# Factors Influencing the Growth and Hydrogen Sulfide Production of *Bilophila wadsworthia*, an Important Member of the Sulfite-Reducing Bacteria in the Gut

Jade Jennifer Davies

Quadram Institute Bioscience

A thesis submitted for the degree of Doctor of Philosophy to the University of East Anglia

December 2023

# Abstract

**Factors influencing the growth and hydrogen sulfide production of *Bilophila wadsworthia*, an important member of the sulfite-reducing bacteria in the gut**

**Background:** Sulfate-reducing bacteria (SRB) reduce sulfate to yield $H_2S$ and are commonly found in the human gut. Excess $H_2S$ has been linked to gut inflammation and is implicated in inflammatory disease pathogenesis. *B. wadsworthia*, a unique SRB member, is correlated with deleterious gut inflammation and disease; despite this, *B. wadsworthia* is present in the commensal gut microbiota of many healthy individuals. Enhancing our understanding of the factors influencing the abundance and $H_2S$ production of *B. wadsworthia* is a valuable research goal.

**Objectives:** This thesis aimed to investigate the effect of other gut microbiota members on the abundance and $H_2S$ production of *B. wadsworthia*.

**Approaches:** *B. wadsworthia* was co-cultured with the common gut commensals *B. thetaiotaomicron* and *E. faecium*. The differential gene expression of the bacterial strains using transcriptomics and the differential metabolome of the cultures using untargeted metabolomics was investigated to unravel the underlying mechanisms behind the microbial interactions observed.

**Results:** *B. thetaiotaomicron* significantly increased *B. wadsworthia*'s $H_2S$ production in co-culture; this interaction was dependent upon *B. thetaiotaomicron* viability and physical proximity between the strains. In co-culture, *B. wadsworthia*'s production of sulfite from taurine was decreased. Additionally, *B. thetaiotaomicron*-derived indole production was decreased. *E. faecium* significantly inhibited *B. wadsworthia*'s growth, which is at least partially mediated via lactic acid production. *In silico* genome screening of *E. faecium* strains revealed the presence of biosynthetic clusters for bacteriocins and absence of clinically-important AMR genes and virulence factors, supporting the potential use of these strains as anti-*B. wadsworthia* probiotics.

**Conclusion:** We hypothesise that in *B. thetaiotaomicron* and *B. wadsworthia* co-culture, co-operative metabolism of sulfate to $H_2S$ via an APS intermediate may

permit excess $H_2S$ production by *B. wadsworthia*. *E. faecium* strains strongly inhibited *B. wadsworthia*'s growth *in vitro*, showing early promise for use of *E. faecium* as a potential anti-*B. wadsworthia* probiotic.

## Access Condition and Agreement

Each deposit in UEA Digital Repository is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the Data Collections is not permitted, except that material may be duplicated by you for your research use or for educational purposes in electronic or print form. You must obtain permission from the copyright holder, usually the author, for any other use. Exceptions only apply where a deposit may be explicitly provided under a stated licence, such as a Creative Commons licence or Open Government licence.

Electronic or print copies may not be offered, whether for sale or otherwise to anyone, unless explicitly stated under a Creative Commons or Open Government license. Unauthorised reproduction, editing or reformatting for resale purposes is explicitly prohibited (except where approved by the copyright holder themselves) and UEA reserves the right to take immediate ‘take down’ action on behalf of the copyright and/or rights holder if this Access condition of the UEA Digital Repository is breached. Any material in this database has been supplied on the understanding that it is copyright material and that no quotation from the material may be published without proper acknowledgement.

# Contents

# List of figures

# List of tables

# Abbreviations

| Abbreviation | Meaning |
|---|---|
| AMR | Antimicrobial resistance |
| APS | Adenosine 5'-phosphosulfate |
| Asr | Assimilatory sulfite reductase |
| BHI + C | Brain Heart Infusion with complements |
| BHI + S | Brain Heart Infusion with supplements |
| BPM | Brain Heart Infusion with complements, Postgate C and de Man-Rogosa Sharpe media (1:1:1 ratio) |
| Bt | *Bacteroides thetaiotaomicron* |
| Bw | *Bilophila wadsworthia* |
| CFU | Colony forming units |
| CIM | Complex Intestinal Media |
| CMM | Colon Model Media |
| DEGs | Differentially expressed genes |
| Dsr | Dissimilatory sulfite reductase |
| EC | Enzyme Classification number |
| Ef | *Enterococcus faecium* |
| FDR | False Discovery Rate |
| $H_2S$ | Hydrogen sulfide |
| HILIC-MS | Hydrophilic interaction chromatography-mass spectrometry |
| LCMS | Liquid Chromatography Mass Spectrometry |
| LLOQ | Lower Limit of Quantification |
| LogFC | Log fold change (gene expression) |
| MAG | Metagenome-assembled genome |
| MRS | de Man-Rogosa-Sharpe |
| $OD_{600}$ | Optical density at 600 nm |
| PAPS | 3'-phosphoadenosine 5'-phosphosulfate |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PLS-DA | Partial least squares-discriminant analysis |
| QIB | Quadram Institute Bioscience |

| | |
|---|---|
| qPCR | Quantitative polymerase chain reaction |
| rpm | Revolutions per minute |
| rRNA | Ribosomal ribonucleic Acid |
| RSD | Relative standard deviation |
| RQN | RNA Quality Number |
| SCFA | Short-chain fatty-acid |
| SIBO | Small intestinal bacterial overgrowth |
| SRB | Sulfate-reducing bacteria |
| $SO_4^{2-}$ | Sulfate |
| $SO_3^{2-}$ | Sulfite |
| TEER | Trans-epithelial electrical resistance |
| up$H_2O$ | Ultrapure water |
| VIP | Variable Importance in Projection |
| ZnS | Zinc sulfide |

# Symbols and units

| Symbol/unit | Meaning |
|---|---|
| % | Percentage |
| ≥ | Equal to or greater than |
| ≤ | Equal to or less than |
| ˚C | Degrees Celsius |
| µM | Micromolar |
| µm | Micrometre |
| g | Gram |
| h | Hour |
| log | Logarithm |
| M | Molar |
| mg | Milligram |
| min | Minute |
| mL | Millilitre |
| mM | Millimolar |
| nM | Nanomolar |
| pH | Potential of hydrogen |
| $R^2$ | Coefficient of correlation |
| s | Second |
| x g | Relative Centrifugal Force (RCF) or g force |
| µL | Microlitre |

# Acknowledgements

I could not have undertaken this journey without Dr. Lizbeth Sayavedra, without whom there quite frankly would not be a PhD. I'm extremely grateful to her for her scientific guidance in many aspects of the project including both the wet and dry lab, but also her unwavering support throughout the ups and downs of the PhD. I cannot thank you enough for your patience, brilliance and friendship these past four years. I wish you the very best and look forward to reading the research from the future Sayavedra group! I sincerely thank the rest of my supervisory team Prof. Arjan Narbad, Dr. Melinda Mayer and Prof. Nathalie Juge for their support, time and scientific guidance over the years. I would also like to acknowledge and thank BBSRC for the funding that made this research possible via the Norwich Research Park Doctoral Training Programme (NRP-DTP).

I'm very grateful to Dr. Mark Philo and Dr. Noemi Tejera Hernandez for their help with all things LC-MS. Thank you for being so friendly and open to collaboration, I learned so much from you both and I really enjoyed working with you. I would also like to thank Dr. Dave Baker for his support with sequencing, your hard work is much appreciated. On a related note, I would be remiss in not thanking the laboratory support team at QIB – you saved me many hours of media preparation for which I am very grateful! Thank you for all the hard work you do in helping the labs run smoothly.

I would like to thank the amazing scientists I have had the opportunity to work with, including past and present members of the Narbad research group – Dr. Jacob Scadden, Dr. Annalisa Altera, Dr. Adam Pattinson, Dr. Lee Kellingray, and Dr. Stefano Romano. Jacob, thank you for being the best bench-mate, the jokes and for putting up with my rants and stresses. Annalisa, firstly thank you for co-parenting Caco-2 cells with me, but more importantly thank you for being such a great friend and for all the 3 pm coffee breaks, brunches and pizza dates – I would have gone mad without you around! Adam, your presence was short and sweet in the group, and I thank you for your help not only with metabolomics but for being such a fun and compos mentis person to be around. Lee, thank you for always cracking jokes and speaking your mind, the group would not be the same without you. Stefano, thank you so much for your scientific guidance over the years, I value your opinion and perspective so highly. I would also like to thank other

members of the Narbad research group – Bushra Schuitemaker, Stephanie Ong, Marnie Barham, Lizzi Coy, Dr. Emad Shehata, Dr. Betul Bozatli, James Choi and Dr. Dan Yara. The experience would not have been the same without you.

Outside of the Narbad group, there are many wonderful people at QIB who shaped my PhD journey. I would like to extend my sincere thanks to Barbora Peck, you are one of the loveliest people I know and I'm so grateful for your friendship – thank you for being such a ray of sunshine in my life. I would like to give a special thank you to Dr. Sian Seaman and Raven Reynolds for your friendship over these past four years, it has made such a difference to have you around and I am so glad to have met you. I would be remiss in not mentioning Dr. Rob James – thank you for being so friendly and down-to-earth, and for always giving great advice. Further thanks to James Docherty, thank you for the laughs and I wish you the best of luck with the rest of your PhD.

I would also like to thank my friends Jack Jordan, Huw Gill and Tess Merrison-Jay for the best group chat, for the endless laughs and for being the best travel buddies I could wish for. You have kept me sane and anchored into life outside of the PhD. I also extend a special thank you to Dr. Katie Riddoch for being such a wonderful person and friend, and for the iconic "PhD playtime" – you understand me so well, and I feel very lucky to know you! Also, it's not a person but I think I should acknowledge the role of Huel in physically fuelling the PhD; it provided me sustenance in the form of four years' worth of lunches and saved the hassle of cooking/buying every day! A true saviour after many hours in the lab or at the computer.

I express a deep, heartfelt thanks to my parents. Mum, thank you for always being there for me and for your endless moral support via messages, day or night! Dad, thank you for looking out for me in every possible way and for being so supportive and understanding. You are the most caring and selfless people I know, and I could not have done this without you. I hope I can make you proud and take care of you both in the way you have done for me. Finally, words cannot express my gratitude to Mark, my ~~partner~~ fiancé. You are my home, and I can't wait to see what's next for us – thank you for giving me stability and love always.

# Chapter 1

## 1. Introduction

## 1.1 The human gut microbiome

Akin to all other organisms on earth, humans have evolved alongside micro-organisms, and this co-evolutionary relationship is largely reflected through mutualism, commensalism or pathogenesis [1]. Microbial populations dominated by bacteria cover almost all human body surfaces, producing distinctive and diverse populations; these are termed the microbiota. Indeed, the human microbiome which describes the collective genetic material of the microbiota has been referred to as our 'second genome', emphasising the profound impact of the microbiota on human health and disease [2].

The gastrointestinal (GI) tract contains by far the largest and most diverse microbial load, the majority of which is found in the colon where approximately $10^{12}$ bacterial cells reside [3]. Much of the gut microbiota consists of bacteria, of which four phyla dominate; *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* [4]. In the GI tract, microbes play pivotal roles in guiding normal immune system function and activity [5], preventing pathogen colonisation [6] and aiding in the breakdown of indigestible fibres to yield beneficial compounds including short-chain fatty-acids (SCFAs) which can be utilised by host colonocytes [7]. Given the overwhelming abundance of bacteria relative to other microbes, the symbiotic interactions involving the gut bacteria are a significant determinant of overall human health. Here, symbiosis is broadly defined as "the living together of unlike organisms" [8].

The importance of maintaining a diverse microbiota population is becoming increasingly acknowledged, as more evidence gathers linking loss of gut microbiota diversity and subsequent pathobiont expansion to disease [9]. These adverse changes to the gut microbiome, often termed dysbiosis, may occur in response to environmental factors such as antibiotic usage, pathogens or diet [10]. Gut microbiome dysbiosis has been increasingly implicated in not only inter-intestinal diseases such as irritable bowel syndrome (IBS) [11] and inflammatory bowel diseases (IBD) [12], but also extra-intestinal diseases such as obesity, insulin resistance [13] and even neurological conditions including depression and anxiety [14], neurodegenerative conditions such as dementia [15] and autism spectrum disorder [16].

## 1.2 Sulfate- and sulfite-reducing bacteria (SRB)

Sulfate- and sulfite-reducing bacteria (SRB) are a taxonomically diverse group of strictly anaerobic bacteria found ubiquitously in anoxic environments and play important ecological roles particularly in marine sediments and the human gut microbiome. SRB utilise inorganic sulfate ($SO_4^{2-}$) or sulfite ($SO_3^{2-}$) as a terminal electron acceptor during energy metabolism, a process which occurs concomitantly with oxidation of molecular hydrogen or organic compounds. The terminal product of sulfate reduction is hydrogen sulfide ($H_2S$) via a process termed dissimilatory sulfate reduction [17]. The largest phylogenetic group of SRB is the class Deltaproteobacteria, within which around 23 Gram-negative genera can be found including *Desulfovibrio, Lawsonia* and *Bilophila.* The second largest group of SRB are Gram-positive, found within the class Clostridia, encompassing genera including *Desulfotomaculum* and *Desulfosporosinus* [18].

### 1.2.1 SRB in the human gut

SRB are found within the colonic mucosa as part of the commensal gut microbiota in at least 50% of humans [19, 20]. Early cultivation-based studies on the SRB taxa in human stool detected *Desulfovibrio* as the most abundant genus, followed by *Desulfobacter*, *Desulfobulbus* and *Desulfomonas* from the class Deltaproteobacteria and *Desulfotomaculum*, a Firmicute [21]. Such studies revealed that *Desulfovibrio* spp. account for approximately 66% of colonic SRB [21]. The SRB in the human colon being mainly from the genus *Desulfovibrio* was confirmed by subsequent culture-independent studies [20, 22, 23]. Additional cultivation-dependent studies revealed the presence of the medically-relevant pathobiont *Bilophila wadsworthia*, which can be readily isolated from the faeces of 59% of healthy individuals [24].

During bacterial fermentation of complex carbohydrates in the human colon, large volumes of hydrogen are produced. However, accumulation of $H_2$ thermodynamically inhibits fermentation [25]. Hydrogenotrophs, including acetogens, methanogens and SRB compete for this resource without mutual exclusivity in the healthy human gut microbiota [22]. The ability to co-exist in the human gut is likely due to the heterogenous environment provided by the intestines; various physiological niches may permit distinct microbial groups, resulting in simultaneous hydrogenotrophic reactions [25]. Additionally, sulfate

availability and intestinal transit time appear to regulate the competitive hydrogenotrophic niche [26, 27]. Although hydrogenotrophs are present in low abundance [25], their function is important for maintenance of efficient fermentation in the gut, demonstrating that abundance does not necessarily equate to functional significance [22]. This mutually beneficial process is termed interspecies $H_2$ transfer [28, 29]. SRB utilise the $H_2$ as an electron donor during dissimilatory sulfite reduction; they also commonly utilise organic compounds for this purpose [18]. Lactate is the most common organic compound used as an electron donor for sulfite reduction, but pyruvate, acetate, propionate or butyrate can also be used, although the ability to utilise these compounds is species-specific [30].

### 1.2.2 *Bilophila wadsworthia*

*Bilophila wadsworthia* is a Gram-negative anaerobic member of the Deltaproteobacteria, first identified in 1989 from gangrenous and perforated appendicitis samples [31, 32]. This bacterium was characterised as a Gram-negative rod being stimulated by bile and pyruvate, strongly catalase positive, urease positive and strictly anaerobic [31]. To date, *B. wadsworthia* is the only species reported within the genus, although a Candidatus *Bilophila faecipullorum* sp. nov. was recently described via metagenomic analysis of the chicken gut [33].

## 1.3 Sulfur metabolism in the human gut

Sulfur is ubiquitous in the biosphere; it is the tenth most abundant element on Earth [34] and the seventh most abundant element in the human body [35]. Sulfur is dynamically exchanged within biological systems as part of the global sulfur cycle. The oxidation state of sulfur ranges from -6 (in sulfate) to -2 (in sulfide and reduced organic sulfur compounds), where the stability of each sulfur state depends on the presence of oxygen; in oxic conditions, sulfur is mostly found as sulfate, whereas the most common state under anoxic conditions is sulfide [36]. As sulfur is found ubiquitously in the environment, it is unsurprising that this ubiquity is mirrored within biological systems; sulfur is essential for life as it contributes to antioxidant molecules [37] and in proteins formed from the sulfated amino acids methionine, cysteine, homocysteine and taurine [38]. Microorganisms capable of reducing sulfate are essential contributors to the global sulfur cycle particularly within marine sediment [39], anoxic marine environments [40], and in the mammalian gut microbiome [41, 42]. Here, sulfur from organosulfur compounds

and/or sulfate is reduced to the lowest oxidation state, sulfide under anaerobic conditions. In this way, microorganisms utilise sulfate as a terminal electron acceptor in anaerobic respiration [43]. There are two main metabolic pathways through which this occurs, termed dissimilatory and assimilatory sulfate reduction respectively.

### 1.3.1 Dissimilatory sulfate reduction

Dissimilatory sulfate reduction describes a type of anaerobic respiration whereby sulfate is used as a terminal electron acceptor in the place of oxygen. This metabolic pathway occurs only under anaerobic conditions and is present in a select group referred to as sulfate-reducing bacteria (SRB). To reduce sulfate, organic compounds such as lactate, pyruvate, acetate, propionate, butyrate or molecular hydrogen are used as electron donors in order to generate energy for ATP production, and the terminal product of this metabolism is $H_2S$ [44]. Dissimilatory sulfate reduction can be further categorised into 'classical' and 'non-classical' pathways.

The 'classical' metabolism is well-characterised in *Desulfovibrio*, and recently the genes encoding the necessary enzymes for this pathway have been identified in a surprisingly diverse range of gut bacteria including *Collinsella*, *Eggerthella*, *Enterococcus*, *Flavonifractor*, *Gordonibacter* and *Roseburia* species [45]. In the 'classical' pathway of sulfate reduction, sulfate is 'activated' by conversion to adenosine 5'-phosphosulfate (APS) by ATP sulfurylase (Sat); APS has a higher redox potential than sulfate, which facilitates the reduction of APS with reduced NADH or ferredoxin [18]. The APS is then reduced by APS reductase (AprAB), resulting in the production of sulfite and adenosine monophosphate (AMP). Finally, sulfite undergoes a six-electron reduction by the dissimilatory sulfite reductase complex DsrABC, using electrons from organic compounds such as lactate and a membrane-associated complex DsrMJKOP [46] to yield $H_2S$, the terminal metabolic product [47]. $H_2S$ freely diffuses across the bacterial cell membrane and is released from the cell [48, 49] (Figure 1).

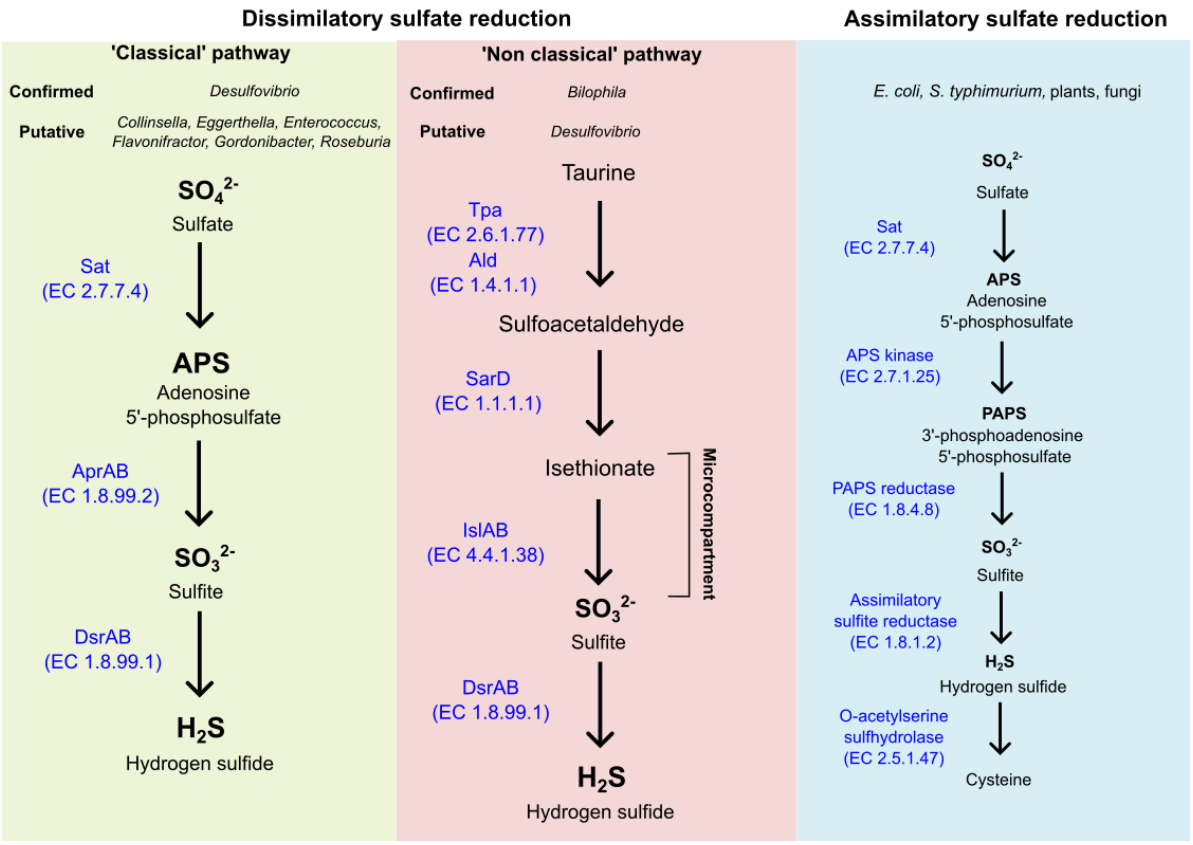



Figure 1*:* Metabolic pathways for sulfate reduction in intestinal bacteria. Dissimilatory sulfate reduction is a strictly anaerobic process performed by sulfate-reducing bacteria (SRB). Assimilatory sulfate reduction occurs under aerobic and anaerobic conditions, and is found in many organisms including bacteria, plants and fungi. Toxic isethionate intermediates are encapsulated within microcompartments in *B. wadsworthia*.

The ‘non-classical’ pathway of sulfate reduction does not utilise organic sulfate, instead using the sulfated amino acid taurine as the terminal electron acceptor (Figure 1). This pathway was first described in *B. wadsworthia*, an SRB unable to directly reduce sulfate [50]. Here, taurine undergoes deamination to sulfoacetaldehyde catalysed by the enzyme taurine:pyruvate aminotransferase (Tpa); the pyruvate of Tpa is subsequently regenerated by an alanine dehydrogenase (Ald) [51]. Sulfoacetaldehyde is then reduced by an alcohol dehydrogenase (SarD) to yield a toxic intermediate isethionate [51]. It has recently been shown that *B. wadsworthia* constructs microcompartments within the cytoplasm to encapsulate the toxic isethionate intermediates [52] (Figure 1); interestingly, expression of these genes was shown to be essential for colonisation in a germ-free mouse model (Sayavedra *et al*., in preparation). Isethionate is cleaved by an isethionate sulfo-lyase enzyme (IslAB) to produce sulfite [53].

Subsequently, as observed in other SRB, sulfite enters the dissimilatory sulfite reduction pathway requiring the enzyme dissimilatory sulfite reductase, using hydrogen, lactate or pyruvate as electron donors [54] to yield $H_2S$ [55] (Figure 1).

Although this pathway was thought to be present exclusively in *B. wadsworthia* [51], the necessary genes were recently identified in *Desulfovibrio spp*. genomes in the human gut [45]. The study employed a genome mining approach of metagenome-assembled genomes (MAGs) from the human microbiome project to investigate the prevalence of microbial sulfur metabolism genes [45]. The six-electron reduction of sulfite to $H_2S$ can be catalysed by dissimilatory sulfite reductase (Dsr), but also anaerobic sulfite reductase (Asr). Anaerobic sulfite reductase (*asr*) genes were more prevalent and widespread across microbial genera than the dissimilatory sulfite reductase (*dsr*) genes, from which the authors concluded that *asr* genes may be more important contributors to sulfate and sulfite reduction in the human gut than *dsr* [45]. Intriguingly, *dsr* genes traditionally associated with sulfate reducing bacteria (SRB) only were found in *Collinsella*, *Eggerthella*, *Enterococcus*, *Flavonifractor*, *Gordonibacter* and *Roseburia* species [45], meaning these genes are more widespread than previously thought (Figure 1). Perhaps more pertinent to this thesis, the genes required for the four-step pathway by which taurine is metabolised into $H_2S$ were identified not only in *Bilophila* but other *Desulfovibrio* spp., although the specific species could not be identified [45]; this is quite significant given that *B. wadsworthia* was the only known bacterium to harbour this metabolism until these findings. Other possible pathways for taurine metabolism were identified including two genera with genes for a complete three-step reduction pathway, and a further 41 unique genera with partially complete three- and four-step pathways; this suggests that taurine metabolism genes in the gut are much more widespread than previously thought, and that co-operative taurine metabolism by different species is likely [45]. It is important to note that the genes were identified by mining metagenome assembled genomes (MAGs) *in silico* only to genus depth so bacterial species could not be resolved [45], it is unknown whether the genes are expressed *in vivo* and indeed the specific isolates have not been obtained for *in vitro* study.

### 1.3.2 Assimilatory sulfate reduction

Assimilatory sulfate reduction is a biosynthetic pathway allowing inorganic sulfate to be incorporated into organic compounds via a series of reduction reactions

yielding cysteine as the terminal metabolic product (Figure 1). This pathway is alike to dissimilatory sulfate reduction in that sulfate is used as a terminal electron acceptor and $H_2S$ is produced, however in the assimilatory pathway $H_2S$ is produced only as an intermediate. Furthermore, assimilatory sulfate reduction is found in a wide range of bacteria including *Escherichia coli* and *Salmonella enterica* serovar Typhimurium [56, 57], and other organisms including plants and yeast [58]. A further distinction between assimilatory and dissimilatory sulfate reduction is that the assimilatory pathway can occur under aerobic or anaerobic conditions, whereas dissimilatory is strictly anaerobic [44].

During assimilatory sulfate reduction, sulfate is activated to APS via ATP sulfurylase (Sat) in the same manner as in the dissimilatory pathway. APS is then converted to 3'-phosphoadenosine 5'-phosphosulfate (PAPS) by APS kinase using ATP. PAPS is then reduced by the enzyme PAPS reductase to produce sulfite, which is further reduced to the intermediate $H_2S$ by assimilatory sulfite reductase. Then, cysteine synthase (o-acetylserine sulfhydrolase) EC 2.5.1.47 reacts $H_2S$ with O-acetylserine to produce the terminal product cysteine [44] (Figure 1). Cysteine synthesis via this pathway is the dominant mechanism for incorporating inorganic sulfur into organic molecules, as cysteine is a proteinogenic amino acid [44]. Due to the highly reactive nature of the sulfhydryl group, cysteine has several biological functions. Cysteine participates in redox reactions, most notably as a precursor in the synthesis of glutathione, an important antioxidant molecule [59]. Cysteine also contributes to proteins involved in metal ion binding including zinc fingers and NiFe hydrogenases [60], and is required for disulfide bond formation in proteins which is often a prerequisite for correct tertiary protein structure and therefore protein functionality [61].

### 1.3.3 Metabolism of *Bilophila wadsworthia*

During reduction of sulfite to $H_2S$, *B. wadsworthia* generates by-products including acetate, succinate [24] ammonia and acetate [62] and is capable of using a range of compounds including formate, lactate, pyruvate and molecular hydrogen as electron donors [54]. It was previously thought that $H_2S$ production by *B. wadsworthia* was taurine-dependent, however recent metabolic studies have revealed that *B. wadsworthia* can also co-operatively utilise sulfoquinovose (SQ), a monosaccharide present in green vegetables, to produce $H_2S$. Hanson *et al*. [63] showed that SQ can be fermented by *Eubacterium rectale* to produce 2,3-

dihydroxypropane-sulfonate (DHPS), which results in near stoichiometric production of $H_2S$ by *B. wadsworthia*. The genes required for SQ metabolism are indeed expressed in human gut metagenomes, although at lower prevalence compared to taurine metabolism genes; this is possibly due to the ubiquitous nature of taurine availability in the colon as a result of taurocholic acid catabolism by microbial bile salt hydrolases [63].

Interestingly, *in vivo* models have provided further insight into SQ metabolism; IL10-deficient mice with genetic susceptibility to colitis were colonised with a simplified human gut consortium (SIHUMI) alongside *B. wadsworthia* and fed cyanobacterial biomass spirulina as a source of SQ, SQ directly, or taurocholate as a positive control for $H_2S$ production by *B. wadsworthia* [64]. *E. coli* strain K12 was confirmed to ferment SQ into DHPS. Severe colitis was not observed in any of the conditions, including the taurocholate positive control which had been shown to induce inflammation in the same mouse model previously [65]. Although some increased expression of pro-inflammatory cytokines were observed in the caecal mucosa of mice fed spirulina or taurocholate, no significant colitis or inflammation was demonstrated and the researchers did not measure $H_2S$ concentration in the caecum or faeces [64]. Therefore, understanding if a metabolic flexibility exists in *B. wadsworthia*, and whether this equates to differential $H_2S$ generation based on availability of DHPS or taurine as substrates remains to be investigated.

The metabolism of taurine and SQ has been subject to *in vitro* study, where these compounds were supplemented to co-cultures of SIHUMI and *B. wadsworthia* [66]. With supplementation of 20 mM taurine, cultures produced approximately 20 mM $H_2S$; in comparison, cultures supplemented with 4 mM SQ produced approximately 1.5 mM $H_2S$ [66]. These initial results could suggest that *B. wadsworthia*-derived $H_2S$ production may differ based on the available substrate, however the culture conditions used were not representative of the gut environment, and bacterial abundance was not measured. Understanding whether these observations are reflecting changes to *B. wadsworthia* cell numbers or metabolism in the presence of either plant-derived SQ or animal-derived taurine merits further study; such discrepancies could provide insight as to why *B. wadsworthia* can be pathogenic and pro-inflammatory when a high-fat, animal-

derived diet is consumed [65, 67], despite being present in the guts of 50-60% of healthy individuals [22-24, 68].

## 1.4 Hydrogen sulfide in the colon: friend or foe?

$H_2S$ is the final product of dissimilatory sulfate reduction by SRB. Characterised by an unpleasant rotten egg odour, $H_2S$ is able to freely diffuse across cell membranes [48, 49] and is perhaps best recognised as a considerable industrial hazard as it is both corrosive and highly toxic to many organs at low concentrations [69]. $H_2S$ is also toxic to SRB themselves, although sensitivity differs between bacteria; more sensitive genera such as *Desulfotomaculum* die at 4-7 mM, whereas the more tolerant *Desulfovibrio vulgaris* can survive above 10 mM [44].

Although SRB contribute to the majority of $H_2S$ in the mammalian gut [70], other gut bacteria are also capable of producing this compound; *Escherichia coli*, *Salmonella enterica*, *Clostridium* spp. and *Enterobacter aerogenes* can generate $H_2S$ from cysteine via cysteine desulfhydrase [71, 72]. Indeed, $H_2S$ production via cysteine degradation was recently found to be highly prevalent in the human gut microbiome, particularly among *Collinsella* and *Prevotella* strains [73]. Intestinal $H_2S$ in healthy adult humans ranges from 0.3 to 3.4 mmol/L [74-78], although it is important to note that a large fraction of this sulfide is bound to faecal components; in humans, it was estimated that 8% of total sulfide was in the unbound form, equating to 24 - 272 µM/L free $H_2S$ [79]. The faecal components appear to have a large binding capacity for $H_2S$; although the compounds responsible remain unknown [80], bismuth, zinc and iron were demonstrated to decrease free sulfide in faecal batch fermentation models [81]. Here, the biological activity of $H_2S$ in the human gut will be discussed.

### 1.4.1 Hydrogen sulfide as an endogenous signalling molecule

Although $H_2S$ is highly toxic, a small amount is produced by human cells as a vital endogenous signalling molecule. The transsulfuration pathway degrades homocysteine into D-cysteine or L-cysteine, which are the only known substrates for endogenous $H_2S$ production in mammalian cells [82, 83]. This pathway is catalysed by two enzymes, cystathionine-β-synthase (CBS) and cystathionine-$\gamma$-lyase (CSE). Both CBS and CSE are found throughout the GI tract expressed by

enteric neurons [84]. The activity of CSE appears to largely define endogenous $H_2S$ production in the mammalian intestine [85].

The role of $H_2S$ as a regulator of gastrointestinal motility is demonstrated by the interaction between enteric neurons and smooth muscle cells in the GI tract. Production of endogenous $H_2S$ by enteric neurons exerts potent relaxant effects upon adjacent smooth muscle cells [86, 87], partially via a stimulatory effect upon $K_{ATP}$ channels. This leads to membrane hyperpolarisation and inactivation of L-type $Ca^{2+}$ channels, ultimately reducing muscle contractility [88]. Additionally, direct inhibition of L-type $Ca^{2+}$ channels by $H_2S$ has been reported [89]. Endogenous $H_2S$ has also been shown to have pro-secretory effects in the colon, principally via stimulation of TRPV1 (transient receptor potential cation channel subfamily V member 1) receptors on primary afferent neuron terminals [84]. In this way, endogenous $H_2S$ neuronally regulates the secretory activity of the colonic mucosa. Furthermore, $H_2S$ acts as a mitochondrial electron donor at low concentrations to stimulate colonocyte bioenergetics and oxygen consumption [90, 91].

Interestingly, the role of $H_2S$ in colonic pain perception (nociception) has been controversial, with conflicting reports from animal studies. An anti-nociceptive effect was reported in response to colonic distension in rats [92], whereas a spontaneous pro-nociceptive effect was presented in mice when sodium hydrosulfide (a $H_2S$ donor) was administered intracolonically [93]. Such discrepancies could potentially be explained by the relaxant effect of $H_2S$ on smooth muscle, where muscle relaxation reduces colonic distension leading to decreased pain response, as opposed to a direct effect upon nociception [94]. However, endogenous $H_2S$ production may have a pro-nociceptive effect; upregulation of expression of the enzyme cystathionine-β-synthase in colonic neurons was implicated in the development of chronic visceral hyperalgesia in rats, an analogue of irritable bowel syndrome [95]. The inconsistencies in available literature highlight the complex role of $H_2S$ in intestinal nociception.

### 1.4.2 Deleterious effects of hydrogen sulfide

The variety of physiological functions exerted by $H_2S$ highlight its importance as an endogenous signalling molecule when produced in small amounts by human cells.

Due to persistent colonisation by SRB, the colonic mucosa is continually exposed to relatively high $H_2S$ concentrations compared to the rest of the human body. Therefore, the mucosa is adapted to this and is capable of detoxifying $H_2S$ through a series of oxidation reactions catalysed by mitochondrial-associated enzymes to produce sulfate, which can be excreted renally [96]. Additionally, low millimolar $H_2S$ concentration may enhance respiration of the colonocytes [97]. An attractive hypothesis is that genotypic differences in enzymes involved in colonic $H_2S$ detoxification may pre-dispose individuals to $H_2S$-mediated inflammatory disorders which, when combined with excessive production of bacterial-derived $H_2S$, results in detrimental outcomes [97, 98].

At higher concentrations, $H_2S$ reduces the ability of colonocytes to oxidise butyrate and other microbial-derived SCFAs, impairing cell metabolism and ultimately reducing colonocyte viability [79, 99, 100]. Furthermore, $H_2S$ is toxic to human intestinal cells; Attene-Ramos *et al*. [101] showed that human intestinal epithelial cells exposed to $H_2S$ overexpressed genes associated with oncogenesis and DNA damage, with genotoxic effects at concentrations as low as 250 µM. Such concentrations are physiologically relevant, as the colonic concentration of free $H_2S$ in healthy humans can reach 272 µM [74-79]. Additionally, $H_2S$ has been shown to induce DNA damage in colonocytes directly through the production of oxidative species [102], with cytotoxic effects upon human intestinal cells at 360 µM when DNA repair is inhibited [103].

## 1.5 SRB in human health and disease

Given the toxic nature of $H_2S$ and the ability of SRB to generate high concentrations of this molecule during their metabolism, it is perhaps expected that they are associated with detrimental health outcomes to the host. Indeed, SRB are the major group of bacteria contributing to intestinal $H_2S$ [70]. It is however important to bear in mind that SRB contribute to the hydrogenotrophic niche in the gut needed to maintain fermentation within the gut microbiota [22]. Here, the evidence linking SRB to human health and disease will be discussed.

### 1.5.1 Inflammatory bowel disease

Inflammatory bowel diseases (IBD) including ulcerative colitis (UC) and Crohn's disease are chronic, relapsing-remitting inflammatory disorders of the GI tract.

Although the aetiology of IBD is poorly understood, a combination of environmental and genetic risk factors has been implicated in the pathogenesis of these inflammatory conditions [104, 105]. The global prevalence of IBD has increased significantly in recent years, particularly in Westernised cultures [106], highlighting environmental factors such as dietary habits and increased visceral fat as potential key players in IBD pathogenesis [107].

SRB and their by-product $H_2S$ have been implicated in IBD; UC patients with active disease showed higher SRB carriage and $H_2S$ concentrations in faeces compared to those with quiescent disease, which could be correlated to clinical severity [108]. Similarly, increased faecal SRB carriage was also found in UC patients compared with healthy controls [109]. Furthermore, removal of foods rich in sulfated amino acids from the diet such as red meat, eggs and dairy can ameliorate UC symptoms [110]. Using PCR, one study demonstrated an association between IBD and increased colonisation by *Desulfovibrio piger* compared to healthy controls [42]. However, other studies suggest that SRB carriage is not significantly increased in IBD; Fite *et al*. [111] used a PCR-based approach to show that *Desulfovibrio* ubiquitously colonised the bowel regardless of disease status, hypothesising that host defects in $H_2S$ detoxification are required for IBD pathogenesis. Further evidence implicating SRB and $H_2S$ in UC comes from pouchitis, an inflammation of the ileal pouch which is an artificial rectum surgically produced during a colectomy for the management of UC or other long term colitis patients [112]. $H_2S$ concentration in UC patients with pouchitis was significantly higher than in patients with pouchitis related to other diseases, implicating $H_2S$ in UC aetiology [113].

More recently, the link between $H_2S$ and IBD has been postulated to be via degradation of the mucus barrier lining the intestinal epithelium. Ijssennagger *et al*. suggested that reduction of disulfide bonds in mucus by $H_2S$ results in increased mucus permeability, allowing micro-organisms and toxic compounds to contact the epithelial surface, initiating a series of inflammatory responses [114]. This is based on evidence demonstrating that mucus barrier function is decreased in the colon of IBD patients [115, 116]. In situations where the colonic mucosa is exposed to high levels of $H_2S$ whether it be due to high SRB carriage, increased metabolic capacity of SRB within the gut, or impaired detoxification pathways, there may be

deleterious outcomes for the intestinal barrier and possibly the wider host. Indeed, intestinal inflammatory disease aetiology may be the result of a combination of these factors. Therefore, an understanding of the factors that influence SRB abundance and $H_2S$ production may provide valuable targets for the prevention of intestinal inflammatory diseases.

### 1.5.2 Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder encompassing a group of symptoms including visceral hypersensitivity and abnormal bowel movements without evidence of physical intestinal pathology. IBS is chronic and highly prevalent, affecting up to 21% of the general population [117]. Given that IBS is largely characterised by abdominal pain, the association of $H_2S$ with intestinal nociception highlights a potential involvement in IBS pathophysiology. Accordingly, the endogenous $H_2S$-producing enzyme CBS has been shown to contribute to abdominal pain in a rat model of IBS [95], reiterating the enigmatic role of $H_2S$ in intestinal nociception. Functional dysbiosis has been reported in IBS patients, with decreased levels of butyrate-producing *Roseburia* species and increased colonisation by SRB relative to controls, linking $H_2S$ with onset of IBS [118].

The most common IBS symptom is postprandial bloating, reported by up to 92% of patients [119]. Small intestinal bacterial overgrowth (SIBO) may explain this common symptom, potentially allowing a unifying pathophysiology for IBS to be identified. Indeed, 78% of IBS patients could be diagnosed with SIBO via hydrogen breath test analysis [120]. Interestingly, eradication of SIBO eliminated IBS symptoms in 48% of patients [120]. Analysis of exhaled $H_2S$ in IBS-D (diarrhoea-dominated symptoms) subjects showed that exhaled $H_2S$ was significantly higher in SIBO-positive patients compared to SIBO-negative patients [121]. Such evidence alludes to a possible role of SRB and their product $H_2S$ in the pathogenesis of IBS-associated SIBO. However, this evidence is tentative and as such, further investigation will be necessary.

### 1.5.3 Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer and second most common cause of cancer deaths worldwide [122]. Over 95% of colorectal cancers

are sporadic, occurring in patients without significant genetic risk [123], meaning that environmental factors are pivotal in CRC pathogenesis. CRC prevalence is highest in Australia, New Zealand, US and Europe [124], and key risk factors include obesity, IBD, consumption of red meat, and lack of exercise. Taken together, CRC pathogenesis appears to involve a strong 'Westernisation' risk factor.

CRC, like other cancers, requires the accumulation of DNA damage in affected cells which permits uncontrolled proliferation. As $H_2S$ has been established as a toxic agent able to induce DNA damage in intestinal epithelial cells [101-103], it becomes a factor of interest in CRC development. Indeed, higher faecal $H_2S$ was observed in patients with CRC compared to healthy age-matched controls [125]. Additionally, decreased $H_2S$ detoxification capacity by the colonic epithelium is found in CRC [126]; this is also observed in IBD as discussed previously, and may explain why IBD is a risk factor for CRC development.

Diet might also play a key role in the aetiology of cancer development, as there is strong epidemiological evidence linking consumption of red meat and saturated fats with CRC development [127-129]. Red meat is high in sulfated amino acids such as taurine, which can promote SRB growth and subsequent $H_2S$ production [130]. Furthermore, exogenously applied $H_2S$ was able to strongly induce *in vitro* CRC cell proliferation [131], suggesting that $H_2S$ may play a propagating role during active CRC development in addition to its role as a genotoxic agent during pathogenesis.

Increased SRB abundance is also linked to CRC development. Yazici *et al*. [132] observed higher rates of intestinal SRB carriage, particularly *B. wadsworthia* in African-Americans compared to non-Hispanic whites. The authors identified this increased abundance of sulfidogenic bacteria as a potential environmental factor contributing to the increased CRC incidence observed in African-Americans [132]. Overall, several studies have associated SRB abundance and resulting $H_2S$ production with pathological intestinal inflammation as observed in IBD and CRC, and they have been tentatively implicated in the pathogenesis of IBS. Therefore, an understanding of the factors influencing SRB abundance and function is an important research question for modern biosciences.

### 1.5.4 Parkinson's Disease

Sulfur metabolism in the gut has also been implicated in the pathogenesis of Parkinson's Disease (PD), a chronic neurodegenerative condition affecting the central nervous system. Early symptoms are often associated with the motor system and include bradykinesia, rigidity and altered walking gait. As the disease progresses, non-motor symptoms emerge impacting cognition, sleep and behaviour. In the later stages Parkinson's Disease dementia is common, affecting up to 78% of those with PD [133]; life expectancy is typically 7-15 years after diagnosis. The commonly accepted pathophysiology of PD involves abnormal aggregates of alpha-synuclein called Lewy bodies in the substantia nigra region of the brain, resulting in progressive cell death of the dopamine-producing neurons [134]. Most cases have no distinguishable cause, although a small subset of cases can be linked to genetic factors [135]. PD is one of the neurogenerative disorders with rapidly increasing prevalence worldwide [136], and affected 6.1 million people globally in 2016 [134]. PD typically affects older adults >60 years old, although early-onset PD is seen in people before the age of 50. Currently, there are no curative treatments and medications aim to manage symptoms. There is an urgent need for preventative therapeutics and a greater understanding of PD pathogenesis [136]. Recently the interplay between the gut microbiota, the gastrointestinal tract and the central nervous system (termed the microbiota-gut-brain axis) has been implicated in the pathogenesis of PD [137]. Generally, decreased abundance of SCFA-producing *Roseburia*, *Fusicatenibacter*, *Blautia*, *Anaerostipes* and *Faecalibacterium* genera is observed in PD compared to controls [138], which corresponds to functional observations of decreased faecal butyrate levels and increased gut permeability [139].

SRB and $H_2S$ in the gut has also been implicated in PD pathogenesis; in a small cohort study, enrichments in *Akkermansia muciniphila* and *B. wadsworthia* were observed in PD compared to controls, in addition to increased secretion potential of $H_2S$ in the PD gut microbiota [140]. Interestingly, higher concentrations of both free and protein-bound $H_2S$ were found in the cerebrospinal fluid of PD patients compared to controls [141]. Furthermore, the abundance of *Desulfovibrio* has been correlated with PD severity [142]; it has been hypothesised that $H_2S$-producing *Desulfovibrio* cause toxicity in alpha-synuclein producing enteroendocrine cells in the intestinal mucosa, which could allow alpha-synuclein

aggregation to seed the central nervous system via the vagus nerve [142]. In mice, increased abundance of *Desulfovibrio* was observed in PD compared to controls, and this was negatively correlated with abundance of N-acetyl-L-leucine (NALL), a neuroprotective metabolite [143]. Overall, gut dysbiosis and inflammation has been associated with PD pathogenesis, and SRB and SRB-derived $H_2S$ may contribute to disease development [144].

## 1.6 *Bilophila wadsworthia* in human health and disease

*B. wadsworthia* is considered to be virulent as it is the third most common anaerobic isolate from appendicitis samples and appears to be clinically important in a variety of anaerobic infections [145, 146]. Furthermore, it exhibits endotoxic activity [147], and is adherent to human embryonic intestinal cells *in vitro* [148]. In the three decades since being reported as a novel genus, correlational studies in humans have linked *B. wadsworthia* enrichment in the gut with many diseases including CRC [149], multiple sclerosis [150, 151], Parkinson's Disease [152], dementia [153], non-alcoholic steatohepatitis [154], intrahepatic cholestasis in pregnancy (ICP) [155], diabetic kidney disease [156] and schizophrenia [157]. Additionally, it was demonstrated that a human stool-derived *B. wadsworthia* strain was able to induce systemic inflammation in specific-pathogen-free mice [67], although no localised intestinal inflammation was identified.

In recent years, the deleterious effects of *B. wadsworthia* are becoming increasingly recognised in the field of human gut microbiota research. An ongoing controlled feeding trial in African Americans with high CRC risk is being conducted, using a crossover design to investigate the impact of a diet high in animal-derived fat on the abundance of pro-inflammatory secondary bile acids and sulfate-reducing bacteria (SRB) such as *B. wadsworthia* [158]. Furthermore, a positive correlation between *Bilophila* abundance and number of concomitant diseases in older adults was recently reported [159]. Additionally, efforts to develop therapeutic inhibitors against the isethionate sulfite-lyase (IslA) of *B. wadsworthia* were reported, where the authors postulate the use of such agents to prevent inflammation and potentially CRC development by reducing intestinal $H_2S$ [160]. The authors also published their efforts towards a targeted vaccine against *B. wadsworthia* [161]. In addition, a randomised 3 month clinical trial assessed the impact of empaglifozin, a novel, orally-administered type 2 diabetes treatment on

the gut microbiota [162]. It was shown that that empaglifozin increased the levels of short-chain fatty-acid (SCFA)-producing bacteria including *Roseburia*, *Eubacterium*, and *Faecalibacterium*, and reduced the abundance of *Escherichia-Shigella*, *Bilophila*, and *Hungatella* [162]. Empaglifozin conferred improvement to cardiovascular disease risk factors compared to metformin treatment, and the authors suggested that the reduced abundance of opportunistic pathogens contributed to this effect via reduction in gut inflammation [162]. Collectively, the growing evidence implicates *B. wadsworthia* as a pathobiont in the human intestine, contributing to inflammatory and pathogenic processes.

Conversely to these indications of pathogenicity, it is of major interest that the presence of *B. wadsworthia* has been frequently demonstrated in the faeces of 50-60% of healthy individuals at $10^3$ – $10^6$ CFU per gram of faeces [22-24, 68], and can also be isolated from buccal and vaginal samples [24]. Using a qPCR-based approach, Scanlan *et al*. showed that *B. wadsworthia* was present ubiquitously in the faeces of healthy individuals [23]. Given that *B. wadsworthia* has been implicated as a potential pathobiont in the human gut, its presence as a commensal in the gut microbiota of healthy individuals highlights the need to understand what triggers *B. wadsworthia* to become pro-inflammatory. *B. wadsworthia* has been largely overlooked in studies of the human gut microbiota, perhaps due to variable phenotype and slow growth when cultured *in vitro* [50]. Therefore, identifying the function of *B. wadsworthia* in the human gut, and the factors that influence abundance and function merit further investigation.

## 1.7 The impact of diet on the human gut microbiota

It is apparent that the gut microbiota plays a key role in human health, and therefore an ability to directly influence the composition and function of the microbiota is a desirable goal. Although the composition of the gut microbiota in healthy adults remains largely stable over time [163], dietary alterations can result in notable shifts. Animal-based diets high in protein and fats and plant-based diets high in fibre can differentially and rapidly alter the gut microbiota composition [164]. Dietary macronutrients appear to be significant determinants of gut microbiota composition; for example, long-term dietary habits have been strongly correlated with gut enterotypes, which describe distinct clusters of gut microbiota composition [165]. A 2011 study associated a *Prevotella*-dominated enterotype with increased carbohydrate consumption, whereas a *Bacteroides*-dominated enterotype was associated with higher intake of animal proteins and fats [166]. Similarly, a comparison of the gut microbiota in African children consuming polysaccharide-rich diets showed significantly higher levels of *Prevotella* in comparison to European children consuming protein-rich diets [167].

The microbes in the large intestine are dependent upon the macronutrients consumed by the host, meaning that nutrition sculpts the gut microbiota composition and function. This, in turn, influences human health, confirming the well-known adage “you are what you eat”. The Western diet, characterised by high sugar, animal fat and protein intake [168, 169], is rising globally. Given that this dietary pattern is increasingly associated with the development of obesity, inflammatory diseases and colorectal cancers [170], understanding how the Western diet affects the gut microbiota, an important determinant of human health, is imperative.

### 1.7.1 Dietary sulfate

In the human gut, SRB compete with methanogenic archaea and acetogens such as *Blautia* for hydrogen oxidation [171]. Dietary sulfate and colonic transit time were shown to be major determinants of this competitive relationship [27]. Colonic sulfate arises from the diet, although free sulfate can be released from the sulfated glycosaminoglycan residues in the host mucins, and from sulfonic acid moieties in bile acids via the activity of microbial sulfatases [19]. Daily dietary intake of sulfate in humans is variable from 2 mM/day up to 16 mM/day [26, 78]. Some sulfate is

absorbed in the small intestine and is used in the synthesis of sulfated compounds including cysteine, methionine, chondroitin sulfate and mucins [172]. The remaining sulfate reaches the colon, where it is available to SRB [57]. Sulfate-rich foods include commercial breads (8-15 µM/g), soya flour (12 µM/g), brassica vegetables including broccoli and brussels sprouts (9 µM/g), and sausages (10 µM/g) [172]. Dried fruits are also high in sulfate, with dried apricots containing up to 40 µM/g [172]. Sulfate is also found in beverages including beer, cider (2.6 µM/mL) and red wine (3.8 µM/mL) [172]. Additionally, drinking water can be a major dietary source of sulfate; some private wells contain up to 20 mM/L [75].

#### 1.7.1.1 Impact of sulfate on the gut microbiota

Literature specifically investigating the impact of dietary sulfate on the gut microbiota is relatively sparse; in one human study, dietary sulfate was shown to increase sulfate availability in the gut which was associated with increased sulfate reduction rate and faecal $H_2S$ [27]. Additionally, sulfate was found to alter anaerobic gut fermentation by acting as an electron sink, allowing bacteria to utilise hydrogen as an electron donor; this results in increased acetate production at the expense of butyrate and lactate production [173].

Although diet contributes towards the colonic bioavailability of inorganic sulfate to the gut microbiota, another major source of free sulfate is from host mucins; in fermentation models, porcine mucin supplementation resulted in increased $H_2S$ production [174]. Furthermore, a mucin-degrading *Bacteroides fragilis* was able to support the growth of *Desulfovibrio desulfuricans* via sulfate generation [174]. Indeed, mucins are an abundant potential source of sulfate; the mucin layers in the large intestine are both thicker [175] and more sulfated [176] than those in the small intestine. Interestingly, higher sulfatase activity was observed in the guts of ulcerative colitis patients compared to controls [177]. Overall, the availability of sulfate to SRB in the human gut depends on both diet and the presence of sulfatase-expressing bacteria. These factors are likely to influence the metabolism and abundance of SRB and in turn the overall $H_2S$ burden in the colon, which has important implications for gut inflammation and potential disease pathogenesis.

### 1.7.2 Dietary taurine

Taurine (2-aminoethanesulfonic acid) is a sulfur-containing amino acid well-known as a constituent of living organisms [178] and is widely distributed throughout the

human body. Taurine exerts many important physiological functions including membrane stabilisation, calcium transport modulation and oxygen radical detoxification [179]. In the mammalian intestine, taurine is a major constituent of bile which facilitates digestion of dietary fats and fat-soluble vitamins. Bile acids are conjugated with glycine or taurine, although glycine-conjugation usually dominates [180].

Taurine is commonly found in the human diet, with the highest concentrations being found in seafood (~9-40 µM/g), followed by red meat (3-4 µM/g) and chicken (1-6 µM/g) [181]. Dairy milk is another rich source of taurine, containing approximately 30 µM per 100 mL [182]. Additionally, high concentrations of taurine can be found in energy drinks, with each serving containing approximately 1 g or 8000 µM [183]. Terrestrial fruits and vegetables contain low amounts of taurine, producing diet-based discrepancies in intake; omnivorous diets provide approximately 40-400 mg of taurine daily [184] and in some cases above 1 g [183], whereas strict vegan diets contain negligible amounts [185]. In the small intestine, dietary taurine is absorbed via taurine transporters (TAUT) and distributed globally to many organs. In the liver, taurine is conjugated to bile acids producing taurocholic acid, which is secreted into the duodenum via the bile duct to aid in solubilisation of fats [186]. Once in the colon, microbial-derived bile salt hydrolases are able to deconjugate taurocholic acid to yield free taurine, which *B. wadsworthia* can utilise for growth [186] (Figure 2). Therefore, bioavailability of taurine in the colon is dependent upon diet, taurine-conjugated bile acids and abundance of microbial bile salt hydrolases (Figure 2).

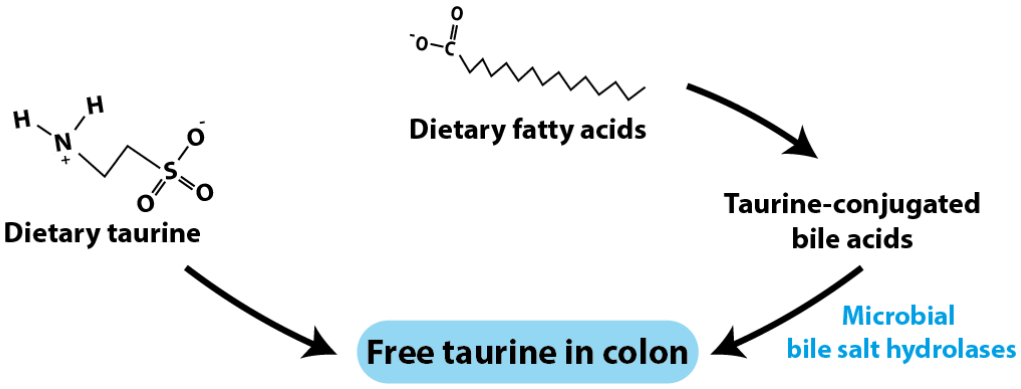



Figure 2: Dietary taurine and dietary fat may contribute towards increased taurine concentration in the colon.

Although literature investigating taurine concentration in the human colon is sparse, one study analysed mucosal taurine concentration in endoscopic biopsy specimens from 10 patients [187]. The average taurine concentration in the colonic mucosa was 2.49 mM/kg, comprising 18.3% of the total free amino acids present [187]. Given that taurine concentration is a determinant of *B. wadsworthia* growth, further analysis of deconjugated taurine concentration in the human colon should be considered.

#### 1.7.2.1 Impact of taurine on the gut microbiota

Global consumption of taurine-rich foods is rising in line with westernisation [188]. Therefore, investigation into how dietary taurine affects the human gut microbiota and by extension, human health, is of considerable interest. Studies investigating the impact of dietary taurine on the colonic gut microbiota are notably few, however one such study observed that in mice supplemented with 165 mg/kg taurine daily for one month, the abundance of *Proteobacteria*, particularly *Helicobacter* was reduced compared to controls [189]. No significant changes to *Bilophila* abundance were identified between groups [189], and investigators concluded that taurine supplementation reduced the abundance of pathogenic bacteria in the mouse gut [189].

Using an *in vitro* fermentation model seeded with human faeces to investigate taurine supplementation on the gut microbiota, Sasaki *et al*. reported no major changes to gut microbiota diversity, and no degradation of taurine under anaerobic conditions [190]. The authors failed to consider that it is therefore unlikely that the faeces contained *B. wadsworthia*, as colonisation is observed in only 50-60% of individuals [22-24]. As this study used faecal samples from only 8 individuals [190], this could explain the lack of anaerobic taurine utilisation reported. The gap in published literature highlights the under-exploration of the impact of dietary taurine on the human gut microbiota; human taurine consumption is increasing globally [191], and therefore characterisation of the resultant impact on the gut microbiota is important for future human health.

Interestingly, a recent study by Stacy *et al*. [192] demonstrated that supplementing taurine to the drinking water of mice increases free taurine concentrations in the distal colon without altering bile acids [192]. This provides supportive evidence to suggest that experimental designs involving taurine supplementation to *in vitro* gut

models do retain physiological relevance. Furthermore, following a challenge with pathogenic *Klebsiella pneumoniae*, mice showed increased colonic abundance of taurine-conjugated bile acids, Deltaproteobacteria, and microbial genes associated with taurine metabolism. These changes to the gut microbiome were closely associated with colonisation resistance against *K. pneumoniae* [192]. Therefore, bile-acid-derived taurine could be beneficial for colonisation resistance through a $H_2S$-dependent mechanism, whereby $H_2S$ inhibits pathogen respiration [192]. In *ex vivo* culture of faeces from taurine-treated mice, significantly more $H_2S$ was produced when taurine was added to the culture compared to the faeces from mice that did not receive taurine supplementation [192]. Furthermore, *B. wadsworthia* was directly implicated in this phenomenon; enhanced resistance to *K. pneumoniae* infection was observed when the gut microbiota of mice was preferentially enriched with *B. wadsworthia* [192]. Overall, these findings suggest that gut microbes able to utilise taurine to produce $H_2S$ (such as *B. wadsworthia*) could provide adaptive benefits to the host by enhancing colonisation resistance to pathogens. Such conclusions challenge much of the available literature, which has largely associated *B. wadsworthia* expansion and metabolism with deleterious outcomes. Indeed, the role of *B. wadsworthia* as a member of the human gut microbiota remains enigmatic.

### 1.7.3 Dietary fat

Most dietary fat (95%) is found as triacylglycerols, which contain three fatty acids attached to a glycerol backbone [193]. The composition of these fatty acids varies by chain length, number of carbon double bonds (saturated, monounsaturated, polyunsaturated) and double bond position (cis or trans) [194]. Plant-based sources usually contain mono- or poly-unsaturated fats, whereas animal sources contain predominantly saturated fats [195]. Bile acids are amphipathic molecules with detergent activity, which facilitate solubilisation required for absorption of fat and fat-soluble vitamins in mammals. Following hepatic synthesis, bile acids are conjugated to glycine or taurine to ensure full ionisation and solubility at postprandial duodenal pH [196, 197]. In humans, most bile acids are conjugated to glycine [180]. Subsequently, conjugated bile acids are secreted into the duodenum via the gallbladder as a major component of bile.

In the duodenum, fats are solubilised by conjugated bile acids to form micelles, allowing pancreatic lipases to degrade the fats at the oil-water interface [197].

These mixed micelles are able to pass through the unstirred water layer covering the intestinal microvilli to reach the epithelial cells, where an acidic environment enables dissociation of the micelles and diffusion of fatty acids into the epithelial cells [198]. Given that global patterns of fat consumption are changing, with particular increase in animal-derived saturated fat intake as part of a Western diet [191], improved understanding of the effect of increased dietary fat on the gut microbiota is important.

#### 1.7.3.1 Impact of fat on gut microbiota

Most dietary fats are efficiently digested and absorbed in the small intestine, with absorption efficiency decreasing with age [199, 200]. However, approximately 7% of ingested fatty acids reach the colon in healthy individuals as measured via spectrometric analysis of stool [201]. Similarly, most bile acids are reabsorbed in the ileum as part of the enterohepatic cycle, but a small fraction remains in the GI tract and passes into the colon, where they can be degraded by the gut microbiota [202].

As diet-derived fatty acids and conjugated bile acids reach the colon, the question arises of whether these compounds can affect the resident gut microbiota. Indeed, amount and type of dietary fat have been established to influence the gut microbiota [193]. As previously mentioned, a dietary intervention study showed that consumption of an animal-derived high-fat diet increased the abundance of bile-tolerant bacteria including *Bilophila*, *Alistipes* and *Bacteroides* with a concurrent decrease in Firmicutes compared to those on a plant-based diet [203]. Such increases in colonic bile acid levels during increased fat consumption have been reported [204], however the direct contribution of dietary fat to these observations cannot be identified, as dietary fibre intake was also different between diet arms [203]. Similarly, another study compared very low-carbohydrate, high-fat diet with high-carbohydrate, low-fat diet. With the low-fat diet, reduced faecal butyrate and decreased *Bifidobacteria* viable counts were observed [205]. However, dietary protein consumption was also significantly higher in the high-fat group, acting as a potential confounding variable [205]. Human dietary intervention studies are susceptible to interference as altered consumption of macronutrients other than fat may influence observations.

Exciting evidence linking dietary fat with *Bilophila* abundance and function was reported by Devkota *et al*. [65]. Consumption of a diet high in animal-derived saturated fat in mice was shown to significantly increase *Bilophila* load in the colon, a phenomenon not observed with unsaturated fat or no fat. Furthermore, the resulting *Bilophila* bloom was capable of inducing inflammation in genetically susceptible mice [65]. The researchers demonstrated that dietary animal-derived fat increased taurine conjugation to bile acids, resulting in increased taurine in the colon allowing *Bilophila* expansion [65]. Such evidence demonstrates a potential cross-talk between dietary fat and taurine as determinants of *Bilophila* abundance in the gut.

More recently, Natividad *et al*. supported these findings, showing that a diet high in milk-derived saturated fats significantly increased *Bilophila* abundance in mice compared with controls [206]. Moreover, the investigators used oral gavage to demonstrate that high-fat diet synergised with *Bilophila wadsworthia* to produce intestinal inflammation and metabolic dysfunction [206]. Additionally, administration of a probiotic *Lacticaseibacillus rhamnosus* strain well-characterised as having anti-inflammatory properties resulted in reduced *Bilophila* abundance and ameliorated the deleterious effects associated with *Bilophila* expansion [206]. Taken together, these studies demonstrate that dietary sources high in animal-derived saturated fat can increase intestinal *Bilophila* abundance in mice, which is directly associated with inflammation and metabolic dysregulation [65, 206]. Given that *Bilophila* is often found as a member of the human gut microbiota, such evidence provides an intriguing hypothesis of *Bilophila*'s potential involvement in the pathogenesis of intestinal inflammatory diseases including IBD and IBS and metabolic disorders such as obesity and diabetes. Furthermore, work by Natividad *et al.* [206] alludes to the use of probiotics as preventative or therapeutic measures against such disorders.

Moreover, several *in vivo* studies using mouse models have identified changes in *Bilophila* abundance being associated with positive or negative health implications in response to specific dietary factors. For example, supplementing high-fat diet-induced obese mice with *Dendrobium officinale* dietary fibre resulted in significantly reduced *Bilophila* abundance [207]. A faecal microbiota transplant (FMT) was performed on high fat diet-induced obese mice, and FMT from mice

that received the dietary fibre supplementation resulted in decreased body weight gain, fat accumulation and improved glucose clearance relative to the control [207]. The authors discussed that the decreased abundance of *Bilophila* “may have a positive effect on obesity, because high levels of *Bilophila* species can aggravate inflammation and metabolic disorders in HFD-induced obese mice” [207]. Similarly, in high-fat diet-induced mice supplemented with sea buckthorn polysaccharide, there was a significantly increased abundance of *Bifidobacterium*, *Bacteroides* and *Alistipes*, and decreased abundance of *Streptococcus* and *Bilophila*; the supplementation significantly reduced body weight gain, serum lipids and liver triglycerides [208]. Further faecal metabolomic analysis revealed an association between decreased *Bilophila* abundance and increased faecal butyrate and acetate [208]. A further study using the high-fat diet-induced obese mouse model supplemented with tempol, a cyclic antioxidant, and assessed the impact on the gut microbiota; interestingly, although tempol ameliorated obesity in the mice, it increased *Bilophila* abundance in the gut 27-fold [209]. The authors suggest that tempol itself “may act to reduce some of the undesirable side effects of an expansion of the *Bilophila* genus”, although this was not investigated [209]. Overall, these studies reiterate previous observations of *Bilophila* expansion in response to high fat diet, but also demonstrate the amelioration of high-fat diet-induced dysbiosis using antioxidants and high fibre supplements.

An inverse correlation between fibre intake and *Bilophila* abundance was also found in humans; a recent study investigating the relationship between dietary factors and the gut microbiota reported that *Bilophila* abundance is inversely correlated to dietary plant protein intake and dietary fibre, and positively correlated to dietary animal protein intake [210]. Another small-scale study involving 12 participants used a dietary intervention model where participants consumed either plant-based, high-fibre diet or animal-based, low-fibre diet for 1 week, separated by a 2-week wash out period [211]. Faecal $H_2S$ levels were quantified *ex vivo*; median $H_2S$ production was higher following the animal-based diet compared to the plant-based diet, but there was substantial interindividual variability [211]. Overall, the evidence demonstrates that several dietary factors influence the abundance of *Bilophila* in the gut; dietary fat can promote *Bilophila* expansion likely by increasing free taurine in the colon, and dietary fibre may prevent or reduce this expansion although the reason why is not currently clear. A greater

understanding of the interplay between diet and the wider gut microbiota in determining *Bilophila* abundance and metabolism within the human gut is therefore an important research goal.

## 1.8 Prevalent species of bacteria in the human gut – *Bacteroides thetaiotaomicron*

*Bacteroides thetaiotaomicron* is a Gram-negative obligate anaerobe within the Bacteroidaceae family, and was the first species within the *Bacteroides* genus to be sequenced [212]. Originally isolated from the faeces of a healthy adult [212], *B. thetaiotaomicron* is highly abundant constituting 1-6% of the total bacteria [213] with a 46% prevalence [214], and is a key commensal member of the human gut microbiota. Given the high abundance and prevalence, *B. thetaiotaomicron* unsurprisingly fulfils important ecological niches. Such roles include modulation of the host mucosal immune system by directly influencing the expression or topology of the host glycans [215] notably by increasing mucosal fucosylation, which reportedly alters the intestinal immune function to provide protection against pathogen colonisation [216]. In germ-free mouse models, *B. thetaiotaomicron* has been shown to modulate the immune system by inducing mucosal IgA production, an important feature of barrier integrity [217]. Additionally, *B. thetaiotaomicron* appears to play a role in regulating intestinal inflammation; this species is able to modulate inflammatory reactions in intestinal epithelial cells via the PPAR-ɣ anti-inflammatory pathway [218], and ameliorate the increased barrier permeability caused by TNF-α and IFN-ɣ [219].

*Bacteroides* spp. play a key role in carbohydrate metabolism in the gut and encode a repertoire of carbohydrate-active enzymes (CAZymes), with the highest number encoded by *B. thetaiotaomicron* strains [220]. Strikingly, up to 18% of the *B. thetaiotaomicron* genome is dedicated to polysaccharide utilisation genes [220] which demonstrates the ecological niche occupied by this commensal species. Gene clusters encoding CAZymes are defined as Polysaccharide Utilisation Loci (PULs); *B. thetaiotaomicron* is predicted to encode 86-96 PULs [220].

*B. thetaiotaomicron* is one of the key degraders of resistant starch, encoding Starch utilisation system (*sus*) genes that can degrade amylose, amylopectin and pullulan [221], which are inducible by maltose [222]; the sus genes were the first PUL to be studied [220]. *B. thetaiotaomicron* has the capacity to degrade the

majority of dietary polysaccharides including β-glucan, fructan, mannan, arabinan, galactan, homogalacturonan, arabinogalactan and rhamnogalacturonan [220]. Similarly, *B. thetaiotaomicron* can also degrade host-derived polysaccharides such as the glycosaminoglycans found in the mucus layer of the colon, including chondroitin sulfate, mucin, hyaluronate and heparan sulfate [223]. Ultimately, the bacterium degrades the polysaccharides to yield simple sugars for fermentation, resulting in the production of beneficial short-chain and organic acids including acetate, lactate, succinate and propionate [224]; *B. thetaiotaomicron* has also been shown to support the growth of butyrate-producing *Anaerostipes caccae in vitro* [224]. SCFAs are a valuable source of energy for human colonocytes and aid in maintaining healthy barrier function [225]. Furthermore, *B. thetaiotaomicron* readily alters CAZyme expression based on nutrient availability [220]; in the fucose utilisation gene cluster, a transcriptional repressor (FucR) induces expression of fucose degradation genes and represses a gene locus that induces host glycan synthesis. This ensures that fucosidases are only expressed when fucose is present. Given that the host mucins are used as an alternative glycan source, this system also ensures that the host synthesis of glycans matches the nutritional needs of the commensals present [226].

Although *B. thetaiotaomicron* is a highly prevalent gut commensal, it can be an opportunistic pathogen in some cases; *B. thetaiotaomicron* was recovered in 70% of cases of perforating and gangrenous appendicitis [32] and was found in the endometria and oviducts of women with moderate to severe pelvic inflammatory disease [227]. Additionally, some enteric pathogens may exploit *B. thetaiotaomicron* as a commensal; in a mouse model, *Citrobacter rodentium* virulence was found to be enhanced by *B. thetaiotaomicron*-derived succinate [228]. Indeed, it has been hypothesised that *Bacteroides* spp. pose a “double-edged sword” in enteric infections due to the high capacity for cross-feeding; if probiotic species are lost as a result of infection or perturbation, *Bacteroides* spp. could exacerbate dysbiosis by cross-feeding to pathogens [229].

*B. thetaiotaomicron* also encodes 28 sulfatases which can cleave sulfated residues of the host glycosaminoglycans to yield free sulfate [220, 230]. Although many SRB encode sulfatases, the additional *B. thetaiotaomicron* sulfatase activity could permit increased sulfate reduction and $H_2S$ production by SRB [78]. Indeed,

the co-colonisation of *B. thetaiotaomicron* and SRB in the gut has been described [231, 232], where *B. thetaiotaomicron* was shown to provide free sulfate to *Desulfovibrio piger* promoting $H_2S$ production both *in vitro* and *in vivo* [233]. Overall, *B. thetaiotaomicron* is a highly abundant and prevalent commensal gut strain which has been demonstrated to influence the metabolism and abundance of SRB and in turn the overall $H_2S$ burden in the colon via cross-feeding of free sulfate. A further important consideration is that *B. thetaiotaomicron* generates hydrogen during polysaccharide fermentation [234] which is used by SRB, including *B. wadsworthia* as an electron donor during dissimilatory sulfate reduction [18, 54]. In this thesis, I establish whether *B. thetaiotaomicron* influences the growth of *B. wadsworthia*, a sulfite-reducing bacterium unable to use sulfate. The results will be discussed in coming chapters.

## 1.9 Prevalent species of bacteria in the human gut – *Enterococcus spp.*

Enterococci are a diverse group of facultatively anaerobic lactic acid bacteria found in many environments including human and animal guts [235], freshwater [236] and soil [237]. They are also important in food preservation [238] and fermentation [239]. Within the phylum Firmicutes, Enterococci are abundant and prevalent within the human gut microbiota constituting up to 1% of the total colonic bacteria in 80% of adults [240], the most common species being *Enterococcus faecalis* and *Enterococcus faecium* [241]. As gut commensals, enterococci are highly adapted for survival in the mammalian intestine where they metabolise nutrients, maintain environmental pH, modulate the mucosal immune system [242], and contribute colonisation resistance against pathogens [243]. Indeed, commensal Enterococci have developed mechanisms to exclude exogenous enterococcal competitors from colonising the gut [244].

Despite their presence in healthy individuals, both *E. faecium* and *E. faecalis* are clinically important pathogens able to cause urinary tract infections, bacteraemia, endocarditis and meningitis [245] with the aid of virulence factors including cytolysin, adhesin and enterococcal surface protein [246]. Importantly, pathogenic enterococci often exhibit high levels of intrinsic resistance, with resistance against last-line antibiotics such as vancomycin becoming increasingly common; Vancomycin Resistant Enterococci (VRE) represent a significant clinical challenge particularly in the US [247]. The serious health burden imposed by pathogenic

enterococci was recognised by the World Health Organisation, where Vancomycin resistant *E. faecium* was placed in “high priority” in the global pathogens list denoting an urgent need for research and development into treatments and prevention [248].

On the other hand, different species and strains of Enterococci are used in food preservation and ripening particularly in dairy, fermented vegetables and meats [249], and are also commercially available as probiotics. Enterocin production by probiotic enterococcal strains display a broad spectrum antimicrobial activity against pathogens including *Listeria* and *Staphylococcus aureus* [250]. In Germany, *E. faecalis* (DSM 16431) is sold under the brand Symbioflor 1 as an oral suspension containing live bacteria for treatment of bronchitis and sinusitis [251], and *E. faecium* SF68 marketed as Bioflorin has been used as a probiotic to ameliorate antibiotic-associated diarrhoea [252, 253]. Furthermore, Bio-three (a mixture of *E. faecalis*, *Bacillus mesentericus* and *Clostridium butyricum*) reduced the duration and severity of acute diarrhoea in children [254]. Additionally, *E. faecium* is used as a probiotic in animals, including pets and livestock [255].

The contrasting roles of Enterococci as commensals, probiotics and pathogens has been extensively discussed in several comprehensive review articles [241, 243, 245, 249, 250]; ultimately, it appears that commensal and pathogenic strains have distinct genomes and can be phylogenetically split into different clades [256]. It was shown that commensal *E. faecium* strains outcompeted hospital-associated strains in a gut colonisation mouse model, and formed phylogenetically distinct groups [256]. Additionally, comparative genomics between the probiotic Symbioflor 1 *E. faecalis* strain and V583, a vancomycin-resistant *E. faecalis* strain showed the absence of virulence factors and antibiotic resistance genes in the probiotic strain [257]. It seems that the presence of specific virulence-associated genes is a key determinant of whether a strain is pathogenic or not [258]. A risk is that use of enterococci as probiotics or in food preservation may inadvertently introduce drug resistance and virulence factors into the human gut leading to disease [259]. However, Biosymflor 1 has been used for decades with no such events reported [260], and the rate of conjugative gene transfer in enterococci is low [241]. Ultimately, the use of comprehensive genome analysis to establish the virulence

factor and drug resistance profile is key during assessment of a potential probiotic strain [243, 255].

Relating to the topics of this thesis, it was reported that *E. faecium* isolated from dairy was capable of inhibiting $H_2S$ production by bacteria associated with meat spoilage; co-culture with washed cells inhibited $H_2S$ production by *Escherichia coli*, *Citrobacter freundii* and *Hafnia alvei* by 48.6% [261]. Based upon this evidence, an initial hypothesis was formed regarding *E. faecium*'s inhibitory effect upon $H_2S$ production, and whether this effect is also observed in *B. wadsworthia*, an SRB capable of substantial $H_2S$ production [51] with deleterious effects in the gut [98, 100, 113, 121, 262]. Given that *B. wadsworthia* and *E. faecium* are common members of the gut microbiota [23, 263], an *E. faecium*-mediated effect on $H_2S$ production has relevance to human gut health.

## 1.10 Aims and Objectives of Thesis

This thesis aimed to investigate the effect of other gut microbiota members on the abundance and $H_2S$ production of *B. wadsworthia*. Following isolation of various bacterial species from human faeces, *Bacteroides thetaiotaomicron* and *Enterococcus faecium* were identified. These species were selected for further study as *B. thetaiotaomicron* has been previously shown to support the growth of other SRB including *D. piger*, whereas *E. faecium* has been shown to inhibit $H_2S$ production by sulfide-producing bacteria including *E. coli*. I therefore hypothesised that *B. thetaiotaomicron* and *E. faecium* would differentially impact *B. wadsworthia*'s abundance and $H_2S$ production, and subsequently used pairwise co-culturing assays to test this.

Subsequently, efforts were focused on one selected strain of *B. wadsworthia* and *B. thetaiotaomicron*, where transcriptomics were utilised to perform differential gene expression analysis alongside metabolomics analysis to identify the underlying alterations in bacterial metabolism and potential metabolites involved in mediating the microbe-microbe interaction. A similar approach was taken to focus on the interaction between *B. wadsworthia* and *E. faecium*. Additionally, I aimed to assess whether the microbe-microbe interactions observed in simple co-culture could be recapitulated in the context of a complex microbiota system more representative of the *in vivo* human gut conditions. To investigate this, an anaerobic fermentation model was seeded with human faeces along with the

selected strains of interest, and the changes in the gut microbiota composition were investigated using metagenomic sequencing. Furthermore, I hoped to assess the impact of co-cultures and mono-cultures of *B. wadsworthia* and *B. thetaiotaomicron* on the human gut barrier function. To investigate this, Caco-2 mono-layer culture was combined with measurements of Trans-Epithelial Electrical Resistance (TEER) in response to applications of the co-cultures and mono-cultures.

# Chapter 2

## 2. Microbe-microbe interaction between *B. wadsworthia* and *B. thetaiotaomicron*

## 2.1 Introduction

Since *B. wadsworthia* was first described in 1989 [31], the majority of research relating to this Gram-negative bacterium involves correlational studies of the human gut microbiota, where enrichment of *Bilophila* is reported in diseases including colorectal cancer [132, 158], multiple sclerosis [150] and Parkinson's Disease [152]. Additionally, murine studies involving oral gavage have established *B. wadsworthia* as a potential pro-inflammatory pathobiont *in vivo* [67, 206]. During the years after initial discovery, *in vitro* studies revealed that *B. wadsworthia* exhibits endotoxic activity [147] and adheres to human embryonic intestinal cells [148]; in recent years, in-depth *in vitro* study of *B. wadsworthia* is relatively sparse. Simplified pair-wise *in vitro* co-culture has been extensively used in microbiological research for studying microbial interactions [264-267]; to the best of my knowledge, the *in vitro* interactions of *B. wadsworthia* with other gut-derived bacterial strains in simplified co-culture have not been studied thus far.

*Bacteroides spp*. are Gram-negative obligate anaerobes that are highly prevalent in the human gut, comprising approximately 30% of cultivable faecal isolates [63]. *Bacteroides thetaiotaomicron* has been established as an important mutualistic commensal with an extensive capacity for degradation of most dietary polysaccharides [65]. *B. thetaiotaomicron* can also degrade host-derived glycans such as chondroitin sulfate and heparin sulfate, releasing sulfated compounds into the extracellular milieu [66]. Bacteria capable of utilising sulfate such as SRB may benefit from this; indeed, the growth and $H_2S$ production of *Desulfovibrio piger* was shown to be increased in co-culture with *B. thetaiotaomicron* due to provision of free sulfate [67]. *B. thetaiotaomicron* produces hydrogen during fermentative metabolism [234] which is an excellent energy source for *B. wadsworthia* [54]. Thus, hydrogen production as a by-product could promote the syntrophic growth of *B. wadsworthia*.

## 2.2 Objectives

The purpose of the work presented in this chapter is to investigate the impact of *B. thetaiotaomicron* on *B. wadsworthia*'s growth and $H_2S$ production using pair-wise anaerobic bacterial co-culture assays. This was based upon previous research demonstrating that *B. thetaiotaomicron* can support the growth of other SRB via provision of free sulfate and molecular hydrogen. It was therefore hypothesised

that *B. thetaiotaomicron* strains would promote *B. wadsworthia*'s growth and $H_2S$ production.

## 2.3 Materials and Methods

### 2.3.1 Cell culture media

All chemicals were purchased from Sigma unless stated otherwise.

Brain Heart Infusion (BHI), de Man, Rogosa and Sharpe (MRS) and Anaerobe Basal Broth (ABB) liquid broth media (Oxoid, Thermo Scientific) were prepared according to manufacturer's instructions. BHI + complements (BHI+C) was prepared by adding 0.5% v/v vitamin K solution in ethanol (10 µL/L), resazurin (1 mg/L), hemin (5 mg/L) and L-cysteine hydrochloride (0.5 g/L) to BHI media prior to autoclaving. BHI + supplements (BHI+S) was prepared by adding hemin (10 mg/L) and yeast extract (5 g/L) prior to autoclaving. Postgate C was prepared as follows: sodium lactate (6 g/L), sodium sulphate (4.5 g/L), ammonium chloride (1 g/L), yeast extract (1 g/L) potassium phosphate (0.5 g/L), sodium citrate tri basic (0.3 g/L), magnesium sulphate 7-hydrate (0.06 g/L), iron sulphate 7-hydrate (4 mg/L), calcium chloride (0.04 g/L), L-cysteine hydrochloride (0.5 g/L), resazurin (0.8 mg/L), distilled water to 1 L [268]. Where required, solid media was made by the addition of 1.5% (w/v) agar to the media prior to autoclaving. All media and culture vessels were maintained under anaerobic conditions using an anaerobic cabinet (Don Whitley, UK) with materials pre-reduced prior to use for at least 18 h in an atmosphere of 5% $CO_2$, 10% $H_2$ in $N_2$ at 37˚C. Anaerobic media was also prepared and maintained using the Hungate tube method [38]. For pairwise co-culture experiments, a mixed media of BHI + complements, Postgate C and MRS was prepared in a 1:1:1 ratio under sterile conditions to produce BPM media. For *B. wadsworthia* cultures, filter-sterilised taurine was added post-autoclave to a final concentration of 10 mM.

### 2.3.2 Organisms and growth conditions

The species and strains used for *in vitro* co-culture experiments are detailed in Table 1. All isolates obtained were retained for long-term storage at -80°C. Briefly, active liquid cultures were centrifuged at 13,000 x g for 5 minutes and supernatant discarded under anaerobic conditions. The pellet was resuspended in anaerobic PBS and washed again. Finally, the pellet was added to a Protect Select

Anaerobe Cryopreservation tube (Technical Service Consultants, UK) and flash frozen on dry ice prior to storage at -80°C.

