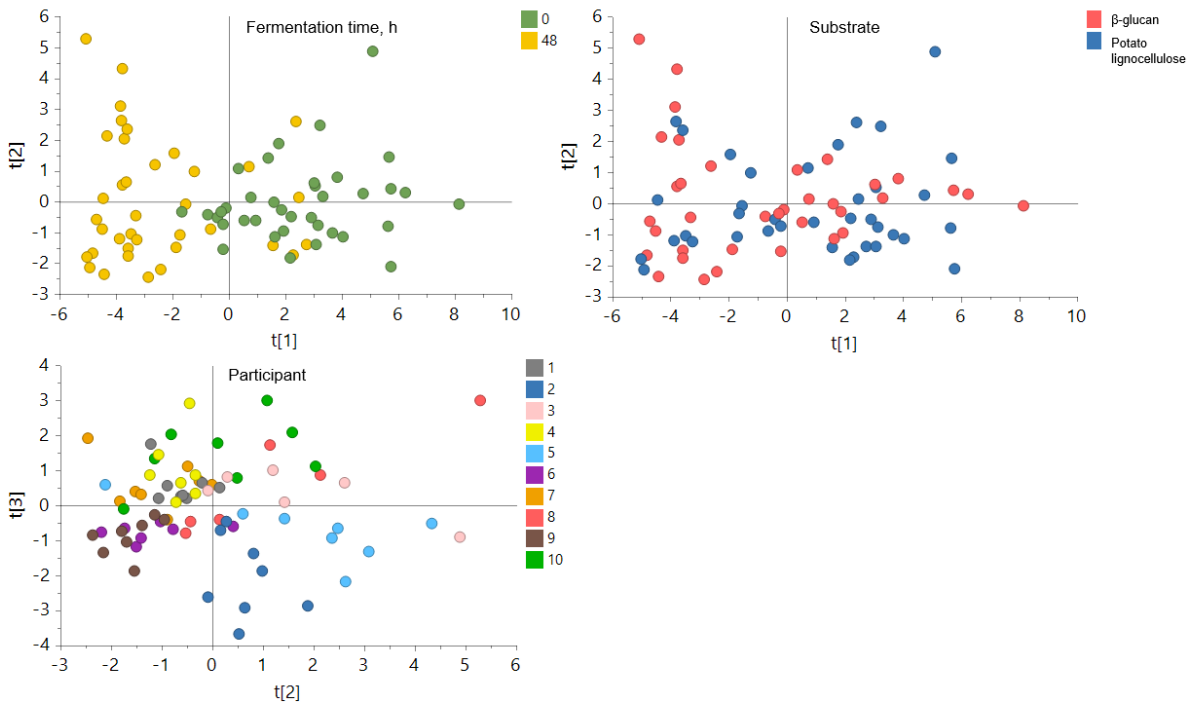


Figure 30 Principal component analysis plots of solid-state nuclear magnetic resonance data coloured by fermentation time, substrate, and participant.
Fermentation time: green 0 h and yellow 48 h, substrates: red β-glucan and blue lignocellulose, participants: grey 1, blue 2, light pink 3, yellow 4, light blue 5, purple 6, orange 7, pink 8, brown 9, and green 10.

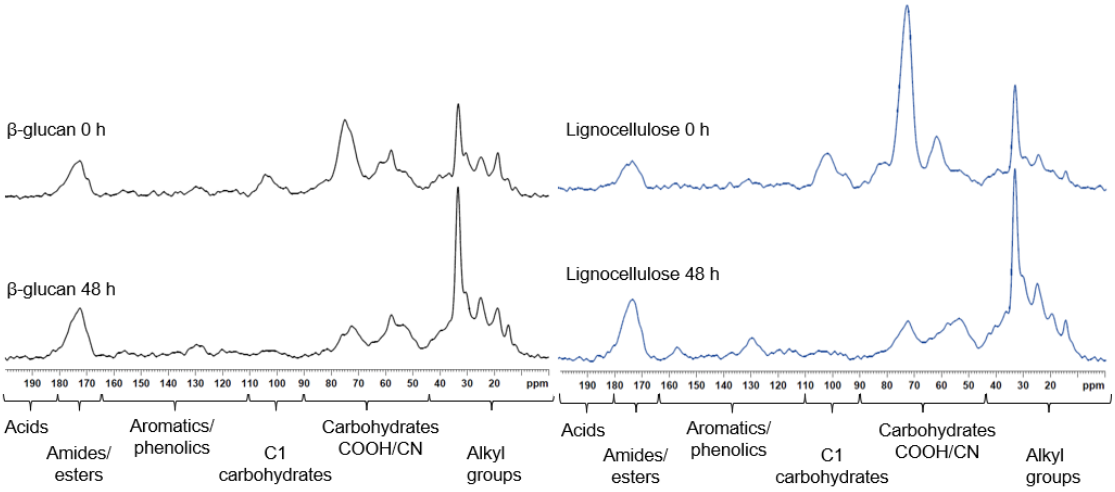


Figure 31 Solid state nuclear magnetic resonance spectra of fermentation pellets from a β-glucan sample (left, black) and a lignocellulose sample (right, blue).
Samples from 0 h (top) and 48 h (lower) fermentation. Featured samples were selected from participant 4, a high breath methane emitter, to show the difference in spectra at 0 and 48 h.

5.5.1.2 Substrate Fermentability

The β-glucan and the lignocellulose samples had the highest organic matter fermentability at 48 h and were alike (37.5 and 37.6 %) (Figure 32). These samples also had similar pH and gas volumes at 48 h. However, the pH of the β-glucan treatment dropped faster than that of the lignocellulose and had greater gas production throughout most fermentation (Figure 33).

The median organic matter fermentability of the two positive blanks was 8 %, and the negative blank was less than 1 % (Figure 32). The positive and negative blanks showed almost no change in pH or gas production over time (Figure 33).

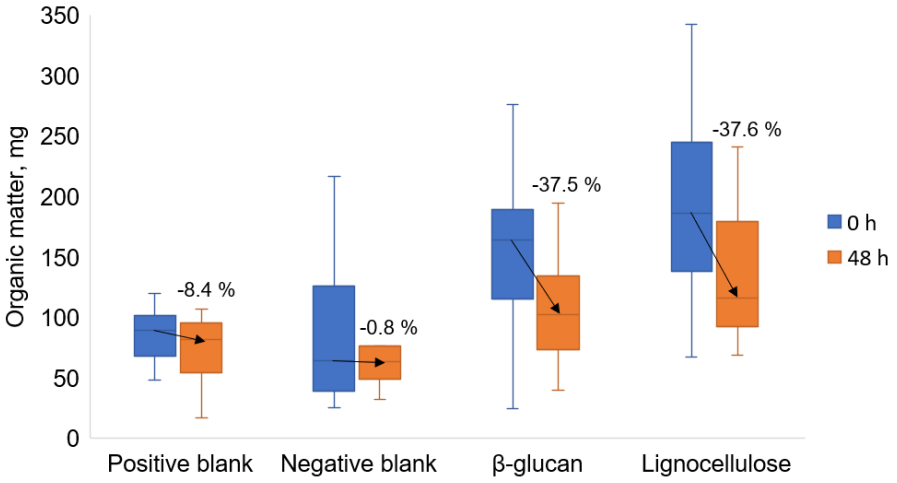


Figure 32 The organic matter concentration of samples from fermentation treatments. Positive blank (β-glucan and lignocellulose combined), negative blank (inoculum only), β-glucan (substrate and inoculum), and lignocellulose (substrate and inoculum) at 0 h (blue) and after 48 h (orange) of fermentation. Box central horizontal line shows the median, box borders show the upper and low quartiles, and box whiskers show 95 % confidence intervals. Arrows and percentages show the change in organic matter (organic matter fermentability).

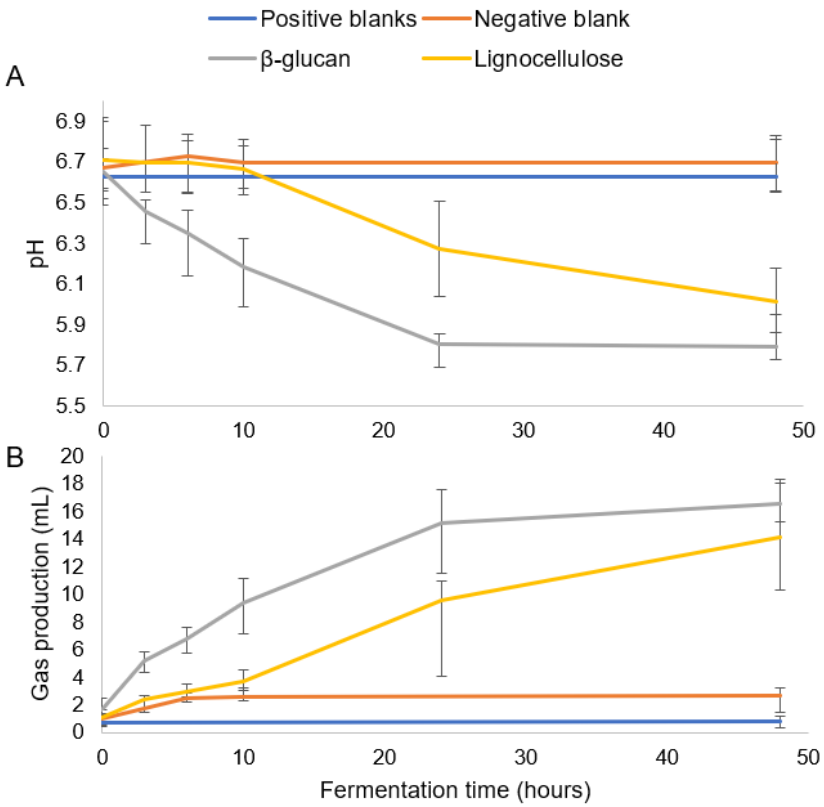


Figure 33 The pH (A) and gas production (B) of fermentation samples over 48 h. Positive blanks (blue; β-glucan and lignocellulose only), negative blanks (orange; inoculum only), β-glucan (grey; substrate and inoculum), or lignocellulose (yellow; substrate and inoculum). Lines are drawn through median values, and error bars are quartiles.

5.5.1.3 Microbial Diversity

Sequencing of fermentation samples provided an average of 7.5 M reads (5.8 to 8.0 M) and the average number of assigned microbial reads was 1.9 M (1.3 to 2.2 M).

Time, substrate, and participant were all drivers of differences in microbial alpha-diversity. At 0 h, samples had the highest diversity, and samples from 24 h had the lowest diversity (Supplementary File 14). Unexpectedly, the negative blank samples had higher microbial alpha-diversity than fermentations with fibre substrates (Supplementary File 14).

Time and substrate explained little of the beta-diversity (R^2 <0.05, p >0.10) as estimated using the Bray-Curtis dissimilarity index, but the participant was an important influencer (R^2 0.71, p <0.001) (Supplementary File 14).

5.5.1.4 Microbial Composition

The non-parametric Kruskal-Wallis Test showed that taxa at the family, genus, and species levels differed according to fermentation time point (Supplementary File 15). At the family level, *Ruminococcaceae* were highest in relative abundance at 0 h and lowest at 24 h (p-adj <0.05). At the genus level, the relative abundance of *Dorea* and *Faecalicatena* decreased over time, yet the relative abundance of *Enterocloster* increased over time (p-adj <0.05) (Supplementary File 16).

From 0 to 24 h of fermentation, there were no differences in the relative abundance of families, genera, or species between β-glucan and lignocellulose samples (p-adj >0.05) (Supplementary File 17). However, at 48 h, *Roseburia* and *Bifidobacterium* genera (specifically *B. adolescentis*) were lowest in relative abundance in the negative blanks and highest in the lignocellulose

samples (p-adj <0.05). The *Dorea* genus showed the opposite pattern, with the highest relative abundance in the negative blanks and the lowest in the lignocellulose samples (p-adj <0.05) (Supplementary File 18).

5.5.1.5 Microbial Functional Potential

At KEGG Levels 2 and 3, the relative abundance of genes related to energy, vitamin and cofactor metabolism decreased over 48 h, yet those related to signalling and cellular processes increased (p-adj <0.05) (Supplementary Files 19 and 20). From 0 to 6 h, the substrate did not affect the relative abundance of KEGG pathways (p-adj >0.05) (Supplementary File 21). However, there was a trend for methane metabolism to be highest in negative blanks and lowest in β-glucan samples at 6 h and 48 h (p <0.05, p-adj >0.05) (Supplementary Files 20 and 21). At 24 h, pathways relating to the biosynthesis of secondary metabolites and oxidative phosphorylation were more abundant in β-glucan samples compared to lignocellulose samples (p-adj <0.01) (Supplementary File 20). At 48 h, there was a trend for sulphur metabolism to be highest in the negative blank and lowest in lignocellulose samples (p <0.05, p-adj >0.05) (Supplementary File 20).

5.5.2 Breath methane concentration and fermentation

5.5.2.1 Characterisation of fermentation samples

Breath methane concentration was a minor contributing variable to differences in chemical composition between fermentation samples (Figure 34). However, LE samples had a higher relative abundance of features relating to aromatic and phenolic compounds than HE samples (p-adj <0.05) (Figure 35).

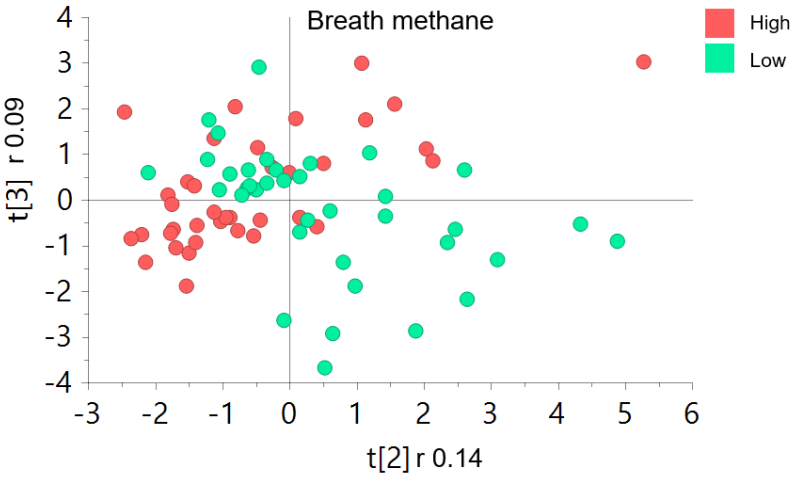


Figure 34 Principal component analysis plot of solid-state nuclear magnetic resonance data of fermentation samples from 0 h and 48 h.
Coloured by participant breath methane concentrations (red high, green low).

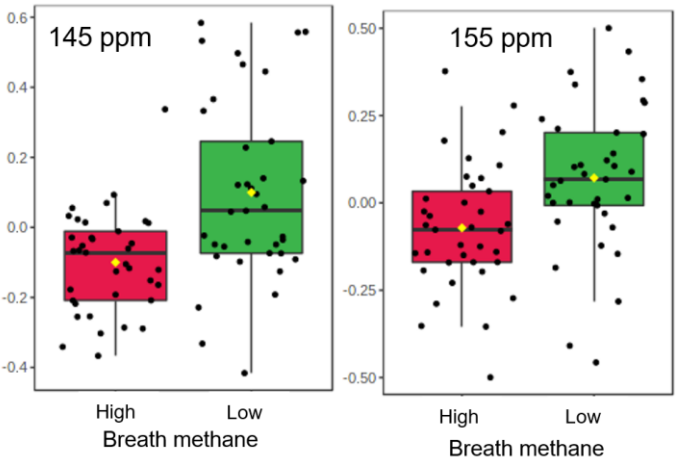


Figure 35 The normalised concentrations of features relating to aromatic and phenolic compounds in fermentation samples from 0 h and 48 h according to breath methane concentrations.
Means are shown by the yellow diamond, medians by the horizontal black line in the centre of the box, upper and lower quartiles by the box borders, and whiskers denote the 95 % confidence intervals. Black dots show the feature concentrations for individual samples. Red, high breath methane samples and green, low breath methane samples.

5.5.2.2 Substrate Fermentability

In LE and HE β-glucan fermentations, organic matter fermentability was 20 and 29 %, respectively. In LE and HE lignocellulose fermentations, organic matter fermentability was 32 and 44 %, respectively. However, all samples showed large variation (Supplementary File 22).

LE samples had higher gas production than HE samples, from 3 to 6 h in β-glucan samples and from 6 to 10 h in lignocellulose samples ($p < 0.01$). At several time points, there were also trends for the pH of LE samples to be lower than that of HE samples ($p < 0.10$) (Figure 36).

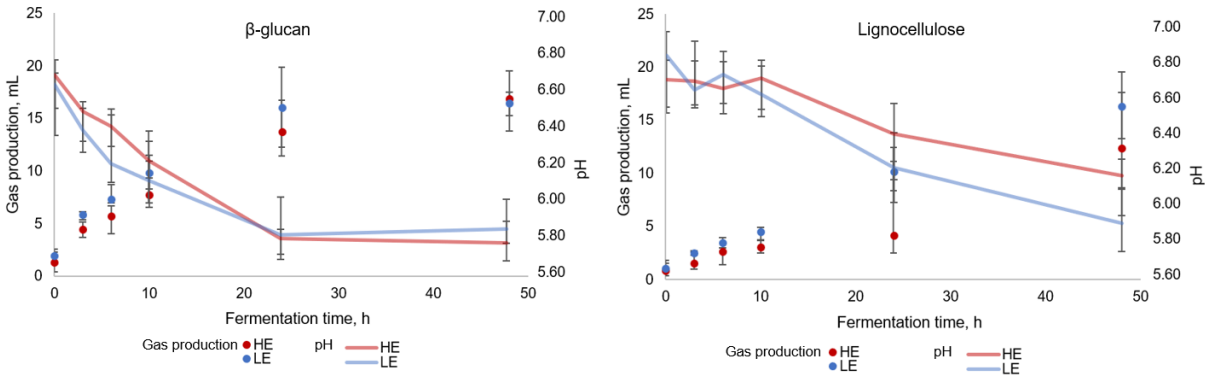


Figure 36 Median gas production (dots) and pH (lines) of fermentation samples across 48 h of fermentation.
Left, β-glucan and right, lignocellulose. Samples were taken at 0, 3, 6, 10, 24, and 48 h. Red dots and lines represent samples from high breath methane emitters, and blue dots and lines show samples from low breath methane emitters. Error bars are quartiles.

5.5.2.3 Microbial Diversity

Alpha-diversity metrics showed variable results. The Shannon Index showed no difference in diversity between HE and LE samples ($p > 0.10$). However, the number of observed taxonomic units and the Simpson Index showed that samples from LE had higher diversity ($p < 0.05$) (Supplementary File 23).

Breath methane concentration explained approximately 11 % of the variation in Bray-Curtis dissimilarity (p <0.001) (Figure 37).

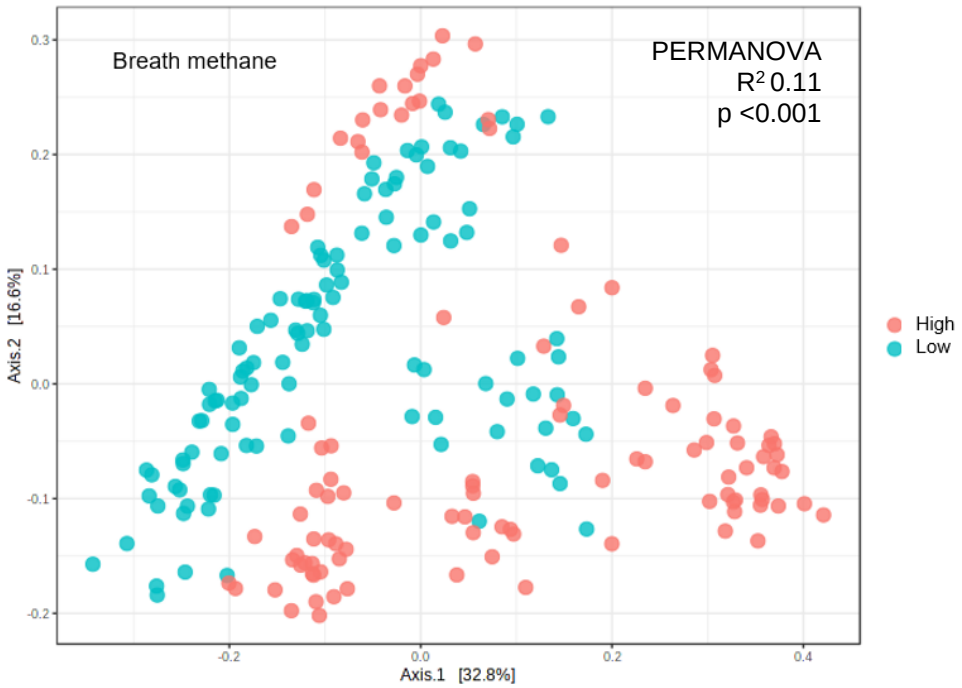


Figure 37 Principal coordinate analysis plot of microbial beta-diversity in fermentation samples using Bray-Curtis dissimilarity.
Axes one and two combined give an R^2 of 0.49. Red-orange data points are fermentation samples from high breath methane emitters, and blue-green data points are related to low breath methane emitters. PERMANOVA analysis gave an R^2 of 0.11 and a p-value of <0.001 for the effect of breath methane on the variance of the Bray-Curtis index.