Table 1*:* Bacterial strains used in *in vitro* experiments

| **Strain** | **Relevant characteristics** | **Growth conditions (enrichment media)** | **Reference/source** |
|---|---|---|---|
| *B. wadsworthia* QI0012 | Isolated from human faeces, colon model study donor CM001B | ABB + 10 mM taurine; anaerobic; static; 37°C | [269] |
| *B. wadsworthia* QI0013 | Human faeces, colon model study donor CM001D | ABB + 10 mM taurine; anaerobic; static; 37°C | [269] |
| *B. wadsworthia* QI0014 | Isolated from human faeces, colon model study donor CM025A | ABB + 10 mM taurine; anaerobic; static; 37°C | [269] |
| *B. wadsworthia* QI0015 | Isolated from human faeces, colon model study donor CM052A | ABB + 10 mM taurine; anaerobic; static; 37°C | [269] |
| *B. thetaiotaomicron* QI0072 (Bt strain 1) | Isolated from human faeces, COMBAT study donor 14 | BHI+C; anaerobic; static; 37°C | [270] |
| *B. thetaiotaomicron* DSM 108160 (Bt strain 2) | Isolated from human faeces | BHI+S; anaerobic; static; 37°C | DSMZ |
| *B. thetaiotaomicron* DSM 108161 (Bt strain 3) | Isolated from human faeces | BHI+S; anaerobic; static; 37°C | DSMZ |
| *E. faecium* and *B. thetaiotaomicron* enrichment (Ef-Bt) | Enrichment of *E. faecium* (QI0436) and *B. thetaiotaomicron* strain 1 (QI0072). Isolated from human faeces, COMBAT study donor 14 | BHI+C; anaerobic; static; 37°C | [270] |

| | | | |
|---|---|---|---|
| *E. faecium* QI0436 | Isolated from human faeces, COMBAT study donor 14 | BHI+C; anaerobic; static; 37°C | [270] |
| *E. faecium* FI 09198 | Isolated from dried milk powder | BHI+C; anaerobic; static; 37°C | QIB culture collection |
| *E. faecium* FI 09206 | Isolated from milk starter culture | BHI+C; anaerobic; static; 37°C | QIB culture collection |
| *E. faecium* FI 09343 | Isolated from human faeces | BHI+C; anaerobic; static; 37°C | QIB culture collection |
| *E. faecium* FI 09344 | Isolated from human faeces | BHI+C; anaerobic; static; 37°C | QIB culture collection |
| *E. faecium* FI 09346 | Isolated from human faeces | BHI+C; anaerobic; static; 37°C | QIB culture collection |
| *E. faecium* FI 09347 | Isolated from human faeces | BHI+C; anaerobic; static; 37°C | QIB culture collection |
| *E. faecium* FI 09934 | Isolated from human infant faeces | BHI+C; anaerobic; static; 37°C | QIB culture collection |
| *E. faecium* FI 10946 | Isolated from human faeces | BHI+C; anaerobic; static; 37°C | QIB culture collection |
| *E. faecalis* FI 09195 | Isolated from cheese | BHI+C; anaerobic; static; 37°C | QIB culture collection |
| *E. faecalis* FI 09197 | | BHI+C; anaerobic; static; 37°C | QIB culture collection |

## 2.3.3 Microbiology

### 2.3.3.1 Bacterial isolation from faeces

Faecal samples were obtained from healthy adults recruited via the QIB Colon model study (ClinicalTrials.gov Identifier: NCT02653001) [269], and healthy adults 50-80 years old recruited via the COMBAT study (ClinicalTrials.gov Identifier: NCT03679533) [270]. A sterile toothpick was used to inoculate the faecal sample into anaerobic ABB liquid broth media supplemented with 10 mM taurine. After 5 days of incubation at 37°C, samples were sub-cultured at 2% (v/v) inoculum into fresh enrichment media. After the second passage, liquid cultures were streaked onto anaerobic agar for colony isolation and incubated under anaerobic conditions at 37°C. For each sample, morphologically distinct colonies were selected and re-streaked onto agar. This was repeated until pure colonies were obtained.

#### 2.3.3.2 Bacterial co-culture experiments

For co-culture experiments, strains were recovered from long-term storage at -80°C by inoculation into 10 mL enrichment media (Table 1) in Hungate tubes. The cultures were incubated overnight at 37°C, and sub-cultured at 2% into fresh enrichment media. After overnight incubation, the second passage cultures were diluted to c. 1 x $10^8$ CFU/mL using a previously standardised cell count per optical density at 600 nm ($OD_{600}$) factor. Experimental cultures were prepared by inoculating 1 x $10^6$ cells/mL into anaerobic BPM media supplemented with 10 mM taurine. Inoculum was diluted and plated onto anaerobic enrichment media agar for actual CFU/mL determination. In sterile universal glass bottles, experimental conditions included negative control (no inoculum), mono-cultures and co-culture with final culture volumes of 10 mL. All cultures were performed in at least triplicate. The experimental cultures were incubated at 37°C under anaerobic conditions, with sampling at 0 and 8 h post-inoculation.

#### 2.3.3.3 Preparation of bacterial cell-free supernatant

To prepare spent supernatant, overnight second passage cultures were grown in BPM media supplemented with 10 mM taurine under anaerobic conditions. Cultures were centrifuged at 13,000 x g for 5 min to pellet cells, and the supernatant was sterilised by filtration through Minisart® 0.22 um Polyethersulfone (PES) syringe filters (Sartorius, UK). The sterile supernatant was diluted 1:1 with anaerobic BPM media supplemented with 10 mM taurine and inoculated with $10^6$ CFU/mL of live culture. Absence of viable cells from the supernatant was confirmed via inoculation onto enrichment media agar and observing absence of growth.

#### 2.3.3.4 Heat-killed bacterial cultures

To prepare killed cultures, overnight second passage cultures were diluted to $10^8$ cells/mL as described in section 2.3.3.2 and heated at 95°C for 10 min. Experimental cultures were prepared by inoculating the equivalent of $10^6$ CFU/mL of the heat-killed culture with $10^6$ CFU/mL of live culture. Efficacy of killing was confirmed via inoculation onto enrichment media agar and observing absence of growth.

#### 2.3.3.5 Co-culture with 0.22 µm membrane separation

For membrane separation co-culture, mono-cultures of *B. wadsworthia* (QI0013) and Bt strain 1 (QI0072) were prepared as described in section 2.3.3.2. To

prepare filter-separated cultures, mono-cultures were transferred into the upper and lower compartments of sterile Merck Ultrafree™-MC Centrifugal Filter Devices with Durapore™ 0.22 µm membrane (Fisher Scientific, UK). For mono-culture controls, mono-culture was placed into the lower compartment and fresh sterile media in the upper compartment. True co-cultures were also prepared by inoculating strains pairwise in a 1:1 ratio as described in section 2.3.3.2, where the co-culture was inoculated into the lower compartment of the filter device with fresh sterile media in the upper compartment (Figure 10). All cultures were performed with a minimum of three technical replicates. The filter devices were incubated at 37°C under anaerobic conditions on a shaker at 180 rpm to facilitate solute transfer across the 0.22 µm membrane.

#### 2.3.3.6 Culture of bacteria in spent supernatant with successive culturing

To prepare cultures grown successively in supernatant, *B. wadsworthia* (QI0013) and Bt strain 1 (QI0072) were recovered from cryobead stocks in their respective anaerobic enrichment media (section 2.3.2). After 18 h of growth, *B. wadsworthia* was sub-cultured into anaerobic BPM media (section 2.3.3.2) supplemented with 10 mM taurine. After 18 h of growth of the 2$^{nd}$ passage *B. wadsworthia*, cell-free supernatant was prepared as described previously (section 2.3.3.3), diluted 1:1 with fresh BPM media + 10 mM taurine and inoculated with $10^6$ CFU/mL Bt strain 1 (QI0072). Heat-killed *B. wadsworthia* cells were prepared from the same culture by diluting the cells to $10^8$ CFU/mL and heating at 95˚C for 10 min. The equivalent of $10^6$ CFU/mL heat-killed cells were inoculated into fresh BPM media + 10 mM taurine with $10^6$ CFU/mL Bt strain 1 (QI0072). The cell-free supernatant and heat-killed cells of *B. wadsworthia* were plated onto anaerobic enrichment media agar to confirm absence of viable cells.

After overnight growth, experimental cultures were prepared by obtaining cell-free supernatant of the overnight Bt strain 1 (QI0072) cultures grown with *B. wadsworthia* heat-killed cells or cell-free supernatant. The prepared conditions were inoculated with $10^6$ CFU/mL *B. wadsworthia* QI0013, alongside mono-culture controls using only fresh sterile media, and co-culture of *B. thetaiotaomicron* and *B. wadsworthia* as positive control. At 8 h post-inoculation, samples were taken for $H_2S$ quantification and DNA extraction for qPCR-based bacterial quantification.

## 2.3.4 Molecular biology

### 2.3.4.1 16S rRNA PCR for bacterial identification

For identification of novel faecal isolates, pure colonies were picked and prepared for PCR of the 16S rRNA gene. Simultaneously, colonies were sub-cultured into fresh media for preservation. Briefly, the colonies were resuspended in 10 µL colony wash buffer (100 mM NaCl, 10 mM Tris pH 7, 1 mM EDTA) and centrifuged at 13,000 x g for 5 min. The pellet was resuspended in 15 µL ultrapure Milli-Q® water (Merck Millipore) and boiled at 95°C for 5 min. Reaction mixtures were prepared containing 1 µL template, 1x GoTaq G2 white buffer (Promega), 0.2 mM each dNTP (Bioline), 0.4 µM AMP_F primer (5'-GAGAGTTTGATYCTGGCTCAG-3'), 0.4 µM AMP_R primer (5'-AAGGAGGTGATCCARCCGCA-3') (Sigma) [271] and 0.25 µL GoTaq G2 DNA polymerase (Promega) to a final volume of 50 µL with up$H_2O$. PCR negative control used 1 µL up$H_2O$ instead of template. Primers targeted conserved regions of the 16S rRNA gene to amplify a ~1.5 kB product containing the V4 and V5 variable regions [271]. Thermal cycling was performed as follows: 95°C for 2 min; 25 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 60 s; 72°C for 5 min. PCR products were checked by gel electrophoresis on 1% agarose [40]. Products were purified using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions and quantified using Nanodrop. Sequencing was performed by Eurofins and the resulting forward and reverse sequences for each isolate were quality checked, trimmed and then assembled using Geneious Prime [41]. Consensus FASTA sequences were compared to the NCBI and EzBioCloud 16S ribosomal RNA sequence database using BLAST with default settings for identification.

## 2.3.5 Colourimetric determination of $H_2S$ concentration

### 2.3.5.1 Determination of stock solution concentrations by titration

To prepare standard curves with known sulfide concentrations, a stock solution of 1 mM zinc sulfide was prepared using 0.2 g sodium sulfide ($Na_2S.9H_2O$) in 1% zinc acetate. The solution was stored in Teflon in the dark at 4˚C. Due to loss of sulfide via oxidation over time, the exact concentration of zinc sulfide was determined on day of use via titration with acidic iodine and thiosulfate. Firstly, 1.98 g of $I_2$ and 15 g KI were added to 1 L of water to prepare an iodine solution, from which an iodine-acid solution was prepared on the day of use by adding 1 volume of 1M HCl to 2 volumes of iodine solution. The true concentration of $I_2$ was

determined in the iodine-acid solution by titrating with sodium thiosulfate ($S_2O_3^{2-}$), where 1 mL iodine-acid solution was diluted to 5 mL in up$H_2O$ with stirring and 25 mM thiosulfate solution was added until the solution turns colourless. A drop of 1% starch solution was added, and the titration continued until the solution turns colourless. The volume of thiosulfate used was recorded. A second titration determined the true concentration of sulfide in the zinc sulfide stock solution via titration against iodine-acid solution. Here, 5 mL zinc sulfide stock solution was added to 2 mL iodine-acid solution with stirring and 25 mM thiosulfate solution was added until the solution turns colourless. A drop of 1% starch solution was added, and the titration continued until the solution turns colourless. The volume of thiosulfate used was recorded. Each titration was performed 5 times to obtain an average volume of 25 mM thiosulfate used.

$I_2$ concentration (mM) is determined according to the equation:

$[I_2]$ (mM) = 12.5 x $V_1$

Where $V_1$ is volume of 25 mM thiosulfate used (mL) to titrate iodine-acid solution.

ZnS concentration (mM) in the zinc sulfide stock solution is determined according to the equation:

$[ZnS]$ (mM) = $((2 \times [I_2]) - (12.5 \times V_2)) / 5$

Where $V_2$ is volume of 25 mM thiosulfate used (mL) to titrate zinc sulfide solution.

#### 2.3.5.2 Determination of $H_2S$ concentration in experimental samples

Determination of $H_2S$ concentration in samples was performed using the methylene blue assay modified from Cline [272]. Briefly, 500 µL of bacterial cultures were taken and immediately fixed 1:1 with 5% zinc acetate and stored at -20°C where sulfide is stabilised as zinc sulfide. For calibration, zinc sulfide solutions were prepared in media diluted 1:200 within concentration range 0 – 40 µM. For analysis, fixed samples were diluted 1:100 in water to a final volume of 1 mL, producing a final sample dilution of 1:200. 80 µL of diamine reagent (250 mL 6M HCl, 1 g N,N-dimethyl-1,4-phenylendiaminsulfate, 1.5 g iron(III)chloride, $FeCl_3 \cdot 6H_2O$) was added to all samples and standards, and stored in the dark to allow methylene blue colour development. After 30 min, the samples were centrifuged at 13,000 x g for 5 min to pellet biomass. 300 µL of supernatant was taken for spectrophotometric absorbance measurement at 670 nm.

The premise of the method is based upon zinc sulfide in the samples reacting with N,N-dimethyl-1,4-phenylendiaminsulfate and iron chloride under acidic conditions to form methylene blue, the absorbance of which can be determined spectrophotometrically at 670 nm. The concentration of methylene blue is proportional to the sulfide in the samples.

For $H_2S$ concentration determination, the reference standard curve of known $H_2S$ concentrations was prepared by plotting blank-adjusted absorbance at 670 nm against $H_2S$ concentration. Linear regression analysis was performed using GraphPad Prism 7 (GraphPad Software, Boston, USA). The diamine reagent coefficient (F) was determined using the reference standard curve of known $H_2S$ concentrations where the coefficient is equal to 1/slope. Then, $H_2S$ concentration in µM was determined according to:

$H_2S$ in original sample (µM) = (blank-corrected absorbance at 670 nm x F) x dilution factor

Statistical significance between culture conditions was established using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests with a significance level set at $\alpha = 0.05$. Results were displayed graphically using GraphPad Prism 7 (GraphPad Software, Boston, USA) showing mean ± standard deviation, and statistical results where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant ($p > 0.05$).

### 2.3.6 DNA extraction from co-cultures for qPCR

For quantification of bacterial cells in experimental cultures, DNA extraction was performed using the Maxwell® RSC Blood DNA kit (Promega) according to the manufacturer's protocol. Prior to extraction, 200 µL samples were boiled at 90°C for 10 min and 150 µL was transferred to a sterile microcentrifuge tube containing 30 µL of proteinase K solution and 300 µL lysis buffer. Each sample was vortexed for 10 s and incubated at 56°C for 20 min.

### 2.3.7 Absolute quantification of bacterial cells via qPCR

Quantification of *B. wadsworthia*, *B. thetaiotaomicron* and *E. faecium* was performed via qPCR using KiCqStart® SYBR® Green qPCR ReadyMix™ with ROX (Sigma-Aldrich) on a StepOnePlus™ Real-Time PCR System (ThermoFisher

Scientific). Absolute quantification of gene copy numbers was performed by comparing samples to calibration standards prepared with known gene copy numbers in the range of 10 – 1 x $10^9$ copies. The number of gene copies per cell was used to calculate the absolute cell counts per mL of culture. All samples and standards were assayed in triplicate. The reaction conditions and primers used are shown in Table 2. Statistical significance between culture conditions was established using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests with a significance level set at $\alpha = 0.05$. Results were displayed graphically using GraphPad Prism 7 (GraphPad Software, Boston, USA) showing mean ± standard deviation, and statistical results where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant ($p > 0.05$).

Table 2: Primers and reaction conditions used for absolute quantification of *B. wadsworthia*, *B. thetaiotaomicron* and *E. faecium* via qPCR

| **Target bacterium** | **Primer name** | **Product size (bp)** | **Primer sequence (5' to 3')** (W=A+T) | **Primer conc. (nM)** | **Reaction conditions** | **Ref** |
|---|---|---|---|---|---|---|
| *B. wadsworthia* | TPA-F | 150 | CAACGTCCC CACCATCAA GTTCTCTG | 100 | 95°C 2 min, 40 cycles of 95°C 15 s, 62°C 30 s. | [206] |
| | TPA-R | | TGAATTCGC GGAAGGAG CGAGAGGTC | 100 | | |
| *B. thetaiotaomicron* | F_Bacter 11 | 131 | CCTWCGAT GGATAGGG GTT | 200 | 95°C 10 min, 40 cycles of 95°C 30 s, 60°C 1 min. | [273] |
| | R_Bacter 08 | | CACGCTACT TGGCTGGTT CAG | 200 | | |
| *E. faecium* | FAEFOR-2 | 94 | ggtacaacccgat tactttgtcccat | 200 | 52°C 2 min, 95°C 10 min, 45 cycles of 95°C 15 s, 60°C 30 s. | [274] |
| | FAEREV-2 | | tctgccgtctacttc ttgaatggt | 200 | | |

### 2.3.9 Genomics

#### 2.3.9.1 DNA extraction from isolates for whole genome sequencing

For whole genome sequencing of pure bacterial cultures, 1 mL of overnight culture was taken and centrifuged at 13,000 x g for 2 min. Pellets were resuspended in 500 µL colony wash buffer (100 mM NaCl, 10 mM Tris pH 7, 1 mM EDTA) and centrifuged at 13,000 x g for 2 min. Pellets were stored at -20°C until extraction. For extraction of high molecular weight DNA, Fire Monkey High Molecular Weight DNA (HMW-DNA) extraction kit was used according to manufacturer's protocol (Revolugen, UK). The additional overnight elution step recommended by the manufacturer was used to maximise DNA yield. For Gram-positive bacteria, the longer incubation, longer vortexing and addition of lysozyme recommended by the manufacturer were used. DNA was quantified using Qubit High Sensitivity DNA Quantification Kit (Thermo Scientific) and Qubit 3.0 fluorometer (Life Technologies).

#### 2.3.9.2 Whole genome sequencing

For whole genome sequencing, a hybrid approach using both short- and long-read sequencing was performed in-house at QIB. After DNA extraction, sequencing was performed by Dave Baker. For short-read sequencing, genomic DNA was normalised to 5 ng/µl with EB buffer (10 mM Tris-HCl) and sequencing was performed at the QIB sequencing facility using an Illumina NextSeq 500 system with 2x150 bp paired-end reads. Libraries were prepared using Bead Linked Transposomes (BLT) (Illumina Catalogue No. 20018704) and P7 and P5 Illumina 9 bp barcodes. Paired-end sequencing reads were received as fastq files. For long-read sequencing, an Oxford Nanopore minION was used (Oxford Nanopore Technologies, Oxford, UK).

For genome assembly, short reads were cleaned using bbduk (BBMap 38.79) [275] to trim the reads and remove sequencing adaptors. FastQC (v 0.11.9) [276] was used to assess the sequence quality, where reads with a Phred score ≥30 were deemed suitable for further analysisLong reads were trimmed using Porechop (v 0.2.3) [277]. Hybrid assembly was performed using Unicycler (v 0.4.9) [278]. The completeness and contamination of the assemblies was checked using CheckM (v 1.0.18) [279]. Genomes were annotated using Prokka (v 1.14.6) [280].

### 2.3.10 Figure preparation

Figures showing experimental data were prepared using GraphPad Prism (GraphPad Software, Boston, USA). Diagrams and schematics were prepared using Adobe Illustrator 2024 (Adobe, USA).

## 2.3 Results

### 2.3.1 Do different *B. thetaiotaomicron* strains affect *B. wadsworthia*'s growth and $H_2S$ production?

To investigate the impact of *B. thetaiotaomicron* strains on *B. wadsworthia*'s growth and $H_2S$ production, pairwise co-cultures were performed with *B. wadsworthia* (QI0013) and three *B. thetaiotaomicron* strains (QI0072, DSM 108160 and DSM 108161) referred to as Bt strain 1, 2 and 3 respectively (Table 1). Bacteria were inoculated at $10^6$ CFU/mL in a 1:1 ratio. At 8 h, significantly higher $H_2S$ concentrations were observed when *B. wadsworthia* was in co-culture with Bt strains 1, 2 and 3 compared to mono-culture (Figure 3A), whereas no $H_2S$ was detected in *B. thetaiotaomicron* mono-cultures (Figure 3A). The abundance of *B. wadsworthia* when co-cultured with Bt strains 1 and 3 was marginally higher compared to mono-culture, and no differences in abundance were observed in co-culture with Bt strain 2 (Figure 3B). $H_2S$ concentration in the cultures was normalised to the *B. wadsworthia* cell counts as determined via qPCR to calculate the $H_2S$ concentration per $10^6$ cells. This provides an indication of *B. wadsworthia*'s metabolic activity in terms of $H_2S$ production under different culture conditions. At 8 h, higher $H_2S$ production per $10^6$ cells was observed in all co-cultures compared to *B. wadsworthia* in mono-culture, although the impact of co-culture with Bt strain 3 was lower than other co-culture conditions (Figure 3D). No differences in the impact of *B. wadsworthia* were observed with respect to the abundance of the different *B. thetaiotaomicron* strains; *B. thetaiotaomicron* abundance was significantly higher in co-culture with *B. wadsworthia* compared to respective mono-cultures of all three strains (Figure 3C). Therefore, in co-culture with Bt strains 1, 2 and 3, *B. wadsworthia* produces significantly more $H_2S$ compared to mono-culture, not only due to increased cell abundance, but also due to an increased $H_2S$ production per cell.

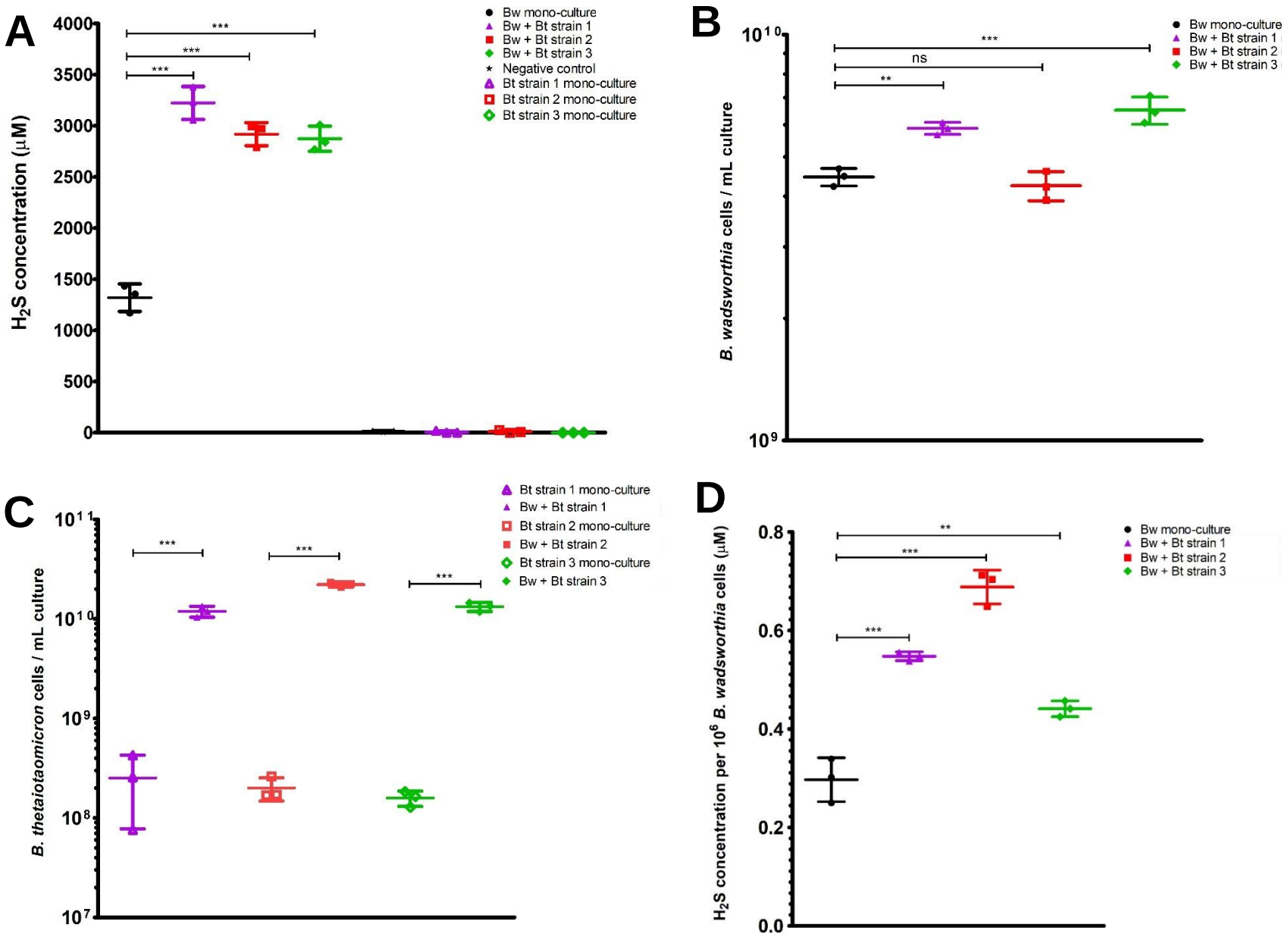

A
$H_2S$ concentration (μM)
4000
3500
3000
2500
2000
1500
1000
500
0
***
***
***
Bw mono-culture
Bw + Bt strain 1
Bw + Bt strain 2
Bw + Bt strain 3
Negative control
Bt strain 1 mono-culture
Bt strain 2 mono-culture
Bt strain 3 mono-culture
B
B. wadsworthia cells / mL culture
$10^{10}$
$10^{9}$
***
ns
**
Bw mono-culture
Bw + Bt strain 1
Bw + Bt strain 2
Bw + Bt strain 3
C
B. thetaiotaomicron cells / mL culture
$10^{11}$
$10^{10}$
$10^{9}$
$10^{8}$
$10^{7}$
***
***
***
Bt strain 1 mono-culture
Bw + Bt strain 1
Bt strain 2 mono-culture
Bw + Bt strain 2
Bt strain 3 mono-culture
Bw + Bt strain 3
D
$H_2S$ concentration per $10^6$ B. wadsworthia cells (μM)
0.8
0.6
0.4
0.2
0.0
**
***
***
Bw mono-culture
Bw + Bt strain 1
Bw + Bt strain 2
Bw + Bt strain 3

Figure 3: Co-culture of three *B. thetaiotaomicron* strains with *B. wadsworthia* (Bw) QI0013. A) $H_2S$ concentrations (µM) in culture conditions at 8 h. Negative control is sterile media. **B)** qPCR-determined *B. wadsworthia* cell counts at 8 h. **C)** qPCR-determined *B. thetaiotaomicron* cell counts at 8 h. **D)** $H_2S$ concentrations were standardised against *B. wadsworthia* cell counts to give $H_2S$ concentration (µM) per $10^6$ cells. Each point represents a technical culture replicate (n=3). Horizontal lines represent average, and error bars represent SD. Results of one-way ANOVA with Tukey's post-hoc test are shown, where ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant ($p > 0.05$).

To investigate whether these observations were primarily mediated by soluble metabolite exchange, cell-free supernatants from the three *B. thetaiotaomicron* strains were added to *B. wadsworthia* cultures. Unlike in co-culture, a substantial increase in $H_2S$ concentration was not observed with supernatant only; Bt strain 1 supernatant elicited an increase of 250 µM $H_2S$ (Figure 4A) which is a modest effect relative to the ~1900 µM increase in $H_2S$ concentration observed when Bt strain 1 cells were co-cultured with *B. wadsworthia* (Figure 3A). Culture with Bt strain 2 supernatant showed similar $H_2S$ concentration to *B. wadsworthia* mono-culture, and Bt strain 3 supernatant resulted in significantly lower $H_2S$ concentration compared to the *B. wadsworthia* mono-culture (Figure 4A), whereas the co-cultures of these Bt strains with *B. wadsworthia* resulted in increased $H_2S$ concentration (Figure 3A). Regarding *B. wadsworthia* abundance, culture with cell-free supernatant from Bt strains 1 and 2 resulted in the same cell counts as mono-culture, whereas Bt strain 3 supernatant resulted in lower *B. wadsworthia* abundance (Figure 4B). The $H_2S$ concentration per $10^6$ cells was not significantly different from the mono-culture in any supernatant condition (Figure 4C), suggesting that the differences in $H_2S$ concentration are caused by the differences in the abundance of *B. wadsworthia* in the culture, not the metabolic activity of the cells.

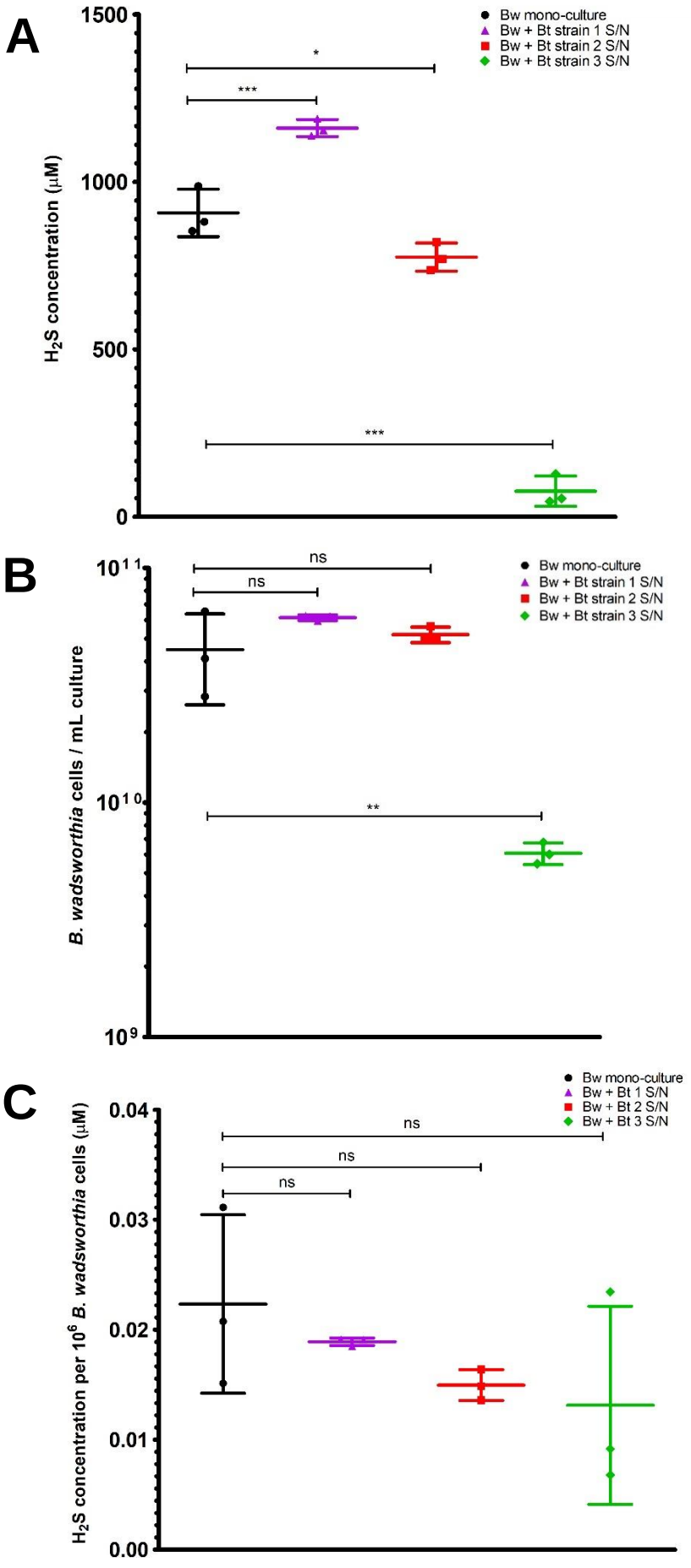

A
1500
1000
500
0
H2S concentration (μM)
Bw mono-culture
Bw + Bt strain 1 S/N
Bw + Bt strain 2 S/N
Bw + Bt strain 3 S/N
*
***
***
B
10 11
10 10
10 9
B. wadsworthia cells / mL culture
ns
ns
**
C
0.04
0.03
0.02
0.01
0.00
H2S concentration per 10^6 B. wadsworthia cells (μM)
Bw mono-culture
Bw + Bt 1 S/N
Bw + Bt 2 S/N
Bw + Bt 3 S/N
ns
ns
ns

Figure 4: *B. wadsworthia* (Bw) QI0013 grown with cell-free supernatant from three *B. thetaiotaomicron* (Bt) strains.

A) $H_2S$ concentrations (µM) in culture conditions at 8 h. B) qPCR-determined *B. wadsworthia* cell counts at 8 h. C) qPCR-determined *B. thetaiotaomicron* cell counts at 8 h. D) $H_2S$ concentration (µM) per $10^6$ cells. Each point represents a technical culture replicate (n=3). Horizontal lines represent average, and error bars represent SD. Results of one-way ANOVA with Tukey's post-hoc test are shown, where * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant ($p > 0.05$).

### 2.3.2 Investigating the $H_2S$-promoting effect of Bt strain 1 on *B. wadsworthia*

#### 2.3.2 1 The $H_2S$-promoting effect of Bt strain 1 on *B. wadsworthia* appears to be dependent on cell proximity

To focus our efforts in understanding the underlying mechanism behind the increased $H_2S$ production by *B. wadsworthia* in co-culture with *B. thetaiotaomicron*, we selected Bt strain 1 as this strain has been directly isolated from faeces obtained from a healthy adult recruited via the COMBAT study (ClinicalTrials.gov Identifier: NCT03679533), whereas Bt strains 2 and 3 were sourced from DSMZ for which the health status is not provided (Table 1). Addressing Bt strain 1, we next hoped to clarify the discrepancies between the effects observed with co-culture of live cells compared to the cell-free supernatant by co-culturing the two strains with physical separation using a permeable 0.22 µm membrane. *B. wadsworthia* mono-culture reached a similar $H_2S$ concentration to the filter-separated culture condition, with significantly increased $H_2S$ observed only in the “true” co-culture condition where *B. wadsworthia* and Bt strain 1 were in physical proximity (Figure 5A). This suggests that the exchange of soluble metabolites between the strains alone does not cause an increase in *B. wadsworthia*’s $H_2S$ production (Figure 5A). A modest amount of $H_2S$ was detectable in the filter-separated Bt strain 1 culture which was not observed in the Bt strain 1 mono-culture (Figure 5A); the presence of $H_2S$ here is indicative of the diffusion of soluble metabolites and molecules below the size cut-off of 0.22 µm without direct interaction between the two species, as confirmed by absence of *B. wadsworthia*-specific genes in the Bt strain 1 filter-separated culture (data not shown).

Furthermore, *B. wadsworthia* abundance was similar between mono-culture and filter-separated culture, and only slightly increased in the ‘true’ co-culture condition at 8 h post-inoculation (Figure 5B), reiterating that the increases in *B. wadsworthia*’s growth and $H_2S$ production are not observed when *B. wadsworthia* and Bt strain 1 share only a soluble metabolite fraction. Conversely, Bt strain 1 abundance is affected by the exchange of soluble metabolites; cell counts were one order of magnitude higher in cultures filter-separated from *B. wadsworthia* when compared to mono-culture, and this abundance increased further when Bt strain 1 was in ‘true’ co-culture with *B. wadsworthia* (Figure 5C). These differences between mono- and filter-separated cultures provide further supportive evidence

that the filters did indeed allow diffusion and metabolite exchange between the cultures whilst preventing physical interaction.

Using $H_2S$ concentration per $10^6$ *B. wadsworthia* cells as an indicator of sulfidogenic activity, $H_2S$ production was slightly increased in the co-culture condition when compared to the mono- or filter-separated *B. wadsworthia* cultures, although this difference was not significant between mono- and co-culture (Figure 5D). This suggests that although there is a modest increase in the sulfidogenic activity of the *B. wadsworthia* cells when in proximity to Bt strain 1, the significant increase in $H_2S$ concentration is primarily driven by increases in *B. wadsworthia* abundance as opposed to impacting the metabolic activity of the cells present. The observation that both species appear to benefit from increased growth when in physical proximity to each other could suggest a beneficial symbiosis between these strains.

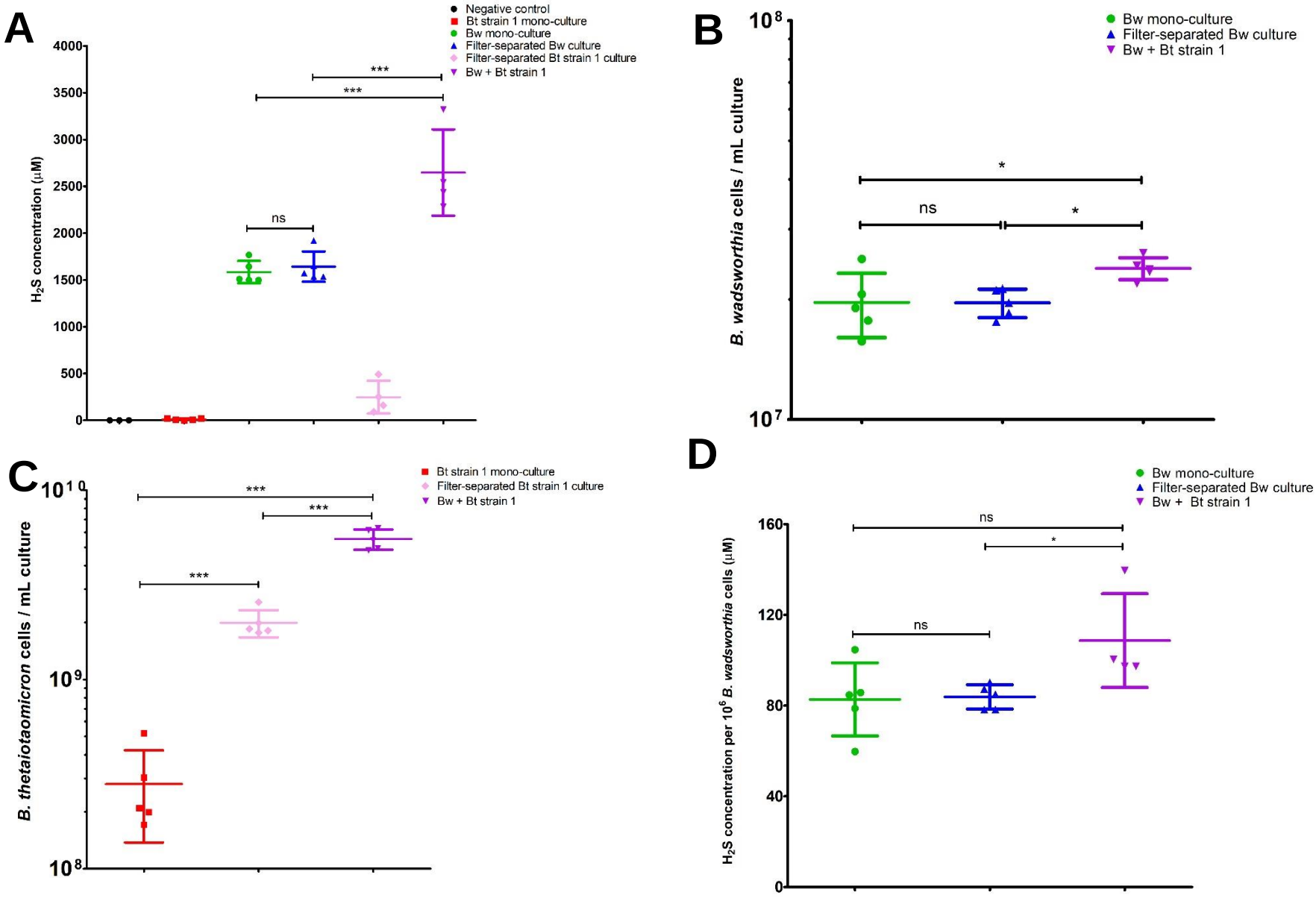

A
Negative control
Bt strain 1 mono-culture
Bw mono-culture
Filter-separated Bw culture
Filter-separated Bt strain 1 culture
Bw + Bt strain 1
H2S concentration (μM)
4000
3500
3000
2500
2000
1500
1000
500
0
***
***
ns
B
Bw mono-culture
Filter-separated Bw culture
Bw + Bt strain 1
B. wadsworthia cells / mL culture
10^8
10^7
*
ns
*
C
Bt strain 1 mono-culture
Filter-separated Bt strain 1 culture
Bw + Bt strain 1
B. thetaiotaomicron cells / mL culture
10^10
10^9
10^8
***
***
***
D
Bw mono-culture
Filter-separated Bw culture
Bw + Bt strain 1
H2S concentration per 10^6 B. wadsworthia cells (μM)
160
120
80
40
0
ns
*
ns

Figure 5: Filter-separated culture of *B. wadsworthia* (Bw) QI0013 and Bt strain 1. A) $H_2S$ concentrations (µM) at 8 h. B) qPCR-determined *B. wadsworthia* cell counts at 8 h. C) qPCR-determined *B. thetaiotaomicron* cell counts at 8 h. D) $H_2S$ concentration (µM) per $10^6$ *B. wadsworthia* cells. Each point represents a technical culture replicate (n=3-5). Horizontal lines represent average, and error bars represent SD. Statistical significance between culture conditions was established using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests with a significance level set at $\alpha = 0.05$. Results show * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant ($p > 0.05$).

#### 2.3.2.2 The heat-killed biomass of Bt strain 1 does not promote *B. wadsworthia*'s $H_2S$ production

After revealing that the $H_2S$-promoting effect of Bt strain 1 appears to depend upon physical proximity between the strains, we next investigated whether this interaction is dependent upon Bt strain 1 viability. Co-cultures of live *B. wadsworthia* and Bt strain 1 were prepared alongside live *B. wadsworthia* and dead Bt strain 1 culture, where the equivalent dead biomass was inoculated. A significantly increased $H_2S$ concentration was observed in the co-culture of live *B. wadsworthia* and Bt strain 1 compared to *B. wadsworthia* mono-culture, reiterating our previous observations (Figure 6A); interestingly, when *B. wadsworthia* was cultured with heat-killed Bt strain 1 cells, no increased $H_2S$ production was observed (Figure 6A). Similarly, *B. wadsworthia* cell abundance was slightly increased in the co-culture with viable Bt strain 1, whereas no such increase was observed with heat-killed Bt strain 1 (Figure 6B). The metabolic activity of *B. wadsworthia* cells in the different conditions was investigated by establishing the $H_2S$ concentration per $10^6$ cells (Figure 6D). An increased metabolic capacity for $H_2S$ generation was observed in *B. wadsworthia* in co-culture with viable Bt strain 1, but not with heat-killed Bt strain 1 (Figure 6C). Overall, this reveals that the heat-killed biomass of Bt strain 1 does not promote increased $H_2S$ production by *B. wadsworthia*. In contrast, Bt strain 1 reached significantly higher cell density at 8 h when co-cultured with heat-killed *B. wadsworthia* cells compared to live co-culture or mono-culture (Figure 6C).This shows that the increased growth of Bt strain 1 in the presence of *B. wadsworthia* does not depend on *B. wadsworthia*'s viability; in fact, the dead biomass of *B. wadsworthia* appears to promote the growth further, perhaps due to cell lysis releasing valuable metabolites to support Bt strain 1's growth. Taken together, it appears that the increased $H_2S$ production by *B. wadsworthia* in the presence of Bt strain 1 is dependent upon Bt strain 1's viability and physical proximity, but Bt strain 1 thrives with heat-killed *B. wadsworthia* biomass.

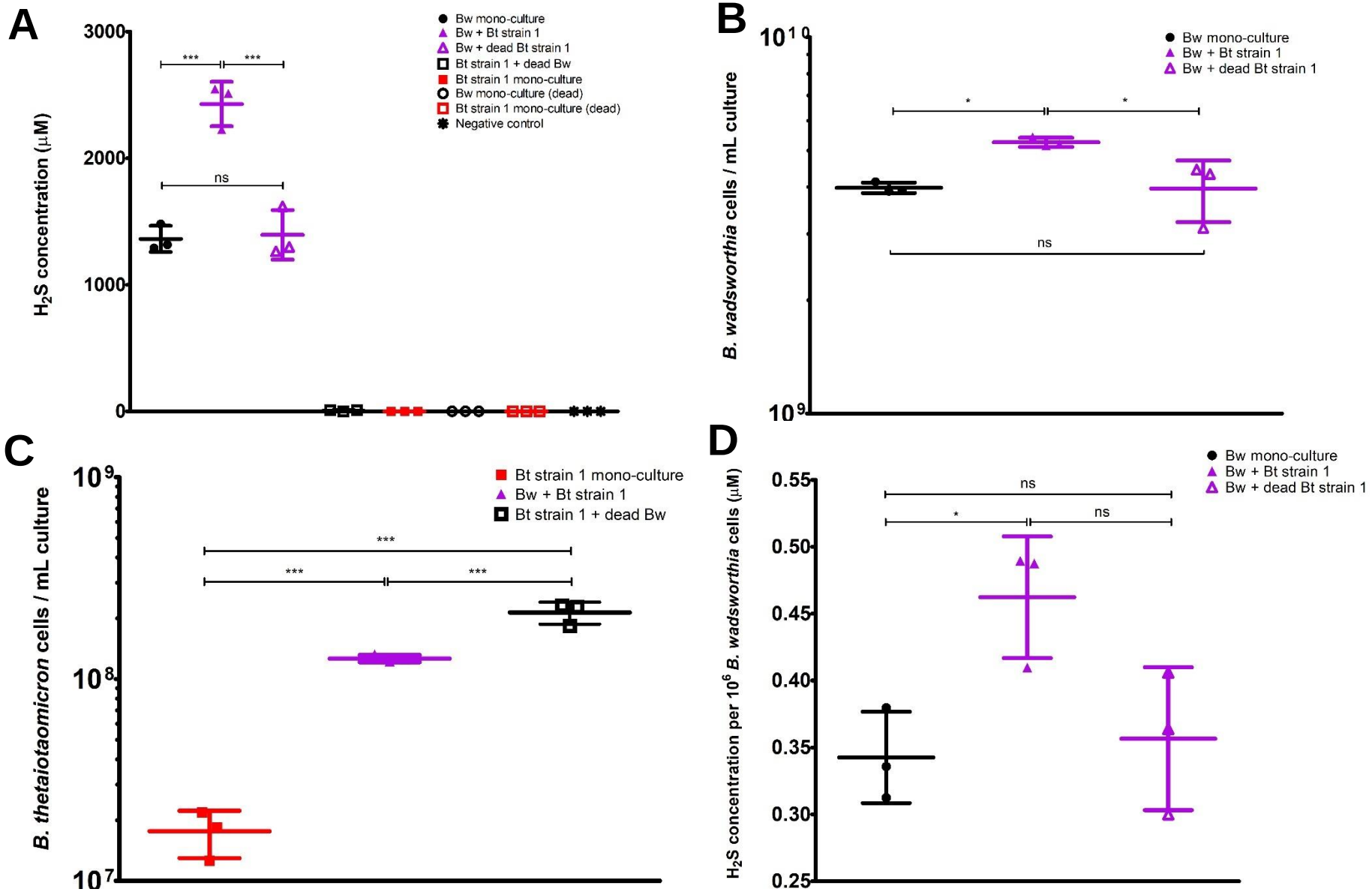

A
3000
2000
1000
0
$H_2S$ concentration (μM)
***
***
ns
Bw mono-culture
Bw + Bt strain 1
Bw + dead Bt strain 1
Bt strain 1 + dead Bw
Bt strain 1 mono-culture
Bw mono-culture (dead)
Bt strain 1 mono-culture (dead)
Negative control
B
$10^{10}$
$10^{9}$
*B. wadsworthia* cells / mL culture
*
*
ns
Bw mono-culture
Bw + Bt strain 1
Bw + dead Bt strain 1
C
$10^{9}$
$10^{8}$
$10^{7}$
*B. thetaiotaomicron* cells / mL culture
***
***
***
Bt strain 1 mono-culture
Bw + Bt strain 1
Bt strain 1 + dead Bw
D
0.55
0.50
0.45
0.40
0.35
0.30
0.25
$H_2S$ concentration per $10^6$ *B. wadsworthia* cells (μM)
ns
*
ns
Bw mono-culture
Bw + Bt strain 1
Bw + dead Bt strain 1

Figure 6: Culture of *B. wadsworthia* (Bw) QI0013 with heat-killed Bt strain 1. A) $H_2S$ concentrations (µM) at 8 h. B) qPCR-determined *B. wadsworthia* cell counts at 8 h. C) qPCR-determined *B. thetaiotaomicron* cell counts at 8 h. D) $H_2S$ concentration (µM) per $10^6$ *B. wadsworthia* cells. Each point represents a technical culture replicate (n=3). Horizontal lines represent average, and error bars represent SD. Statistical significance between culture conditions was established using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests with a significance level set at $\alpha = 0.05$. Results show * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant ($p > 0.05$).

#### 2.3.2.3 Investigating the role of soluble metabolites: sequential supernatant experiments in *B. wadsworthia* and Bt strain 1 co-culture

Although the previous findings suggest that *B. wadsworthia*'s increased $H_2S$ production in the presence of Bt strain 1 is dependent upon physical proximity and viability, we wanted to rule out that there is not a sequential production of a soluble metabolite in the supernatant by Bt strain 1 in response to *B. wadsworthia*'s growth supernatant. To test this, Bt strain 1 was grown with *B. wadsworthia* cell-free supernatant overnight, and the cell-free supernatant of this culture (S/N 2) was added to *B. wadsworthia* mono-culture. In addition, *B. wadsworthia* was grown with Bt 1 mono-culture supernatant (S/N 1), alongside mono-culture and co-culture controls. No increases in $H_2S$ concentration were observed in the *B. wadsworthia* cultures grown with Bt strain 1 supernatant (S/N 1) or with the successive supernatant where Bt strain 1 was grown with *B. wadsworthia* supernatant (S/N 2) (Figure 7A). Similarly, no striking differences in *B. wadsworthia* abundance or $H_2S$ production per $10^6$ cells were observed when grown with S/N 1 or S/N 2 compared to mono-culture (Figure 7B, 7C). This allows us to exclude the possibility that the observed increases in $H_2S$ production in *B. wadsworthia* and Bt strain 1 co-culture are due to Bt strain 1 secreting soluble metabolites in response to *B. wadsworthia*'s supernatant. This successive supernatant experiment further reiterated the importance of physical proximity in co-culture to permit the observed increase in $H_2S$ production by *B. wadsworthia*.

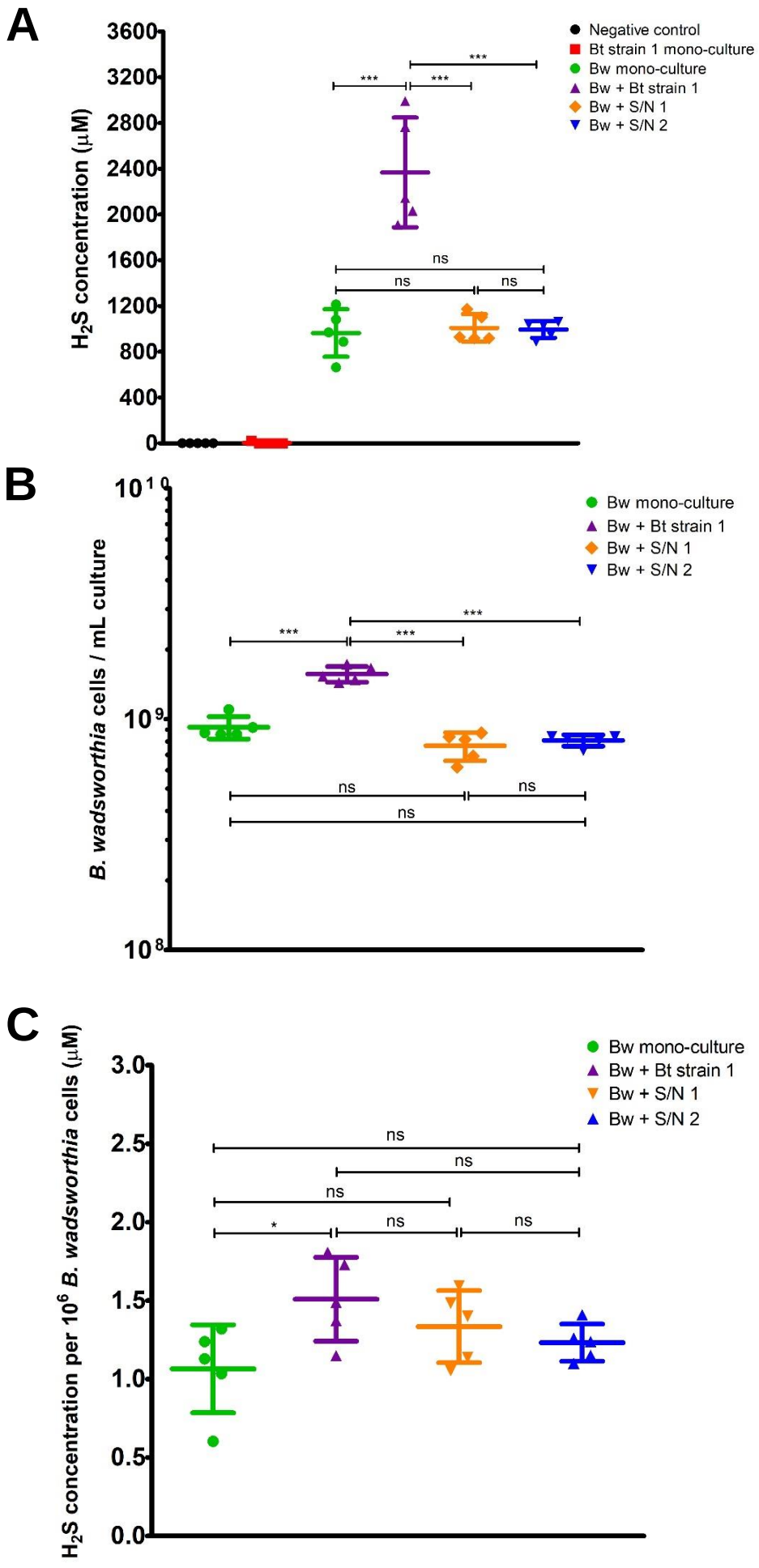

A
3600
3200
2800
2400
2000
1600
1200
800
400
0
H2S concentration (μM)
Negative control
Bt strain 1 mono-culture
Bw mono-culture
Bw + Bt strain 1
Bw + S/N 1
Bw + S/N 2
***
***
***
ns
ns
ns
B
10 10
10 9
10 8
B. wadsworthia cells / mL culture
Bw mono-culture
Bw + Bt strain 1
Bw + S/N 1
Bw + S/N 2
***
***
***
ns
ns
ns
C
3.0
2.5
2.0
1.5
1.0
0.5
0.0
H2S concentration per 10^6 B. wadsworthia cells (μM)
Bw mono-culture
Bw + Bt strain 1
Bw + S/N 1
Bw + S/N 2
ns
ns
ns
*
ns
ns

Figure 7: Culture of *B. wadsworthia* (Bw) QI0013 with supernatant of Bt strain 1 mono-culture (S/N 1) and supernatant of Bt strain 1 grown in *B. wadsworthia* supernatant (S/N 2). A) $H_2S$ concentrations (µM) at 8 h. B) qPCR-determined *B. wadsworthia* cell counts at 8 h. C) $H_2S$ concentration (µM) per $10^6$ *B. wadsworthia* cells. Each point represents a technical culture replicate (n=5). Horizontal line = average. Error bars = SD.

Given the substantial enhancement of Bt strain 1's growth in the presence of heat-killed *B. wadsworthia* biomass (Figure 6C), it was crucial to determine if *B. wadsworthia* biomass could induce Bt strain 1 to produce soluble metabolites, thereby sequentially influencing *B. wadsworthia*'s $H_2S$ production. To investigate this, cell-free supernatant and heat-killed biomass of a *B. wadsworthia* culture was prepared and inoculated into Bt strain 1 mono-cultures. Then, supernatants were prepared from Bt strain 1 grown with *B. wadsworthia* biomass and with *B. wadsworthia* supernatant. *B. wadsworthia* cultures were prepared with each supernatant, alongside mono-culture and co-culture controls. A substantial increase in $H_2S$ concentration was observed only in the co-culture of *B. wadsworthia* and Bt strain 1 (Figure 8A). Culture of *B. wadsworthia* with supernatant of Bt strain 1 that was grown with either *B. wadsworthia* biomass or *B. wadsworthia* supernatant did not result in substantially increased $H_2S$ concentration compared to mono-culture (Figure 8A). Similarly, the co-culture of *B. wadsworthia* and Bt strain 1 resulted in slightly increased *B. wadsworthia* abundance (Figure 8B) and increased $H_2S$ production per $10^6$ cells (Figure 8C) compared to mono-culture, whereas the two successive supernatant treatments did not. In summary, the results contradict the initial hypothesis, revealing that neither the supernatant nor the biomass of *B. wadsworthia* induces Bt strain 1 to produce a soluble metabolite that stimulates *B. wadsworthia*'s growth and $H_2S$ production. Collectively, these findings underscore the necessity of viable Bt strain 1 cells for the observed increase in $H_2S$ production and growth in *B. wadsworthia*.

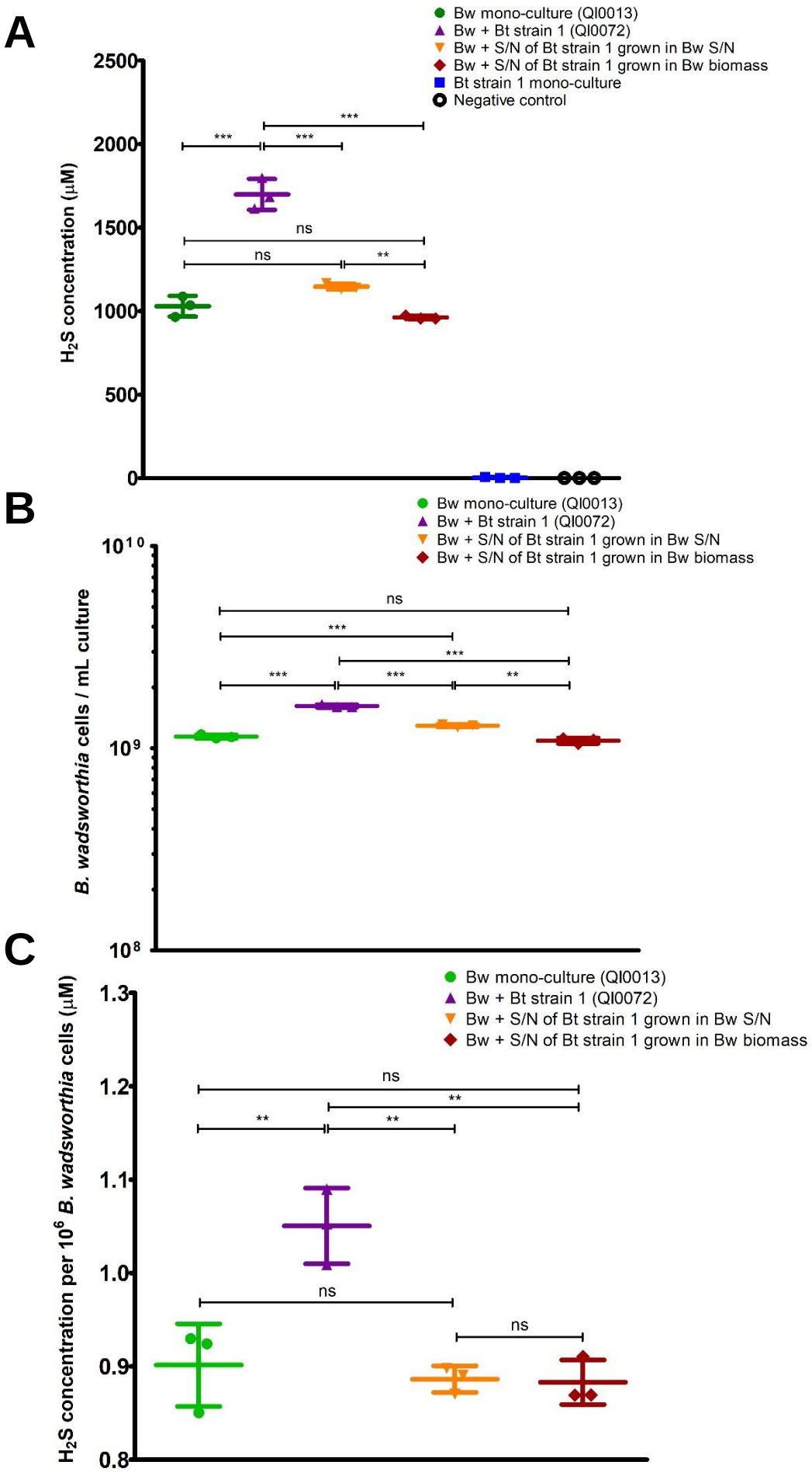

A
Bw mono-culture (QI0013)
Bw + Bt strain 1 (QI0072)
Bw + S/N of Bt strain 1 grown in Bw S/N
Bw + S/N of Bt strain 1 grown in Bw biomass
Bt strain 1 mono-culture
Negative control
H2S concentration (μM)
2500
2000
1500
1000
500
0
***
***
***
ns
ns
**
B
Bw mono-culture (QI0013)
Bw + Bt strain 1 (QI0072)
Bw + S/N of Bt strain 1 grown in Bw S/N
Bw + S/N of Bt strain 1 grown in Bw biomass
B. wadsworthia cells / mL culture
10^10
10^9
10^8
ns
***
***
***
***
**
C
Bw mono-culture (QI0013)
Bw + Bt strain 1 (QI0072)
Bw + S/N of Bt strain 1 grown in Bw S/N
Bw + S/N of Bt strain 1 grown in Bw biomass
H2S concentration per 10^6 B. wadsworthia cells (μM)
1.3
1.2
1.1
1.0
0.9
0.8
ns
**
**
**
ns
ns

Figure 8: Culture of *B. wadsworthia* (Bw) QI0013 with supernatant of Bt strain 1 grown in *B. wadsworthia* supernatant or with heat-killed *B. wadsworthia* cells. A) $H_2S$ concentrations (µM) at 8 h. B) qPCR-determined *B. wadsworthia* cell counts at 8 h. C) $H_2S$ concentration (µM) per $10^6$ *B. wadsworthia* cells. Each point represents a technical culture replicate (n=3). Horizontal line = average. Error bars = SD. Statistical significance between culture conditions was established using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests with a significance level set at α = 0.05. Results show * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001, ns = not significant (p > 0.05).

#### 2.3.2.4 Bt strain 1 promotes *B. wadsworthia*'s $H_2S$ production in a non-strain-dependent fashion

The interactions between Bt strain 1 and *B. wadsworthia* (QI0013) had clearly demonstrated a sulfidogenic interaction in co-culture. Next, we tested if Bt strain 1 would promote the sulfidogenic activity of other *B. wadsworthia* strains. Three additional gut-derived strains of *B. wadsworthia* (QI0012, QI0014, QI0015) were co-cultured with Bt strain 1 alongside mono-culture controls and Bt strain 1 with *B. wadsworthia* (QI0013). Co-culture with Bt strain 1 resulted in significantly increased $H_2S$ concentration in all *B. wadsworthia* strains tested compared to their respective mono-cultures (Figure 9A), suggesting that the sulfidogenic potential of Bt strain 1 is not limited to one *B. wadsworthia* strain. Indeed, QI0014 did not produce detectable $H_2S$ in mono-culture at 8 h, whereas 195.3 ± 18.60 µM was produced in co-culture with Bt strain 1 (Figure 9A). Interestingly, qPCR determination of *B. wadsworthia* cell abundance revealed differences between strains; QI0012 and QI0013 showed slight increases in abundance in co-culture with Bt strain 1, whereas QI0014 and QI0015 showed decreased abundance in co-culture (Figure 9B). When the $H_2S$ concentration was standardised by the *B. wadsworthia* cells, this showed that Bt strain 1 indiscriminately increases $H_2S$ production by *B. wadsworthia* strains, but we cannot rule out that the mechanism may differ between strains (Figure 9). Increased $H_2S$ in QI0012 and QI0013 co-cultures are due to a combined effect of increased abundance and increased $H_2S$ production per cell, whereas with QI0014 and QI0015 it is due to substantially increased $H_2S$ production per *B. wadsworthia* cell (Figure 9D). The abundance of Bt strain 1 was slightly increased in all pairwise co-cultures compared to mono-culture (Figure 9C). Overall, Bt strain 1 showed an ability to increase $H_2S$ production in four *B. wadsworthia* strains.

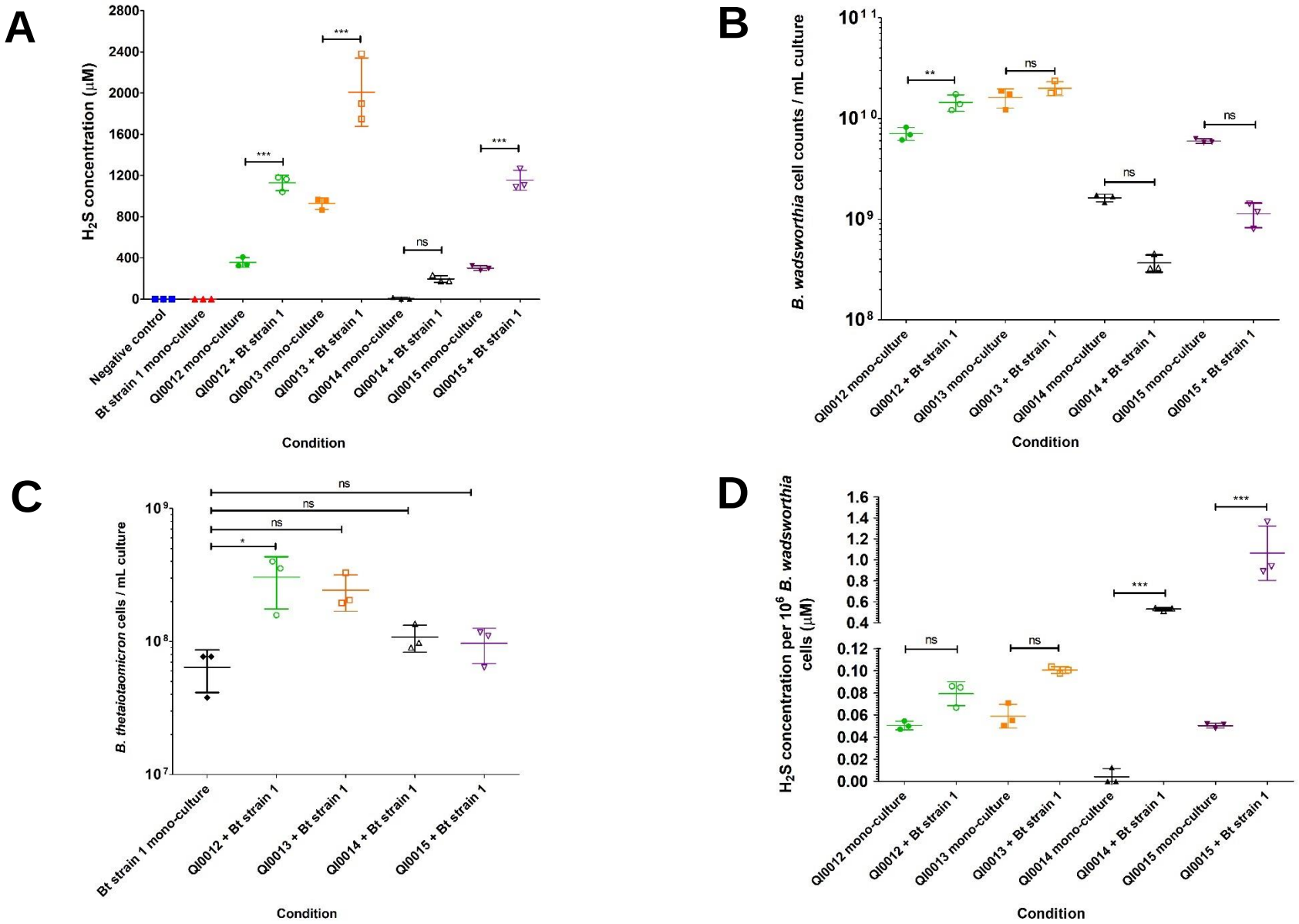

A
$H_2S$ concentration (μM)
2800
2400
2000
1600
1200
800
400
0
***
***
ns
***
Negative control
Bt strain 1 mono-culture
QI0012 mono-culture
QI0012 + Bt strain 1
QI0013 mono-culture
QI0013 + Bt strain 1
QI0014 mono-culture
QI0014 + Bt strain 1
QI0015 mono-culture
QI0015 + Bt strain 1
Condition
B
B. wadsworthia cell counts / mL culture
$10^{11}$
$10^{10}$
$10^{9}$
$10^{8}$
**
ns
ns
ns
QI0012 mono-culture
QI0012 + Bt strain 1
QI0013 mono-culture
QI0013 + Bt strain 1
QI0014 mono-culture
QI0014 + Bt strain 1
QI0015 mono-culture
QI0015 + Bt strain 1
Condition
C
B. thetaiotaomicron cells / mL culture
$10^{9}$
$10^{8}$
$10^{7}$
ns
ns
ns
*
Bt strain 1 mono-culture
QI0012 + Bt strain 1
QI0013 + Bt strain 1
QI0014 + Bt strain 1
QI0015 + Bt strain 1
Condition
D
$H_2S$ concentration per $10^6$ B. wadsworthia cells (μM)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.12
0.10
0.08
0.06
0.04
0.02
0.00
ns
ns
***
***
QI0012 mono-culture
QI0012 + Bt strain 1
QI0013 mono-culture
QI0013 + Bt strain 1
QI0014 mono-culture
QI0014 + Bt strain 1
QI0015 mono-culture
QI0015 + Bt strain 1
Condition

Figure 9: Pairwise co-culture of Bt strain 1 with four *B. wadsworthia* strains (QI0012, QI0013, QI0014, QI0015). A) $H_2S$ concentrations (µM) at 8 h. B) qPCR-determined *B. wadsworthia* cell counts at 8 h. C) qPCR-determined *B. thetaiotaomicron* cell counts at 8 h. D) $H_2S$ concentration (µM) per $10^6$ *B. wadsworthia* cells. Each point represents a technical culture replicate (n=3). Horizontal lines represent average, and error bars represent SD. Statistical significance between culture conditions was established using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests with a significance level set at α = 0.05. Results show * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001, ns = not significant (p > 0.05).

## 2.4 Discussion

Sulfate- and sulfite-reducing bacteria (SRB) such as *B. wadsworthia* can produce significant amounts of $H_2S$ [51], a toxic gas that has been implicated in gastrointestinal disease [98, 100, 113, 121, 262]. Advancing our understanding of the factors affecting the $H_2S$ production and abundance of *B. wadsworthia* in the human gut is therefore a valuable avenue of research. Here, the common gut commensal species *B. thetaiotaomicron* was investigated with respect to impact upon *B. wadsworthia*'s growth and $H_2S$ production. We observed a sulfidogenic interaction between *B. wadsworthia* and *B. thetaiotaomicron* (Figure 3) which was dependent upon cell proximity (Figure 5, 6) and *B. thetaiotaomicron* viability (Figure 7). There is no indication that Bt strain 1 itself can generate $H_2S$; no $H_2S$ was detected in either mono-cultures or when growth was stimulated by *B. wadsworthia* biomass or supernatant (Figures 5, 6). Additionally, the Bt strain 1 genome contains no genes encoding enzymes involved in known $H_2S$ production pathways.

*B. thetaiotaomicron* is a highly prevalent gut commensal able to degrade a range of dietary polysaccharides in the gut [281]. Expression of sulfatases by *B. thetaiotaomicron* permits degradation of host-derived glycans to yield free sulfate, which has been shown to support the growth of the sulfate-reducing bacterium *Desulfovibrio piger* [19]. However, cross-feeding between *B. thetaiotaomicron* and SRB via provision of sulfate cannot resolve the observations reported here with *B. wadsworthia*, as *B. wadsworthia* is not capable of reducing sulfate [50]. During fermentation, *B. thetaiotaomicron* produces hydrogen [234] which is a preferential energy source for *B. wadsworthia* [54]. However, if the $H_2S$-promoting interaction

was driven primarily via hydrogen cross-feeding, it would be expected that increased growth and $H_2S$ production by *B. wadsworthia* would be observed with cell-free supernatant and in filter-separated co-culture with Bt strain 1, which was not the case.

To the best of our knowledge, a species-specific interaction between *B. wadsworthia* and *B. thetaiotaomicron* has not been described, but more general genus-level associations have been reported; abundance of bile-tolerant *Bilophila* and *Bacteroides* spp. has been shown to increase in the gut in response to an animal-based diet [203]. Interestingly, a *Bacteroides*-dominant enterotype is found to be prevalent in individuals consuming a 'western' diet high in animal protein [5], as has been described with *Bilophila* [65, 206, 210]. Here, *B. wadsworthia* and *B. thetaiotaomicron* were studied using *in vitro* co-culture to investigate the microbe-microbe interactions between these species, with a particular interest in the impact upon *B. wadsworthia*-derived $H_2S$ production; the data implies a mutualistic symbiosis between *B. wadsworthia* and *B. thetaiotaomicron in vitro*, where both species appear to benefit from increased growth in co-culture (Figures 3, 6). Ultimately, this work describes a novel species-level interaction which, if occurring in the human gut environment, could contribute towards high levels of $H_2S$ which are associated with inflammation and disease [44, 98, 102, 262, 282] (Figure 10). The mechanism underpinning this interaction is not yet clear. In later chapters, we seek to substantiate the 'how' of the *B. wadsworthia* and *B. thetaiotaomicron* interaction, using transcriptomic analysis of the co-cultures to clarify the gene expression changes in co-culture leading to increased *B. wadsworthia* growth and $H_2S$ production. Additionally, *in vitro* fermentation models were used to simulate the human gut environment to investigate whether this interaction occurs under gut-representative conditions.

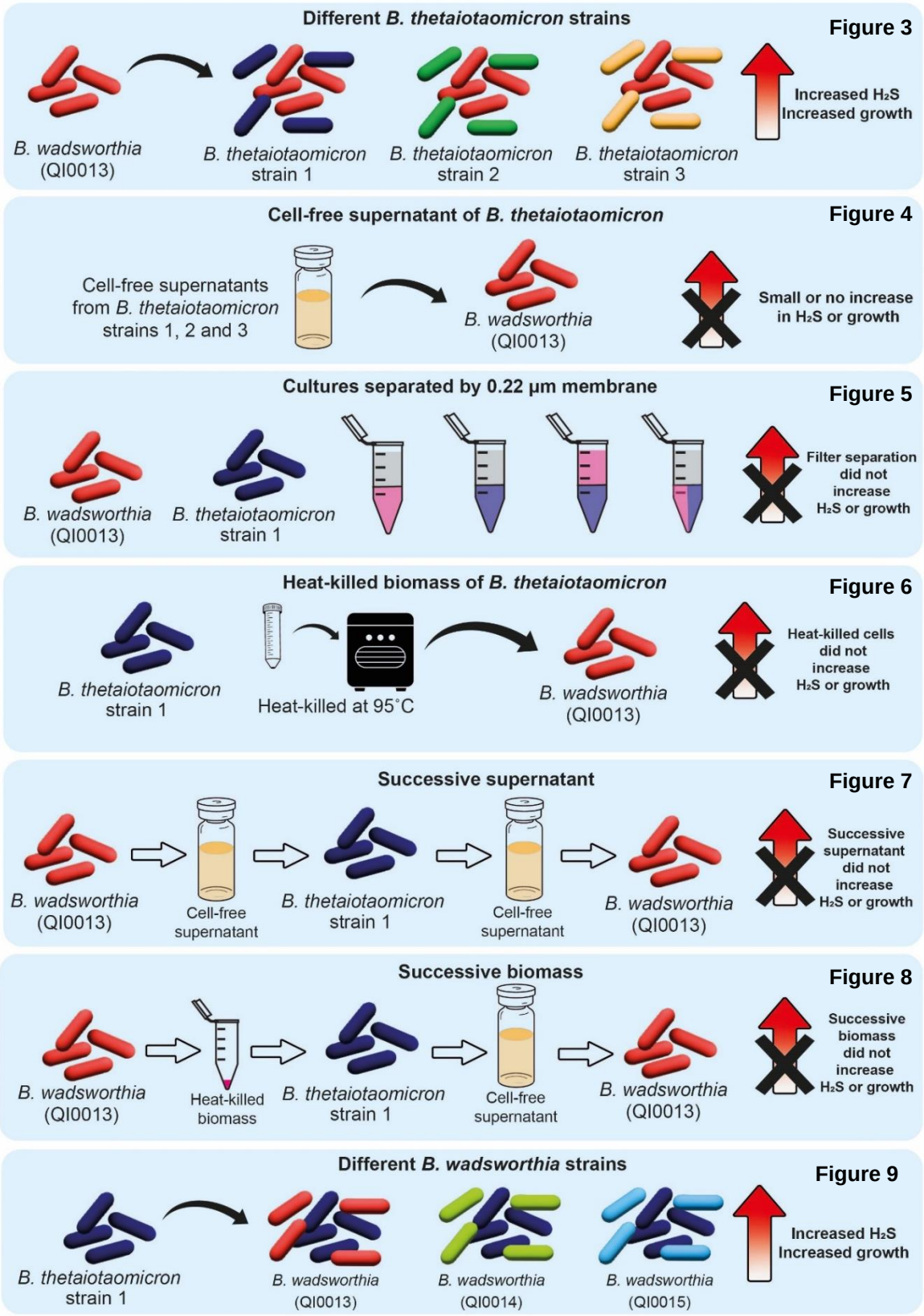



Figure 10: Microbe-microbe interaction between *B. wadsworthia* and *B. thetaiotaomicron*. Schematic outlines the experimental set-ups used and the observations with respect to *B. wadsworthia*'s $H_2S$ production and growth.

# Chapter 3

## 3. Microbe-microbe interaction between *B. wadsworthia* and enterococci

## 3.1 Introduction

*B. wadsworthia* is a pathobiontic member of the human gut microbiota capable of high levels of $H_2S$ production and is often associated with inflammatory diseases [67, 132, 150, 152, 158, 206]. Identifying possible strategies for controlling both the abundance of *B. wadsworthia* as well as $H_2S$ production by this bacterium could aid in combating excess inflammation and pathogenesis in the human gut [45]. Enterococci are a large group of bacteria commonly found in the human and animal gut [235], the most common species being *Enterococcus faecalis* and *Enterococcus faecium* [241]. Enterococci are Gram-positive lactic acid bacteria with complex roles in human health, where some probiotic strains show antimicrobial activity against pathogens [250], whereas other strains are associated with opportunistic infections and are clinically important reservoirs of antibiotic resistance [248]. A dairy-derived probiotic *E. faecium* strain was able to inhibit $H_2S$ production by bacteria associated with meat spoilage; co-culture with washed cells inhibited $H_2S$ production by *Escherichia coli*, *Citrobacter freundii* and *Hafnia alvei* by 48.6% [261]. The ability of enterococcal strains to affect $H_2S$ production by the sulfite-reducing bacterium *B. wadsworthia* was therefore investigated, with a working hypothesis of inhibition of *B. wadsworthia*'s $H_2S$ production in co-culture with *E. faecalis* or *E. faecium*. Furthermore, we assessed the impact of a mixed bacterial enrichment of *E. faecium* (QI0436) and *B. thetaiotaomicron* (QI0072) (Ef-Bt) on the growth and $H_2S$ production of *B. wadsworthia* and identified differentially abundant metabolites between culture conditions using untargeted metabolomics. We also used an *in silico* genome mining approach to investigate the probiotic potential of three *E. faecium* strains derived from the human gut and dairy.

## 3.2 Objectives

The purpose of the work presented in this chapter is to investigate the impact of *Enterococcus faecalis* and *Enterococcus faecium* on *B. wadsworthia*'s growth and $H_2S$ production using pair-wise anaerobic bacterial co-culture assays. This was based upon previous research demonstrating that enterococci can inhibit $H_2S$ production by some sulfide-producing bacteria. It was therefore hypothesised that enterococcal strains would show an inhibitory effect on *B. wadsworthia'*s growth. Additionally, given that *B. thetaiotaomicron* showed a sulfidogenic effect in co-culture with *B. wadsworthia* (Chapter 3), the impact of a mixed bacterial enrichment containing both *B. thetaiotaomicron* and *E. faecium* was investigated

with respect to *B. wadsworthia*'s growth and $H_2S$ production. We also identified differentially abundant metabolites in the cell-free supernatant of the cultures using untargeted metabolomics. Finally, we used an *in silico* genome mining approach to screen two gut-derived *E. faecium* strains (FI 09347 and QI0436) and one dairy-derived *E. faecium* strain (FI 09198) for potential use as anti-*B. wadsworthia* probiotics.

## 3.3 Materials and Methods

*B. wadsworthia*, *E. faecium, E. faecalis* and *B. thetaiotaomicron* strains were cultured using enrichment media and conditions outlined in section 2.3.2. Co-culture experiments and preparation of bacterial cell-free supernatants were performed as described in section 2.3.3.

### 3.3.1 Sample preparation for metabolomic analysis

5 mL of cultures were taken at 6 h post-inoculation and cells were immediately pelleted at 4,000 x g for 10 min. 300 µL of cell-free supernatant was snap frozen on dry ice and transported to Creative Proteomics, USA. Prior to analysis, supernatants were thawed and 100 µL supernatant was added to 300 µL methanol for metabolite extraction. Samples were vortexed for 60 s, sonicated for 30 min at 4°C and stored at -20°C for 1 h. Samples were pelleted at 12,000 x g for 15 min at 4°C. Finally, 200 µL of supernatant and 5 µL of DL-o-Chlorophenylalanine (0.2 mg/mL) was transferred to a vial for LC-MS analysis (Creative Proteomics, New York, USA).

### 3.3.2 LC-MS

All LC-MS analysis was performed by Creative Proteomics, New York, USA. QC samples were prepared by pooling all the samples in triplicate. All samples were injected in triplicate. Separation was performed by ACQUITY UPLC (Waters) combined with Q Exactive MS (Thermo) and screened with ESI-MS. The LC system was comprised of ACQUITY UPLC HSS T3 (100×2.1 mm×1.8 µm) with ACQUITY UPLC (Waters). The mobile phase was composed of solvent A (0.05% formic acid in water) and solvent B (acetonitrile) with a gradient elution (0-1 min, 5% B; 1-12.5 min, 5%-95% B; 12.5-13.5 min, 95% B; 13.5-13.6 min, 95%-5% B; 13.6-16 min, 5% B). The flow rate of the mobile phase was 0.3 mL/min. The column temperature was maintained at 40°C, and the sample manager temperature set at 4°C. Mass spectrometry parameters in ESI+ and ESI- mode were as follows: ESI+: Heater Temp 300°C; Sheath Gas Flow rate, 45 arb; Aux Gas Flow Rate, 15 arb; Sweep Gas Flow Rate, 1 arb; spray voltage, 3.0 KV; Capillary Temp, 350°C; S-Lens RF Level, 30%. ESI-: Heater Temp 300°C, Sheath Gas Flow rate, 45 arb; Aux Gas Flow Rate, 15 arb; Sweep Gas Flow Rate, 1 arb; spray voltage, 3.2 KV; Capillary Temp, 350°C; S-Lens RF Level, 60%.

Metabolites were identified using Compound Discoverer 3.0 (ThermoFisher Scientific, USA) and Progenesis QI v 2.1 (Waters) was used for manual screening of the identified compounds in order to minimise false positive identification results. Data was normalised using Total Ion Count (TIC) method where the peak area of each metabolite divided by the SUM of all metabolites area and then multiplied by one million.

### 3.3.3 Data analysis of metabolome of cell-free supernatant

The metabolite abundance analysed by Creative Proteomics was further analysed as follows: To remove compounds with high analytical variability, compounds with $RSD_{QC}$ >20% were discarded [283]. The relative concentrations in the original samples were back-calculated based on 1:4 dilution with methanol during sample preparation. In positive ion mode, 73% of the identified metabolites remained after quality control (357 left from 489). The metabolomic data was auto-scaled and analysed using Metaboanalyst 5.0 [284], where data was normalised using the reference group of sterile growth media (negative control). Metaboanalyst was then used to obtain the PLS-DA for the global profile changes and Variable Importance in Projection (VIP) compounds that contribute highly to inter-condition differences, and the heatmaps showing feature clustering and inter-condition differences in relative abundance of compounds. The top fifty differentially abundant metabolites were determined using pair-wise t tests between Bw + Ef-Bt and Ef-Bt cultures, $p \leq 0.05$. To investigate the presence of genes related to metabolic pathways for producing or consuming metabolites of interest in the conditions, the Kyoto Encyclopaedia of Genes and Genomes (KEGG) database [285] was used for *B. thetaiotaomicron* VPI-5482 and *E. faecium* DO, and Pathway Tools [286] was used for *B. wadsworthia* QI0013.

### 3.3.4 Screening enterococci genomes for putative bacteriocin biosynthetic gene clusters

Genome assemblies of FI 09198 (3 contigs, N50 2623541, 2.79 Mb, 99.63% completeness, 0.71% contamination), FI 09347 (151 contigs, N50 56613, 2.9 Mb, 100% completeness, 0.23% contamination) and QI0436 (4 contigs, N50 2548291, 2.85 Mb, 98.28% completeness, 0.98% contamination) were obtained as described in section 2.3.9. The genomes were screened for putative bacteriocin biosynthetic clusters using BAGEL4 [287] and antiSMASH 7 [288] to identify regions of interest (ROIs). The ROIs were manually curated by checking for the

presence of post-translational modification (PTM) enzymes, transport proteins and immunity proteins within the predicted biosynthetic gene cluster [289, 290]. Additionally, the amino acid sequence of the predicted proteins encoding bacteriocins, immunity proteins and transport proteins were checked using NCBI blast protein-protein comparison (blastp) [291] and InterProScan [292] to confirm predicted protein function [289].