5.5.2.4 Microbial Composition

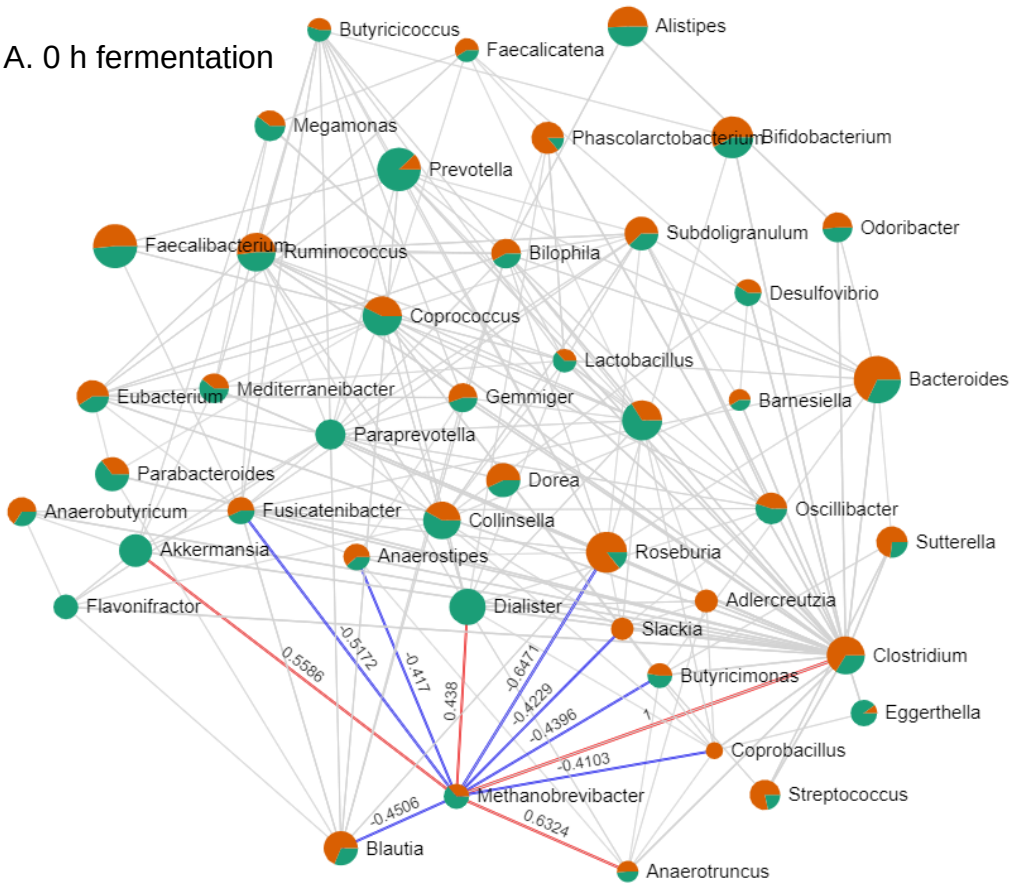
At 0 h, 3 phyla, 13 families, 22 genera, and 37 species differed in relative abundance between HE and LE samples (p-adj <0.05) (Supplementary File 24). At 0 h, HE samples had higher abundances of Euryarchaeota and Verrucomicrobia phyla, which were maintained throughout the 48 h fermentation (p-adj <0.05) (Supplementary File 25). At 0 h, LE samples had a higher abundance of the Firmicutes phylum than HE samples (p-adj <0.05), but this was not observed at subsequent time points (p-adj >0.10). At the genus level, samples from HE had a higher abundance of *Methanobrevibacter*, which

was observed throughout the 48 h fermentation (p-adj <0.05) (Supplementary File 25).

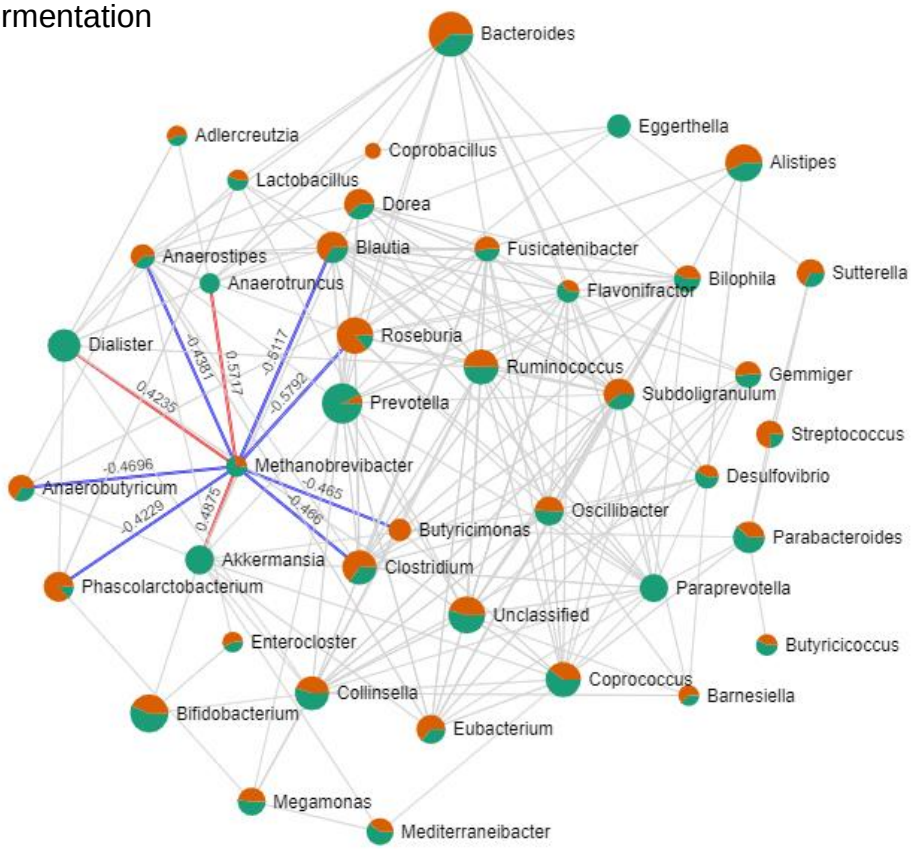
The relative abundance of taxa from the *Methanobrevibacter* genus positively correlated with those from the *Dialister*, *Anaerotruncus* and *Akkermansia* genera throughout the 48 h fermentation (r >0.40, p-adj <0.05) (Figure 38A-D). Additionally, the abundance of the *Methanobrevibacter* genus positively correlated with that of the *Clostridium* genus only at 0 h (Figure 38A) and *Acidaminococcus* and *Hungatella* genera after 24 h of fermentation (r >0.40, p-adj <0.05) (Figure 38C). Conversely, the abundance of the *Methanobrevibacter* genus was negatively correlated with that of the *Blautia*, *Butyricimonas*, and *Anaerobutyricum* genera at several time points of fermentation (Figure 38A-D). However, like the *Methanobrevibacter* genus data in faecal samples from Chapter 4, methanogens were only detected in fermentation samples from two HE (Figure 39).

The species-level data highlighted differences in relative abundances of keystone species for fibre degradation between HE and LE samples (Figure 40). *R. champanellensis* (cellulose degradation) was not detected in fermentation samples (data not shown). In contrast, *B. thetaiotaomicron* (arabinogalactan degradation) occurred in higher abundance in LE samples, and *R. bromii* (RS degradation) occurred in higher abundance in HE samples (Figure 40).

A. 0 h fermentation



B. 6 h fermentation



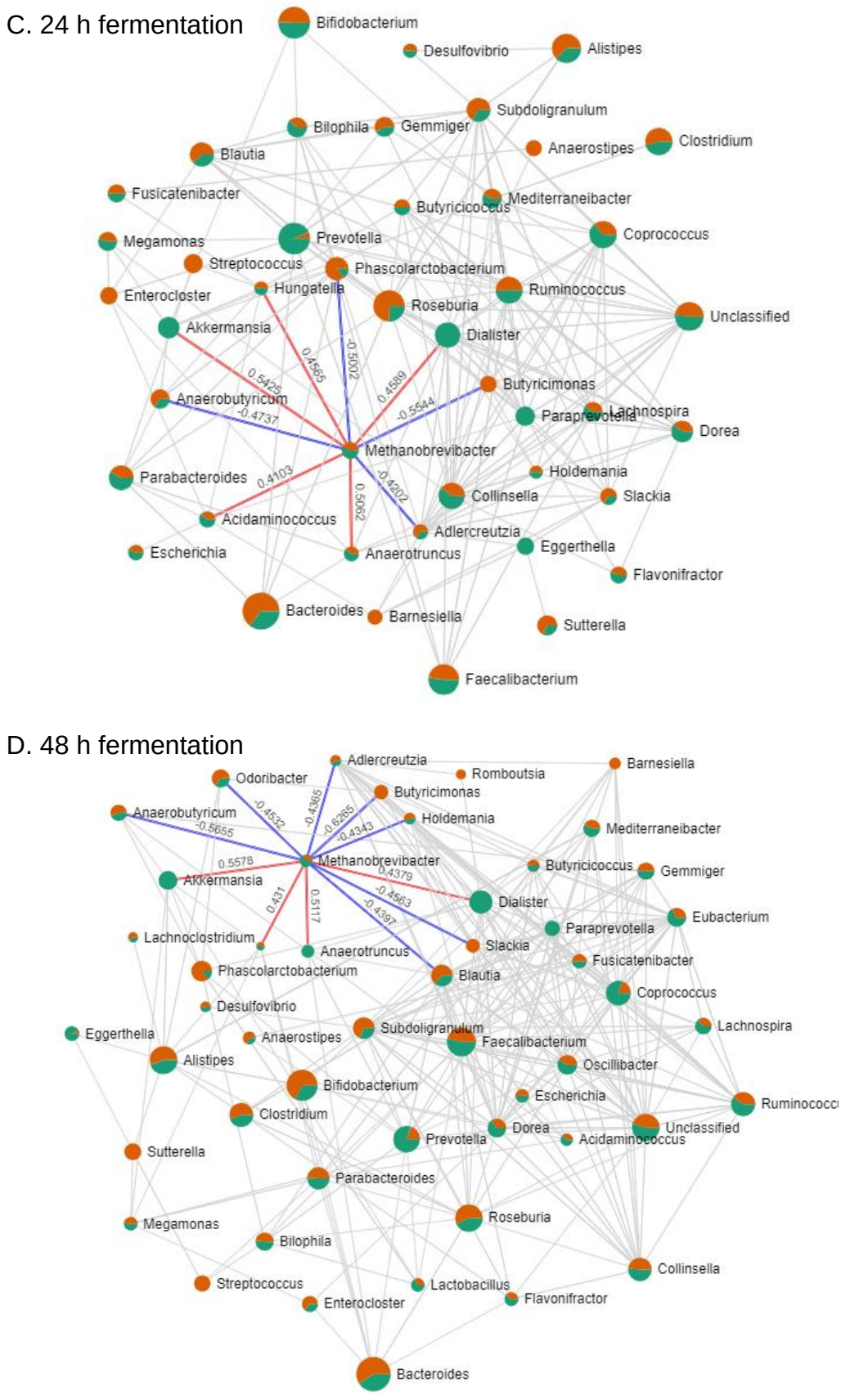


Figure 38 Networks of SparCC correlations showing the proportion of genera in fermentation samples with β-glucan or lignocellulose inoculated with faecal samples from high (orange) and low (green) breath methane emitters at 0 (A), 6 (B), 24 (C) and 48 h (D). Proportions based on median relative abundance. Positive (red lines) and negative (blue lines) correlations with *Methanobrevibacter* genus are annotated with correlation coefficients (r >0.40, p-adj <0.05).

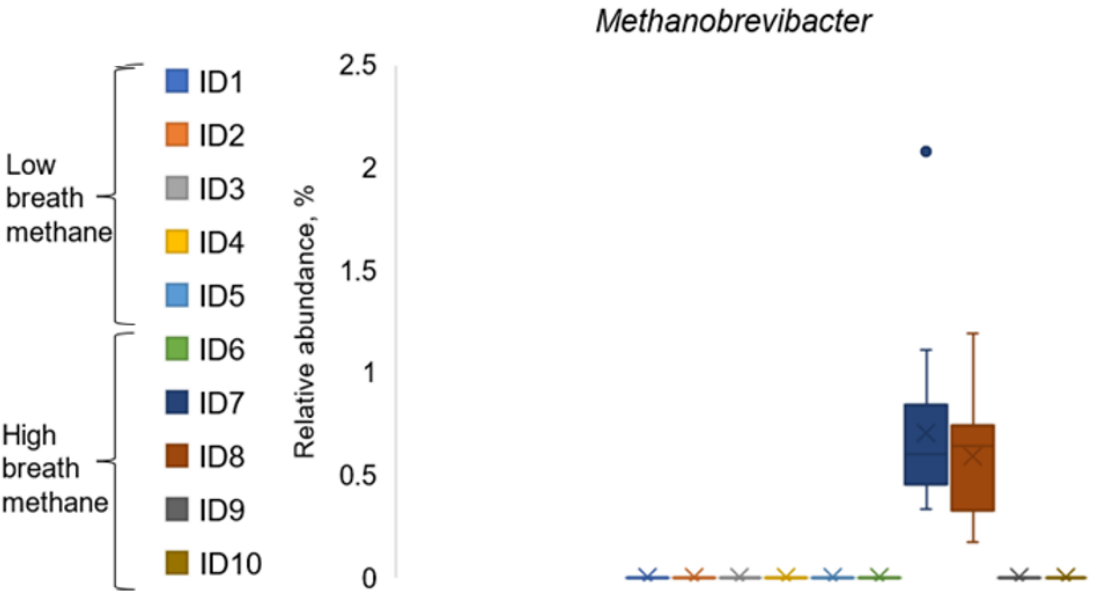


Figure 39 The relative abundance of the *Methanobrevibacter* genus in samples after fermentation with β-glucan or lignocellulose inoculated with faecal samples from high and low breath emitters.

Means are represented by crosses, medians by horizontal lines in the box centre, upper and lower quartiles by box edges, and 95 % confidence intervals by whiskers. Dots show outliers. Participants 1 blue, 2 orange, 3 grey, 4 yellow, 5 light blue, 6 green, 7 dark blue, 8 brown, 9 dark grey, and 10 light brown.

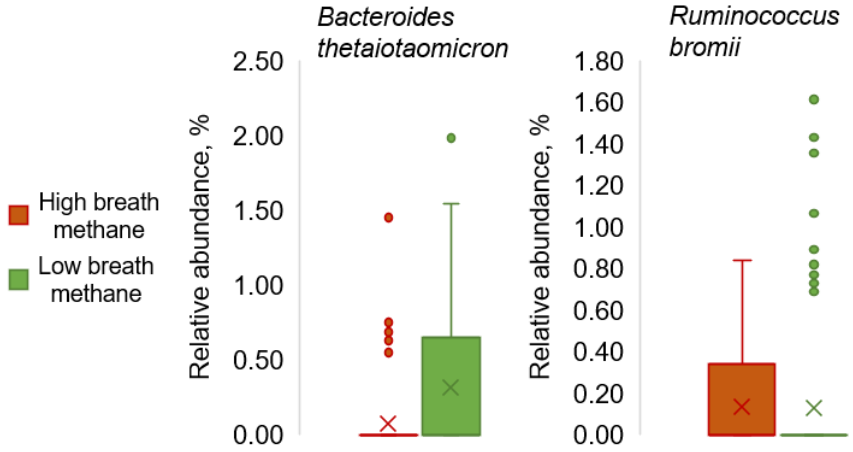


Figure 40 The relative abundance of keystone species (*Bacteroides thetaiotaomicron* and *Ruminococcus bromii*) involved in fibre degradation in samples after fermentation with β-glucan or lignocellulose inoculated with faecal samples from high (red/orange) and low (green) breath methane emitters.

Means are represented by crosses, medians by horizontal lines in the box centre, upper and lower quartiles by box edges, and 95 % confidence intervals by whiskers. Dots show outliers.

In both β-glucan and lignocellulose fermentations, HE samples had a greater relative abundance of *Akkermansia, Coprococcus, Dialister, Eggerthella, Flavonifractor, Parabacteriodes* and *Prevotella* genera compared to LE samples from 24 to 48 h fermentation ($p < 0.05$) (Figure 41). In contrast, LE samples had a greater relative abundance of *Anaerobutyricum, Bacteroides, Blautia, Clostridium, Odoribacter, Phascolarctobacterium*, *Sutterella,* and *Streptococcus* genera at 24 and 48 h fermentation in both β-glucan and lignocellulose fermentations ($p < 0.05$) (Figure 41). Furthermore, different genera increased in relative abundance after fermentation with β-glucan and lignocellulose according to whether inoculum was from HE or LE ($p < 0.05$) (Table 7).

Table 7 Microbial genera with increased relative abundance in fermentation samples from high and low breath methane emitters (p-adj <0.05) at 24 and 48 h of fermentation.

	High breath methane	Low breath methane
β-glucan	↑ *Coprobacillus, Eubacterium*	↑ *Alistipes, Roseburia*, *Subdoligranulum*
Lignocellulose	↑ *Anaerotruncus, Barnesiella, Lachnospira*	↑ *Enterocloster*

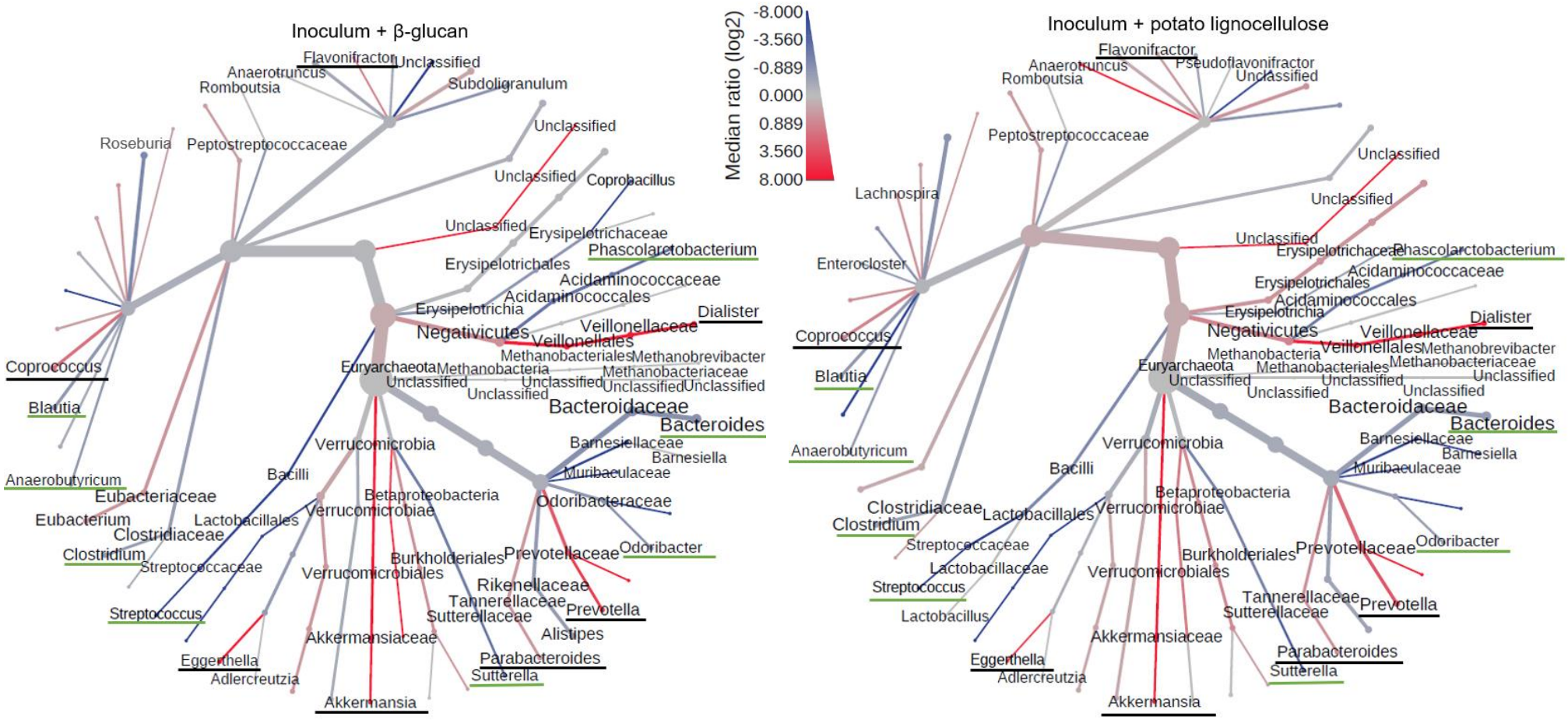


Figure 41 Heat trees showing genera that had increased (red) or decreased (blue) relative abundances in fermentation samples after 24 and 48 h of fermentation from high breath methane emitters (HE) compared to low (LE).

Data from fermentations with β-glucan are shown in the left tree, and data from fermentations with lignocellulose are shown in the right tree. Red lines show positive log2 fold change, blue lines show negative log2 fold change, and grey lines show little to no fold change in HE samples compared to LE. All annotated taxa had different relative abundance in HE and LE samples (p-adj <0.05). Genera that increased in abundance in response to both substrates are underlined in black (genera that increase in HE compared to LE) and green (genera that increased in LE compared to HE).

5.5.2.5 Microbial Functional Potential

At 0 h, 16 pathways were differentially abundant according to participant breath methane concentration, but this number was reduced to five pathways at 48 h (Supplementary file 26). At 0 h and 6 h, HE samples had a greater relative abundance of methane and amino acid metabolism pathways than LE samples (p-adj <0.05) (Figure 42). Furthermore, HE samples maintained a greater relative abundance of amino acid metabolism pathways at 48 h (p-adj <0.05) (Figure 42).

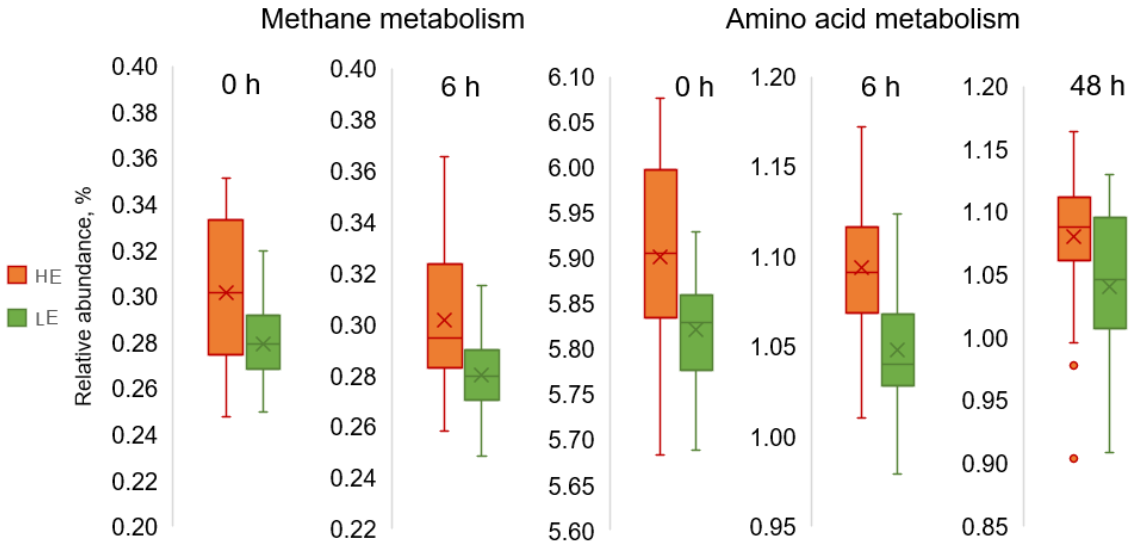


Figure 42 The relative abundance of KEGG pathways in fermentation samples from high (HE, orange) and low (LE, green) breath methane emitters at 0 h, 6 h and 48 h of colonic fermentation.

Means are represented by crosses, medians by horizontal lines in the box centre, upper and lower quartiles by box edges, and 95 % confidence intervals by whiskers. Dots show outliers.

5.5.2.6 Microbial Composition and Functional Potential

There were many associations between microbial genera and potential functions (r >0.40, p-adj <0.05) (Supplementary File 27). The relative abundance of the genus *Methanobrevibacter* was positively associated with functional pathways related to viral infections (r >0.40, p-adj <0.05) (Supplementary File 27). There were no correlations between

Methanobrevibacter abundance and methane or sulphur metabolism (r <0.40, p-adj <0.05). However, methane metabolism was positively associated with taxa from *Bifidobacterium* and *Bilophila* genera and 23 pathways, including nitrogen, lipids, amino acid, cofactor and vitamin, xenobiotic, and glycan metabolism (r >0.40, p-adj <0.05) (Supplementary File 27). Sulphur metabolism was positively associated with the relative abundance of the *Adlercreutzia* genus and amino acid, cofactor and vitamin metabolism, oxidative phosphorylation, and bacterial infection pathways (r >0.40, p-adj <0.05) (Supplementary File 27).

5.6 Discussion

5.6.1 Microbial Fermentation by Time and Substrate

As expected, the data indicated that fibre substrates were fermented over time. The increases in gas volume and decreases in organic matter, pH, and the ratio of carbohydrates to alkyl, amide, and ester, aromatic, and phenolic groups reflect the fermentation of carbohydrates and the production of organic acids and other metabolites.

However, minor changes in microbial communities occurred over the 48 h fermentation. Regardless of the substrate, the main change was decreased relative abundance of taxa from the *Ruminococcaceae* family, which co-occurred with a reduction in the relative abundance of genes related to energy, vitamin, and cofactor metabolism. Long *et al.* (2015) also reported a time-related reduction in the abundance of taxa from the *Ruminococcaceae* family during *in vitro* colonic fermentation using the same basal media as the current study. Indeed, the authors reported that the basal media composition and the

type of fermentable substrate are key determinants of changes in taxa abundance over time.

β-glucan, more accessible to microorganisms for fermentation, was fermented faster than lignocellulose. However, both substrates showed a similar extent of fermentation at 48 h, which was likely due to the unexpected high concentration of RS in the lignocellulose substrate (34 %). RS is expected to increase the fermentability of the lignocellulose substrate, as it has approximately 96 % fermentability in most individuals (Walker *et al.*, 2011). The lignocellulose substrate still had a high concentration of cellulose (49%); however, RS is likely to be preferentially utilised due to its increased fermentability. The deviation in expected and actual composition of the lignocellulose substrate may be attributed to the extraction method used. Different extraction methods are known to impact the final composition of potato substrates (Neeraj et al., 2021). The extraction method used to extract lignocellulose from potato in the current study was a mild and simple process, with the aim of maintaining a substrate that closely resembled the original food structure. As a consequence, this extraction method appeared to be less consistent.

At 48 h, the negative blanks had a lower relative abundance of the *Bifidobacterium* (specifically *B. adolescentis*) and *Roseburia* genera than fermentation with β-glucan and lignocellulose. Strains of *B. adolescentis* have effective carbohydrate utilisation capacity due to carbohydrate-degrading enzymes that cleave starch polymers and β-1,3 and β-1,4 linked glucans (Duranti *et al.*, 2014). Plant-based diets also increase the abundance of the *Roseburia* genus in human faeces (David *et al.*, 2014). Therefore, the growth of

bacteria was limited by the lack of carbohydrate sources in negative blank samples.

Additionally, the relative abundance of the *Dorea* genus and pathways related to sulphur metabolism was higher in the negative blanks than in substrate samples at 48 h. *Dorea* can degrade sialic acids from mucins (Schirmer *et al.*, 2016). Therefore, sialic acids may have been present in the faecal inoculum and provided a fermentable substrate for taxa from the *Dorea* genus in the negative blank.

Finally, co-occurrence of increased abundance of microbial genes related to methane and sulphur metabolism was observed at 48 h, which was unexpected as these pathways are reported to be mutually exclusive in the colon (Macfarlane, 1988; Gibson, Macfarlane and Cummings, 1993). Considering that metagenomics measures the potential function rather than the expression of genes or proteins, this data may reflect a general increase in the abundance of hydrogen utilising microorganisms due to the high availability of hydrogen, without an increase in competing hydrogen utilising pathways. For example, methanogens can use formate instead of hydrogen for methanogenesis, and the *Desulfovibrio* genus can reduce sulphate using lactate instead of hydrogen (Samuel *et al.*, 2007; Steger *et al.*, 2002).

5.6.2 Microbial Communities From High and Low Breath Methane Emitters

5.6.2.1 Hypothesis One: Samples with faecal inoculum from high breath methane emitters have a greater rate and extent of insoluble lignocellulose fermentation than samples with inoculum from low breath methane emitters

The organic matter fermentability data suggested that samples with faecal inoculum from HE had a greater rate and extent of lignocellulose fermentation than samples with inoculum from LE. However, there was a high variability due to the inconsistent inoculation of organic matter from the faecal inoculum. Double-layered cheesecloth was used to filter the inoculum so that fibre-associated microorganisms would be included; however, this step allowed some particulate matter to enter the inoculum, which was not evenly distributed between samples.

The pH and gas production data suggested a greater extent of lignocellulose fermentation in LE compared to HE samples. This discrepancy between organic matter and pH/gas production data may be due to the limited accuracy of organic matter determination. The introduction of particulate matter from the faecal inoculum and/or the use of a relatively crude method for organic matter quantification may have been responsible.

5.6.2.2 Hypothesis Two: Fermentations with faecal inoculum from high breath methane emitters have a higher relative abundance of taxa from the Firmicutes phylum, while fermentations from low breath methane emitters have more taxa from the Bacteroidetes phylum

There were few differences between samples from LE and HE individuals at the phylum level; however, there were differences in the communities associated with fibre fermentation at the genera level within Bacteroidetes and Firmicutes phyla. LE samples after 24 and 48 h of fermentation had increased relative abundance of *Anaerobutyricum, Bacteroides, Blautia, Clostridium, Odoribacter, Phascolarctobacterium, Sutterella,* and *Streptococcus* genera compared to HE samples. However, HE samples had an increased relative abundance of *Akkermansia, Coprococcus, Dialister, Eggerthella, Flavonifractor, Parabacteriodes,* and *Prevotella* genera compared to LE samples after 24 and 48 h of fermentation.

Similarly, Kumpitsch *et al.* (2020) reported differences in the faecal communities of faeces from HE and LE at the genus level. They observed a higher relative abundance of *Blautia* and *Bacteroides* genera in LE samples, which was observed in the current *in vitro* fermentation; however, these genera positively correlated with the *Flavonifractor* genus in Kumpitsch *et al.* (2020), which was in greater abundance in HE samples in the current *in vitro* fermentation.

Like the current study, Hoffmann *et al.* (2013) reported the co-occurrence of *Methanobrevibacter* and *Prevotella* genera. They also reported a positive association between *Bacteroides* and *Parabacteriodes* genera abundance, a

finding that is contrary to the current findings. Hoffmann *et al.* (2013) did not report positive associations of *Dialister*, *Anaerotruncus*, or *Akkermansia* with *Methanobrevibacter* genera found in the current study, nor did they measure the breath methane concentration of the participants.

In the current study, fermentation samples from only two of the five HE participants had detectable *Methanobrevibacter*. Studies have reported highly variable abundances of *Methanobrevibacter* in faecal samples from HE and LE participants (Kumpitsch *et al.*, 2020). Additionally, recent studies have suggested that methanogens in dental plaques may contribute to breath methane emission (Nkamga, Henrissat and Drancourt, 2017; Erdrich *et al.*, 2021). These factors could contribute to low methanogen abundance in HE samples. Additionally, methanogens are oxygen-sensitive and difficult to culture (Traore *et al.*, 2019), and although great care was taken to minimise oxygen exposure at faecal sample collection and culture, some methanogens may have become unviable.

5.6.2.3 Hypothesis Three: *In vitro* fermentations from high breath methane emitters have a higher abundance of genes relating to methane metabolism, whilst fermentations from low breath methane emitters have a higher abundance of genes relating to sulphate reduction

In vitro colonic fermentations with inoculum from HE had a higher relative abundance of genes relating to methane and amino acid metabolism compared to LE samples. However, there was no association between methane metabolism and *Methanobrevibacter* genus abundance. Instead, methane

metabolism positively correlated with the relative abundance of *Bifidobacterium* and *Bilophila* genera.

Taxa of the *Bilophila* genus are known hydrogen sulphide producers (Braccia *et al.*, 2021), a pathway thought to directly compete with methanogenesis for hydrogen (Gibson, Macfarlane, and Cummings, 1993). Some reactions in sulphur and methane metabolic pathways are linked, which may explain this finding. For example, L-serine formed during formaldehyde assimilation in the methane metabolic pathway involves cysteine metabolism in the sulphur metabolic pathway (Kanehisa Laboratories, 2021a) (Figure 43). Metatranscriptomics may help confirm or disprove a correlation between these potential functions.

Whilst there is little literature connecting the *Bifidobacterium* genus and methane metabolism, a clinical study found that administration of a *Bifidobacterium* probiotic strain increased breath methane emissions of humans (Kumar *et al.*, 2018). In addition, KEGG pathways show that some *Bifidobacterium* spp. have genes encoding enzymes involved in methane metabolism, namely formaldehyde assimilation, serine biosynthesis, and methanogenesis (Kanehisa Laboratories, 2021b). Therefore, the metabolism of the *Bifidobacterium* genus might support colonic methanogenesis.

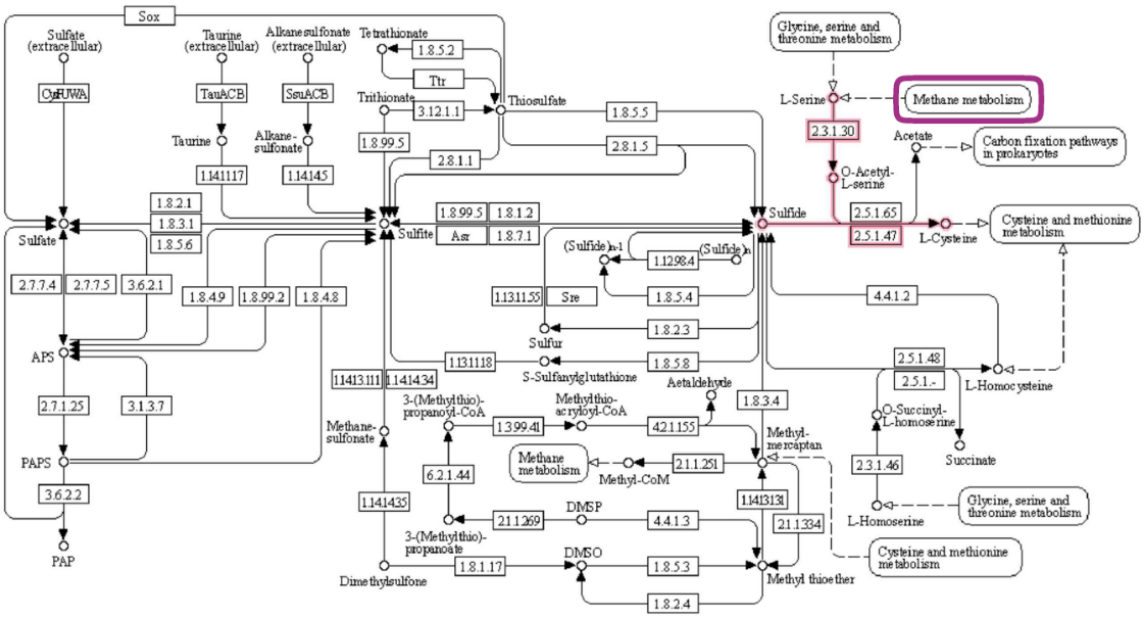


Figure 43 The KEGG sulphur metabolic pathway highlighting the reactions involved in cysteine metabolism (pink lines) and the overlap with methane metabolism (purple circle).

There was no evidence to support the hypothesis that LE samples had a higher abundance of genes related to sulphur metabolism. On the contrary, HE samples had a higher relative abundance of amino acid metabolic pathways, positively associated with sulphur metabolic pathways. Furthermore, the relative abundance of sulphur metabolic pathways was greater in the negative blank compared to the substrate samples. Research has shown that fermentable carbohydrates reduce protein fermentation by approximately 60 % (E. A. Smith and Macfarlane, 1996). Therefore, the lack of fermentable carbohydrates in the negative blank could result in increased amino acid fermentation and the liberation of sulphur from sulphur-containing amino acids (Magee *et al.*, 2000). This observation may also explain the positive association between amino acid and sulphur metabolism pathways in the negative blanks.

5.6.3 Strengths and Limitations

The main strength of the study is the *in vitro* approach that allowed more than 200 samples to be collected during 48 h of fermentation. The analysis of these samples enabled each community to be sampled thoroughly, providing greater accuracy of community composition. Furthermore, applying complementary techniques such as gas pressure, pH, NMR, and metagenomics helped capture the dynamics of fibre fermentation over time. However, these measures are limited by the use of a closed fermentation system, which limits the production of gases and other metabolites as they accumulate in the system.

The microbial composition and NMR data were analysed as separate timepoints to account for temporal autocorrelation; however, the use of the same faecal inocula across different treatments was not accounted for in the statistical model.

A further limitation is that the detection of *Methanobrevibacter* occurred in fewer samples than expected. This result could be inaccurate breath testing, microbial culture from faeces to the batch fermenters, or sequencing bias. It was also difficult to make robust conclusions on microbial functionality based on predictive function, and further analyses would confirm or refute the proposed associations.

5.7 Conclusion

The *in vitro* colonic model was effective for studying differences in faecal microbial community composition and potential function related to breath methane emission. The hypothesis that fermentation samples from HE participants have a higher relative gene abundance of pathways relating to methane metabolism was confirmed. However, these pathways did not relate

to the relative abundance of the *Methanobrevibacter* genus. Connections between the *Bifidobacterium* genus and methane metabolism were observed and warrant further investigation.

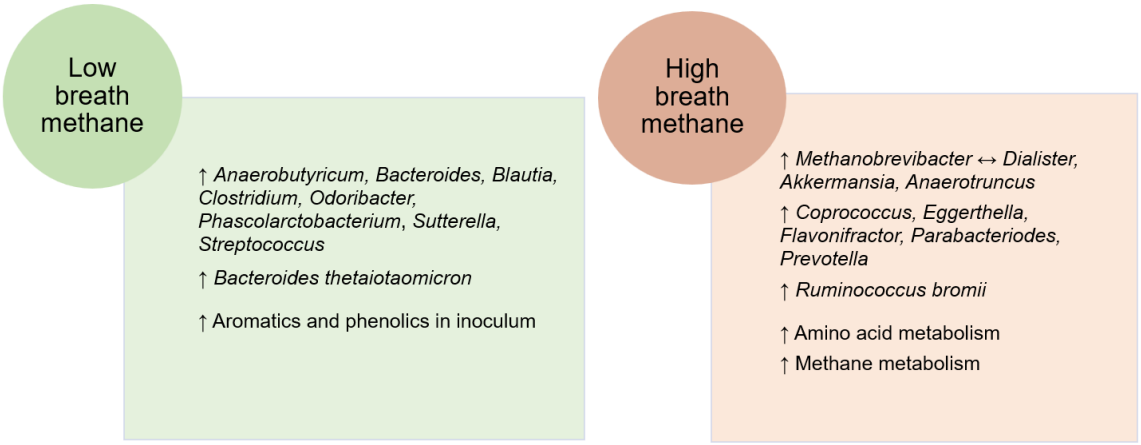


Figure 44 The key differences in rate and extent of fibre fermentation, microbial communities, and pathways in fermentation samples from high (orange) and low (green) breath methane groups.

There were distinct genera associated with fibre fermentation in HE (*Dialister*, *Akkermansia, Coprococcus, Eggerthella, Flavonifractor, Parabacteriodes*, and *Prevotella*) and LE (*Anaerobutyricum, Bacteroides, Blautia, Clostridium, Odoribacter, Phascolarctobacterium, Sutterella*, and *Streptococcus*) samples. However, due to high variability in organic matter quantification, it was difficult to quantify the extent of substrate fermentation.

Overall, the study provided insights regarding the differences in functional potential of the colonic communities between HE and LE samples and how these differences may relate to dietary fibre fermentation. Further analyses into how microbiota functionality in individuals who are HE and LE may relate to fibre fermentation and human health are needed to go beyond the potential function of metagenomics.

References

1. Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society 57, 289–300.

2. Braccia, D.J., Jiang, X., Pop, M., and Hall, A.B. (2021). The Capacity to Produce Hydrogen Sulfide (H2S) via Cysteine Degradation Is Ubiquitous in the Human Gut Microbiome. Frontiers in Microbiology 12, 1–31. https://doi.org/10.3389/fmicb.2021.705583.

3. Chassard, C., Delmas, E., Robert, C., and Bernalier-Donadille, A. (2010). The cellulose-degrading microbial community of the human gut varies according to the presence or absence of methanogens. FEMS Microbiology Ecology 74, 205–213. https://doi.org/10.1111/j.1574-6941.2010.00941.x.

4. Chong, J., Liu, P., Zhou, G., and Xia, J. (2020). Using MicrobiomeAnalyst for comprehensive statistical, functional, and meta-analysis of microbiome data. Nature Protocols, 15(3), 799–821. https://doi.org/10.1038/s41596-019-0264-1.

5. Chng, K. R., Ghosh, T. S., Tan, Y. H., Nandi, T., Lee, I. R., Ng, A. H. Q., Li, C., Ravikrishnan, A., Lim, K. M., Lye, D., Barkham, T., Raman, K., Chen, S. L., Chai, L., Young, B., Gan, Y. H., and Nagarajan, N. (2020). Metagenome-wide association analysis identifies microbial determinants of post-antibiotic ecological recovery in the gut. Nature Ecology and Evolution 4(9), 1256–1267. https://doi.org/10.1038/s41559-020-1236-0.

6. Coenen, A.R., Hu, S.K., Luo, E., Muratore, D., and Weitz, J.S. (2020). A Primer for Microbiome Time-Series Analysis. Frontiers in Genetics 11, 1–15. https://doi.org/10.3389/fgene.2020.00310.

7. Cosma-Grigorov, A., Meixner, H., Mrochen, A., Wirtz, S., Winkler, J., and Marxreiter, F. (2020). Changes in Gastrointestinal Microbiome Composition in PD: A Pivotal Role of Covariates. Frontiers in Neurology 11, 1–13. https://doi.org/10.3389/fneur.2020.01041.

8. David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., Ling, A. v., Devlin, A. S., Varma, Y., Fischbach, M. A., Biddinger, S. B., Dutton, R. J., and Turnbaugh, P. J. (2014). Diet rapidly and reproducibly alters the human gut microbiome. Nature 505(7484), 559–563. https://doi.org/10.1038/nature12820.

9. Duranti, S., Turroni, F., Lugli, G.A., Milani, C., Viappiani, A., Mangifesta, M., Gioiosa, L., Palanza, P., van Sinderen, D., and Ventura, M. (2014). Genomic characterization and transcriptional studies of the starch-utilizing strain Bifidobacterium adolescentis 22L. Applied and Environmental Microbiology 80, 6080–6090. https://doi.org/10.1128/AEM.01993-14.

10. Edwards, C.A., Gibson, G., Champ, M., Jensen, B.-B., Mathers, J.C., Nagengast, F., Rumney, C., and Quehl, A. (1996). *In vitro* Method for Quantification of the Fermentation of Starch by Human Faecal Bacteria. Journal of the Science of Food and Agriculture 71, 209–217. https://doi.org/10.1002/(SICI)1097-0010(199606)71:2<209: AID-JSFA571>3.0.CO;2-4.

11. Emwas, A.H., Saccenti, E., Gao, X., McKay, R.T., dos Santos, V.A.P.M., Roy, R., and Wishart, D.S. (2018). Recommended strategies for spectral processing and post-processing of 1D 1 H-NMR data of biofluids with a particular focus on urine. Metabolomics 14, 1–23. https://doi.org/10.1007/s11306-018-1321-4.

12. Erdrich, S., Tan, E.C.K., Hawrelak, J.A., Myers, S.P., and Harnett, J.E. (2021). Hydrogen–methane breath testing results influenced by oral hygiene. Scientific Reports 11, 1–11. https://doi.org/10.1038/s41598-020-79554-x.

13. Flint, H.J., Duncan, S.H., and Louis, P. (2017). The impact of nutrition on intestinal bacterial communities. Current Opinion in Microbiology 38, 59–65. https://doi.org/10.1016/j.mib.2017.04.005.

14. Foster, Z.S.L., Sharpton, T.J., and Grünwald, N.J. (2017). Metacoder: An R package for visualization and manipulation of community

taxonomic diversity data. PLoS Computational Biology 13. https://doi.org/10.1371/journal.pcbi.1005404.

15. Gibson, G., Macfarlane, G.T., and Cummings, J. (1993). Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut 34, 437–439.

16. Hoffmann, C., Dollive, S., Grunberg, S., Chen, J., Li, H., Wu, G.D., Lewis, J.D., and Bushman, F.D. (2013). Archaea and Fungi of the Human Gut Microbiome: Correlations with Diet and Bacterial Residents. PLoS ONE 8. https://doi.org/10.1371/journal.pone.0066019.