### 3.3.5 Screening enterococci genomes for virulence and antimicrobial resistance genes

FI 09198, FI 09347 and QI0436 were annotated using BV-BRC [293] which integrates functional gene classes and subsystems as well as virulence gene identification using VFDB [294] and Victors [295]. Antimicrobial resistance genes were identified using the Comprehensive Antibiotic Resistance Database (CARD) [296]. The genomes of FI 09347 and QI0436 were manually curated searching for genes relating to amino acid metabolism, vitamin metabolism, protein metabolism, and stress tolerance including acid/bile resistance.

## 3.4 Results

### 3.4.1 Do gut- and dairy-derived enterococci affect *B. wadsworthia*'s growth and $H_2S$ production?

Given that enterococci have been previously reported to inhibit $H_2S$ production by sulfide-producing bacteria associated with meat spoilage [261], we investigated the impact of enterococci on $H_2S$ production by *B. wadsworthia*. An initial screening experiment was performed with eight strains of *E. faecium* and 2 strains of *E. faecalis* derived from the human gut or dairy products sourced from the QIB culture collection (Table 1) in co-culture with *B. wadsworthia*. All co-cultures containing enterococci strains showed significantly decreased $H_2S$ concentration at 8 h post-inoculation compared to *B. wadsworthia* in mono-culture, demonstrating a clear inhibitory effect upon *B. wadsworthia* by *E. faecium* and *E. faecalis* strains (Figure 11).

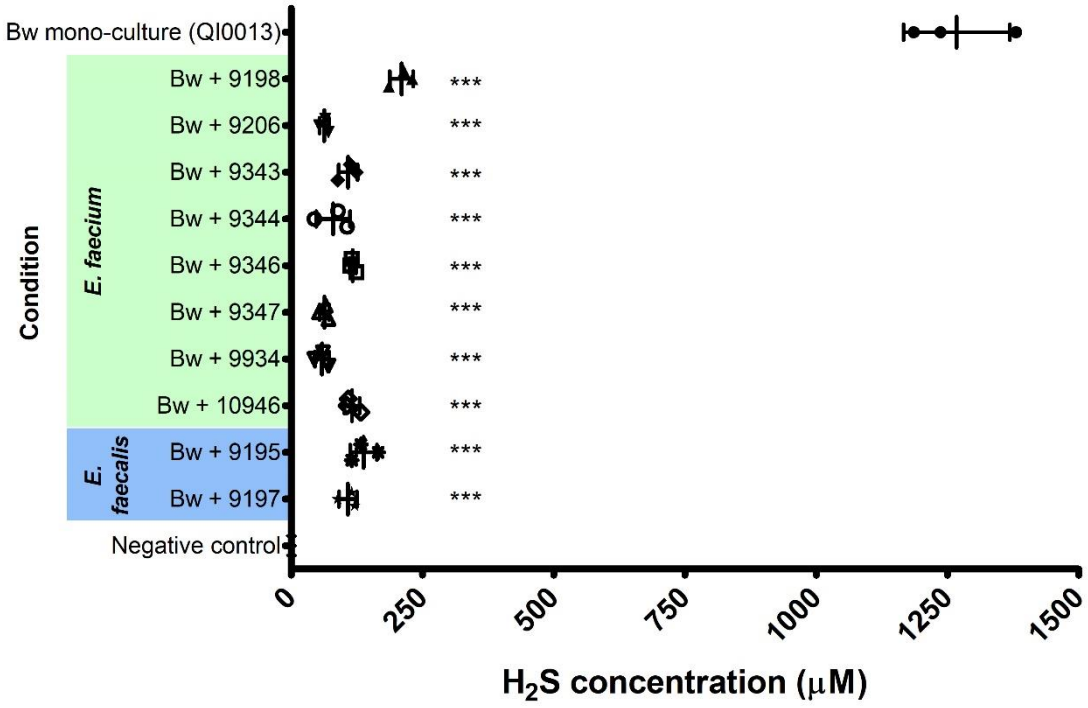


Figure 11: $H_2S$ concentration in pairwise co-cultures of *B. wadsworthia* (Bw) QI0013 with different *E. faecium* and *E. faecalis* strains. $H_2S$ concentrations (µM) at 8 h post-inoculation. Negative control was uninoculated bacterial growth media. Each point represents a technical culture replicate (n=3). Horizontal lines represent average, and error bars represent SD. Statistical significance between culture conditions was established using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests with a significance level set at $\alpha = 0.05$. Results show * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant ($p > 0.05$).

Next, we selected two *E. faecium* strains derived from either dairy (FI 09198) or the human gut (FI 09347) to perform co-cultures with *B. wadsworthia* assessing the resulting impact on $H_2S$ concentration and bacterial abundance. Both *E. faecium* strains significantly reduced $H_2S$ concentration at 8 h in co-culture with *B. wadsworthia* compared to *B. wadsworthia* mono-culture (Figure 12A). *B. wadsworthia* abundance was significantly reduced in co-cultures with *E. faecium*, with approximately one order of magnitude reductions in cell counts observed compared to mono-culture (Figure 12B). *E. faecium* abundance was largely unaffected by the presence of *B. wadsworthia*, as abundance did not differ between mono- and co-cultures (Figure 12C). The $H_2S$ concentration per $10^6$ *B. wadsworthia* cells was significantly increased in the co-cultures compared to *B. wadsworthia* mono-culture (Figure 12D), but the overall phenotypic effect of *E. faecium* was significant inhibition of $H_2S$ concentration driven by decreased *B. wadsworthia* abundance, likely via direct inhibition of *B. wadsworthia*'s growth.

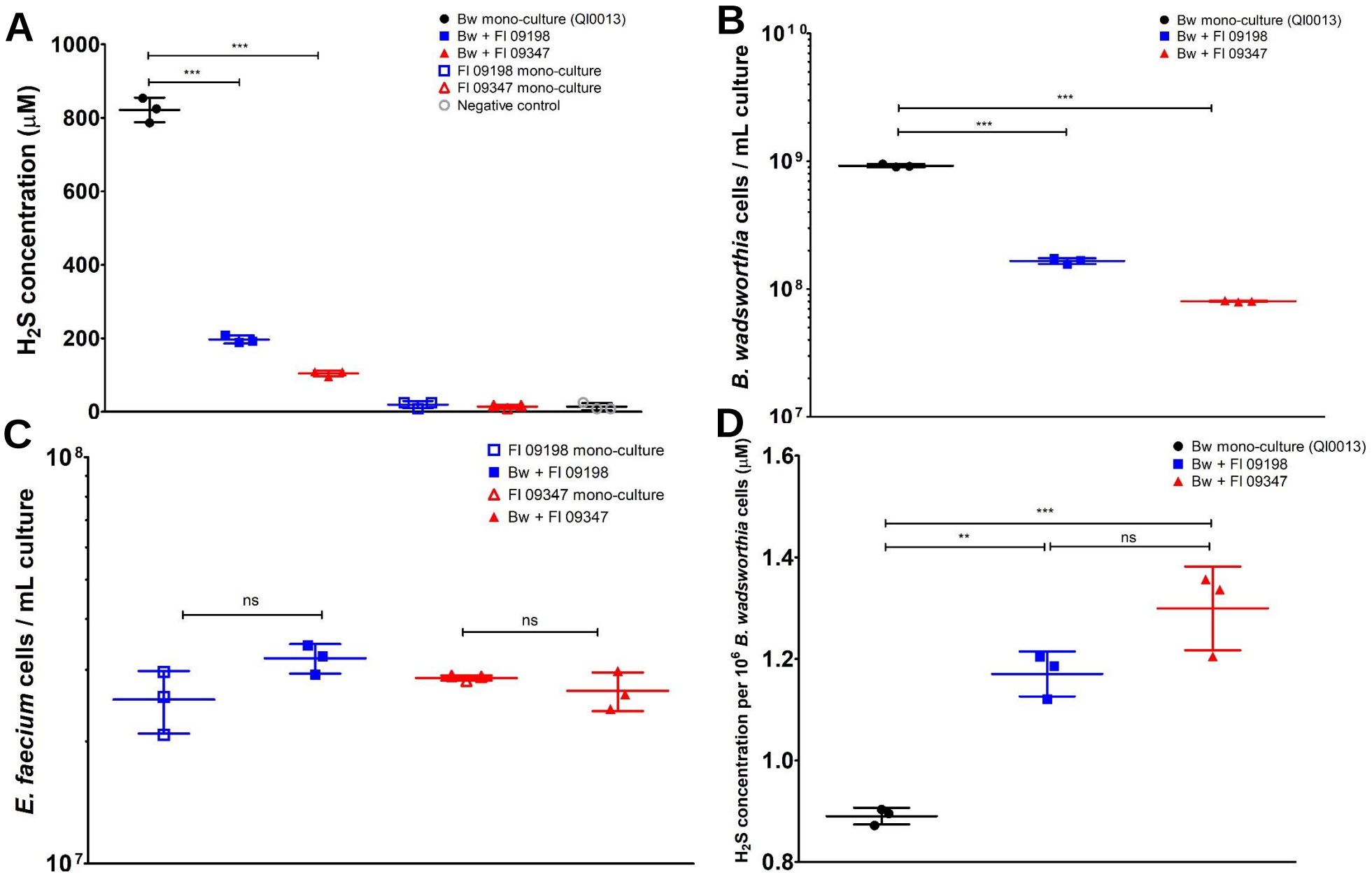

A
$H_2S$ concentration (μM)
1000
800
600
400
200
0
***
***
Bw mono-culture (QI0013)
Bw + FI 09198
Bw + FI 09347
FI 09198 mono-culture
FI 09347 mono-culture
Negative control
B
B. wadsworthia cells / mL culture
$10^{10}$
$10^{9}$
$10^{8}$
$10^{7}$
***
***
Bw mono-culture (QI0013)
Bw + FI 09198
Bw + FI 09347
C
E. faecium cells / mL culture
$10^{8}$
$10^{7}$
ns
ns
FI 09198 mono-culture
Bw + FI 09198
FI 09347 mono-culture
Bw + FI 09347
D
$H_2S$ concentration per $10^6$ B. wadsworthia cells (μM)
1.6
1.4
1.2
1.0
0.8
***
**
ns
Bw mono-culture (QI0013)
Bw + FI 09198
Bw + FI 09347

Figure 12: Pairwise co-culture of *B. wadsworthia* (Bw) QI0013 with dairy-derived (FI 09198) and gut-derived (FI 09347) *E. faecium* strains.

A) $H_2S$ concentrations (µM) at 8 h. B) qPCR-determined *B. wadsworthia* cell counts at 8 h. C) qPCR-determined *E. faecium* cell counts at 8 h. D) $H_2S$ concentration (µM) per $10^6$ *B. wadsworthia* cells. Each point represents a technical culture replicate (n=3). Horizontal lines represent average, and error bars represent SD. Statistical significance between culture conditions was established using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests with a significance level set at α = 0.05. Results show * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001, ns = not significant (p > 0.05).

### 3.4.2 Impact of a mixed culture of *B. thetaiotaomicron* and *E. faecium* on *B. wadsworthia*

The combined influence of *E. faecium* and *B. thetaiotaomicron* on *B. wadsworthia*'s growth and $H_2S$ production was investigated by co-culturing *B. wadsworthia* with a mixed bacterial enrichment containing both *E. faecium* (QI0436) and *B. thetaiotaomicron* (QI0072) (Ef-Bt). Given that significant inhibition of $H_2S$ is observed in *B. wadsworthia* co-cultured with *E. faecium* by 8 h (Figures 12, 13) and the growth dynamics of a mixed enrichment were unknown, time-point sampling was performed at 0, 2, 4, 6, 8 and 26 h post-inoculation to understand the temporal growth dynamics of all strains present. This revealed that *B. wadsworthia* abundance and $H_2S$ production were similar in mono-culture and co-culture with Ef and Bt until 4 h, after which *B. wadsworthia*'s growth was significantly inhibited with concomitant reduction in $H_2S$ (Figure 13A, 13B). By 8 h, $H_2S$ concentration in the co-culture was minimal (Figure 13A). The abundance of both *B. thetaiotaomicron* and *E. faecium* was higher in co-culture with *B. wadsworthia* compared to the absence of this species (Figure 13C, 13D), suggesting that both strains benefitted from *B. wadsworthia*. The initial 4 h delay in the inhibition of *B. wadsworthia*'s growth suggests that the production of inhibitory compounds by *E. faecium* may depend upon cell density and/or growth phase (Figure 13C). An important component of *E. faecium*'s inhibitory effect is related to lactic acid production; the pH of the co-culture decreased to 4.5 by 6 h, suggesting that the inhibition of *B. wadsworthia*'s growth is pH dependent (Figure 13D,

supplementary data section 7.1.1). Potent inhibition of *B. wadsworthia*'s growth and $H_2S$ production was also observed when cultured with cell-free supernatant from the Ef-Bt enrichment culture (Figure 14A, 14B). This is likely to be at least partially mediated by reduction in pH via lactic acid production by *E. faecium,* as the supernatant-containing culture reached pH 5 at 8 h post-inoculation compared to pH 7 in the *B. wadsworthia* mono-culture (Figure 14C). The reduction in pH is likely to result in inhibition of *B. wadsworthia*'s growth, particularly at lower pH range of 4-4.5; however, in the case of co-culture with Bt strain 2 where pH decreased to 5.4, *B. wadsworthia*'s abundance was similar to in mono-culture (Figure 3) where the pH was 6.6 (supplementary data section 7.1.1). The inhibitory effect of *E. faecium* upon *B. wadsworthia*'s growth and $H_2S$ production is likely to be mediated predominantly by lactic acid production, although production of antimicrobial compounds may also play a contributing role.

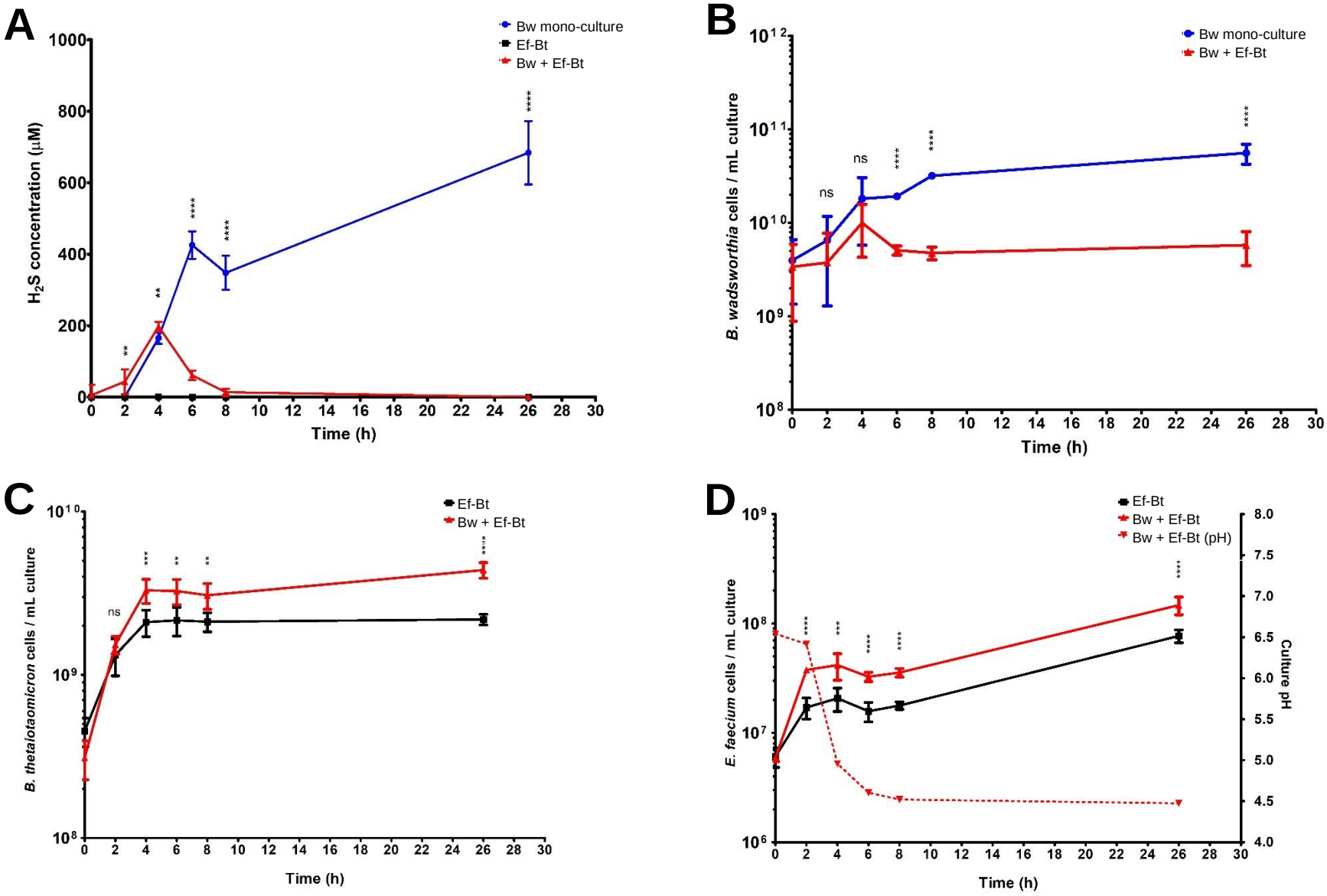

A
Bw mono-culture
Ef-Bt
Bw + Ef-Bt
H2S concentration (μM)
Time (h)
B
Bw mono-culture
Bw + Ef-Bt
B. wadsworthia cells / mL culture
ns
Time (h)
C
Ef-Bt
Bw + Ef-Bt
B. thetaiotaomicron cells / mL culture
ns
Time (h)
D
Ef-Bt
Bw + Ef-Bt
Bw + Ef-Bt (pH)
E. faecium cells / mL culture
Culture pH
Time (h)

Figure 13: Co-culture of *B. wadsworthia (*QI0013) (Bw) with a mixed enrichment of *E. faecium* (QI0436) and *B. thetaiotaomicron* (QI0072) (Ef-Bt). A) $H_2S$ concentrations (µM). B) qPCR-determined *B. wadsworthia* cell counts. C) qPCR-determined *B. thetaiotaomicron* cell counts. D) qPCR-determined *E. faecium* cell counts with culture pH. Each point shows average of technical culture replicates (n=7). Error bars represent SD. Results of unpaired t tests are shown where ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$, ns = not significant ($p > 0.05$).

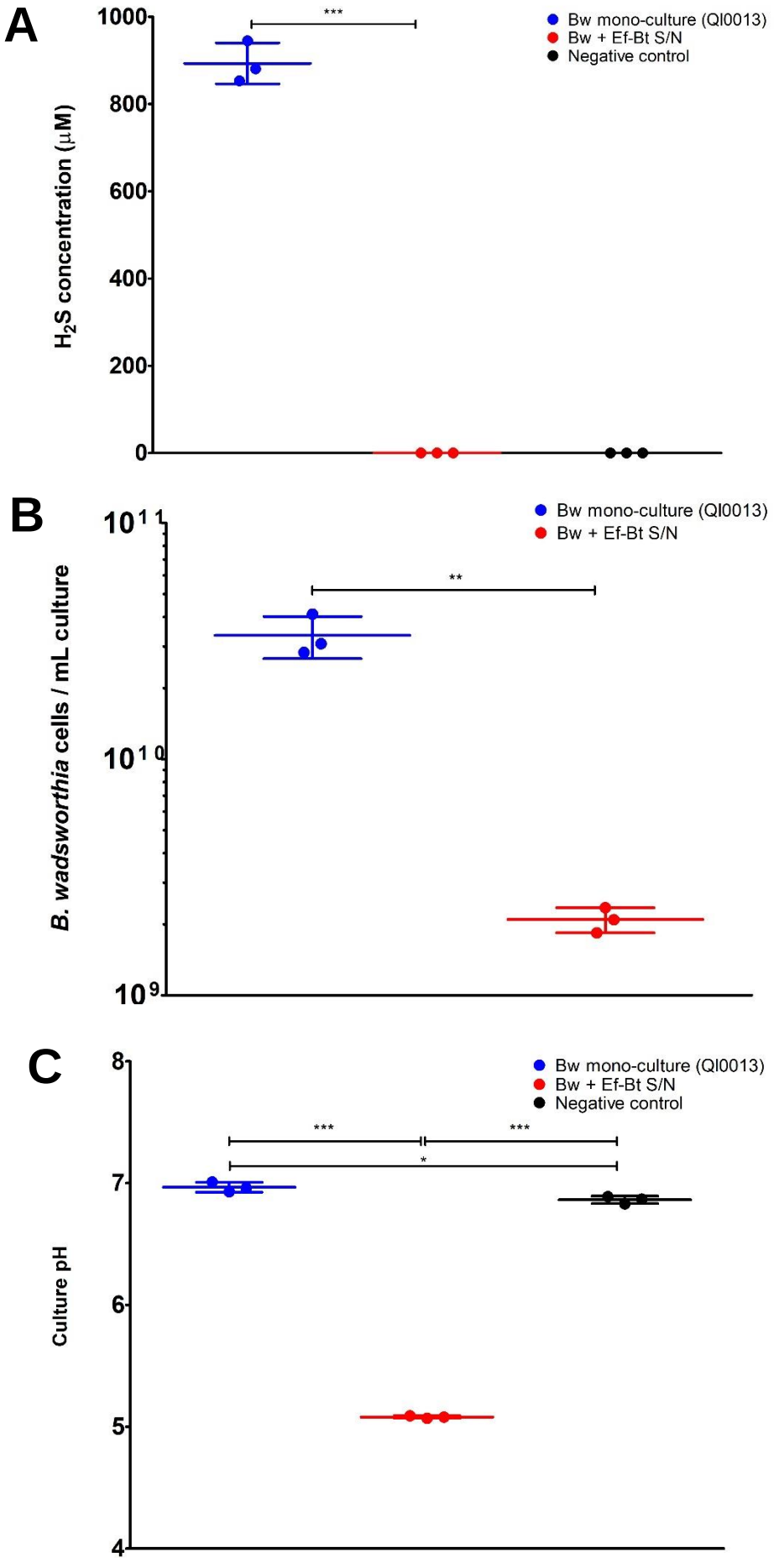

A
1000
800
600
400
200
0
$H_2S$ concentration (μM)
***
Bw mono-culture (QI0013)
Bw + Ef-Bt S/N
Negative control
B
$10^{11}$
$10^{10}$
$10^{9}$
*B. wadsworthia* cells / mL culture
**
Bw mono-culture (QI0013)
Bw + Ef-Bt S/N
C
8
7
6
5
4
Culture pH
***
***
*
Bw mono-culture (QI0013)
Bw + Ef-Bt S/N
Negative control

Figure 14: Culture of *B. wadsworthia* (Bw) QI0013 with Ef + Bt cell-free supernatant (S/N).
A) $H_2S$ concentrations (µM) at 8 h. B) qPCR-determined *B. wadsworthia* cell counts at 8 h. C) pH of cultures. Negative control was sterile growth media. Each point represents a technical culture replicate (n=3). Error bars represent SD. Statistical significance between culture conditions was established using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests with a significance level set at α = 0.05. Results show * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, **** = p ≤ 0.0001, ns = not significant (p > 0.05).

#### 3.4.2.1 Metabolomic analysis of the *B. wadsworthia*, *E. faecium* and *B. thetaiotaomicron* cultures

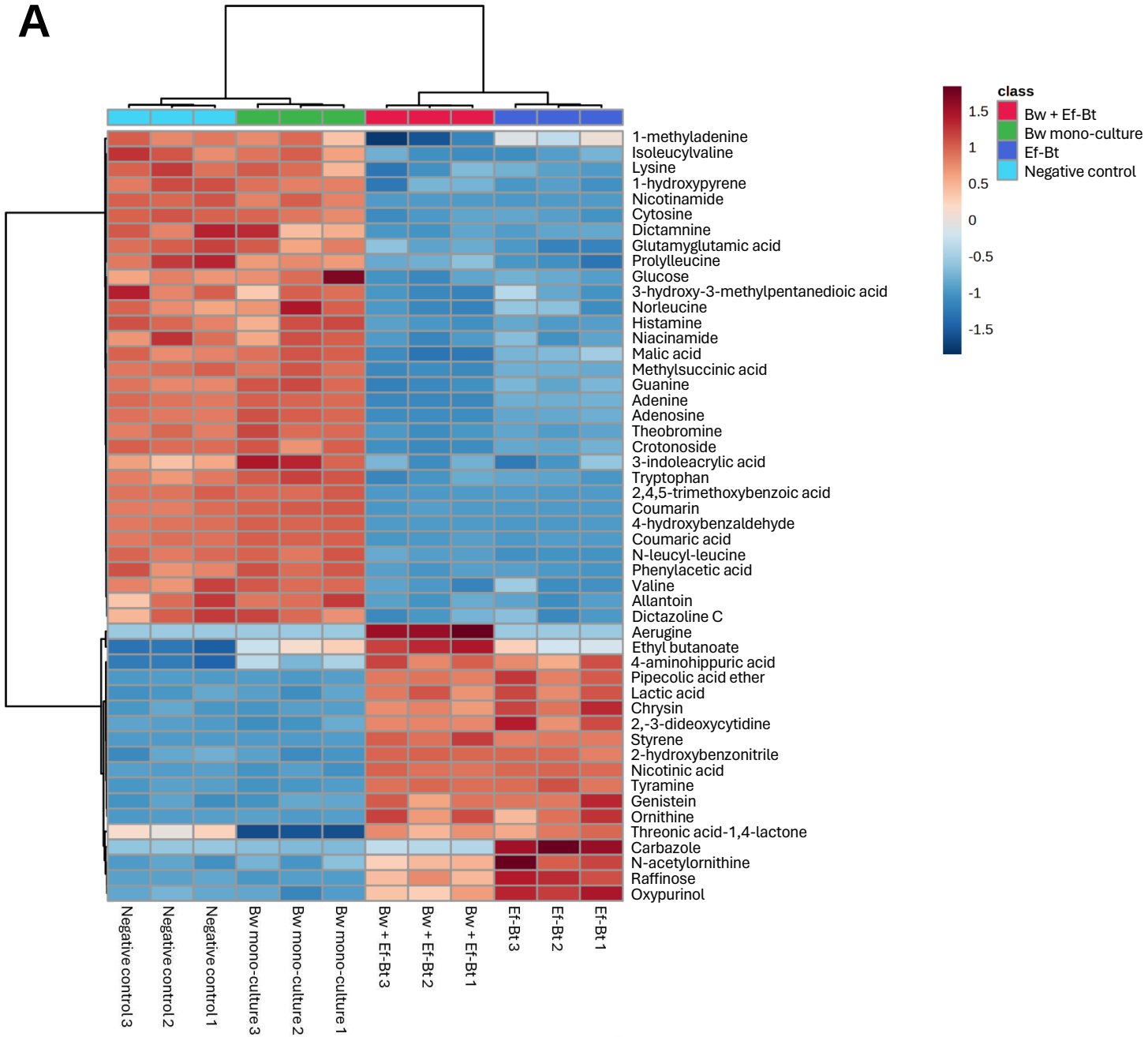
To further investigate the microbial interactions between *B. wadsworthia* and the mixed enrichment of *E. faecium* (QI0436) and *B. thetaiotaomicron* (QI0072) (Ef-Bt) that led to inhibition of *B. wadsworthia*'s growth and $H_2S$ production (Figure 13, Figure 14), we compared the metabolomic profile of the cell-free supernatant from the cultures at 6 h post-inoculation using untargeted LC-MS. Using partial least squares-discriminant analysis (PLS-DA) to compare the overall metabolome of the cultures, the *B. wadsworthia* mono-culture and negative control of sterile growth media clustered distinctly from the other conditions, whereas the Ef-Bt culture and the Bw + Ef-Bt culture were more similar (Figure 15B).

##### 3.4.2.1.1 Decreased abundance of amino acids, vitamin B3 and tryptophan in *E. faecium* and *B. thetaiotaomicron* cultures compared to *B. wadsworthia* mono-culture

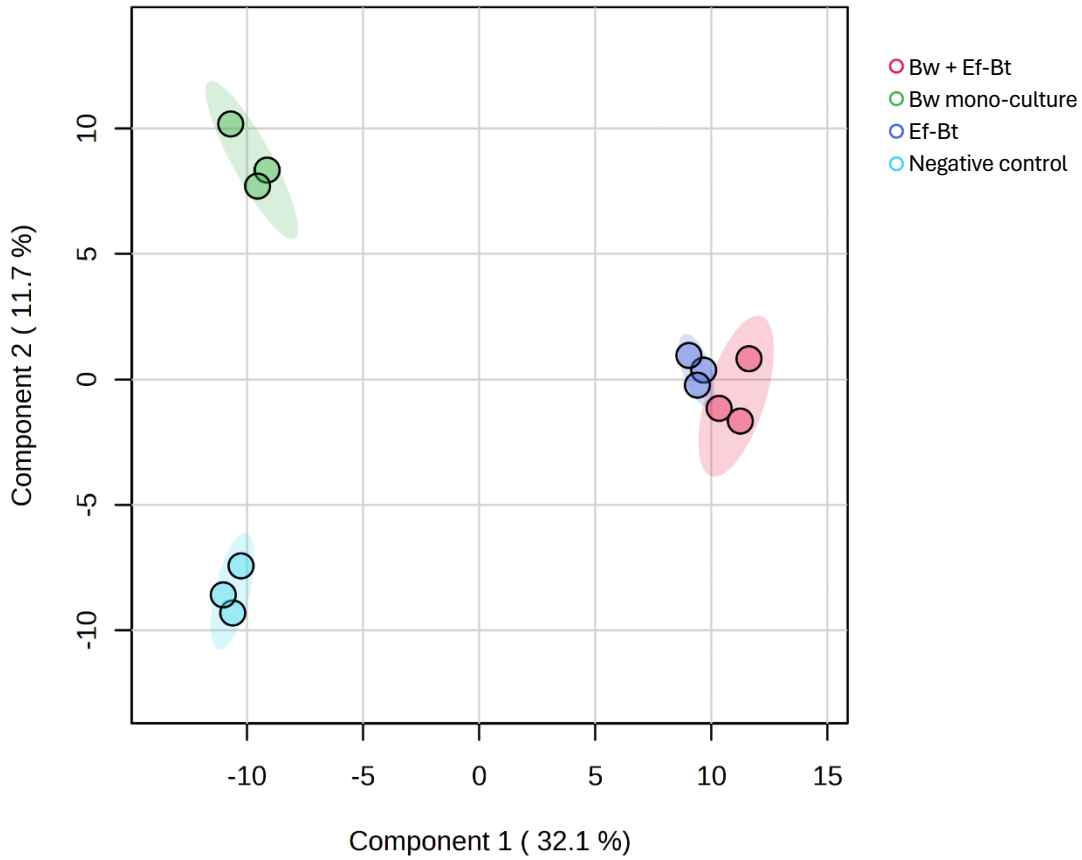
Of the top fifty differentially abundant metabolites, thirty-two were lower in abundance in the Ef-Bt and Bw + Ef-Bt cultures compared to the *B. wadsworthia* mono-culture and the negative control (Figure 15A). These metabolites included the dipeptides isoleucylvaline, glutamylglutamic acid, prolylleucine and leucyl-leucine, and amino acids lysine, norleucine and valine (Figure 15A). This is likely due to direct utilisation of these compounds by either *B. thetaiotaomicron* or *E. faecium*; indeed, leucine is required for the growth of *E. faecium* [297]. The abundance of nucleotide bases guanine, adenine and cytosine and the nucleoside adenosine were also decreased (Figure 15A). This is likely to reflect the increased total cell abundance and growth in these cultures relative to the *B. wadsworthia* mono-culture (Figure 13), leading to increased requirements for purine salvage for

DNA and RNA synthesis [298, 299]. Histamine was also lower in abundance in Ef-Bt culture conditions (Figure 15A). Microbial histamine production genes are widely present in the human gut across a broad taxonomic range including some *Bacteroides* spp. [300], but histamine utilisation by gut bacteria has not been extensively described. Pathogenic *Pseudomonas aeruginosa* can utilise host-derived histamine to induce virulence factor expression during infection [301], but none of the strains used here contain known genes related to histamine uptake, or histidine decarboxylase for histamine production. Allantoin, a nitrogenous compound derived from a purine [302] was decreased in abundance in Ef-Bt culture conditions (Figure 15A, 15E); this is an end product of purine catabolism [303], and decreased abundance may reflect the shift towards increased purine utilisation and anabolism during active cell growth. The ability of *E. faecium* and *B. thetaiotaomicron* to use allantoin is unclear, although *E. faecalis* was shown to be incapable of allantoin utilisation [304]. Interestingly, *B. wadsworthia* encodes an allantoinase able to degrade allantoin to allantoate during purine metabolism; this has been described in *E. coli* to permit the use of allantoin as a nitrogen source [305].

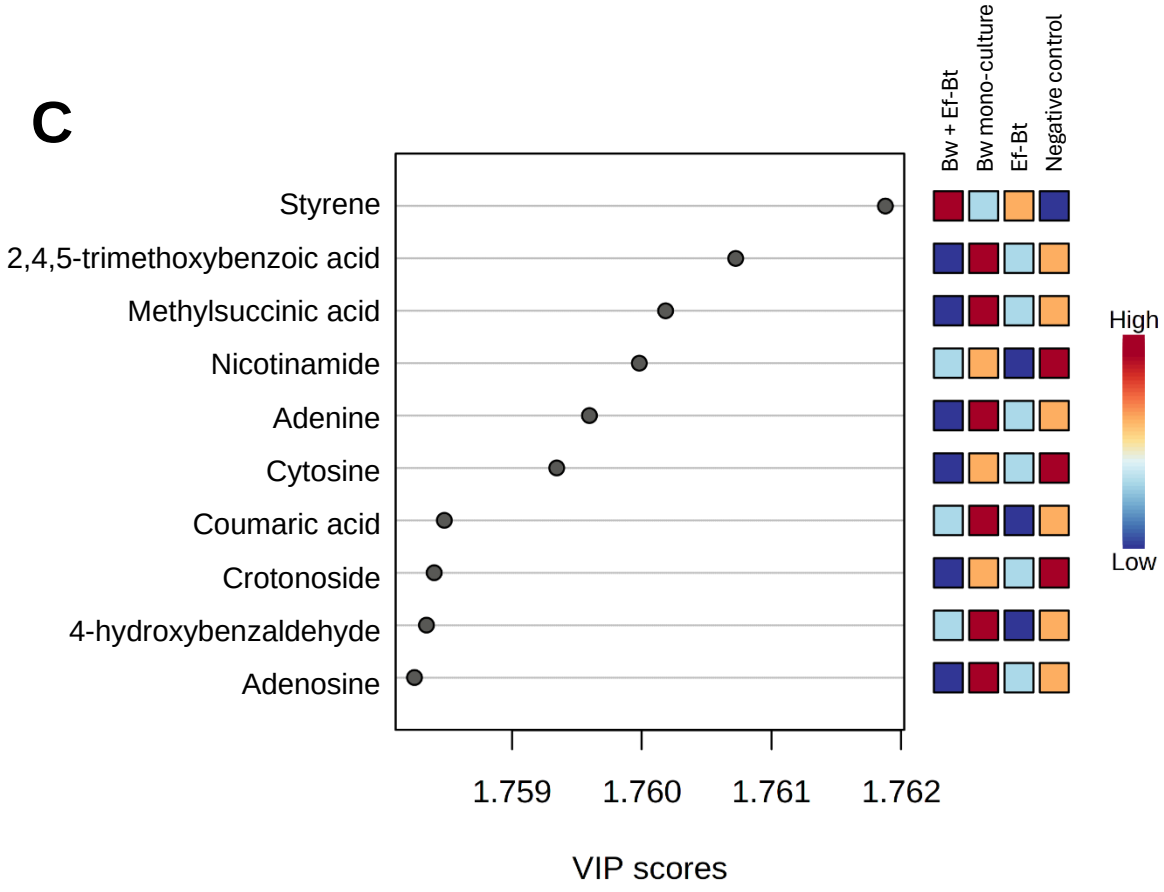
Different forms of vitamin B3 (vitamers) were differentially abundant in the cultures; niacinamide and nicotinamide were lower in abundance in Ef-Bt cultures compared to *B. wadsworthia* mono-culture, whereas nicotinic acid was found in higher abundance in Ef-Bt cultures (Figure 15A, 15E). Furthermore, nicotinamide was one of the top 10 compounds contributing to differences between conditions (Figure 15C). Both *B. thetaiotaomicron* and *E. faecium* have genes encoding nicotinate phosphoribosyltransferase (EC 6.3.4.21) which utilises vitamin B3 for NAD+ biosynthesis, so it is likely that vitamers of vitamin B3 were utilised in the cultures by *B. thetaiotaomicron* and *E. faecium*. Tryptophan and the tryptophan derivative 3-indoleacrylic acid were also decreased in abundance in the Ef-Bt cultures compared to *B. wadsworthia* mono-culture and negative control (Figure 15A). Given that *B. thetaiotaomicron* uses tryptophan during indole production [306], it is possible that *E. faecium* may compete for tryptophan resulting in decreased bioavailability to *B. thetaiotaomicron* for indole production. Alternatively, *B. thetaiotaomicron* may produce less indole when in culture with *E. faecium*.

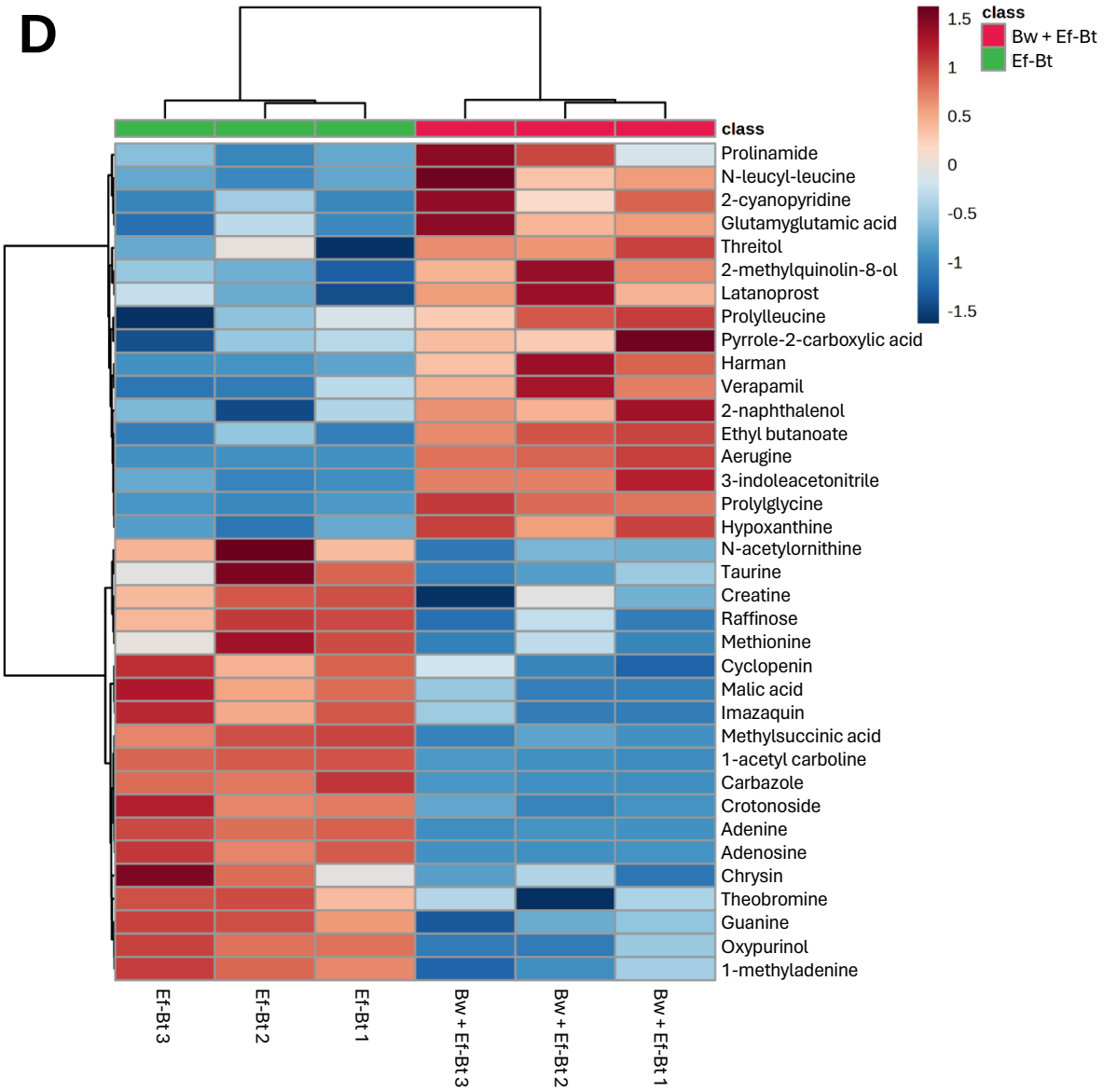

A
class
Bw + Ef-Bt
Bw mono-culture
Ef-Bt
Negative control
1.5
1
0.5
0
-0.5
-1
-1.5
1-methyladenine
Isoleucylvaline
Lysine
1-hydroxypyrene
Nicotinamide
Cytosine
Dictamnine
Glutamyglutamic acid
Prolylleucine
Glucose
3-hydroxy-3-methylpentanedioic acid
Norleucine
Histamine
Niacinamide
Malic acid
Methylsuccinic acid
Guanine
Adenine
Adenosine
Theobromine
Crotonoside
3-indoleacrylic acid
Tryptophan
2,4,5-trimethoxybenzoic acid
Coumarin
4-hydroxybenzaldehyde
Coumaric acid
N-leucyl-leucine
Phenylacetic acid
Valine
Allantoin
Dictazoline C
Aerugine
Ethyl butanoate
4-aminohippuric acid
Pipecolic acid ether
Lactic acid
Chrysin
2,-3-dideoxycytidine
Styrene
2-hydroxybenzonitrile
Nicotinic acid
Tyramine
Genistein
Ornithine
Threonic acid-1,4-lactone
Carbazole
N-acetylornithine
Raffinose
Oxypurinol
Negative control 3
Negative control 2
Negative control 1
Bw mono-culture 3
Bw mono-culture 2
Bw mono-culture 1
Bw + Ef-Bt 3
Bw + Ef-Bt 2
Bw + Ef-Bt 1
Ef-Bt 3
Ef-Bt 2
Ef-Bt 1

**B**


Bw + Ef-Bt
Bw mono-culture
Ef-Bt
Negative control
Component 2 ( 11.7 %)
10
5
0
-5
-10
-10
-5
0
5
10
15
Component 1 ( 32.1 %)

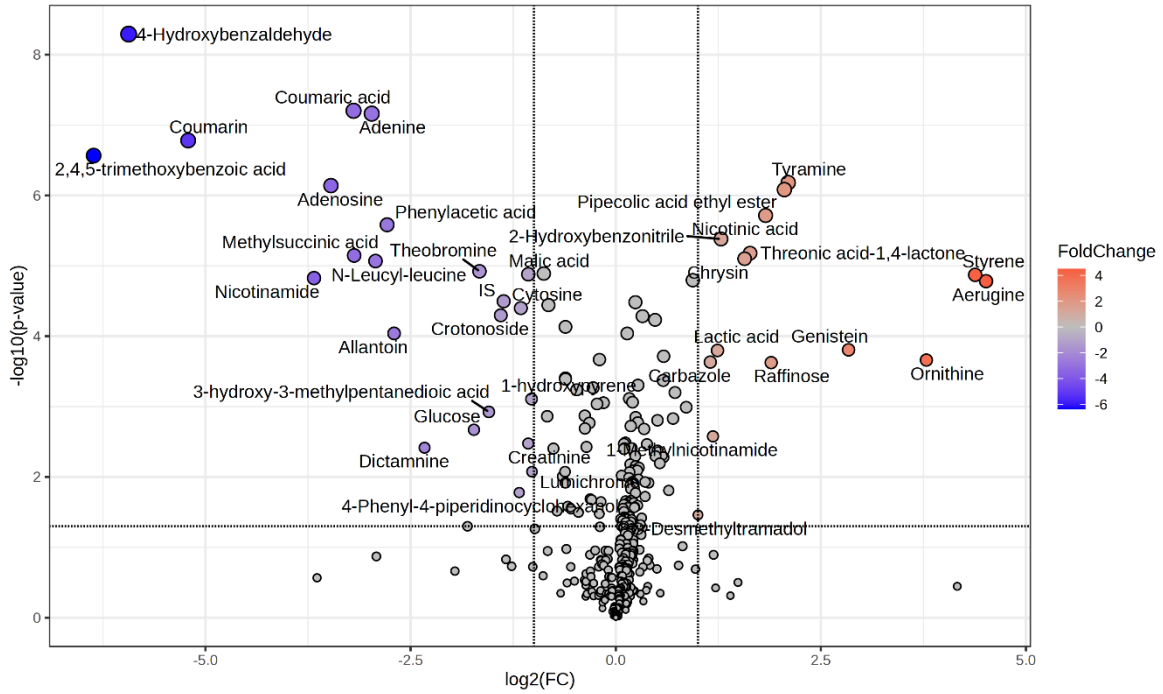
**C**


Bw + Ef-Bt
Bw mono-culture
Ef-Bt
Negative control
Styrene
2,4,5-trimethoxybenzoic acid
Methylsuccinic acid
Nicotinamide
Adenine
Cytosine
Coumaric acid
Crotonoside
4-hydroxybenzaldehyde
Adenosine
High
Low
1.759
1.760
1.761
1.762
VIP scores


D
class
Bw + Ef-Bt
Ef-Bt
1.5
1
0.5
0
-0.5
-1
-1.5
class
Prolinamide
N-leucyl-leucine
2-cyanopyridine
Glutamyglutamic acid
Threitol
2-methylquinolin-8-ol
Latanoprost
Prolylleucine
Pyrrole-2-carboxylic acid
Harman
Verapamil
2-naphthalenol
Ethyl butanoate
Aerugine
3-indoleacetonitrile
Prolylglycine
Hypoxanthine
N-acetylornithine
Taurine
Creatine
Raffinose
Methionine
Cyclopenin
Malic acid
Imazaquin
Methylsuccinic acid
1-acetyl carboline
Carbazole
Crotonoside
Adenine
Adenosine
Chrysin
Theobromine
Guanine
Oxypurinol
1-methyladenine
Ef-Bt 3
Ef-Bt 2
Ef-Bt 1
Bw + Ef-Bt 3
Bw + Ef-Bt 2
Bw + Ef-Bt 1

**E**

## Bw + Ef-Bt vs. Bw mono-culture




**F**

## Bw + Ef-Bt vs. Ef-Bt

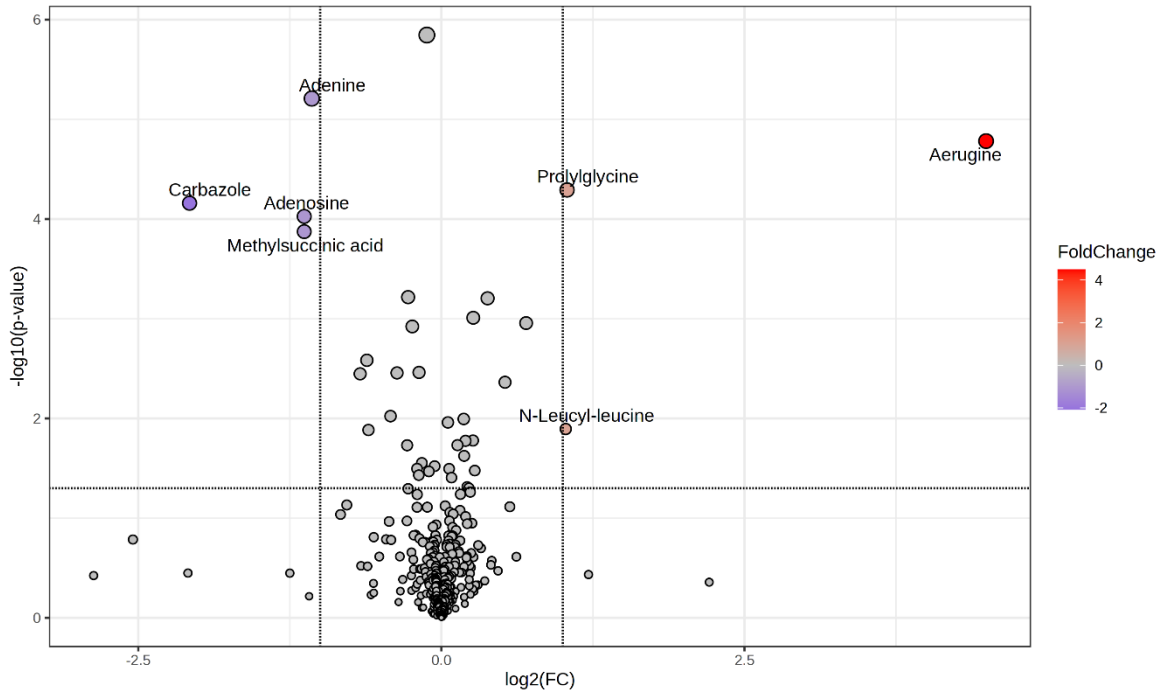


Figure 15: Comparison of the cell-free supernatant metabolome of *B. wadsworthia* mono-culture, Ef-Bt enrichment culture, and Ef-Bt enrichment with *B. wadsworthia* co-culture at 6 h post-inoculation.

A) Heatmap displaying relative abundance of the top 50 differentially abundant metabolites in the culture conditions. The normalised relative abundance is shown in a gradient from blue (low) to red (high). B) PLS-DA plot of metabolites acquired from samples via untargeted LCMS in positive ion mode. C) The top 10 compounds ranked based on the Variable Importance in Projection (VIP) scores. The coloured boxes indicate the relative concentrations of the corresponding metabolite in each group. D) Significantly differentially abundant metabolites between Ef-Bt culture and Ef-Bt culture with *B. wadsworthia*. Significance was determined via t test, where $p \leq 0.05$ was statistically significant. E) Volcano plot displaying pairwise comparison of Bw + Ef-Bt culture with Bw mono-culture, where red displays metabolites higher in abundance in Bw + Ef-Bt culture compared to Bw mono-culture and blue indicates metabolites lower in abundance. Significance was determined via t test, where $p \leq 0.05$ with a 2-fold change in abundance between conditions. F) Volcano plot displaying pairwise comparison of Bw + Ef-Bt culture with Ef-Bt culture, where red displays metabolites higher in abundance in Bw + Ef-Bt culture compared to Ef-Bt culture and blue indicates metabolites lower in abundance. Significance was determined via t test, where $p \leq 0.05$ with a 2-fold change in abundance between conditions.

#### 3.4.2.1.2 Increased abundance of flavonoids, ornithine, and tyramine in Ef-Bt-containing cultures compared to *B. wadsworthia* mono-culture

Of the top fifty differentially abundant metabolites, sixteen were in higher abundance in the *E. faecium* and *B. thetaiotaomicron*-containing culture conditions compared to *B. wadsworthia* mono-culture and negative control (Figure 15A), including 4-aminohippuric acid, pipecolic acid ethyl ester and lactic acid. 4-aminohippuric acid, a metabolite involved in phenylalanine metabolism was found to be positively associated with dietary fibre consumption and negatively associated with metabolic disease risk in the human gut metabolome [307]. Additionally, 4-aminohippuric acid abundance increased in the gut metabolome of cows fed with inulin compared to controls, although no increases of *Bacteroides* or *Enterococcus* were identified [308]; the ability of these strains to produce 4-aminohippuric acid has not been previously reported. Pipecolic acid is a non-proteinogenic amino acid which has been shown to be produced by *E. faecalis in vitro* [309], and has been positively associated with *B. thetaiotaomicron* based on metabolomic study of *in vitro* human faecal fermentation models [310]; the mechanism by which *E. faecium* and/or *B. thetaiotaomicron* produce this compound is unclear. Increased abundance of lactic acid in the Ef-Bt cultures is unsurprising given that lactic acid production by *E. faecium* is well-established [235]. Increased abundance of flavonoid compounds chrysin and genistein were observed in the Ef-Bt cultures (Figure 15A); chrysin is a natural flavone found in plants and has been shown to ameliorate intestinal inflammation in mice [311] and genistein is an isoflavone found in soya beans which can be degraded by *Bacteroides* spp. [312]. Flavonoids are products of secondary plant metabolism and are not microbially synthesised under natural conditions [313]; it may be the case that such flavonoid compounds are present in the bacterial growth media, although no clear plant sources are present within the BPM media used. Metabolites involved in arginine biosynthesis were differentially abundant between the culture conditions; ornithine and N-acetylornithine were increased in abundance in Ef-Bt cultures compared to *B. wadsworthia* mono-culture and negative control, where N-acetylornithine was slightly lower in abundance in the Bw + Ef-Bt culture compared to Ef-Bt culture (Figure 15A).

Tyramine was also increased in abundance in Ef-Bt cultures (Figure 15A, 15E); this could be *E. faecium*-derived, given that tyramine production by *E. faecium* and *E. faecalis* has been previously reported [314]. Raffinose, a trisaccharide found in

plants was increased in abundance in the Ef-Bt cultures (Figure 15A, 15E). Raffinose is indigestible by humans and reaches the colon where it is fermented by α-galactosidase-possessing bacteria [315]. Interestingly, both *E. faecium* and *B. thetaiotaomicron* possess α-galactosidases for raffinose utilisation, but *B. thetaiotaomicron* also has a raffinose synthase (IPR008811) for raffinose production; the increased abundance of raffinose in Ef-Bt cultures may therefore reflect biosynthesis by *B. thetaiotaomicron*. Oxypurinol was increased in abundance in Ef-Bt cultures (Figure 15A); this compound was positively correlated with Firmicutes in the caecum of pigs [316] and is increased in the gut of obese adolescents compared to those of normal weight [317].

#### 3.4.2.1.3 Compounds including styrene, 2,4,5-trimethoxybenzoic acid and methylsuccinic acid contributed highly to differences between the metabolome of the cultures

The Variable Importance of Projection (VIP) values were determined using the PLS-DA model to further identify key differential metabolites between culture conditions (Figure 15C). The highest scoring compound was styrene, which was in relatively low abundance in the negative control and *B. wadsworthia* mono-culture, and higher in conditions containing the Ef-Bt cultures (Figure 15A, 15C, 15E). Anaerobic and aerobic pathways of microbial polystyrene degradation have been identified [318], and the impact of microplastics upon the gut microbiota is an active area of research [319]; it may be possible that *E. faecium* or *B. thetaiotaomicron* are able to produce styrene, but the pathways are not identified or previously reported in literature. A further highly scoring compound was 2,4,5-trimethoxybenzoic acid, which was found in highest abundance in *B. wadsworthia* mono-culture, less abundant in negative control, and the lowest relative abundance in Ef-Bt cultures (Figure 15A), which could suggest utilisation of this compound by *E. faecium* and/or *B. thetaiotaomicron*. A positive association between serum levels of a closely related compound 3,4,5-trimethoxybenzoic acid and colorectal cancer was recently reported [320]. Methylsuccinic acid contributed highly to differences between conditions (Figure 15C), being present in high abundance in the *B. wadsworthia* mono-culture and the negative control compared to Ef-Bt containing cultures (Figure 15A), implying that *E. faecium* and/or *B. thetaiotaomicron* utilised this compound in the culture supernatant. In the literature, methylsuccinic acid was shown to increase in the gut metabolome of rats fed with a probiotic *Bacillus* strain, where supplementation ameliorated gut

dysbiosis [321]. Coumaric acid and 4-Hydroxybenzaldehyde were also highly-scoring VIP compounds, where abundance was relatively high in *B. wadsworthia* mono-culture and negative control and lower abundance in Ef-Bt cultures (Figure 15A, 15C, 15E), suggesting that they may have been utilised by *E. faecium* and/or *B. thetaiotaomicron* in these culture conditions.

#### 3.4.2.1.4 Impact of *B. wadsworthia* presence on the metabolome of Ef-Bt cultures

To investigate the specific effect of *B. wadsworthia*'s presence on the metabolome, we performed pair-wise comparisons between the Bw + Ef-Bt and Ef-Bt cultures. 36 metabolites were identified as significantly differentially abundant between the Bw + Ef-Bt and Ef-Bt cultures (p-value ≤ 0.05, t test) (Figure 15D). 17 metabolites were significantly increased in abundance in the culture supernatant when *B. wadsworthia* was present (Figure 15D, Figure 15F), including dipeptides (N-leucyl-leucine, glutamylglutamic acid, prolylglycine) and 2-cyanopyridine, a compound with a similar structure to a vitamin B3 precursor [322]. An increased abundance of 2-methylquinolin-8-ol was observed when *B. wadsworthia* was present (Figure 15D); this quinolinol compound has been described to have antifungal and antibacterial activity against *Staphylococcus aureus* [323]. Hypoxanthine abundance was increased when *B. wadsworthia* was present (Figure 15D); *B. wadsworthia* contains genes encoding inosine-uridine preferring nucleoside hydrolase, which converts inosine or uridine to hypoxanthine during purine salvage [324] and adenine deaminase which converts adenine to hypoxanthine [325]. Increased abundance of hypoxanthine and decreased abundance of adenine in Bw + Ef-Bt culture compared to Ef-Bt culture (Figure 15A, 15D, 15F) could reflect activity of the adenine deaminase of *B. wadsworthia* under these culture conditions. Two compounds, aerugine and ethyl butanoate, were in higher abundance only in Bw + Ef-Bt culture and were in relatively low abundance in all other conditions (Figure 15A, 15D, 15E, 15F). It was recently hypothesised that microbiota-derived aerugine may play a role in the pathogenesis of idiopathic Parkinson's disease based on toxicity of this compound towards human dopaminergic neurons, and the presence of biosynthetic pathways for aerugine in the human gut microbiota in *Pseudomonas* spp., *E. coli*, *Yersinia* spp., and *Salmonella* spp. [326]. Ethyl butanoate, also known as ethyl butyrate is an ester often used in food flavourings [327] and is produced by a range of lactic acid bacteria including *Lactococcus* and *Lactiplantibacillus* spp. [328]; production by *E. faecium* during cheese fermentation was low [329].

Nineteen metabolites were significantly decreased in abundance in the culture supernatant of Bw + Ef-Bt culture compared to Ef-Bt culture, including N-acetylornithine, raffinose, methylsuccinic acid, adenine, adenosine, chrysin, guanine and oxypurinol (Figure 15D, Figure 15F). Taurine was decreased in abundance when *B. wadsworthia* was present in the co-cultures (Figure 15D); this likely reflects utilisation by *B. wadsworthia* to produce $H_2S$ before growth inhibition at 4 h (Figure 14A). Abundance of creatine was decreased when *B. wadsworthia* was present (Figure 15D); creatine abundance is increased in the gut metabolome of those consuming a high-meat diet, and the breakdown product creatinine is positively associated with *Bilophila* and *Enterococcus* genera in the human gut [330]. This implies that *B. wadsworthia* could degrade creatine to creatinine; indeed, creatinine abundance was higher in *B. wadsworthia* mono-culture compared to Ef-Bt cultures (Figure S1). Microbial creatininase and creatinine deaminase activity has been reported in the human colon [331, 332], although the genomes of the strains used here do not contain genes for known microbial pathways of creatinine utilisation.

Abundance of methionine was also significantly decreased in the presence of *B. wadsworthia* in the co-cultures (Figure 15D). Both *B. wadsworthia* and *B. thetaiotaomicron* are capable of methionine production via 5-methyltetrahydrofolate-homocysteine methyltransferase, which converts homocysteine to methionine. Here, lower abundance of methionine in the Bw + Ef-Bt culture may reflect increased methionine utilisation; all three strains encode a S-Adenosylmethionine synthetase (EC 2.5.1.6) which converts methionine and ATP to S-adenosylmethionine (SAM), a key compound involved in a myriad of cellular processes including DNA/RNA modification and repair [333]. Decreased methionine abundance may reflect increased utilisation by *E. faecium* and/or *B. thetaiotaomicron* in the presence of *B. wadsworthia*.

Malic acid abundance was decreased in the presence of *B. wadsworthia* (Figure 15D); this compound is in the TCA cycle [334] so lower abundance likely reflects the increased number of bacterial cells present compared to the other culture conditions. Abundance of cyclopenin, a fungal-derived benzodiazepine molecule [335] and imazaquin, an imidazolinone molecule were also decreased when *B. wadsworthia* was present in the co-cultures (Figure 15D). A tryptophan metabolite

1-acetyl carboline [336] was decreased in the presence of *B. wadsworthia* (Figure 15D); biosynthesis of this antibiotic molecule was reported in a Gram-positive marine bacterium, but not in the gut microbiota [337]. Theobromine, an alkaloid found in cocoa was significantly decreased in abundance in Bw + Ef-Bt cultures compared to Ef-Bt cultures (Figure 15D); this compound was shown to have antimicrobial activity against *E. faecalis* [338], and oral administration of theobromine-containing cocoa in rats was found to decrease abundance of *Bacteroides* spp. [339]. Given that theobromine was in higher abundance in the negative control (sterile growth media) and the *B. wadsworthia* mono-culture compared to the Ef-Bt condition cultures (Figure 15A), this may suggest possible utilisation of this compound. The abundance of 1-methyladenine was significantly decreased when *B. wadsworthia* was present in the Ef-Bt cultures (Figure 15D); this was in relatively high abundance in the negative control, and the lowest relative abundance in the Bw + Ef-Bt culture (Figure S1). 1-methyladenine can be used by *E. coli* as a substrate for iron(II)- and 2-oxoglutarate (2OG)-dependent dioxygenase AlkB during DNA repair [340]; it may be the case that *B. thetaiotaomicron*, *E. faecium* and/or *B. wadsworthia* also utilise this compound. In the gut metabolome of COVID-19 patients, 1-methyladenine was found to be enriched compared to healthy controls [341, 342]; this raises the possibility that COVID-19 infection affects bacterial groups that degrade 1-methyladenine, such as *B. wadsworthia*.

### 3.4.3 The anti-*B. wadsworthia* potential of *E. faecium*

#### 3.4.3.1 Bacteriocin production potential

Given that three *E. faecium* strains (FI 09198, FI 09347, QI0436) showed an ability to inhibit *B. wadsworthia*'s growth, we explored their probiotic potential using *in silico* genome screening. Firstly, we investigated the bacteriocin production potential of the strains by screening the genomes for biosynthetic gene clusters (BGCs) using BAGEL4 and antiSMASH. In the dairy-derived FI 09198 strain, we identified two and five regions of interest (ROIs) using BAGEL4 and antiSMASH respectively. However, none of the putative biosynthetic gene clusters contained accessory genes for bacteriocin transport, modification, immunity, or regulation, suggesting that these were unlikely to be active biosynthetic gene clusters (Table 3). The screening of gut-derived FI 09347 *E. faecium* strain revealed three and four regions of interest using BAGEL4 and antiSMASH respectively; the only ROI in common was on contig 46 (Table 3). Two other regions on contigs 32 and 56 identified using BAGEL4 were orphan enzymes, where no accessory genes were found within the cluster (Table 3). The cluster on contig 46 contained a biosynthetic gene encoding a class IIa bacteriocin with 98.46% similarity to enterocin A (EntA) as determined using NCBI BLAST, in addition to an EntI immunity protein gene, two genes encoding regulatory proteins EntE and Exu, and four genes encoding transporters including FeoB fusion protein, FeoB, GbuB and OpuAA (Table 3), which could suggest the presence of an active biosynthetic gene cluster. The gut-derived QI0436 *E. faecium* strain was predicted to contain five ROIs by both BAGEL4 and antiSMASH. A region on contig 2 contained a gene encoding a protein with 97.75% similarity to bacteriocin 32, however manual curation showed an absence of accessory genes in the cluster (Table 3). The four other putative BGCs included accessory genes; one BGC on contig 1 encoded a class IIb bacteriocin from the lactobin A/cerein 7B family, whereas three BGCs encoding class II bacteriocins of the leucocin A/sakacin P family were present in contig 1 and at two locations in contig 4 (Table 3). Although BAGEL4 identified this putative gene as encoding enterocin B, a class IId bacteriocin [343], NCBI BLAST protein comparison found 100% similarity to a class IIb bacteriocin in the lactobin A/cerein 7B family (Table 3).

Table 3: Potential biosynthetic gene clusters (BGCs) for bacteriocins identified in *E. faecium* strains FI 09198, FI 09347 and QI0436 using BAGEL4 software. Identified BGCs were manually curated by checking for the presence of accessory genes to evaluate the likelihood of the presence of an active BGC.

| Strain | Contig | Start | End | Bacteriocin class (BAGEL4) | NCBI BLAST protein-protein comparison (blastp) of bacteriocin gene product | Presence of accessory genes (PTM, transport, immunity, regulation) in BGC | Likely to be a fully active BGC? |
|---|---|---|---|---|---|---|---|
| FI 09198 | 2 | 8900 | 29443 | 63.3;Enterolysin_A | Glucosaminidase domain-containing protein [*Enterococcus* faecium] 99.75% | No | No |
| | 2 | 27338 | 47482 | 95.2;Enterocin_SE-K4. Enterocin P | Leucocin A/sakacin P family class II bacteriocin [*Enterococcus*] 98.51% | No | No |
| FI 09347 | 32 | 18962 | 31386 | 63.3;Enterolysin_A | Glucosaminidase domain-containing protein [*Enterococcus* faecium] 100% | No | No |
| | 56 | 2618 | 13083 | 95.2;Enterocin_SE-K4 | Leucocin A/sakacin P family class II bacteriocin [*Enterococcus*] 98.51% | No | No |
| | 46 | 6380 | 17231 | 81.2;Enterocin_A | Class IIa bacteriocin EntA [*Enterococcus* faecium] 98.46% | **1 immunity gene** (immunity protein EntI**)** **2 regulatory genes** (EntF family bacteriocin induction factor, Exu regulon transcriptional regulator) **4 transport genes** | Putative |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | (Fe(2+) transport protein A/Fe(2+) transporter FeoB fusion protein, Fe(2+) transporter FeoB, Glycine betaine/carnitine transport permease protein GbuB, Glycine betaine transport ATP-binding protein OpuAA) | |
| QI0436 | 1 | 987759 | 1010582 | 82.2;Enterocin_B | Class IIb bacteriocin, lactobin A/cerein 7B family [*Enterococcus* faecium] 100% | **1 transport gene** (LanT, lactococcin-G processing and transport ATP-binding protein lagD) **1 bacteriocin signal sequence gene** (IPR010133, Bacteriocin-type signal sequence) | Putative |
| | 1 | 1953893 | 1974034 | 22.2;Bacteriocin_T8 | Leucocin A/sakacin P family class II bacteriocin [*Enterococcus*] 100% | **1 immunity gene** (IPR015046 Lactococcin-A immunity protein-like) **1 transport gene** (IPR030679, ABC-type amino acid transport system, ATPase component, HisP-type) **1 modification gene** (rSAM family heme chaperone HemW) | Putative |
| | 2 | 9011 | 24798 | 14.2;Bac32 | Bacteriocin 32 [*Enterococcus* faecium] 97.75% | No | No |
| | 4 | 255752 | 270352 | 95.2;Enterocin_SE-K4 | Leucocin A/sakacin P family class II bacteriocin [*Enterococcus*] 98.51% | **1 immunity gene** (IPR015046 Lactococcin-A immunity protein-like) **1 transport gene** (LanT, lactococcin-G processing and transport ATP-binding protein lagD) | Putative |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 4 | 58160 | 78319 | 91.2;Enterocin_P | Leucocin A/sakacin P family class II bacteriocin [*Enterococcus* faecium] 100% | **2 immunity genes** (daunorubicin resistance protein DrrC, bacteriocin immunity protein) **2 transport genes** (uvrABC system protein A, ABC transporter) | Putative |

### 3.4.3.2 Presence of virulence and antibiotic resistance genes

To further establish the probiotic potential of the three *E. faecium* strains, the presence of genes associated with virulence and antibiotic resistance was investigated via BV-BRC genome annotation against the VFDB and Victors databases [293]. Virulence genes identified in all three *E. faecium* strains included *purB, clpP, bopD, map, perR, thyA* and *lepA* (Table 4). These were recently reported as being present in a probiotic *Enterococcus durans* strain; the authors noted that although these genes are associated with virulence, they enhance bacterial fitness and survival as opposed to inflicting harm upon the host [344]. The gut-derived strains QI0436 and FI 09347 contained virulence genes not found in the dairy-derived FI 09198 strain, including *fss3* and *ebpC* genes encoding cell wall surface anchor family proteins and *SP_1193* encoding the LacA subunit of galactose-6-phosphate isomerase (Table 4). The two gut-derived *E. faecium* strains also harboured virulence genes not found in the other strains. QI0436-specific genes included *scm* encoding surface protein anchor regions [345], whereas FI 09347-specific genes included *acm* encoding a collagen adhesin precursor [345], *sgrA* encoding a cell wall anchored protein [345], three *esp* genes encoding enterococcal surface protein and *sitA* encoding a manganese ABC transporter (Table 4).

Antibiotic resistance genes were identified in the *E. faecium* strains via BV-BRC genome annotation using the Comprehensive Antibiotic Resistance Database (CARD) [293, 296]. Twelve genes associated with antibiotic resistance were identified in all three *E. faecium* strains (Table 5). These included *aac(6')-Ii*, which encodes an enzyme conferring aminoglycoside resistance [346], a gene encoding translation elongation factor Tu which confers resistance to GE2270A, a thiazolyl peptide family antibiotic [347], and *clsA* which is associated with daptomycin resistance in *E. faecium* [348] (Table 5). Furthermore, *liaS* and *liaR* genes were identified in all three strains which encode LiaRS, a two-component regulatory system that induces expression of the *liaIHGFSR* operon in response to cell stress induced by vancomycin or bacitracin [349], although *liaIHGFSR* genes were not identified in the genomes (Table 5). All three strains contained *dfrE* and *dfrF* genes which encode dihydrofolate reductases with modifications to confer resistance to diaminopyrimidine antibiotics such as trimethoprim [350]. All three strains contained *lsa(A)* which encodes an ABC-F family ribosomal protection protein which confers resistance to antibiotics with ribosomal targets including clindamycin

and lincomycin [351]. Genes related to multidrug efflux pumps were also found in all three *E. faecium* strains including *msrC* [352], *adeC* [353] *efrB* [354] and *efmA* [355] (Table 5). The gut-derived FI 09347 *E. faecium* strain also contained the VanA gene cluster for vancomycin resistance, including *vanRA, vanSA, vanHA, vanA, vanXA, vanYA* and *vanZA* [356] which was not present in either FI 09198 or QI0436 (Table 5). FI 09347 also harboured *ermB*, a gene conferring erythromycin resistance [357] which was not present in the other *E. faecium* strains (Table 5).

Table 4: Virulence genes in the genomes of *E. faecium* strains FI 09347, FI 09198 and QI0436 annotated by both VFDB and Victors database.

| Source | Source ID | Source Organism | Gene | Product | FI 09347 | FI 09198 | QI0436 |
|---|---|---|---|---|---|---|---|
| VFDB | VFG042978 | *Enterococcus faecalis* V583 | *ebpC* | Cell wall surface anchor family protein | No | No | Yes |
| VFDB | VFG043519 | *Enterococcus faecium* DO | *scm* | Surface protein from Gram-positive cocci, anchor region | No | No | Yes |
| VFDB | VFG043518 | *Enterococcus faecium* str. TX2555 | *acm* | Collagen adhesin precursor Acm | Yes | No | No |
| VFDB | VFG043510 | *Enterococcus faecalis* V583 | *fss3* | Cell wall surface anchor family protein | Yes | No | No |
| Victors | 15901058 | *Streptococcus pneumoniae* TIGR4 | *SP_1193* | Galactose-6-phosphate isomerase, LacA subunit (EC 5.3.1.26) | Yes | No | Yes |
| VFDB | VFG043511 | *Enterococcus faecium* DO | *sgrA* | Cell wall anchored protein SgrA | Yes | No | No |
| Victors | 15903537 | Streptococcus pneumoniae R6 | *psaA* | Manganese ABC transporter, periplasmic-binding protein SitA | Yes | No | No |
| Victors | 111380585 | *Enterococcus faecalis* | *esp* | Enterococcal surface protein | Yes | No | No |
| VFDB | VFG043518 | *Enterococcus faecium* str. TX2555 | *acm* | Collagen binding protein Cna | No | Yes | Yes |
| Victors | 116516623 | *Streptococcus pneumoniae* D39 | *purB* | Adenylosuccinate lyase (EC 4.3.2.2) @ SAICAR lyase (EC 4.3.2.2) | Yes | Yes | Yes |
| Victors | 16804506 | *Listeria monocytogenes* EGD-e | *clpP* | ATP-dependent Clp protease proteolytic subunit ClpP (EC 3.4.21.92) | Yes | Yes | Yes |
| VFDB | VFG002197 | *Enterococcus faecalis* V583 | *bopD* | Maltose operon transcriptional repressor MalR, LacI family | Yes | Yes | Yes |
| Victors | 29376708 | *Enterococcus faecalis* V583 | *map* | Methionine aminopeptidase (EC 3.4.11.18) | Yes | Yes | Yes |
| Victors | 67043736 | *Enterococcus faecalis* | *perR* | Peroxide stress regulator PerR, FUR family | Yes | Yes | Yes |
| Victors | 29376139 | *Enterococcus faecalis* V583 | *thyA* | Thymidylate synthase (EC 2.1.1.45) | Yes | Yes | Yes |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Victors | 76788416 | *Streptococcus agalactiae* A909 | *lepA* | Translation elongation factor LepA | Yes | Yes | Yes |

Table 5: Antibiotic resistance genes in the genomes of *E. faecium* strains FI 09347, FI 09198 and QI0436 annotated via CARD database.

| Source ID | Source Organism | Gene | Product | FI 09347 | FI 09198 | QI0436 |
|---|---|---|---|---|---|---|
| AAB63533.1 | *Enterococcus faecium* | AAC(6')-Ii | Aminoglycoside N(6')-acetyltransferase (EC 2.3.1.82) => AAC(6')-Ia (and related AACs) | Yes | Yes | Yes |
| YP_006374661.1 | *Enterococcus faecium DO* | | Translation elongation factor Tu | Yes | Yes | Yes |
| ALL09868 | *Enterococcus faecium* | clsA | Cardiolipin synthase, bacterial type ClsA | Yes | Yes | Yes |
| AFK58561.1 | *Enterococcus faecium DO* | liaS | Cell envelope stress response system LiaFSR, sensor histidine kinase LiaS(VraS) | Yes | Yes | Yes |
| WP_002296175.1 | *Enterococcus* | lsa(A) | ABC-F type ribosomal protection protein => Lsa(A) | Yes | Yes | Yes |
| AAK01167.1 | *Enterococcus faecium* | msrC | ABC-F type ribosomal protection protein => Msr(C) | Yes | Yes | Yes |
| AAD01868.1 | *Enterococcus faecalis* | dfrF | Dihydrofolate reductase (EC 1.5.1.3) | Yes | Yes | Yes |
| AAD01867.1 | *Enterococcus faecalis* | dfrE | Thymidylate synthase (EC 2.1.1.45) | Yes | Yes | Yes |
| AFK60084.1 | *Enterococcus faecium DO* | adeC | Adenine deaminase (EC 3.5.4.2) | Yes | Yes | Yes |
| CDO61516.1 | *Enterococcus faecium* | efrB | Heterodimeric efflux ABC transporter, permease/ATP-binding subunit 2 | Yes | Yes | Yes |
| BAG75524.1 | *Enterococcus faecium* | efmA | Uncharacterized MFS-type transporter | Yes | Yes | Yes |
| AFK58562.1 | *Enterococcus faecium DO* | liaR | Cell envelope stress response system LiaFSR, response regulator LiaR(VraR) | Yes | Yes | Yes |

| | | | | | | |
|---|---|---|---|---|---|---|
| AAA65958.1 | *Enterococcus faecium* | vanYA | D-Ala-D-Ala dipeptidase/carboxypeptidase (EC 3.4.16.4)(EC 3.4.13.22) => VanXY-unclassified | Yes | No | No |
| AAA65956.1 | *Enterococcus faecium* | vanA | D-alanine--(R)-lactate ligase (EC 6.1.2.1) => VanA | Yes | No | No |
| AAA65957.1 | *Enterococcus faecium* | vanXA | D-alanyl-D-alanine dipeptidase (EC 3.4.13.22) of vancomycin resistance => VanX | Yes | No | No |
| AAA65955.1 | *Enterococcus faecium* | vanHA | D-lactate dehydrogenase VanH, associated with vancomycin resistance (EC 1.1.1.28) | Yes | No | No |
| AAA65959.1 | *Enterococcus faecium* | vanZA | Teicoplanin resistance protein VanZ | Yes | No | No |
| AAA65954.1 | *Enterococcus faecium* | vanSA | Vancomycin (or other glycopeptides) histidine kinase VanS => VanA/I/Pt-type | Yes | No | No |
| AAA65953.1 | *Enterococcus faecium* | vanRA | Vancomycin (or other glycopeptides) response regulator VanR => unclassified | Yes | No | No |
| CAA58028.1 | *Enterococcus sp.* | ermB | 23S rRNA (adenine(2058)-N(6))-dimethyltransferase (EC 2.1.1.184) => Erm(B) | Yes | No | No |

#### 3.4.3.3 Presence of desirable genes in potential probiotic *E. faecium* strains FI 09198 and QI0436

We further screened the genomes of *E. faecium* strains FI 09198 and QI0436 for beneficial traits such as acid tolerance for survival in the gastrointestinal tract, and genes related to synthesis of vitamins and amino acids [358]. Potential probiotic strains must be able to survive the gastrointestinal transit, as an ability to tolerate and survive the low pH and bile salts of the stomach and small intestine is required to successfully reach the colon [358]. Both FI 09198 and QI0436 contained genes for acid tolerance; several genes encoding ATP synthase subunits were found (Table 6, Table 7), which allow bacteria to regulate intracellular pH by pumping protons out of the cell [359, 360]. Lactate dehydrogenase catalyses conversion of lactate to pyruvate which enhances the activity of ATP synthase [358]; the gene encoding this enzyme was also found in both strains (Table 6, Table 7). Both strains also encoded *arcD* genes for arginine/ornithine antiporter and Clp protease subunits (Table 6, Table 7) which facilitate bacterial survival at low pH [358, 361]. Furthermore, genes encoding the phosphotransferase system (PTS) were found in the FI 09198 strain which were shown to be required for acid shock survival in *Streptococcus mutans* [362]. Both strains also contained several genes encoding universal stress proteins (Table 6, Table 7) which are important for oxidative and acid stress resistance [363]. During gastrointestinal transit bacteria are subjected to bile salts, which are amphipathic, antimicrobial molecules able to disrupt biological membranes [364]. Potential probiotic bacteria must be able to tolerate exposure to bile salts in the small intestine [360]; both strains also contained genes related to bile resistance, including those encoding chaperone proteins DnaK, DnaJ, GroEL and GroES (Table 6, Table 7) [358, 365].

Using gene mining to investigate the metabolic potential of FI 09198 and QI0436, genes for utilisation of glucose, galactose and fructose were identified in both strains [358] in addition to glycerol metabolism genes which have been reported in probiotic strains [366] (Table 6, Table 7). The two strains also contained several peptidases and peptide transporters, which facilitate uptake and utilisation of different amino acids from the environment [358] (Table 6, Table 7). Furthermore, both strains encoded genes related to synthesis of biotin (vitamin B7) and folate (vitamin B9) (Table 6, Table 7). Genes with beneficial potential were also found in each strain separately. FI 09198 contained genes encoding Opu membrane transporters which confer osmotic stress resistance [367] (Table 6), whereas

QI0436 contained genes for lactate and lipid metabolism, as well as genes related to lysine biosynthesis (Table 7), an essential amino acid in humans.