17. Kalantar-Zadeh, K., Berean, K. J., Ha, N., Chrimes, A. F., Xu, K., Grando, D., Ou, J. Z., Pillai, N., Campbell, J. L., Brkljača, R., Taylor, K. M., Burgell, R. E., Yao, C. K., Ward, S. A., McSweeney, C. S., Muir, J. G., and Gibson, P. R. (2018). A human pilot trial of ingestible electronic capsules capable of sensing different gases in the gut. Nature Electronics 1(1), 79–87. https://doi.org/10.1038/s41928-017-0004-x.

18. Kanehisa Laboratories (2021a). KEGG Reference Pathway- Sulfur metabolism (online). https://www.genome.jp/pathway/map00920.

19. Kanehisa Laboratories (2021b). Methane metabolism - Bifidobacterium bifidum (online). https://www.genome.jp/pathway/bbf00680.

20. Kumar, K., Saadi, M., Ramsey, F. v., Schey, R., and Parkman, H.P. (2018). Effect of Bifidobacterium infantis 35624 on the Lactulose Breath Test for Small Intestinal Bacterial Overgrowth. Digestive Diseases and Sciences 63, 989–995. https://doi.org/10.1007/s10620-018-4945-3.

21. Kumpitsch, C., Fischmeister, F. P. S., Mahnert, A., Lackner, S., Wilding, M., Sturm, C., Holasek, S., Högenauer, C., Berg, I., Schöpf, V., and Moissl-Eichinger, C. (2020). Methane emission of humans is explained by dietary habits, host genetics, local formate availability and a uniform archaeome. BioRxiv 1–45. https://doi.org/10.1101/2020.12.21.423794.

22. De Lacy Costello, B.P.J., Ledochowski, M., and Ratcliffe, N.M. (2013). The importance of methane breath testing: a review. Journal of Breath Research 7, 24001. https://doi.org/10.1088/1752-7155/7/2/024001.

23. Levitt, M.D., Furne, J.K., Kuskowski, M., and Ruddy, J. (2006). Stability of Human Methanogenic Flora Over 35 Years and a Review of Insights Obtained from Breath Methane Measurements. Clinical Gastroenterology and Hepatology 4, 123–129. https://doi.org/10.1016/j.cgh.2005.11.006.

24. Long, W., Xue, Z., Zhang, Q., Feng, Z., Bridgewater, L., Wang, L., Zhao, L., and Pang, X. (2015). Differential responses of gut microbiota to the same prebiotic formula in oligotrophic and eutrophic batch fermentation systems. Scientific Reports 5. https://doi.org/10.1038/srep13469.

25. Macfarlane, G. (1988). Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut. Journal of Applied Bacteriology 65, 103-111.

26. Magee, E.A., Richardson, C.J., Hughes, R., and Cummings, J.H. (2000). Contribution of dietary protein to sulfide production in the large intestine: An *in vitro* and a controlled feeding study in humans. American Journal of Clinical Nutrition 72, 1488–1494. https://doi.org/10.1093/ajcn/72.6.1488.

27. Siddiqui, S., Dalal, N., Srivastva, A., & Pathera, A. K. (2021). Physicochemical, morphological, functional, and pasting properties of potato starch as a function of extraction methods. Journal of Food Measurement and Characterization, 15, 2805-2820.

28. Nkamga, V.D., Henrissat, B., and Drancourt, M. (2017). Archaea: Essential inhabitants of the human digestive microbiota. Human Microbiome Journal 3, 1–8. https://doi.org/10.1016/j.humic.2016.11.005.

29. Pang, Z., Chong, J., Zhou, G., De Lima Morais, D.A., Chang, L., Barrette, M., Gauthier, C., Jacques, P.É., Li, S., and Xia, J. (2021).

MetaboAnalyst 5.0: Narrowing the gap between raw spectra and functional insights. Nucleic Acids Research 49, W388–W396. https://doi.org/10.1093/nar/gkab382.

30. Pham, V.T., and Mohajeri, M.H. (2018). The application of *in vitro* human intestinal models on the screening and development of pre-and probiotics. Beneficial Microbes 9, 725–742. https://doi.org/10.3920/BM2017.0164.

31. Rajilić-Stojanović, M., Biagi, E., Heilig, H.G.H.J., Kajander, K., Kekkonen, R.A., Tims, S., and de Vos, W.M. (2011). Global and deep molecular analysis of microbiota signatures in fecal samples from patients with irritable bowel syndrome. Gastroenterology 141, 1792–1801. https://doi.org/10.1053/j.gastro.2011.07.043.

32. Robert, C., and Bernalier-Donadille, A. (2003). The cellulolytic microflora of the human colon: Evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects. FEMS Microbiology Ecology 46, 81–89. https://doi.org/10.1016/S0168-6496(03)00207-1.

33. Samuel, B. S., Hansen, E. E., Manchester, J. K., Coutinho, P. M., Henrissat, B., Fulton, R., Latreille, P., Kim, K., Wilson, R. K., Gordon, J. I., Performed, K. K., and Contributed, J. K. M. (2007). Genomic and metabolic adaptations of Methanobrevibacter smithii to the human gut. The Proceedings of the National Academy of Sciences, 104, 10643-10648.

34. Schirmer, M., Smeekens, S. P., Vlamakis, H., Jaeger, M., Oosting, M., Franzosa, E. A., Jansen, T., Jacobs, L., Bonder, M. J., Kurilshikov, A., Fu, J., Joosten, L. A. B., Zhernakova, A., Huttenhower, C., Wijmenga, C., Netea, M. G., and Xavier, R. J. (2016). Linking the Human Gut Microbiome to Inflammatory Cytokine Production Capacity. Cell 167(4), 1125-1136.e8. https://doi.org/10.1016/j.cell.2016.10.020.

35. Shahi, S.K., Freedman, S.N., and Mangalam, A.K. (2017). Gut microbiome in multiple sclerosis: The players involved and the roles they play. Gut Microbes 8, 607–615. https://doi.org/10.1080/19490976.2017.1349041.

36. Smith, E.A., and Macfarlane, G.T. (1996). Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism. Journal of Applied Bacteriology 81, 288–302. https://doi.org/10.1111/j.1365-2672.1996.tb04331.x.

37. Sonnenburg, E.D., Smits, S.A., Tikhonov, M., Higginbottom, S.K., Wingreen, N.S., and Sonnenburg, J.L. (2016). Diet-induced extinction in the gut microbiota compounds over generations. Nature 529, 212–215. https://doi.org/10.1038/nature16504.Diet-induced.

38. Steger, J. L., Vincent, C., Ballard, J. D., and Krumholz, L. R. (2002). Desulfovibrio sp. genes involved in the respiration of sulfate during metabolism of hydrogen and lactate. Applied and Environmental Microbiology, 68(4), 1932–1937. https://doi.org/10.1128/AEM.68.4.1932-1937.2002

39. Traore, S.I., Khelaifia, S., Armstrong, N., Lagier, J.C., and Raoult, D. (2019). Isolation and culture of Methanobrevibacter smithii by co-culture with hydrogen-producing bacteria on agar plates. Clinical Microbiology and Infection 25, 1561.e1-1561.e5. https://doi.org/10.1016/j.cmi.2019.04.008.

40. Walker, A. W., Ince, J., Duncan, S. H., Webster, L. M., Holtrop, G., Ze, X., Brown, D., Stares, M. D., Scott, P., Bergerat, A., Louis, P., McIntosh, F., Johnstone, A. M., Lobley, G. E., Parkhill, J., and Flint, H. J. (2011). Dominant and diet-responsive groups of bacteria within the human colonic microbiota. ISME Journal 5(2), 220–230. https://doi.org/10.1038/ismej.2010.118.

41. Williams, B.A., Grant, L.J., Gidley, M.J., and Mikkelsen, D. (2017). Gut fermentation of dietary fibres: Physico-chemistry of plant cell walls and implications for health. International Journal of Molecular Sciences 18. https://doi.org/10.3390/ijms18102203.

42. Ze, X., Le Mougen, F., Duncan, S.H., Louis, P., and Flint, H.J. (2013). Some are more equal than others: The role of “keystone” species in the

degradation of recalcitrant substrates. Gut Microbes 4. https://doi.org/10.4161/gmic.23998.

Chapter 6

The Links Between Human Breath Methane and Colonic Fibre Fermentation Metabolites

The data from this Chapter is being integrated with that of Chapter 5 for publication by Payling, L., Roy, N. C., Hill, S. J., Raymond, L. G., Fraser, K., Gagic, D., Loveday, S. M., Sims, I. M. and McNabb, W. C. The links between human breath methane, microbiota composition, and fibre fermentation in an *in vitro* colonic model. The ISME Journal.

6.1 Abstract

Fibre fermentation is a key function of a healthy colonic microbiota, providing energy for microbial metabolism and beneficial metabolites for host and microbiota health. However, research has indicated that fibre fermentation differs in individuals that are HE compared to LE.

An *in vitro* model of colonic fermentation was used to investigate whether faecal samples from individuals who are HE (5) or LE (5) produce different metabolites in response to β-glucan or lignocellulose fibres. Throughout 48 h of fermentation, supernatant and headspace gas samples were analysed using solution-state NMR for aqueous metabolites, gas chromatography for hydrogen and methane, and spectrophotometry for hydrogen sulphide.

Time and substrate were key drivers of differences in metabolite concentrations up to 24 h of fermentation, but these differences diminished towards 48 h. LE participant samples had higher concentrations of organic acids compared to HE samples ($p < 0.05$). As expected, HE participant samples had more methane in the headspace than LE participant samples; but notably in only two of the five HE fermentations. In a sub-set of samples with low methane production, hydrogen sulphide was not correlated with methane or hydrogen ($r < 0.40$). In addition, there were participant-driven differences in hydrogen sulphide

concentration in faecal inoculum at 0 h, but substrate was the main factor driving differences in hydrogen sulphide concentration at 48 h of fermentation.

In conclusion, the study found that faecal communities from LE participants produced more organic acids, but communities from the HE group produced more methane gas during *in vitro* fibre fermentation.

6.2 Introduction

During colonic fermentation, the microbiota utilises undigested dietary substrates to produce energy. Co-products of these reactions include gases such as hydrogen, nitrogen, carbon dioxide, methane, hydrogen sulphide, and other metabolites, including bile acids, organic acids, branched-chain amino acids, trimethylamine, tryptophan, and indole derivatives (Agus, Clément and Sokol, 2021).

Scientific literature showed that human breath methane concentration might be linked to differences in the colonic microbiota composition. The faecal microbiota of individuals who were HE had a greater relative abundance of fibre-fermenting taxa from the Firmicutes phylum and increased capacity for cellulose fermentation compared to those who were LE (Robert and Bernalier-Donadille, 2003; Chassard *et al.*, 2010). This link may arise from high hydrogen production during fibre fermentation by taxa from the Firmicutes phylum, supporting hydrogen utilisation by hydrogenotrophic methanogenesis (Chassard *et al.*, 2010). Furthermore, as documented in the scientific literature, the increased capacity for fibre fermentation in HE individuals may correspond to increased beneficial organic acid production. Conversely, it is expected that the microbiota of LE individuals have a greater relative abundance of hydrogen utilisation pathways for acetogenesis or hydrogen sulphide production because

of lower methanogenesis (Bernalier *et al.*, 1996). The colonic production of hydrogen, methane, hydrogen sulphide and organic acids is important to human health, but their associations with breath methane concentration remain unclear.

Common methods of measuring aqueous microbial metabolites include mass spectrometry and NMR (Rombouts *et al.*, 2017). NMR is an excellent tool for quantifying abundant metabolites with high reproducibility, detecting 30 to 100 individual compounds, whereas mass spectrometry can detect 300 to 1000 metabolites with high sensitivity (Emwas, 2015; The European Bioinformatics Institute, 2021). As the current study focussed on fibre fermentation metabolites, often represented by a high abundance of low molecular weight compounds, the specific and quantitative range of NMR was most suited to this application.

6.3 Aim and Hypotheses

The analyses conducted here aimed to investigate the links between human breath methane emission and metabolite production during fibre fermentation. An *in vitro* batch culture model of colonic fermentation, described in Chapter 3, was used to study the fermentation of oat β-glucan or potato lignocellulose fibres by faecal microbiota collected from individuals who were HE or LE (Chapter 5). In Chapter 6, solution-state NMR, gas chromatography, and spectrophotometry methods were used to measure the production of aqueous and gaseous metabolites over 48 h of fermentation.

The hypotheses were as follows:

1. There is a greater production of organic acids in fermentation samples from individuals who are HE compared to individuals who are LE, especially for the fermentation of lignocellulose.
2. There is a greater production of methane gas in fermentation samples from individuals who are HE compared to individuals who are LE.
3. There is a negative correlation between methane gas production and hydrogen sulphide production.

6.4 Methods

The methods for *in vitro* colonic fermentations are described in Chapter 3. Below are details of additional analyses relevant to this Chapter.

6.4.1 Solution-State Nuclear Magnetic Resonance

The samples consisted of six time points, two substrates, and ten participants in duplicate (240 samples total). Approximately 5 % of samples were unsuitable for NMR analysis due to low sample volume after syringe-filtering.

Aliquots of supernatants (2 mL) from the β-glucan and lignocellulose fermentations were thawed at 4 °C and filtered through 0.45 µm nylon membrane syringe filters. The filtrate (480 µL) was mixed with 120 µL of D_2O with the sodium salt of 3-(trimethylsilyl)-propionate acid-d4 (TSP) (0.05 % w/v) as an internal standard. The solutions were transferred to 5 mm borosilicate NMR tubes for analysis.

One-dimensional ^1H NMR spectra were acquired on a Bruker Avance III 400 NMR fitted with a five mm Prodigy BBO cryoprobe (Bruker, Switzerland) operating at a ^1H frequency of 400.13 MHz. A standard Bruker "noesygppr1d" pulse sequence with water suppression achieved by application of a 25 Hz

presaturation field at a transmitter frequency offset (o1) of 1881.10 Hz was used. The internal probe temperature was set to 300K with a five-minute temperature stability delay. The spectral data were obtained in 65,000 data points, a relaxation delay of eight seconds and 256 scans. All spectra were Fourier transformed using line broadening of one Hz, phased, baseline corrected, and referenced relative to TSP at zero ppm. Solution-state NMR analyses and data processing were carried out under the guidance of Dr Laura Raymond (Scion, New Zealand), an expert in solution-state NMR.

6.4.2 Headspace Gas Analysis

Vials of headspace gas were analysed on a gas chromatograph (GC) (Shimadzu GC 2010 plus) fitted with a Restek MXT-Molsieve 5 A PLOT column (30 m × 0.53 mm ID x 50 μM) and a helium photoionisation pulsed discharge detector (VICI Valco Instruments, Houston, USA) under the supervision of Dr Stefan Muetzel (AgResearch Ltd., New Zealand), according to his published method (Muetzel *et al.* 2018). The GC had a PAL Combi-xt robot (CTC Analytics AG, Zwingen, Switzerland) fitted with a 32-well tray for samples and a gas-tight 10 μL syringe (Hamilton Ltd., Reno, USA).

Samples (5 μL) were injected into the inlet with a split ratio of 20:1 and analysed as a single run at 85 °C isocratic with a helium flow of 4.76 mL/min through the column. Samples were sequentially injected onto the GC with a 1.5 min delay between injections to separate eluting peaks from the previous injection peaks. All fermentation treatments were analysed, including positive and negative blanks (500 samples total).

The peaks resolved were hydrogen, oxygen, nitrogen, and methane with a retention time of 1.17, 1.34, 1.49 and 1.92 min, respectively. At the beginning of

the run, an air sample and standards containing 2 % methane and 1 % hydrogen in nitrogen and 20 % methane and 10% hydrogen in nitrogen (BOC, Auckland, New Zealand) were injected. Peaks were identified, and peak areas were quantified using LabSolutions software (Shimadzu, Kyoto, Japan) in volts-minute.

6.4.3 Hydrogen Sulphide Determination

Hydrogen sulphide was quantified as described in Payling *et al.* (2021). Frozen samples of fermentation fluid containing zinc acetate (2 % w/v) were thawed at 4 °C. Samples (0.1 mL, in duplicate) were diluted 11x with distilled water and mixed with 0.1 mL "diamine solution" (0.2 % 4-amino-N, -N-dimethylaniline HCl_2 w/v in 20 % H_2SO_4 v/v) and 0.1 mL "Fe solution" (0.5 % $FeNH_4(SO_4)_2$ w/v in 2 % H_2SO_4 v/v). Samples were incubated at room temperature (21 to 27 °C) for ten minutes. The optical density of the samples was measured and recorded on a spectrophotometer at OD 670 (Thermo Fisher Scientific, USA).

Sulphide standards (0.02 to 0.16 μg/mL) were prepared in duplicate and measured as described above for samples. A standard curve was generated using the known concentrations of sulphide and the optical density of standards and used to calculate the sulphide concentration of fermentation samples.

6.4.4 Data Analyses

The NMR spectra were used for untargeted secondary metabolite fingerprinting by binning from 0 to 10.26 ppm, with a bin size of 0.04 ppm using AMIX software (Bruker, Germany). Where pH shifts were observed, bins were widened to include all shifted peaks. The region from 4.60 to 5.08 ppm was excluded to remove variations in the suppression of the water peak. Binned data were normalised to a constant sum, whereby the intensity of each bin was

calculated as a fraction of the total spectrum. This method is a commonly used correction method in NMR, which allows the comparison of samples that may have large variations in peak intensities (Emwas *et al.*, 2018). Data were then Pareto scaled to achieve Gaussian (normal) distribution and evaluated with PCA in SIMCA 16 (Sartorius Stedim Data Analytics AB, Umeå, Sweden) and by ANOVA or t-test in MetaboAnalyst 5.0 (Pang *et al.*, 2021).

Additionally, spectra were loaded into Chenomx NMR Suite 8.5 software (Chenomx Inc., Edmonton, Canada) for metabolite identification. Spectra were referenced to the internal standard, and the software libraries were used to identify and quantify metabolites. Missing values were replaced with zeros. Data were range scaled (mean-centred and divided by the range of each variable) to achieve Gaussian (normal) distribution, then analysed by ANOVA or t-test in MetaboAnalyst 5.0. Unfortunately, data were unbalanced due to the loss of some samples during syringe filtering and therefore unsuitable for a two-way ANOVA analysing the effect of substrate and breath methane. Instead, PCA was used to visualise the formation of four groups representing the two-way interaction between substrate and breath methane. Paired data were analysed by t-test in MetaboAnalyst 5.0.

Solution state NMR data were first analysed as a complete dataset including all time points; however, it was apparent that time point was a major factor driving differences between samples (Section 6.5.4, Figure 45A). Therefore, time points were subdivided according to their natural clusters in PCA to control the time effect. This approach was chosen to focus on the effects of breath methane and substrate and maintain consistency with analyses in Chapter 5, where data were sub-divided by time point.

For headspace gas data, peak areas were divided by 100,000 to improve data readability in tables and figures. Replicates of the peak area data were compared visually using distribution plots, and extreme outliers were removed, totalling approximately 5 % of the data points.

Hydrogen peak area data were normally distributed, whereas methane peak area data had a right skew and were Box-Cox transformed to achieve a Gaussian (normal) distribution. Box-Cox transformations are useful for finding the optimal normalising transformation for a variable when traditional power transformations are ineffective (Box and Cox, 1964; Osborne, 2010). Both data were fitted to Restricted Maximum Likelihood mixed-effects models in Minitab 19 with replicate and GC run as random factors and participant, treatment, time, and participant*treatment as fixed effects. The Satterthwaite approximation was used to test fixed effects, considering degrees of freedom across tests ideal for unbalanced data (IBM, 2021). Fisher's LSD multiple pairwise comparison test at 95 % confidence was used for means separation as it does not assume balanced data (Lee and Lee, 2018). Finally, the Box-Cox transformation was reversed to graphically represent the data.

The hydrogen sulphide data were nonparametric. Therefore, median values were analysed. The association between methane and hydrogen sulphide was tested using Spearman's correlation, and significant correlations were accepted where $r > 0.40$ and $p < 0.05$.

In all datasets where multiple comparisons were made, p-values were adjusted to reduce the likelihood of false-positive results (p-adj) using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

6.5 Results

6.5.1 Solution-State Nuclear Magnetic Resonance

Solution-state NMR spectra were visually assessed. The peaks had clear shape, a flat baseline, and good signal to noise ratio. The residual water peak was under 70 Hz in width, indicating good data quality.

All fermentation samples had similar ^{1}H NMR spectra, with changes in absolute intensity being the key difference observed (Supplementary File 28). Each spectrum was assigned 226 bins from 0 to 10.26 ppm. All major peaks were identified as known metabolites, and there were 10 metabolites present in at least 20 % of samples that were microbial metabolites of fibre fermentation (Table 8). Metabolite concentrations reflected what was expected from the literature.

Table 8 Metabolites identified from spectra of fermentation samples (β-glucan and lignocellulose, all time points) using Chenomx NMR Suite 8.5.
Data includes metabolites in at least 20 % of samples and excludes missing values.

	Concentration, mM		
Metabolite	Lower quartile	Median	Upper quartile
Acetate	14.52	22.94	35.16
Butyrate	4.57	9.26	18.60
Propionate	4.46	7.84	12.88
Ethanol	1.60	3.89	7.24
Caproate	2.13	3.26	8.54
Formate	0.87	1.84	5.98
Valerate	0.67	1.15	1.81
Methanol	0.66	0.85	1.05
Lactate	0.16	0.48	1.15
Succinate	0.02	0.03	0.05

Fermentation samples were subdivided to identify factors affecting sample variation within time points based on clustering samples in the PCA. Samples from 0 h represented the start of fermentation (38 samples in total), samples from 3, 6, and 10 h represented mid-fermentation (115 samples in total), and samples from 24 and 48 h represented the end of fermentation (74 samples in total) (Figure 45A).