Table 6: Genes associated with stress tolerance and potentially beneficial [358] metabolic functions in *E. faecium* FI 09198. Genes were annotated via BV-BRC.

| Functional category | Gene ID | Gene product |
|---|---|---|
| Acid tolerance | FI09198_452 | V-type ATP synthase subunit I (EC 3.6.3.14) |
| | FI09198_453 | V-type ATP synthase subunit K (EC 3.6.3.14) |
| | FI09198_454 | V-type ATP synthase subunit E (EC 3.6.3.14) |
| | FI09198_455 | V-type ATP synthase subunit C (EC 3.6.3.14) |
| | FI09198_456 | V-type ATP synthase subunit F (EC 3.6.3.14) |
| | FI09198_457 | V-type ATP synthase subunit A (EC 3.6.3.14) |
| | FI09198_458 | V-type ATP synthase subunit B (EC 3.6.3.14) |
| | FI09198_459 | V-type ATP synthase subunit D (EC 3.6.3.14) |
| | FI09198_491 | ATP synthase F0 sector subunit a (EC 3.6.3.14) |
| | FI09198_492 | ATP synthase F0 sector subunit c (EC 3.6.3.14) |
| | FI09198_493 | ATP synthase F0 sector subunit b (EC 3.6.3.14) |
| | FI09198_494 | ATP synthase delta chain (EC 3.6.3.14) |
| | FI09198_495 | ATP synthase alpha chain (EC 3.6.3.14) |
| | FI09198_496 | ATP synthase gamma chain (EC 3.6.3.14) |
| | FI09198_497 | ATP synthase beta chain (EC 3.6.3.14) |
| | FI09198_498 | ATP synthase epsilon chain (EC 3.6.3.14) |
| | FI09198_1392 | Arginine/ornithine antiporter ArcD |
| | FI09198_265 | Arginine/ornithine antiporter ArcD |
| | FI09198_362 | Arginine/ornithine antiporter ArcD |
| | FI09198_1078 | L-lactate dehydrogenase (EC 1.1.1.27) |
| | FI09198_2194 | L-lactate dehydrogenase (EC 1.1.1.27) |
| | FI09198_1479 | PTS system, ascorbate-specific IIB component (EC 2.7.1.194) |
| | FI09198_1480 | PTS system, ascorbate-specific IIC component |
| | FI09198_1481 | PTS system, ascorbate-specific IIA component |
| | FI09198_836 | ATP-dependent Clp protease ATP-binding subunit ClpX |
| | FI09198_1800 | ATP-dependent Clp protease proteolytic subunit ClpP (EC 3.4.21.92) |

| | | |
|---|---|---|
| | FI09198_2339 | ATP-dependent Clp protease, ATP-binding subunit ClpC |
| General stress resistance | FI09198_1206 | Universal stress protein family |
| | FI09198_1429 | Universal stress protein family |
| | FI09198_2092 | Universal stress protein family |
| | FI09198_2634 | Universal stress protein family |
| Bile tolerance | FI09198_806 | Chaperone protein DnaK |
| | FI09198_807 | Chaperone protein DnaJ |
| | FI09198_1993 | Heat shock protein 60 kDa family chaperone GroEL |
| | FI09198_1994 | Heat shock protein 10 kDa family chaperone GroES |
| Ribose metabolism | FI09198_2424 | Ribose-5-phosphate isomerase A (EC 5.3.1.6) |
| Glucose/galactose metabolism | FI09198_779 | Glucose 1-dehydrogenase (EC 1.1.1.47) |
| | FI09198_742 | UDP-glucose 4-epimerase (EC 5.1.3.2) |
| | FI09198_1572 | UDP-glucose 4-epimerase (EC 5.1.3.2) |
| | FI09198_1619 | Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49) |
| | FI09198_1592 | Glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24) |
| | FI09198_1578 | dTDP-glucose 4,6-dehydratase (EC 4.2.1.46) |
| | FI09198_1590 | dTDP-glucose 4,6-dehydratase (EC 4.2.1.46) |
| | FI09198_203 | Galactokinase (EC 2.7.1.6) |
| | FI09198_862 | alpha-galactosidase (EC 3.2.1.22) |
| | FI09198_743 | Galactose-1-phosphate uridylyltransferase (EC 2.7.7.10) |
| Fructose metabolism | FI09198_449 | Fructose-1,6-bisphosphatase, Bacillus type (EC 3.1.3.11) |
| | FI09198_1792 | Fructose-bisphosphate aldolase class II (EC 4.1.2.13) |
| Glycerol metabolism | FI09198_90 | Glycerol uptake facilitator protein |
| | FI09198_2497 | Glycerol uptake facilitator protein |
| | FI09198_535 | Glycerol-3-phosphate dehydrogenase [NAD(P)+] (EC 1.1.1.94) |
| | FI09198_1870 | Glycerol-3-phosphate dehydrogenase [NAD(P)+] (EC 1.1.1.94) |
| Opu transporters | FI09198_2571 | Choline ABC transport system, permease protein OpuBB |

| | | |
|---|---|---|
| | FI09198_1333 | Glycine betaine ABC transport system, permease protein OpuAB / Glycine betaine ABC transport system, glycine betaine-binding protein OpuAC |
| | FI09198_2569 | Choline ABC transport system, permease protein OpuBD |
| | FI09198_2570 | Choline ABC transport system, choline-binding protein OpuBC |
| Peptide metabolism | FI09198_1649 | Aminopeptidase YpdF (MP-, MA-, MS-, AP-, NP- specific) |
| | FI09198_1863 | Isoaspartyl aminopeptidase (EC 3.4.19.5) @ Asp-X dipeptidase |
| | FI09198_1598 | Methionine aminopeptidase (EC 3.4.11.18) |
| Biotin synthesis | FI09198_1527 | Biotin carboxylase of acetyl-CoA carboxylase (EC 6.3.4.14) |
| | FI09198_1657 | Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3) |
| | FI09198_1660 | Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3) |
| | FI09198_1663 | Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3) |
| | FI09198_1666 | Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3) |
| | FI09198_1667 | Long-chain-fatty-acid--CoA ligase (EC 6.2.1.3) |
| | FI09198_2318 | Biotin operon repressor / Biotin--protein ligase (EC 6.3.4.9)(EC 6.3.4.10)(EC 6.3.4.11)(EC 6.3.4.15) |
| | FI09198_2505 | Substrate-specific component BioY of biotin ECF transporter |
| Folate synthesis | FI09198_1119 | Formate--tetrahydrofolate ligase (EC 6.3.4.3) |
| | FI09198_1356 | Methylenetetrahydrofolate--tRNA-(uracil-5-)-methyltransferase TrmFO (EC 2.1.1.74) |
| | FI09198_1362 | Substrate-specific component FolT of folate ECF transporter |
| | FI09198_1370 | Dihydrofolate reductase (EC 1.5.1.3) |
| | FI09198_1371 | Thymidylate synthase (EC 2.1.1.45) |
| | FI09198_1646 | Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) / Methylenetetrahydrofolate dehydrogenase (NADP+) (EC 1.5.1.5) |
| | FI09198_1810 | Dihydrofolate synthase (EC 6.3.2.12) @ Folylpolyglutamate synthase (EC 6.3.2.17) |
| | FI09198_1810 | Dihydrofolate synthase (EC 6.3.2.12) @ Folylpolyglutamate synthase (EC 6.3.2.17) |

| | | |
|---|---|---|
| | FI09198_1887 | Substrate-specific component FolT of folate ECF transporter |
| | FI09198_1930 | Serine hydroxymethyltransferase (EC 2.1.2.1) |
| | FI09198_1935 | Thymidine kinase (EC 2.7.1.21) |
| | FI09198_2179 | S-adenosylmethionine synthetase (EC 2.5.1.6) |
| | FI09198_2184 | Cell division-associated, ATP-dependent zinc metalloprotease FtsH |
| | FI09198_2185 | Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) |
| | FI09198_2186 | tRNA(Ile)-lysidine synthetase (EC 6.3.4.19) |
| | FI09198_2187 | tRNA(Ile)-lysidine synthetase (EC 6.3.4.19) |
| | FI09198_2209 | Methionyl-tRNA formyltransferase (EC 2.1.2.9) |
| | FI09198_2458 | ATPase component of general energizing module of ECF transporters |
| | FI09198_2459 | ATPase component of general energizing module of ECF transporters |
| | FI09198_2460 | Transmembrane component of general energizing module of ECF transporters |
| | FI09198_648 | Dihydrofolate reductase (EC 1.5.1.3) |
| | FI09198_728 | Dihydrofolate reductase (EC 1.5.1.3) |
| | FI09198_734 | 5-formyltetrahydrofolate cyclo-ligase (EC 6.3.3.2) |

Table 7: Genes associated with stress tolerance and potentially beneficial [358] metabolic functions in *E. faecium* QI0436. Genes were annotated via BV-BRC.

| Functional category | Gene ID | Gene product |
|---|---|---|
| Acid tolerance | QI0436_2290 | ATP synthase epsilon chain (EC 3.6.3.14) |
| | QI0436_2291 | ATP synthase beta chain (EC 3.6.3.14) |
| | QI0436_2292 | ATP synthase gamma chain (EC 3.6.3.14) |
| | QI0436_2293 | ATP synthase alpha chain (EC 3.6.3.14) |
| | QI0436_2294 | ATP synthase delta chain (EC 3.6.3.14) |
| | QI0436_2295 | ATP synthase F0 sector subunit b (EC 3.6.3.14) |
| | QI0436_2296 | ATP synthase F0 sector subunit c (EC 3.6.3.14) |
| | QI0436_2297 | ATP synthase F0 sector subunit a (EC 3.6.3.14) |
| | QI0436_2328 | V-type ATP synthase subunit D (EC 3.6.3.14) |
| | QI0436_2329 | V-type ATP synthase subunit B (EC 3.6.3.14) |
| | QI0436_2330 | V-type ATP synthase subunit A (EC 3.6.3.14) |
| | QI0436_2331 | V-type ATP synthase subunit F (EC 3.6.3.14) |
| | QI0436_2332 | V-type ATP synthase subunit C (EC 3.6.3.14) |
| | QI0436_2333 | V-type ATP synthase subunit E (EC 3.6.3.14) |
| | QI0436_2334 | V-type ATP synthase subunit K (EC 3.6.3.14) |
| | QI0436_2335 | V-type ATP synthase subunit I (EC 3.6.3.14) |
| | QI0436_2336 | V-type ATP synthase subunit G (EC 3.6.3.14) |

| | | |
|---|---|---|
| | QI0436_619 | L-lactate dehydrogenase (EC 1.1.1.27) |
| | QI0436_1626 | L-lactate dehydrogenase (EC 1.1.1.27) |
| | QI0436_1411 | D-lactate dehydrogenase (EC 1.1.1.28) |
| | QI0436_1328 | Arginine/ornithine antiporter ArcD |
| | QI0436_2433 | Arginine/ornithine antiporter ArcD |
| | QI0436_2531 | Arginine/ornithine antiporter ArcD |
| General stress resistance | QI0436_1287 | Universal stress protein family |
| | QI0436_1498 | Universal stress protein family |
| | QI0436_205 | Universal stress protein family |
| | QI0436_2744 | Universal stress protein family |
| | QI0436_713 | Universal stress protein family |
| Bile tolerance | QI0436_811 | Heat shock protein 10 kDa family chaperone GroES |
| | QI0436_812 | Heat shock protein 60 kDa family chaperone GroEL |
| Glucose/galactose metabolism | QI0436_2027 | Glucose 1-dehydrogenase (EC 1.1.1.47) |
| | QI0436_1135 | Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49) |
| | QI0436_1161 | Glucose-1-phosphate thymidylyltransferase (EC 2.7.7.24) |
| | QI0436_1163 | dTDP-glucose 4,6-dehydratase (EC 4.2.1.46) |
| | QI0436_1181 | UDP-glucose 4-epimerase (EC 5.1.3.2) |
| | QI0436_1181 | UDP-glucose 4-epimerase (EC 5.1.3.2) |
| | QI0436_1930 | alpha-galactosidase (EC 3.2.1.22) |
| | QI0436_2068 | Galactose operon repressor, GalR-LacI family of transcriptional regulators |

| | | |
|---|---|---|
| | QI0436_2069 | Galactose-1-phosphate uridylyltransferase (EC 2.7.7.10) |
| | QI0436_2070 | UDP-glucose 4-epimerase (EC 5.1.3.2) |
| | QI0436_23 | Aldose 1-epimerase (EC 5.1.3.3) |
| | QI0436_2590 | Aldose 1-epimerase (EC 5.1.3.3) |
| | QI0436_2591 | Galactokinase (EC 2.7.1.6) |
| | QI0436_54 | Aldose 1-epimerase (EC 5.1.3.3) |
| Fructose metabolism | QI0436_2338 | Fructose-1,6-bisphosphatase, Bacillus type (EC 3.1.3.11) |
| | QI0436_1043 | Fructose-bisphosphate aldolase class II (EC 4.1.2.13) |
| | QI0436_1258 | 6-phosphofructokinase (EC 2.7.1.11) |
| Lactate metabolism | QI0436_1411 | D-lactate dehydrogenase (EC 1.1.1.28) |
| | QI0436_1626 | L-lactate dehydrogenase (EC 1.1.1.27) |
| | QI0436_2035 | Xylulose-5-phosphate phosphoketolase (EC 4.1.2.9) @ Fructose-6-phosphate phosphoketolase (EC 4.1.2.22) |
| | QI0436_2409 | Phosphate acetyltransferase (EC 2.3.1.8) |
| | QI0436_256 | Acetaldehyde dehydrogenase (EC 1.2.1.10) / Alcohol dehydrogenase (EC 1.1.1.1) |
| | QI0436_619 | L-lactate dehydrogenase (EC 1.1.1.27) |
| | QI0436_707 | Acetate kinase (EC 2.7.2.1) |
| Glycerol metabolism | QI0436_2255 | Glycerol-3-phosphate dehydrogenase [NAD(P)+] (EC 1.1.1.94) |
| | QI0436_936 | Glycerol-3-phosphate dehydrogenase [NAD(P)+] (EC 1.1.1.94) |
| | QI0436_336 | Glycerol uptake facilitator protein |
| | QI0436_80 | Glycerol uptake facilitator protein |

| | | |
|---|---|---|
| Protein metabolism | QI0436_1033 | ATP-dependent Clp protease proteolytic subunit ClpP (EC 3.4.21.92) |
| | QI0436_474 | ATP-dependent Clp protease, ATP-binding subunit ClpC |
| | QI0436_1982 | ATP-dependent Clp protease ATP-binding subunit ClpX |
| | QI0436_628 | Cell division-associated, ATP-dependent zinc metalloprotease FtsH |
| Peptide transport | QI0436_2833 | Dipeptide ABC transporter, permease protein DppB (TC 3.A.1.5.2) |
| | QI0436_2834 | Dipeptide ABC transporter, permease protein DppC (TC 3.A.1.5.2) |
| | QI0436_2835 | Dipeptide ABC transporter, permease protein DppC (TC 3.A.1.5.2) |
| | QI0436_2837 | Oligopeptide ABC transporter, ATP-binding protein OppF (TC 3.A.1.5.1) |
| | QI0436_359 | Oligopeptide ABC transporter, permease protein OppC (TC 3.A.1.5.1) |
| | QI0436_361 | Oligopeptide ABC transporter, ATP-binding protein OppF (TC 3.A.1.5.1) |
| | QI0436_362 | Oligopeptide ABC transporter, ATP-binding protein OppD (TC 3.A.1.5.1) |
| | QI0436_908 | Oligopeptide ABC transporter, permease protein OppC (TC 3.A.1.5.1) |
| | QI0436_909 | Oligopeptide ABC transporter, ATP-binding protein OppD (TC 3.A.1.5.1) |
| | QI0436_910 | Oligopeptide ABC transporter, ATP-binding protein OppF (TC 3.A.1.5.1) |
| | QI0436_911 | Oligopeptide ABC transporter, ATP-binding protein OppF (TC 3.A.1.5.1) |
| Peptide metabolism | QI0436_1106 | Aminopeptidase YpdF (MP-, MA-, MS-, AP-, NP- specific) |
| | QI0436_1155 | Methionine aminopeptidase (EC 3.4.11.18) |
| | QI0436_943 | Isoaspartyl aminopeptidase (EC 3.4.19.5) @ Asp-X dipeptidase |
| | QI0436_207 | Glutamyl aminopeptidase (EC 3.4.11.7); Deblocking aminopeptidase |
| | QI0436_1035 | Glycerophosphoryl diester phosphodiesterase (EC 3.1.4.46) |

| | | |
|---|---|---|
| Lipid metabolism (esterases) | QI0436_2096 | membrane-anchoring domain / Glycerophosphoryl diester phosphodiesterase (EC 3.1.4.46) |
| | QI0436_1795 | Glycerophosphoryl diester phosphodiesterase (EC 3.1.4.46) |
| Biotin synthesis | QI0436_1233 | Biotin carboxylase of acetyl-CoA carboxylase (EC 6.3.4.14) |
| | QI0436_328 | Substrate-specific component BioY of biotin ECF transporter |
| | QI0436_486 | Biotin operon repressor / Biotin--protein ligase (EC 6.3.4.9)(EC 6.3.4.10)(EC 6.3.4.11)(EC 6.3.4.15) |
| Folate synthesis | QI0436_1023 | Dihydrofolate synthase (EC 6.3.2.12) @ Folylpolyglutamate synthase (EC 6.3.2.17) |
| | QI0436_1109 | Methenyltetrahydrofolate cyclohydrolase (EC 3.5.4.9) / Methylenetetrahydrofolate dehydrogenase (NADP+) (EC 1.5.1.5) |
| | QI0436_1351 | Thymidylate synthase (EC 2.1.1.45) |
| | QI0436_1352 | Dihydrofolate reductase (EC 1.5.1.3) |
| | QI0436_1360 | Substrate-specific component FolT of folate ECF transporter |
| | QI0436_1366 | Methylenetetrahydrofolate--tRNA-(uracil-5-)-methyltransferase TrmFO (EC 2.1.1.74) |
| | QI0436_1593 | Formate-tetrahydrofolate ligase (EC 6.3.4.3) |
| | QI0436_2080 | 5-formyltetrahydrofolate cyclo-ligase (EC 6.3.3.2) |
| | QI0436_2085 | Dihydrofolate reductase (EC 1.5.1.3) |
| | QI0436_2147 | Dihydrofolate reductase (EC 1.5.1.3) |
| | QI0436_372 | Transmembrane component of general energizing module of ECF transporters |
| | QI0436_373 | ATPase component of general energizing module of ECF transporters |

| | | |
|---|---|---|
| | QI0436_374 | ATPase component of general energizing module of ECF transporters |
| | QI0436_605 | Methionyl-tRNA formyltransferase (EC 2.1.2.9) |
| | QI0436_626 | tRNA(Ile)-lysidine synthetase (EC 6.3.4.19) |
| | QI0436_627 | Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8) |
| | QI0436_628 | Cell division-associated, ATP-dependent zinc metalloprotease FtsH |
| | QI0436_633 | S-adenosylmethionine synthetase (EC 2.5.1.6) |
| | QI0436_875 | Thymidine kinase (EC 2.7.1.21) |
| | QI0436_880 | Serine hydroxymethyltransferase (EC 2.1.2.1) |
| | QI0436_925 | Substrate-specific component FolT of folate ECF transporter |
| Lysine synthesis | QI0436_2517 | FIG138056: a glutathione-dependent thiol reductase |
| | QI0436_2599 | Aromatic amino acid aminotransferase gamma (EC 2.6.1.57) @ N-acetyl-L,L-diaminopimelate aminotransferase (EC 2.6.1.-) |
| | QI0436_2600 | N-acetyl-L,L-diaminopimelate deacetylase (EC 3.5.1.47) |
| | QI0436_2601 | Aspartokinase (EC 2.7.2.4) |
| | QI0436_2602 | Aspartate-semialdehyde dehydrogenase (EC 1.2.1.11) |
| | QI0436_2603 | 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase (EC 2.3.1.89) |
| | QI0436_2604 | 4-hydroxy-tetrahydrodipicolinate reductase (EC 1.17.1.8) |
| | QI0436_2605 | 4-hydroxy-tetrahydrodipicolinate synthase (EC 4.3.3.7) |
| | QI0436_2606 | Diaminopimelate epimerase (EC 5.1.1.7) |
| | QI0436_2607 | Diaminopimelate decarboxylase (EC 4.1.1.20) |

## 3.5 Discussion

Sulfate- and sulfite-reducing bacteria (SRB) such as *B. wadsworthia* can produce significant amounts of $H_2S$ [51], a toxic gas that has been implicated in gastrointestinal disease [98, 100, 113, 121, 262]. Understanding the factors affecting the $H_2S$ production and abundance of *B. wadsworthia* in the human gut is therefore a critical avenue of research. Here, strains of *E. faecium* and *E. faecalis* isolated from the human gut or dairy sources were demonstrated to inhibit *B. wadsworthia*'s growth and $H_2S$ production.

### 3.5.1 Metabolomic analysis of *E. faecium* and *B. thetaiotaomicron* (Ef-Bt) cultures that inhibit *B. wadsworthia*'s growth

We compared the metabolomic profile of cell-free supernatant from *B. wadsworthia* mono-cultures, enrichments of *E. faecium* and *B. thetaiotaomicron* (Ef-Bt) and *B. wadsworthia* with *E. faecium* and *B. thetaiotaomicron* (Bw + Ef-Bt) at 6 h post-inoculation, where *B. wadsworthia*'s growth and $H_2S$ production had been significantly inhibited (Figure 14). Given that *B. thetaiotaomicron* is a known indole producer [306], the decreased abundance of tryptophan and tryptophan metabolites 3-indoleacrylic acid and 1-acetyl carboline [336] in Ef-Bt cultures (Figure 15A) could reflect competition for tryptophan in the co-culture. Additionally, raffinose abundance was increased in Ef-Bt cultures (Figure 15A), which may result from the activity of raffinose synthase in *B. thetaiotaomicron*; raffinose is a probiotic that promotes the growth of health-promoting lactic acid bacteria and increases the abundance of short-chain fatty-acids in the gut [368].

Using pair-wise comparisons of Bw + Ef-Bt and Ef-Bt cultures to assess the specific impact of *B. wadsworthia* presence upon the metabolome, we observed increased abundance of hypoxanthine and decreased abundance of adenine when *B. wadsworthia* was present (Figure 15D, Figure 15F). *B. wadsworthia* encodes adenine deaminase which converts adenine to hypoxanthine, the activity of which could explain the differential abundance of these compounds in the culture conditions. Increased abundance of hypoxanthine may be beneficial in the gut, as this has been shown to play an important role in maintaining intestinal barrier function [369].

### 3.5.2 Investigating the probiotic, anti-*B. wadsworthia* potential of *E. faecium* strains

Enterococci are a controversial group within the lactic acid bacteria, as some strains exhibit probiotic potential whereas others are clinically important pathogens [370]. It is therefore important to screen potential probiotic candidates within this group to establish the absence of virulence and antibiotic resistance genes, and to identify potentially beneficial probiotic properties such as capacity to produce bacteriocins, amino acids and vitamins [358]. The next section will discuss the potential of *E. faecium* strains QI0436, FI 09198 and FI 09347 for use as probiotics.

### 3.5.3 Gut-derived FI 09347 and QI0436 *E. faecium* strains contained putative bacteriocin biosynthetic genes

Whilst exploring the probiotic potential of the three *E. faecium* strains using BAGEL4 and antiSMASH, we investigated their well-documented ability to produce bacteriocins [371], which are antimicrobial compounds gaining prominence as promising alternatives to traditional antibiotics in the context of antimicrobial resistance [372]. Notably, *E. faecium* strain FI 09198 did not contain any identified biosynthetic gene clusters (Table 3). This absence could be linked to the environmental origin of the strain; bacteriocins typically confer adaptive advantages in densely populated environments with high microbial competition [373], and the dairy setting may not exert as strong a selective pressure as the human gut environment. Conversely, in the gut-derived FI 09347 *E. faecium* strain, a putative biosynthetic gene cluster was detected, encoding enterocin A along with two regulatory genes, one immunity gene, and four transporter genes (Table 3). Enterocin A, a class IIa bacteriocin, exhibits a relatively broad spectrum of activity against Gram-positive bacteria, including the foodborne pathogen *Listeria monocytogenes* [372]. Noteworthy for its commercial potential as a food additive to mitigate *Listeria* contamination [374], enterocin A stands out in food preservation, inhibiting pathogen growth without impeding the proliferation of beneficial lactic acid bacteria [372].

The gut-derived QI0436 strain genome contained four potential BGCs after manual curation; one encoded a class IIb bacteriocin in the lactobin A/cerein 7B family, and the other three encoded class IIa bacteriocins of the leucocin A/sakacin P family (Table 3). Class IIb bacteriocins are two-peptide bacteriocins which form membrane pores in target cells [375]. Generally, enterocins exhibit

activity against closely related Gram-positive bacteria including potential pathogens such as *Listeria* and *Bacillus* [376], but have also been demonstrated to have antimicrobial activity against Gram-negative species including *E. coli* [377] and *Salmonella* [378]. The activity of bacteriocins against gut-derived bacterial strains and the human gut microbiota is an active area of research, as studies have often focused on testing the susceptibility of only a few selected laboratory strains [379]. Therefore, establishing whether these bacteriocins could inhibit Gram-negative species including *B. wadsworthia* is yet to be investigated and is worthy of study. Overall, several putative BGCs were identified across the gut-derived *E. faecium* strains, but none in the dairy-derived strain. Establishing whether these clusters produce active bacteriocins, and whether these exert inhibitory impact on *B. wadsworthia* separately from *E. faecium* cells and associated pH reduction due to lactic acid, remains to be determined.

### 3.5.4 *E. faecium* strains FI 09198 and QI0436 contained virulence factors that enhance fitness, but FI 09347 carried potentially pathogenic virulence genes

All three *E. faecium* strains contained virulence genes *purB, clpP, bopD, map, perR, thyA* and *lepA* (Table 4), which are associated with bacterial fitness and survival as opposed to inflicting damage to the host [344]. The QI0436 and FI 09198 strains did not contain further virulence genes associated with pathogenicity, however the gut-derived FI 09347 harboured *acm*, *sgrA* and four *esp* genes (Table 4). These encode surface proteins associated with virulence; a study comparing virulence factor distribution between hospital-associated (clade A) and community-associated (clade B) *E. faecium* isolates found that *acm*, *sgrA* and *esp* genes were more frequently found in clinical ampicillin-resistant strains [345], and the authors suggested *sgrA* as a possible marker for assessing the safety of *E. faecium* strains [345]. This may therefore indicate that FI 09347 is not a suitable probiotic candidate based on the presence of these specific virulence markers in the genome.

### 3.5.5 *E. faecium* strains FI 09198 and QI0436 contained intrinsic antimicrobial resistance genes, but FI 09347 harboured vancomycin resistance

Antimicrobial resistance genes were detected in all three *E. faecium* strains conferring resistance to aminoglycosides via *aac(6')-Ii* [346], daptomycin via *clsA*, diaminopyrimidine via *dfrE* and *dfrF* [350], and macrolides via *efmA* [355]. A

probiotic *E. lactis* strain also contained *aac(6')-Ii* and *efrA*, which are intrinsic resistance genes in enterococcal strains [380]. Furthermore, despite expressing *efmA*, the strain showed sensitivity to ciprofloxacin and levofloxacin *in vitro* [380]. Therefore, the presence of intrinsic resistance genes and the absence of high-level acquired resistance genes permit QI0436 and FI 09198 to remain as potential probiotic candidates; future work would require fully establishing the antibiotic resistance profile *in vitro*.

Notably, the gut-derived FI 09347 *E. faecium* strain contained a complete VanA gene cluster required for vancomycin resistance [381] (Table 5). Vancomycin Resistant Enterococci (VRE) were first identified in 1987 and have since contributed a growing and significant clinical burden, particularly in the US [247]. Interestingly, colonisation with VRE in healthy humans and farm animals was common in Europe, which is largely attributed to the historical use of avoparcin in animal husbandry up until 1996, which conferred cross-resistance to vancomycin [382]. Since the avoparcin ban, colonisation frequency of healthy humans by VRE has decreased. In 2000, 6% of intestinal carriage by VanA-containing enterococci was reported in faecal samples from healthy humans in Spain [383], later studies in 2005 detected it in 3% of samples [384], and in 2012, VRE was not detected in human faecal samples [385]. The vanA gene cluster is mainly found in *E. faecium* [381], and permits the replacement of the terminal D-Ala of peptidoglycan precursors with D-Lac which reduces the affinity of vancomycin 1,000-fold [386]. The cluster encodes 9 polypeptides where VanR and VanS form a two-component regulatory system, VanH and VanA synthesise the D-Ala-D-Lac precursors, VanX and VanY hydrolyse the D-Ala-D-Ala precursors [381]. VanZ has an unknown function [381]. The detection of vancomycin resistance genes here in the FI 09347 strain reiterates the importance of screening potential probiotic candidates at the strain level [380]; the presence of these genes firmly rules out the use of FI 09347 as a probiotic, as this poses a serious risk as a highly-resistant opportunistic pathogen [382], in addition to the possibility of horizontal transfer of these resistance genes to other members of the gut microbiota [387].

### 3.5.6 Genome mining of *E. faecium* strains FI 09347 and QI0436 revealed genes beneficial for survival in the gastrointestinal tract

Both FI 09198 and QI0436 contained genes encoding ATP synthases and phosphotransferases for acid tolerance, chaperone proteins for bile tolerance and

universal stress proteins for general stress resistance [358] (Table 6, Table 7). The two strains also contained genes related to the metabolism of peptides, and synthesis of biotin (vitamin B7) and folate (vitamin B9) which could confer nutritional benefit to the host and wider microbiota as functional probiotics [388, 389] (Table 6, Table 7). Overall, the two *E. faecium* strains with probiotic potential contained a range of genes allowing utilisation of a range of extracellular substrates which could facilitate adaptation to the nutritional environment within the gut [358], genes for tolerance to bile and acidic conditions, and contain the genomic potential to produce beneficial compounds including folate and biotin.

## 3.6 Conclusion

In this study, we explored the inhibitory effects of enterococcal strains, specifically *E. faecium* and *E. faecalis*, on the growth and $H_2S$ production of *B. wadsworthia in vitro*. The findings indicated a robust ability of these enterococcal strains, particularly in the form of cell-free supernatant, to inhibit *B. wadsworthia* growth, primarily through pH reduction via lactic acid production. The Ef-Bt mixed bacterial enrichment, featuring *E. faecium* and *B. thetaiotaomicron* demonstrated potent inhibition of *B. wadsworthia*, accompanied by a notable decrease in pH and minimal $H_2S$ production. Considering the potential therapeutic implications, the ability to control *B. wadsworthia* abundance and $H_2S$ production in the human gut holds promise for mitigating inflammation and disease pathogenesis associated with their enrichment [67, 145, 146, 152-154]. Lactic acid emerged as a key factor in *B. wadsworthia* growth inhibition, while increased levels of raffinose and altered purine metabolites suggested intricate interactions within the microbial community. Furthermore, our exploration of using a commensal gut bacterial enrichment, particularly with *E. faecium* and *B. thetaiotaomicron*, highlighted the potential benefits of regulating *B. wadsworthia* growth while promoting the production of beneficial compounds such as hypoxanthine [369], lactic acid [390] and raffinose [368]. This approach could offer therapeutic advantages if implemented in the human gut environment, positioning *E. faecium* as a promising candidate for anti-*B. wadsworthia* probiotic applications.

# Chapter 4

## 4. Why does *Bacteroides thetaiotaomicron* increase *Bilophila wadsworthia*'s $H_2S$ production? An integrated 'Omics approach

## 4.1 Introduction

*B. wadsworthia* is a taurine-utilising member of the sulfate-reducing bacteria (SRB) found in the gut of 50-60% of healthy individuals [22-24, 68], but has been associated with gut inflammation in animal models [67, 206] and human disease [145, 149, 150]. *B. wadsworthia* colonisation results in production of $H_2S$, a toxic gas that can cause inflammation in high concentrations in the gut environment [66, 79, 99, 100, 144, 391]. *B. thetaiotaomicron* is a highly prevalent and abundant member of the human gut microbiota [214] occupying a key niche of polysaccharide fermentation, which produces short-chain fatty acids which exert important modulatory effects on both the gut microbiota and the human enterocytes to maintain gut barrier function [220]. In previous chapters, we showed that *B. thetaiotaomicron* and *B. wadsworthia* exhibit a mutually beneficial interaction in co-culture, where growth of both strains is supported and *B. wadsworthia*'s $H_2S$ production is significantly increased. Further *in vitro* study of the interaction between *B. wadsworthia* (QI0013) and Bt strain 1 (QI0072) revealed that the sulfidogenic interaction was not observed using heat-killed Bt strain 1, cell-free supernatant from Bt strain 1 either grown in mono-culture or in *B. wadsworthia* supernatant, or when the species were separated by a permeable 0.22 µm membrane. This implied that increased $H_2S$ production by *B. wadsworthia* occurred only in the presence of viable Bt strain 1 cells. To investigate the mechanism underpinning the sulfidogenic interaction between *B. wadsworthia* and *B. thetaiotaomicron*, we used an integrated 'omics approach combining transcriptomics and metabolomics to compare co-culture of *B. wadsworthia* and Bt strain 1 with respective mono-cultures. Given the apparent importance of the physical cell proximity of the two species and the inability of cell-free supernatant to exert a sulfidogenic effect on *B. wadsworthia*, we opted to study the endometabolome by extracting intracellular metabolites from mono-cultures and co-culture instead of using "traditional" metabolomics of the extracellular supernatant. Although study of the endometabolome is relatively new, it has been employed to successfully interrogate microbial co-cultures of bacteria and yeast [392], diatoms and marine bacteria [393] and bacteria-bacteria interactions [394, 395], as well as co-cultures of bacterial and human cells [396].

In this chapter, we explore the interaction between *B. wadsworthia* (QI0013) and *B. thetaiotaomicron* strain 1 (QI0072) in greater depth using transcriptomic analysis to study the differential gene expression of each bacterial strain in co-

culture and mono-culture alongside metabolomic analysis of the endometabolome between the different culture conditions.

## 4.2 Objectives

The purpose of the work in this chapter was to elucidate the molecular mechanism behind the excess $H_2S$ production by *B. wadsworthia* in the presence of Bt strain 1. We aimed to identify potential candidate genes required for the sulfidogenic interaction between Bt strain 1 and *B. wadsworthia* using RNA-seq to identify differentially expressed genes between co-cultures and mono-cultures. Additionally, the intracellular metabolome of Bt strain 1 and *B. wadsworthia* was compared between co-culture and mono-cultures to provide further evidence for potential metabolic pathways important for the sulfidogenic interaction inferred by the transcriptomic data. Overall, this work aimed towards an integrated 'omics approach using both transcriptomics and metabolomics to understand the underlying metabolic changes within *B. wadsworthia* and Bt strain 1 leading to increased $H_2S$ production.

## 4.3 Methods

Bacteria were inoculated and cultured as described previously in section 2.3.3.2. After 8 h, material was obtained for transcriptomic and metabolomic analysis.

### 4.3.1 RNA extraction

5 mL of cultures were taken at 8 h post-inoculation and cells were immediately pelleted at 15,000 x g for 2 min. All supernatant was removed, and cell pellets were snap frozen on dry ice prior to storage at -80°C. RNA was extracted using RNeasy® Mini Kit (Qiagen) according to manufacturer's protocol [397] with on-column DNase digestion. RNA concentration was determined via Qubit RNA High Sensitivity kit (ThermoFisher Scientific, UK). Total RNA underwent rRNA depletion using the RiboMinus™ Bacteria 2.0 Transcriptome Isolation Kit (ThermoFisher Scientific, UK) and library preparation prior to sequencing via an Illumina NovaSeq 6000 instrument with 2x150 bp configuration using a sequencing depth of 20 M paired-end reads per sample (Azenta Genewiz, Germany).

### 4.3.2 Transcriptomic analysis

The *B. wadsworthia* (QI0013) genome (3 contigs, N50 3619022, 100% completeness, 0% contamination) and *B. thetaiotaomicron* (QI0072) genome (8

contigs, N50 6645989, 99.41% completeness, 0.73% contamination) were used as reference genomes. Six technical replicates for *B. wadsworthia* (QI0013) and *B. thetaiotaomicron* (QI0072) mono-cultures and co-culture were taken for transcriptomic analysis. For differential expression analysis, raw data were analysed as previously described [398]. In summary, reads were cleaned to remove sequencing adapters and residual ribosomal RNA sequences using the BBDuk function in the BBMap package [41]. FastQC (v 0.11.9) [276] was used to assess the sequence quality, where reads with a Phred score ≥30 were deemed suitable for further analysis. Cleaned reads were mapped to the QI0013 and QI0072 reference genomes using the BBSplit function of the BBMap package. The number of transcripts per gene was estimated using featureCounts (v.2.0) [399]. Reference genomes were annotated with the BV-BRC comprehensive genome analysis tool [293]. Differentially expressed genes were identified using edgeR with TMM normalisation [400] using the Trinity RNASeq package [401], with a P value cut-off of 0.01, False Discovery Rate (FDR) ≤ 0.05 and a logFC of 1 equating to a 2-fold change in gene expression. In the case of specific genes of interest where there was no functional annotation, InterProScan [292] was used to assign predicted protein function based on domains where possible using the predicted amino acid sequence. Additionally, Pathway Tools v23.0 [286] was used for preliminary pathway enrichment analysis and pathway predictions using the reference genomes.

### 4.3.3 Sample preparation for endometabolome analysis

5 mL of cultures were taken at 8 h post-inoculation and cells were immediately pelleted at 4,000 x g for 10 min. All supernatant was removed, and cell pellets were snap frozen on dry ice and transported to Creative Proteomics, USA. Prior to analysis, bacterial pellets were thawed and 240 µL methanol added for metabolite extraction. Samples were vortexed for 60 s, sonicated for 30 min at 4°C and stored at -20°C for 1 h. Samples were pelleted at 12,000 x g for 15 min at 4°C. Finally, 200 µL of supernatant and 5 µL of DL-o-Chlorophenylalanine (0.2 mg/mL) was transferred to a vial for LC-MS analysis (Creative Proteomics, New York, USA).

### 4.3.4 LC-MS

All LC-MS analysis was performed by Creative Proteomics, New York, USA. QC samples were prepared by pooling all the samples in triplicate. All samples were injected in triplicate. Separation was performed by ACQUITY UPLC (Waters)

combined with Q Exactive MS (Thermo) and screened with ESI-MS. The LC system was comprised of ACQUITY UPLC HSS T3 (100×2.1 mm×1.8 µm) with ACQUITY UPLC (Waters). The mobile phase was composed of solvent A (0.05% formic acid water) and solvent B (acetonitrile) with a gradient elution (0-1 min, 5% B; 1-12.5 min, 5%-95% B; 12.5-13.5 min, 95% B; 13.5-13.6 min, 95%-5% B; 13.6-16 min, 5% B). The flow rate of the mobile phase was 0.3 mL/min. The column temperature was maintained at 40°C, and the sample manager temperature set at 4°C. Mass spectrometry parameters in ESI+ and ESI- mode were as follows: ESI+: Heater Temp 300°C; Sheath Gas Flow rate, 45 arb; Aux Gas Flow Rate, 15 arb; Sweep Gas Flow Rate, 1 arb; spray voltage, 3.0 KV; Capillary Temp, 350°C; S-Lens RF Level, 30%. ESI-: Heater Temp 300°C, Sheath Gas Flow rate, 45 arb; Aux Gas Flow Rate, 15 arb; Sweep Gas Flow Rate, 1 arb; spray voltage, 3.2 KV; Capillary Temp,350°C; S-Lens RF Level, 60%.

Metabolites were identified using Compound Discoverer 3.0 (ThermoFisher Scientific, USA) and Progenesis QI v 2.1 (Waters) was used for manual screening of the identified compounds in order to minimise false positive identification results. Data was normalised using Total Ion Count (TIC) method where the peak area of each metabolite divided by the SUM of all metabolites area and then multiplied by one million. The resulting data was supplied directly from Creative Proteomics, and I performed the next steps of data analysis.

### 4.3.5 Data analysis of endometabolome

To remove compounds with high analytical variability, compounds with $RSD_{QC}$ >20% were discarded [283]. Endometabolomic data was standardised by calculating the concentration (uM) per $10^9$ bacterial cells present as measured via qPCR, in order to account for cell density differences in mono-cultures and co-cultures [393, 394, 396]. Compounds at low concentrations across all samples (<2 µM per $10^9$ cells) were removed. In positive ion mode, 65% of the identified metabolites remained after quality control (320 left from 490), and in negative mode 70% remained (267 left from 377). Given the higher peak intensity and number of reported compounds, positive mode was used for further analysis. The metabolomic data was auto-scaled and analysed using Metaboanalyst 5.0 [284], to obtain the PLS-DA for the global profile changes and Variable Importance in Projection (VIP) compounds that contribute highly to inter-condition differences, and the heatmaps showing feature clustering and inter-condition differences in

relative abundance of compounds. For pairwise comparisons between conditions of interest, volcano plots were visualised where significantly differentially abundant metabolites were determined using unpaired t tests with p value cut-off at 0.1 and a 2-fold change in metabolite abundance.

### 4.3.6 *In silico* analysis of sulfur metabolism genes

For genes of interest, amino acid sequences were obtained from *B. wadsworthia* (QI0013) and Bt strain 1 (QI0072) genomes in addition to those from reference genomes *D. desulfuricans* subsp. *desulfuricans* DSM 642, *D. gigas* DSM 1382 and *D. alaskensis* G20 via BV-BRC [293]. InterProScan [292] was used to assign predicted protein function based on domains. Protein-protein comparison was performed using blastp on NCBI-BLAST using default settings [291].

## 4.4 Results and Discussion

### 4.4.1 Co-culture of *B. wadsworthia* and Bt strain 1

*B. wadsworthia* (QI0013) and *B. thetaiotaomicron* strain 1 (QI0072) were grown in mono-cultures and co-culture inoculated at $10^6$ CFU/mL in a 1:1 ratio. Significantly increased $H_2S$ concentration was observed in the co-culture of *B. wadsworthia* and Bt strain 1 compared to *B. wadsworthia* mono-culture at 8 h post-inoculation (Figure 16A). *B. wadsworthia* abundance was not substantially different between co-culture and mono-culture (Figure 16B), demonstrating that the increased $H_2S$ was the result of significantly increased $H_2S$ production per *B. wadsworthia* cell in co-culture with Bt strain 1 compared to *B. wadsworthia* mono-culture (Figure 16D). During the culture period, the abundance of Bt strain 1 was significantly higher in co-culture with *B. wadsworthia* compared to mono-culture (Figure 16C). At 8 h, the cultures were sampled for transcriptomic and metabolomic analysis.

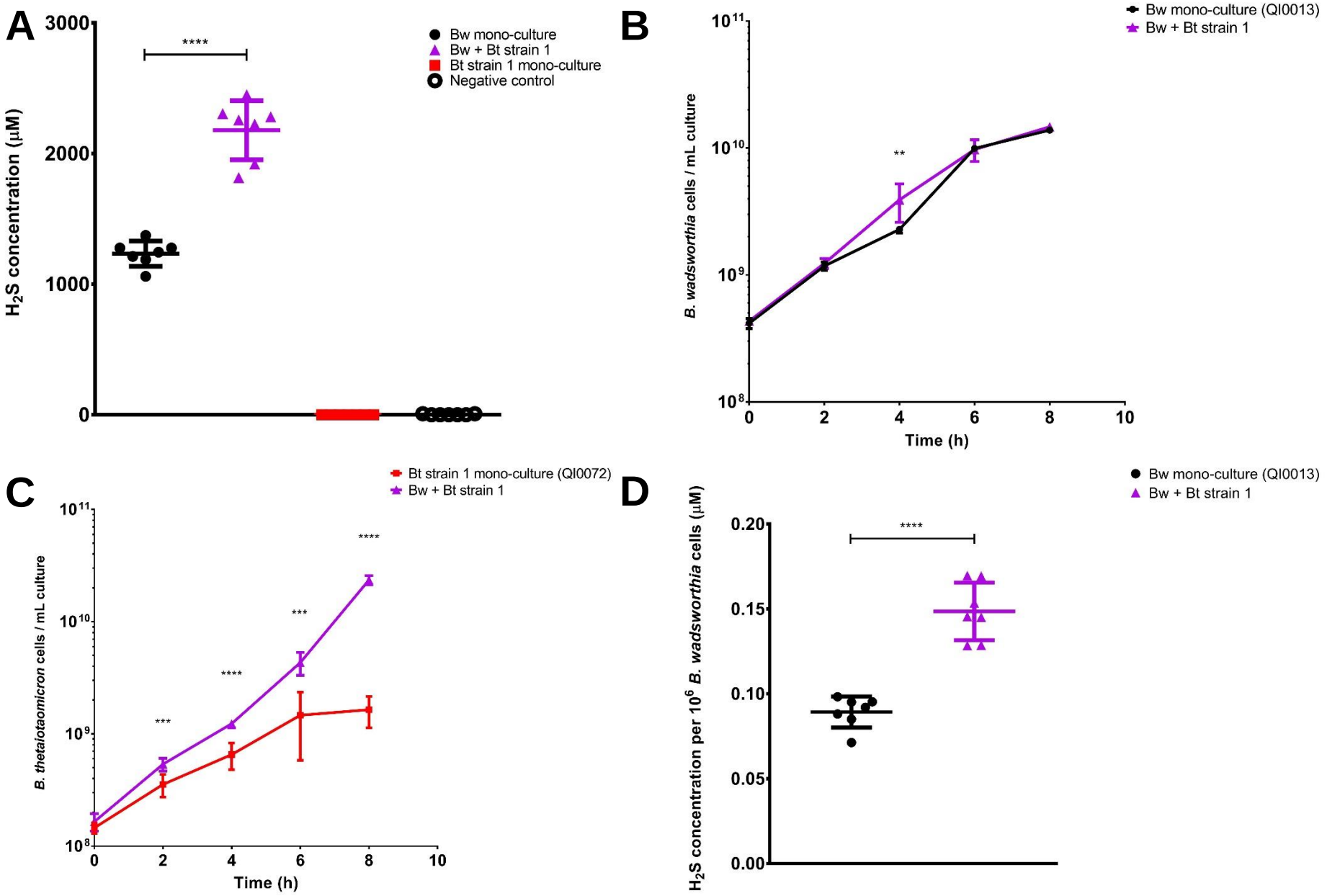

A
3000
2000
1000
0
$H_2S$ concentration (μM)
****
Bw mono-culture
Bw + Bt strain 1
Bt strain 1 mono-culture
Negative control
B
$10^{11}$
$10^{10}$
$10^{9}$
$10^{8}$
*B. wadsworthia* cells / mL culture
**
0
2
4
6
8
10
Time (h)
Bw mono-culture (QI0013)
Bw + Bt strain 1
C
$10^{11}$
$10^{10}$
$10^{9}$
$10^{8}$
*B. thetaiotaomicron* cells / mL culture
***
****
***
****
0
2
4
6
8
10
Time (h)
Bt strain 1 mono-culture (QI0072)
Bw + Bt strain 1
D
0.20
0.15
0.10
0.05
0.00
$H_2S$ concentration per $10^6$ *B. wadsworthia* cells (μM)
****
Bw mono-culture (QI0013)
Bw + Bt strain 1

Figure 16: Co-culture of *B. wadsworthia* (Bw) QI0013 and Bt strain 1. A) $H_2S$ concentrations (µM) at 8 h. B) qPCR-determined *B. wadsworthia* cell counts during culture. C) qPCR-determined *B. thetaiotaomicron* cell counts during culture. D) $H_2S$ concentration (µM) per $10^6$ *B. wadsworthia* cells at 8 h. Each point represents a technical culture replicate (n=7). Horizontal lines represent average, and error bars represent SD. Results of unpaired t tests are shown where ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

### 4.4.2 Transcriptome of *B. wadsworthia* – co-culture with Bt strain 1 vs. mono-culture

Transcriptomic data was quality controlled by trimming to remove residual sequencing adaptors and residual rRNA sequences. All samples with an RNA Quality Number (RQN) of ≥9.0 were taken for further analysis.

#### 4.4.2.1 Respiration of sulfite using hydrogen or lactate was increased in co-culture

Four genes associated with sulfur metabolism were increased in expression in *B. wadsworthia* in co-culture compared to mono-culture (Figure 17); importantly, these genes are involved in $H_2S$ generation by this bacterium. Genes encoding some proteins of the dissimilatory sulfite reductase protein complex DsrMKJOP were increased in expression, including *dsrM* (QI0013_1671), *dsrK* (QI0013_1670), *dsrO* (QI0013_1668), and *dsrP* (QI0013_1667). During dissimilatory sulfite reduction in *B. wadsworthia*, sulfite is generated from taurine [51]. DsrAB is the dissimilatory sulfite reductase enzyme that reduces sulfite ($SO_3^{2-}$) to $H_2S$; this is not a membrane-associated protein, and the energy conservation mechanism for this process was unresolved until Santos *et al*. showed that DsrC is a substrate for DsrAB and forms a trisulfide as an intermediate step [17]. They showed that the six-electron reduction from sulfite to $H_2S$ via the DsrABC complex first utilises 2 electrons from DsrAB and then 2 electrons from DsrC [17]. Critically, the DsrC trisulfide is then reduced by 2 electrons to yield $H_2S$ and recycle DsrC [17]. The authors suggest that this final two electron reduction of DsrC is likely performed by the membrane-associated DsrMKJOP complex; the DsrK contains a [4Fe-4S] cluster known to perform disulfide reductions and can interact with DsrC [402]. Taken together, the DsrMKJOP complex allows the cytoplasmic DsrAB sulfite reduction process to be coupled with ΔpH generation across the membrane and energy conservation, and the electrons required likely originate from

menaquinone [17]. Expression of *dsrMKJOP* is increased in $H_2$-rich growth conditions [403]; this suggests that there is a higher bioavailability of $H_2$ to *B. wadsworthia* under co-culture conditions compared to mono-culture. It is known that *B. thetaiotaomicron* generates hydrogen during polysaccharide fermentation [234]; therefore, *B. wadsworthia* could directly utilise this as an electron donor during dissimilatory sulfate reduction [18, 54].

The increased expression of *dsrMKJOP* raises the question of whether this membrane-associated complex could contribute to the increased $H_2S$ production observed, possibly by increased electron flow to DsrAB, allowing a greater capacity of sulfite to $H_2S$ conversion. Membrane-associated redox complexes such as DsrMKJOP utilise the quinone pool for electron shuttling [46]. Quinones are lipophilic molecules that can accept up to two electrons upon reduction; Bt strain 1 can generate ubiquinone via ubiquinone oxidase, though the type of quinone used by DsrMKJOP is unclear [46]. The quinol pool is re-oxidised by cytochrome bd quinol oxidases; in *B. wadsworthia*, cytochrome d ubiquinol oxidase subunit I (QI0013_4172) and subunit II (QI0013_4173) were both decreased in expression in co-culture compared to mono-culture. A potential hypothesis is that Bt strain 1 provides *B. wadsworthia* with reduced ubiquinone to support electron flow across the DsrMKJOP complex, leading to increased capacity of the conversion of sulfite to $H_2S$.

Interestingly, two genes encoding lactate permeases were also increased in expression in co-culture (QI0013_2306, QI0013_357); this is a marker of bacterial lactate uptake and utilisation, although this enzyme can also import glycolate in *E. coli* [404]. Lactate is a major electron donor for the dsr [30, 51]; combined with the observation of increased expression of DsrMKJOP complex genes, this could permit greater enzymatic activity of the DsrABC complex in *B. wadsworthia* under co-culture conditions, leading to higher $H_2S$ production. Taken together, the transcriptomic data revealed an increased capacity for uptake and utilisation of hydrogen and lactate in *B. wadsworthia* in co-culture with Bt strain 1; both compounds are key electron donors for dissimilatory sulfite reduction. This corresponds to the phenotypic observations of increased $H_2S$ concentration and increased $H_2S$ per *B. wadsworthia* cell in co-culture with Bt strain 1 compared to mono-culture (Figure 16).

The first gene cluster involved in taurine metabolism in *B. wadsworthia* encodes the enzymes taurine pyruvate dehydrogenase (*tpa*), alanine dehydrogenase (*ald*) and an alcohol dehydrogenase (*sarD*) [51] (Figure 18A). All of these genes had decreased expression in co-culture compared to mono-culture, despite the observations of increased $H_2S$ in the co-culture condition (alanine dehydrogenase, QI0013_950; taurine-pyruvate aminotransferase, QI0013_949; alcohol dehydrogenase, QI0013_947). Tpa catalyses the conversion of taurine to sulfoacetaldehyde, and the associated Ald regenerates the amino group acceptor for Tpa [51] (Figure 1). It has been shown that expression of these enzymes is high in *B. wadsworthia* cells grown in taurine, but not in isethionate-grown cells [51]. One possibility could be that *B. wadsworthia* receives isethionate in co-culture; although isethionate sulfite-lyase *islA* and isethionate sulfite-lyase activating protein *islB* were not among the differentially expressed genes, acetaldehyde dehydrogenase (*adhE*), which encodes an enzyme able to convert acetaldehyde (a product of isethionate degradation) to acetyl coA was decreased in expression in co-culture (QI0013_1941). Downregulation of *tpa* and *sarD* could imply that production of isethionate via taurine in *B. wadsworthia* is decreased in co-culture, but increased expression of the DsrMKJOP complex could support increased capacity of the DsrABC to convert sulfite to $H_2S$. As a whole, this implies that *B. wadsworthia* may be utilising sulfite without the requirement for taurine degradation in co-culture with Bt strain 1 (Figure 18A).

Further evidence for an alternative supply of sulfite comes from the observation that sulfolactate sulfo-lyase genes encoding the alpha subunit (QI0013_772) and beta subunit (QI0013_771) of the enzyme are decreased in expression in co-culture; this enzyme converts sulfolactate to sulfite and pyruvate. An additional gene involved in sulfite production, Rhodanese-domain-containing inner membrane protein *ygaP* which generates sulfite from thiosulfate was decreased in expression in co-culture compared to mono-culture (QI0013_2882), further suggesting an alternative sulfite source for *B. wadsworthia* in co-culture with Bt strain 1 (Figure 18A).

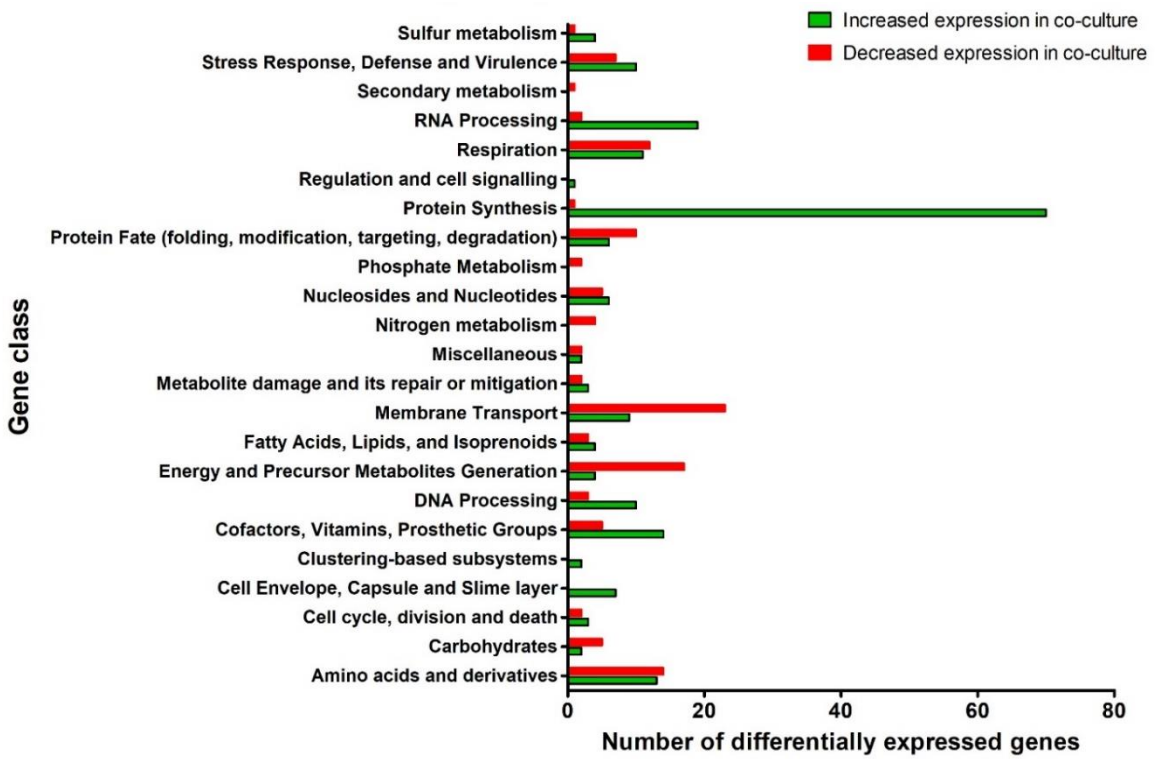



Figure 17: Differentially expressed genes (DEGs) of *B. wadsworthia* (Bw) QI0013 in co-culture with Bt strain 1 vs. mono-culture.
Numbers of DEGs in *B. wadsworthia* increased and decreased in expression in each functional gene class as annotated in BV-BRC. Green indicates genes that are increased in expression in co-culture, and red indicates decreased expression in co-culture relative to the *B. wadsworthia* mono-culture.

#### 4.4.2.2 Sulfur metabolism in *B. wadsworthia* and Bt strain 1 – why is more $H_2S$ generated in the co-culture?

In co-culture with Bt strain 1, *B. wadsworthia*'s sulfur metabolism appeared to be altered where genes required for taurine degradation to sulfite showed decreased expression. Given that genes associated with the dissimilatory sulfite reductase enzyme were increased in expression and $H_2S$ concentration was higher in the co-culture, this raised a question: what is the source of sulfite for *B. wadsworthia* in the co-culture?

In the majority of sulfate-reducing bacteria (but not *B. wadsworthia*), sulfate is reduced by sulfate adenylyltransferase (Sat) which converts sulfate to adenosine 5'-phosphosulfate (APS), followed by the conversion of APS to sulfite by adenylylsulfate reductase (AprAB) which consists of an alpha and beta subunit [405, 406], and the sulfite produced then enters the dissimilatory sulfite reduction

pathway (Figure 1). It has been previously shown that *B. wadsworthia* is unable to utilise sulfate as an electron acceptor [31, 407] instead using taurine or sulfoquinovose to produce sulfite [51, 63]. Although *B. wadsworthia* cannot directly utilise sulfate, it was interesting to observe that sulfate permease (QI0013_1830) and sulfatase (QI0013_1848) genes were increased in expression in *B. wadsworthia* in co-culture with Bt strain 1, indicative of increased sulfate bioavailability (Figure 18A).

Given that *B. wadsworthia* cannot utilise sulfate in the “traditional” sulfate reduction pathway, it was assumed that the genes for these enzymes are absent from the genome [52]. Investigating the protein domains of query genes using Pathway Tools [286] and BV-BRC [293], it was therefore surprising to find that *B. wadsworthia* QI0013 contains genes predicted to encode adenylylsulfate reductase alpha and beta subunits at two separate gene loci (QI0013_309, QI0013_310, QI0013_741, QI0013_742) (Table S2, Table S3). Using NCBI BLAST protein-protein comparison, the protein sequence of the alpha subunit shows low similarity (~30% similarity) to adenylylsulfate reductase (AprAB) genes from *Desulfovibrio gigas*, *Desulfovibrio desulfuricans* DSM 642 and *Desulfovibrio alaskensis* G20 (Table S4). However, protein domain function prediction using InterProScan [292] showed similarities across the sequence, including FAD-dependent oxidoreductase 2, FAD binding domain (IPR003953), FAD/NAP(P)binding domain (IPR036188), Succinate dehydrogenase/fumarate reductase flavoprotein, catalytic domain superfamily (IPR027477) and Fumarate reductase/succinate dehydrogenase flavoprotein-like, C-terminal domain superfamily (IPR037099) domains (Table S5). Indeed, the genes in *B. wadsworthia* were associated with AprAB activity based on protein function predictions. Given that the putative *B. wadsworthia* adenylylsulfate reductase genes share similar predicted protein domains to characterised genes in these *Desulfovibrio* strains, the gene may also encode a functional enzyme.

The *B. wadsworthia* (QI0013) genome did not contain genes encoding the Sat enzyme necessary for sulfate reduction; this agrees with previous experimental observations that *B. wadsworthia* cannot directly utilise sulfate [31, 407]. The presence of adenylylsulfate reductase genes in *B. wadsworthia* has not been previously described, and, if proved active, would allow *B. wadsworthia* to convert

adenosine 5’-phosphosulfate (APS) to sulfite which then enters the dissimilatory sulfite reduction pathway. Interestingly, *aprAB* genes were predicted to be present in additional *B. wadsworthia* strains QI0012, QI0014, QI0015, and QI0016 using BV-BRC annotation. This suggests that other *B. wadsworthia* strains may also be capable of using APS as a substrate for sulfite acquisition, which has been previously overlooked.

#### 4.4.2.3 Bt strain 1: providing adenosine 5’-phosphosulfate (APS), a sulfite precursor, to *B. wadsworthia*?

When in co-culture with *B. wadsworthia*, Bt strain 1 showed decreased expression of eight genes encoding arylsulfatases; these enzymes hydrolyse arylsulfate esters to yield phenol and sulfate [408]. It has been previously shown that Bt strain 1 is capable of releasing sulfate via mucin degradation [233], and expression of arylsulfatases is strongly repressed by the presence of inorganic sulfate [408, 409], which suggests a higher sulfate bioavailability in co-culture compared to mono-culture.

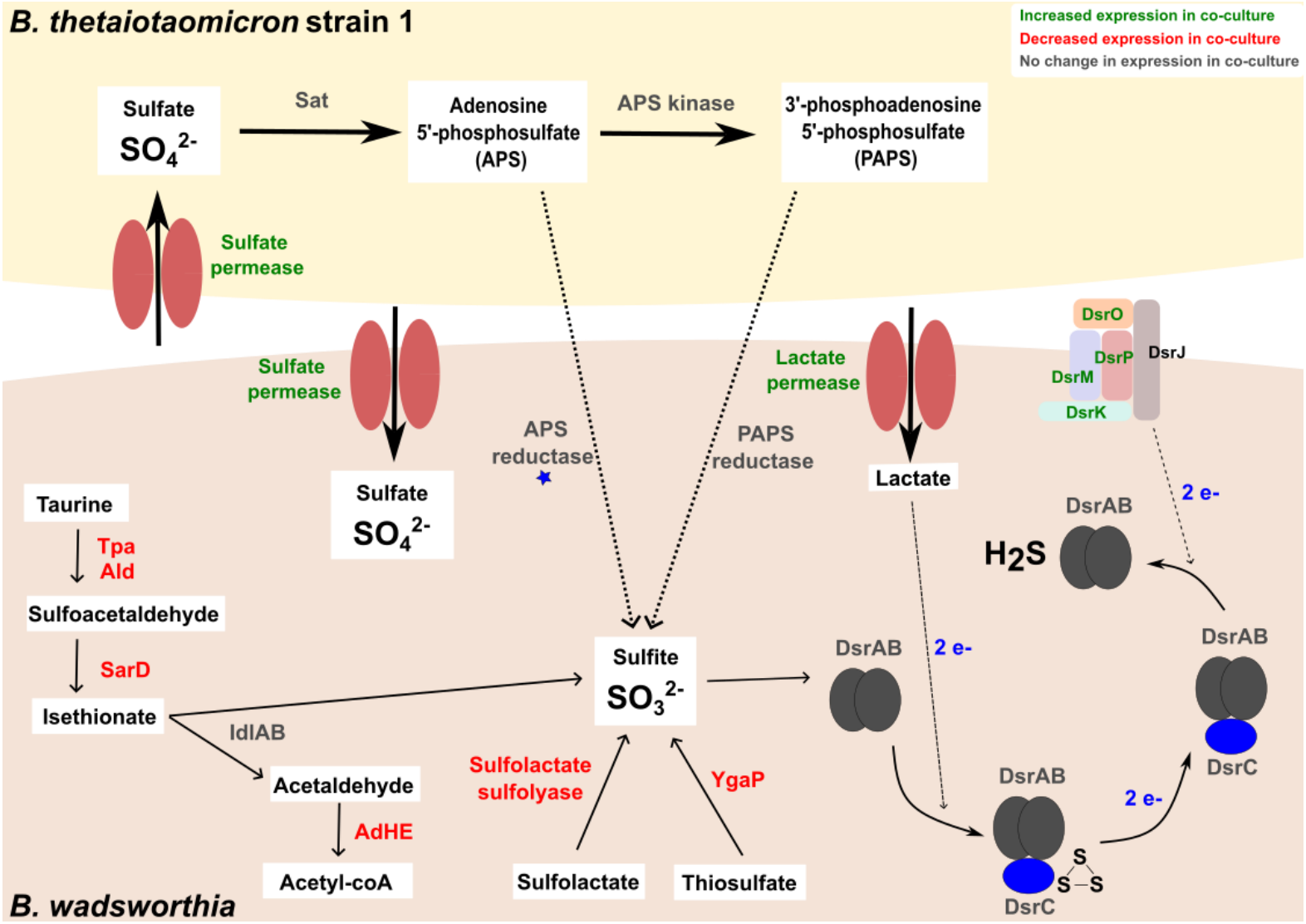
We next investigated whether Bt strain 1 contains any genes related to the assimilatory sulfite reduction pathway. This involves the reduction of sulfate to APS via sulfate adenylyltransferase (Sat) (EC 2.7.7.4), which is then converted to phosphoadenosine phosphosulfate (PAPS) via adenylylsulfate kinase / APS kinase (EC 2.7.1.25). PAPS is then reduced to sulfite by phosphoadenosine phosphosulfate (PAPS) reductase (EC 1.8.4.8), and the sulfite is converted to $H_2S$ by assimilatory sulfite reductase (EC 1.8.1.2) [47] (Figure 1). Interestingly, the Bt strain 1 genome encodes some enzymes involved in assimilatory sulfite reduction, including sulfate adenylyltransferase (Sat) (QI0072_1554, QI0072_1555) and adenylylsulfate kinase / APS kinase (QI0072_1556) (Table S1), which would facilitate conversion of sulfate to both APS and PAPS. However, Bt strain 1 does not possess the enzymes PAPS reductase or assimilatory sulfite reductase. *B. wadsworthia* encodes both phosphoadenosine phosphosulfate (PAPS) reductase (QI0013_194), which reduces PAPS to sulfite, in addition to the putative AprAB which reduces APS to sulfite (Table S2).

We hypothesise that Bt strain 1 produces sulfate from sulfated saccharides via sulfatases [410] an activity which has been previously described to support the growth of *Desulfovibrio piger* via cross-feeding [233], and then reduces the sulfate

to APS and/or PAPS. Then, Bt-derived APS and PAPS could be utilised directly by *B. wadsworthia*, where either AprAB or PAPS reductase yield sulfite, which then enters the dissimilatory reduction pathway (Figure 18A, 18B). In this way, Bt strain 1 may provide *B. wadsworthia* with an alternative source of sulfite, which may be energetically favourable over taurine degradation, or utilised when taurine or other organosulfur compounds are depleted. This hypothesis is supported by the decreased expression of genes associated with sulfite generation via taurine, thiosulfate and sulfolactate under co-culture conditions, and the phenotypic observation of increased $H_2S$ concentration in the co-culture (Figure 18).

As the genes involved in this potential interaction were not differentially expressed in co-culture compared to mono-culture to a logFC ≥1, the TMM-normalised read counts were interrogated for each of these genes. The genes encoding putative adenylylsulfate reductase alpha and beta subunits and PAPS reductase were expressed in *B. wadsworthia* both in co-culture with Bt strain 1 and mono-culture, although only at low levels (Table S2). In Bt strain 1, expression of genes encoding sulfate adenylyltransferase subunits 1 and 2 and adenylylsulfate kinase was found both in co-culture with *B. wadsworthia* and mono-culture, with no differences in expression levels between conditions (Table S1). This suggests that APS and PAPS were likely present within Bt strain 1 under these conditions. It is important to note that this hypothesis is yet to be experimentally proven; it is unknown whether APS or PAPS could be utilised by *B. wadsworthia* for growth in the absence of taurine, whether these intermediate molecules are imported/exported, and which transporters are used (Figure 18).

A


B. thetaiotaomicron strain 1
Increased expression in co-culture
Decreased expression in co-culture
No change in expression in co-culture
Sulfate
$SO_4^{2-}$
Sat
Adenosine 5'-phosphosulfate (APS)
APS kinase
3'-phosphoadenosine 5'-phosphosulfate (PAPS)
Sulfate permease
Sulfate permease
Sulfate
$SO_4^{2-}$
APS reductase
PAPS reductase
Lactate permease
Lactate
DsrO
DsrP
DsrJ
DsrM
DsrK
2 e-
DsrAB
$H_2S$
Taurine
Tpa
Ald
Sulfoacetaldehyde
SarD
Isethionate
IdlAB
Acetaldehyde
AdHE
Acetyl-coA
Sulfite
$SO_3^{2-}$
Sulfolactate sulfolyase
YgaP
Sulfolactate
Thiosulfate
DsrAB
2 e-
DsrAB
DsrC
DsrAB
2 e-
S
S—S
DsrC
B. wadsworthia

**B**

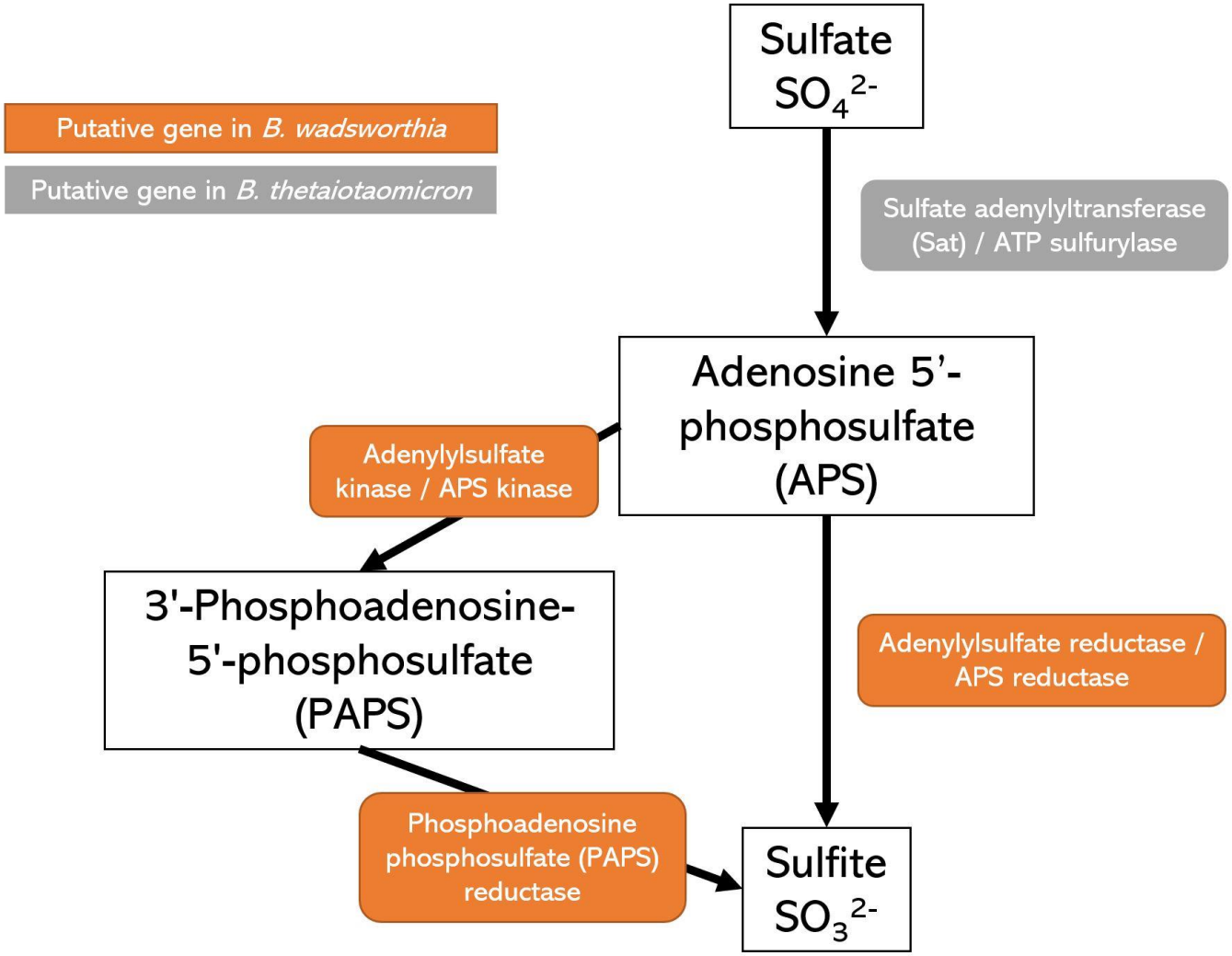

Putative gene in B. wadsworthia
Putative gene in B. thetaiotaomicron
Sulfate
$SO_4^{2-}$
Sulfate adenylyltransferase (Sat) / ATP sulfurylase
Adenosine 5'-phosphosulfate (APS)
Adenylylsulfate kinase / APS kinase
3'-Phosphoadenosine-5'-phosphosulfate (PAPS)
Adenylylsulfate reductase / APS reductase
Phosphoadenosine phosphosulfate (PAPS) reductase
Sulfite
$SO_3^{2-}$

Figure 18: Summary schematic diagram of the putative cross-feeding interaction between *B. wadsworthia* and Bt strain 1. A) Enzymes for each reaction step are shown in colours corresponding to transcriptomic data where green showed increased expression of the encoding gene in co-culture compared to mono-culture; red showed decreased expression in co-culture vs. mono-culture; grey showed no change in expression between co-culture and mono-culture. The blue star represents a putative enzyme where functionality is unproven. The dotted line represents a putative cross-feeding mechanism. Dashed lines represent electron transfer. B) Summary schematic of the putative co-operative metabolism of sulfate to sulfite in *B. wadsworthia* and *B. thetaiotaomicron* co-culture based on genes with putative function.

#### 4.4.2.4 *B. wadsworthia* virulence is tamed in co-culture with Bt strain 1

Genes associated with the type VI secretion system (T6SS) were decreased in expression in *B. wadsworthia* in co-culture with Bt strain 1 compared to mono-culture, including sheath and tube components *tssB*/*C* and *tssD/Hcp*, baseplate components *tssE*, *tssF*, *tssG*, membrane complex components *tssL* and *tssM*, as well as spike components *vgrG* and a gene encoding PAAR-repeat protein (Table 8). The T6SS forms a bacteriophage-like needle structure along with a membrane complex to deliver effector molecules directly into target cells. Found in Gram-negative bacteria [411], the T6SS appears to serve a primary role of inter-bacterial competition, where toxic effectors are injected into rival bacterial cells [412]. T6SS effectors typically have broad spectrum antibacterial activity, from which genetically identical cells are shielded via immunity proteins [412]. The T6SS can also contribute to bacterial virulence; T6SS effectors in *Pseudomonas aeruginosa* have been shown to have activity against both bacterial and eukaryotic cells [413]. Given that the T6SS assembly and employment is likely to be energetically expensive, expression is probably highly regulated; indeed, quorum sensing may play a regulatory role via the global regulator TsrA in *Vibrio cholerae* [414]. The T6SS may also play a contact-independent role in abiotic cell stress conditions; T6SS can facilitate extracellular $Zn^{2+}$ scavenging [415], and manganese, copper, molybdenum and iron uptake under abiotic cell stress conditions [416]. Much of the literature has focused on increased expression of T6SS in inter-bacterial competition, however the phenomenon described here has not been extensively discussed. It seems probable that the decreased T6SS expression in *B. wadsworthia* when in co-culture with Bt strain 1 reflects a decreased cell stress environment and may demonstrate reduced inter-bacterial competition, showing the mutualistic symbiotic interaction between these strains under these conditions.

Four genes associated with the type III secretion system (T3SS) were decreased in expression in *B. wadsworthia* in co-culture compared to mono-culture. Like the T6SS, the T3SS is found in Gram-negative bacteria and forms an injectosome complex which allows the delivery of toxic effectors to target eukaryotic cells [417]. The genes decreased in expression in *B. wadsworthia* encoded a YscD-like base apparatus structure protein [418], an SseC-like effector protein involved in pore formation in target membrane (YopB) [419], SycD, a protein chaperone for pore-forming YopD [420] and a YopN protein involved in facilitating effector translocation into target cells [421] (Table 8). These genes are mainly related to

the translocon proteins of the T3SS which aid in pore formation and transport of effectors directly into the target eukaryotic cell [417]. Given that the T3SS is a well-established virulence mechanism used by many bacterial pathogens [422, 423], decreased expression by *B. wadsworthia* in co-culture could reflect decreased virulence in the presence of Bt strain 1. The environmental inducers of T3SS expression are still under study, but include direct host cell contact, low $Ca^{2+}$ concentration and serum albumin in the case of *Pseudomonas aeruginosa* [424]. It could be speculated that altered $Ca^{2+}$ concentration in the co-culture contributes to the observed decreased expression of T3SS genes in *B. wadsworthia* in co-culture, however this has not been confirmed. Expression of the T3SS and T6SS is associated with bacterial antagonism [425-427], so a decreased expression supports the premise that *B. wadsworthia*'s interaction with Bt strain 1 is more symbiotic than competitive/antagonistic. Furthermore, these secretion systems are often related to bacterial virulence; decreased expression of these systems in *B. wadsworthia* could reflect a loss of virulence when co-cultured with Bt strain 1.

Table 8: Differentially expressed genes (DEGs) related to secretion systems in *B. wadsworthia* (QI0013) in co-culture with Bt strain 1 (QI0072) compared to mono-culture. Differentially expressed genes were identified with a P value cut-off of 0.01, FDR ≤ 0.05 and a logFC of 1 equating to a 2-fold change in gene expression.

| | Gene locus ID | Gene annotation | LogFC in co-culture vs. mono-culture |
|---|---|---|---|
| Type VI Secretion System (T6SS) | QI0013_477 | T6SS component TssG (ImpH/VasB) | -1.81 |
| | QI0013_476 | T6SS component TssF (ImpG/VasA) | -1.83 |
| | QI0013_467 | T6SS component TssM (IcmF/VasK) | -1.33 |
| | QI0013_469 | T6SS component TssK (ImpJ/VasE) | -1.21 |
| | QI0013_468 | T6SS outer membrane component TssL (ImpK/VasF) | -1.55 |
| | QI0013_475 | T6SS lysozyme-like component TssE | -2.05 |
| | QI0013_480 | T6SS PAAR-repeat protein | -1.91 |
| | QI0013_474 | T6SS component Hcp | -1.95 |
| | QI0013_473 | T6SS component TssB (ImpB/VipA) / T6SS component TssC (ImpC/VipB) | -2.17 |

| | | | |
|---|---|---|---|
| | QI0013_479 | VgrG protein | -1.71 |
| Type III Secretion System (T3SS) | QI0013_4411 | Type III secretion chaperone protein for YopD (SycD) | -1.20 |
| | QI0013_4414 | Type III secretion outer membrane contact sensing protein (yopN, Yop4b, CLcrE) | -1.53 |
| | QI0013_4403 | Type III secretion inner membrane protein (YscD, Chomologous to flagellar export components) | -2.27 |
| | QI0013_4410 | SseC-like effector protein (YopB) | -1.32 |

Additionally, *B. wadsworthia* showed decreased expression of the virulence-associated urease in co-culture with Bt strain 1; genes encoding the urease enzyme were among the top 50 decreased DEGs including the alpha (QI0013_3507), beta (QI0013_3508) and gamma subunits (QI0013_3509). In the wider dataset, other genes in this cluster including those encoding urease accessory proteins *ureE* (QI0013_3505), *ureF* (QI0013_3504) and *ureG* (QI0013_3503) were also decreased in expression. Urease is expressed in a broad range of bacteria; the importance of urease as a virulence factor has been particularly well established in *H. pylori*, the causative organism of peptic ulcer disease. In *H. pylori*, urease is essential for pathogenesis, as the pH increase caused by ammonia production is postulated to firstly allow *H. pylori* to persist at the epithelial lining of the stomach, and secondly to cause tissue damage via production of ammonium hydroxide [428]. Urease is pro-inflammatory, cytotoxic to human cells, and may damage epithelial tight junctions in the case of *H. pylori*-induced gastritis [429]. Taken together, decreased expression of T6SS, T3SS and urease in *B. wadsworthia* in co-culture with Bt strain 1 suggests that *B. wadsworthia* adopts a lower virulence phenotype under these conditions.

#### 4.4.2.5 *B. wadsworthia* showed altered expression of genes associated with cell stress in co-culture

In *B. wadsworthia*, ten genes associated with the stress response, defence and virulence gene class were increased in expression in co-culture with Bt strain 1 compared to mono-culture, and seven genes showed decreased expression (Figure 17). Several upregulated genes were antibiotic targets for protein synthesis, including transcription termination factor Rho (QI0013_2576),

translation elongation factors G (QI0013_1296) and Tu (QI0013_1311) and isoleucyl-tRNA synthetase (QI0013_2252); this logically follows the trend towards increased expression of genes associated with protein synthesis observed in *B. wadsworthia* in co-culture with Bt strain 1 (Figure 17). Five genes encoding universal stress proteins (USPs) showed decreased expression in *B. wadsworthia* in co-culture compared to mono-culture; USP expression is increased in response to environmental stressors such as nutrient scarcity [430], so decreased expression alludes towards lower stress conditions for *B. wadsworthia* when in co-culture with Bt strain 1.

#### 4.4.2.6 *B. wadsworthia* increases expression of genes for nucleotide synthesis and cell division in co-culture

Several genes associated with nucleotide biosynthesis were increased in expression in *B. wadsworthia* in co-culture with Bt strain 1 (Figure 17). The pentose phosphate pathway uses glucose-6-phosphate to produce NADPH, pentose sugars and ribose 5-phosphate, which is a precursor for nucleotide synthesis. In co-culture with Bt strain 1, *B. wadsworthia* showed increased expression of four genes encoding enzymes associated with the pentose phosphate pathway including ribose-phosphate pyrophosphokinase (QI0013_2010) which converts ribose 5-phosphate to phosphoribosyl pyrophosphate (PRPP) in the first committed step of purine biosynthesis, as well as amidophosphoribosyltransferase (*purF*) (QI0013_4357) phosphoribosylformylglycinamidine synthase (*purS*) (QI0013_4263) and adenylosuccinate lyase (*purB*) (QI0013_2185) which catalyse various steps to generate intermediates in the biosynthetic pathway for purines; a useful schematic summary of this pathway was presented by Goncheva *et al.* [431]. Not only is this biosynthetic pathway important for dinucleotide production for DNA replication, but it also produces ATP and GTP to serve as key cellular energy sources [431]. Additionally, four genes encoding subunits of xanthine dehydrogenases (QI0013_3046, QI0013_3305, QI0013_799, QI0013_913) were decreased in expression in *B. wadsworthia* in co-culture compared to mono-culture; this enzyme converts hypoxanthine to xanthine, and xanthine to uric acid in purine catabolism [432]. Furthermore, one gene encoding a carbamoyl-phosphate synthase large chain (QI0013_4359) was increased in expression in *B. wadsworthia* in co-culture with Bt strain 1 compared to mono-culture; this uses either ammonia or glutamine to produce carbamoyl phosphate in the first committed step of pyrimidine

biosynthesis [431, 433]. Overall, this suggests that purine and pyrimidine levels are increased in *B. wadsworthia* in co-culture with Bt strain 1.

Cell division associated genes were also differentially expressed in *B. wadsworthia* in co-culture with Bt strain 1; genes encoding rod shape-determining proteins MreC and MreD were significantly increased in expression (QI0013_887, QI0013_886). These genes are necessary for shape maintenance in rod-shaped bacteria, and *ΔmreC* mutants are non-viable [434]. Other rod-associated genes were also increased in expression including *rodA* (QI0013_884) and *mreB* (QI0013_888). Sometimes called “prokaryotic cytoskeletons”, *mreB* genes are found in almost all non-coccoid bacteria where they encode proteins within the actin superfamily involved in organisation of cell wall synthesis [435]. Rod-shape determining proteins MreBCD and RodA form part of the elongasome, a protein complex that directs peptidoglycan insertion into the lateral cell wall, therefore promoting cylindrical growth. Interestingly, penicillin binding proteins (PBPs) which are important elongasome components were not differentially expressed. It has been shown that cell wall stress upregulates elongasome activity through $\sigma^{M}$-mediated increase in expression of *mreBCD* and *rodA* [436]. Overall, this suggests that the elongasome of *B. wadsworthia* could be more biosynthetically active under co-culture conditions, possibly in response to cell wall stress. Additionally, *ftsK* (QI0013_4472) was increased in expression in *B. wadsworthia* in co-culture; this encodes DNA translocase which coordinates DNA segregation into daughter cells as part of the divisome complex during cell division and is an essential gene in *E. coli* [437]. Overall, the transcriptomic data indicates increased purine and pyrimidine biosynthesis and increased expression of cell division associated genes in *B. wadsworthia* under co-culture conditions; this alludes to increased metabolic activity in *B. wadsworthia* in co-culture with Bt strain 1.

#### 4.4.2.7 *B. wadsworthia* increases production of vitamin B6 and glutamate in co-culture

*B. wadsworthia* showed increased expression of genes for vitamin B6 biosynthesis in co-culture with Bt strain 1; genes encoding the glutaminase and synthase subunits of pyridoxal 5’-phosphate synthase were increased in expression (QI0013_4335, QI0013_4334) in addition to pyridoxal kinase (QI0013_638). These enzymes produce pyridoxal 5’-phosphate, the bioactive form of vitamin B6. This enzyme utilises a pentose sugar (aldehydo-D-ribose 5-phosphate), a triose sugar

(D-glyceraldehyde 3-phosphate) and glutamine to produce glutamate and pyridoxal 5'-phosphate (PLP) [438]. Vitamin B6 (PLP) is the cofactor of enzymes catalysing a large variety of chemical reactions mainly involved in amino acid metabolism [439], and is an essential nutrient in the human diet [440]. During vitamin B6 biosynthesis, the glutaminase subunit of the enzyme first hydrolyses L-glutamine to yield PLP, L-glutamate and ammonia. The ammonia is then channelled to the synthase subunit where it is reacted with D-ribose 5-phosphate and D-glyceraldehyde 3-phosphate in a complex series of reactions to yield PLP. Expression of glutamate synthase large chain (QI0013_4438) and small chain (QI0013_916) is decreased in *B. wadsworthia* in co-culture, likely reflecting the increased glutamate abundance due to increased vitamin B6 biosynthesis. Similarly, expression of proline dehydrogenase (QI0013_750) which catalyses the oxidation of proline to glutamate is also reduced in co-culture.

The transcriptomic data indicates a higher production of glutamate in *B. wadsworthia* in the co-culture; this can be utilised in the citric acid cycle via conversion to 2-oxo-glutarate by glutamate dehydrogenase. Expression of the gene encoding glutamate dehydrogenase was higher in co-culture in *B. wadsworthia* (QI0013_2400), suggesting that the glutamate produced as a result of increased PLP synthesis is utilised for the citric acid cycle. Glutamate dehydrogenase generates ammonia; this is likely to be utilised by carbamoyl phosphate synthase in pyrimidine biosynthesis. Overall, *B. wadsworthia* appears to increase vitamin B6 biosynthesis in co-culture with Bt strain 1, leading to increased glutamate production as an additional product of this reaction which is in turn utilised during pyrimidine synthesis.

#### 4.4.2.8 Reduced expression of branched chain amino acid transport proteins in *B. wadsworthia* co-cultured with Bt strain 1

Genes associated with membrane transport and nutrient uptake were differentially expressed in *B. wadsworthia* in co-culture with Bt strain 1 compared to mono-culture. Of the 50 genes most decreased in expression in *B. wadsworthia* in co-culture, several were associated with ABC transporters for branched chain amino acids (BCAAs), including *livF*, *livG, livH*, *livM* and three *livJ* genes. Some Liv genes were also observed as being increased in expression in co-culture, including two *livG*, two *livH*, two *livM* and two *livF*. Together, the *livJHMGF* complex encode a high-affinity LIV-I ATP-binding cassette (ABC) transporter in

Gram-negative bacteria [441]. BCAA transport via this complex is mediated by 2 substrate binding proteins; LivJ, which binds all 3 BCAAs and LivK which is leucine-specific [441]. Expression of *liv* genes was shown to be non-essential for *Campylobacter* colonisation in chicken gut with the exception of *livJ* and *livK* which were essential; this suggests that these genes may have additional function besides branched chain amino acid transport [442]. Furthermore, a *ΔlivJHMGF* mutant *Streptococcus pneumoniae* strain showed no growth defects *in vitro*, but reduced virulence in pneumonia and septicaemia models [443]. Changes in expression of branched chain amino acid transport proteins could reflect alterations in availability of leucine, isoleucine and valine to *B. wadsworthia* in the co-culture compared to mono-culture but may also have effects on the virulence of this *B. wadsworthia* strain.

#### 4.4.2.9 *B. wadsworthia* utilises hydrogen as an energy source in co-culture

Given that the ability of *B. thetaiotaomicron* to produce hydrogen during fermentation is well-established [234] and hydrogen is an excellent energy source for *B. wadsworthia* [54], we expected that *B. wadsworthia* would readily utilise Bt-derived hydrogen when co-cultured with Bt strain 1. Indeed, *B wadsworthia*'s hydrogen utilisation was increased in co-culture; energy-conserving hydrogenase subunits BCDEF were increased in expression (QI0013_1929, QI0013_1928, QI0013_1927, QI0013_1926, QI0013_1925). Hydrogenases convert molecular hydrogen to protons and are coupled to the electrochemical ion gradients generated across membranes in bacteria and archaea to allow utilisation of hydrogen as a cellular energy source [444]. Hydrogenase expression has been suggested to enhance *B. wadsworthia* virulence, as is the case in other hydrogenase-expressing pathogenic bacteria [54]. It appears that in co-culture with Bt strain 1, *B. wadsworthia* may have greater capacity for hydrogen utilisation to access additional energy for growth and metabolic activity.

### 4.4.3 Transcriptome of Bt strain 1 – co-culture with *B. wadsworthia* vs. mono-culture

#### 4.4.3.1 $H_2S$-utilising amino acid biosynthetic pathways were reduced in expression in Bt strain 1

Given the sulfidogenic interaction between *B. wadsworthia* and Bt strain 1, we first used Pathway Tools to establish the capacity of Bt strain 1 to produce or utilise $H_2S$. The genome of Bt strain 1 does not encode known genes for $H_2S$ production, but it does have the capacity to utilise $H_2S$ during the biosynthesis of amino acids including cysteine and homocysteine. In Bt strain 1, twelve genes associated with amino acid metabolism were increased in expression, and nine genes were decreased in co-culture with *B. wadsworthia* compared to mono-culture (Figure 19). Interestingly, cysteine synthase (o-acetylserine sulfhydrolase) EC 2.5.1.47 (QI0072_3844) was highly decreased in expression in Bt strain 1 in co-culture compared to mono-culture; this catalyses the conversion of O3-acetyl-L-serine and $H_2S$ to L-cysteine and acetate, utilising pyridoxal-5'-phosphate (vitamin B6) as a co-factor [445] (Figure 1). This is relevant as this shows that although Bt strain 1 has the capacity to utilise *B. wadsworthia*-derived $H_2S$, the gene encoding this enzyme is heavily decreased in expression in co-culture with *B. wadsworthia*. This therefore indirectly contributes to the high $H_2S$ concentration observed in the co-culture. Similarly, two genes involved in $H_2S$-utilising homocysteine biosynthesis were also differentially expressed; one gene encoding O-acetylhomoserine sulfhydrylase (EC 2.5.1.49) O-succinylhomoserine sulfhydrylase (EC 2.5.1.48) was heavily decreased in expression in co-culture (QI0072_3143), whereas another gene encoding this enzyme was increased in expression in co-culture (QI0072_2658), although not to the same fold change as the decreased gene. This enzyme catalyses the conversion of O-acylhomoserine and $H_2S$ to homocysteine [446, 447]. Overall, decreased homocysteine and cysteine biosynthesis by Bt strain 1 in co-culture with *B. wadsworthia* results in lower $H_2S$ utilisation by Bt strain 1, which indirectly contributes towards the overall phenotype of high $H_2S$ concentration in the co-culture.


Figure 19: Differentially expressed genes (DEGs) of Bt strain 1 (QI0072) in co-culture with *B. wadsworthia* vs. mono-culture.

Numbers of DEGs in Bt strain 1 increased and decreased in expression in each functional gene class as annotated in BV-BRC. Green indicates genes that are increased in expression in co-culture, and red indicates decreased expression in co-culture relative to the Bt strain 1 mono-culture.

#### 4.4.3.2 Bt strain 1 showed reduced expression of polysaccharide binding and utilisation genes in co-culture

Genes associated with carbohydrates were notably affected in Bt strain 1 in co-culture with *B. wadsworthia* compared to mono-culture; three genes were increased in expression, whereas twenty-three were decreased in expression (Figure 19). Many of the genes decreased in expression were associated with sugar utilisation including three α-galactosidases (QI0072_3829, QI0072_3896, QI0072_4365) and a UDP-glucose 4-epimerase (QI0072_600) associated with degrading glycosphingolipids or glycoproteins to galactose, and ten β-galactosidases (QI0072_1151, QI0072_1160, QI0072_1161, QI0072_1166, QI0072_1492, QI0072_2325, QI0072_281, QI0072_3398, QI0072_3661, QI0072_899) for lactose utilisation [448]. Additionally, six genes encoding α-mannosidases (QI0072_2924, QI0072_3023, QI0072_3693, QI0072_4673, QI0072_4830, QI0072_4832) were decreased in expression; this enzyme is

involved in mannose degradation [449]. This suggests that polysaccharide utilisation and degradation is reduced in Bt strain 1 when in co-culture with *B. wadsworthia* compared to mono-culture. Additionally, genes associated with membrane transport were distinctly affected in Bt strain 1 when in co-culture with *B. wadsworthia* compared to mono-culture, with fourteen genes showing increased expression and ninety-six decreased in expression (Figure 19). Perhaps unsurprisingly given the decreased expression of genes for carbohydrate utilisation, genes associated with polysaccharide binding and import were also decreased in expression. The starch utilisation system (Sus) protein family showed a striking pattern of decreased expression, including one *susA* gene encoding α-amylase, forty-one *susC* genes which encode outer membrane Ton-B dependent transporters, forty-four *susD* genes for cell surface glycan-binding lipoproteins, as well as s*usE* and *susF* which encode outer membrane proteins. The Sus proteins are involved in cell surface polysaccharide binding in *Bacteroides spp*. as part of the Polysaccharide Utilisation Loci (PULs), where SusC and SusD primarily function to bind starch to the outer membrane, with SusE stabilising binding [450]. The utilisation and breakdown of polysaccharides by *B. thetaiotaomicron* is largely responsible for the prevalence of this species in the gut human gut microbiota, as this serves important ecological function in the human gut [451]. It is possible that due to the higher growth rate of Bt strain 1 in co-culture, the polysaccharides were utilised more rapidly and at 8 h decreased gene expression is due to scarcity in the co-culture by this time-point. If occurring in the human gut environment, this may also be interesting as decreased fermentation by this strain in the presence of *B. wadsworthia* could have a wider impact on the gut microbiota and the host.

#### 4.4.3.3 Decreased vitamin B6 biosynthesis and increased folate biosynthesis by Bt strain 1 in co-culture

Twenty-eight genes associated with cofactors and vitamin synthesis were decreased in expression in Bt strain 1 when in co-culture with *B. wadsworthia* compared to mono-culture, compared to only eight genes increased in expression (Figure 19). Of those decreased in expression, three were associated with vitamin B6 biosynthesis, including pyridoxal kinase (QI0072_5367) and pyridoxamine 5'-phosphate oxidase (QI0072_2263) which generate pyridoxal 5'-phosphate (PLP), the bioactive form of vitamin B6 from precursor molecules, and 1-deoxy-D-xylulose 5-phosphate synthase (QI0072_2069) which generates pyridoxine 5'-phosphate, a

precursor for PLP. Given that Bt strain 1 showed increased growth in co-culture and that vitamin B6 is an important co-factor for many cellular processes [440], it seems likely that Bt strain 1 is able to source the required PLP in the co-culture. As *B. wadsworthia* showed increased vitamin B6 biosynthesis, Bt strain 1 may directly utilise this.

Folate biosynthesis appears to be increased in Bt strain 1 in co-culture with *B. wadsworthia*, as genes encoding enzymes in the tetrahydrofolate (THF) pathway showed increased expression including dihydrofolate synthase (QI0072_1984), formate--tetrahydrofolate ligase (QI0072_878), and methenyltetrahydrofolate cyclohydrolase (QI0072_2306). Tetrahydrofolate (vitamin B9) is an important co-factor in the synthesis of purines, pyrimidines and methionine [452]; increased synthesis likely represents the increased metabolic activity of Bt strain 1 when in co-culture with *B. wadsworthia*, where there is more rapid growth (Figure 16C).

#### 4.4.3.4 Shifts in the central metabolism – decreased expression of genes associated with pentose phosphate pathway, TCA cycle and respiration in Bt strain 1

Several genes encoding enzymes involved in the pentose phosphate pathway were decreased in expression in Bt strain 1 in co-culture with *B. wadsworthia*; this pathway utilises glucose to generate NADPH, pentose sugars and ribose 5'-phosphate for anabolic reactions such as lipid and nucleotide biosynthesis [453]. Genes decreased in expression included glucose-6-phosphate dehydrogenase (QI0072_1443), 6-phosphogluconolactonase (QI0072_1442), 6-phosphogluconate dehydrogenase (QI0072_1445) and ribose-5-phosphate isomerase A (QI0072_2729). Additionally, genes for enzymes that catalyse the conversion of ribose-5-phosphate to glyceraldehyde-3-phosphate for glycolysis were also decreased in expression in co-culture, including transkelotases (QI0072_1990, QI0072_1989) and transaldolase (QI0072_2361). However, the gene for ribose-phosphate pyrophosphokinase, which converts the pentose phosphate pathway product ribose-5-phophate to PRPP was increased in expression (QI0072_890); this catalyses the first committed step of nucleotide biosynthesis, and likely reflects the increased growth and division of Bt strain 1 in co-culture with *B. wadsworthia*.

Several genes encoding TCA cycle enzymes were decreased in expression in Bt strain 1 in co-culture, including those of the anaerobic module where acetyl coA is

converted to 2-oxo-glutarate by citrate synthase (QI0072_2888), aconitate hydratase (QI0072_2890) and isocitrate dehydrogenase (QI0072_2889). Genes encoding enzymes which convert 2-oxo-glutarate to succinate were also decreased in expression, including dihydrolipoamide dehydrogenases (QI0072_2225, QI0072_3477) 2-oxoglutarate ferredoxin oxidoreductase beta and gamma subunits (QI0072_3562, QI0072_3563) and succinyl coA ligase alpha and beta chains (QI0072_984, QI0072_985). Overall, this pattern is similar to that observed in *B. wadsworthia*. Although both species show increased metabolic activity and/or growth in co-culture, there is a seemingly paradoxical observation of decreased expression of enzymes involved in central metabolism in both strains. Nonetheless, this suggests that *B. wadsworthia* and Bt strain 1 have a co-operative, mutualistic interaction in co-culture.

Several respiration-associated genes were decreased in expression in Bt strain 1 in co-culture with *B. wadsworthia*, including nine genes co-localised in an operon encoding $F_0$-$F_1$ ATP synthase components (Table 9). Membrane-bound $F_0$-$F_1$ ATP synthases are able to synthesise ATP as well as utilise ATP to generate a transmembrane ion gradient [454, 455], and generate the majority of cellular ATP [456]. The decreased expression of ATP synthase in Bt strain 1 under co-culture conditions together with the observation of increased growth rate implies that Bt strain 1 can access an alternative ATP source in co-culture with *B. wadsworthia*.

Table 9: Differentially expressed genes related to ATP synthase in Bt strain 1 (QI0072) in co-culture with *B. wadsworthia* (QI0013) compared to mono-culture. Differentially expressed genes were identified with a P value cut-off of 0.01, FDR ≤ 0.05 and a logFC of 1 equating to a 2-fold change in gene expression.

| Gene locus ID | Gene annotation | LogFC |
|---|---|---|
| QI0072_843 | ATP synthase beta chain (EC 3.6.3.14) | -2.08 |
| QI0072_844 | ATP synthase epsilon chain (EC 3.6.3.14) | -2.14 |
| QI0072_845 | ATP synthase protein I | -2.57 |
| QI0072_846 | ATP synthase F0 sector subunit a (EC 3.6.3.14) | -2.46 |
| QI0072_847 | ATP synthase F0 sector subunit c (EC 3.6.3.14) | -2.05 |
| QI0072_848 | ATP synthase F0 sector subunit b (EC 3.6.3.14) | -2.30 |
| QI0072_849 | ATP synthase delta chain (EC 3.6.3.14) | -2.27 |
| QI0072_850 | ATP synthase alpha chain (EC 3.6.3.14) | -2.33 |

| | | |
|---|---|---|
| QI0072_851 | ATP synthase gamma chain (EC 3.6.3.14) | -2.41 |

#### 4.4.3.5 Bt strain 1 showed increased protein synthesis in co-culture

Genes involved in protein synthesis were differentially expressed in Bt strain 1 when in co-culture with *B. wadsworthia* compared to mono-culture, where thirty-two genes were increased in expression compared to only five decreased in expression (Figure 19). Of those increased in expression, many were associated with the ribosome including 20 LSU ribosomal proteins and 5 SSU ribosomal proteins. Additionally, genes associated with translation showed increased expression such as translation initiation factor SUI1-related protein (QI0072_4693), elongation factor Ts (QI0072_4692) and ribosome recycling factor (QI0072_3072). Furthermore, genes associated with amino acid recruitment to the ribosome were also increased in expression, including asparagine synthetase (QI0072_1641), histidyl-tRNA synthetase (QI0072_2558), phenylalanyl-tRNA synthetase (QI0072_618) and valyl-tRNA synthetase (QI0072_5242). As ribosomal protein expression has been shown to be increased during rapid bacterial growth [457], this increased expression of ribosomal proteins in Bt strain 1 agrees with the phenotypic observation of increased growth and indicates favourable, nutrient-dense conditions for Bt strain 1 in co-culture with *B. wadsworthia*.

#### 4.4.3.6 Cell stress genes are decreased in expression in Bt strain 1 in co-culture

Nineteen genes associated with cell stress showed decreased expression in Bt strain 1 in co-culture with *B. wadsworthia* compared to mono-culture (Figure 19), including *opuAA*, *opuAB* and *opuAC* genes associated with glycine betaine ABC transport system. Expression of this transporter is induced by stress conditions [458], where increased glycine betaine uptake has osmoprotective effects in high osmolarity environments [459]. This supports the premise that Bt strain 1 is under more favourable growth conditions when in co-culture with *B. wadsworthia* compared to mono-culture.

#### 4.4.3.7 Altered expression of genes associated with bacterial conjugation and bacteriophage in Bt strain 1

Increased expression of genes encoding conjugative transposon proteins of the Tra system was observed in Bt strain 1 in co-culture with *B. wadsworthia*. These included *traA* (QI0072_5616), *traE* (QI0072_1699) two *traF* genes (QI0072_1700, QI0072_5649), *traG* (QI0072_1701) and *traM* (QI0072_1710). These genes allow

bacterial conjugation, a major mechanism of horizontal gene transfer where DNA is directly transferred from one bacterium to another using a pilus assembly [460]. TraA, TraE, TraF and TraG are involved in pilus assembly; TraA forms the repeating protein to form the F pilus [461], TraE and TraG are involved in tip assembly, and TraF plays a role in pilus extension [462]. TraM is thought to be associated with DNA transfer and relaxasome formation by being to the origin of transfer (oriT) [463]. Expression of the conjugative transfer proteins is usually low, as constitutive expression would entail a fitness cost [460], therefore bacteria employ various sensing strategies to induce or inhibit expression such as quorum sensing, secretion of donor-specific peptides or activation by specific nutrients [464]. This allows bacterial conjugation to occur at appropriate cell densities; the increased expression of Tra genes is perhaps expected given that Bt strain 1 achieves significantly higher abundance in co-culture with *B. wadsworthia* compared to mono-culture (Figure 16C). Horizontal gene transfer is a hallmark of bacterial evolution and contributes to the clinically important transfer of multi-drug resistance genes and pathogenicity islands both within and between species [465, 466]. If occurring in the human gut, an increased frequency of gene transfer events due to increased expression of bacterial conjugation genes by Bt strain 1 could contribute to clinically important bacterial evolution such as the transfer of multi-drug resistance.

Several phage-associated genes were differentially expressed in Bt strain 1 in co-culture with *B. wadsworthia* compared to mono-culture, including a phage protein and a putative phage-related transcriptional regulator that were increased in expression in Bt strain 1 in co-culture (QI0072_1419, QI0072_5619), and eight phage-associated genes that were decreased in expression (Table 10). This suggests that lysogenic phage induction in Bt strain 1 may be reduced when in co-culture with *B. wadsworthia*, providing more evidence in support of a symbiotic interaction between these strains where both bacteria are under reduced stress when in co-culture.

Table 10: Differentially expressed genes related to bacteriophage in Bt strain 1 (QI0072) in co-culture with *B. wadsworthia* (QI0013) compared to mono-culture. Differentially expressed genes were identified with a P value cut-off of 0.01, FDR ≤ 0.05 and a logFC of 1 equating to a 2-fold change in gene expression.

| Gene locus ID | Gene annotation | LogFC |
|---|---|---|
| QI0072_3496 | Bacteriophage lysis protein, putative | -1.23 |
| QI0072_1440 | Phage protein | -3.49 |
| QI0072_4797 | Phage protein | -3.77 |
| QI0072_700 | Phage endolysin | -1.70 |
| QI0072_702 | Prophage Clp protease-like protein | -1.57 |
| QI0072_704 | Phage protein | -1.41 |
| QI0072_705 | Bacteriophage Mu, Gene product J (IPR009752) | -1.38 |
| QI0072_706 | Phage protein Mup29 H | -1.63 |

#### 4.4.3.8 Bt strain 1 increases expression of vitamin B12 transporters in co-culture

Genes involved in vitamin B12 transport were increased in expression in Bt strain 1 in co-culture with *B. wadsworthia* including *btuB* (QI0072_2693), *btuC* (QI0072_2691), *btuD* (QI0072_2690) and two *btuF* genes (QI0072_2692, QI0072_2917). The BtuBCDF system allows bacterial vitamin B12 uptake; BtuB encodes a TonB-dependent outer membrane transporter found in Gram-negative bacteria, BtuF encodes a periplasmic B12 binding protein and BtuCD form the ABC transporter [467]. Interestingly, a previous study revealed that the *B. thetaiotaomicron* genome encodes 3 B12 transporters where each transporter exhibits a preference for a different corrinoid group; this provides *B. thetaiotaomicron* with a competitive advantage in terms of adaptability to grow with specific corrinoids [468]. The expression of Btu systems in *B. thetaiotaomicron* was also shown to be important for bacterial fitness both *in vitro* and during gut colonisation of gnotobiotic mice [468]; this therefore suggests that the higher expression of vitamin B12 transporters by Bt strain 1 in co-culture with *B. wadsworthia* enhances bacterial fitness and could permit this strain to colonise the human gut more successfully compared to mono-culture grown strains. It may also imply that *B. wadsworthia* is able to produce vitamin B12, although this is yet to be demonstrated. This further highlights the mutualistic interaction between Bt strain 1 and *B. wadsworthia* in co-culture.

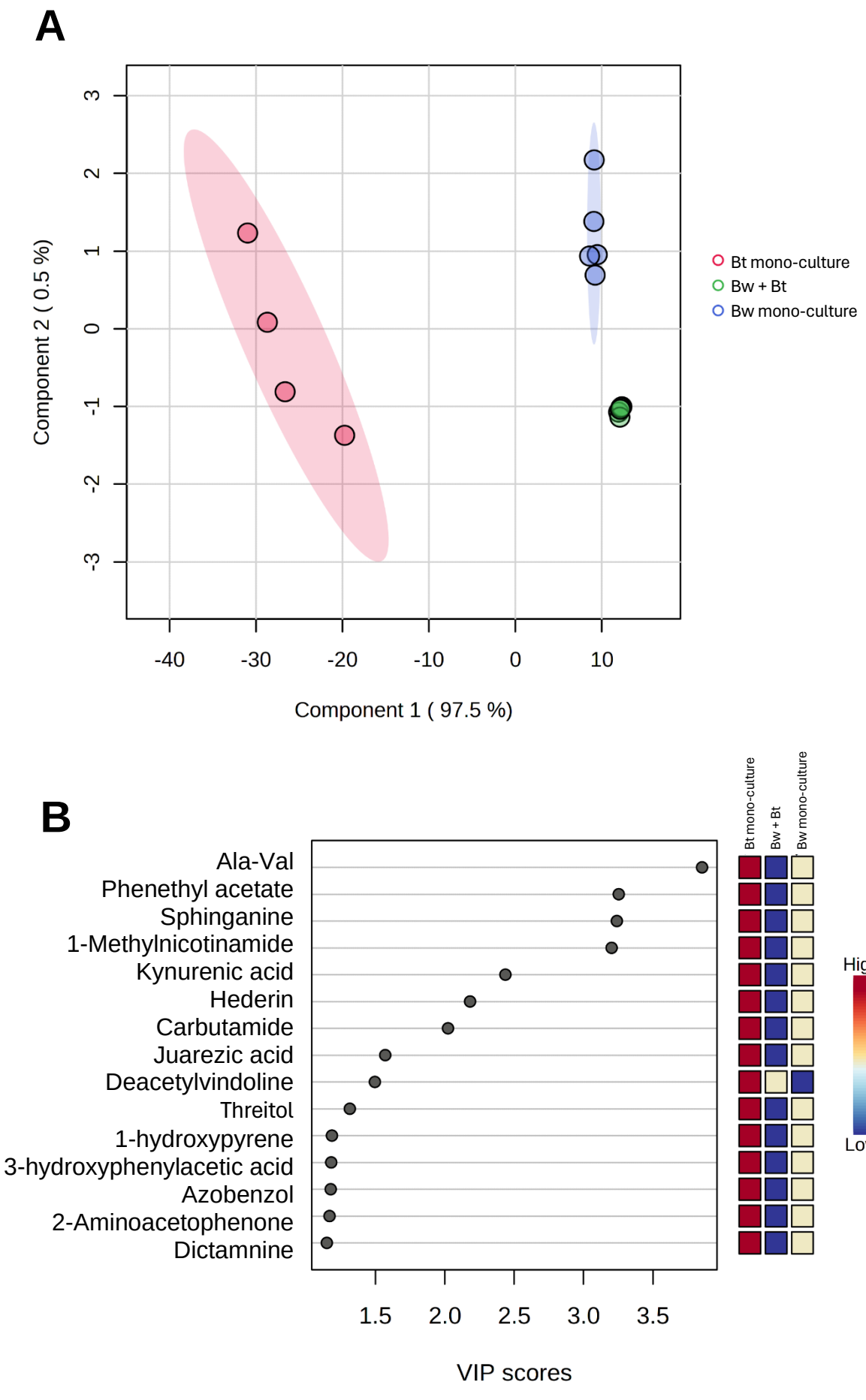
### 4.4.4 Analysis of the endometabolome of *B. wadsworthia* and Bt strain 1

The endometabolome of the *B. wadsworthia* and Bt strain 1 co-culture was analysed at 8 h post-inoculation, alongside mono-cultures. In the PLS-DA plot the Bt strain 1 mono-culture condition clustered distinctly from the *B. wadsworthia* mono-culture and co-culture conditions, with the first and second principal components explaining 97.5% and 0.5% of the data variance respectively (Figure 20A).

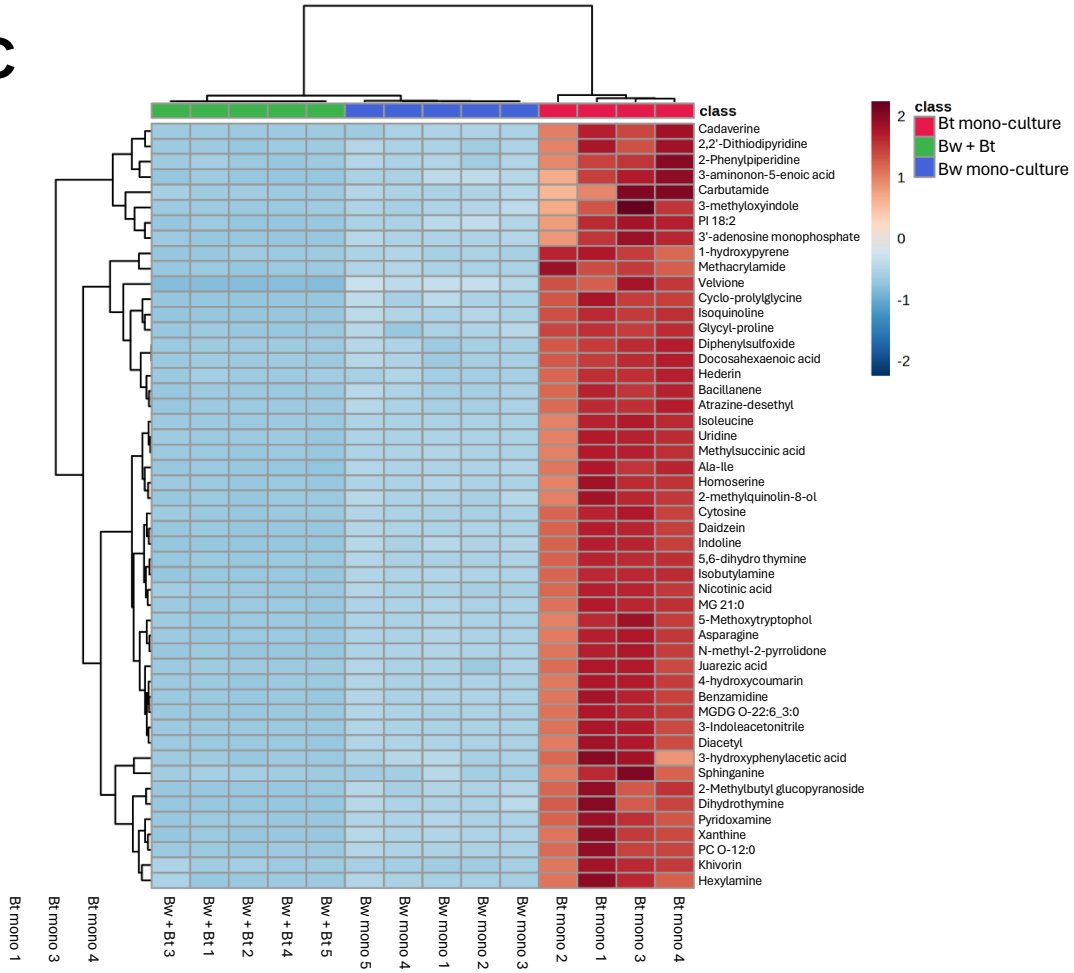
The Variable Importance in Projection (VIP) scores showed that the top 15 compounds contributing to differences between conditions were relatively high abundance in the Bt strain 1 mono-culture and relatively low abundance in the co-culture (Figure 20B); this suggests that these compounds are produced by Bt strain 1 and either 1) not produced by Bt strain 1 in co-culture with *B. wadsworthia* or 2) consumed by *B. wadsworthia*. The highest scoring compound was Ala-Val, a dipeptide demonstrated to be produced in *E. coli* specifically engineered for dipeptide synthesis [469] (Figure 20B). Phenethyl acetate was also in relatively high abundance in the Bt strain 1 endometabolome compared to the other conditions (Figure 20B); this ester has been described as a fermentation product during yeast maturation of cheese [470]. Sphinganine was also one of the highest scoring compounds in relatively high abundance in Bt strain 1 (Figure 20B); production of this compound by *Bacteroides* strains in the gut was reported previously [471]. A nicotinic compound 1-Methylnicotinamide was also in relatively high abundance in Bt strain 1 (Figure 20B); this metabolite was found to be positively correlated with *Limosilactobacillus mucosae* abundance in the piglet gut, a bacterial strain associated with gastrointestinal health [472]. Kynurenic acid was a highly scoring compound, indicating that this compound significantly contributes to the differences between conditions (Figure 20B). Kynurenic acid is a product of tryptophan degradation in mammals [473], however *E. coli* has been demonstrated to produce kynurenic acid from L-kynurenine in the rat small intestine [474] and in humans, kynurenic acid concentration is high in the distal colon relative to other body sites, suggesting that the gut microbiota may also produce this compound [475]. Kynurenic acid was in relatively low abundance within the cells in the co-culture compared to Bt strain 1 mono-culture (Figure 20B), suggesting that tryptophan metabolism via this pathway is lessened in co-culture. The heatmap displaying the top 50 differentially abundant metabolites showed clear differences

between the conditions, most notably when Bt strain 1 mono-culture is compared to the other culture conditions (Figure 20C).

Specific metabolites of interest based on the gene expression data obtained for the cultures were manually curated based upon the transcriptomic data and displayed as a heatmap (Figure 20D). Branched chain amino acids (BCAAs) were implicated as metabolites of interest by the transcriptomic data, which revealed differential expression of Liv system genes encoding ABC transporters for BCAAs in *B. wadsworthia* (Figure 17); these gene expression changes could reflect altered bioavailability of leucine, isoleucine and valine to *B. wadsworthia* in the co-culture compared to mono-culture. Indeed, valine and isoleucine were detected in the endometabolome in all culture conditions, in addition to dipeptides of leucine including threonylleucine, prolylleucine, N-leucyl-leucine and leucyl-leucine. In all cases, relative abundance was high in Bt strain 1 mono-culture, lower in *B. wadsworthia* mono-culture and the lowest abundance in the co-culture condition (Figure 20D). Taken together, this suggests lower bioavailability of branched chain amino acids in the endometabolome of *B. wadsworthia* and Bt strain 1 in co-culture at 8 h compared to mono-cultures.


A
Component 2 ( 0.5 %)
3
2
1
0
-1
-2
-3
-40
-30
-20
-10
0
10
Component 1 ( 97.5 %)
Bt mono-culture
Bw + Bt
Bw mono-culture
B
Ala-Val
Phenethyl acetate
Sphinganine
1-Methylnicotinamide
Kynurenic acid
Hederin
Carbutamide
Juarezic acid
Deacetylvindoline
Threitol
1-hydroxypyrene
3-hydroxyphenylacetic acid
Azobenzol
2-Aminoacetophenone
Dictamnine
1.5
2.0
2.5
3.0
3.5
VIP scores
Bt mono-culture
Bw + Bt
Bw mono-culture
High
Low

C


class
Cadaverine
2,2'-Dithiodipyridine
2-Phenylpiperidine
3-aminonon-5-enoic acid
Carbutamide
3-methyloxyindole
PI 18:2
3'-adenosine monophosphate
1-hydroxypyrene
Methacrylamide
Velvione
Cyclo-prolylglycine
Isoquinoline
Glycyl-proline
Diphenylsulfoxide
Docosahexaenoic acid
Hederin
Bacillanene
Atrazine-desethyl
Isoleucine
Uridine
Methylsuccinic acid
Ala-Ile
Homoserine
2-methylquinolin-8-ol
Cytosine
Daidzein
Indoline
5,6-dihydro thymine
Isobutylamine
Nicotinic acid
MG 21:0
5-Methoxytryptophol
Asparagine
N-methyl-2-pyrrolidone
Juarezic acid
4-hydroxycoumarin
Benzamidine
MGDG O-22:6_3:0
3-Indoleacetonitrile
Diacetyl
3-hydroxyphenylacetic acid
Sphinganine
2-Methylbutyl glucopyranoside
Dihydrothymine
Pyridoxamine
Xanthine
PC O-12:0
Khivorin
Hexylamine
Bt mono 1
Bt mono 3
Bt mono 4
Bw + Bt 3
Bw + Bt 1
Bw + Bt 2
Bw + Bt 4
Bw + Bt 5
Bw mono 5
Bw mono 4
Bw mono 1
Bw mono 2
Bw mono 3
Bt mono 2
Bt mono 1
Bt mono 3
Bt mono 4
2
1
0
-1
-2
class
Bt mono-culture
Bw + Bt
Bw mono-culture

D

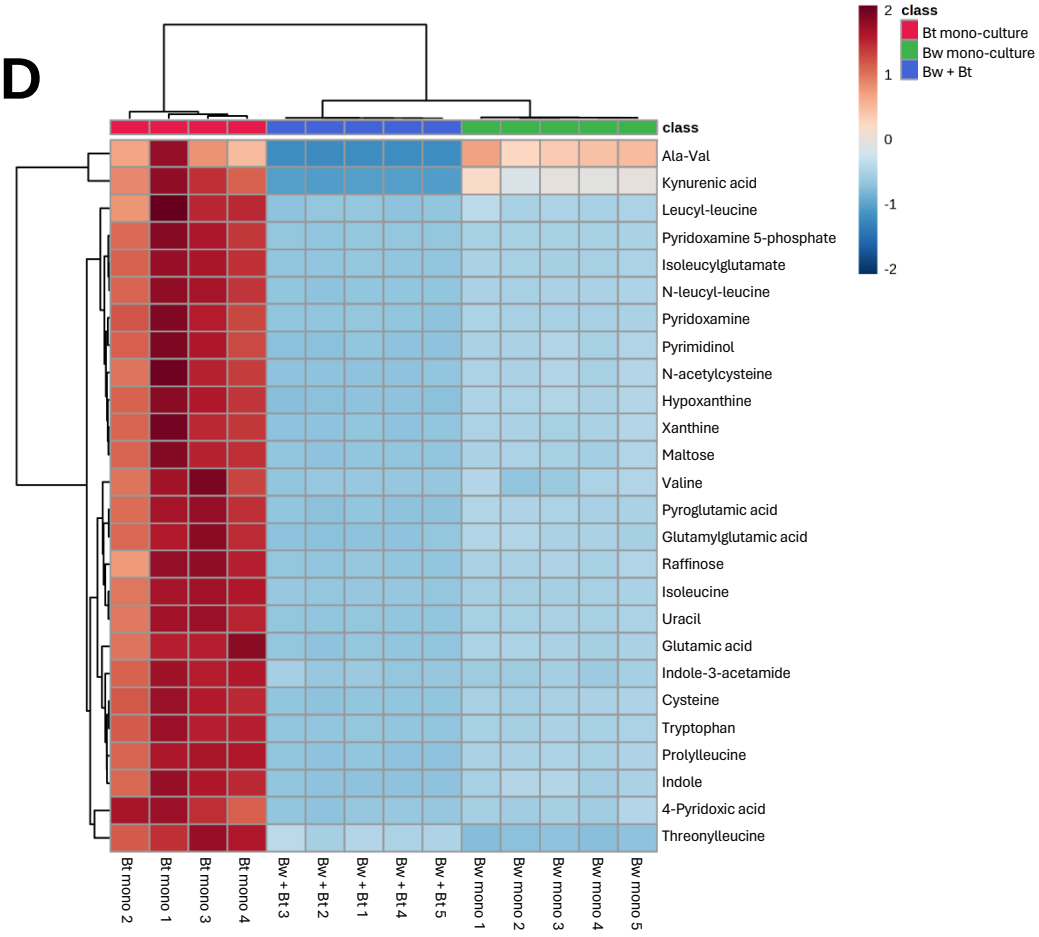

class
Ala-Val
Kynurenic acid
Leucyl-leucine
Pyridoxamine 5-phosphate
Isoleucylglutamate
N-leucyl-leucine
Pyridoxamine
Pyrimidinol
N-acetylcysteine
Hypoxanthine
Xanthine
Maltose
Valine
Pyroglutamic acid
Glutamylglutamic acid
Raffinose
Isoleucine
Uracil
Glutamic acid
Indole-3-acetamide
Cysteine
Tryptophan
Prolylleucine
Indole
4-Pyridoxic acid
Threonylleucine
Bt mono 2
Bt mono 1
Bt mono 3
Bt mono 4
Bw + Bt 3
Bw + Bt 2
Bw + Bt 1
Bw + Bt 4
Bw + Bt 5
Bw mono 1
Bw mono 2
Bw mono 3
Bw mono 4
Bw mono 5
2
1
0
-1
-2
class
Bt mono-culture
Bw mono-culture
Bw + Bt

Figure 20: Comparisons of the endometabolome of *B. wadsworthia* and Bt strain 1 in co-culture (Bw + Bt) with mono-cultures (Bw_mono, Bt_mono).

A) PLS-DA plot of metabolites acquired from samples via untargeted LCMS in positive ion mode. B) The top compounds ranked based on the Variable Importance in Projection (VIP) scores. The coloured boxes on the right indicate the relative concentrations of the corresponding metabolite in each group. C) Heatmap displaying relative abundance of top 50 differentially abundant metabolites in the culture conditions. D) Specific metabolites of interest manually curated from the compound list detected via untargeted LCMS.

### 4.4.5 Utilisation of glutamate and glutamine was increased in the co-culture

The transcriptomic data suggested that glutamine utilisation by *B. wadsworthia* is increased in co-culture, as this is used as substrate for pyridoxal 5'-phosphate synthase during vitamin B6 biosynthesis, carbamoyl phosphate during pyrimidine biosynthesis and amidophosphoribosyltransferase (purF) during purine biosynthesis, all of which show increased gene expression in co-culture. A logical next step is to query where glutamine is sourced to facilitate these processes; indeed, expression of glutamine ABC transporter permease protein *glnP* (QI0013_1829) was increased in expression in *B. wadsworthia* in co-culture, along with 3 other ABC transporter permease proteins (QI0013_2582, QI0013_1696, QI0013_1694) and 3 ATP-binding proteins (QI0013_1826, QI0013_2581, QI0013_1695) of cluster 3, which facilitate uptake of polar amino acids including glutamine and glutamate [476]. Interestingly, glutamine transporters are required for virulence of group B streptococci and *S. enterica* serovar Typhimurium [477]. This suggests that there is increased extracellular availability of glutamine leading to increased expression of transporters allowing uptake under co-culture conditions, however this is speculative.

Interestingly, glutamine utilisation also appeared to be increased in Bt strain 1 in co-culture compared to mono-culture. Expression of genes encoding GMP synthase (QI0072_3179), imidazole glycerol phosphate synthase (QI0072_2039, QI0072_2041) and asparagine synthetase (QI0072_1641) were increased in Bt strain 1 in co-culture, all of which are capable of converting glutamine to glutamate via different pathways [478-480]. Additionally, glutamate synthase large chain (QI0072_1639) and small chain (QI0072_1640) were increased in expression, which uses glutamine to synthesise glutamate. On the contrary, a gene encoding a glutamine synthetase type III was decreased in expression in Bt strain 1 in co-culture with *B. wadsworthia* compared to mono-culture (QI0072_1649); therefore the source of glutamine for increased utilisation by both *B. wadsworthia* and Bt strain 1 under co-culture conditions is unclear. Glutamate utilisation also appeared to be increased in Bt strain 1 in co-culture; dihydrofolate synthase was increased in expression which utilises glutamate in folate biosynthesis [481]. Overall, there appears to be a higher metabolic demand for glutamine from both species in co-culture compared to the respective mono-cultures, although the source of glutamine as a substrate to support this demand is unclear.

Expression of both glutamate decarboxylase and glutaminase were significantly decreased in Bt strain 1 in co-culture with *B. wadsworthia* compared to mono-culture (QI0072_3377, QI0072_3378); these enzymes catalyse the conversion of glutamine to glutamate to gamma-amino butyric acid (GABA) [482]. Alterations in faecal GABA concentrations have been implicated in modulating mental health disorders in humans [482] Interestingly, 96% of *Bacteroides spp*. in the gut are able to produce GABA from glutamine, which could contribute to the regulation of the GABAergic system in the gut [482]. In this case, it appears that glutamate conversion to GABA by Bt strain 1 is decreased when in co-culture with *B. wadsworthia*, implying that GABA production is lower in the co-culture. This could have implications for the human gut, as lower abundance of GABA-producing species and lower faecal GABA has been associated with depressive disorders [483].

In summary, the transcriptomic data suggested that glutamine utilisation was higher in both bacteria under co-culture conditions compared to respective mono-cultures, and glutamate utilisation by Bt strain 1 appears to be greater in the co-culture condition compared to mono-culture. Although glutamine was detected in the endometabolome, the $RSD_{QC}$ was >20% and therefore excluded from further analysis. However, glutamic acid (of which the ionic form is glutamate) and pyroglutamic acid (a cyclic lactam of glutamic acid) showed high abundance in Bt strain 1 mono-culture relative to *B. wadsworthia* mono-culture, and the lowest abundance in the co-culture condition (Figure 20D). Similar patterns in relative abundance were observed for glutamylglutamic acid (glu-glu dipeptide) and isoleucylglutamate (ile-gly dipeptide) (Figure 20D). This aligns with the transcriptomic dataset to demonstrate higher glutamate utilisation in the co-culture, as the relative abundance of this metabolite per $10^9$ cells is lower under co-culture conditions compared to respective mono-cultures.

### 4.4.6 Reduced intracellular abundance of sulfated amino acids in co-culture

Sulfated amino acids cysteine and N-acetylcysteine were less abundant per cell in the co-culture compared to either of the mono-cultures (Figure 20D). Bt strain 1 showed reduced expression of cysteine synthase (QI0072_3844) which catalyses the conversion of O3-acetyl-L-serine and $H_2S$ to L-cysteine and acetate, utilising pyridoxal-5'-phosphate (vitamin B6) as a co-factor [445]; the metabolomic data supports this observation.

### 4.4.7 Vitamin B6 in the endometabolome of the co-culture

Transcriptomic analysis alluded to alterations in vitamin B6 metabolism in the co-culture, where *B. wadsworthia* showed increased expression of genes associated with vitamin B6 biosynthesis and Bt strain 1 showed decreased expression compared to respective mono-cultures. Several vitamers were detected via endometabolome analysis, including pyridoxamine 5'-phosphate (PNP) and pyridoxamine which are produced during vitamin B6 salvage [484] and 4-pyridoxic acid, the breakdown product of pyridoxal 5'-phosphate (PLP), the bioactive form of vitamin B6 [485]. These three metabolites showed decreased abundance per $10^9$ cells in the co-culture compared to the mono-cultures, and the bioactive form of vitamin B6 was not detected (Figure 20D); this suggests that the vitamin B6 produced by *B. wadsworthia* was utilised in the co-culture.

### 4.4.8 Decreased availability of xanthine and hypoxanthine in the co-culture

The transcriptomic data revealed alterations to xanthine and uracil uptake in the two species in co-culture; *B. wadsworthia* showed decreased xanthine-uracil permease expression in co-culture (QI0013_799), whereas Bt strain 1 showed increased expression of the genes encoding xanthine and uracil permeases (QI0072_5381, QI0072_818). Additionally, Bt strain 1 showed increased expression of uracil phosphoribosyltransferase (QI0072_3515), which catalyses the conversion of uracil and phosphoribosylpyrophosphate (PRPP) to UMP [486], as well as uridine kinase (QI0072_406), which catalyses the conversion of uridine to UMP [487]. Together, this suggests enhanced uptake and utilisation of uracil and xanthine as part of the pyrimidine salvage pathway in Bt strain 1 in co-culture. Furthermore, expression of hypoxanthine-guanine phosphoribosyltransferase was increased (QI0072_5276); this enzyme converts PRPP and either hypoxanthine or guanine to either IMP or GMP respectively as part of purine salvage [488]. Therefore, Bt strain 1 appears to increase purine and pyrimidine salvage to support nucleotide synthesis in co-culture with *B. wadsworthia* compared to mono-culture. This suggests altered bioavailability of these nitrogenous compounds in the co-culture; indeed, xanthine, hypoxanthine and pyrimidinol were relatively high abundance within the cells of Bt strain 1 in mono-culture, lower abundance in *B. wadsworthia* mono-culture and the lowest abundance in the co-culture endometabolome (Figure 20D). The transcriptomic and metabolomic data corroborate a decreased intracellular bioavailability of xanthine and hypoxanthine

in the co-culture, which likely leads to the decreased expression of xanthine dehydrogenases in *B. wadsworthia* within the co-culture.

### 4.4.9 Decreased abundance of raffinose and maltose in co-culture

The transcriptomic analysis of Bt strain 1 showed that starch utilisation system (sus) genes were decreased in expression under co-culture conditions. Interestingly, the presence of maltose is required for Sus gene expression [489]; the metabolomic data reveals that the intracellular abundance of maltose is relatively low in the co-culture compared to Bt strain 1 mono-culture (Figure 20D), possibly explaining why these genes are downregulated under co-culture conditions. Additionally, *B. thetaiotaomicron* possesses α-galactosidases for raffinose degradation [490]; in Bt strain 1, three α-galactosidases were decreased in expression in co-culture with *B. wadsworthia* compared to mono-culture. Raffinose relative abundance was much lower in the co-culture endometabolome compared to Bt strain 1 mono-culture (Figure 20D). This therefore aligns with the gene expression data, suggesting that raffinose utilisation by Bt strain 1 occurred earlier in the co-culture compared to mono-culture, highlighting that the cells are more metabolically active.

### 4.4.10 Alterations to Bt strain 1's tryptophan and indole metabolism in co-culture

Differences in tryptophan, indole and indole-3-acetamide relative concentrations were also observed in the endometabolome of Bt strain 1 and *B. wadsworthia* mono-cultures compared with co-culture (Figure 20D). Indole-producing members of the human gut microbiota include *E. coli*, *Proteus vulgaris*, *Paracolobactrum coliforme*, *Achromobacter liquefaciens* and *Bacteroides spp*. [491]. These strains are able to degrade tryptophan to indole, pyruvate and ammonia via the enzyme tryptophanase (TnaA), the activity of which is induced by tryptophan and inhibited by glucose [491]. Interestingly, indole production is commonly observed in *B. thetaiotaomicron* [306], and Bt-derived indole has been shown to inhibit virulence in EPEC and *Vibrio cholerae* by inhibiting T3SS expression [306]. Given that *B. wadsworthia* does not produce indole [492], it is assumed that indole and indole derivatives present in these culture conditions are derived from *B. thetaiotaomicron*. The relative abundance of these metabolites is lower in the co-culture per $10^9$ cells compared to the Bt strain 1 mono-culture (Figure 20D), although this may simply reflect the higher number of cells present in the co-culture as opposed to changes in Bt strain 1's tryptophan consumption and indole

production. Therefore, the indole and tryptophan relative abundances were determined in the Bt strain 1 mono-culture and co-culture per $10^9$ Bt strain 1 cells only; this showed a decreased relative abundance of indole in the endometabolome of the co-culture compared to Bt strain 1 mono-culture (Figure 21). This suggests that *B. wadsworthia* either utilises the Bt-derived indole or utilises the tryptophan directly meaning less is available for Bt strain 1 (Figure 21). The transcriptomic data revealed that expression of a tryptophan-specific transport protein in *B. wadsworthia* is increased in co-culture compared to mono-culture (QI0013_2980), indicating that *B. wadsworthia*'s uptake of tryptophan is increased, and a tryptophanyl-tRNA synthetase (EC 6.1.1.2) gene was also increased in expression (QI0013_2967) which adds tryptophan to its cognate tRNA molecule during translation [493]. Together, the metabolomic data showing decreased concentrations of both tryptophan, indole and indole derivatives in the co-culture compared to Bt strain 1 mono-culture (Figure 20D, Figure 21) and the transcriptomic data suggests that under co-culture conditions *B. wadsworthia*'s tryptophan uptake and incorporation into new proteins is increased compared to respective mono-culture, meaning less tryptophan is available for indole production by Bt strain 1. Indeed, although tryptophanase (*tnaA*) was not differentially expressed in Bt strain 1, a trend towards decreased expression was observed in the co-culture compared to mono-culture (Figure 22).

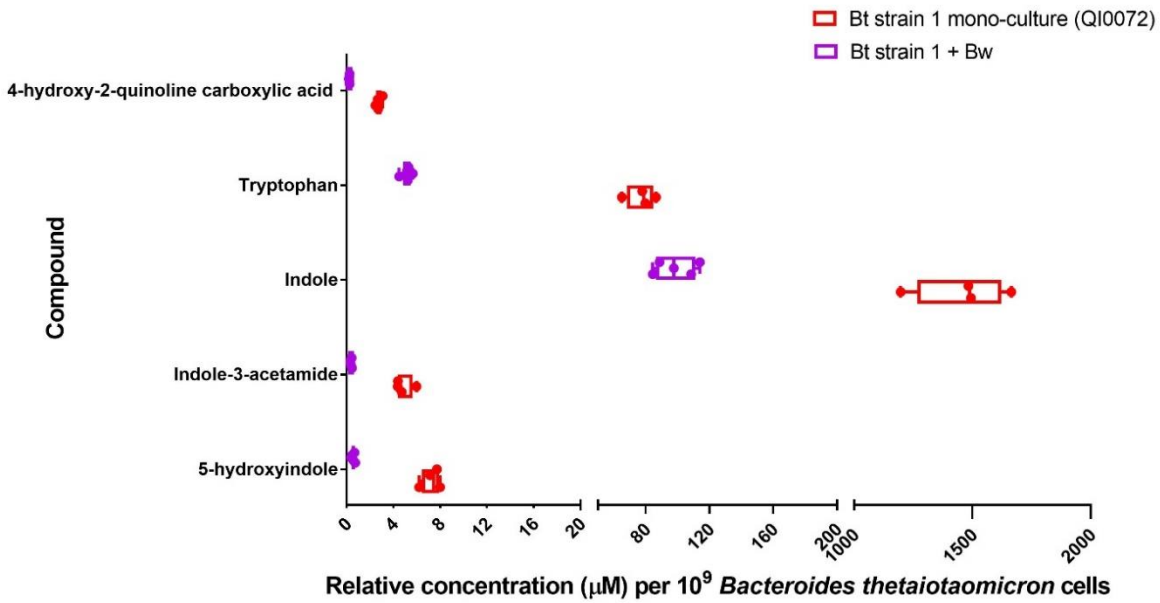



Figure 22: Relative intracellular concentrations of compounds related to tryptophan metabolism.

Compound concentration is standardised to Bt strain 1 cell counts. Each point represents a technical culture replicate. Box and whisker plots show line at mean, box at 25$^{th}$ – 75$^{th}$ percentile, whiskers to minimum and maximum.

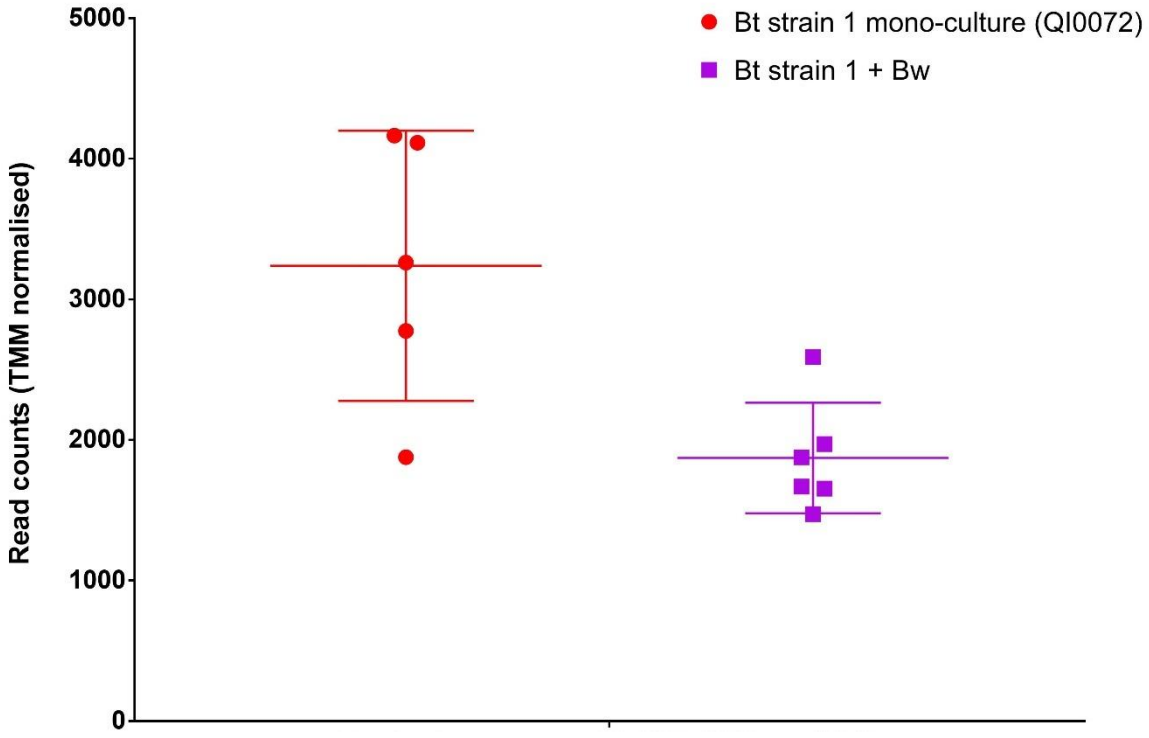



Figure 21: Relative expression of the tryptophanase gene (*tnaA*) in Bt strain 1 in mono-culture and co-culture with *B. wadsworthia*.

TMM-normalised gene expression data obtained via RNA-seq of mono-cultures and co-cultures. Each point represents a technical culture replicate. Error bars show standard deviation.

## 4.5 Summary

In *B. wadsworthia*, the transcriptomic data revealed decreased expression of genes required for sulfite production from taurine, thiosulfate and sulfolactate (Figure 18A), suggesting an alternative source of sulfite was present in the co-culture. Integrating transcriptomic and genomic data from both species, we hypothesise that Bt strain 1 provides *B. wadsworthia* with APS or PAPS, which *B. wadsworthia* converts to sulfite. Additionally, uptake of lactate was increased in *B. wadsworthia*, which is likely used as an electron donor during dissimilatory sulfite reduction. Taken together, we propose a putative APS-mediated cross-feeding to explain the sulfidogenic interaction between *B. wadsworthia* and Bt strain 1 (Figure 18A, 18B), where *B. wadsworthia* preferentially utilises Bt strain 1-derived APS as a sulfite source for dissimilatory sulfite reduction to yield $H_2S$ as opposed to degrading taurine to isethionate. As Bt strain 1 encodes the necessary enzymes for sulfate conversion to APS, and *B. wadsworthia* encodes a putative enzyme capable of converting APS to sulfite, the full enzymatic pathway allowing conversion of sulfate to $H_2S$ is only complete in the co-culture condition. This hypothesis is yet to be tested, but if proved true this would have important implications for our understanding of *B. wadsworthia*, the interactions of $H_2S$-producing bacteria with other gut microbiota members, as well as for the wider sulfate metabolism in the human gut. In conclusion, the increased $H_2S$ production by *B. wadsworthia* in addition to the reduced $H_2S$ utilisation by Bt strain 1 due to decreased cysteine synthase expression underpins the phenotypic observation of increased $H_2S$ in the co-culture.

In addition to changes to sulfur metabolism, factors influencing *B. wadsworthia*'s virulence appears to be affected during co-culture with Bt strain 1 compared to mono-culture as the expression of the virulence factors urease, T6SS and T3SS was decreased [412, 422, 429]. The observation of reduced T3SS expression in *B. wadsworthia* in co-culture with Bt strain 1 could be explained by the presence of indole; indole production is commonly observed in *B. thetaiotaomicron* [306], and Bt-derived indole has been shown to inhibit virulence in EPEC and *Vibrio cholerae* by inhibiting T3SS expression [306]. Urease activity varies between *B. wadsworthia* strains [31], with approximately 75% of strains being urease positive [24]. Although both urease-positive and urease-negative strains have been associated with clinical disease [145], genomic differences between strains have not been investigated beyond confirmation that the 16S rRNA gene sequences

remain highly similar [494]. Furthermore, the impact of urease activity in *B. wadsworthia* pathogenesis has not been described. The expression of urease in *B. wadsworthia* and the resulting impact of this upon virulence could be included in future work to better understand the factors involved in *B. wadsworthia* virulence and pathogenesis. Here, it could be hypothesised that decreased expression of urease under co-culture conditions compared to mono-culture reduces *B. wadsworthia*'s pro-inflammatory and endotoxic properties. In co-culture with Bt strain 1, the *B. wadsworthia* transcriptome revealed increased vitamin B6 biosynthesis, protein and nucleotide synthesis, and decreased universal stress proteins which overall alludes to more favourable conditions for *B. wadsworthia* in co-culture.

We observed decreased indole production by Bt strain 1 under co-culture conditions; this is potentially due to decreased tryptophan availability to Bt strain 1, as *B. wadsworthia* showed increased expression of genes for tryptophan import and utilisation. This could have important implications if occurring in the human gut; microbial indole production in the human gut ameliorates intestinal inflammation [495, 496], and modulates intestinal IL-22 production which encourages antimicrobial peptide production, preventing colonisation by pathogens [497]. The overall shift in the co-culture towards decreased indole production and increased $H_2S$ production could have notable pro-inflammatory effects if occurring in the human gut environment.

# Chapter 5

## 5. *B. wadsworthia* in the human gut environment

## 5.1 Introduction

In previous chapters, we showed that *B. wadsworthia*'s $H_2S$ production is significantly increased in co-culture with *B. thetaiotaomicron*, whereas co-culture with either *E. faecium* or an enrichment of *E. faecium* and *B. thetaiotaomicron* (Ef-Bt) showed a significant inhibitory effect on *B. wadsworthia*'s growth. This work was performed using simplified mono-culture and co-cultures in optimal growth media. Establishing whether these microbial interactions could also occur in the human gut is a logical next step, given that these gut-derived strains are common members of the gut microbiota in the majority of healthy individuals [23, 213, 240]. To investigate this, we aimed to represent the complex gut microbiota using simplified *in vitro* models.

Firstly, we developed methods for tracking the metabolism of *B. wadsworthia* in the context of the human faecal matrix diluted in rich bacterial growth media. We focused on two metrics of *B. wadsworthia*'s metabolism which can be readily measured: taurine utilisation and $H_2S$ production. In subsection 1, the method development of a Hydrophilic interaction chromatography-mass spectrometry (HILIC-MS) method for quantifying the sulfated amino acids taurine, cysteine, and homocysteine is described. Taurine is utilised directly by *B. wadsworthia* during dissimilatory sulfite reduction to produce $H_2S$ [51]. An ability to quantify other sulfated amino acids was also of interest as cysteine is an important proteinogenic amino acid that is formed from $H_2S$ during assimilatory sulfate reduction in the gut microbiota [44], and can be degraded to $H_2S$ via desulfhydration [498]. Additionally, both homocysteine and cysteine can form disulfide bridges *in vivo* resulting in $H_2S$ production [499]. Furthermore, a study of the rumen digesta showed extensive microbial degradation of cysteine and methionine to $H_2S$ [500]. The accurate quantification of these sulfated amino acids can therefore provide wider insight into sulfur metabolism and sources of $H_2S$ in the human gut environment. We also investigated the use of a colourimetric methylene blue assay [272] for quantifying $H_2S$ in experimental samples containing the human faecal matrix diluted with either PBS or complex growth media used in *in vitro* colon models [501].

Secondly, we investigated the impact of *B. wadsworthia* in the gut environment as described in subsection 2 by utilising two *in vitro* models, one involving human cell

culture and one based on *ex vivo* faecal culture. The first model used differentiated Caco-2 cell mono-layers grown in transwells to represent the human gut colonic epithelium. Such models have been extensively used for studying host-microbiota interactions [6, 502] and host responses to microbial metabolites [495, 503, 504]. We applied cell-free supernatant of *B. wadsworthia* mono-culture and co-culture with *B. thetaiotaomicron* strain QI0072 (Bt strain 1), and *B. wadsworthia* cells alone and in combination with probiotic *Lactiplantibacillus plantarum* strains EKN4 and OOY9 to the Caco-2 mono-layers. The probiotic health benefits of *L. plantarum* strains have been well-described [505], and supplementation of a related *Lacticaseibacillus rhamnosus* strain was shown to reduce *B. wadsworthia*'s growth, and in turn reduce inflammation in the mouse gut [206]. The impact on barrier integrity was measured using Transepithelial Electrical Resistance (TEER). We hypothesised that the high levels of $H_2S$ found in the cell-free supernatant of *B. wadsworthia* and Bt strain 1 co-culture would decrease barrier integrity due to oxidative damage to the cells [101, 102].

The second model comprised a simplified microbiological representation of the gut microbiota utilising human faeces obtained via the QIB Colon Model study in an *ex vivo* faecal culture. Traditional *in vitro* microbiological models for studying the interactions of the gut microbiota comprise a complex rich media designed to replicate the gut contents and are seeded with human faeces to recapitulate the gut microbiota [501, 506, 507]. We used Complex Intestinal Media, which has previously been demonstrated to support the growth of several keystone taxa in the human gut [508], and contains fermentative compounds including starch, arabinogalactan, xylo-oligosaccharide and inulin [508]. The media was inoculated with human faeces to recapitulate a complex gut microbiota *in vitro*, where experiments were performed in triplicate using faeces from three different donors. We also aimed to supplement a representative dose of taurine; based upon an oral dose of 4000 mg taurine as found in 2 standard "energy drinks" [183, 509], taurine colonic bioavailability of approximately 7% [510] and human colonic liquid volume of 400 mL [511], 5.6 mM taurine was determined as a physiologically relevant concentration to supplement to the *in vitro* cultures. We hypothesised that the microbe-microbe interactions previously observed in simplified co-cultures would be recapitulated in the more complex model used here, where *B.*

*wadsworthia*'s $H_2S$ production would be increased in the presence of Bt strain 1, and growth inhibited in the presence of the Ef-Bt culture.

## 5.2 Objectives

In this chapter, we aimed to investigate the growth and $H_2S$ production of *B. wadsworthia* in gut-representative *in vitro* experimental models. Firstly, as described in subsection 1, we aimed to develop methods for quantifying sulfated amino acids and $H_2S$ concentration in samples obtained from *in vitro* microbiological models, and within the human faecal matrix, in order to achieve robust quantification of these compounds of interest within complex sample matrices. Secondly, as described in subsection 2, we investigated the impact of *B. wadsworthia* in the gut environment using two *in vitro* models. The first model used Caco-2 mono-layers to investigate the impact of *B. wadsworthia* cells and cell-free supernatant on epithelial barrier integrity using TEER measurement. We hypothesised that the high levels of $H_2S$ produced in *B. wadsworthia* and Bt strain 1 co-culture would decrease epithelial barrier integrity. The second model comprised *ex vivo* faecal cultures from three human donors, where *B. wadsworthia* was supplemented alone or in a 1:1 ratio with either the $H_2S$-promoting Bt strain 1, or the inhibitory Ef-Bt enrichment. The $H_2S$ concentration, total bacterial cell counts and metagenomic composition were determined at 0- and 8 h post-inoculation. We hypothesised that the microbe-microbe interactions observed in simplified co-cultures would also be observed in the more complex microbiological model.

## 5.3 Materials and Methods

### 5.3.1 Subsection 1 methods

#### 5.3.1.1 Preparation of faecal matrices

Ten grams of fresh faecal sample obtained from a healthy volunteer (donor ID CM001) was diluted 1:10 in anaerobic phosphate buffered saline (PBS) and homogenised using a Stomacher 400 (Seward) at 230 rpm for 45 s within 4 h of stool collection to prepare faecal water. Faecal water was also diluted 1:10 with colon model media to simulate samples obtained from *in vitro* microbiological fermentation models seeded with human faeces [501]. Colon model media was prepared as follows: peptone water 2 g/L, yeast extract 2 g/L, NaCl 0.1 g/L, $K_2HPO_4$ 0.04 g/L, $KH_2PO_4$ 0.04 g/L, $MgSO_4.7H_2O$ 0.01 g/L, $CaCl_2.2H_2O$ 0.01 g/L, $NaHCO_3$ 2 g/L, L-cysteine hydrochloride 0.5 g/L, bile salts 0.5 g/L, tween-80 2 mL/L, hemin 0.02 g/L, vitamin K 10 µL/L, glucose 10 g/L pH 7.0 [501]. Aliquots of the two matrices were then prepared for analysis.

#### 5.3.1.2 Quantification of sulfated amino acids in faecal matrices

##### 5.3.1.2.1 Preparation of stock solutions

For external standards, analytical grade taurine, L-cysteine, homocysteine and methionine were sourced from Sigma. A stock solution containing 4 mM analytical grade taurine, L-cysteine, homocysteine and methionine was prepared in 0.1 M HCl. For internal standards, $^{13}C$-labelled taurine and L-cysteine sourced from CK Isotopes were prepared as a 1 mM stock in 0.1 M HCl. Stock solutions were filtered through a 0.22 µm membrane, and 500 µL aliquots were stored at -20°C.

##### 5.3.1.2.2 Preparation of samples for HILIC-MS analysis

Standard curves were prepared using 0.1 M HCl as reference, fresh faecal water to investigate the faecal matrix effect, or fresh faecal water diluted 1:10 with colon model media to simulate samples taken from colon model. To prepare the standard curve, 100 µL aliquots were prepared of each matrix and spiked with external and internal standard solutions and diluted to 200 µL using 0.1 M HCl. Solvent extraction was performed by diluting samples 1:1 with Solvent A (20 mM ammonium formate, 0.5% formic acid in $H_2O$). Litmus paper was used to check the pH was ≤4 in all samples. Samples were centrifuged at 14,000 x g for 10 min and supernatant was filtered through 0.22 µm membrane prior to transfer into LCMS

vials for analysis. The final standard curve samples contained 0 – 200 µM external standard, 50 µM internal standard.

#### 5.3.1.2.3 LC-MS

A HILIC-MS method was used modified from Prinsen *et al*. [512] (Table 15). All samples were injected 3 times using injection volume of 1 µL. Chromatographic separation was achieved using a Acquity UPLC-system (Waters, Manchester, UK) with a BEH Amide 150 x 2.1, 1.7 column set to 35˚C [512]. A flow-rate of 0.5 mL/min was used with a gradient of solvent A and solvent B (Table 16).

Table 11: Settings used during HILIC-MS analysis of four sulfated amino acids [512].

| Amino acid | Retention time (min) | MRM-transition (m/z) | Cone voltage (V) | Collision energy (V) | Internal standard |
|---|---|---|---|---|---|
| Taurine | 4.6 | 128.0 → 110.0 | 26 | 11 | 1,2-$^{13}$C-taurine |
| L-Cysteine | 4.9 | 122.0 → 76.0 | 12 | 10 | 1-$^{13}$C-L-cysteine |
| Methionine | 3.5 | 150.0 →104.0 | 16 | 10 | 1-$^{13}$C-L-cysteine |
| Homocysteine | 3.9 | 136 → 90 | 15 | 12 | 1,2-$^{13}$C-taurine |

Table 12: Chromatographic separation conditions used, modified from Prinsen *et al*. [512].

| Time (min) | 0 | 6 | 6.1 | 11 | 14 | 14.1 | 18 |
|---|---|---|---|---|---|---|---|
| Solvent A (%) 10 mM Ammonium formate, 0.15 % formic acid in 85% acetonitrile | 100 | 100 | 94 | 83 | 60 | 100 | 100 |
| Solvent B (%) 10 mM Ammonium formate, 0.15% formic acid in milliQ- $H_2O$ | 0 | 0 | 6 | 17 | 40 | 0 | 0 |

The column was linked to a Xevo-TQ MS triple quadrupole mass spectrometer with an electrospray ionization (ESI) source. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode with settings were as previously described [512]: ESI-positive mode, capillary voltage 1.00 kV, desolvation

temperature 550 °C, source temperature 150 °C, cone gas flow 50 L/h, desolvation gas flow 1000 L/h. Quantification and spectra were obtained and analysed using MassLynx 4.2 software (Waters, UK). Graphs and linear regression analysis for $R^2$ values were prepared using GraphPad Prism.

#### 5.3.1.2.4 Data analysis

##### Internal calibrations using $^{13}$C-labelled cysteine and taurine

In each of the matrices, internal standard calibration curves were constructed for each amino acid by dividing the area under peak obtained for the external standard by the area under peak obtained for the respective internal standard obtained on the same injection to determine the external/internal area ratio. The determined concentration in the faecal matrices were calculated using the graph equation obtained from the reference standard curve in 0.1 M HCl, according to y = mx, where m is the slope of the calibration curve. The determined concentration for the external and internal amino acids were calculated and expressed as the external/internal concentration ratio.

##### Faecal matrices standard curves

A blank sample of each faecal matrix was prepared with only internal standard added. The blank samples were used to adjust the spiked samples to remove background amino acids for the purpose of assessing method precision, accuracy and matrix effect.

##### Method precision, accuracy, and matrix effect

The precision, accuracy and matrix effect were determined in the faecal matrices to assess the quality of the quantification method. Precision (intra-sample variability) was determined in blank-adjusted faecal matrix samples spiked with known amino acid concentrations by determining the mean and relative standard deviation (RSD). Percentage accuracy was determined by dividing the determined concentration in the blank-adjusted faecal matrix samples by the spiked concentration and multiplying by 100. The matrix effect in each faecal matrix was determined using the blank-adjusted area ratio divided by the area ratio obtained in the reference standard curve (0.1 M HCl) multiplied by 100 to express as a percentage. The Lower Limit of Quantification (LLOQ) was defined as the minimum concentration at which each amino acid could be reliably quantified with a precision ≤ 20% and an 80–120% accuracy [513].

#### 5.3.1.3 Quantification of $H_2S$ in faecal matrices using methylene blue colourimetric assay

##### 5.3.1.3.1 Preparation of zinc sulfide-spiked samples for analysis

A stock solution of zinc sulfide was prepared, and true concentration was determined as previously described (section 2.3.5). Standard curves of known sulfide concentrations were prepared by adding 0 – 400 µM zinc sulfide to aliquots of three matrices: 1) faecal water diluted 1:10 with PBS, 2) faecal water diluted 1:10 with colon model media, and 3) up$H_2O$ as reference. All samples were fixed by adding 5% zinc acetate solution to a 1:1 ratio. Samples were then diluted 1:1 with up$H_2O$ for analysis. All samples were prepared in triplicate.

##### 5.3.1.3.2 Methylene blue assay

The colourimetric assay based was performed as described previously (section 2.3.5). Diamine reagents of differing concentrations were used to test a quantification range of 0 – 100 µM $H_2S$. Quantification in the range of 2 – 40 µM was performed using a lower concentration diamine reagent (250 mL 6M HCl, 1 g N,N-dimethyl-1,4-phenylendiaminsulfate, 1.5 g iron(III)chloride, $FeCl_3 \cdot 6H_2O$), whereas 40 – 100 µM was quantified using a higher concentration reagent (250 mL 6M HCl, 4 g N,N-dimethyl-1,4-phenylendiaminsulfate, 6 g iron(III)chloride, $FeCl_3 \cdot 6H_2O$) [272].

##### 5.3.1.3.3 Sample blanks for methylene blue assay

To correct for turbidity and absorbance introduced by the faecal matrix, sample blanks were prepared in triplicate as described by Strocchi *et al*. [514]. Briefly, aliquots of faecal water diluted 1:10 with PBS and faecal water diluted 1:10 with colon model media were prepared in the same way as the un-spiked blank sample, but up$H_2O$ was added instead of 5% zinc acetate prior to storage at -20°C. To deplete residual $H_2S$, 80 µL of 6 M HCl was added to the sample blanks and stirred under the fume hood for 1 h. The samples were centrifuged at 13,000 x g for 5 min to pellet biomass, and 300 µL of supernatant was taken for spectrophotometric absorbance measurement at 670 nm.

##### 5.3.1.3.4 Data analysis

For $H_2S$ quantification, the raw absorbance values were corrected for the matrix effect using the absorbance values obtained from the sample blanks in each matrix. $H_2S$ concentration was determined using the blank-adjusted absorbance

values multiplied by the diamine reagent coefficient as previously described (section 2.3.5). To determine the accuracy of the method, samples spiked with known concentrations of ZnS were blanked by subtracting the $H_2S$ detected in the un-spiked faecal matrix. Linear regression analysis was performed in GraphPad Prism 7 (GraphPad Software, Boston, USA). Percentage accuracy of the method was determined by (determined concentration / spiked concentration) * 100. Method precision was determined using the relative standard deviation between the three technical replicates for each spiked concentration.

### 5.3.2 Subsection 2 methods

#### 5.3.2.1 Caco-2 cell culture

Caco-2 cells originally isolated from a human adenocarcinoma were purchased from Public Health England (ECACC catalogue no. 09042001). All manipulations of Caco-2 cells were performed under strict sterile conditions using a BioMAT 2 Class II biological safety cabinet (CAS, UK). Caco-2 cells were propagated in Eagle's Minimum Essential Medium (EMEM) with 10% foetal bovine serum and the following additions: 0.1 mM non-essential amino acids (NEAA), 2 mM L-glutamine, 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% $CO_2$. Cells were counted using a Countess II FL chamber (ThermoFisher Scientific) and inoculated into culture flasks at a seeding density of $10^5$ cells/$cm^2$. Growth was checked daily, and cells were passaged at 70-80% confluence. Passaging was performed by incubating the adherent cells with Trypsin-EDTA solution (T4049, Merck) for 10 min, followed by the inactivation of trypsinisation by adding complete media. Cell viability and counts were obtained, and the cells were passaged.

##### 5.3.2.1.1 Establishment of Caco-2 mono-layer

Caco-2 cells (passage number 6) were inoculated into PET transwells with 0.4 µm pore diameter (662640, Greiner Bio-One) in tissue culture plates. Each transwell was seeded with 1 x $10^5$ cells in 250 µL media. Caco-2 cells were allowed to polarise and differentiate in the transwells for 21 days. Apical compartment media was changed every 2 days, basal compartment media was changed every 4 days and TEER measurements were obtained every 2 days using an EVOM2 Epithelial voltohmeter machine (World Precision Instruments). TEER results were multiplied by the filter size to obtain the results in Ohm per $cm^2$ ($\Omega$ $cm^2$).

#### 5.3.2.1.2 Addition of bacterial cells or supernatant to Caco-2 mono-layer

After 21 days, individual transwells where strong mono-layer was formed (TEER value ≥800 Ω $cm^2$ for ≥4 days) were selected for experimental use. Overnight second passage bacterial cultures of *B. wadsworthia* (QI0013) and Bt strain 1 (QI0072) were prepared in enrichment media and inoculated at $10^6$ CFU/mL in mono-culture or co-culture in 1:1 ratio in anaerobic BPM media supplemented with 10 mM taurine as previously described (section 2.3.3.2). After 8 h of growth, cultures were pelleted by centrifuging at 4,000 x g for 5 min. Supernatant was filtered through 0.22 µm membrane to prepare cell-free culture supernatant. The bacterial pellets were resuspended in anaerobic PBS. Additionally, cell-free supernatant were prepared from overnight cultures of *Lactiplantibacillus plantarum* OOY9 and EKN4 grown in enrichment media (Table 17).

Table 13: *Lactiplantibacillus plantarum* strains used in Caco-2 cell experiments.

| **Strain** | **Relevant characteristics** | **Growth conditions** | **Reference/source** |
|---|---|---|---|
| *L. plantarum* OOY9 (ULAG11) | Isolated from African fermented cereals ogi | MRS; aerobic; static; 37°C | [515] |
| *L. plantarum* EKN4 (ULAG24) | Isolated from African fermented cereals kunu-zaki | MRS; aerobic; static; 37°C | [515] |

Experimental conditions were prepared by removing media from the apical compartment and replacing with fresh antibiotic-free media as blank, or media with 30% resuspended bacterial cell pellets or cell-free supernatant (Table 18). A negative control was prepared using 14% ethanol to disrupt the barrier [504, 516-518], and 10 mM propionic acid was used as a positive control [518] (Table 18). All conditions were prepared in triplicate. Material for $H_2S$ quantification was taken at 0, 2, 18 and 24 h. After 24 h, TEER values were obtained.

Table 14: Experimental conditions used and their preparation for Caco-2 cell experiment.

| Condition | Preparation |
|---|---|
| Blank | Complete media without penicillin streptomycin |
| Negative control | 14% ethanol |
| Positive control | 10 mM propionic acid |
| *B. wadsworthia* S/N | 30% 8 h *B. wadsworthia* cell-free supernatant |
| *B. wadsworthia* + Bt S/N | 30% 8 h *B. wadsworthia* + Bt strain 1 co-culture cell-free supernatant |
| EKN4 | 30% L. plantarum EKN4 washed cells |
| OOY9 | 30% L. plantarum OOY9 washed cells |
| EKN4 + OOY9 | 15% *L. plantarum* EKN4 washed cells<br>15% *L. plantarum* OOY9 washed cells |
| *B. wadsworthia* cells | 30% *B. wadsworthia* washed cells |
| EKN4 + OOY9 + *B. wadsworthia* cells | 10% *B. wadsworthia* washed cells<br>10% *L. plantarum* EKN4 washed cells<br>10% *L. plantarum* OOY9 washed cells |

#### 5.3.2.1.3 Sampling for $H_2S$ concentration determination in from Caco-2 mono-layer culture

Briefly, 20 µL media was removed from the apical compartment of the Caco-2 cell mono-layer and immediately fixed 1:1 with 5% zinc acetate. $H_2S$ concentration was determined as described previously (section 2.3.5).

#### 5.3.2.2 *B. wadsworthia*, *B. thetaiotaomicron* and *E. faecium* interactions in *ex vivo* faecal cultures

##### 5.3.2.2.1 Preparation of faecal matrices

Fresh faecal samples were obtained from three donors recruited via the QIB Colon Model Study (Table 19). Samples were processed within 4 h of collection. Ten grams of each faecal sample were diluted 1:10 in anaerobic phosphate buffered saline (PBS) and homogenised using a Stomacher 400 (Seward) at 230 rpm for 45 s to prepare faecal water. The faecal water was flash frozen on dry ice [507], and sealed inside an air-tight bag with an Oxoid™ AnaeroGen™ anaerobic sachet (ThermoFisher Scientific, UK) to maintain anaerobic conditions. Faecal water was stored at -80˚C prior to use and used within one week [507].

Table 15: Metadata of the faecal sample donors recruited via the QIB colon model study

| Donor | Donor ID | Age | Gender | BMI | Dietary pattern |
|---|---|---|---|---|---|
| 1 | CM046 | 39 | M | 29.8 | Omnivore |
| 2 | CM072 | 54 | F | 21.4 | Omnivore |
| 3 | CM053 | 35 | F | 25.1 | Omnivore |

##### 5.3.2.2.2 Preparation of experimental cultures

On the day of experiment, the faecal water was thawed on wet ice. Cultures of *B. wadsworthia* (QI0013), *B. thetaiotaomicron* strain 1 (QI0072), and *E. faecium-B. thetaiotaomicron* enrichment (Ef-Bt) were prepared as previously described (section 2.3.3.2). Experimental cultures were prepared by inoculating 10% faecal water (1% final concentration of faeces) and $10^8$ CFU/mL of *B. wadsworthia* (QI0013) alone, or in a 1:1 ratio with either *B. thetaiotaomicron* (QI0072) or and *E. faecium-B. thetaiotaomicron* enrichment (Ef-Bt) into universal bottles containing Complex Intestinal Media (CIM) [508] as described in Table 20. All experimental conditions were prepared in triplicate. After preparation, filter-sterilised taurine solution was added to a final concentration of 5.6 mM. Cultures were incubated under anaerobic conditions at 37˚C using an anaerobic cabinet (Don Whitley, UK) and samples taken at 0- and 8 h post-inoculation.

Complex Intestinal Media (CIM) was prepared as follows: arabinogalactan from larch wood 2 g/L, bile salts 0.5 g/L, $CaCl_2.2H_2O$ 0.01 g/L, pancreatic casein peptone 4.3 g/L, $K_2HPO_4$ 0.04 g/L, $KH_2PO_4$ 0.04 g/L, hemin 0.005 g/L, inulin 1 g/L, L-cysteine hydrochloride 0.5 g/L, $MgSO_4.7H_2O$ 0.02 g/L, menadione (vitamin K3) 0.001 g/L, porcine gastric mucin type II 4 g/L, pectin from citrus peel 2 g/L, NaCl 0.72 g/L, $NaHCO_3$ 2 g/L, starch from wheat 5 g/L, xylo-oligosaccharide from corn 2 g/L, yeast extract 2 g/L, pH 7.2 [508].

Table 16: Preparation of experimental cultures for modelling complex gut microbiota *in vitro*.

| **Experimental conditions** | **CIM media volume (mL)** | **Faecal water volume (mL)** | ***B. wadsworthia* volume (mL)** | **QI0072 volume (mL)** | **Ef-Bt volume (mL)** | **Anaerobic PBS volume (mL)** |
|---|---|---|---|---|---|---|
| **Bw** *B. wadsworthia* (QI0013) | 7 | 1 | 1 | 0 | 0 | 1 |
| **Bw + Bt** *B. wadsworthia* (QI0013) + *B. thetaiotaomicron* (QI0072) | 7 | 1 | 1 | 1 | 0 | 0 |
| **Bw + Ef-Bt** *B. wadsworthia* (QI0013) + *E. faecium-B. thetaiotaomicron* enrichment (Ef-Bt) | 7 | 1 | 1 | 0 | 1 | 0 |
| **Control** (faecal inoculum only) | 7 | 1 | 0 | 0 | 0 | 2 |

#### 5.3.2.2.3 Determination of total cell counts and culture pH

The total number of bacterial cells present at 0 and 8 h were determined using flow cytometry. Briefly, culture aliquots were analysed using a Guava® easyCyte™ HT System (Luminex Corporation) to determine the cell number. Cultures were prepared by diluting 1:1000 in filter-sterile PBS, and filtering through a 0.45 µm membrane to remove cell aggregates. Additional dilutions were prepared at 1:10,000 and 1:100,000. Cells were stained using SYBR® Green I nucleic acid gel stain (Sigma) diluted to a working solution of 100x and analysed in a V-bottom 96 well plate format on the flow cytometer according to the Standard Operating Procedure (Quadram Institute Bioscience). The culture pH was determined using a LE422 pH electrode (Mettler Toledo) calibrated using pH calibration solutions at pH 4, 7 and 10 (Merck-Millipore).

#### 5.3.2.2.4 Metagenomic sequencing

##### DNA extraction and sequencing

DNA extractions were performed using Maxwell® RSC Fecal Microbiome DNA Kit (Promega, UK) according to manufacturer's protocol, with an additional cell lysis step by bead-beating in Lysing Matrix E tubes (MP Biomedicals, USA) at 6.5 m/s for 60 s twice using a FastPrep-24™ machine (MP Biomedicals, USA). Extracted DNA was quantified using Qubit High Sensitivity DNA Quantification Kit (Thermo Scientific) and Qubit 3.0 fluorometer (Life Technologies). DNA was normalised to 5 ng/µL and a pooled DNA library was prepared by the QIB sequencing facility using the Illumina DNA Prep kit and the IDT 8bp UDIs indexing kit. Library clean-up was performed using AMPure XP beads (Beckman Coulter Life Sciences, USA). This produced a final library with unique dual indexing where each of the 72 samples in the library were assigned a unique i7 and i5 index sequence. The library was quantified as 12.5 ng/µL and sequenced with Illumina® NovaSeq, generating paired-end reads with 2x150bp length (Azenta-Genewiz, Germany).

##### Microbiome data analysis

Reads were cleaned using bbduk (BBMap 38.79) [275] to trim the reads and remove sequencing adaptors. FastQC (v 0.11.9) [276] was used to assess the sequence quality, where reads with a Phred score ≥30 were deemed suitable for further analysis. Human contamination was removed by mapping the reads to the human genome (GRCh38) using bbsplit (BBMap 38.79) [275] and discarding mapped reads [519, 520]. Metagenomic composition as relative abundance of taxa was determined using metaphlan 4.0 [521]. The data was normalised using the total cell counts determined via flow cytometry to determine the absolute abundance of each taxon expressed as cells/mL of culture.

Statistical analysis and data visualisation was performed using R (v4.1.2) and the ggplot2 package [522]. For beta diversity, Bray-Curtis distances were determined and visualised via NMDS plot using the vegan package (v2.6.4) [523] using the average of culture triplicates per condition. PERMANOVA (adonis) tests were performed using Bray-Curtis distances to identify differential composition between groups based on the variable of interest (time, experimental condition, or donor) [523].

To test for statistically significant differences in taxa abundance, the absolute abundance data was first log(10) transformed. Then, non-parametric tests were

performed using the coin package (v1.4.3) [524]; Wilcoxon test was used to compare differential abundance due to time-point (two groups), whereas Kruskal-Wallis test was used to compare differential abundance due to condition and donor (four and three groups respectively). P values were corrected for multiple testing (Benjamini-Hochberg) to reduce the false discovery rate [525]. For all statistical analyses, p-values $\leq 0.05$ were considered significant. Statistical testing for differential taxa was performed using all replicates for each condition. Differentially abundant taxa due to time-point and donor were ranked by absolute abundance, and the top 10 taxa were displayed graphically. For differential taxa due to condition, conditions were compared pair-wise using Mann Whitney U tests with Benjamini-Hochberg correction using the stats package in R (v4.1.2).

## 5.4 Results

### 5.4.1 Subsection 1 – Method development for tracking *B. wadsworthia* metabolism and sulfur metabolism in *in vitro* gut models

#### 5.4.1.1 Development of HILIC-MS method for quantifying sulfated amino acids in faecal matrices

We aimed to develop a HILIC-MS method for quantification of the sulfated amino acids methionine, cysteine, taurine and homocysteine within complex matrices containing human faeces obtained from *in vitro* colon models. Reference standard curves were prepared with known concentrations of amino acids in 0.1 M HCl. Linear regression analyses of the reference standard curves for methionine, cysteine, taurine and homocysteine performed well, showing a strong relationship between concentration and area under peak within the concentration range tested ($R^2 \geq 0.97$) (Figure 23).