6.5.2 Headspace Gases

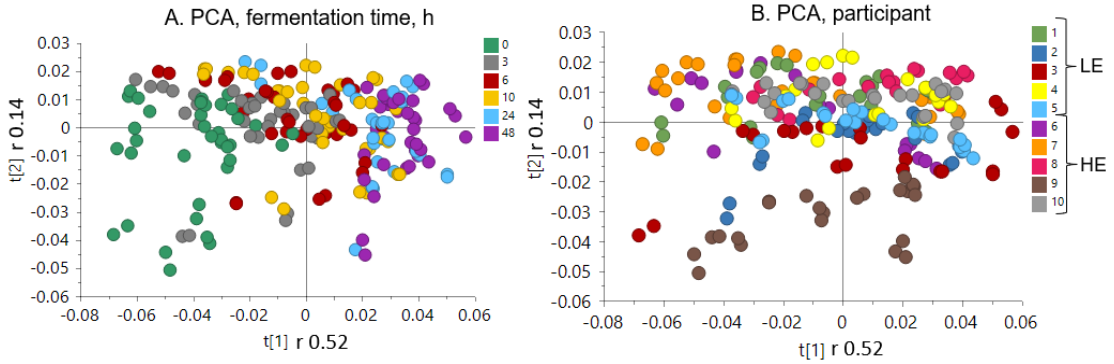
Hydrogen and methane were identified, and peak areas were quantified from gas chromatography spectra (Supplementary File 28). Interpolating peak area data on the standard curve showed methane concentrations ranged from 365 ppm to 70,732 ppm and hydrogen concentrations ranged from 144 to 32,411 ppm.

6.5.3 Hydrogen Sulphide

No hydrogen sulphide was detected in fermentation samples from seven of the ten participants. These samples had been frozen at -80 °C for six months due to laboratory lockdown during the SARS-CoV-2 pandemic. It is known that the viability of frozen samples for sulphide determination is three months (Janssen, 1975, unpublished), so it was unsurprising that sulphide was not detected in these samples. Thus, the data described relates to samples from the remaining three participants (3, 5, 10; two LE and one HE) where samples were collected post-lockdown.

6.5.4 Time, Participant, and Substrate Affected Fermentation Metabolites

The PCA plots showed that time point was a major variable explaining variation on the t1 axis (r 0.52) (Figure 45A), and the participant effect explained much variation on the t2 axis (r 0.14) (Figure 45B). An ANOVA confirmed that 73 % of metabolites varied with time point (p-adj <0.05) (Supplementary File 29). Concentrations of acetate and propionate increased over time (p-adj <0.001), and there was a trend for formate concentration to increase over time (p-adj <0.10) (Supplementary File 30). However, concentrations of succinate and lactate had the highest concentrations at the start of fermentation (p-adj <0.03) (Supplementary File 30).



LE Low breath methane emitter
HE High breath methane emitter

Figure 45 Principal component analysis plot of aqueous metabolites in fermentation samples coloured by fermentation time (left) and participant (right).
Left, time points are coloured as 0 h green, 3 h grey, 6 h red, 10 h yellow, 24 h blue, and 48 h purple. Right, participants are coloured 1 green, 2 blue, 3 red, 4 yellow, 5 bright blue, 6 purple, 7 orange, 8 pink, 9 brown, and 10 grey.

Conversely, time did not affect the concentration of gaseous metabolites, methane, and hydrogen, in the fermentation samples (p >0.10). Yet participant variation affected methane and hydrogen concentrations (p <0.02). Participants 7 and 8 had the highest peak area for methane, and there was no

significant production in samples from other participants (Figure 47). Participants 7 and 8 were the only participants that had detectable *Methanobrevibacter* abundance in Chapters 4 and 5.

Interactions between time, participants and substrate were observed for hydrogen sulphide concentration in the fermentation samples (Figure 46). Participants had a different sulphide concentration in fermentation samples at 0 h but were similar at 48 h (Figure 46, left), whereas similar sulphide concentration was observed at 0 h but differed at 48 h between substrates (Figure 46, right).

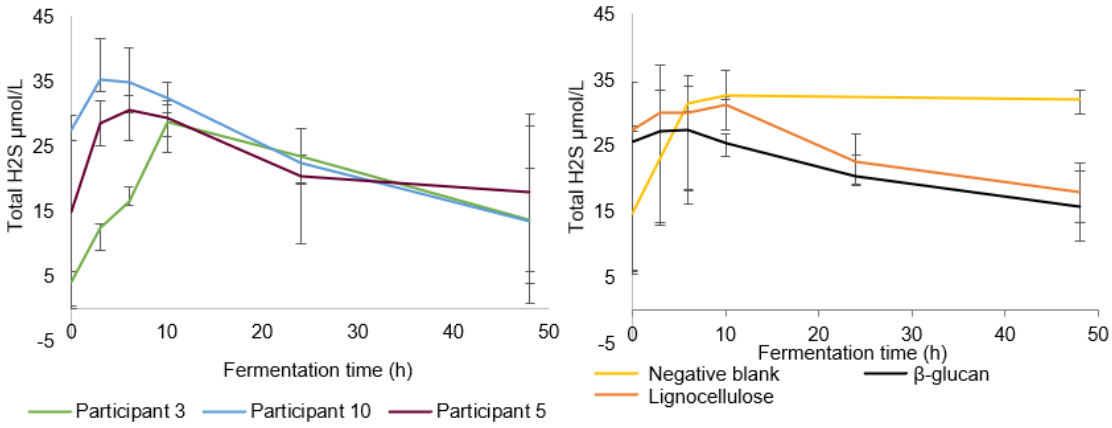


Figure 46 The median concentration of hydrogen sulphide in fermentation samples over 48 h of fermentation.
Left according to participants (3 green, 5 purple, 10 blue). Right according to substrates (yellow negative blank, orange lignocellulose and inoculum, and dark brown β-glucan and inoculum). Error bars are quartiles.

For some participants, the hydrogen peak area did not change over time in any treatments, others increased according to substrates compared to positive controls, and others had a decreased hydrogen peak area according to substrates compared to positive controls (Figure 47). Methane concentration for participants 7 and 8 was highest in the negative blank and substrate samples ($p < 0.05$), but there was no difference between β-glucan and lignocellulose (p

>0.10) (Figure 47). Conversely, aqueous metabolites differed according to the two substrates. β-glucan samples had higher concentrations of acetate, propionate, butyrate, formate, lactate, and succinate compared to lignocellulose samples (p-adj <0.05) from 0 to 10 h (Supplementary File 30). However, at the end of fermentation (24 to 48 h), only acetate and propionate remained more abundant in β-glucan samples (p-adj <0.05) (Supplementary File 30). Throughout fermentation, methanol was more abundant in lignocellulose samples compared to β-glucan samples (p-adj <0.05), and formate was also higher in lignocellulose samples at the end of fermentation (24 to 48 h) (p-adj <0.05) (Supplementary File 30).

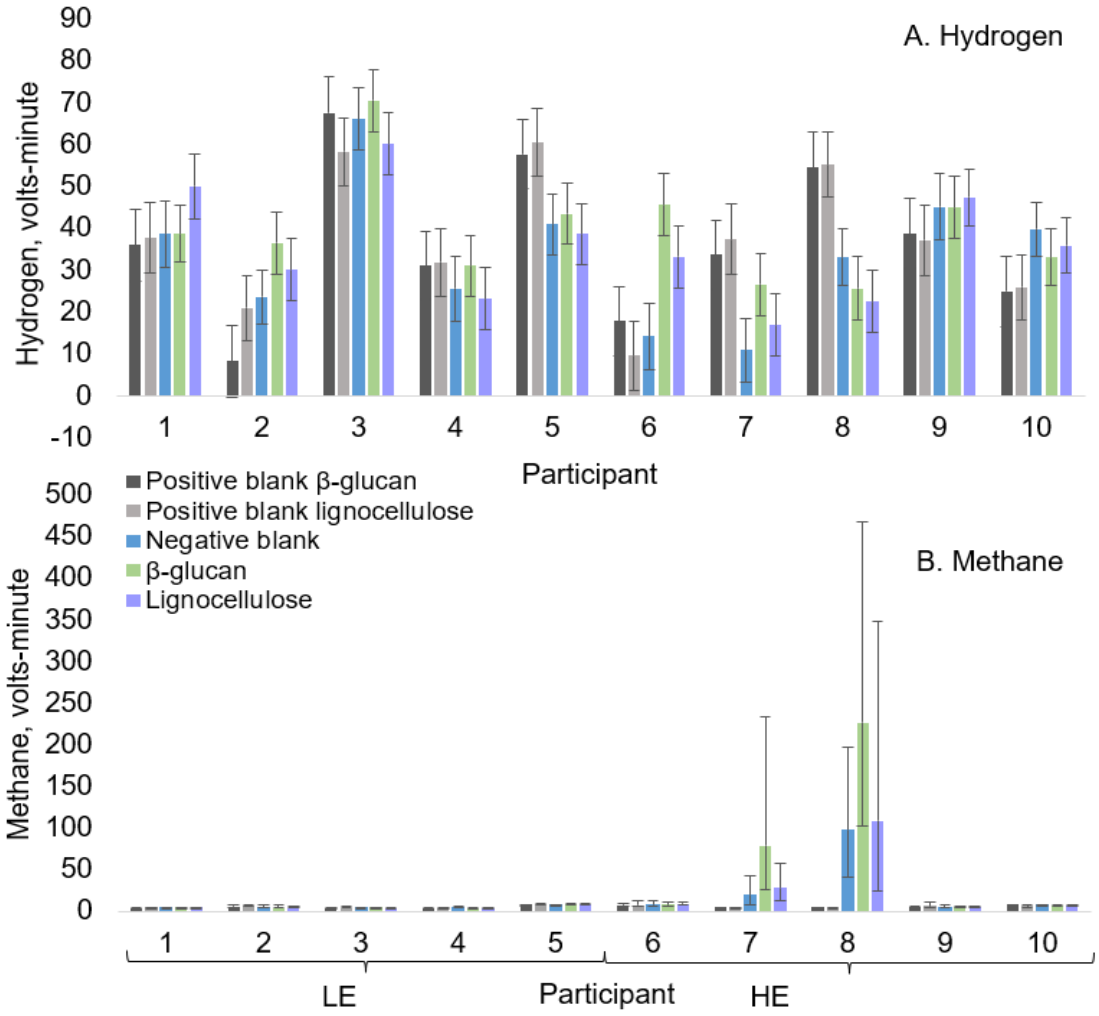


Figure 47 The participant × treatment interaction effect on hydrogen (A.) and methane (B.) peak area in fermentation samples (all time points).

Participants 1 to 5 were low-breath methane emitters (LE), and participants 6 to 10 were high-breath methane emitters (HE). Each coloured bar shows a different fermentation treatment: dark grey positive blank with a β-glucan, light grey positive blank with lignocellulose, blue negative blank (inoculum only), green β-glucan (substrate and inoculum), and light purple lignocellulose (substrate and inoculum). Data were modelled by Restricted Maximum Likelihood mixed effects and means separation generated using Fisher's LSD. Hydrogen peak area values are means with error bars as standard errors. Methane peak area data were Box-Cox transformed, but the values shown are reversed-transformed (medians with error bars as upper and lower quartiles).

6.5.5 Metabolites in Fermentation Samples from Individuals who Were High and Low Breath Methane Emitters

At 0 h, there was a greater concentration of butyrate in samples from LE participants compared to HE participants (p-adj <0.05), and there were trends for acetate, propionate and caproate to be higher in LE participant samples compared to the HE group (p-adj <0.05; p-adj <0.10), irrespective of the substrate (Figure 48). At 3 to 10 h, concentrations of acetate, propionate, butyrate and caproate remained higher in LE participant samples compared to the HE group (p-adj <0.05) (Figure 48). At 24 to 48 h, LE participant samples had higher concentrations of acetate, butyrate, caproate and formate compared to the HE group samples (p-adj <0.05). These results reflected those at other time points, except LE participant samples, which had a higher formate concentration, and no difference in propionate concentration (Figure 48). Furthermore, PCA showed that the interaction between breath methane and substrate explained a large amount of variation in the t2 and t3 axes (r 0.26) of aqueous metabolite data (Figure 49).

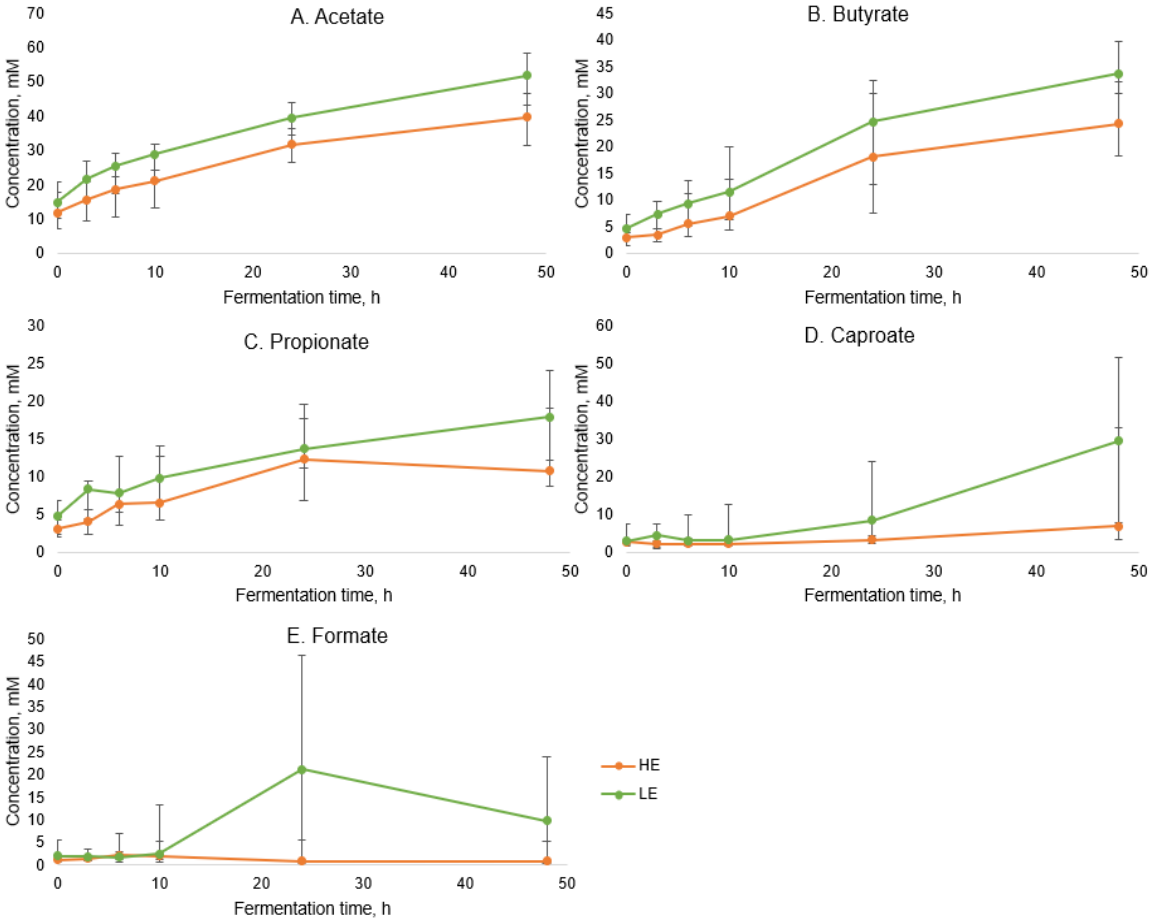


Figure 48 The median concentrations of acetate (A.), butyrate (B.), propionate (C.), caproate (D.), and formate (E.) in fermentation samples from 0 to 48 h of fermentation.
Orange lines represent fermentation samples (both substrates) from high breath methane emitters (HE), and green lines show fermentation samples (both substrates) from low breath methane emitters (LE). Error bars are quartiles.

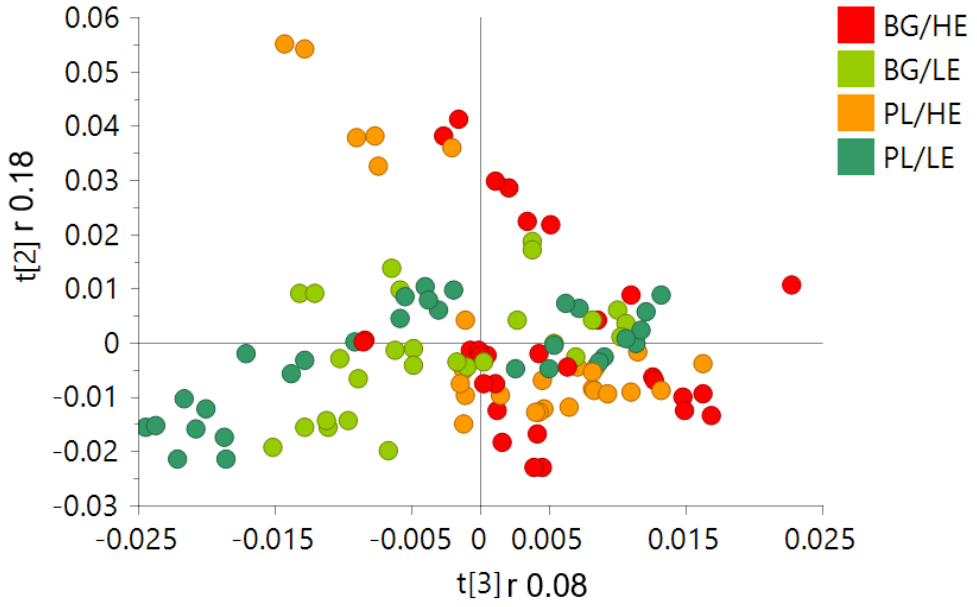


Figure 49 Principal component analysis plot of fermentation samples (all time points) coloured by substrate and breath methane.
Samples from high breath methane emitters (HE) with β-glucan (BG) and lignocellulose (PL) are shown in red and orange, respectively. Samples from low breath methane emitters (LE) with β-glucan (BG) and lignocellulose (PL) are shown in light green and dark green, respectively.

Samples from LE participants had a greater hydrogen peak area compared to samples from the HE group ($p < 0.001$), whereas samples from HE participants had a higher methane peak area than samples from the LE group ($p < 0.001$) (Figure 50) (Supplementary File 31). However, the methane peak area of the HE participant samples was driven by two of five participants, as shown by the high standard error bars (Figure 50). Samples from participants 7 and 8 had a final methane peak area of 336 volts-minute compared to 7.02 volts-minute for the rest of the HE participants. LE participants had a final methane peak area of 5.70 volts-minute (Supplementary File 31).

Spearman's correlation showed that there was no association between methane and hydrogen sulphide concentrations (total 216 samples) ($p > 0.05$, 95 % confidence intervals -0.026 to 0.303, r 0.14).

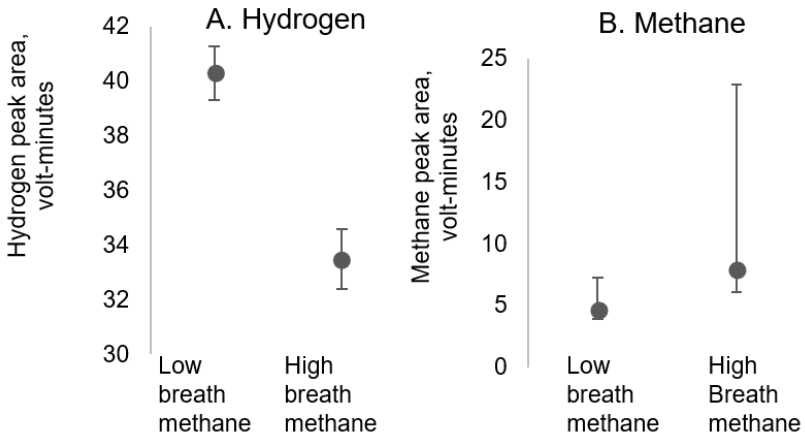


Figure 50 Hydrogen and methane peak area of fermentation samples with inoculum from low and high breath methane emitters.
Hydrogen data are means, and error bars are standard errors of the mean. Methane data are medians, and error bars are upper and lower quartiles.

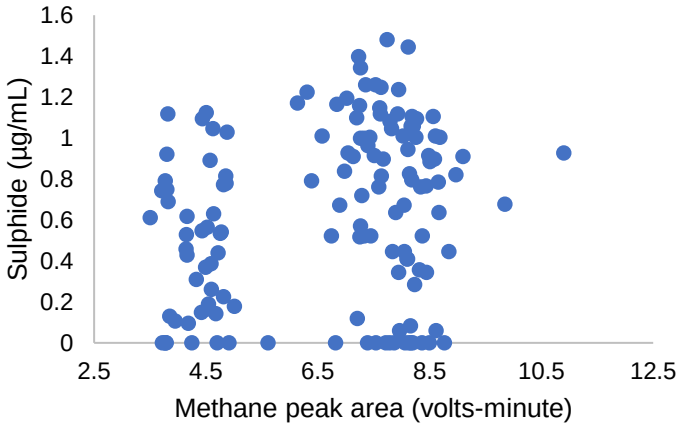


Figure 51 Headspace methane gas concentration and aqueous hydrogen sulphide concentration from fermentation samples (all fermentation treatments and all time points).

6.6 Discussion

6.6.1 Current Data Compared to the Scientific Literature

The most abundant metabolites in β-glucan and lignocellulose fermentations (all time points) were the organic acids acetate, propionate, and butyrate (>10 mM). Using median values at 0 h and multiplying by the inoculum dilution factor, the estimated concentrations of acetate, propionate, and butyrate in faecal samples were 80, 24, and 26 mM, respectively. These findings agree with published studies. Acetate, propionate, and butyrate are the most abundant metabolites (3:1:1) in the human colon (Topping and Clifton, 2001; Krautkramer, Fan and Bäckhed, 2021; Cummings *et al.*, 1987; Mowat and Agace, 2014) with concentrations in faecal samples of 40 to 115 mM for acetate and 12 to 27 mM for propionate and butyrate (Topping and Clifton, 2001; Machiels *et al.*, 2014). Ethanol, caproate, and formate were present at lower concentrations (5 to 10 mM), with minor concentrations of valerate, methanol, lactate, and succinate (<2 mM) (substrate treatments, all time points).