To assess the ability of the HILIC-MS method to accurately quantify the amino acids of interest within the faecal matrix, standard curves were also prepared in faecal water and faecal water diluted 1:10 with colon model media (Figure 24). Background amino acids were removed by adjusting spiked samples by the blank where only internal standards were added. Using the area under peak obtained for the external and internal standards in the faecal matrices, the external/internal area ratio obtained was used to calculate the determined concentration of amino acids in the spiked samples. Linear regression analyses for each amino acid in each faecal matrix showed good linearity in the concentration range tested, with the lowest $R^2$ value being 0.95 for quantification of methionine in faecal water (Figure 24).

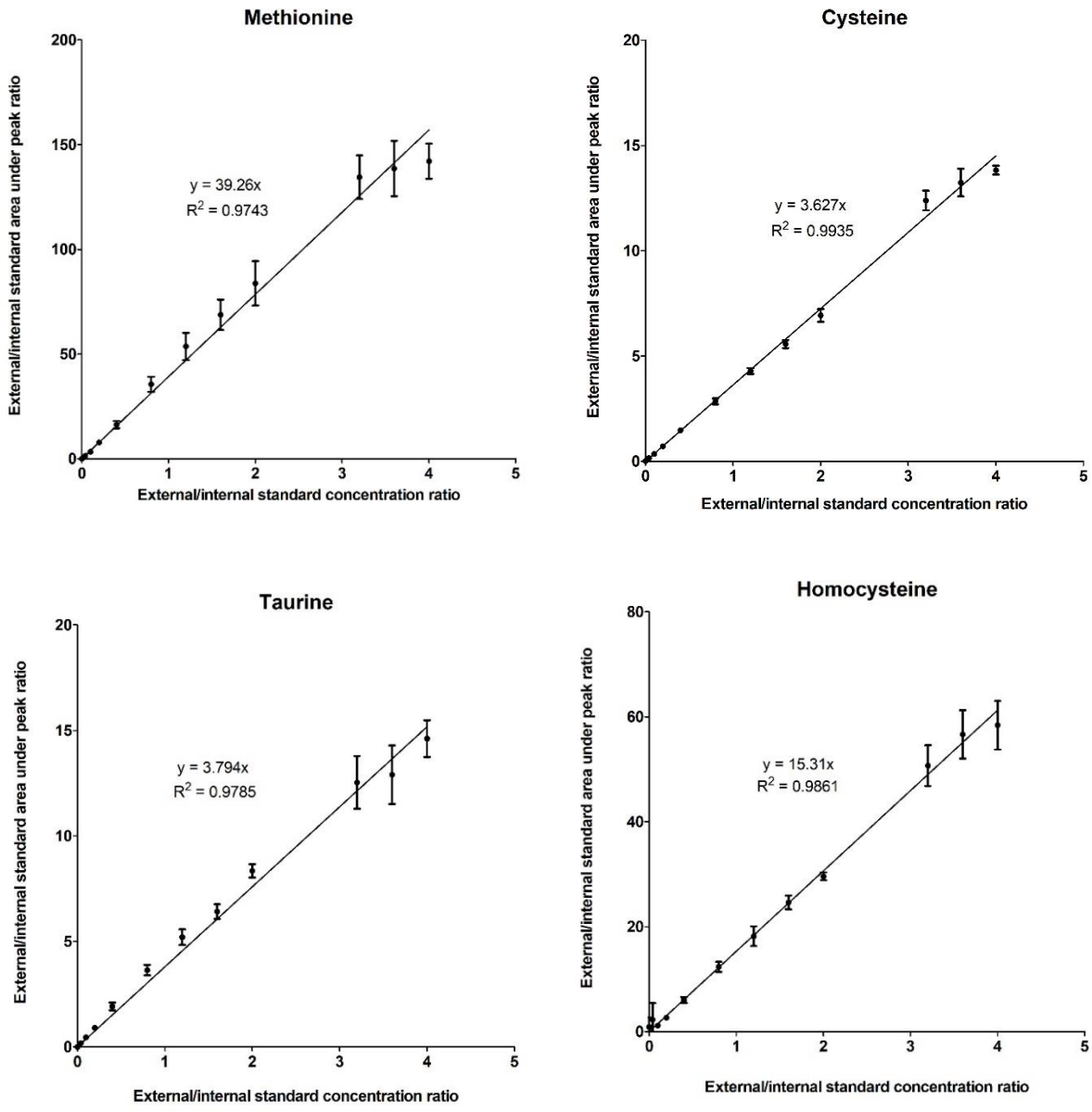
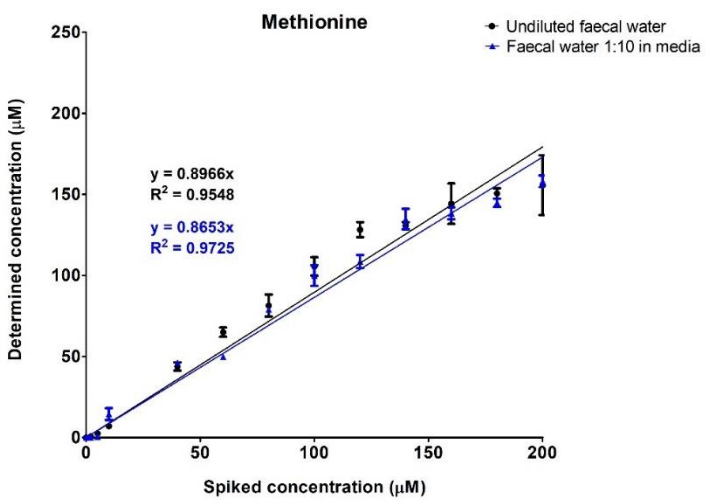
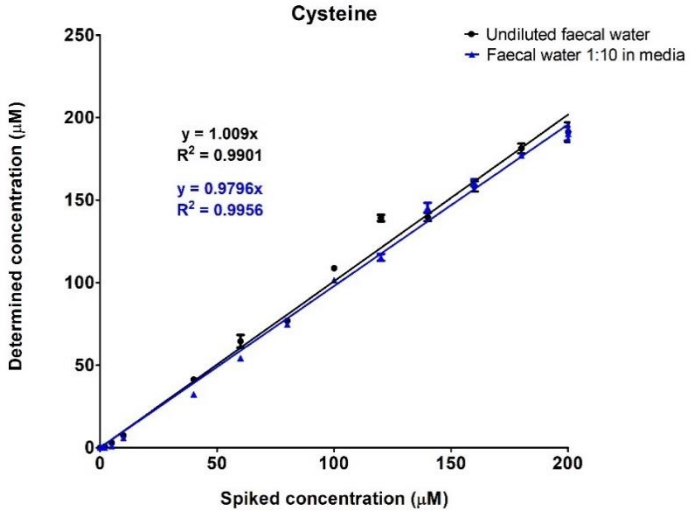
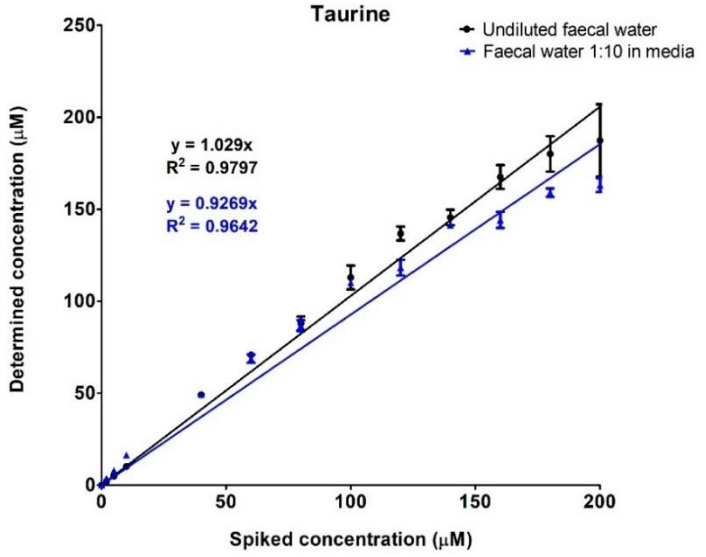



Figure 23: Reference standard curves of methionine, cysteine, taurine, and homocysteine prepared in 0.1 M HCl.

Analytical grade amino acids were diluted 0-200 µM in 0.1 M HCl and spiked with 50 µM internal standards. Area under peak and concentration ratios were determined for external and respective internal standard in each injection replicate (n=3). Error bars represent SD. A linear regression through the origin was performed, and the $R^2$ and graph equation are displayed.


Methionine
Undiluted faecal water
Faecal water 1:10 in media
y = 0.8966x
R² = 0.9548
y = 0.8653x
R² = 0.9725
Determined concentration (µM)
Spiked concentration (µM)
0
50
100
150
200
250


Cysteine
Undiluted faecal water
Faecal water 1:10 in media
y = 1.009x
R² = 0.9901
y = 0.9796x
R² = 0.9956
Determined concentration (µM)
Spiked concentration (µM)
0
50
100
150
200
250


Taurine
Undiluted faecal water
Faecal water 1:10 in media
y = 1.029x
R² = 0.9797
y = 0.9269x
R² = 0.9642
Determined concentration (µM)
Spiked concentration (µM)
0
50
100
150
200
250

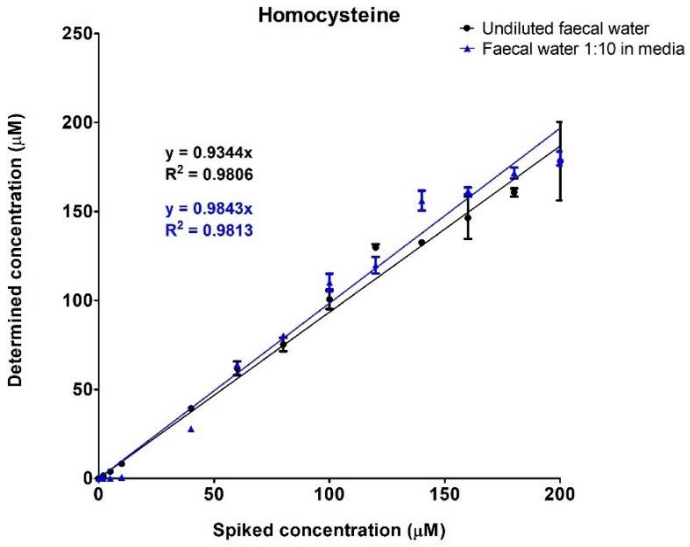
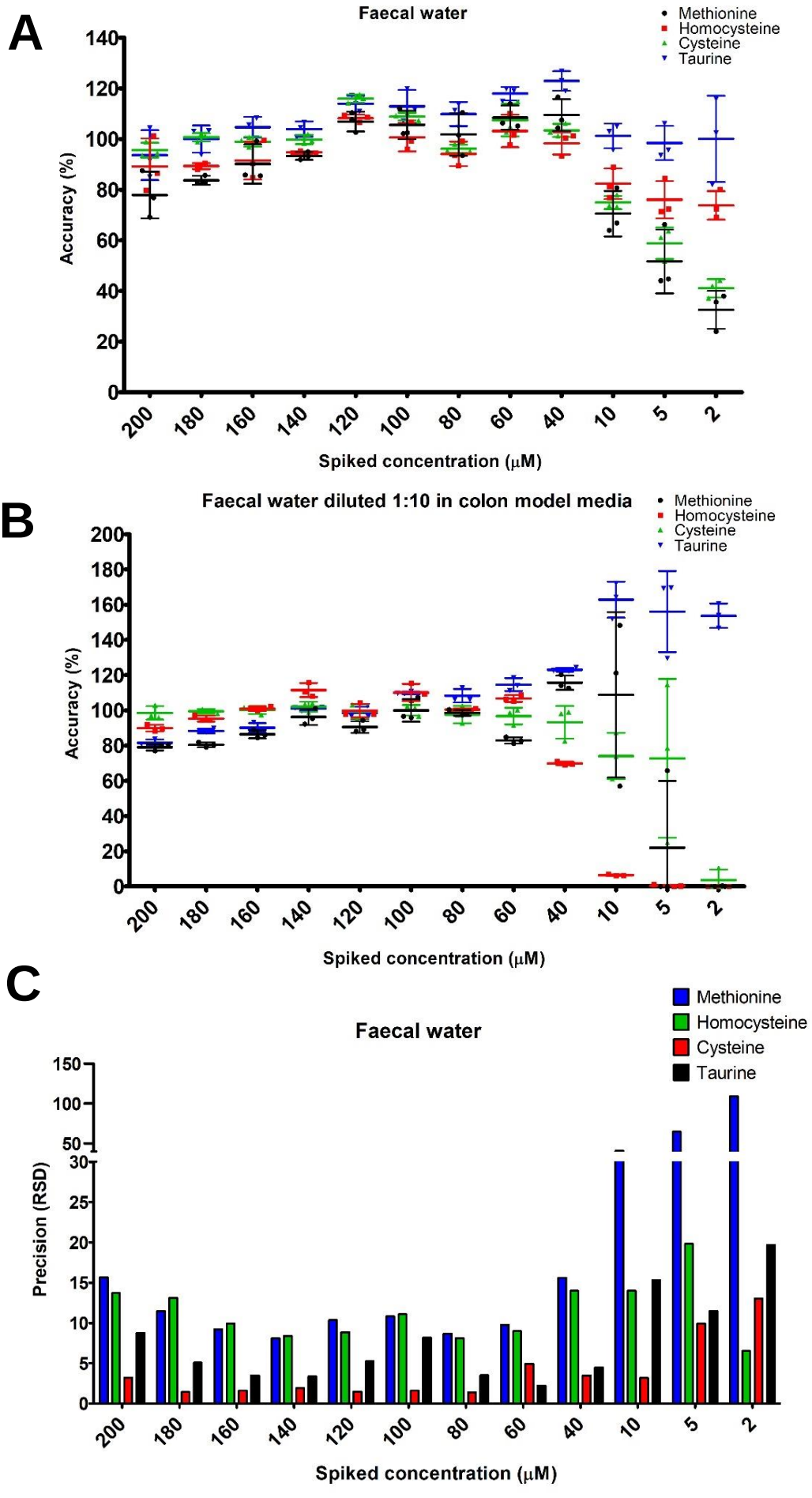

Homocysteine
Undiluted faecal water
Faecal water 1:10 in media
y = 0.9344x
R² = 0.9806
y = 0.9843x
R² = 0.9813
Determined concentration (µM)
Spiked concentration (µM)
0
50
100
150
200
250

Figure 24: Determined concentration (µM) of amino acids in faecal water and faecal water diluted 1:10 with colon model media spiked with known concentrations.
Analytical grade amino acids were diluted 0-200 µM in each faecal matrix and spiked with 50 µM internal standards. Determined concentration was determined in each injection replicate (n=3) using the reference standard curve prepared in 0.1 M HCl. Error bars represent SD. A linear regression through the origin was performed, and the $R^2$ and graph equation are displayed.

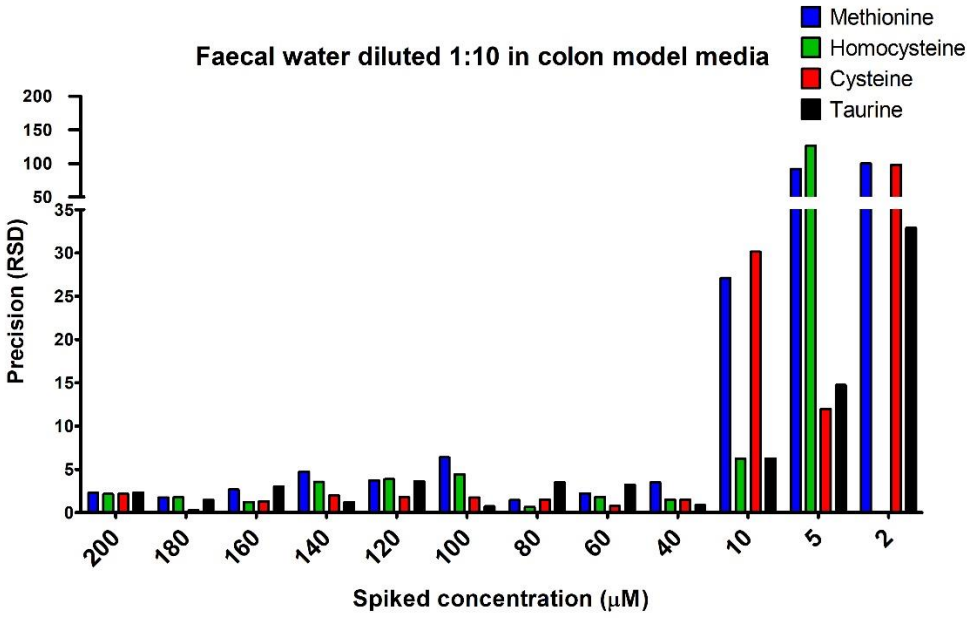
To assess the performance of the quantification method, we determined the percentage accuracy, precision and matrix effect in the faecal matrices (Figure 25). Accuracy of quantification was acceptable for all four amino acids in the faecal water matrix from 200 – 40 µM, below which the accuracy for quantification of methionine, homocysteine and cysteine decreased (Figure 25A). Taurine could be accurately quantified at the lowest concentration tested (2 µM) in the faecal water matrix (Figure 25A). Faecal water diluted 1:10 with colon model media similarly showed good quantification accuracy in the 200 – 40 µM range for all amino acids, with decreased accuracy at 10 µM and below (Figure 25B). An accuracy of 80 - 120% is an ideal range [513, 526, 527]; using this method, the four amino acids can be accurately quantified in both matrices at 40 µM and above, and taurine quantification is accurate to 2 µM in faecal water (Figure 25A, 25B). The precision of the method was determined using relative standard deviation, where RSD ≤15% is considered an acceptable cut-off for inter-injection variability [528, 529]. The four amino acids could be quantified with good precision between 40 – 200 µM in both matrices (Figure 25C, 25D). In faecal water, precision of methionine quantification was decreased at 10 µM and below, whereas cysteine, homocysteine and taurine retained good precision at these lower concentrations (Figure 25C). In faecal water diluted 1:10 with media, precision was poor for quantification of all amino acids at 5 and 2 µM (Figure 25D). The matrix effect was also determined to assess the ion suppression or enhancement induced by the matrices [530] by comparing the area ratios obtained at each spiked concentration in blank-adjusted faecal matrix samples to those obtained in the reference standard curve in 0.1 M HCl. Matrix effect of 70-120% constitutes an acceptable range [531]. In both faecal water and faecal water diluted 1:10 with media, homocysteine showed a significant matrix effect across the concentration range tested (Figure 25E, 25F). For

methionine, taurine and cysteine, the matrix effect was within acceptable range from 10 – 200 µM in faecal water (Figure 25E) and 40 – 200 µM in faecal water diluted 1:10 with media (Figure 25F), with increasing ionisation suppression being observed at lower concentrations (Figure 25E, 25F).

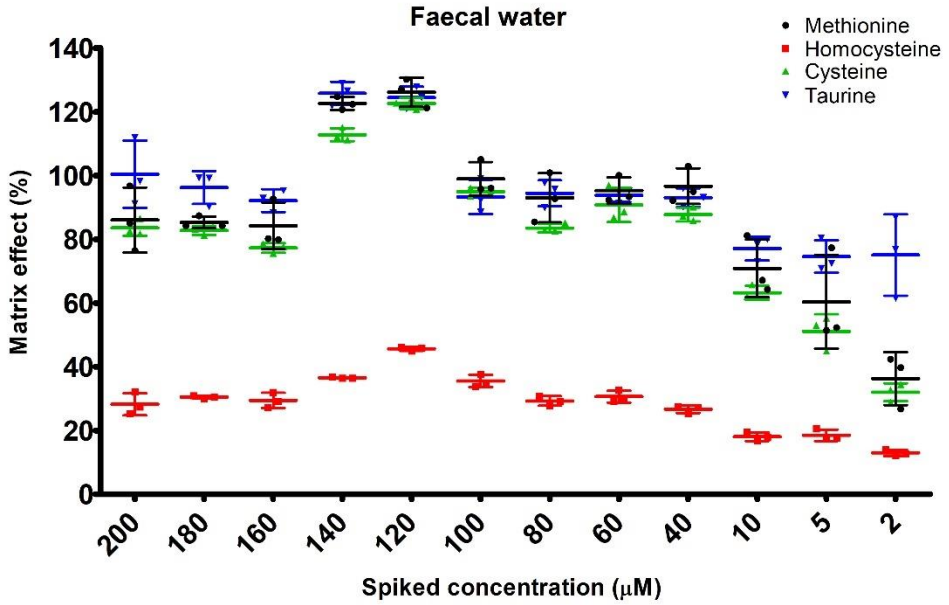
The Lower Limit of Quantification (LLOQ) was determined for each amino acid in each matrix; LLOQ was 40 µM for all amino acids in each matrix except for taurine, which could be accurately quantified to 2 µM in faecal water (Table 11). Concentrations of the sulfated amino acids was determined in the “blank” samples where only internal amino acid standard were added for quantification. Methionine and homocysteine were detectable in both faecal water and faecal water diluted 1:10 with colon model media, although this was below the LLOQ meaning accurate quantification was not possible (Table 12). Homocysteine was not detected in faecal water and was quantified below LLOQ in faecal water diluted with media (Table 12). Taurine was detected below the LLOQ in faecal water diluted with media but was detected within the accurate quantification range in faecal water at 5.13 µM equating to 207.33 µM per gram of wet weight faeces (Table 12).


A
Faecal water
Methionine
Homocysteine
Cysteine
Taurine
Accuracy (%)
Spiked concentration (µM)
B
Faecal water diluted 1:10 in colon model media
Methionine
Homocysteine
Cysteine
Taurine
Accuracy (%)
Spiked concentration (µM)
C
Faecal water
Methionine
Homocysteine
Cysteine
Taurine
Precision (RSD)
Spiked concentration (µM)

D


Faecal water diluted 1:10 in colon model media
Methionine
Homocysteine
Cysteine
Taurine
Precision (RSD)
200
150
100
50
35
30
25
20
15
10
5
0
200
180
160
140
120
100
80
60
40
10
5
2
Spiked concentration (µM)

E


Faecal water
Methionine
Homocysteine
Cysteine
Taurine
Matrix effect (%)
140
120
100
80
60
40
20
0
200
180
160
140
120
100
80
60
40
10
5
2
Spiked concentration (µM)

F

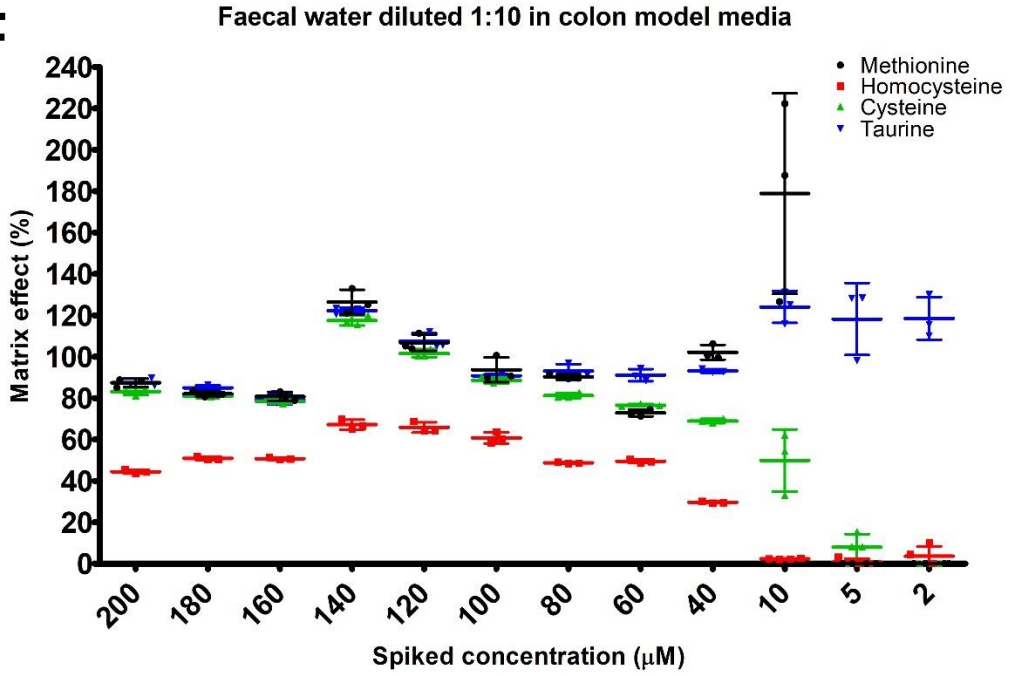

Faecal water diluted 1:10 in colon model media
Methionine
Homocysteine
Cysteine
Taurine
Matrix effect (%)
240
220
200
180
160
140
120
100
80
60
40
20
0
200
180
160
140
120
100
80
60
40
10
5
2
Spiked concentration (µM)

Figure 25: Performance metrics of the HILIC-MS method in the two faecal matrices.

A) Percentage accuracy of quantification in faecal water matrix determined as (determined concentration/spiked concentration) multiplied by 100. Each datapoint represents an injection replicate. Error bars represent SD.

B) Percentage accuracy of quantification in faecal water diluted with colon model media matrix determined as (determined concentration/spiked concentration) multiplied by 100. Each datapoint represents an injection replicate. Error bars represent SD.

C) Precision in faecal water matrix expressed as relative standard deviation (RSD) calculated from injection replicates (n=3).

D) Precision in faecal water matrix diluted with colon model media expressed as relative standard deviation (RSD) calculated from injection replicates (n=3).

E) Matrix effect in faecal water matrix calculated by comparing raw data obtained in faecal matrix to 0.1 M HCl for each spiked concentration. Each datapoint represents an injection replicate. Error bars represent SD.

F) Matrix effect in faecal water matrix diluted with colon model media calculated by comparing raw data obtained in faecal matrix to 0.1 M HCl for each spiked concentration. Each datapoint represents an injection replicate. Error bars represent SD.

Table 17: Lower Limit of Quantification (LLOQ) of amino acids using HILIC-MS method in faecal matrices

| **Amino acid** | **Faecal water** | **Faecal water diluted 1:10 in colon model media** |
|---|---|---|
| Methionine | 40 µM | 40 µM |
| Homocysteine | 40 µM | 40 µM |
| Cysteine | 40 µM | 40 µM |
| Taurine | 2 µM | 40 µM |

Table 18: Determined concentrations of sulfated amino acids in blank samples with no additional external standard spike-in. Value shown is the average obtained from 3 injection replicates.

| | **Determined concentration in sample (µM)** | | **Determined concentration in faeces (µM per g wet weight faeces)** | |
|---|---|---|---|---|
| **Amino acid** | Faecal water | Faecal water diluted 1:10 in colon model media | Faecal water | Faecal water diluted 1:10 in colon model media |
| Methionine | 7.48 µM (Below LLOQ) | 31.65 µM (Below LLOQ) | N/A | N/A |
| Homocysteine | Not detected | 0.32 µM (Below LLOQ) | Not detected | N/A |
| Cysteine | 0.45 µM (Below LLOQ) | 13.55 µM (Below LLOQ) | N/A | N/A |
| Taurine | **5.18 µM** | 0.85 µM (Below LLOQ) | **207.33 µM** | N/A |

### 5.4.1.2 Use of a colourimetric methylene blue method for quantification of $H_2S$ in faecal matrices

To enable the determination of $H_2S$ concentration in experimental samples containing human faeces, we investigated the use of the methylene blue colourimetric assay with two diamine reagents able to quantify $H_2S$ between the concentration ranges of 2 – 40 µM and 40 – 100 µM respectively. A reference standard curve was prepared in up$H_2O$ spiked with known concentrations of zinc sulfide to determine the diamine reagent coefficient required for calculating $H_2S$ concentration in unknown samples for each diamine reagent. Diamine reagent coefficients were calculated as 1/slope of calibration curve [272]; the lower concentration (2 – 40 µM) diamine reagent had a coefficient of 37.79, whereas the higher concentration (40 – 100 µM) reagent had a coefficient of 47.98 (Figure 26A, 26B). Both standard curves showed a high linearity ($R^2$ ≥0.96) within the absorbance range tested, indicating a strong correlation between $H_2S$ concentration and absorbance confirming that the diamine reagents could accurately quantify $H_2S$ within the respective concentration ranges (Figure 26A, 26B).

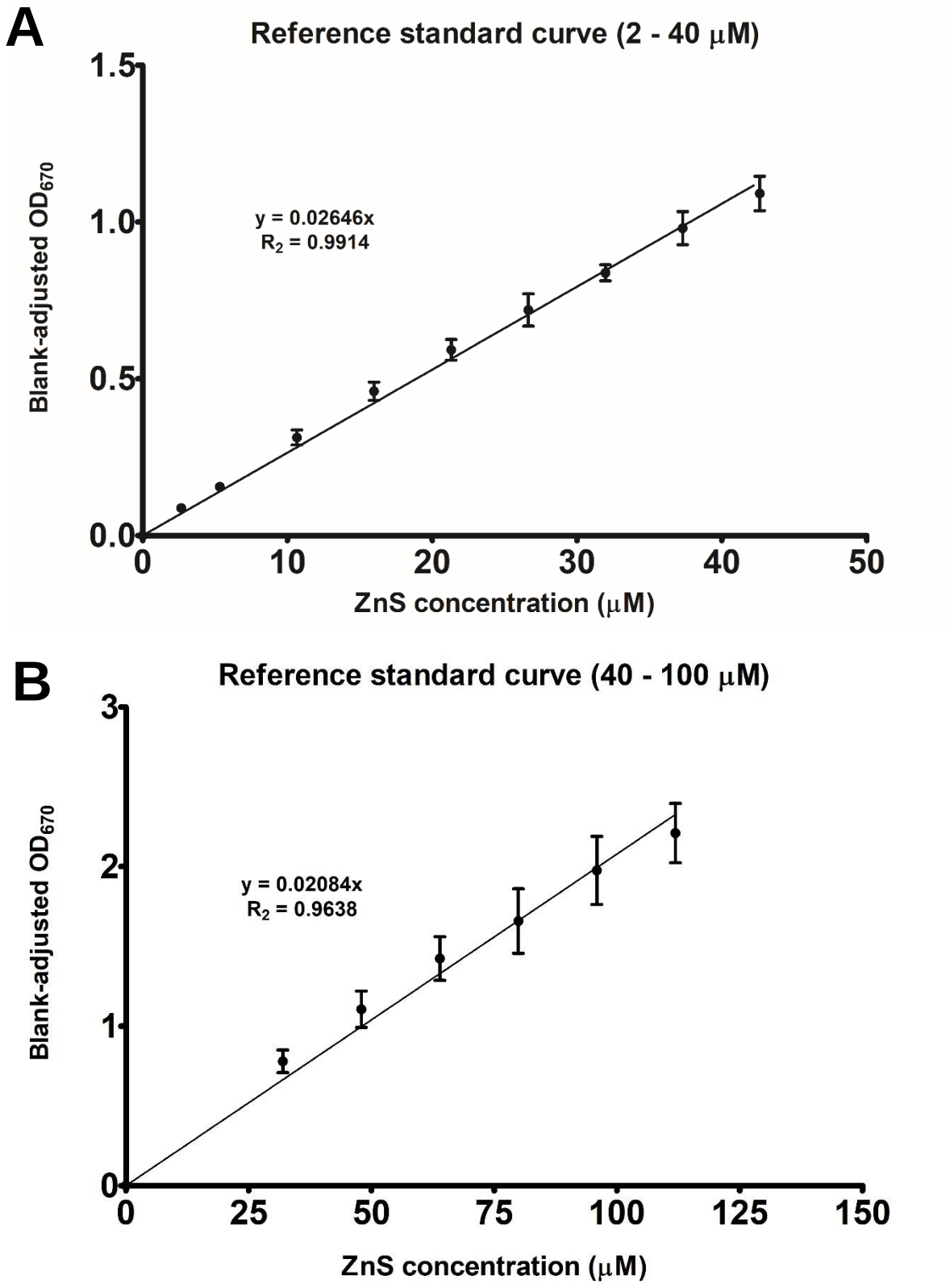



Figure 26: Reference standard curves of known zinc sulfide (ZnS) concentrations in up$H_2O$ for spectrophotometric determination of $H_2S$ concentration.
A) Standard curve with lower concentration diamine reagent for quantifying 2 – 40 µM $H_2S$. B) Standard curve with higher concentration diamine reagent for quantifying 40 – 100 µM $H_2S$. Error bars represent SD. Data-point shows mean of two technical replicates.

Faecal water diluted 1:10 with PBS or colon model media (CMM) were spiked with ZnS in the concentration ranges of the two diamine reagents and the absorbance measured alongside the reference standard curves. To account for absorbance-influencing factors attributable to the faecal matrix, sample blanks of each matrix were prepared where $H_2S$ was depleted from the samples, the absorbance of which was subtracted from the un-spiked and spiked faecal matrix samples. For assessment of the method's performance, the absorbance of the un-spiked faecal matrix samples was subtracted from the spiked samples so that the only $H_2S$ present is the known concentration added to the sample. Both diamine reagents performed well across the linear range in both faecal matrices; 2 – 40 µM diamine reagent had an $R^2$ value of 0.9889 in PBS and 0.9846 in CMM (Figure 27A), whereas the 40 – 100 µM diamine reagent had $R^2$ values of 0.9571 in PBS and 0.9950 in CMM respectively (Figure 27B).

To determine the performance of the two diamine reagents in the different faecal matrices, the percentage accuracy and precision of the quantification at each spiked concentration was determined. Regarding method accuracy, the determined $H_2S$ concentration was higher than expected in all cases; the 2 – 40 µM reagent had average accuracy of 133% and 123% in faecal water diluted 1:10 with PBS and CMM respectively, and the 40 – 100 µM reagent had an average accuracy of 130% and 124% in in faecal water diluted 1:10 with PBS and CMM respectively (Figure 27C). No differences in accuracy were observed between the two diamine reagents (Figure 27C). The precision of the method was determined using the relative standard deviation (RSD) between three technical replicates at each concentration; a high level of precision (RSD ≤ 15%) [528, 531] was observed across the concentration range in all matrices with both diamine reagents (Figure 27D). The highest RSD observed was 18% in faecal water diluted 1:10 with PBS spiked with 41 µM $H_2S$ quantified using the 40 – 100 µM diamine reagent (Figure 27D), however this was still within an acceptable range [528, 531].

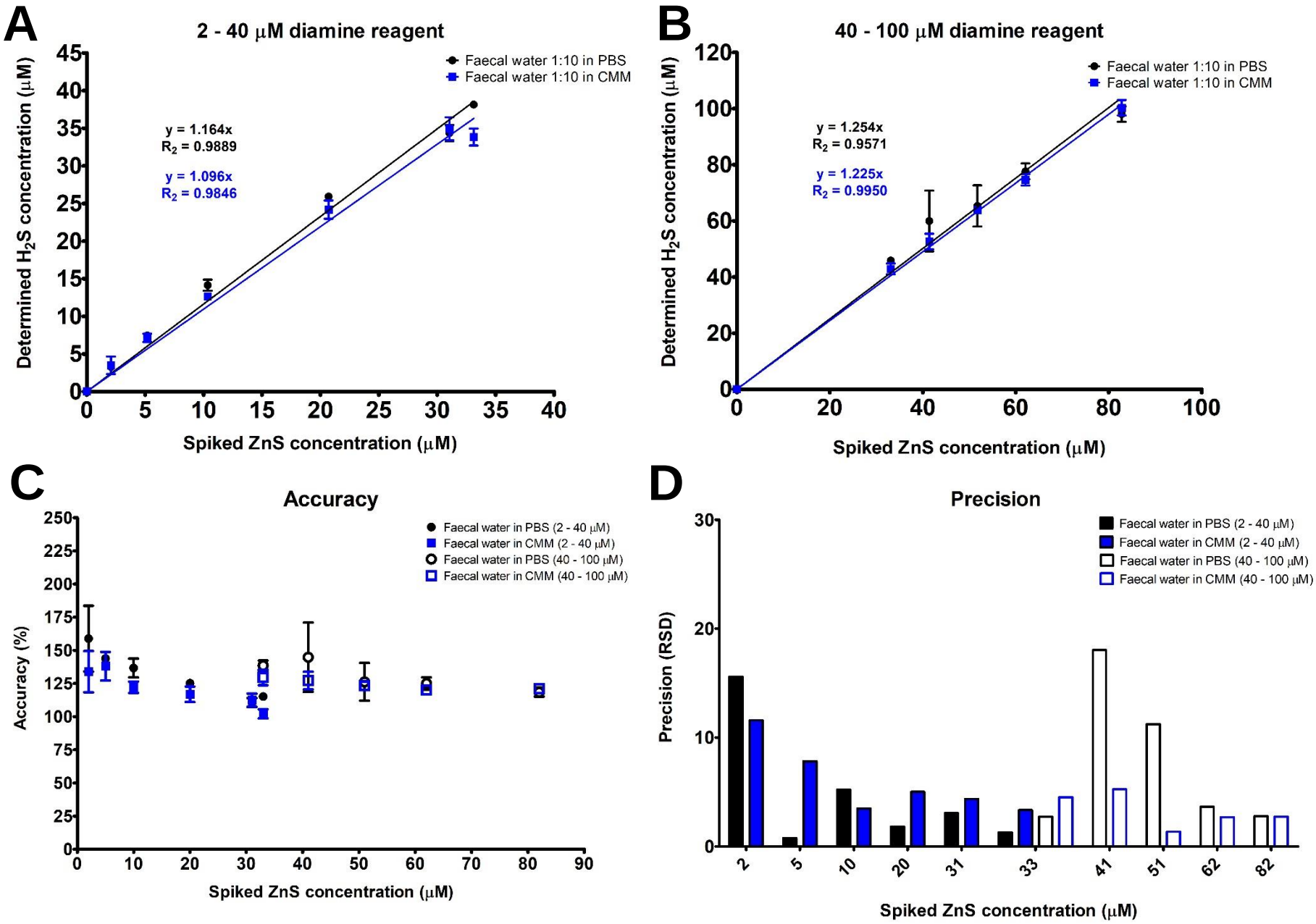

A
2 - 40 μM diamine reagent
Faecal water 1:10 in PBS
Faecal water 1:10 in CMM
y = 1.164x
R2 = 0.9889
y = 1.096x
R2 = 0.9846
Determined H2S concentration (μM)
Spiked ZnS concentration (μM)
B
40 - 100 μM diamine reagent
Faecal water 1:10 in PBS
Faecal water 1:10 in CMM
y = 1.254x
R2 = 0.9571
y = 1.225x
R2 = 0.9950
Determined H2S concentration (μM)
Spiked ZnS concentration (μM)
C
Accuracy
Faecal water in PBS (2 - 40 μM)
Faecal water in CMM (2 - 40 μM)
Faecal water in PBS (40 - 100 μM)
Faecal water in CMM (40 - 100 μM)
Accuracy (%)
Spiked ZnS concentration (μM)
D
Precision
Faecal water in PBS (2 - 40 μM)
Faecal water in CMM (2 - 40 μM)
Faecal water in PBS (40 - 100 μM)
Faecal water in CMM (40 - 100 μM)
Precision (RSD)
Spiked ZnS concentration (μM)

Figure 27: Performance of colourimetric assay for determining $H_2S$ concentration in two matrices - faecal water diluted 1:10 with PBS or faecal water diluted 1:10 with colon model media (CMM).
A) Determined $H_2S$ concentration with diamine reagent for 2 – 40 µM $H_2S$. B) Determined $H_2S$ concentration with diamine reagent for 40 – 100 µM $H_2S$. C) Percentage accuracy of quantification in faecal matrices. D) Method precision expressed as relative standard deviation (RSD) of three technical replicates. Data-point shows mean of three technical replicates. Error bars represent SD.

$H_2S$ concentration in the original faecal sample was determined using the lower concentration diamine reagent as the estimated quantification in the assayed sample was 0.1 µM, which is well below the range of the higher concentration diamine reagent (40 – 100 µM) (Table 13). Back-calculating from a 1:400 dilution from original faeces, the determined $H_2S$ concentration was 40 µM per g wet weight faeces (Table 13).

Table 19: $H_2S$ concentration determined in faecal water diluted in PBS quantified using the 2 – 40 µM diamine reagent. The $H_2S$ concentration per gram (g) wet weight faeces was determined via back-calculation.

| | $H_2S$ concentration determined in assay (µM) | $H_2S$ concentration per g wet weight faeces (µM) |
|---|---|---|
| Replicate 1 | 0.101 | 40.312 |
| Replicate 2 | 0.025 | 10.078 |
| Replicate 3 | 0.176 | 70.547 |
| **Average** | **0.101** | **40.312** |

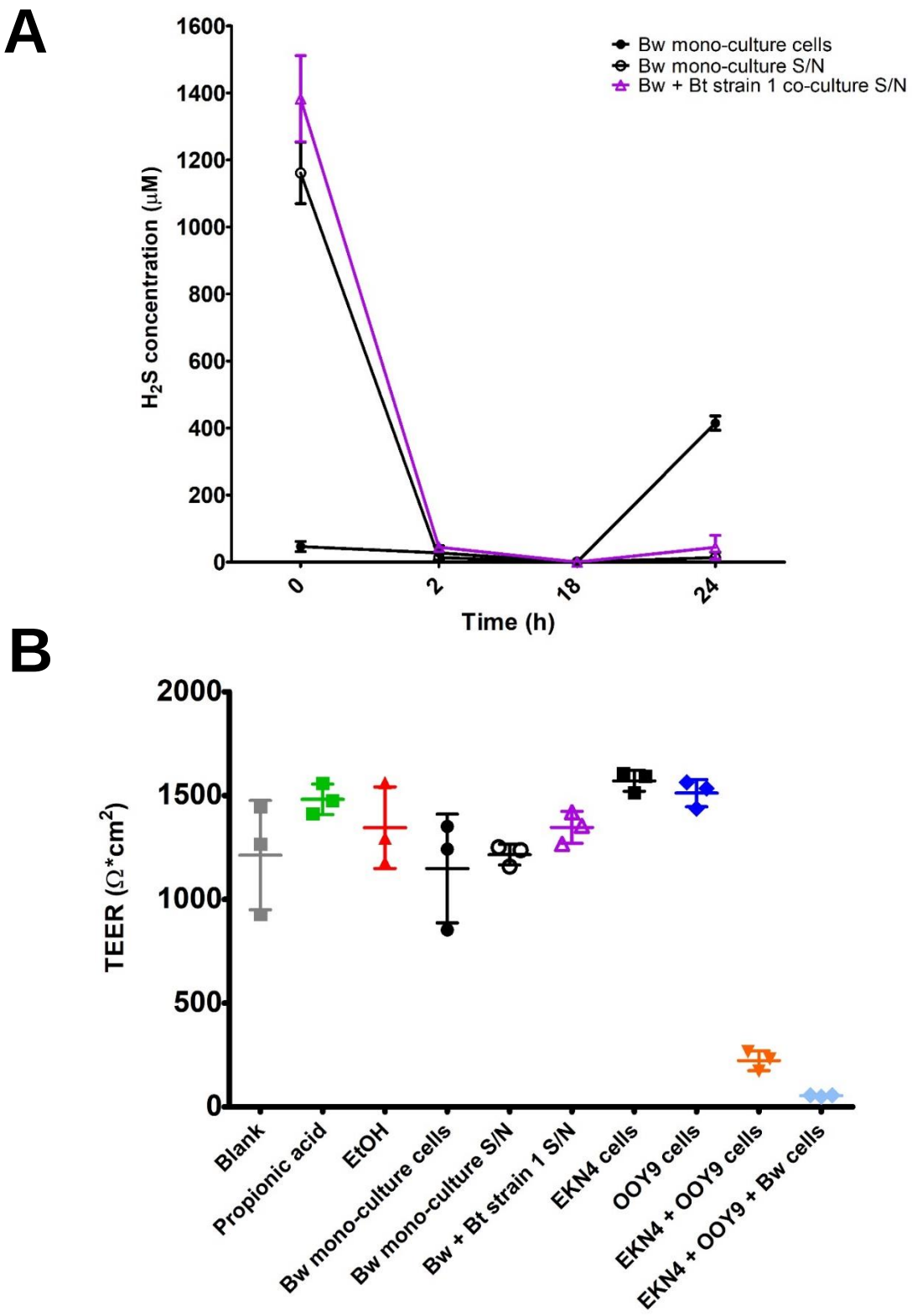
### 5.4.2 Subsection 2 – Investigating the impact of *B. wadsworthia* in gut representative conditions *in vitro*

#### 5.4.2.1 Impact of *B. wadsworthia* on Caco-2 cell mono-layer

Caco-2 cell mono-layers established in transwells for 21 days were inoculated with *B. wadsworthia* (QI0013) cells and cell-free supernatant, or cell-free supernatant from a co-culture of *B. wadsworthia* and Bt strain 1. Additionally, washed cells of probiotic *Lactiplantibacillus plantarum* strains EKN4 and OOY9 were added to the Caco-2 cells alone, together or in combination with *B. wadsworthia*. The resulting impact on the Caco-2 barrier integrity was measured using TEER after 24 h.

Given that $H_2S$ readily oxidises upon exposure to air at physiological pH [532], the $H_2S$ concentration was determined immediately following addition of the *B. wadsworthia*-containing conditions to the Caco-2 cells. Here, mM levels of $H_2S$ were present in cell-free supernatants of *B. wadsworthia* mono-culture and *B. wadsworthia* and Bt strain 1 co-culture, and this was readily oxidised by 2 h (Figure 28A). Interestingly, conditions containing *B. wadsworthia* cells showed slight increase in $H_2S$ concentration between 18 and 24 h (Figure 28A).

The positive control of 10 mM propionic acid showed only a modest increase in barrier integrity relative to the blank media control, whereas the negative control (14% ethanol) did not decrease TEER relative to the blank (Figure 28B). Furthermore, no significant changes were observed with respect to barrier integrity by the addition of *B. wadsworthia* cells or cell-free supernatant after 24 h (Figure 28B). Similarly, cell-free supernatant from *B. wadsworthia* (Bw) and Bt strain 1 co-culture did not affect barrier integrity (Figure 28B). Addition of washed *L. plantarum* EKN4 and OOY9 cells did not affect the barrier alone, however when combined they induced a 75% decrease in TEER relative to blank (Figure 28C). The addition of *B. wadsworthia* washed cells further exacerbated this loss of integrity (Figure 28C).


A
Bw mono-culture cells
Bw mono-culture S/N
Bw + Bt strain 1 co-culture S/N
H2S concentration (μM)
1600
1400
1200
1000
800
600
400
200
0
0
2
18
24
Time (h)
B
TEER (Ω*cm2)
2000
1500
1000
500
0
Blank
Propionic acid
EtOH
Bw mono-culture cells
Bw mono-culture S/N
Bw + Bt strain 1 S/N
EKN4 cells
OOY9 cells
EKN4 + OOY9 cells
EKN4 + OOY9 + Bw cells

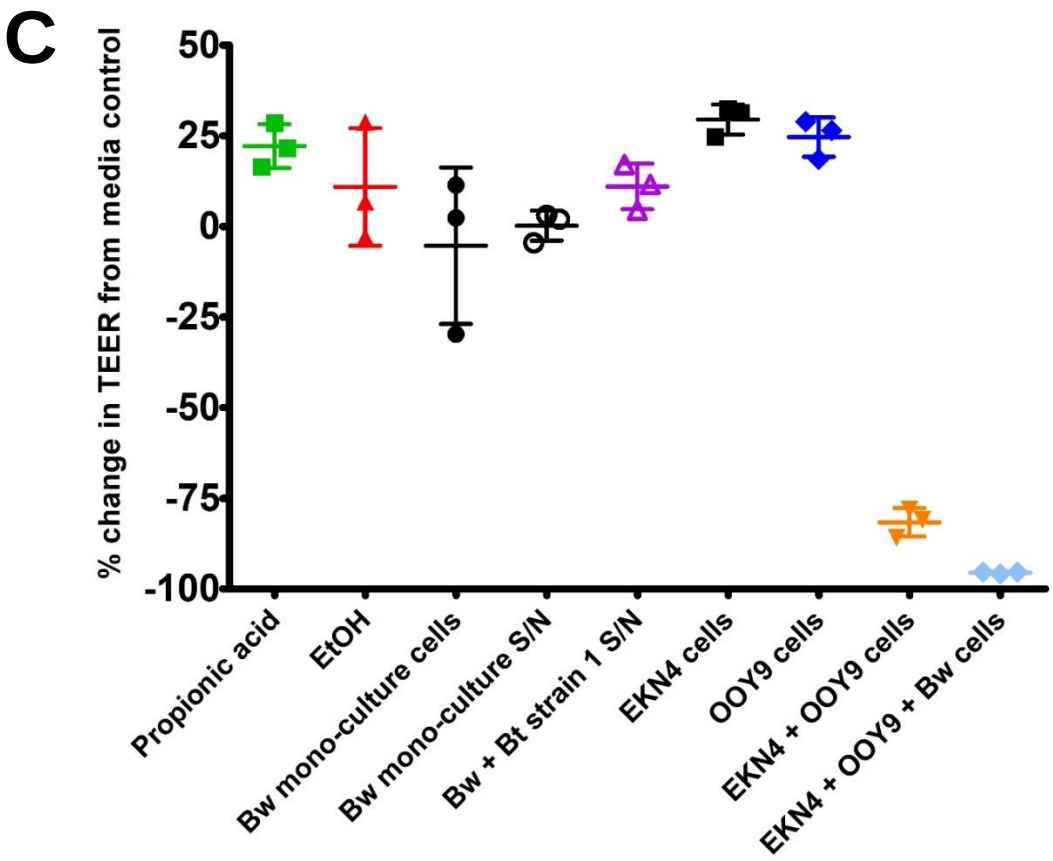


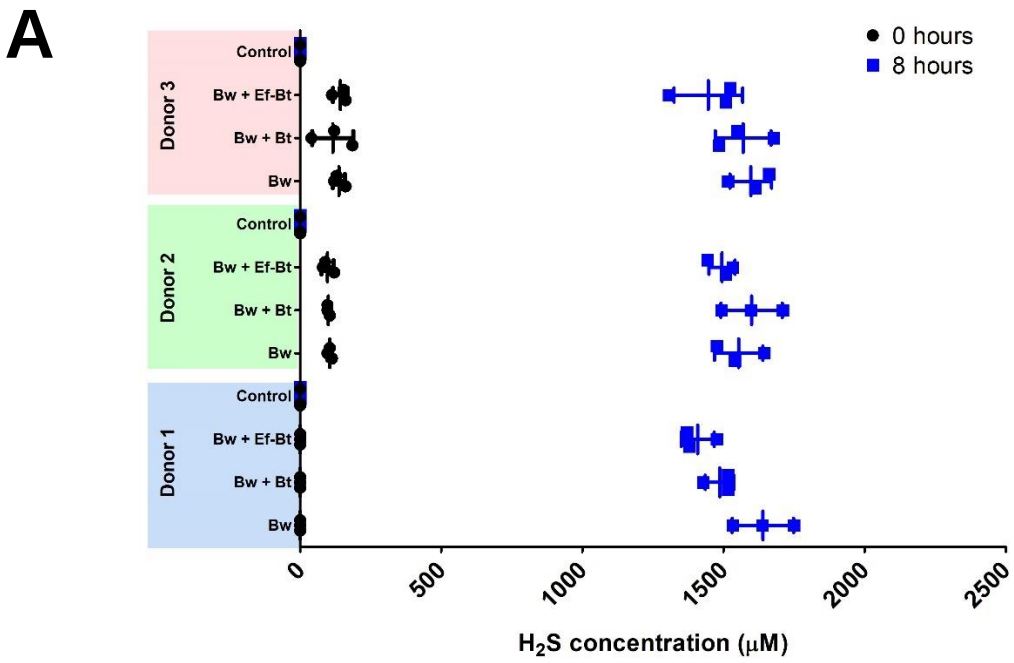

Figure 28: Impact of bacterial cells and supernatant on Caco-2 cell mono-layer.

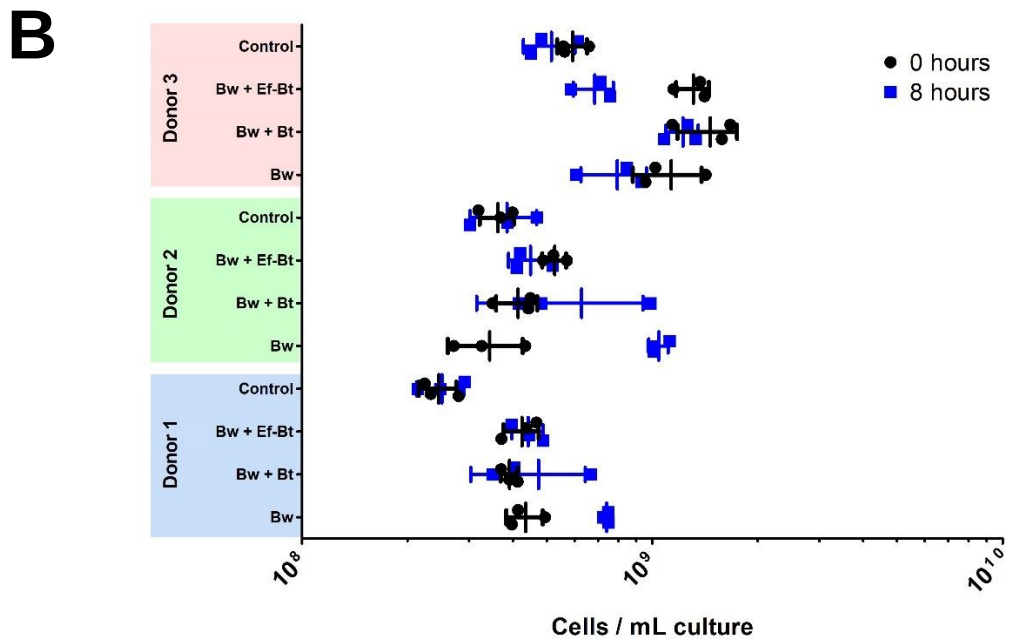
A) $H_2S$ concentration in Caco-2 cell mono-layer apical media at 0-, 2-, 18- and 24-h post-inoculation. B) Transepithelial electrical resistance (TEER) values in Caco-2 cell mono-layers exposed to different conditions after 24 h. C) Percentage change in TEER values relative to blank in Caco-2 cell mono-layers exposed to different conditions after 24 h. Blank is media only control, 10 mM propionic acid is positive control and EtOH is 14% ethanol negative control. S/N = supernatant. Datapoints represent technical culture replicates (n=3).

### 5.4.2.2 Modelling the impact of *B. wadsworthia* supplementation to a complex faeces-derived microbiota *in vitro*

We investigated the $H_2S$ production by *B. wadsworthia* in the context of a complex gut microbiota and gut-representative nutritional conditions by seeding human faeces into Complex Intestinal Media supplemented with 5.6 mM taurine and adding $10^8$ CFU/mL *B. wadsworthia* (QI0013). To investigate whether the sulfidogenic effects of Bt strain 1 and inhibitory effects of *E. faecium-B. thetaiotaomicron* enrichment (Ef-Bt) could be recapitulated in the gut-representative conditions, we supplemented *B. wadsworthia* (Bw) to the model with either Bt strain 1 or Ef-Bt in a 1:1 ratio. The experiment was performed in triplicate, using faecal inoculum from QIB Colon Model study donors 1, 2 and 3 (Table 19). Samples were taken at 0- and 8 h post-inoculation to determine the $H_2S$ concentration, total bacterial cell counts and for metagenomic analysis of the microbiota composition.

No $H_2S$ was detected in the control samples at 0 or 8 h post-inoculation, where 1% faeces were inoculated with no additional bacterial strain supplementation (Figure 29A). This indicates that $H_2S$-producing bacteria were either non-viable, or the growth media and conditions used did not permit substantial growth of $H_2S$-producers. However, Bw-containing conditions showed significant $H_2S$ production of approximately 1.5 mM by 8 h post-inoculation in all three experiments seeded by different faecal donors (Figure 29A). *B. wadsworthia*-derived $H_2S$ concentrations were not differentially affected by the presence of either the $H_2S$-promoting Bt strain 1 or the inhibitory Ef-Bt enrichment (Figure 29A). Total bacterial cell counts were determined using flow cytometry; cell numbers remained largely stable between 0 and 8 h with no changes in order of magnitude between these time-points (Figure 29B). In donors 1 (CM046) and 2 (CM072), supplementation with Bw alone resulted in increased cell number at 8 h compared to 0 h (Figure 29B), indicating increased growth; this is likely to be attributable to *B. wadsworthia* directly as $H_2S$ concentration increased to 8 h in these cultures (Figure 29A). Culture pH decreased substantially in all conditions from pH 7.2 to pH 5 – 6 after 8 h (Figure 29C), likely reflecting the metabolic activity of lactic acid bacteria present in the faecal samples.


A
0 hours
8 hours
Donor 3
Donor 2
Donor 1
Control
Bw + Ef-Bt
Bw + Bt
Bw
0
500
1000
1500
2000
2500
H2S concentration (μM)


B
0 hours
8 hours
Donor 3
Donor 2
Donor 1
Control
Bw + Ef-Bt
Bw + Bt
Bw
10^8
10^9
10^10
Cells / mL culture

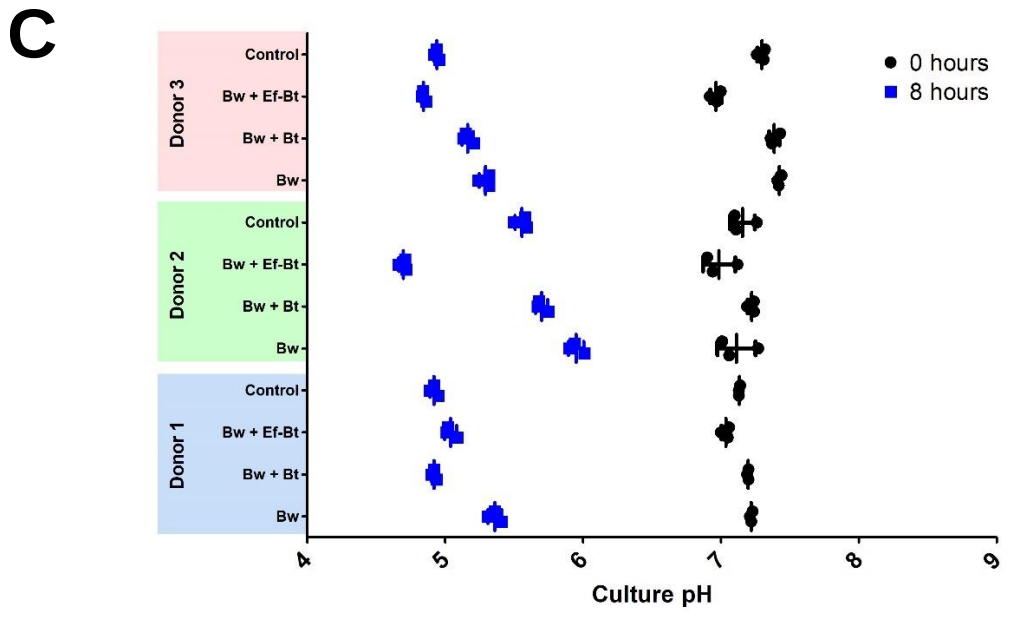

C
0 hours
8 hours
Donor 3
Donor 2
Donor 1
Control
Bw + Ef-Bt
Bw + Bt
Bw
4
5
6
7
8
9
Culture pH

Figure 29: Impact of supplementation of *B. wadsworthia* alone (Bw), *B. wadsworthia* with Bt strain 1 (Bw + Bt) and *B. wadsworthia* with Ef-Bt enrichment (Bw + Ef-Bt) on an *in vitro* complex gut microbiota seeded with faeces from 3 different donors.
Faecal inoculum with no additional bacterial strain supplementation was performed as control. A) $H_2S$ concentration at 0- and 8 h post-inoculation. B) Total bacterial cell numbers per mL culture determined via flow cytometry at 0- and 8 h post-inoculation. C) Culture pH at 0- and 8 h post-inoculation. Data-points show each technical culture replicate (n=3). Line shows mean, and error bars show SD.

Next, we investigated the impact of supplementing *B. wadsworthia* alone (Bw), in combination with *B. thetaiotaomicron* (Bw + Bt) or in combination with a mixed enrichment of *E. faecium* and *B. thetaiotaomicron* (Bw + Ef-Bt) on the composition of the complex gut microbiota *in vitro*. We used shotgun metagenomic sequencing to analyse the microbial composition of samples taken at 0- and 8 h post-inoculation, using metaphlan 4.0 combined with flow cytometry data to determine the absolute abundance to species level. The absolute abundance of Proteobacteria increased from 0- to 8 h in all conditions where *B. wadsworthia* was supplemented (Figure 30A), likely reflecting increased abundance of *B. wadsworthia*. This pattern was observed with all three donors, except for donor 3 supplemented with *B. wadsworthia* and the *E. faecium* and *B. thetaiotaomicron* enrichment (Bw + Ef-Bt), where the Proteobacteria abundance decreased from 0- to 8 h (Figure 30A). In the control cultures where no additional strains were supplemented, it is interesting to note that donor 3 was the only sample where Proteobacteria were present at both 0- and 8 h (Figure 30A); this was due to the presence of *B. wadsworthia* and *D. piger* in the faeces of this donor (supplementary data, section 7.1.3). The differences in microbiota composition between samples were investigated using beta diversity, looking for changes in composition due to time-point, experimental condition, and donor (Figure 30B). The NMDS analysis stress score was 0.18; this was below the generally-accepted cut-off of ≤0.2 [533, 534] indicating that the reduced-dimension model provided a reliable representation of the data. There was clear clustering of samples based

on donor with no overlap, indicating that microbial composition was distinct between donors (Figure 30B). Compositional shifts due to sampling time-point were also apparent in donors 1 and 3, whereas samples from donor 2 appeared more similar at both 0- and 8 h (Figure 30B). Compositional differences due to experimental condition were relatively less pronounced, but still apparent (Figure 30B). We then used PERMANOVA to test if differences in microbial composition were statistically significant, where the null hypothesis was that there were no differences in microbial composition due to time-point, experimental condition, or donor; differences in microbial composition were statistically significant ($p \leq 0.05$) due to these factors (Table 14). However, $R^2$ values were relatively low for both time-point and condition, explaining approximately 16% and 26% of the variance respectively (Table 14) [535]. The faecal donor appeared to be the main determinant of microbial composition, accounting for ~ 44% of data variance (Table 14). Taken together, this shows that most of the variance in microbial composition was due to the faecal donor.

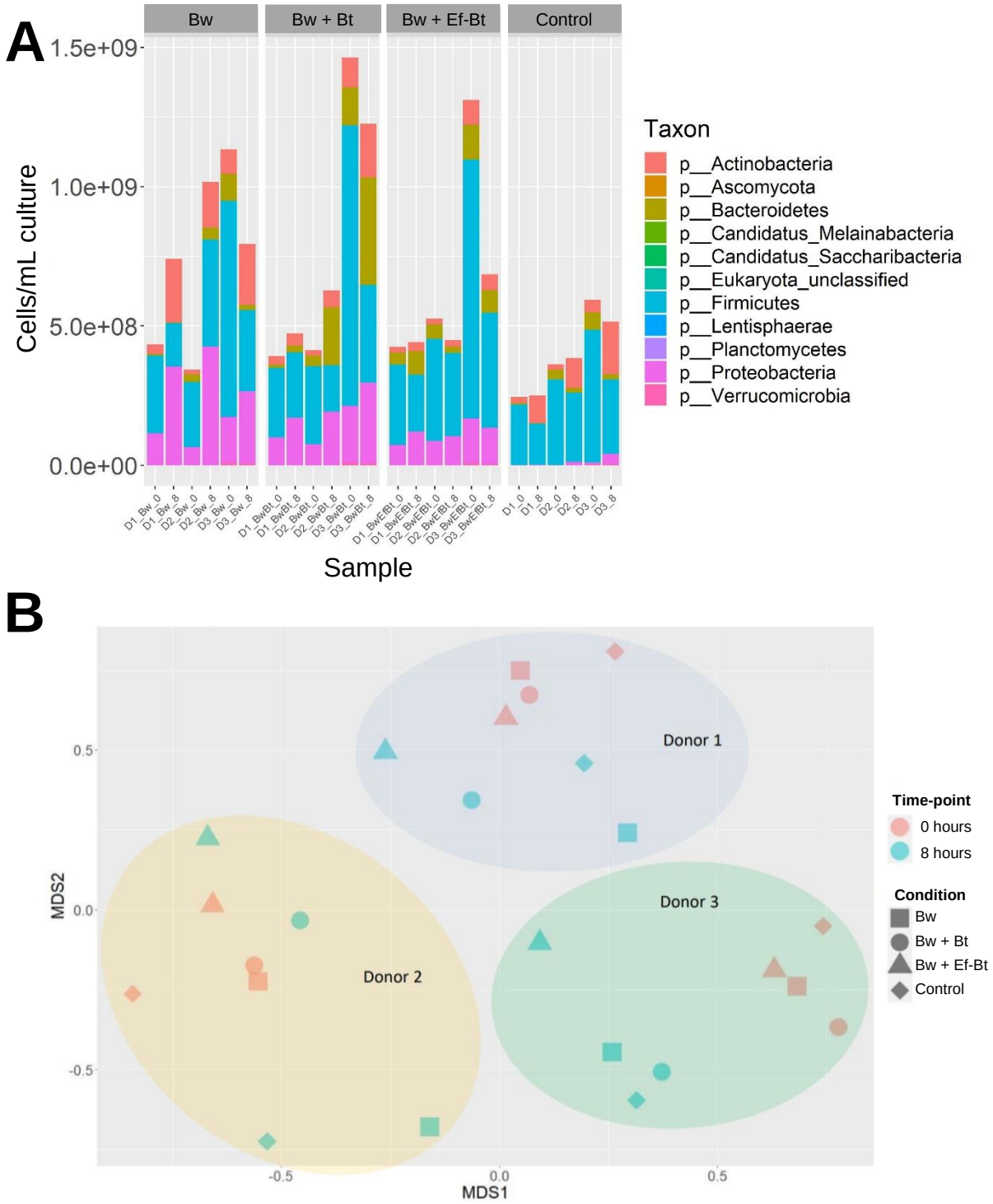



Figure 30: Microbial composition of *in vitro* models of the complex gut microbiota supplemented with *B. wadsworthia* QI0013 (Bw), *B. wadsworthia* with *B. thetaiotaomicron* (Bw + Bt), or *B. wadsworthia* with an *E. faecium-B. thetaiotaomicron* enrichment (Bw + Ef-Bt).
Faecal inoculum with no additional bacterial strain supplementation was performed as control. A) Phyla-level microbial composition of samples inoculated with faeces from donor 1, 2 or 3 (D1, D2, D3) at 0- and 8 h post-inoculation clustered by condition. Average values of culture triplicates for each condition are shown, expressed as absolute abundance (cells/mL culture). B) NMDS plot representing beta diversity using Bray-Curtis distances to compare sample composition by donor, condition, and sampling time-point.

Table 20: Permutational analysis of variance (PERMANOVA) scores using the adonis package.

| Variable | $R^2$ value | P value |
|---|---|---|
| Time-point | 0.16459 | 0.001 *** |
| Condition | 0.26442 | 0.003 ** |
| Donor | 0.43628 | 0.001 *** |

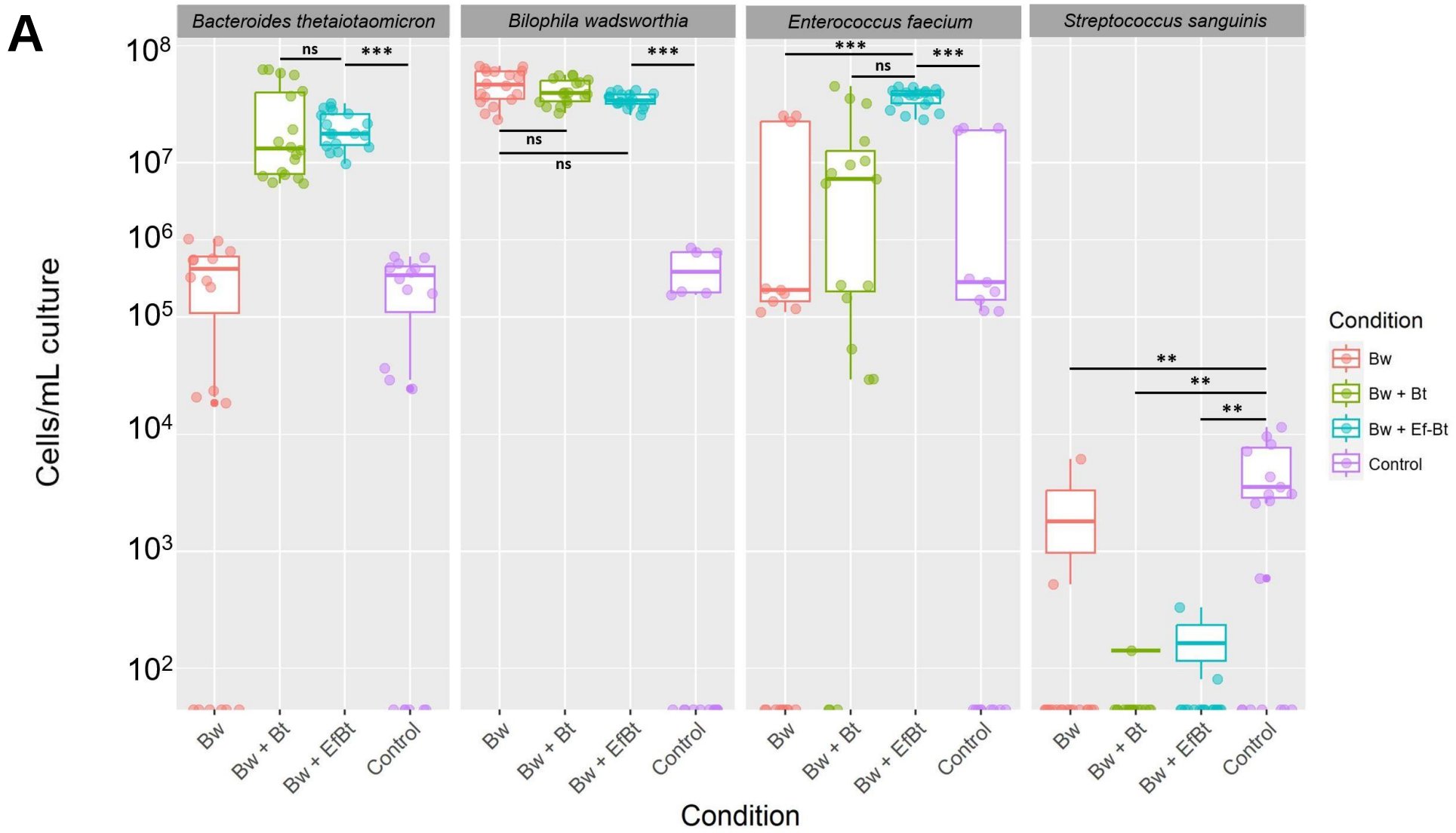
Following investigation of the overall data structure, we then explored the differentially abundant species due to condition, donor, and time-point (Figure 32). Non-parametric testing was used with Benjamini-Hochberg correction for multiple hypothesis testing [525], where remaining species were identified as being significantly differentially abundant between groups. Four species were identified as differentially abundant due to condition, 122 species due to time-point and 381 species due to donor. For visualisation, differentially abundant species identified by time-point and donor were ranked by absolute abundance and the top ten were displayed, as these were the most likely to have biological importance.

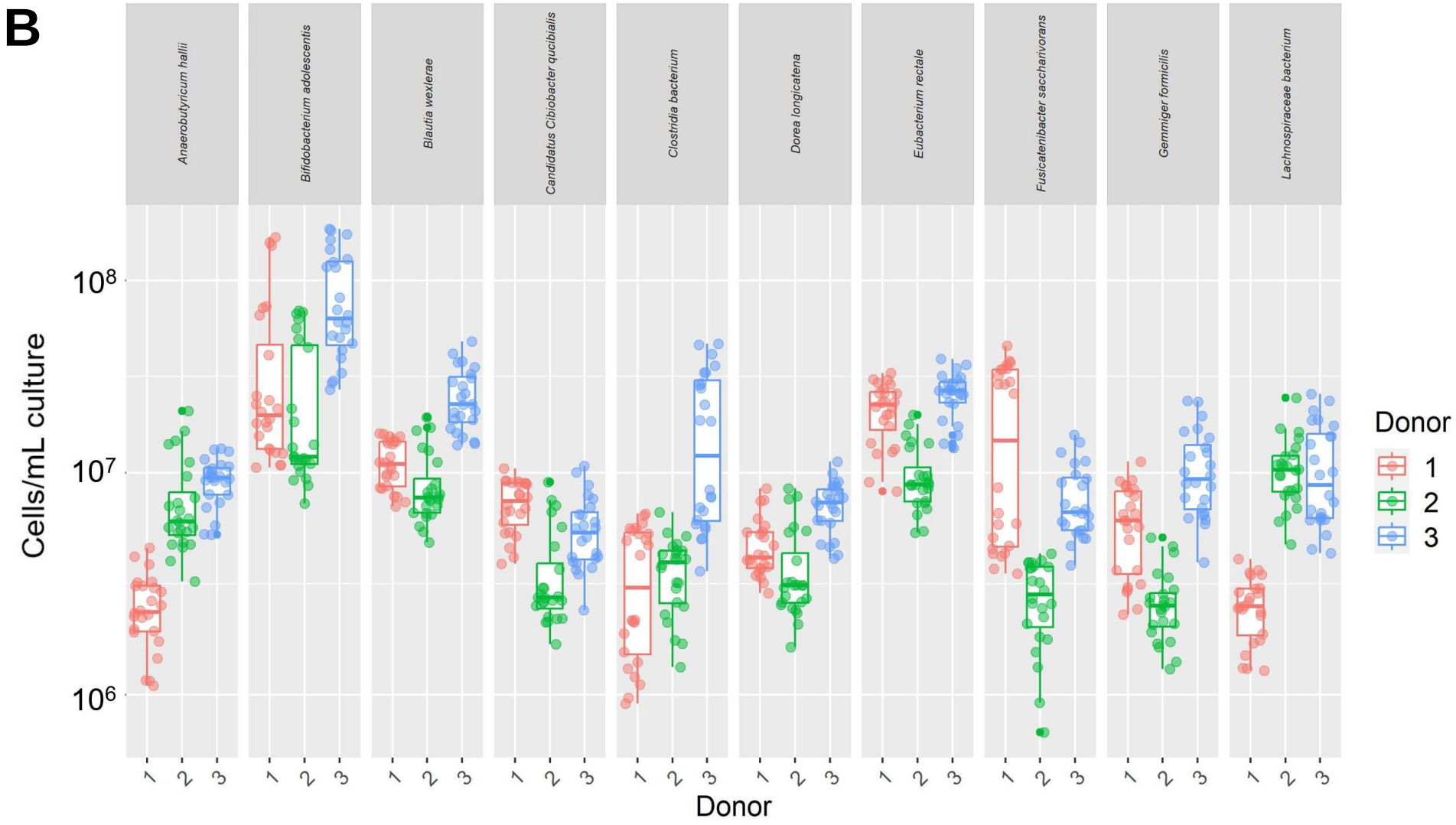
The four species identified as differentially abundant due to condition included *B. wadsworthia*, *E. faecium* and *B. thetaiotaomicron*; this is expected given that these strains were supplemented to their respective experimental conditions (Figure 31A). *B. wadsworthia* abundance was not significantly different when supplemented alone (Bw) compared to together with the $H_2S$-promoting Bt strain 1 (Bw + Bt) or when supplemented with the Ef-Bt enrichment (Bw + Ef-Bt) (Figure 31A). However, there was a trend towards decreased *B. wadsworthia* abundance when together with Ef-Bt enrichment; the average abundance of *B. wadsworthia* was $2 \times 10^8$ cells/mL when supplemented alone (Bw), compared to $1 \times 10^8$ cells/mL in Bw + Ef-Bt (Figure 31A). *B. thetaiotaomicron* abundance was not significantly affected by the presence of *E. faecium* (Bw + Ef-Bt) compared to the absence of this supplemented strain (Bw + Bt) (Figure 31A). The ability of this *E. faecium* strain to slightly reduce *B. wadsworthia* abundance without affecting *B. thetaiotaomicron* could potentially confer benefits in the gut environment. *Streptococcus sanguinis* was also identified as significantly differentially abundant between conditions (Figure 31A); this strain was present in low abundance ($10^3$ – $10^4$ cells/mL culture) in control cultures from donors 1 and 2 and significantly

decreased in abundance in supplemented cultures (Figure 31A). The strain was not present in donor 3 cultures (supplementary data, section 7.1.3). *S. sanguinis* is associated with the oral microbiota [536] and was found to be enriched in the faecal microbiota of chronic fatigue patients compared to controls where it is associated with significant lactic acid production [537]. It seems that supplementation with *B. wadsworthia* alone or in combination with Bt or Ef-Bt here significantly reduced the abundance of this strain (Figure 31A).

Of the 381 significantly differentially abundant species due to faecal donor, the ten most abundant were displayed graphically (Figure 31B). Several species were more abundant in donor 3 compared to donors 1 and 2, including *Bifidobacterium adolescentis*, *Blautia wexlerae*, *Clostridia* bacterium, *Dorea longicatena*, *Eubacterium rectale* and *Gemmiger formicilis* (Figure 31B). This data highlights the heterogeneity of the gut microbiota composition between individuals and emphasises the importance of considering such variation when designing experiments investigating the effect of specific inventions on the gut microbiota composition.

A total of 122 species were identified as significantly differentially abundant due to sampling time-point; the ten most abundant were displayed here (Figure 31C). Several species decreased in abundance between 0 and 8 h, including *Faecalibacterium prausnitzii*, *Fusicatenibacter saccharivorans*, *Gemmiger formicilis*, *Clostridia* bacterium, Clostridiales bacterium KLE1615 and a Lachnospiraceae spp. (Figure 31C). This could reflect the inability of these strains to maintain their population in the *in vitro* environment, possibly due to out-competition or an absence of specific growth requirements. Under the conditions used, *Bifidobacterium adolescentis, Bifidobacterium longum* and *Collinsella aerofaciens* increased in abundance during the experimental period (Figure 31C), suggesting that the conditions were conducive to growth of these strains. *B. wadsworthia* was also identified as significantly increasing in abundance between 0 and 8 h (Figure 31C); this corresponds to the observations of significantly increased $H_2S$ concentration in *B. wadsworthia*-supplemented cultures at 8 h (Figure 29A) and shows that *B. wadsworthia* can successfully establish in the context of a complex gut microbiota *in vitro*.


A
Bacteroides thetaiotaomicron
Bilophila wadsworthia
Enterococcus faecium
Streptococcus sanguinis
Cells/mL culture
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
$10^3$
$10^2$
ns
***
**
Bw
Bw + Bt
Bw + EfBt
Bw + Ef-Bt
Control
Condition


B
Anaerobutyricum hallii
Bifidobacterium adolescentis
Blautia wexlerae
Candidatus Cibiobacter qucibialis
Clostridia bacterium
Dorea longicatena
Eubacterium rectale
Fusicatenibacter saccharivorans
Gemmiger formicilis
Lachnospiraceae bacterium
Cells/mL culture
$10^8$
$10^7$
$10^6$
1
2
3
Donor
Donor

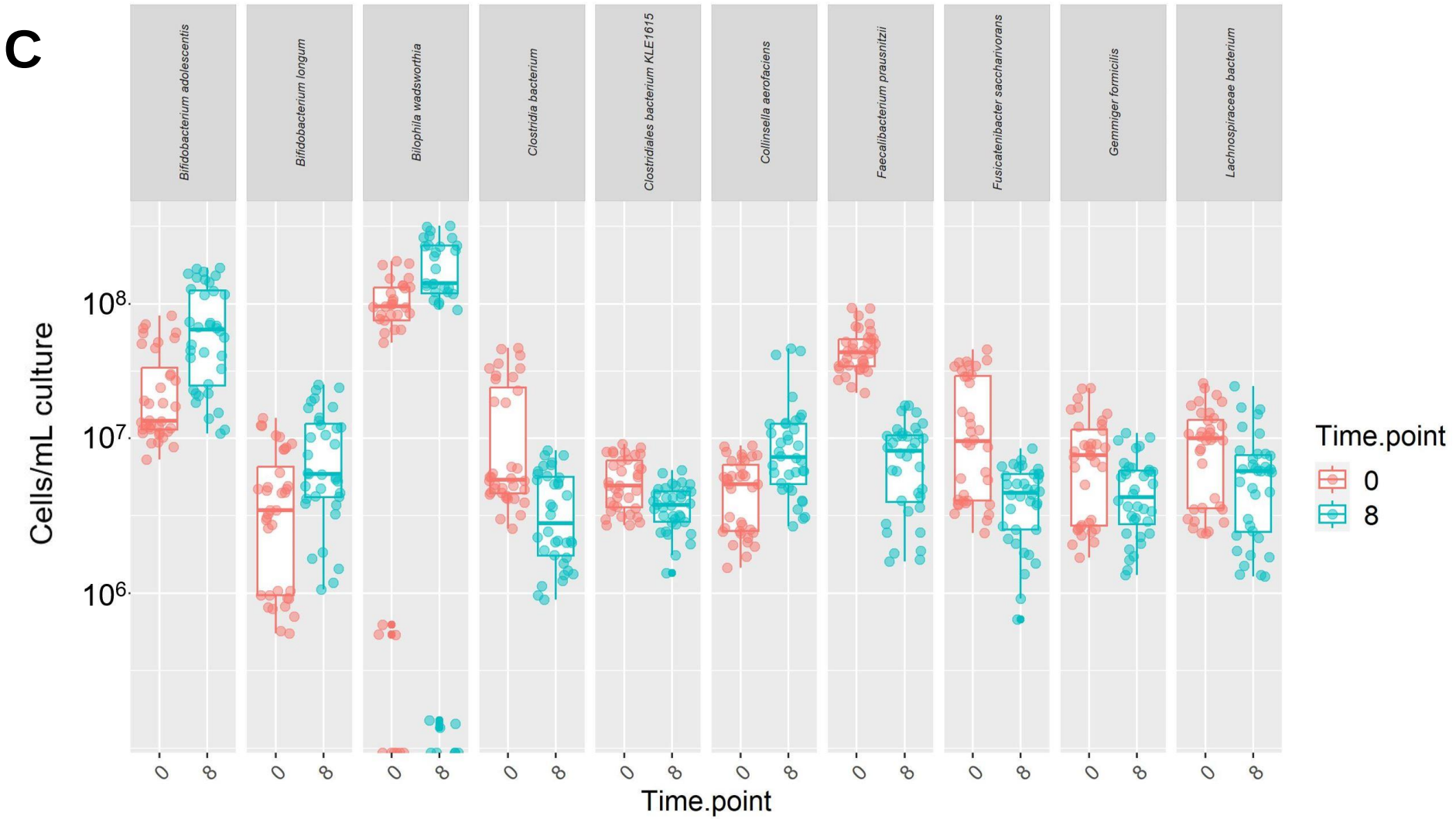

C
Bifidobacterium adolescentis
Bifidobacterium longum
Bilophila wadsworthia
Clostridia bacterium
Clostridiales bacterium KLE1615
Collinsella aerofaciens
Faecalibacterium prausnitzii
Fusicatenibacter saccharivorans
Gemmiger formicilis
Lachnospiraceae bacterium
Cells/mL culture
$10^8$
$10^7$
$10^6$
0
8
Time.point
Time.point
0
8

Figure 31: Differentially abundant taxa in *in vitro* models of the complex gut microbiota supplemented with *B. wadsworthia* QI0013 (Bw), *B. wadsworthia* with *B. thetaiotaomicron* (Bw + Bt), or *B. wadsworthia* with an *E. faecium-B. thetaiotaomicron* enrichment (Bw + Ef-Bt).
Faecal inoculum with no additional bacterial strain supplementation was performed as control. Boxplots display the interquartile range (Q1 – Q3), whiskers display minimum and maximum values (1.5x IQR), line at median. A) Differentially abundant taxa due to experimental condition. All culture replicates are shown for 0- and 8- h post-inoculation. Results of pair-wise Mann Whitney U tests are shown where ns = not significant, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$. B) Differentially abundant taxa due to donor. The top 10 taxa by absolute abundance are displayed. All culture replicates are shown for 0- and 8-h post-inoculation. C) Differentially abundant taxa due to time-point. The top 10 taxa by absolute abundance are displayed. All culture replicates are shown.

## 5.5 Discussion

### 5.5.1 Sulfated amino acid quantification – method development

We investigated the use of a HILIC-MS (LC-MS/MS) method to quantify sulfated amino acids in human faecal matrices. This method can be used to determine sulfated amino acid concentrations when using a standard curve prepared in the same matrix. The LLOQ was relatively high; for utilisation with unknown samples, it may be beneficial to spike externally added amino acids to bring samples into the accurate quantification range. Future method development could investigate alternative sample preparation methods such as liquid or solid phase extraction to improve removal of extraneous matrix components prior to analysis to reduce LLOQ [538]. This would be particularly beneficial for homocysteine which was subject to a significant matrix effect (Figure 25C). Although matrix effect can be mitigated against by preparing a standard curve in the matrix of interest from which unknown samples can be directly compared, this can be problematic if compounds are low-abundance. Here, the only amino acid to be quantified above the LLOQ was taurine in faecal water. This was determined as 5.18 µM as an average of three injection replicates, equating to 207.33 µM per gram wet weight of faeces (Table 12). The expected concentrations of sulfated amino acids in human faeces are difficult to predict due to a lack of published literature

investigating the amino acid composition in the human colon. Amino acid analysis of mucosa from the descending colon showed 2.63 mM taurine and 0.05 mM methionine per kg biopsy weight, but the researchers could not analyse cysteine or homocysteine [187], and another study found methionine at 0.06 mM per kg biopsy weight in human ileal mucosal specimens [539]. One study quantified cysteine at 1.8 nM and homocysteine at 1.2 nM per mg of protein in the distal colonic mucosa of healthy patients [540]. However, the researchers performed amino acid analysis after protein precipitation of the mucosal samples, making comparisons between studies difficult [540]. Overall, the preliminary method development has shown that this HILIC-MS approach can be used to accurately quantify four sulfated amino acids in faecal matrices within a known concentration range; this could be used to track amino acid utilisation and production during fermentation in *in vitro* colon models, and to gather data on sulfated amino acid concentration in human faeces.

### 5.5.2 Evaluating the colourimetric methylene blue method for $H_2S$ quantification in faecal matrices

We investigated the accuracy and precision of the colourimetric methylene blue assay for determining $H_2S$ concentration in matrices containing human faeces. Both diamine reagents performed linearly across the concentration ranges tested (Figure 26, Figure 27A, Figure 27B), quantified $H_2S$ with an accuracy of 120-130% in both faecal matrices (Figure 27C) and showed a high level of precision between technical replicates (Figure 27D). A generally accepted percentage accuracy range for LCMS-based metabolite quantification is 85-115% [526, 541, 542]; there was consistent over-quantification in both matrices across the concentration range of both diamine reagents (Figure 27C). Although this method perhaps cannot be directly compared to the stringent accuracy guidelines of LCMS-based quantification methods, the higher-than-expected $H_2S$ concentration identifies a clear target for method development. Future work could seek to reduce this by initially ensuring that residual $H_2S$ in the sample blanks used to standardise the samples is fully depleted using additional stirring, although Strocchi *et al*. showed that 20 minutes of stirring with atmospheric exposure and acidification was sufficient to remove 98% faecal sulfide from the sample blanks [514].

Both diamine reagents performed similarly with regards to quantification accuracy and precision, however it is recommended that the diamine reagent absorbance

values do not exceed 1.0, as aqueous solutions of methylene blue may not strictly conform to Beer's law at higher concentrations [272]. The lower concentration diamine reagent suitable for quantifying 2 – 40 µM $H_2S$ showed lower absorbance values (Figure 26A) compared to the higher concentration reagent (Figure 26B); the lower concentration reagent is more suitable for $H_2S$ quantification here. It is therefore recommended that samples of interest are first tested at varying dilutions to ensure that the samples are within the absorbance range of the diamine reagent; use of the lower concentration reagent has the additional benefits of further dilution which reduces potential absorbance interference introduced by the sample matrix. Quantification of the $H_2S$ present in the original faecal sample was performed using the 2 – 40 µM diamine reagent, where the average determined concentration in the assayed samples was 0.1 µM (Table 1), which is well below the recommended concentration range of the higher concentration diamine reagent. The faeces were diluted 1:400, meaning the estimated $H_2S$ concentration was 40 µM per g wet weight faeces (Table 13). This is considerably higher than previous studies which reported $H_2S$ concentrations in human faeces as 1 µM per g [74], 0.35 µM per g [76], and 3.38 µM per g with high sulfate diet [77]. For accurate quantification of $H_2S$ in faeces, a lower final dilution should be considered; here a 1:400 dilution reduced the assayed $H_2S$ concentration to 0.1 µM, which is too low for accurate quantification. However, this method is suitable for accurate quantification in the 2 – 100 µM $H_2S$ range in experimental samples obtained in faecal water matrices containing either PBS or colon model media. This colourimetric method for determining $H_2S$ concentration is a precise and accurate method for use with experimental samples at the tested dilution ranges of matrices containing human faeces diluted with either PBS or colon model media.

### 5.5.3 Impact of bacterial strains on gut barrier integrity

We investigated the impact of cells and cell-free supernatant from bacterial strains on the barrier integrity of Caco-2 cell mono-layers; treatments included cell-free supernatant of *B. wadsworthia* mono-culture and co-culture with Bt strain 1, and *B. wadsworthia* cells alone and in combination with probiotic *L. plantarum* EKN4 and OOY9 strains. Although the impact of $H_2S$ on the intestinal barrier has not been extensively studied *in vitro*, one study using sodium hydrosulfide, a fast-release $H_2S$ donor showed an ability to partially ameliorate TNF-α and IFN-γ induced intestinal epithelial barrier dysfunction [503], and the slow-release $H_2S$ donor GYY4137 improved barrier integrity in Caco-2 cell mono-layers [543, 544]. In all

cases, the authors did not appear to consider $H_2S$ oxidation under aerobic conditions as an important factor. Here, we showed the rapid oxidation of $H_2S$ by 2 h under physiological conditions used widely for Caco-2 cell culture (Figure 28A); this is an important consideration when utilising fast-release $H_2S$ donors to study the effect on the intestinal barrier. The use of slow-release $H_2S$ donors may offer a more physiologically accurate dose and exposure to Caco-2 cells, in addition to circumventing the issue of oxidation under aerobic conditions. However, the problem persists for study of anaerobic and $H_2S$-producing bacteria here, as the bacterial viability and $H_2S$ concentration are significantly affected by the oxic culture conditions used for Caco-2 cell propagation. *In vitro* models allowing simultaneous co-culture of strict anaerobes and intestinal epithelial cells is an actively developing research field [545-547], and are likely to constitute more meaningful models for studying anaerobe-host interactions.

Here, significant barrier integrity loss was only observed in Caco-2 cells cultured with washed cells of *L. plantarum* EKN4 and OOY9 (Figure 28C). Given the ability of *L. plantarum* to produce lactic acid under aerobic conditions [548], it is probable that the loss of barrier integrity is due to pH reduction, although this is speculative. Interestingly, the most significant loss of barrier integrity was observed with a combination of EKN4, OOY9 and *B. wadsworthia* cells (Figure 28C). At 24 h, elevated $H_2S$ concentration was found in cultures containing *B. wadsworthia* washed cells (Figure 28A); the extensive loss of barrier integrity observed in the combined cell treatment could reflect the additive impact of lactic acid and $H_2S$ production upon the Caco-2 cells. This preliminary data suggests that supernatant from *B. wadsworthia* and Bt strain 1 supernatant does not negatively impact the barrier, but *B. wadsworthia* cells in combination with washed cells of probiotic strains could exert deleterious impacts on the gut barrier integrity. Critically, no significant decrease in TEER was observed here with the negative control treatment with 14% ethanol which has been previously reported to impair barrier integrity [504, 516-518]. In this case, the Caco-2 cells were matured for a longer period of time prior to experiment start and were selected based on high barrier integrity (≥800 Ω $cm^2$); future work should seek to validate these results using a higher concentration of ethanol or an alternative compound such as TNF-α [503, 549] as a negative control for barrier integrity.

### 5.5.4 Investigating the impact of *B. wadsworthia* supplementation to a complex gut microbiota *in vitro*

In previous chapters, *B. wadsworthia*'s $H_2S$ production and growth was shown to be differentially affected by co-culture with *B. thetaiotaomicron* and *E. faecium*, where *B. thetaiotaomicron* showed an $H_2S$-promoting effect (Figure 16) and *E. faecium* was inhibitory (Figure 13). Given that these bacteria are gut-derived strains found in the majority of healthy individuals [23, 213, 240], we investigated whether these interactions are also observed in conditions more closely representing those found in the human gut, and in the context of a complex human gut microbiota. Our hypothesis was disproven; no differences in $H_2S$ concentration were observed due to *B. thetaiotaomicron* or Ef-Bt supplementation (Figure 29A). However, we showed that *B. wadsworthia* could contribute significant levels of $H_2S$ in the context of a faecal matrix and complex gut microbiota with a physiologically relevant taurine dose; this was observed in all conditions in all three faecal samples (Figure 29A). If occurring in the human gut environment, the contribution of *B. wadsworthia*-derived $H_2S$ in the mM range could be pro-inflammatory [98]. Metagenomic analysis of the impact of *B. wadsworthia* supplementation on microbial composition revealed that the majority of variance in composition was attributed to the faecal donor, with smaller contributions from sampling time-point and experimental condition (Table 14, Figure 30B). This concurs with previously published literature demonstrating the considerable inter-individual variation in the gut microbiota composition [165, 550, 551]. *B. wadsworthia* abundance was slightly reduced by the addition of the Ef-Bt enrichment, although this was not statistically significant. Nonetheless, the ability of *E. faecium* to modestly reduce *B. wadsworthia* abundance without affecting *B. thetaiotaomicron* abundance (Figure 31A) is worthy of further investigation.

Substantial decreases in culture pH were observed in all conditions, where cultures reached 5 – 6 by 8 h (Figure 29C). This likely reflects lactic acid production by bacteria present in the faecal samples. Changes in pH are likely to influence bacterial growth and viability; it is therefore important to acknowledge that this study is preliminary and future work could seek to investigate this hypothesis using a growth medium with increased buffering capacity, or by using an *in vitro* model system with integrated pH control such as the colon model [501]. However, the pH range observed here is not necessarily below physiological range. Typically the human colonic mucosa surface pH is around 6 – 7 [552], and

luminal pH is around 6.4 due to production of short chain fatty acids and hydrogen ions by colonic bacteria [553]. However, pathology may induce acidification in the gut; small-scale studies found an association between colonic luminal pH and severity of ulcerative colitis, where pH can range from 4.7 in those with active disease to 2.3 – 3.4 in severe cases requiring surgery [553]. Therefore, the use of gut model systems where pH is not strictly controlled may have merit in allowing exploration of potential mechanisms leading to colonic acidification. A further consideration for future work is the use of freshly prepared human faeces for seeding experimental models. Here, faecal samples were processed anaerobically, flash frozen and stored at -80˚C; it was shown that freezing at -80˚C without added cryoprotectant preserved the microbial ecosystem more efficiently compared to freezing with 10% glycerol or lyophilisation, using fresh stool as a benchmark [507]. Fresh faecal samples are the benchmark for seeding *in vitro* gut model systems but are highly time- and oxygen-sensitive with respect to viability of anaerobes after collection. To minimise these variables impacting stool viability, the faecal samples were processed identically by immediately flash freezing and storing on the same day, whilst achieving a compromise on microbial ecosystem preservation [507]. It would undeniably be valuable to investigate the interaction between *B. wadsworthia* and other bacterial strains using *in vitro* models seeded with fresh faecal samples. In summary, the current study showed the capacity of *B. wadsworthia* to produce significant levels of $H_2S$ from a physiological taurine dose within a complex faeces-derived microbiota in the presence of the faecal matrix and gut-representative growth media [508]. Future work should seek to utilise increased pH buffering capacity and fresh human faeces to seed the gut microbiota model to overcome acidification and potential bacterial viability loss observed with this preliminary study.

# Chapter 6

## 6. Discussion

## 6.1 Summary

*B. wadsworthia* is a unique member of the SRB, able to utilise taurine for $H_2S$ production [51]. Since its discovery in 1989, research into *B. wadsworthia* has implicated this bacterium in disease pathogenesis and inflammation [67, 145, 146, 149-157], but it is commonly found within the commensal gut microbiota of 50-60% of healthy individuals [22-24, 68]. This thesis aimed to investigate the enigmatic nature of *B. wadsworthia* with respect to its interactions with other common human gut microbiota members, to better establish the factors influencing *B. wadsworthia*'s growth and $H_2S$ production potential within the human gut. Here, the main hypotheses, experiments and findings of the thesis are summarised.

## 6.2 *B. thetaiotaomicron* can promote *B. wadsworthia*'s growth and $H_2S$ production

In co-culture with *B. thetaiotaomicron*, *B. wadsworthia* displays significantly increased $H_2S$ production. This interaction appears to depend upon the physical proximity of the strains (Figure 5) and viability of *B. thetaiotaomicron* (Figure 6). In co-culture, indole production by *B. thetaiotaomicron* appeared to be decreased (Figure 20). The sulfidogenic interaction is potentially mediated via cross-feeding of APS, a metabolite that could potentially be used by *B. wadsworthia* for $H_2S$ production (Figure 18). To our knowledge, the beneficial mutualistic interaction between *B. wadsworthia* and *B. thetaiotaomicron* has not been previously identified. This work therefore describes a novel species-level interaction which, if occurring in the human gut environment, could contribute towards high levels of $H_2S$ which are associated with inflammation and disease [44, 98, 102, 262, 282].

### 6.2.1 Future work

A key aspect to consider for future research is identifying whether *B. wadsworthia* contains a functional putative adenylylsulfate reductase; this is a pivotal test for the hypothesis described here, where *B. wadsworthia* and *B. thetaiotaomicron* can co-operatively convert sulfate to $H_2S$ via an APS intermediate. If *B. wadsworthia* produces $H_2S$ in response to APS supplementation, this would suggest the presence of an active adenylylsulfate reductase. A further possible avenue of research could involve isolation of the putative adenylylsulfate reductase for study of the structure and function via proteomic methods such as gel electrophoresis and mass spectrometry [554]. Furthermore, establishing whether *B. thetaiotaomicron* can produce APS from sulfate is an important aspect for future research. This could be investigated by supplementing sulfate to *B.*

*thetaiotaomicron*, and measuring the resulting APS production possibly via LC-MS based quantification; an established method for measuring PAPS via LC-MS has been reported [555], providing a starting point for method development for quantification of APS in bacterial cultures.

The apparent shift towards decreased indole production by *B. thetaiotaomicron* in co-culture with *B. wadsworthia* also provides an opportunity for follow-up in future research. Here, we explored only the endometabolome, limiting our metabolomic study to the differential metabolites within the bacterial cells. Future work could substantiate these observations by investigating the cell-free supernatant metabolome of co-cultures and mono-cultures to explore the relative abundance of tryptophan, indole and indole derivatives. This would establish whether *B. thetaiotaomicron*'s capacity for indole production is indeed decreased in co-culture with *B. wadsworthia*. Given that microbe-derived indole confers beneficial effects in the human gut by ameliorating intestinal inflammation [495, 496], and modulating intestinal IL-22 production to prevent pathogen colonisation [497], establishing whether indole production by *B. thetaiotaomicron*, a key indole producer [306, 491] is decreased by *B. wadsworthia* in the human gut environment is of importance for human health. Future work could investigate this by modelling the *B. wadsworthia* and *B. thetaiotaomicron* interaction in a gut-representative system using *in vitro* microbiological fermentation models seeded with human faeces, and quantifying tryptophan and indole metabolites. This would enhance our understanding of this mutualistic interaction between *B. wadsworthia* and *B. thetaiotaomicron* resulting in increased $H_2S$ and decreased indole production.

Expression of the virulence factor urease was significantly decreased in *B. wadsworthia* in co-culture with *B. thetaiotaomicron*. Urease activity varies between *B. wadsworthia* strains [31], with approximately 75% of strains being urease positive [24]. Although both urease-positive and urease-negative strains have been associated with disease [145], genomic differences between strains have not been investigated beyond confirmation that the 16S rRNA gene sequences remain highly similar [494]. Furthermore, the impact of urease activity in *B. wadsworthia* pathogenesis has not been described. The expression of urease in *B. wadsworthia* and the resulting impact on virulence could be included in future work to better understand the factors involved in *B. wadsworthia* virulence and pathogenesis.

Here, it could be hypothesised that decreased expression of urease in co-culture reduces *B. wadsworthia*'s pro-inflammatory and endotoxic properties. Future work could seek to test this hypothesis using *in vitro* models combining both the human epithelial mono-layer such as Caco-2 cells to represent the gut barrier, in addition to recapitulating the anaerobic environment of the gut lumen conducive to growth of strict anaerobes including *B. wadsworthia* and *B. thetaiotaomicron*, such as gut-on-a-chip [556] or vertical diffusion chamber models [557]. Using such systems, the impact of high-urease-expressing and low-urease-expressing *B. wadsworthia* could be compared with respect to impact on inflammatory cytokine expression and gut barrier integrity. This would allow us to examine whether urease expression by *B. wadsworthia* affects the virulence of this bacterium.

## 6.3 Enterococcal strains inhibit *B. wadsworthia*'s growth and prevent $H_2S$ production

Enterococcal strains caused potent inhibition of *B. wadsworthia*'s $H_2S$ production (Figure 11) and growth (Figure 12). Growth inhibition was also observed using cell-free supernatant from *E. faecium*, suggesting the inhibition is at least in part due to pH reduction via lactic acid production (Figure 14, Figure 15). Use of a *B. thetaiotaomicron* and *E. faecium* enrichment to control growth and $H_2S$ production by *B. wadsworthia* whilst facilitating production of beneficial compounds such as hypoxanthine [369], lactic acid [390] and raffinose [368] (Figure 15) could confer benefit if occurring in the human gut environment. As such, *E. faecium* could be an attractive preliminary candidate for use as an anti-*B. wadsworthia* probiotic.

### 6.3.1 Future work

The inhibitory effect of *E. faecium* upon *B. wadsworthia* appears to be at least partially mediated by pH reduction, but the relative contributions cannot be established in these experiments as the pH was not controlled. To unpick the contribution of lactic acid to the inhibitory effect, future work could co-culture *B. wadsworthia* with washed cells, or pH-neutralised cell-free supernatant of *E. faecium*. Alternatively, the co-culture could be performed in a pH-controlled setting, such as the microMatrix fermentation system [558] or the *in vitro* colon model system [501] to allow identification of a pH-independent inhibitory effect of *E. faecium* on *B. wadsworthia*.

Analysis of the cell-free supernatant metabolome of *B. wadsworthia* in co-culture with the mixed enrichment of *E. faecium* and *B. thetaiotaomicron* (Ef-Bt) revealed differentially abundant metabolites of interest, including increased abundance of chrysin and genistein. Microbial production of these compounds has not been reported in the literature; future work could substantiate these observations by measuring production of these compounds in bacterial mono-cultures and co-cultures, either using untargeted LC-MS or NMR-based metabolomic approaches. Alternatively, targeted quantification methods could be developed to quantify the absolute abundance of compounds of interest via comparison of unknown samples to a standard curve. For example, the metabolomic analysis revealed increased abundance of raffinose in Ef-Bt cultures, which we postulated as being due to raffinose synthase activity by *B. thetaiotaomicron*. Future work could test this hypothesis by measuring raffinose in the cell-free supernatant of *B. thetaiotaomicron* mono-cultures over time, or by investigating expression and activity of the raffinose synthase gene. Furthermore, styrene was identified as the top VIP compound (VIP score 1.762) contributing to differences between experimental conditions; styrene was in higher abundance in Ef-Bt cultures compared to *B. wadsworthia* mono-culture and negative control. To the best of our knowledge, styrene metabolism in *E. faecium* and *B. thetaiotaomicron* has not been previously explored; quantification of styrene in cell-free supernatants of *E. faecium* and *B. thetaiotaomicron* cultures would help substantiate whether these strains are indeed capable of styrene production.

An ability to control *B. wadsworthia* abundance and $H_2S$ production in the human gut could have therapeutic benefit, as *B. wadsworthia* enrichment and high $H_2S$ concentrations have been associated with inflammation [67, 145, 146] and disease pathogenesis [152-154]. Further screening of the two potential probiotic *E. faecium* strains (FI 09198 and QI0436) would be a crucial next step. The candidate strains should be screened for their bacteriocin production capacity *in vitro*, using growth assays with indicator strains such as *Listeria* or *E. coli* [559]. Furthermore, exploration of the impact of *E. faecium*-derived bacteriocins directly upon *B. wadsworthia* whilst excluding the effects of other inhibitory compounds such as lactic acid would be a pertinent next step. This could be achieved by co-culturing the strains on agar plates buffered with sodium bicarbonate to identify inhibition independent of lactic acid production [560]. The activity of bacteriocins against gut-

derived bacterial strains and the human gut microbiota is an active area of research, as traditionally bacteriocin assays focus on susceptibility testing using a few selected laboratory indicator strains [379]. Therefore, establishing whether these bacteriocins could inhibit Gram-negative species including *B. wadsworthia* is yet to be determined and is worthy of study. Additionally, the two candidate strains did not harbour clinically important antibiotic resistance genes based on genome screening, but confirmation using conventional *in vitro* minimum inhibitory concentration (MIC) assays and screening for specific AMR genes via PCR [561] would provide additional supportive evidence and scientific rationale for pursuing these strains as potential probiotic candidates. A further aspect of studying these candidate strains could investigate the presence of endotoxic or pro-inflammatory traits, using models combining anaerobic bacterial culture with human colonic epithelium such as gut-on-a-chip [556] or vertical diffusion chamber models [557].

## 6.4 *B. wadsworthia* in the human gut environment

The impact of *B. wadsworthia* cells and cell-free supernatant on the barrier integrity of Caco-2 cell mono-layers was investigated. Significant barrier integrity loss was only observed in Caco-2 cells cultured with washed cells of *L. plantarum* EKN4 and OOY9 (Figure 28); this loss of barrier integrity was likely mediated by reduced culture pH due to lactic acid production. Interestingly, the most significant loss of barrier integrity was observed with a combination of EKN4, OOY9 and *B. wadsworthia* cells. This suggested that supernatant from *B. wadsworthia* and Bt strain 1 supernatant does not negatively impact the barrier, but *B. wadsworthia* cells in combination with washed cells of probiotic strains could exert deleterious impacts on the gut barrier integrity.

Using *ex vivo* faecal cultures to investigate *B. wadsworthia*'s impact on the wider gut microbiota when supplemented together with *B. thetaiotaomicron* or Ef-Bt, we found no differences in $H_2S$ concentration at 8 h post-inoculation due to the addition of specific bacterial strains (Figure 29). However, we showed that *B. wadsworthia* could contribute significant levels of $H_2S$ in the context of a faecal matrix and complex gut microbiota with a physiologically relevant taurine dose; this was observed in all *B. wadsworthia*-containing conditions in all three faecal samples (Figure 29). If occurring in the human gut environment, the contribution of *B. wadsworthia*-derived $H_2S$ in the mM range could be pro-inflammatory [98]. Furthermore, metagenomic analysis of the impact of *B. wadsworthia*

supplementation on microbial composition revealed that the majority of variance in microbial composition was attributed to the faecal donor (Table 4, Figure 30B). Despite this, small differences in *B. wadsworthia* abundance were observed between experimental conditions; the presence of *E. faecium* modestly reduced *B. wadsworthia* abundance without affecting *B. thetaiotaomicron* abundance (Figure 31A) but did not inhibit $H_2S$ production (Figure 29A). An ability to control *B. wadsworthia* abundance in the gut environment is a desirable goal for reducing gut inflammation and pathogenesis associated with high $H_2S$ levels [161]; in this experimental system, we did not observe a significant inhibition of *B. wadsworthia* by *E. faecium* as we saw in simplified co-cultures. The use of alternative gut model systems could be considered for further investigation into the efficacy of *E. faecium* as a potential anti-*B. wadsworthia* probiotic

### 6.4.1 Future work

The HILIC-MS method for quantifying sulfated amino acids in the human faecal matrix would benefit from further method development, particularly with respect to reducing the Lower Limit of Quantification (LLOQ). A simple solution to overcome the relatively high LLOQ of 40 µM would be to perform spike-in of known concentrations of external standard amino acids to bring the unknown samples into the accurate quantification range. However, future method development could investigate alternative sample preparation methods such as liquid or solid phase extraction to improve removal of extraneous matrix components prior to analysis to reduce LLOQ [538]. This would be particularly beneficial for homocysteine which was subject to a significant matrix effect. Additionally, the use of $^{13}C$-labelled internal standards for both homocysteine and methionine may improve quantification accuracy of these compounds. A further key aspect of future work is direct application of this method to human faeces. Literature delineating the concentrations of sulfated amino acids in the human colon is relatively sparse, although some studies have reported amino acid concentrations per kg weight based upon biopsies of the descending colon mucosa [539, 540]. To our knowledge, no studies thus far have investigated sulfated amino acid concentrations in human faeces, leaving a notable research gap. Given that these sulfated amino acids contribute to $H_2S$ concentrations *in vivo* [44, 51, 498-500], and increased $H_2S$ burden is associated with inflammation and pathogenesis in the gut [44, 98, 102, 262, 282], gathering data on sulfated amino acid

concentrations in human stool would contribute towards a better understanding of colonic sulfur metabolism in human health and disease.

The determination of $H_2S$ concentration in the human faecal matrices was accurate and precise within the 2 – 100 µM range, however the method could not be used for accurate quantification of the original $H_2S$ concentration in the faeces as the 1:400 dilution used was too high. In order to accurately quantify $H_2S$ in human faeces using this method, future work could aim to investigate the impact of lower dilution upon the quantification accuracy and precision, using a 1:200 dilution as a starting point [514]. A valuable future aspiration for this method development would be to quantify $H_2S$ in a range of human faecal samples where metadata is available regarding dietary habits. To our knowledge, studies into the $H_2S$ concentration in the colon and the relationship with diet are restricted to small-scale studies involving a limited number of participants [74, 76, 77]. This is likely in part due to the volatile nature of $H_2S$ introducing difficulties with quantification, as it is readily oxidised if the samples are exposed to oxygen without zinc acetate fixation [272, 514]. Future work could exploit the accessibility of fresh human faeces from the QIB colon model study (ClinicalTrials.gov Identifier: NCT02653001) and the MOTION study (ClinicalTrials.gov Identifier: NCT04199195) to be able to glean a large dataset of faecal $H_2S$ concentrations in human adults.

Regarding the Caco-2 cell culture experiment, a key weakness of the study was the absence of an effective negative control for decreased epithelial barrier integrity; no significant decrease in TEER was observed with 14% ethanol treatment which has been previously reported to impair barrier integrity [504, 516-518]. A potential explanation was that the Caco-2 cell mono-layer used here underwent a longer period of differentiation prior to experiment start and had higher starting barrier integrity (≥800 Ω $cm^2$); future work should seek to validate these results using a higher concentration of ethanol or an alternative compound such as TNF-α [503, 549] as an effective negative control for barrier integrity. Future work could also utilise alternative systems for investigating the impact of strict anaerobes such as *B. wadsworthia* and *B. thetaiotaomicron* on the human gut epithelial layer. A general disadvantage of conventional Caco-2 cell culture is that the conditions are not conducive to the growth of anaerobic bacteria, and as

such do not accurately model the anaerobic mucosa and lumen of the human gastrointestinal tract. Novel technologies permitting viable co-culture of anaerobic bacteria and human cells are currently being developed and optimised, such as gut-on-a-chip technology [556], vertical diffusion chamber models [557] and 3D tissue culture models [562] among others; future work could employ such systems to more accurately model the impact of the sulfidogenic *B. wadsworthia* and *B. thetaiotaomicron* interaction upon the epithelial layer of the human gut.

In *ex vivo* faecal culture experiments, an important consideration of this model is the lack of pH control; substantial decreases in culture pH were observed in all conditions, where cultures reached 5 – 6 by 8 h likely due to the lactic acid bacteria present within the faeces. Changes in pH are likely to affect bacterial growth and viability; it is therefore important to acknowledge that this study is preliminary and future work could seek to investigate these bacterial interactions using a model with increased media buffering capacity, or by using an *in vitro* model system with integrated pH control such as the colon model [501] or microMatrix [563]. A further consideration for future work is the use of freshly prepared human faeces for seeding experimental models. Here, faecal samples were processed anaerobically, flash frozen and stored at -80˚C; it was shown that freezing at -80˚C without added cryoprotectant preserved the microbial ecosystem more efficiently compared to freezing with 10% glycerol or lyophilisation, using fresh stool as a benchmark [507]. However, it would undeniably be valuable to investigate the interaction between *B. wadsworthia* and other bacterial strains using *in vitro* models seeded with fresh faecal samples, as this is the gold standard for *in vitro* recapitulation of the human gut microbiota [507]. Furthermore, future work could seek to investigate the impact of different dietary taurine doses upon *B. wadsworthia* in the context of the human gut microbiota; dietary taurine intake varies widely between omnivores and vegans. Additionally, *B. wadsworthia* was recently shown to utilise the plant-derived sulfoquinovose for $H_2S$ production [63]; a comparison of *B. wadsworthia* response to vegan diet and omnivorous diet would be beneficial in order to better understand possible dietary strategies for controlling the abundance and $H_2S$ production by this potentially pathogenic gut bacterium.

# Bibliography


1. Ghosh A: **Appraisal of microbial evolution to commensalism and pathogenicity in humans**. *Clin Med Insights Gastroenterol* 2013, **6**:1-12.
2. Grice E, Segre J: **The Human Microbiome: Our Second Genome**. *Annual Review of Genomics and Human Genetics* 2012, **13**(1):151-170.
3. Sender R, Fuchs S, Milo R: **Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans**. *Cell* 2016, **164**(3):337-340.
4. Eckburg P, Bik E, B., Purdom E, Dethlefsen L, Sargent M, Gill S, Nelson K, Relman D: **Diversity of the Human Intestinal Microbial Flora**. *Science* 2005, **308**(5728):1635.
5. Wu H, Wu E: **The role of gut microbiota in immune homeostasis and autoimmunity**. *Gut Microbes* 2012, **3**(1):4-14.
6. Candela M, Perna F, Carnevali P, Vitali B, Ciati R, Gionchetti P, Rizzello F, Campieri M, Brigidi P: **Interaction of probiotic *Lactobacillus* and *Bifidobacterium* strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production**. *International Journal of Food Microbiology* 2008, **125**(3):286-292.
7. den Besten G, van Eunen K, Groen A, Venema K, Reijngoud D, Bakker B: **The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism**. *Journal of Lipid Research* 2013, **54**(9):2325-2340.
8. Martin B, Schwab E: **Symbiosis: "Living together" in chaos**. *Studies in the History of Biology* 2012, **4**(4):7-25.
9. Petersen C, Round J: **Defining dysbiosis and its influence on host immunity and disease**. *Cellular Microbiology* 2014, **16**(7):1024-1033.
10. Carding S, Verbeke K, Vipond D, Corfe B, Owen L: **Dysbiosis of the gut microbiota in disease**. *Microbial Ecology in Health and Disease* 2015, **26**(1):26191.
11. Chong P, Chin V, Looi C, Wong W, Madhavan P, Yong V: **The Microbiome and Irritable Bowel Syndrome – A Review on the Pathophysiology, Current Research and Future Therapy**. *Frontiers in Microbiology* 2019, **10**(1136).
12. Tong M, Li X, Wegener Parfrey L, Roth B, Ippoliti A, Wei B, Borneman J, McGovern D, Frank D, Li E *et al*: **A Modular Organization of the Human Intestinal Mucosal Microbiota and Its Association with Inflammatory Bowel Disease**. *PloS One* 2013, **8**(11):e80702.
13. Belizário J, Faintuch J, Garay-Malpartida M: **Gut Microbiome Dysbiosis and Immunometabolism: New Frontiers for Treatment of Metabolic Diseases**. *Mediators Inflamm* 2018, **2018**:2037838-2037838.
14. Luna R, Foster J: **Gut brain axis: diet microbiota interactions and implications for modulation of anxiety and depression**. *Current Opinion in Biotechnology* 2015, **32**:35-41.
15. Łuc M, Misiak B, Pawłowski M, Stańczykiewicz B, Zabłocka A, Szcześniak D, Pałęga A, Rymaszewska J: **Gut microbiota in dementia. Critical review of novel findings and their potential application**. *Progress in Neuro-Psychopharmacology and Biological Psychiatry* 2021, **104**:110039.
16. Vuong H, Hsiao E: **Emerging Roles for the Gut Microbiome in Autism Spectrum Disorder**. *Biological Psychiatry* 2017, **81**(5):411-423.

17. Santos A, Venceslau S, Grein F, Leavitt W, Dahl C, Johnston D, Pereira I: **A protein trisulfide couples dissimilatory sulfate reduction to energy conservation**. *Science* 2015, **350**(6267):1541.
18. Muyzer G, Stams A: **The ecology and biotechnology of sulphate-reducing bacteria**. *Nature Reviews Microbiology* 2008, **6**(6):441-454.
19. Rey F, Gonzalez M, Cheng J, Wu M, Ahern P, Gordon J: **Metabolic niche of a prominent sulfate-reducing human gut bacterium**. *Proc Natl Acad Sci U S A* 2013, **110**(33):13582-13587.
20. Stewart J, Chadwick V, Murray A: **Carriage, quantification, and predominance of methanogens and sulfate-reducing bacteria in faecal samples**. *Letters in Applied Microbiology* 2006, **43**(1):58-63.
21. Gibson G, Macfarlane S, Macfarlane G: **Metabolic interactions involving sulphate-reducing and methanogenic bacteria in the human large intestine**. *FEMS Microbiology Ecology* 1993, **12**(2):117-125.
22. Nava G, Carbonero F, Croix J, Greenberg E, Gaskins H: **Abundance and diversity of mucosa-associated hydrogenotrophic microbes in the healthy human colon**. *The ISME journal* 2012, **6**(1):57-70.
23. Scanlan P, Shanahan F, Marchesi J: **Culture-independent analysis of desulfovibrios in the human distal colon of healthy, colorectal cancer and polypectomized individuals**. *FEMS Microbiology Ecology* 2009, **69**(2):213-221.
24. Baron E, Curren M, Henderson G, Jousimies-Somer H, Lee K, Lechowitz K, Strong C, Summanen P, Tunér K, Finegold S: ***Bilophila wadsworthia* isolates from clinical specimens**. *J Clin Microbiol* 1992, **30**(7):1882-1884.
25. Nakamura N, Lin H, McSweeney C, Mackie R, Gaskins H: **Mechanisms of Microbial Hydrogen Disposal in the Human Colon and Implications for Health and Disease**. *Annual Review of Food Science and Technology* 2010, **1**(1):363-395.
26. Blachier F, Davila A, Mimoun S, Benetti P, Atanasiu C, Andriamihaja M, Benamouzig R, Bouillaud F, Tomé D: **Luminal sulfide and large intestine mucosa: friend or foe?** *Amino Acids* 2010, **39**(2):335-347.
27. Lewis S, Cochrane S: **Alteration of Sulfate and Hydrogen Metabolism in the Human Colon by Changing Intestinal Transit Rate**. *Official journal of the American College of Gastroenterology | ACG* 2007, **102**(3).
28. Bryant M, Wolin E, Wolin M, Wolfe R: ***Methanobacillus omelianskii*, a symbiotic association of two species of bacteria**. *Archiv für Mikrobiologie* 1967, **59**(1):20-31.
29. Iannotti E, Kafkewitz D, Wolin M, Bryant M: **Glucose Fermentation Products of *Ruminococcus albus* Grown in Continuous Culture with *Vibrio succinogenes*: Changes Caused by Interspecies Transfer of $H_2S$**. *Journal of Bacteriology* 1973, **114**(3):1231.
30. Qian Z, Tianwei H, Mackey HR, van Loosdrecht MCM, Guanghao C: **Recent advances in dissimilatory sulfate reduction: From metabolic study to application**. *Water Research* 2019, **150**:162-181.
31. Baron E, Summanen P, Downes J, Roberts M, Wexler H, Finegold S: ***Bilophila wadsworthia*, gen. nov. and sp. nov., a Unique Gram-negative Anaerobic Rod Recovered from Appendicitis Specimens and Human Faeces**. *Microbiology* 1989, **135**(12):3405-3411.
32. Bennion R, Baron E, Thompson J, Downes J, Summanen P, Talan D, Finegold S: **The bacteriology of gangrenous and perforated appendicitis--revisited**. *Annals of Surgery* 1990, **211**(2):165-171.
33. Gilroy R, Ravi A, Getino M, Pursley I, Horton DL, Alikhan N-F, Baker D, Gharbi K, Hall N, Watson M *et al*: **Extensive microbial diversity within**

**the chicken gut microbiome revealed by metagenomics and culture**. *PeerJ* 2021, **9**:e10941-e10941.
34. Charlson RJ, Anderson TL, McDuff RE: **13 The Sulfur Cycle**. In: *International Geophysics.* Edited by Butcher SS, Charlson RJ, Orians GH, Wolfe GV, vol. 50: Academic Press; 1992: 285-300.
35. Ingenbleek Y, Kimura H: **Nutritional essentiality of sulfur in health and disease**. *Nutr Rev* 2013, **71**(7):413-432.
36. Dahl C: **A biochemical view on the biological sulfur cycle**. *Environmental Technologies to Treat Sulfur Pollution: Principles and Engineering* 2020, **2**:55-96.
37. Mukwevho E, Ferreira Z, Ayeleso A: **Potential Role of Sulfur-Containing Antioxidant Systems in Highly Oxidative Environments**. In: *Molecules.* vol. 19; 2014: 19376-19389.
38. Yadav M, Verma MK, Chauhan NS: **A review of metabolic potential of human gut microbiome in human nutrition**. *Archives of Microbiology* 2018, **200**(2):203-217.
39. Moran MA, Durham BP: **Sulfur metabolites in the pelagic ocean**. *Nature Reviews Microbiology* 2019, **17**(11):665-678.
40. Zhou L, Ou P, Zhao B, Zhang W, Yu K, Xie K, Zhuang W-Q: **Assimilatory and dissimilatory sulfate reduction in the bacterial diversity of biofoulant from a full-scale biofilm-membrane bioreactor for textile wastewater treatment**. *Science of The Total Environment* 2021, **772**:145464.
41. Carbonero F, Gaskins H: **Sulfate-Reducing Bacteria in the Human Gut Microbiome**. In: *Encyclopedia of Metagenomics.* Edited by Nelson KE. New York, NY: Springer New York; 2013: 1-3.
42. Loubinoux J, Bronowicki J, Pereira I, Mougenel J, Le Faou A: **Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases**. *FEMS Microbiology Ecology* 2002, **40**(2):107-112.
43. Wagner M, Roger A, Flax J, Brusseau G, Stahl D: **Phylogeny of dissimilatory sulfite reductases supports an early origin of sulfate respiration**. *Journal of Bacteriology* 1998, **180**(11):2975-2982.
44. Kushkevych I, Dordević D, Vítězová M: **Possible synergy effect of hydrogen sulfide and acetate produced by sulfate-reducing bacteria on inflammatory bowel disease development**. *Journal of Advanced Research* 2021, **27**:71-78.
45. Wolf PG, Cowley ES, Breister A, Matatov S, Lucio L, Polak P, Ridlon JM, Gaskins HR, Anantharaman K: **Diversity and distribution of sulfur metabolic genes in the human gut microbiome and their association with colorectal cancer**. *Microbiome* 2022, **10**(1):64.
46. Duarte AG, Barbosa ACC, Ferreira D, Manteigas G, Domingos RM, Pereira IAC: **Redox loops in anaerobic respiration - The role of the widespread NrfD protein family and associated dimeric redox module**. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 2021, **1862**(7):148416.
47. Kushkevych I, Cejnar J, Treml J, Dordević D, Kollar P, Vítězová M: **Recent Advances in Metabolic Pathways of Sulfate Reduction in Intestinal Bacteria**. *Cells* 2020, **9**(3).
48. Riahi S, Rowley CN: **Why can hydrogen sulfide permeate cell membranes?** *J Am Chem Soc* 2014, **136**(43):15111-15113.
49. Mathai JC, Missner A, Kügler P, Saparov SM, Zeidel ML, Lee JK, Pohl P: **No facilitator required for membrane transport of hydrogen sulfide**.

*Proceedings of the National Academy of Sciences* 2009, **106**(39):16633-16638.
50. Baron E: ***Bilophila wadsworthia*: a Unique Gram-negative Anaerobic Rod**. *Anaerobe* 1997, **3**(2):83-86.
51. Peck S, Denger K, Burrichter A, Irwin S, Balskus E, Schleheck D: **A glycyl radical enzyme enables hydrogen sulfide production by the human intestinal bacterium *Bilophila wadsworthia***. *Proceedings of the National Academy of Sciences* 2019, **116**(8):3171.
52. Burrichter AG, Dörr S, Bergmann P, Haiß S, Keller A, Fournier C, Franchini P, Isono E, Schleheck D: **Bacterial microcompartments for isethionate desulfonation in the taurine-degrading human-gut bacterium Bilophila wadsworthia**. *BMC Microbiology* 2021, **21**(1):340.
53. Xing M, Wei Y, Zhou Y, Zhang J, Lin L, Hu Y, Hua G, N. Nanjaraj Urs A, Liu D, Wang F *et al*: **Radical-mediated C-S bond cleavage in C2 sulfonate degradation by anaerobic bacteria**. *Nature Communications* 2019, **10**(1):1609.
54. da Silva S, Venceslau S, Fernandes C, Valente F, Pereira I: **Hydrogen as an energy source for the human pathogen *Bilophila wadsworthia***. *Antonie van Leeuwenhoek* 2008, **93**(4):381-390.
55. Laue H, Friedrich M, Ruff J, Cook A: **Dissimilatory Sulfite Reductase (Desulfoviridin) of the Taurine-Degrading, Non-Sulfate-Reducing Bacterium *Bilophila wadsworthia* RZATAU Contains a Fused DsrB-DsrD Subunit**. *Journal of Bacteriology* 2001, **183**(5):1727.
56. Kopriva S, Koprivova A: **Sulphate Assimilation: A Pathway Which Likes to Surprise**. In: *Sulphur in Plants.* Edited by Abrol YP, Ahmad A. Dordrecht: Springer Netherlands; 2003: 87-112.
57. Carbonero F, Benefiel A, Alizadeh-Ghamsari A, Gaskins H: **Microbial pathways in colonic sulfur metabolism and links with health and disease**. *Front Physiol* 2012, **3**:448-448.
58. Schiff JA: **Pathways of assimilatory sulphate reduction in plants and microorganisms**. *Ciba Found Symp* 1979(72):49-69.
59. Loi VV, Rossius M, Antelmann H: **Redox regulation by reversible protein S-thiolation in bacteria**. *Frontiers in Microbiology* 2015, **6**.
60. Blokesch M, Rohrmoser M, Rode S, Böck A: **HybF, a Zinc-Containing Protein Involved in NiFe Hydrogenase Maturation**. *Journal of Bacteriology* 2004, **186**(9):2603-2611.
61. Bulaj G: **Formation of disulfide bonds in proteins and peptides**. *Biotechnology Advances* 2005, **23**(1):87-92.
62. Laue H, Cook A: **Biochemical and molecular characterization of taurine:pyruvate aminotransferase from the anaerobe *Bilophila wadsworthia***. *European Journal of Biochemistry* 2000, **267**(23):6841-6848.
63. Hanson B, Kits K, Löffler J, Burrichter A, Fiedler A, Denger K, Frommeyer B, Herbold C, Rattei T, Karcher N: **Sulfoquinovose is a select nutrient of prominent bacteria and a source of hydrogen sulfide in the human gut**. 2020.
64. Burkhardt W, Rausch T, Klopfleisch R, Blaut M, Braune A: **Impact of dietary sulfolipid-derived sulfoquinovose on gut microbiota composition and inflammatory status of colitis-prone interleukin-10-deficient mice**. *International Journal of Medical Microbiology* 2021, **311**(3):151494.
65. Devkota S, Wang Y, Musch M, Leone V, Fehlner-Peach H, Nadimpalli A, Antonopoulos D, Jabri B, Chang E: **Dietary-fat-induced taurocholic acid**

**promotes pathobiont expansion and colitis in Il10-/- mice**. *Nature* 2012, **487**(7405):104-108.
66. Haange SB, Groeger N, Froment J, Rausch T, Burkhardt W, Gonnermann S, Braune A, Blaut M, von Bergen M, Rolle-Kampczyk U: **Multiplexed Quantitative Assessment of the Fate of Taurine and Sulfoquinovose in the Intestinal Microbiome**. *Metabolites* 2020, **10**(11).
67. Feng Z, Long W, Hao B, Ding D, Ma X, Zhao L, Pang X: **A human stool-derived *Bilophila wadsworthia* strain caused systemic inflammation in specific-pathogen-free mice**. *Gut Pathog* 2017, **9**:59-59.
68. Efimov B, Chaplin A, Sokolova S, Chernaia Z, Pikina A, Savilova A, Kafarskaya L: **Application of culture-based, mass spectrometry and molecular methods to the study of gut microbiota in children**. *Bulletin of RSMU (Biomedical Journal of Pirogov University)*.
69. Guidotti T: **Hydrogen Sulphide**. *Occupational Medicine* 1996, **46**(5):367-371.
70. Singh SB, Lin HC: **Hydrogen Sulfide in Physiology and Diseases of the Digestive Tract**. In: *Microorganisms.* vol. 3; 2015: 866-889.
71. Awano N, Wada M, Mori H, Nakamori S, Takagi H: **Identification and functional analysis of Escherichia coli cysteine desulfhydrases**. *Appl Environ Microbiol* 2005, **71**(7):4149-4152.
72. Kumagai H, Sejima S, Choi Y-J, Tanaka H, Yamada H: **Crystallization and properties of cysteine desulfhydrase from Aerobacter aerogenes**. *FEBS Letters* 1975, **52**(2):304-307.
73. Braccia DJ, Jiang X, Pop M, Hall AB: **The Capacity to Produce Hydrogen Sulfide (H2S) via Cysteine Degradation Is Ubiquitous in the Human Gut Microbiome**. *Frontiers in Microbiology* 2021, **12**.
74. Macfarlane G, Gibson G, Cummings J: **Comparison of fermentation reactions in different regions of the human colon**. *Journal of Applied Bacteriology* 1992, **72**(1):57-64.
75. Deplancke B, Finster K, Graham W, Collier C, Thurmond J, Gaskins H: **Gastrointestinal and microbial responses to sulfate-supplemented drinking water in mice**. *Experimental Biology and Medicine (Maywood, New Jersey)* 2003, **228**(4):424-433.
76. Gibson G, Cummings J, Macfarlane G, Allison C, Segal I, Vorster H, Walker A: **Alternative pathways for hydrogen disposal during fermentation in the human colon**. *Gut* 1990, **31**(6):679-683.
77. Magee E, Richardson C, Hughes R, Cummings J: **Contribution of dietary protein to sulfide production in the large intestine: an *in vitro* and a controlled feeding study in humans**. *The American Journal of Clinical Nutrition* 2000, **72**(6):1488-1494.
78. Dordević D, Jančíková S, Vítězová M, Kushkevych I: **Hydrogen sulfide toxicity in the gut environment: Meta-analysis of sulfate-reducing and lactic acid bacteria in inflammatory processes**. *Journal of Advanced Research* 2021, **27**:55-69.
79. Jørgensen J, Mortensen PB: **Hydrogen sulfide and colonic epithelial metabolism**. *Digestive diseases and sciences* 2001, **46**:1722-1732.
80. Medani M, Collins D, Docherty NG, Baird AW, O'Connell PR, Winter DC: **Emerging role of hydrogen sulfide in colonic physiology and pathophysiology**. *Inflammatory Bowel Diseases* 2011, **17**(7):1620-1625.
81. Mitsui T, Edmond LM, Magee EA, Cummings JH: **The effects of bismuth, iron, zinc and nitrate on free sulfide in batch cultures seeded with fecal flora**. *Clin Chim Acta* 2003, **335**(1-2):131-135.

82. Shibuya N, Koike S, Tanaka M, Ishigami-Yuasa M, Kimura Y, Ogasawara Y, Fukui K, Nagahara N, Kimura H: **A novel pathway for the production of hydrogen sulfide from D-cysteine in mammalian cells**. *Nature Communications* 2013, **4**(1):1366.
83. Abe K, Kimura H: **The possible role of hydrogen sulfide as an endogenous neuromodulator**. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 1996, **16**(3):1066-1071.
84. Schicho R, Krueger D, Zeller F, Von Weyhern C, Frieling T, Kimura H, Ishii I, De Giorgio R, Campi B, Schemann M: **Hydrogen Sulfide Is a Novel Prosecretory Neuromodulator in the Guinea-Pig and Human Colon**. *Gastroenterology* 2006, **131**(5):1542-1552.
85. Barbara R: **Hydrogen Sulfide Generation in Mammals: The Molecular Blology of Cystathionine-β-synthase (CBS) and Cystathionine-β-lyase (CSE)**. *Inflammation and Allergy - Drug Targets* 2011, **10**:85-91.
86. Teague B, Asiedu S, Moore P: **The smooth muscle relaxant effect of hydrogen sulphide *in vitro*: evidence for a physiological role to control intestinal contractility**. *British Journal of Pharmacology* 2002, **137**(2):139-145.
87. Gil V, Gallego D, Jimenez M: **Effects of inhibitors of hydrogen sulphide synthesis on rat colonic motility**. *British Journal of Pharmacology* 2011, **164**(2b):485-498.
88. Gallego D, Clave P, Donovan J, Rahmati R, Grundy D, Jimenez M, Beyak M: **The gaseous mediator, hydrogen sulphide, inhibits in vitro motor patterns in the human, rat and mouse colon and jejunum**. *Neurogastroenterology & Motility* 2008, **20**(12):1306-1316.
89. Quan X, Luo H, Liu Y, Xia H, Chen W, Tang Q: **Hydrogen sulfide regulates the colonic motility by inhibiting both L-type calcium channels and BKCa channels in smooth muscle cells of rat colon**. *PloS one* 2015, **10**(3).
90. Szabo C, Ransy C, Módis K, Andriamihaja M, Murghes B, Coletta C, Olah G, Yanagi K, Bouillaud F: **Regulation of mitochondrial bioenergetic function by hydrogen sulfide. Part I. Biochemical and physiological mechanisms**. *British Journal of Pharmacology* 2014, **171**(8):2099-2122.
91. Goubern M, Andriamihaja M, Nübel T, Blachier F, Bouillaud F: **Sulfide, the first inorganic substrate for human cells**. *The FASEB Journal* 2007, **21**(8):1699-1706.
92. Distrutti E, Sediari L, Mencarelli A, Renga B, Orlandi S, Russo G, Caliendo G, Santagada V, Cirino G, Wallace J: **5-Amino-2-hydroxybenzoic acid 4-(5-thioxo-5H-[1, 2]dithiol-3yl)-phenyl ester (ATB-429), a hydrogen sulfide-releasing derivative of mesalamine, exerts antinociceptive effects in a model of postinflammatory hypersensitivity**. *Journal of Pharmacology and Experimental Therapeutics* 2006, **319**(1):447-458.
93. Matsunami M, Tarui T, Mitani K, Nagasawa K, Fukushima O, Okubo K, Yoshida S, Takemura M, Kawabata A: **Luminal hydrogen sulfide plays a pronociceptive role in mouse colon**. *Gut* 2009, **58**(6):751-761.
94. Schemann M, Grundy D: **Role of hydrogen sulfide in visceral nociception**. *Gut* 2009, **58**(6):744.
95. Xu G, Winston J, Shenoy M, Zhou S, Chen J, Pasricha P: **The Endogenous Hydrogen Sulfide Producing Enzyme Cystathionine-β Synthase Contributes to Visceral Hypersensitivity in a Rat Model of Irritable Bowel Syndrome**. *Molecular Pain* 2009, **5**:1744-8069-1745-1744.
96. Kabil O, Banerjee R: **Redox biochemistry of hydrogen sulfide**. *Journal of Biological Chemistry* 2010, **285**(29):21903-21907.

97. Blachier F, Beaumont M, Kim E: **Cysteine-derived hydrogen sulfide and gut health: a matter of endogenous or bacterial origin**. *Current Opinion in Clinical Nutrition & Metabolic Care* 2019, **22**(1).
98. Guo F, Yu T, Hong J, Fang J: **Emerging Roles of Hydrogen Sulfide in Inflammatory and Neoplastic Colonic Diseases**. *Front Physiol* 2016, **7**:156-156.
99. Linden D: **Hydrogen Sulfide Signaling in the Gastrointestinal Tract**. *Antioxidants & Redox Signaling* 2013, **20**(5):818-830.
100. Babidge W, Millard S, Roediger W: **Sulfides impair short chain fatty acid β-oxidation at acyl-CoA dehydrogenase level in colonocytes: Implications for ulcerative colitis**. *Molecular and Cellular Biochemistry* 1998, **181**(1):117-124.
101. Attene-Ramos M, Nava GM, Muellner MG, Wagner ED, Plewa MJ, Gaskins H: **DNA damage and toxicogenomic analyses of hydrogen sulfide in human intestinal epithelial FHs 74 Int cells**. *Environmental and Molecular Mutagenesis* 2010, **51**(4):304-314.
102. Attene-Ramos M, Wagner ED, Gaskins H, Plewa MJ: **Hydrogen Sulfide Induces Direct Radical-Associated DNA Damage**. *Molecular Cancer Research* 2007, **5**(5):455.
103. Attene-Ramos M, Wagner ED, Plewa M, Gaskins H: **Evidence That Hydrogen Sulfide Is a Genotoxic Agent**. *Molecular Cancer Research* 2006, **4**(1):9.
104. Pierik M, Yang H, Barmada M, Cavanaugh J, Annese V, Brant S, Cho J, Duerr R, Hugot J, McGovern D *et al*: **The IBD international genetics consortium provides further evidence for linkage to IBD4 and shows gene-environment interaction**. *Inflamm Bowel Dis* 2005, **11**(1):1-7.
105. Loddo I, Romano C: **Inflammatory Bowel Disease: Genetics, Epigenetics, and Pathogenesis**. *Frontiers in Immunology* 2015, **6**:551-551.
106. Molodecky N, Soon I, Rabi D, Ghali W, Ferris M, Chernoff G, Benchimol E, Panaccione R, Ghosh S, Barkema H *et al*: **Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review**. *Gastroenterology* 2012, **142**(1):46-54.e42; quiz e30.
107. Ghosh S, Almadi M: **Inflammatory bowel disease: a global disease**. *Saudi Journal of Gastroenterology: Official Journal of the Saudi Gastroenterology Association* 2013, **19**(1):1-2.
108. Pitcher MC, Beatty ER, Cummings JH: **The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis**. *Gut* 2000, **46**(1):64-72.
109. Gibson G, Cummings J, Macfarlane G: **Growth and activities of sulphate-reducing bacteria in gut contents of healthy subjects and patients with ulcerative colitis**. *FEMS Microbiology Letters* 1991, **86**(2):103-111.
110. Tilg H, Kaser A: **Diet and relapsing ulcerative colitis: take off the meat?** *Gut* 2004, **53**(10):1399-1401.
111. Fite A, Macfarlane G, Cummings J, Hopkins M, Kong S, Furrie E, Macfarlane S: **Identification and quantitation of mucosal and faecal desulfovibrios using real time polymerase chain reaction**. *Gut* 2004, **53**(4):523.
112. Mahadevan U, Sandborn W: **Diagnosis and management of pouchitis**. *Gastroenterology* 2003, **124**(6):1636-1650.
113. Ohge H, Furne J, Springfield J, Rothenberger D, Madoff R, Levitt M: **Association Between Fecal Hydrogen Sulfide Production and Pouchitis**. *Diseases of the Colon & Rectum* 2005, **48**(3):469-475.

114. Ijssennagger N, van der Meer R, van Mil S: **Sulfide as a mucus barrier-breaker in inflammatory bowel disease?** *Trends in Molecular Medicine* 2016, **22**(3):190-199.
115. Johansson M, Gustafsson J, Holmén-Larsson J, Jabbar K, Xia L, Xu H, Ghishan F, Carvalho F, Gewirtz A, Sjövall H *et al*: **Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis**. *Gut* 2014, **63**(2):281.
116. Swidsinski A, Loening-Baucke V, Theissig F, Engelhardt H, Bengmark S, Koch S, Lochs H, Dörffel Y: **Comparative study of the intestinal mucus barrier in normal and inflamed colon**. *Gut* 2007, **56**(3):343.
117. Chey W, Kurlander J, Eswaran S: **Irritable bowel syndrome: a clinical review**. *Jama* 2015, **313**(9):949-958.
118. Chassard C, Dapoigny M, Scott K, Del'Homme C, Dubray C, Eschalier A, Flint H, Bernalier-Donadille A: **The intestinal microbiota of irritable bowel syndrome patients is characterized by functional dysbiosis**. *Gastroenterology* 2009, **136**(5, Supplement 1):A214-A214.
119. Lin H: **Small Intestinal Bacterial Overgrowth: A Framework for Understanding Irritable Bowel Syndrome**. *JAMA* 2004, **292**(7):852-858.
120. Pimentel M, Chow E, Lin H: **Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome**. *The American Journal of Gastroenterology* 2000, **95**(12):3503-3506.
121. Banik G, De A, Som S, Jana S, Daschakraborty S, Chaudhuri S, Pradhan M: **Hydrogen sulphide in exhaled breath: a potential biomarker for small intestinal bacterial overgrowth in IBS**. *Journal of Breath Research* 2016, **10**(2):026010.
122. **Cancer** [https://www.who.int/news-room/fact-sheets/detail/cancer]
123. Watson A, Collins P: **Colon cancer: a civilization disorder**. *Digestive Diseases* 2011, **29**(2):222-228.
124. Mustafa M, Menon J, Muniandy R, Illzam E, Shah M, Sharifa A: **Colorectal cancer: pathogenesis, management and prevention**. *Journal of Dental and Medical Sciences* 2016, **15**:94-100.
125. Kanazawa K, Konishi F, Mitsuoka T, Terada A, Itoh K, Narushima S, Kumemura M, Kimura H: **Factors influencing the development of sigmoid colon cancer: Bacteriologic and biochemical studies**. *Cancer* 1996, **77**(8):1701-1706.
126. Ramasamy S, Singh S, Taniere P, Langman M, Eggo M: **Sulfide-detoxifying enzymes in the human colon are decreased in cancer and upregulated in differentiation**. *American Journal of Physiology - Gastrointestinal Liver Physiology* 2006, **291**(2):G288-296.
127. Norat T, Lukanova A, Ferrari P, Riboli E: **Meat consumption and colorectal cancer risk: dose-response meta-analysis of epidemiological studies**. *International Journal of Cancer* 2002, **98**(2):241-256.
128. Reddy B: **Types and amount of dietary fat and colon cancer risk: Prevention by omega-3 fatty acid-rich diets**. *Environ Health Prev Med* 2002, **7**(3):95-102.
129. Bradbury KE, Murphy N, Key TJ: **Diet and colorectal cancer in UK Biobank: a prospective study**. *International Journal of Epidemiology* 2020, **49**(1):246-258.
130. Geypens B, Claus D, Evenepoel P, Hiele M, Maes B, Peeters M, Rutgeerts P, Ghoos Y: **Influence of dietary protein supplements on the formation of bacterial metabolites in the colon**. *Gut* 1997, **41**(1):70.

131. Cai W, Wang M, Ju L, Wang C, Zhu Y: **Hydrogen sulfide induces human colon cancer cell proliferation: Role of Akt, ERK and p21**. *Cell Biology International* 2010, **34**(6):565-572.
132. Yazici C, Wolf P, Kim H, Cross T, Vermillion K, Carroll T, Augustus G, Mutlu E, Tussing-Humphreys L, Braunschweig C *et al*: **Race-dependent association of sulfidogenic bacteria with colorectal cancer**. *Gut* 2017, **66**(11):1983.
133. Gomperts SN: **Lewy Body Dementias: Dementia With Lewy Bodies and Parkinson Disease Dementia**. *CONTINUUM: Lifelong Learning in Neurology* 2016, **22**(2):435-463.
134. Bloem BR, Okun MS, Klein C: **Parkinson's disease**. *The Lancet* 2021, **397**(10291):2284-2303.
135. Samii A, Nutt JG, Ransom BR: **Parkinson's disease**. *Lancet* 2004, **363**(9423):1783-1793.
136. Zhu M, Liu X, Ye Y, Yan X, Cheng Y, Zhao L, Chen F, Ling Z: **Gut Microbiota: A Novel Therapeutic Target for Parkinson's Disease**. *Frontiers in Immunology* 2022, **13**.
137. Li Q, Meng L-b, Chen L-j, Shi X, Tu L, Zhou Q, Yu J-l, Liao X, Zeng Y, Yuan Q-y: **The role of the microbiota-gut-brain axis and intestinal microbiome dysregulation in Parkinson's disease**. *Frontiers in Neurology* 2023, **14**.
138. Romano S, Savva GM, Bedarf JR, Charles IG, Hildebrand F, Narbad A: **Meta-analysis of the Parkinson's disease gut microbiome suggests alterations linked to intestinal inflammation**. *npj Parkinson's Disease* 2021, **7**(1):27.
139. Schwiertz A, Spiegel J, Dillmann U, Grundmann D, Bürmann J, Faßbender K, Schäfer K-H, Unger MM: **Fecal markers of intestinal inflammation and intestinal permeability are elevated in Parkinson's disease**. *Parkinsonism & related disorders* 2018, **50**:104-107.
140. Hertel J, Harms AC, Heinken A, Baldini F, Thinnes CC, Glaab E, Vasco DA, Pietzner M, Stewart ID, Wareham NJ *et al*: **Integrated Analyses of Microbiome and Longitudinal Metabolome Data Reveal Microbial-Host Interactions on Sulfur Metabolism in Parkinson's Disease**. *Cell Rep* 2019, **29**(7):1767-1777.e1768.
141. Greco V, Neri C, Pieragostino D, Spalloni A, Persichilli S, Gastaldi M, Mercuri NB, Longone P, Urbani A: **Investigating Different Forms of Hydrogen Sulfide in Cerebrospinal Fluid of Various Neurological Disorders**. In: *Metabolites.* vol. 11; 2021.
142. Murros KE, Huynh VA, Takala TM, Saris PEJ: **Desulfovibrio Bacteria Are Associated With Parkinson's Disease**. *Frontiers in Cellular and Infection Microbiology* 2021, **11**.
143. Xu Z, Lian C, Pan L, Lai W, Zhang F, Peng L, Zhou S, Zhao G, Yang X, Zhang G *et al*: **N-acetyl-L-leucine protects MPTP-treated Parkinson's disease mouse models by suppressing Desulfobacterota via the gut-brain axis**. *Brain Research Bulletin* 2023:110729.
144. Murros KE: **Hydrogen Sulfide Produced by Gut Bacteria May Induce Parkinson's Disease**. In: *Cells.* vol. 11; 2022.
145. Finegold S, P. S, Hunt Gerardo S., E. B: **Clinical Importance of *Bilophila wadsworthia***. *European Journal of Clinical Microbiology and Infectious Diseases* 1992, **11**:1058-1063.
146. Schumacher U, Eiring P, Häcker F: **Incidence of *Bilophila wadsworthia* in appendiceal, peritoneal and fecal samples from children**. *Clinical Microbiology and Infection* 1997, **3**(1):134-136.

147. Mosca A, D'Alagni M, del Prete R, de Michele G, Summanen P, Finegold S, Miragliotta G: **Preliminary evidence of endotoxic activity of *Bilophila wadsworthia***. *Anaerobe* 1995, **1**(1):21-24.
148. Hunt Gerardo S, Garcia M, Wexler H, Finegold S: **Adherence of *Bilophila wadsworthia* to Cultured Human Embryonic Intestinal Cells**. *Anaerobe* 1998, **4**(1):19-27.
149. Yazici C, Wolf P, Carroll T, Mutlu E, Xicola R, Llor X, Jung B, Ellis N, Gaskins H: ***Bilophila wadsworthia* Is More Abundant in the Colonic Microbiome of Colorectal Cancer Cases Compared to Healthy Controls**. *Gastroenterology* 2015, **148**(4):S-100.
150. Tremlett H, Fadrosh D, Faruqi A, Zhu F, Hart J, Roalstad S, Graves J, Lynch S, Waubant E: **Gut microbiota in early pediatric multiple sclerosis: a case-control study**. *Eur J Neurol* 2016, **23**(8):1308-1321.
151. Kozhieva M, Naumova N, Alikina T, Boyko A, Vlassov V, Kabilov M: **Primary progressive multiple sclerosis in a Russian cohort: relationship with gut bacterial diversity**. *BMC Microbiology* 2019, **19**(1):309.
152. Baldini F, Hertel J, Sandt E, Thinnes CC, Neuberger-Castillo L, Pavelka L, Betsou F, Krüger R, Thiele I, Aguayo G *et al*: **Parkinson's disease-associated alterations of the gut microbiome predict disease-relevant changes in metabolic functions**. *BMC Biol* 2020, **18**(1):62.
153. Sheng C, Yang K, He B, Du W, Cai Y, Han Y: **Combination of gut microbiota and plasma amyloid-β as a potential index for identifying preclinical Alzheimer's disease: a cross-sectional analysis from the SILCODE study**. *Alzheimer's Research & Therapy* 2022, **14**(1):35.
154. Jiao N, Baker SS, Chapa-Rodriguez A, Liu W, Nugent CA, Tsompana M, Mastrandrea L, Buck MJ, Baker RD, Genco RJ *et al*: **Suppressed hepatic bile acid signalling despite elevated production of primary and secondary bile acids in NAFLD**. *Gut* 2018, **67**(10):1881.
155. Li G-H, Huang S-J, Li X, Liu X-S, Du Q-L: **Response of gut microbiota to serum metabolome changes in intrahepatic cholestasis of pregnant patients**. *World J Gastroenterol* 2020, **26**(46):7338-7351.
156. He X, Sun J, Liu C, Yu X, Li H, Zhang W, Li Y, Geng Y, Wang Z: **Compositional Alterations of Gut Microbiota in Patients with Diabetic Kidney Disease and Type 2 Diabetes Mellitus**. *Diabetes Metab Syndr Obes* 2022, **15**:755-765.
157. Thirion F, Speyer H, Hansen TH, Nielsen T, Fan Y, Le Chatelier E, Fromentin S, Berland M, Plaza Oñate F, Pons N *et al*: **Alteration of Gut Microbiome in Patients With Schizophrenia Indicates Links Between Bacterial Tyrosine Biosynthesis and Cognitive Dysfunction**. *Biological Psychiatry Global Open Science* 2022.
158. Wolf PG, Gaskins HR, Ridlon JM, Freels S, Hamm A, Goldberg S, Petrilli P, Schering T, Vergis S, Gomez-Perez S *et al*: **Effects of taurocholic acid metabolism by gut bacteria: A controlled feeding trial in adult African American subjects at elevated risk for colorectal cancer**. *Contemporary Clinical Trials Communications* 2020, **19**:100611.
159. Leite G, Pimentel M, Barlow GM, Chang C, Hosseini A, Wang J, Parodi G, Sedighi R, Rezaie A, Mathur R: **Age and the aging process significantly alter the small bowel microbiome**. *Cell Reports* 2021, **36**(13):109765.
160. Waqas M, Halim SA, Ullah A, Ali AA, Khalid A, Abdalla AN, Khan A, Al-Harrasi A: **Multi-Fold Computational Analysis to Discover Novel Putative Inhibitors of Isethionate Sulfite-Lyase (Isla) from Bilophila**

**wadsworthia: Combating Colorectal Cancer and Inflammatory Bowel Diseases**. In: *Cancers.* vol. 15; 2023.
161. Khan S, Aziz S, Waqas M, Kakar MA, Ahmad S: **Targeted vaccine development against Bilophila wadsworthia to curb colon diseases: A multiepitope approach based on reverse vaccinology and computational analysis**. *International Journal of Biological Macromolecules* 2023, **250**:126002.
162. Deng X, Zhang C, Wang P, Wei W, Shi X, Wang P, Yang J, Wang L, Tang S, Fang Y *et al*: **Cardiovascular Benefits of Empagliflozin Are Associated With Gut Microbiota and Plasma Metabolites in Type 2 Diabetes**. *The Journal of Clinical Endocrinology & Metabolism* 2022:dgac210.
163. Costello E, Lauber C, Hamady M, Fierer N, Gordon J, Knight R: **Bacterial community variation in human body habitats across space and time**. *Science* 2009, **326**(5960):1694-1697.
164. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA *et al*: **Diet rapidly and reproducibly alters the human gut microbiome**. *Nature* 2014, **505**(7484):559-563.
165. Arumugam M, Raes J, Pelletier E, Le Paslier D, Yamada T, Mende DR, Fernandes GR, Tap J, Bruls T, Batto J-M *et al*: **Enterotypes of the human gut microbiome**. *Nature* 2011, **473**(7346):174-180.
166. Wu G, Chen J, Hoffmann C, Bittinger K, Chen Y, Keilbaugh S, Bewtra M, Knights D, Walters W, Knight R *et al*: **Linking long-term dietary patterns with gut microbial enterotypes**. *Science* 2011, **334**(6052):105-108.
167. De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet J, Massart S, Collini S, Pieraccini G, Lionetti P: **Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa**. *Proc Natl Acad Sci U S A* 2010, **107**(33):14691-14696.
168. Adlercreutz H: **Western diet and Western diseases: Some hormonal and biochemical mechanisms and associations**. *Scandinavian Journal of Clinical and Laboratory Investigation* 1990, **50**(sup201):3-23.
169. Hariharan D, Vellanki K, Kramer H: **The Western Diet and Chronic Kidney Disease**. *Current Hypertension Reports* 2015, **17**(3):16.
170. Arnold M, Sierra, M., Laversanne, M., Soerjomataram, I., Jemal, A., Bray, F.: **Global patterns and trends in colorectal cancer incidence and mortality**. *Gut* 2017, **66**(4):683.
171. Hoegenauer C, Hammer HF, Mahnert A, Moissl-Eichinger C: **Methanogenic archaea in the human gastrointestinal tract**. *Nature Reviews Gastroenterology & Hepatology* 2022, **19**(12):805-813.
172. Florin THJ, Neale G, Goretski S, Cummings JH: **The Sulfate Content of Foods and Beverages**. *Journal of Food Composition and Analysis* 1993, **6**(2):140-151.
173. Macfarlane S, Macfarlane GT: **Regulation of short-chain fatty acid production**. *Proceedings of the Nutrition Society* 2003, **62**(1):67-72.
174. Willis CL, Cummings JH, Neale G, Gibson GR: **In VitroEffects of Mucin Fermentation on the Growth of Human Colonic Sulphate-Reducing Bacteria: ECOLOGY**. *Anaerobe* 1996, **2**(2):117-122.
175. Allen A, Flemström G: **Gastroduodenal mucus bicarbonate barrier: protection against acid and pepsin**. *American Journal of Physiology-Cell Physiology* 2005, **288**(1):C1-C19.
176. Liau YH, Horowitz MI: **The importance of PAPS in determining sulfation in gastrointestinal mucosa**. *Digestion* 1976, **14 4**:372-375.

177. Tsai HH, Dwarakanath AD, Hart CA, Milton JD, Rhodes JM: **Increased faecal mucin sulphatase activity in ulcerative colitis: a potential target for treatment**. *Gut* 1995, **36**(4):570-576.
178. Jacobsen J, Smith L: **Biochemistry and physiology of taurine and taurine derivatives**. *Physiological Reviews* 1968, **48**(2):424-511.
179. Ghandforoush-Sattari M, Mashayekhi S, Krishna C, Thompson J, Routledge P: **Pharmacokinetics of Oral Taurine in Healthy Volunteers**. *Journal of Amino Acids* 2010, **2010**.
180. Sjövall J: **Dietary glycine and taurine on bile acid conjugation in man; Bile acids and steroids 75**. *Proceedings of the Society for Experimental Biology and Medicine* 1959, **100**(4):676-678.
181. Pasantes-Morales H, Quesada O, Alcocer L, Olea R: **Taurine content in foods**. *Nutrition reports international (USA)* 1989.
182. Laidlaw S, Grosvenor M, Kopple J: **The taurine content of common foodstuffs**. *Journal of Parenteral and Enteral Nutrition* 1990, **14**(2):183-188.
183. Adeva-Andany M. F-CR, Lopez-Pereiro Y., Fernandez-Fernandez C., and Rodriguez-Martinez T.: **Taurine Metabolism in Humans**. *Journal of Food Nutrition and Dietetics* 2018, **2**:104.
184. Roe D: **Taurine Intolerance in Psoriasis**. *Journal of Investigative Dermatology* 1966, **46**(4):420-430.
185. Rana S, Sanders T: **Taurine concentrations in the diet, plasma, urine and breast milk of vegans compared with omnivores**. *British Journal of Nutrition* 1986, **56**(1):17-27.
186. Merheb M, Daher R, Nasrallah M, Sabra R, Ziyadeh F, Barada K: **Taurine Intestinal Absorption and Renal Excretion Test in Diabetic Patients**. *Diabetes Care* 2007, **30**(10):2652.
187. Ahlman B, Leijonmarck, C., Lind, C., Vinnars, E., Wernerman, J.: **Free Amino Acids in Biopsy Specimens from the Human Colonic Mucosa**. *Journal of Surgical Research* 1993, **55**(6):647-653.
188. **Global and regional food consumption patterns and trends - Availability and changes in consumption of animal products** [https://www.who.int/nutrition/topics/3_foodconsumption/en/index4.html]
189. Yu H, Guo Z, Shen S, Shan W: **Effects of taurine on gut microbiota and metabolism in mice**. *Amino Acids* 2016, **48**(7):1601-1617.
190. Sasaki K, Sasaki D, Okai N, Tanaka K, Nomoto R, Fukuda I, Yoshida K, Kondo A, Osawa R: **Taurine does not affect the composition, diversity, or metabolism of human colonic microbiota simulated in a single-batch fermentation system**. *PloS one* 2017, **12**(7):e0180991-e0180991.
191. Popkin B, Adair L, Ng S: **Global nutrition transition and the pandemic of obesity in developing countries**. *Nutr Rev* 2012, **70**(1):3-21.
192. Stacy A, Andrade-Oliveira V, McCulloch JA, Hild B, Oh JH, Perez-Chaparro PJ, Sim CK, Lim AI, Link VM, Enamorado M *et al*: **Infection trains the host for microbiota-enhanced resistance to pathogens**. *Cell* 2021, **184**(3):615-627.e617.
193. Mokkala K, Houttu N, Cansev T, Laitinen K: **Interactions of dietary fat with the gut microbiota: Evaluation of mechanisms and metabolic consequences**. *Clinical Nutrition* 2019.
194. Forouhi N, Krauss R, Taubes G, Willett W: **Dietary fat and cardiometabolic health: evidence, controversies, and consensus for guidance**. *The BMJ* 2018, **361**:k2139.

195. **Nutrition Fact Sheet: Lipids** [https://web.archive.org/web/20110720014201/nuinfo-proto4.northwestern.edu/nutrition/factsheets/lipids.html]
196. Hofmann A: **The Continuing Importance of Bile Acids in Liver and Intestinal Disease**. *Archives of Internal Medicine* 1999, **159**(22):2647-2658.
197. Honda A, Ikegami T, Matsuzaki Y: **Intestinal Digestion and Absorption**. In: *Bile Acids in Gastroenterology: Basic and Clinical.* Edited by Tazuma S, Takikawa H. Tokyo: Springer Japan; 2017: 27-41.
198. Thomson A, Schoeller C, Keelan M, Smith L, Clandinin M: **Lipid absorptions passing through the unstirred layers, brush-border membrane, and beyond**. *Canadian Journal of Physiology and Pharmacology* 1993, **71**(8):531-555.
199. Webster S, Wilkinson E, Gowland E: **A Comparison of Fat Absorbtion in Young and Old Subjects**. *Age and Ageing* 1977, **6**(2):113-117.
200. Ryder J: **Steatorrhoea in the elderly**. *Gerontologia Clinica* 1963, **5**(1):30-37.
201. Gabert L, Vors C, Louche-Pélissier C, Sauvinet V, Lambert-Porcheron S, Drai J, Laville M, Désage M, Michalski M: **$^{13}$C tracer recovery in human stools after digestion of a fat-rich meal labelled with [1,1,1-$^{13}$C3]tripalmitin and [1,1,1-$^{13}$C3]triolein**. *Rapid Communications in Mass Spectrometry* 2011, **25**(19):2697-2703.
202. Barrasa J, Olmo N, Lizarbe M, Turnay J: **Bile acids in the colon, from healthy to cytotoxic molecules**. *Toxicology in Vitro* 2013, **27**(2):964-977.
203. David L, Maurice C, Carmody R, Gootenberg D, Button J, Wolfe B, Ling A, Devlin A, Varma Y, Fischbach M *et al*: **Diet rapidly and reproducibly alters the human gut microbiome**. *Nature* 2014, **505**(7484):559-563.
204. Cummings J, Wiggins H, Jenkins D, Houston H, Jivraj T, Drasar B, Hill M: **Influence of diets high and low in animal fat on bowel habit, gastrointestinal transit time, fecal microflora, bile acid, and fat excretion**. *J Clin Invest* 1978, **61**(4):953-963.
205. Brinkworth G, Noakes M, Clifton P, Bird A: **Comparative effects of very low-carbohydrate, high-fat and high-carbohydrate, low-fat weight-loss diets on bowel habit and faecal short-chain fatty acids and bacterial populations**. *British Journal of Nutrition* 2009, **101**(10):1493-1502.
206. Natividad J, Lamas B, Pham H, Michel M, Rainteau D, Bridonneau C, da Costa G, van Hylckama Vlieg J, Sovran B, Chamignon C *et al*: ***Bilophila wadsworthia* aggravates high fat diet induced metabolic dysfunctions in mice**. *Nature Communications* 2018, **9**(1):2802-2802.
207. Zheng H, Ji H, Fan K, Xu H, Huang Y, Zheng Y, Xu Q, Li C, Zhao L, Li Y *et al*: **Targeting Gut Microbiota and Host Metabolism with Dendrobium officinale Dietary Fiber to Prevent Obesity and Improve Glucose Homeostasis in Diet-Induced Obese Mice**. *Molecular Nutrition & Food Research* 2022, **n/a**(n/a):2100772.
208. Lan Y, Sun Q, Ma Z, Peng J, Zhang M, Wang C, Zhang X, Yan X, Chang L, Hou X *et al*: **Seabuckthorn polysaccharide ameliorates high-fat diet-induced obesity by gut microbiota-SCFAs-liver axis**. *Food & Function* 2022, **13**(5):2925-2937.
209. Choudhuri R, Sowers AL, Chandramouli GVR, Gamson J, Krishna MC, Mitchell JB, Cook JA: **The antioxidant tempol transforms gut microbiome to resist obesity in female C3H mice fed a high fat diet**. *Free Radical Biology and Medicine* 2022, **178**:380-390.

210. Wu YT, Shen SJ, Liao KF, Huang CY: **Dietary Plant and Animal Protein Sources Oppositely Modulate Fecal Bilophila and Lachnoclostridium in Vegetarians and Omnivores**. *Microbiol Spectr* 2022, **0**(0):e0204721.
211. Teigen L, Mathai PP, Lopez S, Matson M, Elkin B, Kozysa D, Kabage AJ, Hamilton M, Vaughn BP, Sadowsky MJ *et al*: **Differential hydrogen sulfide production by a human cohort in response to animal- and plant-based diet interventions**. *Clinical Nutrition* 2022.
212. Xu J, Bjursell Magnus K, Himrod J, Deng S, Carmichael Lynn K, Chiang Herbert C, Hooper Lora V, Gordon Jeffrey I: **A Genomic View of the Human-Bacteroides thetaiotaomicron Symbiosis**. *Science* 2003, **299**(5615):2074-2076.
213. Hong P-Y, Wu J-H, Liu W-T: **Relative Abundance of Bacteroides spp. in Stools and Wastewaters as Determined by Hierarchical Oligonucleotide Primer Extension**. *Appl Environ Microbiol* 2008, **74**(9):2882-2893.
214. Human Microbiome Project C: **Structure, function and diversity of the healthy human microbiome**. *Nature* 2012, **486**(7402):207-214.
215. Freitas M, Axelsson L-G, Cayuela C, Midtvedt T, Trugnan G: **Indigenous microbes and their soluble factors differentially modulate intestinal glycosylation steps in vivo**. *Histochemistry and Cell Biology* 2005, **124**(5):423-433.
216. Garber JM, Hennet T, Szymanski CM: **Significance of fucose in intestinal health and disease**. *Molecular Microbiology* 2021, **115**(6):1086-1093.
217. Shroff KE, Meslin K, Cebra JJ: **Commensal enteric bacteria engender a self-limiting humoral mucosal immune response while permanently colonizing the gut**. *Infect Immun* 1995, **63**(10):3904-3913.
218. Kelly D, Campbell JI, King TP, Grant G, Jansson EA, Coutts AG, Pettersson S, Conway S: **Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-γ and RelA**. *Nature immunology* 2004, **5**(1):104-112.
219. Resta–Lenert S, Barrett KE: **Probiotics and Commensals Reverse TNF-α– and IFN-γ–Induced Dysfunction in Human Intestinal Epithelial Cells**. *Gastroenterology* 2006, **130**(3):731-746.
220. Ye M, Yu J, Shi X, Zhu J, Gao X, Liu W: **Polysaccharides catabolism by the human gut bacterium -Bacteroides thetaiotaomicron: advances and perspectives**. *Critical Reviews in Food Science and Nutrition* 2021, **61**(21):3569-3588.
221. Cerqueira FM, Photenhauer AL, Pollet RM, Brown HA, Koropatkin NM: **Starch Digestion by Gut Bacteria: Crowdsourcing for Carbs**. *Trends in Microbiology* 2020, **28**(2):95-108.
222. Reeves AR, Wang GR, Salyers AA: **Characterization of four outer membrane proteins that play a role in utilization of starch by Bacteroides thetaiotaomicron**. *Journal of Bacteriology* 1997, **179**(3):643-649.
223. Hooper LV, Gordon JI: **Glycans as legislators of host–microbial interactions: spanning the spectrum from symbiosis to pathogenicity**. *Glycobiology* 2001, **11**(2):1R-10R.
224. Chia LW, Mank M, Blijenberg B, Aalvink S, Bongers RS, Stahl B, Knol J, Belzer C: **Bacteroides thetaiotaomicron Fosters the Growth of Butyrate-Producing Anaerostipes caccae in the Presence of Lactose and Total Human Milk Carbohydrates**. In: *Microorganisms.* vol. 8; 2020.

225. Porter Nathan T, Larsbrink J, Avci Fikri Y: **Investigation and Alteration of Organic Acid Synthesis Pathways in the Mammalian Gut Symbiont Bacteroides thetaiotaomicron**. *Microbiology Spectrum*, **10**(1):e02312-02321.
226. Hooper LV, Xu J, Falk PG, Midtvedt T, Gordon JI: **A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem**. *Proceedings of the National Academy of Sciences* 1999, **96**(17):9833-9838.
227. Heinonen PK, Leinonen M: **Fecundity and morbidity following acute pelvic inflammatory disease treated with doxycycline and metronidazole**. *Arch Gynecol Obstet* 2003, **268**(4):284-288.
228. Curtis MM, Hu Z, Klimko C, Narayanan S, Deberardinis R, Sperandio V: **The gut commensal Bacteroides thetaiotaomicron exacerbates enteric infection through modification of the metabolic landscape**. *Cell host & microbe* 2014, **16**(6):759-769.
229. Bornet E, Westermann AJ: **The ambivalent role of Bacteroides in enteric infections**. *Trends in Microbiology* 2022, **30**(2):104-108.
230. Benjdia A, Martens EC, Gordon JI, Berteau O: **Sulfatases and a Radical S-Adenosyl-L-methionine (AdoMet) Enzyme Are Key for Mucosal Foraging and Fitness of the Prominent Human Gut Symbiont, Bacteroides thetaiotaomicron**. *Journal of Biological Chemistry* 2011, **286**(29):25973-25982.
231. Gibson GR, Macfarlane GT, Cummings JH: **Occurrence of sulphate-reducing bacteria in human faeces and the relationship of dissimilatory sulphate reduction to methanogenesis in the large gut**. *J Appl Bacteriol* 1988, **65**(2):103-111.
232. Croix JA, Carbonero F, Nava GM, Russell M, Greenberg E, Gaskins HR: **On the relationship between sialomucin and sulfomucin expression and hydrogenotrophic microbes in the human colonic mucosa**. *PLoS One* 2011, **6**(9):e24447.
233. Rey Federico E, Gonzalez Mark D, Cheng J, Wu M, Ahern Philip P, Gordon Jeffrey I: **Metabolic niche of a prominent sulfate-reducing human gut bacterium**. *Proceedings of the National Academy of Sciences* 2013, **110**(33):13582-13587.
234. Traore SI, Khelaifia S, Armstrong N, Lagier JC, Raoult D: **Isolation and culture of Methanobrevibacter smithii by co-culture with hydrogen-producing bacteria on agar plates**. *Clinical Microbiology and Infection* 2019, **25**(12):1561.e1561-1561.e1565.
235. Lebreton F, Willems RJ, Gilmore MS: **Enterococcus diversity, origins in nature, and gut colonization**. *Enterococci: from commensals to leading causes of drug resistant infection [Internet]* 2014.
236. Sadowy E, Luczkiewicz A: **Drug-resistant and hospital-associated Enterococcus faecium from wastewater, riverine estuary and anthropogenically impacted marine catchment basin**. *BMC microbiology* 2014, **14**(1):1-15.
237. Micallef SA, Goldstein RER, George A, Ewing L, Tall BD, Boyer MS, Joseph SW, Sapkota AR: **Diversity, distribution and antibiotic resistance of Enterococcus spp. recovered from tomatoes, leaves, water and soil on US Mid-Atlantic farms**. *Food Microbiology* 2013, **36**(2):465-474.
238. Kasimin ME, Shamsuddin S, Molujin AM, Sabullah MK, Gansau JA, Jawan R: **Enterocin: Promising Biopreservative Produced by Enterococcus sp**. In: *Microorganisms.* vol. 10; 2022.