The pattern of hydrogen sulphide production during the fermentation of β-glucan or lignocellulose in the current study reflected that seen in other studies. Karppinen *et al.* (2000) saw increased sulphide concentrations during the first 3 to 4 h of *in vitro* polysaccharide fermentations, followed by a decline to the end of fermentation (Figure 52). The mechanism leading to decreases in hydrogen sulphide concentration in the latter part of the fermentation is unclear. Assimilatory sulphate reduction is where hydrogen sulphide is converted to cysteine using the enzyme O-acetylserine sulfhydrolase, a pathway abundant in bacteria and yeast (Kushkevych *et al.*, 2020). Furthermore, promoting assimilatory sulphate reduction might be a mechanism for reducing

potentially harmful hydrogen sulphide accumulation in the human colon (Kushkevych *et al.*, 2020). Further study will confirm whether fermentable dietary fibres promote assimilatory sulphate reduction.

Compared to the current study, other studies have reported higher concentrations of hydrogen sulphide during colonic fermentations with faecal inocula (Magee *et al.*, 2000; Gibson *et al.*, 1990). However, these were fermentations with protein instead of fibre, and protein fermentation produces higher concentrations of hydrogen sulphide than carbohydrate fermentation due to the provision of sulphur-containing amino acids (Magee *et al.*, 2000).

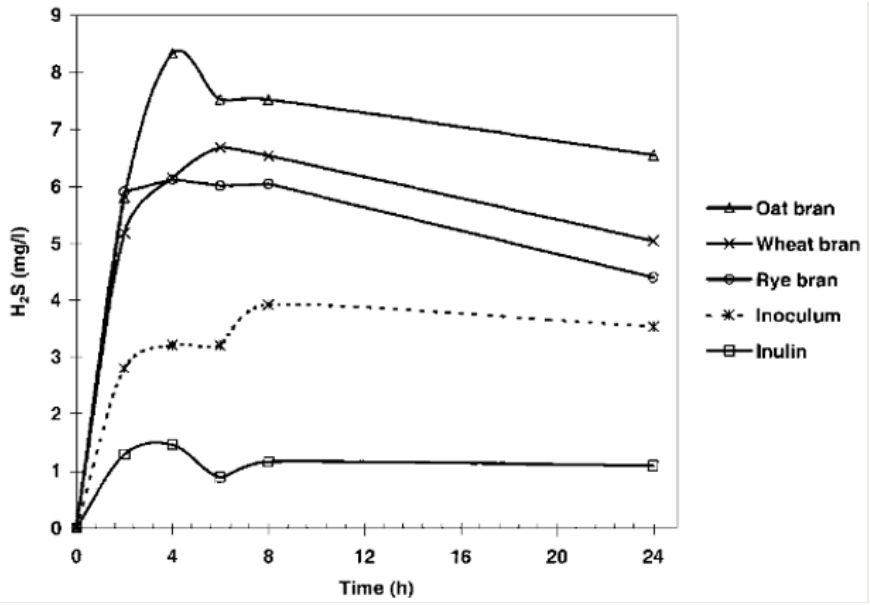


Figure 52 Hydrogen sulphide gas measured in the headspace of *in vitro* colonic fermentations with different substrates: oat bran, wheat bran, rye bran and inulin.
Data from Karppinen *et al.* (2000).

6.6.2 Participant Breath Methane and Fermentation Metabolites

6.6.2.1 Hypothesis One: There is a greater production of organic acids in samples from high breath methane emitters compared to low breath methane emitters

The current data disproved this hypothesis. In contrast, fermentation samples from LE participants had greater concentrations of acetate, butyrate, caproate, and occasionally propionate and formate than fermentation samples from HE participants, irrespective of the substrate. This finding is surprising because several studies suggest that individuals who were HE had a microbiota that was better adapted for fibre fermentation compared to LE individuals (Robert and Bernalier-Donadille, 2003; Chassard *et al.*, 2010; Kumpitsch *et al.*, 2020). The microbiota of individuals who were HE contained keystone species specialised for cellulose fermentation which were not present in LE individuals (Robert and Bernalier-Donadille, 2003; Chassard *et al.*, 2010). Organic acids are key metabolites of fibre fermentation, and their concentrations were expected to positively correlate with fibre fermentation, especially with lignocellulose fermentation. However, the lignocellulose used here also contained RS (see Chapter 5). Therefore, it is likely that the current organic acid data reflect some RS fermentation.

A similar study that measured organic acid concentrations during *in vitro* colonic fermentation with faecal inoculum from HE and LE individuals found that breath methane was associated with differences in lactulose fermentation but no other substrates. With lactulose, LE samples had the highest acetate concentration after 5 h, but by 24 h, samples from HE individuals had the

highest acetate concentration (Fernandes, Rao and Wolever, 2000). The authors suggested that breath methane was primarily linked to differences in organic acid concentration when substrates were fermented to acetate. However, they did not measure the composition of microbial communities, and there are hundreds of bacterial strains in the human colon that can utilise lactulose (Mao *et al.*, 2014). The current study did not corroborate the hypothesis of Fernandes *et al.* (2000) that breath methane affects acetate concentration.

There are several reasons why LE fermentation samples resulted in higher organic acid concentrations compared to HE samples. Firstly, an increased rate and extent of substrate fermentation in samples from LE individuals could lead to a higher production of organic acids. Secondly, there might be differences in metabolite cross-feeding that affected the net concentration of organic acids. For example, increased methanogenesis in HE participants could result in more acetate utilisation (Hobson and Stewart, 1997; reviewed by Payling *et al.*, 2020), lowering acetate concentration in the HE group samples. Furthermore, increased acetogenesis and sulphate reduction in LE samples could result in more acetate production (den Besten *et al.*, 2013; Flint *et al.*, 2015; Ríos-Covián *et al.*, 2016; Smith *et al.*, 2018; Payling *et al.*, 2020). Similarly, the concentration of propionate may have been greater in LE samples due to increased activity of the main propionate production pathway that converts succinate to propionate (Louis, Hold and Flint, 2014; Payling *et al.*, 2020).

6.6.2.2 Hypothesis Two: There is a greater production of methane gas in samples from high breath methane emitters compared to low breath methane emitters

It was expected that hydrogen would be produced from the substrate during the fermentation and that most of the hydrogen would be used for methanogenesis in HE, but not LE samples. However, there was no overall change in hydrogen concentration during the colonic fermentation. Some fermentations in the current study showed a decrease or plateau in hydrogen concentration over time. This effect of net hydrogen production may be due to hydrogen utilisation rather than hydrogen production. Hydrogen tension is one factor that determines hydrogen utilisation. Other fermentations with a hydrogen tension of 1 to 10 % showed that hydrogen concentration reduced by 25 % over time (Strocchi and Levitt, 1992). In the current study, the starting hydrogen tension was 5 %, above the expected levels reported for the colon of 3.5 % (Kalantar-Zadeh *et al.*, 2018).

Furthermore, higher hydrogen tension could have disadvantaged methanogens in competing for hydrogen. Hydrogen utilisation by methanogens occurs at a lower hydrogen tension than hydrogen utilisation by hydrogenotrophic bacteria (Strocchi and Levitt, 1992). In future studies, more advanced systems may be available where the hydrogen tension can be adjusted to accurately reflect colonic levels.

Other studies have reported that fermentations with faecal inoculum from HE individuals had a greater hydrogen consumption than those from LE individuals (Bauchop and Mountfort, 1981; Strocchi and Levitt, 1992). Indeed, samples from HE individuals had a lower hydrogen concentration than those

from LE individuals in the current study, which may reflect greater hydrogen consumption in HE samples.

It was surprising that inconsistent methane production was observed in samples from HE participants. Whilst methane production was observed in four HE participants and one LE participant, only two HE samples showed large increases in methane. The NMR metabolite data did not explain these observations. One explanation is that the pH of the samples (started at approximately 6.6 and reduced over the 48 h of fermentation, Chapter 5) was too low for optimal methanogen growth and activity, which occurs at pH 6.8 to 7.2 (Reungsang, Pattra and Sittijunda, 2012).

In addition, studies reported variable abundances of *M. smithii* in faecal samples from HE and LE individuals (Kumpitsch *et al.*, 2020), which could allow for a low abundance of methanogens in some samples from HE individuals. Furthermore, methanogens are oxygen-sensitive and, therefore, difficult to culture (Traore *et al.*, 2019), so they could have been cultured inconsistently in the current study.

6.6.2.3 Hypothesis Three: There is a negative correlation between methane gas production and hydrogen sulphide production

The data obtained here did not support the hypothesis that there was a negative correlation between methane gas production and hydrogen sulphide production. In the late eighties, Macfarlane *et al.* showed that SRB were only enumerated in samples that did not produce methane during *in vitro* colonic fermentation with LE faecal inoculum (Macfarlane, 1988; Gibson, Macfarlane, and Cummings, 1993). However, Braccia *et al.* (2021) found that microbial

genes for hydrogen sulphide production and methanogenesis were co-expressed in approximately 20 % of human colonic samples. These observations were confirmed with data from other habitats like sediment and wastewater, where there is coexistence and synergy of sulphate reducing bacteria and methanogens (Oremland and Polcin, 1982; Konhauser *et al.*, 2017; Shi *et al.*, 2020). These authors suggested that SRB outcompete methanogens for hydrogen and acetate but do not compete for methanol, trimethylamine, or methionine (Oremland and Polcin, 1982).

Together the observations from this study and other studies suggest that future research should focus on the association between methane and hydrogen sulphide in a larger cohort of HE and LE individuals. Methods should also be used to understand the substrate utilisation strategies of methanogens and sulphate-reducers in the colonic environment.

6.6.3 Strengths and Limitations

The current study is the first to use a multi-faceted approach, including solution-state NMR, gas chromatography, and spectrophotometry, to characterise the production of aqueous and gaseous metabolites during the colonic fermentation of faecal HE and LE microbiota in the presence of β-glucan and lignocellulose. Since fibre fermentation is an important function of the colonic microbiota, these data could impact the ongoing investigation of whether breath methane and methanogen abundance are markers of health or disease (Pimentel *et al.*, 2003; Ghavami *et al.*, 2018).

However, using the hydrogen sulphide data to compare production in HE and LE samples during *in vitro* colonic fermentation was impossible. Only three participants were included in the measurement due to unforeseen laboratory

disruptions related to the COVID-19 pandemic. Furthermore, the detection of high methane production in samples from only two of five individuals who were HE during *in vitro* colonic fermentation reduced the strength of comparison of the fermentation profiles of HE and LE samples.

Breath methane was not an accurate predictor of *in vitro* methane gas production, and selecting individuals based on their baseline microbiota composition may allow a better match to the desired phenotype (Johnson *et al.*, 2020). The concentration of gas standards was also selected based on published research; however, the peak area data was below the lowest standard concentration, resulting in a sub-optimal standard curve. Therefore, headspace gas data was presented as the original peak area data, which was difficult to compare to published concentration data.

6.7 Conclusion

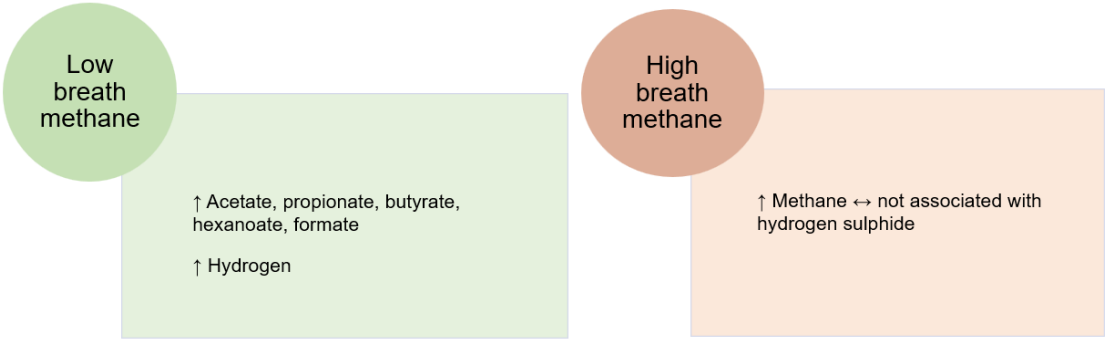


Figure 53 The key differences in gaseous and aqueous metabolites in fermentation samples with faecal inoculum from high breath methane emitters (orange) and low breath methane emitters (green).

The analysis of aqueous and gaseous metabolites from *in vitro* colonic fermentations with faecal inocula from HE and LE participants showed differences in fibre fermentation metabolites between the two groups (Figure 53). Firstly, fermentation samples from LE individuals had a greater abundance

of organic acids compared to samples from HE individuals, which was consistent across substrate and time point. Secondly, fermentation samples from LE individuals had a higher hydrogen concentration than samples from HE individuals, and samples from HE individuals had a greater methane concentration than samples from LE individuals, although methane concentration was inconsistent. Methane concentration in the fermentation samples was not associated with hydrogen sulphide concentration, which could be due to the small sample size.

These findings showed some unexpected differences between the metabolites produced by the faecal microbiota of HE and LE participants during *in vitro* colonic fermentation. However, the measurement of these metabolites during *in vitro* colonic fermentation only captures part of the complex and dynamic cross-feeding of metabolites occurring in the microbial community. Integration of the metabolite data with the relative abundance of taxa from the microbial communities might help to identify characteristics of the microbiota that are associated with differences in fibre fermentation in individuals who are LE and HE.

References

1. Agus, A., Clément, K., and Sokol, H. (2021). Gut microbiota-derived metabolites as central regulators in metabolic disorders. Gut 70, 1174–1182. https://doi.org/10.1136/gutjnl-2020-323071.
2. Bauchop, T., and Mountfort, D.O. (1981). Cellulose fermentation by a rumen anaerobic fungus in both the absence and the presence of rumen methanogens. Applied and Environmental Microbiology 42, 1103–1110. https://doi.org/10.1128/aem.42.6.1103-1110.1981.

3. Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society 57(1), 289–300.

4. Bernalier, A., Lelait, M., Rochet, V., Grivet, J.-P., Gibson, G. R., and Durand, M. (1996). Acetogenesis from H2 and CO2 by methane- and non-methane-producing human colonic bacterial communities. FEMS Microbiology Ecology, 19(3), 193–202. https://doi.org/10.1111/j.1574-6941.1996.tb00212.x.

5. den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.-J., and Bakker, B.M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. Journal of Lipid Research 54, 2325–2340. https://doi.org/10.1194/jlr.R036012.

6. Box, G.E., and Cox, D.R. (1964). An analysis of transformations. Journal Of the Royal Statistical Society 26, 211–252. https://doi.org/10.1080/01621459.1982.10477788.

7. Braccia, D.J., Jiang, X., Pop, M., and Hall, A.B. (2021). The Capacity to Produce Hydrogen Sulfide (H2S) via Cysteine Degradation Is Ubiquitous in the Human Gut Microbiome. Frontiers in Microbiology 12, 1–31. https://doi.org/10.3389/fmicb.2021.705583.

8. Chassard, C., Delmas, E., Robert, C., and Bernalier-Donadille, A. (2010). The cellulose-degrading microbial community of the human gut varies according to the presence or absence of methanogens. FEMS Microbiology Ecology 74, 205–213. https://doi.org/10.1111/j.1574-6941.2010.00941.x.

9. Cummings, J.H., Pomare, E.W., Branch, W.J., Naylor, C.P.E., and Macfarlane, G.T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut 28, 1221–1227.

10. Emwas, A.-H.M. (2015). The Strengths and Weaknesses of NMR Spectroscopy and Mass Spectrometry with Particular Focus on Metabolomics Research. In Metabonomics: Methods and Protocols, J.T. Bjerrum, ed. (New York, NY: Springer New York), pp. 161–193.

11. Emwas, A.H., Saccenti, E., Gao, X., McKay, R.T., dos Santos, V.A.P.M., Roy, R., and Wishart, D.S. (2018). Recommended strategies for spectral processing and post-processing of 1D 1 H-NMR data of biofluids with a particular focus on urine. Metabolomics 14, 1–23. https://doi.org/10.1007/s11306-018-1321-4.

12. Fernandes, J., Rao, A.V., and Wolever, T.M.S. (2000). Different substrates and methane producing status affect short-chain fatty acid profiles produced by vitro fermentation of human feces. Journal of Nutrition 130, 1932–1936. https://doi.org/10.1093/jn/130.8.1932.

13. Flint, H.J., Duncan, S.H., Scott, K.P., and Louis, P. (2015). Links between diet, gut microbiota composition and gut metabolism. Proceedings of the Nutrition Society 74, 13–22. https://doi.org/10.1017/S0029665114001463.

14. Ghavami, S.B., Rostami, E., Sephay, A.A., Shahrokh, S., Balaii, H., Aghdaei, H.A., and Zali, M.R. (2018). Alterations of the human gut Methanobrevibacter smithii as a biomarker for inflammatory bowel diseases. Microbial Pathogenesis 117, 285–289. https://doi.org/10.1016/j.micpath.2018.01.029.

15. Gibson, G., Macfarlane, G.T., and Cummings, J. (1993). Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut 34, 437–439.

16. Gibson, G.R., Cummings, J.H., Macfarlane, G.T., Allison, C., Segal, I., Vorster, H.H., and Walker, A.R. (1990). Alternative pathways for hydrogen disposal during fermentation in the human colon. Gut 31, 679–683. https://doi.org/10.1136/gut.31.6.679.

17. Hobson, P.N., and Stewart, C.S. (1997). The Rumen Microbial Ecosystem (Dordrecht: Springer).

18. IBM (2021). Linear Mixed Models Estimation (online). https://www.ibm.com/docs/en/spss-statistics/28.0.0?topic=models-linear-mixed-estimation.

19. Janssen, P. (1975). A modified method for measuring hydrogen sulphide with spectrophotometry (Unpublished laboratory records, Palmerston North, New Zealand).

20. Johnson, A.J., Zheng, J.J., Kang, J.W., Saboe, A., Knights, D., and Zivkovic, A.M. (2020). A Guide to Diet-Microbiome Study Design. Frontiers in Nutrition 7, 1–16. https://doi.org/10.3389/fnut.2020.00079.

21. Kalantar-Zadeh, K., Berean, K.J., Ha, N., Chrimes, A.F., Xu, K., Grando, D., Ou, J.Z., Pillai, N., Campbell, J.L., Brkljača, R., *et al.* (2018). A human pilot trial of ingestible electronic capsules capable of sensing different gases in the gut. Nature Electronics 1, 79–87. https://doi.org/10.1038/s41928-017-0004-x.

22. Konhauser, K.O., van Hullebusch, E.D., Egger, M., Sela-Adler, M., Ronen, Z., Herut, B., Antler, G., Vigderovich, H., Eckert, W., and Sivan, O. (2017). Co-existence of Methanogenesis and Sulfate Reduction with Common Substrates in Sulfate-Rich Estuarine Sediments. Frontiers in Microbiology 8, 766. https://doi.org/10.3389/fmicb.2017.00766.

23. Kumpitsch, C., Fischmeister, F. P. S., Mahnert, A., Lackner, S., Wilding, M., Sturm, C., Holasek, S., Högenauer, C., Berg, I., Schöpf, V., and Moissl-Eichinger, C. (2020). Methane emission of humans is explained by dietary habits, host genetics, local formate availability and a uniform archaeome. BioRxiv 1–45. https://doi.org/10.1101/2020.12.21.423794.

24. Kushkevych, I., Cejnar, J., Treml, J., Dordević, D., Kollar, P., and Vítězová, M. (2020). Recent Advances in Metabolic Pathways of Sulfate Reduction in Intestinal Bacteria. Cells 9, 1–16. https://doi.org/10.3390/cells9030698.

25. Lee, S., and Lee, D.K. (2018). What is the proper way to apply the multiple comparison test? Korean Journal of Anesthesiology 71, 353–360. https://doi.org/10.4097/kja.d.18.00242.

26. Louis, P., Hold, G.L., and Flint, H.J. (2014). The gut microbiota, bacterial metabolites and colorectal cancer. Nature Reviews Microbiology 12, 661–672. https://doi.org/10.1038/nrmicro3344.

27. Macfarlane, G. (1988). Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut. Journal of Applied Bacteriology 65, 103-111.

28. Machiels, K., Joossens, M., Sabino, J., de Preter, V., Arijs, I., Eeckhaut, V., Ballet, V., Claes, K., van Immerseel, F., Verbeke, K., Ferrante, M., Verhaegen, J., Rutgeerts, P., and Vermeire, S. (2014). A decrease of the butyrate-producing species roseburia hominis and faecalibacterium prausnitzii defines dysbiosis in patients with ulcerative colitis. Gut, 63(8), 1275–1283. https://doi.org/10.1136/gutjnl-2013-304833.

29. Magee, E.A., Richardson, C.J., Hughes, R., and Cummings, J.H. (2000). Contribution of dietary protein to sulfide production in the large intestine: An *in vitro* and a controlled feeding study in humans. American Journal of Clinical Nutrition 72, 1488–1494. https://doi.org/10.1093/ajcn/72.6.1488.

30. Mao, B., Li, D., Zhao, J., Liu, X., Gu, Z., Chen, Y.Q., Zhang, H., and Chen, W. (2014). *In vitro* fermentation of lactulose by human gut bacteria. Journal of Agricultural and Food Chemistry 62, 10970–10977. https://doi.org/10.1021/jf503484d.

31. Mowat, A.M., and Agace, W.W. (2014). Regional specialization within the intestinal immune system. Nature Reviews Immunology 14, 667–685. https://doi.org/10.1038/nri3738.

32. Muetzel, S., Ronimus, R.S., Lunn, K., Kindermann, M., Duval, S., and Tavendale, M. (2018). A small-scale rumen incubation system to screen chemical libraries for potential methane inhibitors. Animal Feed Science and Technology 244, 88–92. https://doi.org/10.1016/j.anifeedsci.2018.08.001.

33. Oremland, R.S., and Polcin, S. (1982). Methanogenesis and Sulfate Reduction: Competitive and Noncompetitive Substrates in Estuarine Sediments. Applied and Environmental Microbiology 44(6), 1270-1276.

34. Osborne, J.W. (2010). Improving your data transformations: Applying the Box-Cox transformation. Practical Assessment, Research and Evaluation 15, 12. https://doi.org/10.7275/qbpc-gk17.

35. Pang, Z., Chong, J., Zhou, G., De Lima Morais, D.A., Chang, L., Barrette, M., Gauthier, C., Jacques, P.É., Li, S., and Xia, J. (2021). MetaboAnalyst 5.0: Narrowing the gap between raw spectra and functional insights. Nucleic Acids Research 49, W388–W396. https://doi.org/10.1093/nar/gkab382.

36. Payling, L., Fraser, K., Loveday, S.M., Sims, I., Roy, N., and McNabb, W. (2020). The effects of carbohydrate structure on the composition and functionality of the human gut microbiota. Trends in Food Science and Technology 97, 233–248. https://doi.org/10.1016/j.tifs.2020.01.009.

37. Payling, L., Roy, N.C., Fraser, K., Loveday, S.M., Sims, I.M., Stefan, J., Laura, G., and Warren, C. (2021). A protocol combining breath testing and ex vivo fermentations to study the human gut microbiome. STAR Protocols 100227. https://doi.org/10.1016/j.xpro.2020.100227.

38. Pimentel, M., Mayer, A.G., Park, S., Chow, E.J., Hasan, A., and Kong, Y. (2003). Methane production during lactulose breath test is associated with gastrointestinal disease presentation. Digestive Diseases and Sciences 48, 86–92. https://doi.org/10.1023/A:1021738515885.

39. Reungsang, A., Pattra, S., and Sittijunda, S. (2012). Optimization of key factors affecting methane production from acidic effluent coming from the sugarcane juice hydrogen fermentation process. Energies (Basel) 5, 4746–4757. https://doi.org/10.3390/en5114746.

40. Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de los Reyes-Gavilán, C.G., and Salazar, N. (2016). Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. Frontiers in Microbiology 7, 1–9. https://doi.org/10.3389/fmicb.2016.00185.

41. Robert, C., and Bernalier-Donadille, A. (2003). The cellulolytic microflora of the human colon: Evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects. FEMS Microbiology Ecology 46, 81–89. https://doi.org/10.1016/S0168-6496(03)00207-1.

42. Rombouts, C., Hemeryck, L.Y., Van Hecke, T., De Smet, S., De Vos, W.H., and Vanhaecke, L. (2017). Untargeted metabolomics of colonic digests reveals kynurenine pathway metabolites, dityrosine and 3-dehydroxycarnitine as red versus white meat discriminating metabolites. Scientific Reports 7, 1–13. https://doi.org/10.1038/srep42514.

43. Shi, X., Gao, G., Tian, J., Wang, X.C., Jin, X., and Jin, P. (2020). Symbiosis of sulfate-reducing bacteria and methanogenic archaea in sewer systems. Environment International 143. https://doi.org/10.1016/j.envint.2020.105923.

44. Smith, N.W., McNabb, W.C., Roy, N.C., Altermann, E.H., and Shorten, P.R. (2018). Hydrogen cross-feeders of the human gastrointestinal tract. Gut Microbes 1–19. https://doi.org/10.1080/19490976.2018.1546522.

45. Strocchi, A., and Levitt, M.D. (1992). Factors affecting hydrogen production and consumption by human fecal flora: The critical roles of hydrogen tension and methanogenesis. Journal of Clinical Investigation 89, 1304–1311. https://doi.org/10.1172/JCI115716.

46. The European Bioinformatics Institute (2021). Comparison of NMR and MS (online). https://www.ebi.ac.uk/training/online/courses/metabolomics-introduction/designing-a-metabolomics-study/comparison-of-nmr-and-ms/.

47. Topping, D.L., and Clifton, P.M. (2001). Short-chain fatty acids and human colonic function: Roles of resistant starch and nonstarch polysaccharides. Physiological Reviews 81, 1031–1064. https://doi.org/10.1152/physrev.2001.81.3.1031.

Chapter 7
General Discussion

7.1 Introduction

By contributing to the knowledge of colonic microbiota ecology and function, this thesis aimed to generate scientific evidence that can be used to modulate the microbiota for human health. The colonic microbiota is critical for human health (Sommer and Bäckhed, 2013), and diet is a key driver of microbiota variation. However, the mechanisms by which dietary fibre components impact the colonic microbiota, and the subsequent effects on the production of important microbial metabolites, are not well understood (Johnson *et al.*, 2020).

The literature review in Chapter 2 highlighted that breath methane might be linked to dietary factors and the composition and function of the colonic microbiota (Robert and Bernalier-Donadille, 2003; Chassard *et al.*, 2010; Hoffmann *et al.*, 2013; Kumpitsch *et al.*, 2020). Therefore, this research aimed to use a multi-faceted approach to investigate the links between human breath methane and the composition and fibre fermentation function of the colonic microbiota. The research will aid the understanding of breath methane in humans, which may help to clarify whether it is associated with health or disease.

Faecal samples were used as a proxy for the colonic microbiota, a commonly used approach due to the invasive nature of colonic sampling. Faeces do not reflect the variation in microbiota along the colon, but general compositional similarities between faecal microbiota and the distal colon microbiota make this method suitable for the current research (Flint *et al.*, 2012; Venema, 2015). The key methods used were breath testing, *in vitro* colonic fermentation, shotgun metagenomics and solid- and solution-state NMR. The findings

discussed in this Chapter are graphically represented in Figure 54.

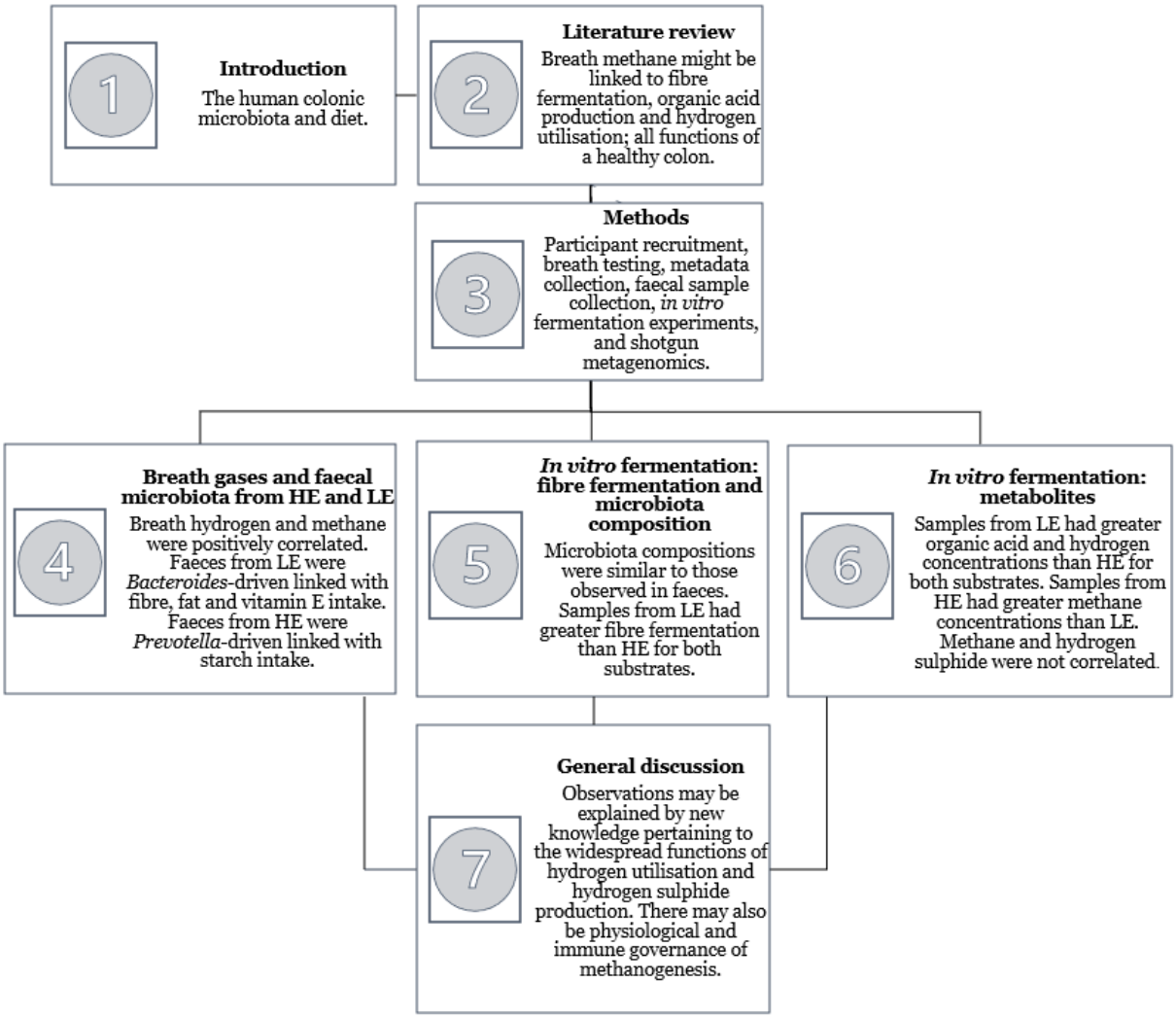


Figure 54 The main findings of the thesis.

7.2 Breath Methane and Microbiota Composition

Several studies have shown that individuals who are HE have higher dietary fibre and carbohydrate intakes, and their faecal samples have a greater abundance of taxa from the Firmicutes phylum and an increased capacity for cellulose fermentation compared to individuals who are LE (Robert and Bernalier-Donadille, 2003; Chassard *et al.*, 2010; Kumpitsch *et al.*, 2020; Ruaud *et al.*, 2020). However, the current study did not find that faecal samples

from HE had a greater abundance of taxa from the Firmicutes phylum or greater fibre fermentation.

The faecal and fermentation samples described in Chapters 4 and 5 showed that microbial communities from HE and LE were *Prevotella*- and *Bacteroides*-driven, respectively. These genera are both from the Bacteroidetes phylum. The findings were aligned with the dietary data in that the *Bacteroides*-driven composition of LE samples was linked with higher dietary fat intakes, and the *Prevotella*-driven composition of HE samples was linked with higher starch intakes. Such associations are frequently reported in the literature since high-fat and -protein diets promote bile-tolerant taxa from the *Bacteroides* genus, whilst taxa from the *Prevotella* genus utilise dietary starch and fibre (Flint and Stewart, 1999; Arumugam *et al.*, 2011; Rinninella *et al.*, 2019). Furthermore, high-starch diets allow non-bile tolerant taxa from the *Ruminococcus* genus to thrive (Arumugam *et al.*, 2011; Cho and Blaser, 2012; Rinninella *et al.*, 2019). In the current study, HE individuals had greater dietary starch intake and higher abundances of non-bile tolerant *R. bromii* during *in vitro* colonic fermentation; this bacterium is a known utiliser of dietary starch (Ze *et al.*, 2012, 2015).

In the current study, solid-state NMR showed that the faecal inocula from LE individuals had a greater abundance of aromatic compounds than the faecal inoculum from HE individuals at 0 and 48 h of fermentation, suggesting differences in amino acid concentrations (Parthasarathy *et al.*, 2018). Approximately 3.5 g of endogenous protein enters the colon daily, but most of it is metabolised by the resident microbiota (Starck, Wolfe and Moughan, 2018). The metagenome of faeces from LE individuals and *in vitro*

fermentation samples showed a decreased gene abundance of amino acid metabolic pathways relative to HE counterparts. These findings might explain the higher concentration of amino acids in samples from LE individuals at 0 and 48 h.

A major limitation of the research was that only two of five HE participants had a detectable level of the *Methanobrevibacter* genus in faeces or *in vitro* colonic fermentation samples. It is accepted that breath methane emission is stable in individuals over the long term, and this stability has been shown for 73 to 93 % of cohorts in other studies (Levitt and Slavin, 1991; le Marchand *et al.*, 1992; Strocchi *et al.*, 1994). However, other studies have argued that stability depends on the intake of carbohydrates and fibre (Levitt and Slavin, 1991; Hoffmann *et al.*, 2013). Therefore, the high breath methane detected in HE individuals may not have represented a long-term phenotype in all participants.

In a cross-over study of 24 participants, Levitt and Slavin (1991) attempted to modulate breath methane emission using high dietary fibre intakes. The breath methane concentrations of all participants changed, but only 17 % of participants were reclassified based on the 5-ppm cut-off. Furthermore, the exhalation of gases in breath depends upon gas accumulation in the colon. When colonic gas accumulation is low, approximately 90 % of colonic gases are exhaled in breath, but as colonic gas accumulation increases, this exhalation in breath reduces to approximately 20 %, with most being expelled in flatus (Hammer, 1993).

Changes in colonic gas excretion patterns could contribute to decreased breath methane associated with increasing dietary fibre (Levitt and Slavin, 1991). Furthermore, inter-individual differences in dietary fibre intake and transit

time potentially impacted gas accumulation in the colon and the proportion of gas excreted in breath compared to flatus. For example, some participants may have consumed low-fibre diets and/or had a long transit time, reducing colonic gas accumulation after 12 h of fasting and resulting in high breath methane concentration relative to the abundance of colonic methanogens. In addition, recent studies have suggested that methanogens in dental plaques can contribute to breath methane emission (Nkamga, Henrissat and Drancourt, 2017; Erdrich *et al.*, 2021), which could inflate breath methane emission compared to the abundance of colonic methanogens.

Recent studies have classified HE and LE individuals based on faecal methanogen counts rather than breath (Ozato *et al.*, 2020), which may be more accurate for future studies. This observation aligns with the approach Johnson *et al.* (2020) described, where the baseline faecal microbiota composition is assessed to select participants that form a homogeneous group. Based on that approach, the current study would have two HE participants with detectable methanogens and eight LE participants with no detectable methanogens.

Another useful approach would be to control for fibre intake between individuals that are HE and LE. Previous studies have found that individuals who are HE have higher dietary fibre intake and a microbiota composition suited for insoluble fibre fermentation. However, the current study found that individuals who were LE had greater dietary fibre intake and greater fermentation of insoluble substrates *in vitro*. Therefore, it is necessary to control fibre intake in future studies to better separate the effects of dietary fibre and human breath methane.

7.3 Breath Methane and Fibre Fermentation

The pH and total gas production data reported in Chapter 5 suggested that faecal inocula from LE samples were able to ferment lignocellulose to a greater extent and had a faster initial rate of β-glucan fermentation compared to inoculum from HE over 48 h of fermentation. The metabolite data from Chapter 6 supported this observation with consistently higher concentrations of acetate, propionate, butyrate, caproate, formate, and hydrogen in samples from LE participants with both fermentation substrates. This finding was unexpected, as other studies found that HE individuals had a microbiota better adapted for cellulose fermentation than LE individuals (Robert and Bernalier-Donadille, 2003; Chassard *et al.*, 2010). However, most studies have used isolated cultures or sequencing of one faecal sample, which have a limited representation of complex or metabolically-active communities. The current study is the first to analyse microbial communities and metabolites in samples from HE and LE individuals over 48 h of *in vitro* colonic fibre fermentation. Novel findings were observed between HE and LE groups concerning fermentations with β-glucan or lignocellulose.

A possible mechanism for the increased fibre fermentation in the LE group during *in vitro* colonic fermentation relates to higher dietary fibre intakes (Chapter 4) and faecal amino acid concentrations (Chapter 5) compared to the HE group. Pig studies have shown that increased soluble dietary fibre reduces the retention time and thus digestibility of protein in the small intestine and delivers twice as much undigested protein to the colon as diets without soluble fibre (Zhang *et al.*, 2015; Gidley, 2013). Furthermore, dietary fibre reduces the flow and mixing of digesta, thereby reducing macronutrient digestion and

increasing the delivery of undigested protein to the colon (Lentle and Janssen, 2008). When combined with fermentable fibre, more protein in the colon increases the abundance of fibre-degrading microorganisms by supporting cellular replication (Belobrajdic *et al.*, 2012; Gidley, 2013) (Figure 55).

7.4 Hydrogen Utilisation

The breath hydrogen and *in vitro* hydrogen data suggested that methanogenesis was a hydrogen sink in some individuals who were HE but not in individuals who were LE. The expectation was that sulphate reduction would be a key hydrogen sink in individuals who were LE, but the limited dataset made this difficult to test. Interestingly, the *Prevotella*-driven microbiota of individuals who were HE co-occurred with known sulphate-reducing genera *Desulfovibrio* and *Bilophila* in faecal samples and *in vitro,* as shown by also others (Arumugam *et al.*, 2011; Rinninella *et al.*, 2019). The co-occurrence of sulphate reduction and methanogenesis contradicts the widely-accepted notion that these processes are competing and mutually exclusive pathways in the colon (Macfarlane, 1988; Gibson, Macfarlane, and Cummings, 1993).

Recent literature has suggested that dissimilatory sulphate reduction by taxa from the *Desulfovibrio* and *Bilophila* genera may not be the dominant hydrogen sulphide-producing pathway in the human colon but that cysteine fermentation may be more important. Bioinformatic exploration of the human colonic metagenome found that more than 18 % of species had genes for cysteine fermentation compared to less than 1 % for sulphate reduction (Braccia *et al.*, 2021). Furthermore, faecal samples from more than 6,000 healthy individuals had a higher relative abundance of cysteine-degrading bacteria than SRB (Braccia *et al.*, 2021). Therefore, previous sulphate reduction and

methanogenesis findings may be irrelevant to total hydrogen sulphide production. Indeed, the current study did not find any association between methane and hydrogen sulphide. In addition, Braccia *et al.* (2021) found that microbial genes for hydrogen sulphide production and methanogenesis were co-expressed in approximately 20 % of samples. Together these findings support the small but growing body of literature challenging the exclusivity of hydrogen sulphide and methane production in the human colon.

Wolf *et al.* (2016) suggested that methanogenesis, sulphate reduction and acetogenesis are only fractional sinks of colonic hydrogen, contrary to common belief. The most common hydrogenases in the human colon were related to electron bifurcation and hydrogen sensing, of which more than 70 % occurred in taxa from the Bacteroidetes phylum. Whilst acetogenesis also utilises electron bifurcation, acetogens are of the Firmicutes phylum, constituting less than 25 % of the hydrogenase genes in the human colon. Furthermore, most hydrogen might be immediately recycled without entering the colonic hydrogen pool (Wolf *et al.*, 2016). This finding calls for novel approaches to monitor subtle changes in hydrogen that occur without affecting net gas concentrations. High-throughput hydrogenase assays may support quantifying hydrogenase activity in the colonic microbiota (Lacasse *et al.*, 2019).

Finally, rumen data have suggested alternative physiological strategies that may affect methanogenesis in the human colon. Leng (2018) suggested that gas production is endogenously monitored and controlled by the immune system in ruminants that are HE compared to LE. In humans, microbial biofilms develop in association with the mucosal lymphoid tissue of the appendix, and these biofilms inoculate digesta passing through the colon (Bollinger *et al.*,

2007). It has been hypothesised that these biofilms contain moieties that kill or inhibit methanogens: antimicrobial peptides, immunoglobulins, innate lymphoid cells, and mucin (Leng, 2018). Furthermore, innate lymphoid cells, which are known to have a role in maintaining an optimal symbiotic microbiota composition, undergo cell population expansion in response to organic acids produced from fibre fermentation (Sepahi *et al.*, 2020) (Figure 55). In ruminants, it has already been suggested that innate lymphoid cells have a role in suppressing methanogenesis (Leng, 2018).

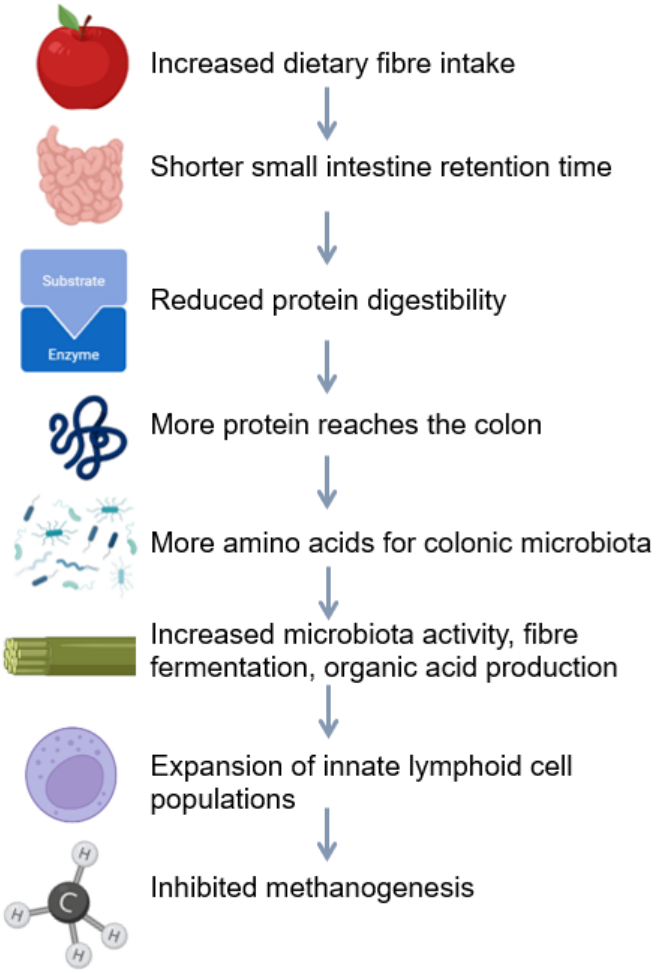


Figure 55 The hypothesised mechanism for differences in the dietary patterns, colonic microbiota fermentation, and methane production in individuals who were low breath methane emitters compared to high breath methane emitters.

Bang *et al.* (2014) showed that methanogens are recognised by the innate immune system of humans, causing the release of proinflammatory cytokines, activation of the adaptive immune response, and increased expression of antimicrobial peptides. Through gas sensing, the colon may receive feedback of excessive gas, which can stimulate colonic immune secretions to suppress the organisms responsible for gas generation (Leng, 2018). Currently, there is little research to support this hypothesis. However, Wolf *et al.* (2016) found that genes for hydrogen sensing were highly prevalent in the human colonic microbiota. The hypothesis may also help to explain the positive association often observed between methanogen abundance and constipation symptoms. If immune agents secreted from the colonic mucosa or appendix are responsible for reductions in methanogen abundance and activity, their effectiveness would be affected by digesta mixing. Presumably, firmer digesta would reduce the distribution and efficacy of anti-methanogenic compounds.

The postulations of Leng (2018) suggested that methane abundance and activity would be increased after appendectomy due to the loss of a biofilm reservoir capable of producing anti-methanogenic compounds. However, an observational study of nearly 5,000 patients showed that post-appendectomy patients were more likely to be LE than those who did not undergo appendectomy (Takakura *et al.*, 2020). Although contradictory in hypotheses, these studies suggest that research considering biofilms, the immune system, and the appendix may help uncover the physiological mechanisms behind LE and HE in humans.

7.5 Future Perspectives

The research undertaken in this PhD dissertation found a positive correlation between breath hydrogen and methane in healthy individuals but no association between methane and hydrogen sulphide production *in vitro*. This finding challenged traditional concepts but aligned with emerging hypotheses proposed in the scientific literature. In addition, the finding that LE samples had greater *in vitro* colonic fibre fermentation and organic acid production than HE samples challenges current assumptions and warrants further investigation due to the importance of these functions to human health.

Future investigations need to take a different approach to that used here and in prior studies. It is now known that more than 400 species in the human colonic microbiota have the genetic capacity to produce hydrogen sulphide, but most species have not been tested in a wet-laboratory setting (Braccia *et al.*, 2021). Most of this capacity relates to cysteine fermentation rather than sulphate reduction, so approaches using isotopically labelled cysteine, for example, would be more beneficial than monitoring the activity and abundance of *Desulfovibrio* and *Bilophila* genera. Furthermore, the findings from the current study were consistent with assimilatory sulphate reduction in the colonic microbiota. Different methods, including RNA microarrays, sequencing, and enzyme assays, have been used to measure the expression of key enzymes responsible for assimilatory sulphate reduction in bacteria (Becker, Kredich and Tomkins, 1969; Wei *et al.*, 2001; Fernández *et al.*, 2002; Bogicevic *et al.*, 2012).

The current study and much literature unknowingly missed the vast hydrogen-utilising potential of the colonic microbiota by focussing on sulphate reduction,

methanogenesis and acetogenesis. It is now proposed that taxa from the Bacteroidetes phylum are the main hydrogen utilisers, and their mechanisms for doing so are largely unexplored. Furthermore, their hydrogenases often co-occur with hydrogen sensing systems, which may contribute to the important gas sensing functions of the colon (Wolf *et al.*, 2016).

Gas sensing in the colon may relate to the release of immunomodulatory secretions from the appendix or biofilms that affect the composition and function of the microbiota, including methanogens. It is known that individuals with irritable bowel syndrome have impaired colonic gas management and tolerance, together with increased faecal methanogen abundance (Serra, Azpiroz and Malagelada, 2001; Pimentel *et al.*, 2003). Further investigation of the relationship between gas sensing and methanogens is warranted.

Molecular studies investigating the location and function of microbial hydrogenases and hydrogen-sensing mechanisms have been conducted in samples from environmental and ruminal habitats (Lenz *et al.*, 1997, 2002; Zheng *et al.*, 2014). However, these studies are lacking in the human colonic microbiota. New technologies such as *in vivo* gas sensing capsules provide useful tools to measure the concentration of different gases in the human GIT (Kalantar-Zadeh *et al.*, 2018). Magnetic Resonance Imaging is also useful for studying GIT processing, emptying and secretions (Spiller and Marciani, 2019). These approaches can complement gas management and sensing research without the tenuous extrapolation of *in vitro* models.

In conclusion, gas management is an essential function of a healthy colon and is associated with the colonic microbiota, including methanogens. However, investigations have been limited. Future studies should consider the molecular

strategies of microorganisms to sense and manage gas concentrations and their interaction with the host immune system and physiology. These investigations may be possible with traditional molecular methods, enzymatic analyses, and new *in vivo* technologies.

This research will be important to clarify the role of methanogens in human health and the mechanisms behind colonic gas management. Exciting work is already progressing in this space, including water enriched with dissolved hydrogen to improve colonic hydrogen homeostasis and promote butyrate production (Ostojic, 2021b, 2021a), and trimethylamine-consuming methanogenic archaea as the next-generation probiotics (Fadhlaoui *et al.*, 2020; de La Cuesta-Zuluaga *et al.*, 2021).

7.6 Conclusion

The current research has confirmed links between human breath methane and the composition and function of the colonic microbiota during fibre fermentation. It was established that individuals who were LE had higher dietary fibre intake, an increased abundance of faecal amino acids, a *Bacteroides*-driven microbiota, increased insoluble fibre fermentation, and a lower relative abundance of amino acid metabolism pathways in the metagenome, compared to individuals who were HE. These results are aligned with the current mechanistic understanding of the relationship between diet, protein, and fibre fermentation; however, it was impossible to determine whether these effects were specific to breath methane phenotypes or were consequences of different diets between the two groups.

References

1. Arumugam, M., Raes, J., Pelletier, E., le Paslier, D., Yamada, T., Mende, D. R., Fernandes, G. R., Tap, J., Bruls, T., Batto, J. M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Bork, P. (2011). Enterotypes of the human gut microbiome. Nature 473(7346), 174–180. https://doi.org/10.1038/nature09944.
2. Bang, C., Weidenbach, K., Gutsmann, T., Heine, H., and Schmitz, R.A. (2014). The intestinal archaea Methanosphaera stadtmanae and Methanobrevibacter smithii activate human dendritic cells. PLoS ONE 9. https://doi.org/10.1371/journal.pone.0099411.
3. Becker, M.A., Kredich, N.M., and Tomkins, G.M. (1969). The purification and characterization of O-acetylserine sulfhydrylase-A from Salmonella typhimurium. Journal of Biological Chemistry 244, 2418–2427. https://doi.org/10.1016/s0021-9258(19)78240-4.
4. Belobrajdic, D.P., Bird, A.R., Conlon, M.A., Williams, B.A., Kang, S., McSweeney, C.S., Zhang, D., Bryden, W.L., Gidley, M.J., and Topping, D.L. (2012). An arabinoxylan-rich fraction from wheat enhances caecal fermentation and protects colonocyte DNA against diet-induced damage in pigs. British Journal of Nutrition, 107, 1274–1282. https://doi.org/10.1017/S0007114511004338.
5. Bogicevic, B., Berthoud, H., Portmann, R., Meile, L., and Irmler, S. (2012). CysK from Lactobacillus casei encodes a protein with O-acetylserine sulfhydrylase and cysteine desulfurization activity. Applied Microbiology and Biotechnology 94, 1209–1220. https://doi.org/10.1007/s00253-011-3677-5.
6. Braccia, D.J., Jiang, X., Pop, M., and Hall, A.B. (2021). The Capacity to Produce Hydrogen Sulfide (H2S) via Cysteine Degradation Is Ubiquitous in the Human Gut Microbiome. Frontiers in Microbiology 12, 1–31. https://doi.org/10.3389/fmicb.2021.705583.
7. Chassard, C., Delmas, E., Robert, C., and Bernalier-Donadille, A. (2010). The cellulose-degrading microbial community of the human gut varies

according to the presence or absence of methanogens. FEMS Microbiology Ecology 74, 205–213. https://doi.org/10.1111/j.1574-6941.2010.00941.x.

8. Cho, I., and Blaser, M.J. (2012). Applications of Next-Generation Sequencing: The human microbiome: at the interface of health and disease. Nature Publishing Group 13, 260–270. https://doi.org/10.1038/nrg3182.
9. Erdrich, S., Tan, E.C.K., Hawrelak, J.A., Myers, S.P., and Harnett, J.E. (2021). Hydrogen–methane breath testing results influenced by oral hygiene. Scientific Reports 11, 1–11. https://doi.org/10.1038/s41598-020-79554-x.
10. Fadhlaoui, K., Arnal, M.-E., Martineau, M., Camponova, P., Ollivier, B., O'Toole, P.W., and Brugère, J.-F. (2020). Archaea, specific genetic traits, and development of improved bacterial live biotherapeutic products: another face of next-generation probiotics. Applied Microbiology and Biotechnology 104, 4705–4716. https://doi.org/10.1007/s00253-020-10599-8.
11. Fernández, M., Kleerebezem, M., Kuipers, O.P., Siezen, R.J., and van Kranenburg, R. (2002). Regulation of the metC-cysK operon, involved in sulfur metabolism in Lactococcus lactis. Journal of Bacteriology 184, 82–90. https://doi.org/10.1128/JB.184.1.82-90.2002.
12. Flint, H.J., and Stewart, C.S. (1999). Bacteroides and Prevotella. Encyclopedia of Food Microbiology 198–203. https://doi.org/10.1006/RWFM.1999.0160.
13. Flint, H.J., Scott, K.P., Duncan, S.H., Louis, P., and Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. Gut Microbes 3. https://doi.org/10.4161/gmic.19897.
14. Gibson, G., Macfarlane, G.T., and Cummings, J. (1993). Sulphate reducing bacteria and hydrogen metabolism in the human large intestine. Gut 34, 437–439.

15. Gidley, M. J. (2013). Hydrocolloids in the digestive tract and related health implications. Current Opinion in Colloid and Interface Science, 18(4), 371–378. https://doi.org/10.1016/J.COCIS.2013.04.003.

16. Hammer, H.F. (1993). Colonic hydrogen absorption: Quantification of its effect on hydrogen accumulation caused by bacterial fermentation of carbohydrates. Gut 34, 818–822. https://doi.org/10.1136/gut.34.6.818.

17. Hoffmann, C., Dollive, S., Grunberg, S., Chen, J., Li, H., Wu, G.D., Lewis, J.D., and Bushman, F.D. (2013). Archaea and Fungi of the Human Gut Microbiome: Correlations with Diet and Bacterial Residents. PLoS ONE 8. https://doi.org/10.1371/journal.pone.0066019.

18. Johnson, A.J., Zheng, J.J., Kang, J.W., Saboe, A., Knights, D., and Zivkovic, A.M. (2020). A Guide to Diet-Microbiome Study Design. Frontiers in Nutrition 7, 1–16. https://doi.org/10.3389/fnut.2020.00079.

19. Kumpitsch, C., Fischmeister, F. P. S., Mahnert, A., Lackner, S., Wilding, M., Sturm, C., Holasek, S., Högenauer, C., Berg, I., Schöpf, V., and Moissl-Eichinger, C. (2020). Methane emission of humans is explained by dietary habits, host genetics, local formate availability and a uniform archaeome. BioRxiv 1–45. https://doi.org/10.1101/2020.12.21.423794.

20. de la Cuesta-Zuluaga, J., Spector, T. D., Youngblut, N. D., and Ley, R. E. (2021). Genomic Insights into Adaptations of Trimethylamine-Utilizing Methanogens to Diverse Habitats, Including the Human Gut. MSystems 6(1). https://doi.org/10.1128/mSystems.00939-20.

21. Lacasse, M.J., Sebastiampillai, S., Côté, J.P., Hodkinson, N., Brown, E.D., and Zamble, D.B. (2019). A whole-cell, high-throughput hydrogenase assay to identify factors that modulate [NiFe]-hydrogenase activity. Journal of Biological Chemistry 294, 15373–15385. https://doi.org/10.1074/jbc.RA119.008101.

22. Leng, R.A. (2018). Unravelling methanogenesis in ruminants, horses and kangaroos: The links between gut anatomy, microbial biofilms and host immunity. Animal Production Science 58, 1175–1191. https://doi.org/10.1071/AN15710.

23. Lentle, R. G., and Janssen, P. W. M. (2008). Physical characteristics of digesta and their influence on flow and mixing in the mammalian intestine: a review. Journal of Comparative Physiology B, 178(6), 673–690. https://doi.org/10.1007/s00360-008-0264-x.

24. Lenz, O., Strack, A., Tran-Betcke, A., Ba¨, B., and Friedrich, B. (1997). A Hydrogen-Sensing System in Transcriptional Regulation of Hydrogenase Gene Expression in Alcaligenes Species. Journal of Bacteriology 179(5), 1655-1663.

25. Lenz, O., Bernhard, M., Buhrke, T., Schwartz, E., and Rbel Friedrich, B. (2002). The Hydrogen-Sensing Apparatus in Ralstonia eutropha. Journal of. Molecular Microbiology and. Biotechnology. 4, 255–262.

26. Levitt, M.D., and Slavin, J.L. (1991). Methane Production and Bowel Function Parameters in Healthy Subjects on Low- and High-Fiber Diets. Nutrition and Cancer 16, 85–92. https://doi.org/10.1080/01635589109514147.

27. Macfarlane, G. (1988). Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut. Journal of Applied Bacteriology 65, 103-111.

28. le Marchand, L., Wilkens, L.R., Harwood, P., and Cooney, R. (1992). Use of Breath Hydrogen and Methane as Markers of Colonic Fermentation in Epidemiological Studies: Circadian Patterns of Excretion. Environmental Health Perspectives 98, 199–202. https://doi.org/10.1289/ehp.9298199.

29. Nkamga, V.D., Henrissat, B., and Drancourt, M. (2017). Archaea: Essential inhabitants of the human digestive microbiota. Human Microbiome Journal 3, 1–8. https://doi.org/10.1016/j.humic.2016.11.005.

30. Ostojic, S.M. (2021a). Hydrogen-rich water as a modulator of gut microbiota? Journal of Functional Foods 78. https://doi.org/10.1016/j.jff.2021.104360.

31. Ostojic, S.M. (2021b). Does drinking water rich in hydrogen gas revive brain hypometabolism in neurodegeneration by SCFAs upregulation? European Journal of Clinical Nutrition 75, 212–213. https://doi.org/10.1038/s41430-020-0680-x.

32. Ozato, N., Saito, S., Yamaguchi, T., Katashima, M., Tokuda, I., Sawada, K., Katsuragi, Y., Kakuta, M., Imoto, S., Ihara, K., and Nakaji, S. (2020). Association between breath methane concentration and visceral fat area: A population-based cross-sectional study. Journal of Breath Research 14(2). https://doi.org/10.1088/1752-7163/ab61c6.

33. Parthasarathy, A., Cross, P.J., Dobson, R.C.J., Adams, L.E., Savka, M.A., and Hudson, A.O. (2018). A Three-Ring circus: Metabolism of the three proteogenic aromatic amino acids and their role in the health of plants and animals. Frontiers in Molecular Biosciences 5. https://doi.org/10.3389/fmolb.2018.00029.

34. Pimentel, M., Mayer, A.G., Park, S., Chow, E.J., Hasan, A., and Kong, Y. (2003). Methane production during lactulose breath test is associated with gastrointestinal disease presentation. Digestive Diseases and Sciences 48, 86–92. https://doi.org/10.1023/A:1021738515885.

35. Randal Bollinger, R., Barbas, A.S., Bush, E.L., Lin, S.S., and Parker, W. (2007). Biofilms in the large bowel suggest an apparent function of the human vermiform appendix. Journal of Theoretical Biology 249, 826–831. https://doi.org/10.1016/j.jtbi.2007.08.032.

36. Rinninella, E., Raoul, P., Cintoni, M., Franceschi, F., Miggiano, G., Gasbarrini, A., and Mele, M. (2019). What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases. Microorganisms 7(1), 14. https://doi.org/10.3390/microorganisms7010014.

37. Robert, C., and Bernalier-Donadille, A. (2003). The cellulolytic microflora of the human colon: Evidence of microcrystalline cellulose-degrading bacteria in methane-excreting subjects. FEMS Microbiology Ecology 46, 81–89. https://doi.org/10.1016/S0168-6496(03)00207-1.

38. Ruaud, A., Esquivel-Elizondo, S., de la Cuesta-Zuluaga, J., Waters, J.L., Angenent, L.T., Youngblut, N.D., and Ley, R.E. (2020). Syntrophy via Interspecies H 2 Transfer between Christensenella and Methanobrevibacter Underlies Their Global Cooccurrence in the Human Gut. MBio 11, 1–16. https://doi.org/10.1128/mBio.03235-19.

39. Sepahi, A., Liu, Q., Friesen, L., and Kim, C. H. (2021). Dietary fiber metabolites regulate innate lymphoid cell responses. Mucosal Immunology, 14(2), 317–330. https://doi.org/10.1038/s41385-020-0312-8.

40. Serra, J., Azpiroz, F., and Malagelada, J.R. (2001). Impaired transit and tolerance of intestinal gas in the irritable bowel syndrome. Gut 48, 14–19. https://doi.org/10.1136/gut.48.1.14.

41. Spiller, R., and Marciani, L. (2019). Intraluminal Impact of Food: New Insights from MRI. Nutrients, 11(5), 1147. https://doi.org/10.3390/nu11051147.

42. Starck, C.S., Wolfe, R.R., and Moughan, P.J. (2018). Endogenous Amino Acid Losses from the Gastrointestinal Tract of the Adult Human - A Quantitative Model. Journal of Nutrition 148, 1871–1881. https://doi.org/10.1093/jn/nxy162.

43. Strocchi, A., Ellis, C.J., Furne, J.K., and Levitt, M.D. (1994). Study of constancy of hydrogen-consuming flora of human colon. Digestive Diseases and Sciences 39, 494–497. https://doi.org/10.1007/BF02088333.

44. Takakura, W., Oh, S.J., Singer-Englar, T., Mirocha, J., Leite, G., Fridman, A., Pimentel, M., Mathur, R., Pichetshote, N., and Rezaie, A. (2020). Comparing the rates of methane production in patients with and without appendectomy: results from a large-scale cohort. Scientific Reports 10. https://doi.org/10.1038/s41598-020-57662-y.

45. Venema, K. (2015). The TNO *In vitro* Model of the Colon (TIM-2). In The Impact of Food Bioactives on Health: *In vitro* and Ex Vivo Models, K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A.

Mackie, T. Requena, D. Swiatecka, and H. Wichers, eds. (Cham: Springer International Publishing), pp. 293–304.

46. Wei, Y., Lee, J.M., Richmond, C., Blattner, F.R., Rafalski, J.A., and Larossa, R.A. (2001). High-density microarray-mediated gene expression profiling of Escherichia coli. Journal of Bacteriology 183, 545–556. https://doi.org/10.1128/JB.183.2.545.

47. Wolf, P.G., Biswas, A., Morales, S.E., Greening, C., and Gaskins, H.R. (2016). H2 metabolism is widespread and diverse among human colonic microbes 7, 235–245. https://doi.org/10.1080/19490976.2016.1182288.

48. Ze, X., Duncan, S.H., Louis, P., and Flint, H.J. (2012). Ruminococcus bromii is a keystone species for the degradation of resistant starch in the human colon. ISME Journal 6, 1535–1543. https://doi.org/10.1038/ismej.2012.4.

49. Ze, X., ben David, Y., Laverde-Gomez, J. A., Dassa, B., Sheridan, P. O., Duncan, S. H., Louis, P., Henrissat, B., Juge, N., Koropatkin, N. M., Bayer, E. A., and Flint, H. J. (2015). Unique Organization of Extracellular Amylases into Amylosomes in the Resistant Starch-Utilizing Human Colonic Firmicutes Bacterium Ruminococcus bromii. American Society for Microbiology 6(5), e01058-15. https://doi.org/10.1128/mbio.01058-15.

50. Zhang, D., Williams, B. A., Mikkelsen, D., Li, X., Keates, H. L., Lisle, A. T., Collins, H. M., Fincher, G. B., Bird, A. R., Topping, D. L., Gidley, M. J., and Bryden, W. L. (2015). Soluble arabinoxylan alters digesta flow and protein digestion of red meat-containing diets in pigs. Nutrition 31(9), 1141–1147. https://doi.org/10.1016/j.nut.2015.03.006.

51. Zheng, Y., Kahnt, J., Kwon, I.H., Mackie, R.I., and Thauer, R.K. (2014). Hydrogen formation and its regulation in Ruminococcus albus: Involvement of an electron-bifurcating [FeFe]-hydrogenase, of a non-electron-bifurcating [FeFe]-hydrogenase, and of a putative hydrogen-sensing [FeFe]-hydrogenase. Journal of Bacteriology 196, 3840–3852. https://doi.org/10.1128/JB.02070-14.

Appendix 1

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Laura Payling
Name/title of Primary Supervisor:	Professor Warren McNabb
In which chapter is the manuscript /published work:	Chapter 2

Please select one of the following three options:

(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Payling, L., Fraser, K., Loveday, S.M., Sims, I., Roy, N., and McNabb, W. (2020). The effects of carbohydrate structure on the composition and functionality of the human gut microbiota. Trends in Food Science and Technology 97, 233–248.

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:

- The percentage of the manuscript/published work that was contributed by the candidate: 60.00

- Describe the contribution that the candidate has made to the manuscript/published work:

The candidate completed the literature searches, drafting, reviewing, editing and finalisation of the manuscript. The candidate also completed submission of the manuscript to the Journal for publication and satisfying the reviewers comments.

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Laura Payling Digitally signed by Laura Payling Date: 2022.09.18 16:42:01 +01'00'
Date:	18-Sep-2022
Primary Supervisor's Signature:	Warren McNabb Digitally signed by Warren McNabb Date: 2022.09.19 10:50:33 +12'00'
Date:	19-Sep-2022

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.

Appendix 2

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Laura Payling
Name/title of Primary Supervisor:	Professor Warren McNabb
In which chapter is the manuscript /published work:	Chapter 3

Please select one of the following three options:

(●) The manuscript/published work is published or in press

- Please provide the full reference of the Research Output:

Payling, L., Roy, N. C., Fraser, K., Loveday, S. M., Sims, I. M., Stefan, J., Laura, G., and McNabb, W. C. (2021). A protocol combining breath testing and ex vivo fermentations to study the human gut microbiome. STAR Protocols 2, 100227. https://doi.org/10.1016/j.xpro.2020.100227.

( ) The manuscript is currently under review for publication – please indicate:

- The name of the journal:

- The percentage of the manuscript/published work that was contributed by the candidate: 60.00

- Describe the contribution that the candidate has made to the manuscript/published work:

The candidate constructed the protocol of methods based on current literature and expertise of others in the field. The candidate also completed the drafting, reviewing, editing and finalisation of the manuscript. The candidate completed submission of the manuscript to the Journal for publication and satisfying the reviewers comments.

( ) It is intended that the manuscript will be published, but it has not yet been submitted to a journal

Candidate's Signature:	Laura Payling Digitally signed by Laura Payling Date: 2022.09.18 16:47:42 +01'00'
Date:	18-Sep-2022
Primary Supervisor's Signature:	Warren McNabb Digitally signed by Warren McNabb Date: 2022.09.19 10:51:14 +12'00'
Date:	19-Sep-2022

This form should appear at the end of each thesis chapter/section/appendix submitted as a manuscript/ publication or collected as an appendix at the end of the thesis.