239. Graham K, Stack H, Rea R: **Safety, beneficial and technological properties of enterococci for use in functional food applications – a review**. *Critical Reviews in Food Science and Nutrition* 2020, **60**(22):3836-3861.
240. Qin J, Li R, Raes J, Arumugam M, Burgdorf KS, Manichanh C, Nielsen T, Pons N, Levenez F, Yamada T: **A human gut microbial gene catalogue established by metagenomic sequencing**. *nature* 2010, **464**(7285):59-65.
241. Suvorov A: **What Is Wrong with Enterococcal Probiotics?** *Probiotics and Antimicrobial Proteins* 2020, **12**(1):1-4.
242. Wan LYM, Chen ZJ, Shah NP, El-Nezami H: **Modulation of Intestinal Epithelial Defense Responses by Probiotic Bacteria**. *Critical Reviews in Food Science and Nutrition* 2016, **56**(16):2628-2641.
243. Krawczyk B, Wityk P, Gałęcka M, Michalik M: **The Many Faces of Enterococcus spp.—Commensal, Probiotic and Opportunistic Pathogen**. In: *Microorganisms.* vol. 9; 2021.
244. Dubin K, Pamer Eric G: **Enterococci and Their Interactions with the Intestinal Microbiome**. *Microbiology Spectrum* 2017, **5**(6):10.1128/microbiolspec.bad-0014-2016.
245. Agudelo Higuita NI, Huycke MM: **Enterococcal Disease, Epidemiology, and Implications for Treatment**. In: *Enterococci: From Commensals to Leading Causes of Drug Resistant Infection.* Edited by Gilmore MS, Clewell DB, Ike Y, Shankar N. Boston: Massachusetts Eye and Ear Infirmary; 2014.
246. Bin-Asif H, Ali SA: **The genus Enterococcus and its associated virulent factors**. *Microorganisms* 2019:109-130.
247. Turner AM, Lee JYH, Gorrie CL, Howden BP, Carter GP: **Genomic Insights Into Last-Line Antimicrobial Resistance in Multidrug-Resistant Staphylococcus and Vancomycin-Resistant Enterococcus**. *Front Microbiol* 2021, **12**:637656.
248. **WHO priority pathogens list for R&D of new antibiotics** [https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed]
249. Ben Braiek O, Smaoui S: **Enterococci: between emerging pathogens and potential probiotics**. *BioMed research international* 2019, **2019**.
250. Hanchi H, Mottawea W, Sebei K, Hammami R: **The genus Enterococcus: between probiotic potential and safety concerns—an update**. *Frontiers in microbiology* 2018, **9**:1791.
251. Kitz R, Martens U, Zieseniß E, Enck P, Rose M: **Probiotic E.faecalis — adjuvant therapy in children with recurrent rhinosinusitis**. *Open Medicine* 2012, **7**(3):362-365.
252. Ouwehand AC, Salminen S, Isolauri E: **Probiotics: an overview of beneficial effects**. *Antonie van Leeuwenhoek* 2002, **82**(1):279-289.
253. Buydens P, Debeuckelaere S: **Efficacy of SF 68 in the Treatment of Acute Diarrhea a Placebo-Controlled Trial**. *Scandinavian Journal of Gastroenterology* 1996, **31**(9):887-891.
254. Chen C-C, Kong M-S, Lai M-W, Chao H-C, Chang K-W, Chen S-Y, Huang Y-C, Chiu C-H, Li W-C, Lin P-Y *et al*: **Probiotics Have Clinical, Microbiologic, and Immunologic Efficacy in Acute Infectious Diarrhea**. *The Pediatric Infectious Disease Journal* 2010, **29**(2).
255. Additives EPo, Products or Substances used in Animal F: **Guidance on the safety assessment of Enterococcus faecium in animal nutrition**. *EFSA Journal* 2012, **10**(5):2682.

256. Montealegre MC, Singh KV, Murray BE: **Gastrointestinal tract colonization dynamics by different Enterococcus faecium clades**. *The Journal of infectious diseases* 2016, **213**(12):1914-1922.
257. Domann E, Hain T, Ghai R, Billion A, Kuenne C, Zimmermann K, Chakraborty T: **Comparative genomic analysis for the presence of potential enterococcal virulence factors in the probiotic Enterococcus faecalis strain Symbioflor 1**. *International Journal of Medical Microbiology* 2007, **297**(7):533-539.
258. Vebø HC, Solheim M, Snipen L, Nes IF, Brede DA: **Comparative genomic analysis of pathogenic and probiotic Enterococcus faecalis isolates, and their transcriptional responses to growth in human urine**. *PloS one* 2010, **5**(8):e12489.
259. Chajęcka-Wierzchowska W, Zadernowska A, Łaniewska-Trokenheim Ł: **Virulence factors of Enterococcus spp. presented in food**. *LWT* 2017, **75**:670-676.
260. Fritzenwanker M, Kuenne C, Billion A, Hain T, Zimmermann K, Goesmann A, Chakraborty T, Domann E: **Complete Genome Sequence of the Probiotic Enterococcus faecalis Symbioflor 1 Clone DSM 16431**. *Genome Announcements* 2013, **1**(1):10.1128/genomea.00165-00112.
261. Liu X, Gong C, Jiang X: **Inhibitory effects of enterococci on the production of hydrogen sulfide by hydrogen sulfide–producing bacteria in raw meat**. *Journal of Applied Microbiology* 2011, **111**(1):83-92.
262. Libiad M, Vitvitsky V, Bostelaar T, Bak D, Lee H, Sakamoto N, Fearon E, Lyssiotis C, Weerapana E, Banerjee R: **Hydrogen sulfide perturbs mitochondrial bioenergetics and triggers metabolic reprogramming in colon cells**. *Journal of Biological Chemistry* 2019, **294**(32):12077-12090.
263. Silva N, Igrejas G, Gonçalves A, Poeta P: **Commensal gut bacteria: distribution of Enterococcus species and prevalence of Escherichia coli phylogenetic groups in animals and humans in Portugal**. *Annals of Microbiology* 2012, **62**(2):449-459.
264. Tognon M, Köhler T, Luscher A, van Delden C: **Transcriptional profiling of Pseudomonas aeruginosa and Staphylococcus aureus during in vitro co-culture**. *BMC Genomics* 2019, **20**(1):30.
265. Kim H, Jeong Y, Kang S, You HJ, Ji GE: **Co-Culture with Bifidobacterium catenulatum Improves the Growth, Gut Colonization, and Butyrate Production of Faecalibacterium prausnitzii: In Vitro and In Vivo Studies**. In: *Microorganisms.* vol. 8; 2020.
266. Chaveerach P, Lipman LJA, van Knapen F: **Antagonistic activities of several bacteria on in vitro growth of 10 strains of Campylobacter jejuni/coli**. *International Journal of Food Microbiology* 2004, **90**(1):43-50.
267. Trejo FM, Perez PF, De Antoni GL: **Co-culture with potentially probiotic microorganisms antagonises virulence factors of Clostridium difficile in vitro**. *Antonie Van Leeuwenhoek* 2010, **98**(1):19-29.
268. Postgate John R: **Versatile Medium for the Enumeration of Sulfate-Reducing Bacteria**. *Applied Microbiology* 1963, **11**(3):265-267.
269. **The QIB colon model (NCT02653001)** [https://classic.clinicaltrials.gov/ct2/show/NCT02653001]
270. **The Impact of Cranberries On the Microbiome and the Brain in Healthy Ageing sTudy (COMBAT) (COMBAT) (NCT03679533)** [https://clinicaltrials.gov/study/NCT03679533]
271. Baker G, Smith J, Cowan D: **Review and re-analysis of domain-specific 16S primers**. *Journal of Microbiological Methods* 2003, **55**(3):541-555.

272. Cline J: **Spectrophotometric determination of hydrogen sulphide in natural waters**. *Limnology and Oceanography* 1969, **14**(3):454-458.
273. Furet J-P, Firmesse O, Gourmelon M, Bridonneau C, Tap J, Mondot S, Doré J, Corthier G: **Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR**. *FEMS Microbiology Ecology* 2009, **68**(3):351-362.
274. Furtado I, Xavier PC, Tavares LV, Alves F, Martins SF, Martins Ade S, Palhares DB: **Enterococcus faecium and Enterococcus faecalis in blood of newborns with suspected nosocomial infection**. *Rev Inst Med Trop Sao Paulo* 2014, **56**(1):77-80.
275. Bushnell B: **BBTools**. 2022.
276. Andrews S: **FastQC: a quality control tool for high throughput sequence data**. In.: Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom; 2010.
277. Wick RR, Judd LM, Gorrie CL, Holt KE: **Completing bacterial genome assemblies with multiplex MinION sequencing**. *Microbial genomics* 2017, **3**(10).
278. Wick RR, Judd LM, Gorrie CL, Holt KE: **Unicycler: resolving bacterial genome assemblies from short and long sequencing reads**. *PLoS computational biology* 2017, **13**(6):e1005595.
279. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW: **CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes**. *Genome Res* 2015, **25**(7):1043-1055.
280. Seemann T: **Prokka: rapid prokaryotic genome annotation**. *Bioinformatics* 2014, **30**(14):2068-2069.
281. Salyers AA: **Bacteroides of the human lower intestinal tract**. *Annu Rev Microbiol* 1984, **38**:293-313.
282. Beaumont M, Andriamihaja M, Lan A, Khodorova N, Audebert M, Blouin J, Grauso M, Lancha L, Benetti P, Benamouzig R *et al*: **Detrimental effects for colonocytes of an increased exposure to luminal hydrogen sulfide: The adaptive response**. *Free Radical Biology and Medicine* 2016, **93**:155-164.
283. Sánchez-Illana Á, Piñeiro-Ramos JD, Sanjuan-Herráez JD, Vento M, Quintás G, Kuligowski J: **Evaluation of batch effect elimination using quality control replicates in LC-MS metabolite profiling**. *Analytica Chimica Acta* 2018, **1019**:38-48.
284. Pang Z, Chong J, Zhou G, de Lima Morais DA, Chang L, Barrette M, Gauthier C, Jacques P-É, Li S, Xia J: **MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights**. *Nucleic Acids Research* 2021, **49**(W1):W388-W396.
285. Okuda S, Yamada T, Hamajima M, Itoh M, Katayama T, Bork P, Goto S, Kanehisa M: **KEGG Atlas mapping for global analysis of metabolic pathways**. *Nucleic Acids Res* 2008, **36**(Web Server issue):W423-426.
286. Karp PD, Midford PE, Billington R, Kothari A, Krummenacker M, Latendresse M, Ong WK, Subhraveti P, Caspi R, Fulcher C *et al*: **Pathway Tools version 23.0 update: software for pathway/genome informatics and systems biology**. *Briefings in Bioinformatics* 2021, **22**(1):109-126.
287. van Heel AJ, de Jong A, Song C, Viel JH, Kok J, Kuipers OP: **BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins**. *Nucleic acids research* 2018, **46**(W1):W278-W281.
288. Blin K, Shaw S, Augustijn HE, Reitz ZL, Biermann F, Alanjary M, Fetter A, Terlouw BR, Metcalf WW, Helfrich EJ: **antiSMASH 7.0: New and**

**improved predictions for detection, regulation, chemical structures and visualisation**. *Nucleic acids research* 2023:gkad344.
289. Fernandez-Cantos MV, Garcia-Morena D, Yi Y, Liang L, Gómez-Vázquez E, Kuipers OP: **Bioinformatic mining for RiPP biosynthetic gene clusters in Bacteroidales reveals possible new subfamily architectures and novel natural products**. *Frontiers in Microbiology* 2023, **14**.
290. Elnar AG, Kim GB: **Complete genome sequence of Clostridium perfringens B20, a bacteriocin-producing pathogen**. *J Anim Sci Technol* 2021, **63**(6):1468-1472.
291. Johnson M, Zaretskaya I, Raytselis Y, Merezhuk Y, McGinnis S, Madden TL: **NCBI BLAST: a better web interface**. *Nucleic Acids Research* 2008, **36**(suppl_2):W5-W9.
292. Blum M, Chang H-Y, Chuguransky S, Grego T, Kandasaamy S, Mitchell A, Nuka G, Paysan-Lafosse T, Qureshi M, Raj S *et al*: **The InterPro protein families and domains database: 20 years on**. *Nucleic Acids Research* 2021, **49**(D1):D344-D354.
293. Olson RD, Assaf R, Brettin T, Conrad N, Cucinell C, Davis JJ, Dempsey DM, Dickerman A, Dietrich EM, Kenyon RW: **Introducing the bacterial and viral bioinformatics resource center (BV-BRC): a resource combining PATRIC, IRD and ViPR**. *Nucleic acids research* 2023, **51**(D1):D678-D689.
294. Chen L, Yang J, Yu J, Yao Z, Sun L, Shen Y, Jin Q: **VFDB: a reference database for bacterial virulence factors**. *Nucleic acids research* 2005, **33**(suppl_1):D325-D328.
295. Sayers S, Li L, Ong E, Deng S, Fu G, Lin Y, Yang B, Zhang S, Fa Z, Zhao B: **Victors: a web-based knowledge base of virulence factors in human and animal pathogens**. *Nucleic acids research* 2019, **47**(D1):D693-D700.
296. Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, Huynh W, Nguyen A-LV, Cheng AA, Liu S *et al*: **CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database**. *Nucleic Acids Research* 2020, **48**(D1):D517-D525.
297. Deguchi Y, Morishita T: **Nutritional Requirements in Multiple Auxotrophic Lactic Acid Bacteria: Genetic Lesions Affecting Amino Acid Biosynthetic Pathways in Lactococcus lactis, Enterococcus faecium, and Pediococcus acidilactici**. *Biosci Biotechnol Biochem* 1992, **56**(6):913-918.
298. Hochstadt-Ozer J, Stadtman ER: **The Regulation of Purine Utilization in Bacteria: III. THE INVOLVEMENT OF PURINE PHOSPHORIBOSYLTRANSFERASES IN THE UPTAKE OF ADENINE AND OTHER NUCLEIC ACID PRECURSORS BY INTACT RESTING CELLS**. *Journal of Biological Chemistry* 1971, **246**(17):5312-5320.
299. Magasanik B, Karibian D: **Purine Nucleotide Cycles and Their Metabolic Role**. *Journal of Biological Chemistry* 1960, **235**(9):2672-2681.
300. Mou Z, Yang Y, Hall AB, Jiang X: **The taxonomic distribution of histamine-secreting bacteria in the human gut microbiome**. *BMC Genomics* 2021, **22**(1):695.
301. Wang Y, Cao Q, Cao Q, Gan J, Sun N, Yang C-G, Bae T, Wu M, Lan L: **Histamine activates HinK to promote the virulence of Pseudomonas aeruginosa**. *Science Bulletin* 2021, **66**(11):1101-1118.
302. Wang P, Kong C, Sun B, Xu X: **Allantoin-induced changes of microbial diversity and community in rice soil**. *Plant and Soil* 2010, **332**(1):357-368.

303. Byerley LO, Gallivan KM, Christopher CJ, Taylor CM, Luo M, Dowd SE, Davis GM, Castro HF, Campagna SR, Ondrak KS: **Gut Microbiome and Metabolome Variations in Self-Identified Muscle Builders Who Report Using Protein Supplements**. In: *Nutrients.* vol. 14; 2022.
304. Young EG, Hawkins WW: **The Decomposition of Allantoin by Intestinal Bacteria**. *J Bacteriol* 1944, **47**(4):351-353.
305. Kim K, Kim M-I, Chung J, Ahn J-H, Rhee S: **Crystal Structure of Metal-Dependent Allantoinase from Escherichia coli**. *Journal of Molecular Biology* 2009, **387**(5):1067-1074.
306. Gorelik O, Rogad A, Holoidovsky L, Meijler MM, Sal-Man N: **Indole intercepts the communication between enteropathogenic E. coli and Vibrio cholerae**. *Gut Microbes* 2022, **14**(1):2138677.
307. Pallister T, Jackson MA, Martin TC, Zierer J, Jennings A, Mohney RP, MacGregor A, Steves CJ, Cassidy A, Spector TD: **Hippurate as a metabolomic marker of gut microbiome diversity: Modulation by diet and relationship to metabolic syndrome**. *Sci Rep* 2017, **7**(1):13670.
308. Wang Y, Nan X, Zhao Y, Jiang L, Wang H, Zhang F, Hua D, Liu J, Yao J, Yang L *et al*: **Consumption of Supplementary Inulin Modulates Milk Microbiota and Metabolites in Dairy Cows with Subclinical Mastitis**. *Appl Environ Microbiol* 2022, **88**(4):e02059-02021.
309. Kart D, Yabanoğlu Çiftçi S, Nemutlu E: **Metabolomics-driven Approaches on Interactions Between Enterococcus faecalis and Candida albicans Biofilms**. *Turk J Pharm Sci* 2021, **18**(5):557-564.
310. Duan H, Yu Q, Ni Y, Li J, Fan L: **Effect of Agaricus bisporus Polysaccharides on Human Gut Microbiota during In Vitro Fermentation: An Integrative Analysis of Microbiome and Metabolome**. In: *Foods.* vol. 12; 2023.
311. Shin EK, Kwon H-S, Kim YH, Shin H-K, Kim J-K: **Chrysin, a natural flavone, improves murine inflammatory bowel diseases**. *Biochemical and Biophysical Research Communications* 2009, **381**(4):502-507.
312. Steer TE, Johnson IT, Gee JM, Gibson GR: **Metabolism of the soyabean isoflavone glycoside genistin in vitro by human gut bacteria and the effect of prebiotics**. *British Journal of Nutrition* 2003, **90**(3):635-642.
313. Chouhan S, Sharma K, Guleria S, Koffas MA: **Microbial production of flavonoids**. *Systems and Synthetic Biotechnology for Production of Nutraceuticals* 2019:93-128.
314. Pugin B, Barcik W, Westermann P, Heider A, Wawrzyniak M, Hellings P, Akdis CA, O'Mahony L: **A wide diversity of bacteria from the human gut produces and degrades biogenic amines**. *Microbial Ecology in Health and Disease* 2017, **28**(1):1353881.
315. Mao B, Tang H, Gu J, Li D, Cui S, Zhao J, Zhang H, Chen W: **In vitro fermentation of raffinose by the human gut bacteria**. *Food Funct* 2018, **9**(11):5824-5831.
316. Yang Y, Chen N, Sun L, Zhang Y, Wu Y, Wang Y, Liao X, Mi J: **Short-term cold stress can reduce the abundance of antibiotic resistance genes in the cecum and feces in a pig model**. *Journal of Hazardous Materials* 2021, **416**:125868.
317. Liu Y, Chen L, Liu L, Zhao S-S, You J-Q, Zhao X-J, Liu H-X, Xu G-W, Wen D-L: **Interplay between dietary intake, gut microbiota, and metabolic profile in obese adolescents: Sex-dependent differential patterns**. *Clinical Nutrition* 2022, **41**(12):2706-2719.
318. Tsochatzis E, Berggreen IE, Tedeschi F, Ntrallou K, Gika H, Corredig M: **Gut Microbiome and Degradation Product Formation during**

**Biodegradation of Expanded Polystyrene by Mealworm Larvae under Different Feeding Strategies**. In: *Molecules.* vol. 26; 2021.
319. Fackelmann G, Sommer S: **Microplastics and the gut microbiome: How chronically exposed species may suffer from gut dysbiosis**. *Marine Pollution Bulletin* 2019, **143**:193-203.
320. Guo J, Pan Y, Chen J, Jin P, Tang S, Wang H, Su H, Wang Q, Chen C, Xiong F *et al*: **Serum metabolite signatures in normal individuals and patients with colorectal adenoma or colorectal cancer using UPLC-MS/MS method**. *Journal of Proteomics* 2023, **270**:104741.
321. Huang J, Xiao N, Sun Y, Wu S, Tian W, Lai Y, Li P, Du B: **Supplementation of Bacillus sp. DU-106 reduces hypercholesterolemia and ameliorates gut dysbiosis in high-fat diet rats**. *Applied Microbiology and Biotechnology* 2021, **105**(1):287-299.
322. Umar Y: **A comparative study on the molecular structures and vibrational spectra of 2-, 3-and 4-cyanopyridines by density functional theory**. *IOSR Journal of Applied Chemistry (IOSR-JAC)* 2015, **8**(7):44-55.
323. Lam K-H, Gambari R, Lee KK-H, Chen Y-X, Kok SH-L, Wong RS-M, Lau F-Y, Cheng C-H, Wong W-Y, Bian Z-X: **Preparation of 8-hydroxyquinoline derivatives as potential antibiotics against Staphylococcus aureus**. *Bioorganic & medicinal chemistry letters* 2014, **24**(1):367-370.
324. Arivett B, Farone M, Masiragani R, Burden A, Judge S, Osinloye A, Minici C, Degano M, Robinson M, Kline P: **Characterization of inosine–uridine nucleoside hydrolase (RihC) from Escherichia coli**. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* 2014, **1844**(3):656-662.
325. Matsui H, Shimaoka M, Kawasaki H, Takenaka Y, Kurahashi O: **Adenine deaminase activity of the yicP gene product of Escherichia coli**. *Biosci Biotechnol Biochem* 2001, **65**(5):1112-1118.
326. Ückert A-K, Rütschlin S, Gutbier S, Wörz NC, Miah MR, Martins AC, Hauer I, Holzer A-K, Meyburg B, Mix A-K *et al*: **Identification of the bacterial metabolite aerugine as potential trigger of human dopaminergic neurodegeneration**. *Environment International* 2023, **180**:108229.
327. Xu Y, Minhazul KAHM, Li X: **The occurrence, enzymatic production, and application of ethyl butanoate, an important flavor constituent**. *Flavour and Fragrance Journal* 2020, **35**(6):601-615.
328. Liu SQ, Holland R, Crow VL: **Ethyl Butanoate Formation by Dairy Lactic Acid Bacteria**. *International Dairy Journal* 1998, **8**(7):651-657.
329. Centeno JA, Lorenzo JM, Carballo J: **Effects of autochthonous Kluyveromyces lactis and commercial Enterococcus faecium adjunct cultures on the volatile profile and the sensory characteristics of short-ripened acid-curd Cebreiro cheese**. *Food Microbiology* 2022, **108**:104101.
330. Jain A, Li XH, Chen WN: **An untargeted fecal and urine metabolomics analysis of the interplay between the gut microbiome, diet and human metabolism in Indian and Chinese adults**. *Sci Rep* 2019, **9**(1):9191.
331. Dunn SR, Gabuzda GM, Superdock KR, Kolecki RS, Schaedler RW, Simenhoff ML: **Induction of creatininase activity in chronic renal failure: Timing of creatinine degradation and effect of antibiotics**. *American Journal of Kidney Diseases* 1997, **29**(1):72-77.
332. Wyss M, Kaddurah-Daouk R: **Creatine and Creatinine Metabolism**. *Physiological Reviews* 2000, **80**(3):1107-1213.
333. Loenen WAM: **S-Adenosylmethionine: jack of all trades and master of everything?** *Biochemical Society Transactions* 2006, **34**(2):330-333.

334. Robertson RC, Seira Oriach C, Murphy K, Moloney GM, Cryan JF, Dinan TG, Ross RP, Stanton C: **Deficiency of essential dietary n-3 PUFA disrupts the caecal microbiome and metabolome in mice**. *British Journal of Nutrition* 2017, **118**(11):959-970.
335. Cutler HG, Crumley FG, Cox RH, Wells JM, Cole RJ: **The biological properties of cyclopenin and cyclopenol**. *Plant and cell physiology* 1984, **25**(2):257-263.
336. Nemet I, Varga-Defterdarovic L: **The role of methylglyoxal in the non-enzymatic conversion of tryptophan, its methyl ester and tryptamine to 1-acetyl-beta-carbolines**. *Bioorg Med Chem* 2008, **16**(8):4551-4562.
337. Kang JD, Myers CJ, Harris SC, Kakiyama G, Lee I-K, Yun B-S, Matsuzaki K, Furukawa M, Min H-K, Bajaj JS: **Bile acid 7α-dehydroxylating gut bacteria secrete antibiotics that inhibit Clostridium difficile: role of secondary bile acids**. *Cell chemical biology* 2019, **26**(1):27-34. e24.
338. Lakshmi A, Vishnurekha C, Baghkomeh PN: **Effect of theobromine in antimicrobial activity: An in vitro study**. *Dent Res J (Isfahan)* 2019, **16**(2):76-80.
339. Martín-Peláez S, Camps-Bossacoma M, Massot-Cladera M, Rigo-Adrover M, Franch À, Pérez-Cano FJ, Castell M: **Effect of cocoa's theobromine on intestinal microbiota of rats**. *Molecular Nutrition & Food Research* 2017, **61**(10):1700238.
340. van den Born E, Bekkelund A, Moen MN, Omelchenko MV, Klungland A, Falnes PØ: **Bioinformatics and functional analysis define four distinct groups of AlkB DNA-dioxygenases in bacteria**. *Nucleic Acids Research* 2009, **37**(21):7124-7136.
341. He F, Zhang T, Xue K, Fang Z, Jiang G, Huang S, Li K, Gu Z, Shi H, Zhang Z *et al*: **Fecal multi-omics analysis reveals diverse molecular alterations of gut ecosystem in COVID-19 patients**. *Analytica Chimica Acta* 2021, **1180**:338881.
342. Yan Z, He F, He H, Li D, Pang P, Fang Z, Jiang G, Huang S, Li K, Gu Z: **Molecular Phenomics of Gut Ecosystem in COVID-19 Patients**. 2021.
343. Pérez-Ramos A, Madi-Moussa D, Coucheney F, Drider D: **Current Knowledge of the Mode of Action and Immunity Mechanisms of LAB-Bacteriocins**. *Microorganisms* 2021, **9**(10).
344. Enuh BM, Gedikli S, Aytar Celik P, Cabuk A: **Genome sequence and probiotic potential of newly isolated Enterococcus durans strain MN187066**. *Lett Appl Microbiol* 2023, **76**(3):ovad035.
345. Freitas AR, Tedim AP, Novais C, Coque TM, Peixe L: **Distribution of putative virulence markers in Enterococcus faecium: towards a safety profile review**. *J Antimicrob Chemother* 2018, **73**(2):306-319.
346. Costa Y, Galimand M, Leclercq R, Duval J, Courvalin P: **Characterization of the chromosomal aac(6')-Ii gene specific for Enterococcus faecium**. *Antimicrob Agents Chemother* 1993, **37**(9):1896-1903.
347. Miele A, Goldstein BP, Bandera M, Jarvis C, Resconi A, Williams RJ: **Differential susceptibilities of enterococcal species to elfamycin antibiotics**. *J Clin Microbiol* 1994, **32**(8):2016-2018.
348. Morley VJ, Sim DG, Penkevich A, Woods RJ, Read AF: **An orally administered drug prevents selection for antibiotic-resistant bacteria in the gut during daptomycin therapy**. *Evolution, Medicine, and Public Health* 2022, **10**(1):439-446.
349. Mascher T, Zimmer Sara L, Smith T-A, Helmann John D: **Antibiotic-Inducible Promoter Regulated by the Cell Envelope Stress-Sensing**

**Two-Component System LiaRS of Bacillus subtilis**. *Antimicrob Agents Chemother* 2004, **48**(8):2888-2896.
350. Bergmann R, van der Linden M, Chhatwal GS, Nitsche-Schmitz DP: **Factors that cause trimethoprim resistance in Streptococcus pyogenes**. *Antimicrob Agents Chemother* 2014, **58**(4):2281-2288.
351. Sharkey LK, Edwards TA, O'Neill AJ: **ABC-F Proteins Mediate Antibiotic Resistance through Ribosomal Protection**. *mBio* 2016, **7**(2):e01975.
352. Molale LG, Bezuidenhout CC: **Antibiotic resistance, efflux pump genes and virulence determinants in Enterococcus spp. from surface water systems**. *Environ Sci Pollut Res Int* 2016, **23**(21):21501-21510.
353. Coyne S, Courvalin P, Périchon B: **Efflux-mediated antibiotic resistance in Acinetobacter spp**. *Antimicrob Agents Chemother* 2011, **55**(3):947-953.
354. Lavilla Lerma L, Benomar N, Valenzuela AS, Casado Munoz Mdel C, Galvez A, Abriouel H: **Role of EfrAB efflux pump in biocide tolerance and antibiotic resistance of Enterococcus faecalis and Enterococcus faecium isolated from traditional fermented foods and the effect of EDTA as EfrAB inhibitor**. *Food Microbiol* 2014, **44**:249-257.
355. Nishioka T, Ogawa W, Kuroda T, Katsu T, Tsuchiya T: **Gene cloning and characterization of EfmA, a multidrug efflux pump, from Enterococcus faecium**. *Biol Pharm Bull* 2009, **32**(3):483-488.
356. Dezfulian A, Aslani MM, Oskoui M, Farrokh P, Azimirad M, Dabiri H, Salehian MT, Zali MR: **Identification and Characterization of a High Vancomycin-Resistant Staphylococcus aureus Harboring VanA Gene Cluster Isolated from Diabetic Foot Ulcer**. *Iran J Basic Med Sci* 2012, **15**(2):803-806.
357. Wan TW, Hung WC, Tsai JC, Lin YT, Lee H, Hsueh PR, Lee TF, Teng LJ: **Novel Structure of Enterococcus faecium-Originated ermB-Positive Tn1546-Like Element in Staphylococcus aureus**. *Antimicrob Agents Chemother* 2016, **60**(10):6108-6114.
358. Mazhar S, Khokhlova E, Colom J, Simon A, Deaton J, Rea K: **In vitro and in silico assessment of probiotic and functional properties of Bacillus subtilis DE111**. *Front Microbiol* 2022, **13**:1101144.
359. Ryan S, Hill C, Gahan CGM: **Chapter 3 - Acid Stress Responses in Listeria monocytogenes**. In: *Advances in Applied Microbiology.* Edited by Laskin AI, Sariaslani S, Gadd GM, vol. 65: Academic Press; 2008: 67-91.
360. Ruiz L, Ruas-Madiedo P, Gueimonde M, de Los Reyes-Gavilan CG, Margolles A, Sanchez B: **How do bifidobacteria counteract environmental challenges? Mechanisms involved and physiological consequences**. *Genes Nutr* 2011, **6**(3):307-318.
361. Duport C, Jobin M, Schmitt P: **Adaptation in Bacillus cereus: From Stress to Disease**. *Front Microbiol* 2016, **7**:1550.
362. Wu X, Hou J, Chen X, Chen X, Zhao W: **Identification and functional analysis of the L-ascorbate-specific enzyme II complex of the phosphotransferase system in Streptococcus mutans**. *BMC Microbiology* 2016, **16**(1):51.
363. Seifart Gomes C, Izar B, Pazan F, Mohamed W, Mraheil MA, Mukherjee K, Billion A, Aharonowitz Y, Chakraborty T, Hain T: **Universal Stress Proteins Are Important for Oxidative and Acid Stress Resistance and Growth of Listeria monocytogenes EGD-e In Vitro and In Vivo**. *PLOS ONE* 2011, **6**(9):e24965.
364. Horáčková Š, Plocková M, Demnerová K: **Importance of microbial defence systems to bile salts and mechanisms of serum cholesterol reduction**. *Biotechnology Advances* 2018, **36**(3):682-690.

365. Azcarate-Peril MA, Altermann E, Goh Yong J, Tallon R, Sanozky-Dawes Rosemary B, Pfeiler Erika A, O'Flaherty S, Buck BL, Dobson A, Duong T *et al*: **Analysis of the Genome Sequence of Lactobacillus gasseri ATCC 33323 Reveals the Molecular Basis of an Autochthonous Intestinal Organism**. *Appl Environ Microbiol* 2008, **74**(15):4610-4625.
366. Rivaldi JD, Sousa Silva MLC, Duarte LC, Ferreira AEN, Cordeiro C, de Almeida Felipe MdG, de Ponces Freire A, de Mancilha IM: **Metabolism of biodiesel-derived glycerol in probiotic Lactobacillus strains**. *Applied Microbiology and Biotechnology* 2013, **97**(4):1735-1743.
367. Rath H, Reder A, Hoffmann T, Hammer E, Seubert A, Bremer E, Völker U, Mäder U: **Management of Osmoprotectant Uptake Hierarchy in Bacillus subtilis via a SigB-Dependent Antisense RNA**. *Frontiers in Microbiology* 2020, **11**.
368. Anggraeni AA: **Mini-Review: The potential of raffinose as a prebiotic**. *IOP Conference Series: Earth and Environmental Science* 2022, **980**(1):012033.
369. Lee JS, Wang RX, Alexeev EE, Lanis JM, Battista KD, Glover LE, Colgan SP: **Hypoxanthine is a checkpoint stress metabolite in colonic epithelial energy modulation and barrier function**. *Journal of Biological Chemistry* 2018, **293**(16):6039-6051.
370. Popovic N, Dinic M, Tolinacki M, Mihajlovic S, Terzic-Vidojevic A, Bojic S, Djokic J, Golic N, Veljovic K: **New Insight into Biofilm Formation Ability, the Presence of Virulence Genes and Probiotic Potential of Enterococcus sp. Dairy Isolates**. *Front Microbiol* 2018, **9**:78.
371. Nes IF, Diep DB, Holo H: **Bacteriocin diversity in Streptococcus and Enterococcus**. *J Bacteriol* 2007, **189**(4):1189-1198.
372. Hill C, O'Keeffe T, Ross P: **Bacteriocins**. In: *Encyclopedia of Dairy Sciences.* Edited by Roginski H. Oxford: Elsevier; 2002: 128-135.
373. Choi S, Baek M-g, Chung M-J, Lim S, Yi H: **Distribution of bacteriocin genes in the lineages of Lactiplantibacillus plantarum**. *Sci Rep* 2021, **11**(1):20063.
374. Aymerich T, Jofré A, Bover-Cid S: **Enterocin A-based antimicrobial film exerted strong antilisterial activity in sliced dry-cured ham immediately and after 6 months at 8 °C**. *Food Microbiology* 2022, **105**:104005.
375. Nissen-Meyer J, Oppegård C, Rogne P, Haugen HS, Kristiansen PE: **Structure and Mode-of-Action of the Two-Peptide (Class-IIb) Bacteriocins**. *Probiotics and Antimicrobial Proteins* 2010, **2**(1):52-60.
376. Strompfová V, Marciňáková M, Simonová M, Gancarčíková S, Jonecová Z, Sciranková Ľ, Koščová J, Buleca V, Čobanová K, Lauková A: **Enterococcus faecium EK13—an enterocin A-producing strain with probiotic character and its effect in piglets**. *Anaerobe* 2006, **12**(5-6):242-248.
377. Ewida R, El-Prince E: **INHIBITORY EFFECT OF ENTEROCIN WITH SOME FOOD ADDITIVES ON ESCHERICHIA COLI O18 IN UHT MILK**. *Assiut Veterinary Medical Journal* 2018, **64**(159):178-186.
378. Lauková A, Guba P, Nemcová R, Vasilková Z: **Reduction of Salmonella in Gnotobiotic Japanese Quails Caused by the Enterocin A-producing EK13 Strain of Enterococcus faecium**. *Veterinary Research Communications* 2003, **27**(4):275-280.
379. Heilbronner S, Krismer B, Brötz-Oesterhelt H, Peschel A: **The microbiome-shaping roles of bacteriocins**. *Nature Reviews Microbiology* 2021, **19**(11):726-739.

380. Fu X, Lyu L, Wang Y, Zhang Y, Guo X, Chen Q, Liu C: **Safety assessment and probiotic characteristics of Enterococcus lactis JDM1**. *Microb Pathog* 2022, **163**:105380.
381. Courvalin P: **Vancomycin resistance in gram-positive cocci**. *Clin Infect Dis* 2006, **42 Suppl 1**(Supplement_1):S25-34.
382. Bonten MJ, Willems R, Weinstein RA: **Vancomycin-resistant enterococci: why are they here, and where do they come from?** *Lancet Infect Dis* 2001, **1**(5):314-325.
383. Robredo B, Singh KV, Torres C, Murray BE: **Streptogramin resistance and shared pulsed-field gel electrophoresis patterns in vanA-containing Enterococcus faecium and Enterococcus hirae isolated from humans and animals in Spain**. *Microb Drug Resist* 2000, **6**(4):305-311.
384. Poeta P, Costa D, Rodrigues J, Torres C: **Study of faecal colonization by vanA-containing Enterococcus strains in healthy humans, pets, poultry and wild animals in Portugal**. *Journal of Antimicrobial Chemotherapy* 2005, **55**(2):278-280.
385. López M, Tenorio C, Torres C: **Study of Vancomycin Resistance in Faecal Enterococci from Healthy Humans and Dogs in Spain a Decade after the Avoparcin Ban in Europe**. *Zoonoses and Public Health* 2013, **60**(2):160-167.
386. Arthur M, Courvalin P: **Genetics and mechanisms of glycopeptide resistance in enterococci**. *Antimicrob Agents Chemother* 1993, **37**(8):1563-1571.
387. Zirakzadeh A, Patel R: **Vancomycin-resistant enterococci: colonization, infection, detection, and treatment**. In: *Mayo Clinic Proceedings: 2006*. Elsevier: 529-536.
388. Pompei A, Cordisco L, Amaretti A, Zanoni S, Matteuzzi D, Rossi M: **Folate Production by Bifidobacteria as a Potential Probiotic Property**. *Appl Environ Microbiol* 2007, **73**(1):179-185.
389. Gu Q, Li P: **Biosynthesis of vitamins by probiotic bacteria**. *Probiotics and prebiotics in human nutrition and health* 2016:135-148.
390. Dicks LM, Botes M: **Probiotic lactic acid bacteria in the gastro-intestinal tract: health benefits, safety and mode of action**. *Benef Microbes* 2010, **1**(1):11-29.
391. Ridlon JM, Wolf PG, Gaskins HR: **Taurocholic acid metabolism by gut microbes and colon cancer**. *Gut Microbes* 2016, **7**(3):201-215.
392. Ogawa M, García-Martínez T, Bisson L, Mauricio JC, Moreno J, Moreno-García J: **Mapping the intracellular metabolome of yeast biocapsules - Spherical structures of yeast attached to fungal pellets**. *New Biotechnology* 2020, **58**:55-60.
393. Olofsson M, Ferrer-González FX, Uchimiya M, Schreier JE, Holderman NR, Smith CB, Edison AS, Moran MA: **Growth-stage-related shifts in diatom endometabolome composition set the stage for bacterial heterotrophy**. *ISME Communications* 2022, **2**(1):28.
394. Zhang J, Luo W, Wang Z, Chen X, Lv P, Xu J: **A novel strategy for D-psicose and lipase co-production using a co-culture system of engineered Bacillus subtilis and Escherichia coli and bioprocess analysis using metabolomics**. *Bioresources and Bioprocessing* 2021, **8**(1):77.
395. Dahal S, Hurst GB, Chourey K, Engle NL, Burdick LH, Morrell-Falvey JL, Tschaplinski TJ, Doktycz MJ, Pelletier DA: **Mechanism for Utilization of**

**the Populus-Derived Metabolite Salicin by a Pseudomonas—Rahnella Co-Culture**. In: *Metabolites.* vol. 13; 2023.
396. Veyrat-Durebex C, Corcia P, Piver E, Devos D, Dangoumau A, Gouel F, Vourc'h P, Emond P, Laumonnier F, Nadal-Desbarats L *et al*: **Disruption of TCA Cycle and Glutamate Metabolism Identified by Metabolomics in an In Vitro Model of Amyotrophic Lateral Sclerosis**. *Molecular Neurobiology* 2016, **53**(10):6910-6924.
397. **Purification of total RNA from bacteria using the RNeasy® Mini Kit** [https://www.qiagen.com/us/resources/resourcedetail?id=60ec1159-4828-4f27-b4e6-b98772bdf7e1&lang=en]
398. Sayavedra L, Li T, Bueno Batista M, Seah BKB, Booth C, Zhai Q, Chen W, Narbad A: **Desulfovibrio diazotrophicus sp. nov., a sulfate-reducing bacterium from the human gut capable of nitrogen fixation**. *Environmental Microbiology* 2021, **23**(6):3164-3181.
399. Liao Y, Smyth GK, Shi W: **featureCounts: an efficient general purpose program for assigning sequence reads to genomic features**. *Bioinformatics* 2014, **30**(7):923-930.
400. Robinson MD, Oshlack A: **A scaling normalization method for differential expression analysis of RNA-seq data**. *Genome Biology* 2010, **11**(3):R25.
401. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q *et al*: **Full-length transcriptome assembly from RNA-Seq data without a reference genome**. *Nature biotechnology* 2011, **29**(7):644-652.
402. Venceslau SS, Stockdreher Y, Dahl C, Pereira IAC: **The "bacterial heterodisulfide" DsrC is a key protein in dissimilatory sulfur metabolism**. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 2014, **1837**(7):1148-1164.
403. Keon RG, Fu R, Voordouw G: **Deletion of two downstream genes alters expression of the hmc operon of Desulfovibrio vulgaris subsp. vulgaris Hildenborough**. *Archives of Microbiology* 1997, **167**(6):376-383.
404. Louis P, Duncan SH, Sheridan PO, Walker AW, Flint HJ: **Microbial lactate utilisation and the stability of the gut microbiome**. *Gut Microbiome* 2022, **3**:e3.
405. Parey K, Demmer U, Warkentin E, Wynen A, Ermler U, Dahl C: **Structural, biochemical and genetic characterization of dissimilatory ATP sulfurylase from Allochromatium vinosum**. *PLoS One* 2013, **8**(9):e74707.
406. Dostal Webster A, Staley C, Hamilton MJ, Huang M, Fryxell K, Erickson R, Kabage AJ, Sadowsky MJ, Khoruts A: **Influence of short-term changes in dietary sulfur on the relative abundances of intestinal sulfate-reducing bacteria**. *Gut Microbes* 2019, **10**(4):447-457.
407. Denger K, Laue H, Cook A: **Anaerobic taurine oxidation: a novel reaction by a nitrate-reducing Alcaligenes sp**. *Microbiology* 1997, **143**(6):1919-1924.
408. Beil S, Kehrli H, James P, Staudenmann W, Cook AM, Leisinger T, Kertesz MA: **Purification and Characterization of the Arylsulfatase Synthesized by Pseudomonas aeruginosa PAO During Growth in Sulfate-Free Medium and Cloning of the Arylsulfatase Gene (atsA)**. *European Journal of Biochemistry* 1995, **229**(2):385-394.
409. Kertesz MA: **Riding the sulfur cycle – metabolism of sulfonates and sulfate esters in Gram-negative bacteria**. *FEMS Microbiology Reviews* 2000, **24**(2):135-175.

410. Luis AS, Jin C, Pereira GV, Glowacki RWP, Gugel SR, Singh S, Byrne DP, Pudlo NA, London JA, Baslé A *et al*: **A single sulfatase is required to access colonic mucin by a gut bacterium**. *Nature* 2021, **598**(7880):332-337.
411. Silverman JM, Brunet YR, Cascales E, Mougous JD: **Structure and Regulation of the Type VI Secretion System**. *Annual Review of Microbiology* 2012, **66**(1):453-472.
412. Coulthurst S: **The Type VI secretion system: a versatile bacterial weapon**. *Microbiology* 2019, **165**(5):503-515.
413. Jiang F, Waterfield Nicholas R, Yang J, Yang G, Jin Q: **A Pseudomonas aeruginosa Type VI Secretion Phospholipase D Effector Targets Both Prokaryotic and Eukaryotic Cells**. *Cell Host & Microbe* 2014, **15**(5):600-610.
414. Zheng J, Shin OS, Cameron DE, Mekalanos JJ: **Quorum sensing and a global regulator TsrA control expression of type VI secretion and virulence in Vibrio cholerae**. *Proc Natl Acad Sci U S A* 2010, **107**(49):21128-21133.
415. DeShazer D: **A novel contact-independent T6SS that maintains redox homeostasis via Zn2+ and Mn2+ acquisition is conserved in the Burkholderia pseudomallei complex**. *Microbiological Research* 2019, **226**:48-54.
416. Lin J, Xu L, Yang J, Wang Z, Shen X: **Beyond dueling: roles of the type VI secretion system in microbiome modulation, pathogenesis and stress resistance**. *Stress Biology* 2021, **1**(1):11.
417. Izoré T, Job V, Dessen A: **Biogenesis, Regulation, and Targeting of the Type III Secretion System**. *Structure* 2011, **19**(5):603-612.
418. Ross Julia A, Plano Gregory V: **A C-Terminal Region of Yersinia pestis YscD Binds the Outer Membrane Secretin YscC**. *Journal of Bacteriology* 2011, **193**(9):2276-2289.
419. Nikolaus T, Deiwick J, Rappl C, Freeman Jeremy A, Schröder W, Miller Samuel I, Hensel M: **SseBCD Proteins Are Secreted by the Type III Secretion System of Salmonella Pathogenicity Island 2 and Function as a Translocon**. *Journal of Bacteriology* 2001, **183**(20):6036-6045.
420. Neyt C, Cornelis GR: **Role of SycD, the chaperone of the Yersinia Yop translocators YopB and YopD**. *Molecular Microbiology* 1999, **31**(1):143-156.
421. Bamyaci S, Ekestubbe S, Nordfelth R, Erttmann SF, Edgren T, Forsberg Å: **YopN Is Required for Efficient Effector Translocation and Virulence in Yersinia pseudotuberculosis**. *Infect Immun* 2018, **86**(8).
422. Charro N, Mota LJ: **Approaches targeting the type III secretion system to treat or prevent bacterial infections**. *Expert Opin Drug Discov* 2015, **10**(4):373-387.
423. Duncan Miles C, Linington Roger G, Auerbuch V: **Chemical Inhibitors of the Type Three Secretion System: Disarming Bacterial Pathogens**. *Antimicrob Agents Chemother* 2012, **56**(11):5433-5441.
424. Kim J, Ahn K, Min S, Jia J, Ha U, Wu D, Jin S: **Factors triggering type III secretion in Pseudomonas aeruginosa**. *Microbiology* 2005, **151**(11):3575-3587.
425. Jani AJ, Cotter PA: **Type VI Secretion: Not Just for Pathogenesis Anymore**. *Cell Host & Microbe* 2010, **8**(1):2-6.
426. Singh RP, Kumari K: **Bacterial type VI secretion system (T6SS): an evolved molecular weapon with diverse functionality**. *Biotechnology Letters* 2023, **45**(3):309-331.

427. Hotinger JA, Pendergrass HA, May AE: **Molecular Targets and Strategies for Inhibition of the Bacterial Type III Secretion System (T3SS); Inhibitors Directly Binding to T3SS Components**. In: *Biomolecules.* vol. 11; 2021.
428. Cox GM, Mukherjee J, Cole GT, Casadevall A, Perfect JR: **Urease as a virulence factor in experimental cryptococcosis**. *Infect Immun* 2000, **68**(2):443-448.
429. Konieczna I, Zarnowiec P, Kwinkowski M, Kolesinska B, Fraczyk J, Kaminski Z, Kaca W: **Bacterial urease and its role in long-lasting human diseases**. *Curr Protein Pept Sci* 2012, **13**(8):789-806.
430. Tkaczuk KL, A. Shumilin I, Chruszcz M, Evdokimova E, Savchenko A, Minor W: **Structural and functional insight into the universal stress protein family**. *Evolutionary Applications* 2013, **6**(3):434-449.
431. Goncheva MI, Chin D, Heinrichs DE: **Nucleotide biosynthesis: the base of bacterial pathogenesis**. *Trends in Microbiology* 2022, **30**(8):793-804.
432. Chen MM, Guo W, Chen SM, Guo XZ, Xu L, Ma XY, Wang YX, Xie C, Meng LH: **Xanthine dehydrogenase rewires metabolism and the survival of nutrient deprived lung adenocarcinoma cells by facilitating UPR and autophagic degradation**. *Int J Biol Sci* 2023, **19**(3):772-788.
433. Holden HM, Thoden JB, Raushel FM: **Carbamoyl phosphate synthetase: an amazing biochemical odyssey from substrate to product**. *Cellular and Molecular Life Sciences CMLS* 1999, **56**(5):507-522.
434. Martins A, Contreras-Martel C, Janet-Maitre M, Miyachiro MM, Estrozi LF, Trindade DM, Malospirito CC, Rodrigues-Costa F, Imbert L, Job V *et al*: **Self-association of MreC as a regulatory signal in bacterial cell wall elongation**. *Nature Communications* 2021, **12**(1):2987.
435. Wagstaff J, Löwe J: **Prokaryotic cytoskeletons: protein filaments organizing small cells**. *Nature Reviews Microbiology* 2018, **16**(4):187-201.
436. Patel Y, Zhao H, Helmann JD: **A regulatory pathway that selectively up-regulates elongasome function in the absence of class A PBPs**. *eLife* 2020, **9**:e57902.
437. Chan H, Mohamed AMT, Grainge I, Rodrigues CDA: **FtsK and SpoIIIE, coordinators of chromosome segregation and envelope remodeling in bacteria**. *Trends in Microbiology* 2022, **30**(5):480-494.
438. Strohmeier M, Raschle T, Mazurkiewicz J, Rippe K, Sinning I, Fitzpatrick TB, Tews I: **Structure of a bacterial pyridoxal 5′-phosphate synthase complex**. *Proceedings of the National Academy of Sciences* 2006, **103**(51):19284-19289.
439. Amadasi A, Bertoldi M, Contestabile R, Bettati S, Cellini B, Luigi di Salvo M, Borri-Voltattorni C, Bossa F, Mozzarelli A: **Pyridoxal 5-Phosphate Enzymes as Targets for Therapeutic Agents**. *Current Medicinal Chemistry* 2007, **14**(12):1291-1324.
440. Stach K, Stach W, Augoff K: **Vitamin B6 in Health and Disease**. In: *Nutrients.* vol. 13; 2021.
441. Kaiser JC, Heinrichs, David E. : **Branching Out: Alterations in Bacterial Physiology and Virulence Due to Branched-Chain Amino Acid Deprivation**. *mBio* 2018, **9**(5):10.1128/mbio.01188-01118.
442. Ribardo Deborah A, Hendrixson David R: **Analysis of the LIV System of Campylobacter jejuni Reveals Alternative Roles for LivJ and LivK in Commensalism beyond Branched-Chain Amino Acid Transport**. *Journal of Bacteriology* 2011, **193**(22):6233-6243.
443. Basavanna S, Khandavilli S, Yuste J, Cohen Jonathan M, Hosie Arthur HF, Webb Alexander J, Thomas Gavin H, Brown Jeremy S: **Screening of**

**Streptococcus pneumoniae ABC Transporter Mutants Demonstrates that LivJHMGF, a Branched-Chain Amino Acid ABC Transporter, Is Necessary for Disease Pathogenesis**. *Infect Immun* 2009, **77**(8):3412-3423.
444. Schoelmerich MC, Muller V: **Energy-converting hydrogenases: the link between H2 metabolism and energy conservation**. *Cell Mol Life Sci* 2020, **77**(8):1461-1481.
445. Benoni R, De Bei O, Paredi G, Hayes CS, Franko N, Mozzarelli A, Bettati S, Campanini B: **Modulation of Escherichia coli serine acetyltransferase catalytic activity in the cysteine synthase complex**. *FEBS Letters* 2017, **591**(9):1212-1224.
446. Guédon E, Martin-Verstraete I: **Cysteine metabolism and its regulation in bacteria**. In: *Amino acid biosynthesis~ pathways, regulation and metabolic engineering.* Springer; 2006: 195-218.
447. Kulikova VV, Revtovich SV, Bazhulina NP, Anufrieva NV, Kotlov MI, Koval VS, Morozova EA, Hayashi H, Belyi YF, Demidkina TV: **Identification of O-acetylhomoserine sulfhydrylase, a putative enzyme responsible for methionine biosynthesis in Clostridioides difficile: Gene cloning and biochemical characterizations**. *IUBMB Life* 2019, **71**(11):1815-1823.
448. Sonnenburg ED, Zheng H, Joglekar P, Higginbottom SK, Firbank SJ, Bolam DN, Sonnenburg JL: **Specificity of Polysaccharide Use in Intestinal Bacteroides Species Determines Diet-Induced Microbiota Alterations**. *Cell* 2010, **141**(7):1241-1252.
449. Panwar D, Shubhashini A, Kapoor M: **Complex alpha and beta mannan foraging by the human gut bacteria**. *Biotechnology Advances* 2023, **66**:108166.
450. Shipman Joseph A, Berleman James E, Salyers Abigail A: **Characterization of Four Outer Membrane Proteins Involved in Binding Starch to the Cell Surface of Bacteroides thetaiotaomicron**. *Journal of Bacteriology* 2000, **182**(19):5365-5372.
451. Cheng J, Hu J, Geng F, Nie S: **Bacteroides utilization for dietary polysaccharides and their beneficial effects on gut health**. *Food Science and Human Wellness* 2022, **11**(5):1101-1110.
452. Bodnar LM, Himes KP, Venkataramanan R, Chen J-Y, Evans RW, Meyer JL, Simhan HN: **Maternal serum folate species in early pregnancy and risk of preterm birth**. *The American Journal of Clinical Nutrition* 2010, **92**(4):864-871.
453. TeSlaa T, Ralser M, Fan J, Rabinowitz JD: **The pentose phosphate pathway in health and disease**. *Nature Metabolism* 2023, **5**(8):1275-1289.
454. Deckers-Hebestreit G, Altendorf K: **The F0F1 ATP Synthases of Bacteria: Structure and Function of the F0 Complex**. *Annual Review of Microbiology* 1996, **50**(1):791-824.
455. Vlasov AV, Osipov SD, Bondarev NA, Uversky VN, Borshchevskiy VI, Yanyushin MF, Manukhov IV, Rogachev AV, Vlasova AD, Ilyinsky NS *et al*: **ATP synthase FOF1 structure, function, and structure-based drug design**. *Cellular and Molecular Life Sciences* 2022, **79**(3):179.
456. Kühlbrandt W: **Structure and Mechanisms of F-Type ATP Synthases**. *Annual Review of Biochemistry* 2019, **88**(1):515-549.
457. Bosdriesz E, Molenaar D, Teusink B, Bruggeman FJ: **How fast-growing bacteria robustly tune their ribosome concentration to approximate growth-rate maximization**. *The FEBS Journal* 2015, **282**(10):2029-2044.
458. Herrou J, Willett JW, Czyż DM, Babnigg G, Kim Y, Crosson S: **Conserved ABC Transport System Regulated by the General Stress Response**

**Pathways of Alpha- and Gammaproteobacteria**. *J Bacteriol* 2017, **199**(5).
459. Kempf B, Bremer E: **OpuA, an Osmotically Regulated Binding Protein-dependent Transport System for the Osmoprotectant Glycine Betaine in Bacillus subtilis(∗)**. *Journal of Biological Chemistry* 1995, **270**(28):16701-16713.
460. Virolle C, Goldlust Kelly, Djermoun Sarah, Bigot Sarah, Lesterlin Christian: **Plasmid Transfer by Conjugation in Gram-Negative Bacteria From the Cellular to the Community Level**. In: *Genes.* vol. 11; 2020.
461. Costa TRD, Ilangovan A, Ukleja M, Redzej A, Santini JM, Smith TK, Egelman EH, Waksman G: **Structure of the Bacterial Sex F Pilus Reveals an Assembly of a Stoichiometric Protein-Phospholipid Complex**. *Cell* 2016, **166**(6):1436-1444.e1410.
462. Lawley TD, Klimke WA, Gubbins MJ, Frost LS: **F factor conjugation is a true type IV secretion system**. *FEMS Microbiology Letters* 2003, **224**(1):1-15.
463. Howard MT, Nelson WC, Matson SW: **Stepwise Assembly of a Relaxosome at the F Plasmid Origin of Transfer (*)**. *Journal of Biological Chemistry* 1995, **270**(47):28381-28386.
464. Koraimann G, Wagner MA: **Social behavior and decision making in bacterial conjugation**. *Frontiers in Cellular and Infection Microbiology* 2014, **4**.
465. Devanga Ragupathi NK, Muthuirulandi Sethuvel DP, Gajendran R, Anandan S, Walia K, Veeraraghavan B: **Horizontal Transfer of Antimicrobial Resistance Determinants Among Enteric Pathogens Through Bacterial Conjugation**. *Current Microbiology* 2019, **76**(6):666-672.
466. Soucy SM, Huang J, Gogarten JP: **Horizontal gene transfer: building the web of life**. *Nature Reviews Genetics* 2015, **16**(8):472-482.
467. Balabanova L, Averianova L, Marchenok M, Son O, Tekutyeva L: **Microbial and Genetic Resources for Cobalamin (Vitamin B12) Biosynthesis: From Ecosystems to Industrial Biotechnology**. In: *International Journal of Molecular Sciences.* vol. 22; 2021.
468. Degnan Patrick H, Barry Natasha A, Mok Kenny C, Taga Michiko E, Goodman Andrew L: **Human Gut Microbes Use Multiple Transporters to Distinguish Vitamin B12 Analogs and Compete in the Gut**. *Cell Host & Microbe* 2014, **15**(1):47-57.
469. Yagasaki M, Hashimoto S-i: **Synthesis and application of dipeptides; current status and perspectives**. *Applied Microbiology and Biotechnology* 2008, **81**(1):13-22.
470. Andrade RP, Oliveira DR, Lopes ACA, de Abreu LR, Duarte WF: **Survival of Kluyveromyces lactis and Torulaspora delbrueckii to simulated gastrointestinal conditions and their use as single and mixed inoculum for cheese production**. *Food Research International* 2019, **125**:108620.
471. Johnson EL, Heaver SL, Waters JL, Kim BI, Bretin A, Goodman AL, Gewirtz AT, Worgall TS, Ley RE: **Sphingolipids produced by gut bacteria enter host metabolic pathways impacting ceramide levels**. *Nature Communications* 2020, **11**(1):2471.
472. Li Z, Zhang W, Su L, Huang Z, Zhang W, Ma L, Sun J, Guo J, Wen F, Mei K *et al*: **Difference analysis of intestinal microbiota and metabolites in piglets of different breeds exposed to porcine epidemic diarrhea virus infection**. *Front Microbiol* 2022, **13**:990642.

473. Dalgliesh C: **Biological degradation of tryptophan**. *Quarterly Reviews, Chemical Society* 1951, **5**(3):227-244.
474. Kuc D, Zgrajka W, Parada-Turska J, Urbanik-Sypniewska T, Turski WA: **Micromolar concentration of kynurenic acid in rat small intestine**. *Amino Acids* 2008, **35**(2):503-505.
475. Turski MP, Turska M, Paluszkiewicz P, Parada-Turska J, Oxenkrug GF: **Kynurenic Acid in the Digestive System–-New Facts, New Challenges**. *International Journal of Tryptophan Research* 2013, **6**:IJTR.S12536.
476. Dassa E, Bouige P: **The ABC of ABCs: a phylogenetic and functional classification of ABC systems in living organisms**. *Research in Microbiology* 2001, **152**(3):211-229.
477. Tanaka KJ, Song S, Mason K, Pinkett HW: **Selective substrate uptake: The role of ATP-binding cassette (ABC) importers in pathogenesis**. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 2018, **1860**(4):868-877.
478. Bhat Javaid Y, Shastri Brahmanaspati G, Balaram H: **Kinetic and biochemical characterization of Plasmodium falciparum GMP synthetase**. *Biochemical Journal* 2007, **409**(1):263-273.
479. Wurm JP, Sung S, Kneuttinger AC, Hupfeld E, Sterner R, Wilmanns M, Sprangers R: **Molecular basis for the allosteric activation mechanism of the heterodimeric imidazole glycerol phosphate synthase complex**. *Nature Communications* 2021, **12**(1):2748.
480. Erfle JD, Sauer FD, Mahadevan S: **Effect of Ammonia Concentration on Activity of Enzymes of Ammonia Assimilation and on Synthesis of Amino Acids by Mixed Rumen Bacteria in Continuous Culture1**. *Journal of Dairy Science* 1977, **60**(7):1064-1072.
481. Bermingham A, Derrick JP: **The folic acid biosynthesis pathway in bacteria: evaluation of potential for antibacterial drug discovery**. *BioEssays* 2002, **24**(7):637-648.
482. Otaru N, Ye K, Mujezinovic D, Berchtold L, Constancias F, Cornejo FA, Krzystek A, de Wouters T, Braegger C, Lacroix C *et al*: **GABA Production by Human Intestinal Bacteroides spp.: Prevalence, Regulation, and Role in Acid Stress Tolerance**. *Frontiers in Microbiology* 2021, **12**.
483. Altaib H, Nakamura K, Abe M, Badr Y, Yanase E, Nomura I, Suzuki T: **Differences in the Concentration of the Fecal Neurotransmitters GABA and Glutamate Are Associated with Microbial Composition among Healthy Human Subjects**. *Microorganisms* 2021, **9**(2):378.
484. Richts B, Commichau FM: **Underground metabolism facilitates the evolution of novel pathways for vitamin B6 biosynthesis**. *Applied Microbiology and Biotechnology* 2021, **105**(6):2297-2305.
485. Ge F, Yagi T: **Topology of 4-Pyridoxic acid dehydrogenase in transformed Escherichia coli cells**. *The Journal of Biochemistry* 2010, **147**(2):291-295.
486. Martinussen J, Hammer K: **Cloning and characterization of upp, a gene encoding uracil phosphoribosyltransferase from Lactococcus lactis**. *J Bacteriol* 1994, **176**(21):6457-6463.
487. Tozzi MG, Camici M, Mascia L, Sgarrella F, Ipata PL: **Pentose phosphates in nucleoside interconversion and catabolism**. *The FEBS Journal* 2006, **273**(6):1089-1101.
488. Ueda S, Hirata T, Sakasegawa S-i: **Hypoxanthine-guanine phosphoribosyltransferase is activated via positive cooperativity between guanine and IMP**. *FEBS Letters* 2022, **596**(8):1072-1080.

489. Cho Kyu H, Cho D, Wang G-R, Salyers Abigail A: **New Regulatory Gene That Contributes to Control of Bacteroides thetaiotaomicron Starch Utilization Genes**. *Journal of Bacteriology* 2001, **183**(24):7198-7205.
490. Chaudet MM, Allen J-L, Rose DR: **Expression and purification of two Family GH31 α-glucosidases from Bacteroides thetaiotaomicron**. *Protein Expression and Purification* 2012, **86**(2):135-141.
491. Smith EA, Macfarlane GT: **Formation of Phenolic and Indolic Compounds by Anaerobic Bacteria in the Human Large Intestine**. *Microbial Ecology* 1997, **33**(3):180-188.
492. Claros MC, Schumacher U, Jacob M, Hunt Gerardo S, Kleinkauf N, Goldstein EJC, Finegold SM, Rodloff AC: **Characterization of Bilophila wadsworthia Isolates Using PCR Fingerprinting**. *Anaerobe* 1999, **5**(6):589-593.
493. Ahn YH, Oh SC, Zhou S, Kim TD: **Tryptophanyl-tRNA Synthetase as a Potential Therapeutic Target**. *Int J Mol Sci* 2021, **22**(9).
494. Sapico FL, Reeves D, Wexler HM, Duncan J, Wilson KH, Finegold SM: **Preliminary study using species-specific oligonucleotide probe for rRNA of Bilophila wadsworthia**. *J Clin Microbiol* 1994, **32**(10):2510-2513.
495. Bansal T, Alaniz RC, Wood TK, Jayaraman A: **The bacterial signal indole increases epithelial-cell tight-junction resistance and attenuates indicators of inflammation**. *Proceedings of the national academy of sciences* 2010, **107**(1):228-233.
496. Whitfield-Cargile CM, Cohen ND, Chapkin RS, Weeks BR, Davidson LA, Goldsby JS, Hunt CL, Steinmeyer SH, Menon R, Suchodolski JS: **The microbiota-derived metabolite indole decreases mucosal inflammation and injury in a murine model of NSAID enteropathy**. *Gut Microbes* 2016, **7**(3):246-261.
497. Levy M, Blacher E, Elinav E: **Microbiome, metabolites and host immunity**. *Current Opinion in Microbiology* 2017, **35**:8-15.
498. Stipanuk MH: **Metabolism of Sulfur-Containing Amino Acids: How the Body Copes with Excess Methionine, Cysteine, and Sulfide**. *The Journal of Nutrition* 2020, **150**:2494S-2505S.
499. Kumar A, Palfrey HA, Pathak R, Kadowitz PJ, Gettys TW, Murthy SN: **The metabolism and significance of homocysteine in nutrition and health**. *Nutrition & Metabolism* 2017, **14**(1):78.
500. Nader C, Walker D: **Metabolic fate of cysteine and methionine in rumen digesta**. *Applied microbiology* 1970, **20**(5):677-681.
501. Parmanand B, Kellingray L, Le Gall G, Basit A, Fairweather-Tait S, Narbad A: **A decrease in iron availability to human gut microbiome reduces the growth of potentially pathogenic gut bacteria; an *in vitro* colonic fermentation study**. *The Journal of Nutritional Biochemistry* 2019, **67**:20-27.
502. Cho S-H, Cho Y-J, Park J-H: **The human symbiont Bacteroides thetaiotaomicron promotes diet-induced obesity by regulating host lipid metabolism**. *Journal of Microbiology* 2022, **60**(1):118-127.
503. Chen S-w, Zhu J, Zuo S, Zhang J-l, Chen Z-y, Chen G-w, Wang X, Pan Y-s, Liu Y-c, Wang P-y: **Protective effect of hydrogen sulfide on TNF-α and IFN-γ-induced injury of intestinal epithelial barrier function in Caco-2 monolayers**. *Inflammation Research* 2015, **64**(10):789-797.
504. Che S-y, Yuan J-w, Zhang L, Ruan Z, Sun X-m, Lu H: **Puerarin prevents epithelial tight junction dysfunction induced by ethanol in Caco-2 cell model**. *Journal of Functional Foods* 2020, **73**:104079.

505. Garcia-Gonzalez N, Battista N, Prete R, Corsetti A: **Health-promoting role of Lactiplantibacillus plantarum isolated from fermented foods**. *Microorganisms* 2021, **9**(2):349.
506. MacFarlane GT, MacFarlane S, Gibson GR: **Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon**. *Microbial Ecology* 1998, **35**(2):180-187.
507. Deschamps C, Fournier E, Uriot O, Lajoie F, Verdier C, Comtet-Marre S, Thomas M, Kapel N, Cherbuy C, Alric M *et al*: **Comparative methods for fecal sample storage to preserve gut microbial structure and function in an in vitro model of the human colon**. *Applied Microbiology and Biotechnology* 2020, **104**(23):10233-10247.
508. Krause JL, Schaepe SS, Fritz-Wallace K, Engelmann B, Rolle-Kampczyk U, Kleinsteuber S, Schattenberg F, Liu Z, Mueller S, Jehmlich N *et al*: **Following the community development of SIHUMIx – a new intestinal in vitro model for bioreactor use**. *Gut Microbes* 2020, **11**(4):1116-1129.
509. Hohmann M, Felbinger C, Christoph N, Wachter H, Wiest J, Holzgrabe U: **Quantification of taurine in energy drinks using 1H NMR**. *Journal of Pharmaceutical and Biomedical Analysis* 2014, **93**:156-160.
510. Sved DW, Godsey JL, Ledyard SL, Mahoney AP, Stetson PL, Ho S, Myers NR, Resnis P, Renwick AG: **Absorption, tissue distribution, metabolism and elimination of taurine given orally to rats**. *Amino Acids* 2007, **32**(4):459-466.
511. Sender R, Fuchs S, Milo R: **Revised Estimates for the Number of Human and Bacteria Cells in the Body**. *PLoS Biol* 2016, **14**(8):e1002533.
512. Prinsen H, Schiebergen-Bronkhorst B, Roeleveld M, Jans J, de Sain-van der Velden M, Visser G, van Hasselt P, Verhoeven-Duif N: **Rapid quantification of underivatized amino acids in plasma by hydrophilic interaction liquid chromatography (HILIC) coupled with tandem mass-spectrometry**. *J Inherit Metab Dis* 2016, **39**(5):651-660.
513. Rathmann D, Rijntjes E, Lietzow J, Köhrle J: **Quantitative analysis of thyroid hormone metabolites in cell culture samples using LC-MS/MS**. *European thyroid journal* 2015, **4**(1_Suppl):51-58.
514. Strocchi A, Furne JK, Levitt MD: **A modification of the methylene blue method to measure bacterial sulfide production in feces**. *Journal of Microbiological Methods* 1992, **15**(2):75-82.
515. Oguntoyinbo FA, Narbad A: **Molecular characterization of lactic acid bacteria and in situ amylase expression during traditional fermentation of cereal foods**. *Food Microbiol* 2012, **31**(2):254-262.
516. Catalioto RM, Festa C, Triolo A, Altamura M, Maggi CA, Giuliani S: **Differential Effect of Ethanol and Hydrogen Peroxide on Barrier Function and Prostaglandin E2 Release in Differentiated Caco-2 Cells: Selective Prevention by Growth Factors**. *Journal of Pharmaceutical Sciences* 2009, **98**(2):713-727.
517. Bellos DA, Sharma D, McMullen MR, Wat J, Saikia P, de la Motte CA, Nagy LE: **Specifically Sized Hyaluronan (35 kDa) Prevents Ethanol-Induced Disruption of Epithelial Tight Junctions Through a layilin-Dependent Mechanism in Caco-2 Cells**. *Alcoholism: Clinical and Experimental Research* 2019, **43**(9):1848-1858.
518. Gaudioso G, Weil T, Marzorati G, Solovyev P, Bontempo L, Franciosi E, Bertoldi L, Pedrolli C, Tuohy KM, Fava F: **Microbial and metabolic characterization of organic artisanal sauerkraut fermentation and**

**study of gut health-promoting properties of sauerkraut brine**. *Frontiers in Microbiology* 2022, **13**:929738.
519. Ilett EE, Jørgensen M, Noguera-Julian M, Daugaard G, Murray DD, Helleberg M, Paredes R, Lundgren J, Sengeløv H, MacPherson C: **Gut microbiome comparability of fresh-frozen versus stabilized-frozen samples from hospitalized patients using 16S rRNA gene and shotgun metagenomic sequencing**. *Sci Rep* 2019, **9**(1):13351.
520. Ye Z, Zhang N, Wu C, Zhang X, Wang Q, Huang X, Du L, Cao Q, Tang J, Zhou C *et al*: **A metagenomic study of the gut microbiome in Behcet's disease**. *Microbiome* 2018, **6**(1):135.
521. Blanco-Míguez A, Beghini F, Cumbo F, McIver LJ, Thompson KN, Zolfo M, Manghi P, Dubois L, Huang KD, Thomas AM *et al*: **Extending and improving metagenomic taxonomic profiling with uncharacterized species using MetaPhlAn 4**. *Nature Biotechnology* 2023, **41**(11):1633-1644.
522. Wickham H: **ggplot2**. *Wiley interdisciplinary reviews: computational statistics* 2011, **3**(2):180-185.
523. Oksanen J, Blanchet F, Kindt R, Legendre P, Minchin P, O'hara R, Simpson G, Solymos P, Stevens M, Wagner H: **Package 'vegan'—Community ecology package. 2019**. *View Article* 2018.
524. Hothorn T, Hornik K, Van De Wiel MA, Zeileis A: **Implementing a class of permutation tests: the coin package**. *Journal of statistical software* 2008, **28**(8):1-23.
525. Hu Y-L, Pang W, Huang Y, Zhang Y, Zhang C-J: **The gastric microbiome is perturbed in advanced gastric adenocarcinoma identified through shotgun metagenomics**. *Frontiers in cellular and infection microbiology* 2018, **8**:433.
526. Chhonker YS, Kanvinde S, Ahmad R, Singh AB, Oupický D, Murry DJ: **Simultaneous Quantitation of Lipid Biomarkers for Inflammatory Bowel Disease Using LC–MS/MS**. In: *Metabolites.* vol. 11; 2021.
527. Ramsauer B, Sterz K, Hagedorn H-W, Engl J, Scherer G, McEwan M, Errington G, Shepperd J, Cheung F: **A liquid chromatography/tandem mass spectrometry (LC-MS/MS) method for the determination of phenolic polycyclic aromatic hydrocarbons (OH-PAH) in urine of non-smokers and smokers**. *Analytical and Bioanalytical Chemistry* 2011, **399**(2):877-889.
528. Sulyok M, Stadler D, Steiner D, Krska R: **Validation of an LC-MS/MS-based dilute-and-shoot approach for the quantification of > 500 mycotoxins and other secondary metabolites in food crops: challenges and solutions**. *Analytical and Bioanalytical Chemistry* 2020, **412**(11):2607-2620.
529. Gonzalez O, Alonso RM, Ferreirós N, Weinmann W, Zimmermann R, Dresen S: **Development of an LC–MS/MS method for the quantitation of 55 compounds prescribed in combined cardiovascular therapy**. *Journal of Chromatography B* 2011, **879**(3):243-252.
530. Van Eeckhaut A, Lanckmans K, Sarre S, Smolders I, Michotte Y: **Validation of bioanalytical LC–MS/MS assays: Evaluation of matrix effects**. *Journal of Chromatography B* 2009, **877**(23):2198-2207.
531. Steiner D, Krska R, Malachová A, Taschl I, Sulyok M: **Evaluation of Matrix Effects and Extraction Efficiencies of LC–MS/MS Methods as the Essential Part for Proper Validation of Multiclass Contaminants in Complex Feed**. *Journal of Agricultural and Food Chemistry* 2020, **68**(12):3868-3880.

532. Hughes MN, Centelles MN, Moore KP: **Making and working with hydrogen sulfide: The chemistry and generation of hydrogen sulfide in vitro and its measurement in vivo: A review**. *Free Radical Biology and Medicine* 2009, **47**(10):1346-1353.
533. Taylor CA: **Two new species of freshwater crayfish of the genus Faxonius (Decapoda: Cambaridae) from the Ozark Highlands of Arkansas and Missouri**. *Zootaxa* 2018, **4399**(4):491–520-491–520.
534. Albini D, Lester L, Sanders P, Hughes JMR, Jackson MC: **Early detection and environmental drivers of sewage fungus outbreaks in rivers**. *Ecological Solutions and Evidence* 2023, **4**(3):e12277.
535. Nearing Jacob T, DeClercq V, Van Limbergen J, Langille Morgan GI: **Assessing the Variation within the Oral Microbiome of Healthy Adults**. *mSphere* 2020, **5**(5):10.1128/msphere.00451-00420.
536. Kreth J, Giacaman RA, Raghavan R, Merritt J: **The road less traveled – defining molecular commensalism with Streptococcus sanguinis**. *Molecular Oral Microbiology* 2017, **32**(3):181-196.
537. Sheedy JR, Wettenhall RE, Scanlon D, Gooley PR, Lewis DP, Mcgregor N, Stapleton DI, Butt HL, De Meirleir KL: **Increased d-lactic acid intestinal bacteria in patients with chronic fatigue syndrome**. *in vivo* 2009, **23**(4):621-628.
538. Stone J: **A practical guide to sample preparation for liquid chromatography-tandem mass spectrometry in clinical research and toxicology**. *Spectrosc Eur* 2018, **30**:15-21.
539. Adibi S, Mercer, D.: **Protein Digestion in Human Intestine as Reflected in Luminal, Mucosal, and Plasma Amino Acid Concentrations after Meals**. *J Clin Invest* 1973, **52**(7):1586-1594.
540. Morgenstern I, Raijmakers M, Peters W, Hoensch H, Kirch W: **Homocysteine, Cysteine, and Glutathione in Human Colonic Mucosa: Elevated Levels of Homocysteine in Patients with Inflammatory Bowel Disease**. *Digestive Diseases and Sciences* 2003, **48**(10):2083-2090.
541. Guironnet A, Sanchez-Cid C, Vogel TM, Wiest L, Vulliet E: **Aminoglycosides analysis optimization using ion pairing liquid chromatography coupled to tandem mass spectrometry and application on wastewater samples**. *Journal of Chromatography A* 2021, **1651**:462133.
542. Kafle B, Baak J, Brede C: **Quantification by LC–MS/MS of astragaloside IV and isoflavones in Astragali radix can be more accurate by using standard addition**. *Phytochemical Analysis* 2021, **32**(4):466-473.
543. Zhao H, Yan R, Zhou X, Ji F, Zhang B: **Hydrogen sulfide improves colonic barrier integrity in DSS-induced inflammation in Caco-2 cells and mice**. *International Immunopharmacology* 2016, **39**:121-127.
544. Chen S, Bu D, Ma Y, Zhu J, Sun L, Zuo S, Ma J, Li T, Chen Z, Zheng Y *et al*: **GYY4137 ameliorates intestinal barrier injury in a mouse model of endotoxemia**. *Biochemical Pharmacology* 2016, **118**:59-67.
545. Sasaki N, Miyamoto K, Maslowski KM, Ohno H, Kanai T, Sato T: **Development of a Scalable Coculture System for Gut Anaerobes and Human Colon Epithelium**. *Gastroenterology* 2020, **159**(1):388-390 e385.
546. Umehara Y, Aoyagi H: **Development of a simple cultured cell–anaerobic microbial co-culture system using liquid paraffin**. *Journal of Bioscience and Bioengineering* 2023, **135**(6):487-492.
547. Sen A, Nishimura T, Yoshimoto S, Yoshida K, Gotoh A, Katoh T, Yoneda Y, Hashimoto T, Xiao J-Z, Katayama T: **Comprehensive analysis of metabolites produced by co-cultivation of Bifidobacterium breve**

**MCC1274 with human iPS-derived intestinal epithelial cells**. *Frontiers in Microbiology* 2023, **14**:1155438.
548. Kim S-H, Singh D, Kim S-A, Kwak MJ, Cho D, Kim J, Roh J-H, Kim W-G, Han NS, Lee CH: **Strain-specific metabolomic diversity of Lactiplantibacillus plantarum under aerobic and anaerobic conditions**. *Food Microbiology* 2023, **116**:104364.
549. Xiao K, Cao S, Jiao L, Song Z, Lu J, Hu C: **TGF-β1 protects intestinal integrity and influences Smads and MAPK signal pathways in IPEC-J2 after TNF-α challenge**. *Innate Immunity* 2017, **23**(3):276-284.
550. Fontana A, Panebianco C, Picchianti-Diamanti A, Laganà B, Cavalieri D, Potenza A, Pracella R, Binda E, Copetti M, Pazienza V: **Gut Microbiota Profiles Differ among Individuals Depending on Their Region of Origin: An Italian Pilot Study**. *International Journal of Environmental Research and Public Health* 2019, **16**(21):4065.
551. Radjabzadeh D, Boer CG, Beth SA, van der Wal P, Kiefte-De Jong JC, Jansen MAE, Konstantinov SR, Peppelenbosch MP, Hays JP, Jaddoe VWV *et al*: **Diversity, compositional and functional differences between gut microbiota of children and adults**. *Sci Rep* 2020, **10**(1):1040.
552. McNeil NI, Ling KL, Wager J: **Mucosal surface pH of the large intestine of the rat and of normal and inflamed large intestine in man**. *Gut* 1987, **28**(6):707.
553. Nugent SG, Kumar D, Rampton DS, Evans DF: **Intestinal luminal pH in inflammatory bowel disease: possible determinants and implications for therapy with aminosalicylates and other drugs**. *Gut* 2001, **48**(4):571-577.
554. Gasperskaja E, Kučinskas V: **The most common technologies and tools for functional genome analysis**. *Acta Med Litu* 2017, **24**(1):1-11.
555. Dowood RK, Adusumalli R, Tykesson E, Johnsen E, Lundanes E, Prydz K, Wilson SR: **Determination of 3′-phosphoadenosine-5′-phosphosulfate in cells and Golgi fractions using hydrophilic interaction liquid chromatography–mass spectrometry**. *Journal of Chromatography A* 2016, **1470**:70-75.
556. Jalili-Firoozinezhad S, Gazzaniga F, Calamari E, Camacho D, Fadel C, Bein A, Swenor B, Nestor B, Cronce M, Tovaglieri A *et al*: **A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip**. *Nature Biomedical Engineering* 2019, **3**(7):520-531.
557. McGrath CJ, Laveckis E, Bell A, Crost E, Juge N, Schüller S: **Development of a novel human intestinal model to elucidate the effect of anaerobic commensals on Escherichia coli infection**. *Dis Model Mech* 2022, **15**(4).
558. D'ambrosio S, Ventrone M, Alfano A, Schiraldi C, Cimini D: **Microbioreactor (micro-Matrix) potential in aerobic and anaerobic conditions with different industrially relevant microbial strains**. *Biotechnology Progress* 2021, **37**(5):e3184.
559. Yaakoubi K, Benkerroum N, Wiorowski F, Sanson F, Haydersah J, Chevallier I: **Development of a Multiwell Antagonistic Activity Assay for the Detection of Bacteriocin Production by Lactic Acid Bacteria**. *J Rapid Meth Aut Mic* 2009, **17**(1):32-45.
560. Lim E-S: **Inhibitory effect of bacteriocin-producing lactic acid bacteria against histamine-forming bacteria isolated from Myeolchi-jeot**. *Fisheries and Aquatic Sciences* 2016, **19**:1-10.
561. Anjum MF: **Screening methods for the detection of antimicrobial resistance genes present in bacterial isolates and the microbiota**. *Future microbiology* 2015, **10**(3):317-320.

562. Chen Y, Rudolph SE, Longo BN, Pace F, Roh TT, Condruti R, Gee M, Watnick PI, Kaplan DL: **Bioengineered 3D tissue model of intestine epithelium with oxygen gradients to sustain human gut microbiome**. *Advanced Healthcare Materials* 2022, **11**(16):2200447.
563. Lawrence GW, McCarthy N, Walsh CJ, Kunyoshi TM, Lawton EM, O'Connor PM, Begley M, Cotter PD, Guinane CM: **Effect of a bacteriocin-producing Streptococcus salivarius on the pathogen Fusobacterium nucleatum in a model of the human distal colon**. *Gut Microbes* 2022, **14**(1):2100203.

# Supplementary data

## 7. Appendix

## 7.1 Supplementary data

Supplementary data can be accessed at https://github.com/jade-j-davies/thesis-2023-supplementary-data.git:

### 7.1.1 Chapter 3

- Table of 358 metabolites identified via untargeted LC-MS in positive ion mode in the culture supernatant of *B. wadsworthia* mono-culture, Ef-Bt culture and Bw + Ef-Bt culture at 6 h post-inoculation.
- Graph showing the culture pH of *B. wadsworthia* mono-cultures and co-cultures with *B. thetaiotaomicron* strains, or cell-free supernatant of *B. thetaiotaomicron* strains.
- Graph showing the culture pH of *B. wadsworthia* mono-culture and co-culture with Ef-Bt enrichment.
- Genomes of *E. faecium* strains (FI 09347, FI 09198, QI0436), including genomic sequences (FASTA format) and annotated genomes (GenBank format).

### 7.1.2 Chapter 4

- Table of 319 metabolites identified via untargeted LC-MS in positive ion mode in the endometabolome of *B. wadsworthia* mono-culture, *B. thetaiotaomicron* mono-culture and co-culture at 8 h post-inoculation.
- Table of differentially expressed genes identified in *B. wadsworthia* (QI0013) in co-culture with *B. thetaiotaomicron* (QI0072) compared to mono-culture at 8 h post-inoculation.
- Table of differentially expressed genes identified in *B. thetaiotaomicron* (QI0072) in co-culture with *B. wadsworthia* (QI0013) compared to mono-culture at 8 h post-inoculation.

### 7.1.3 Chapter 5

- Table showing the microbiota composition in all samples obtained from *in vitro* complex gut microbiota model experiments where *B. wadsworthia* was supplemented alone (Bw), together with Bt strain 1 (Bw + Bt) or with the *E. faecium* and *B. thetaiotaomicron* enrichment (Bw + Ef-Bt).

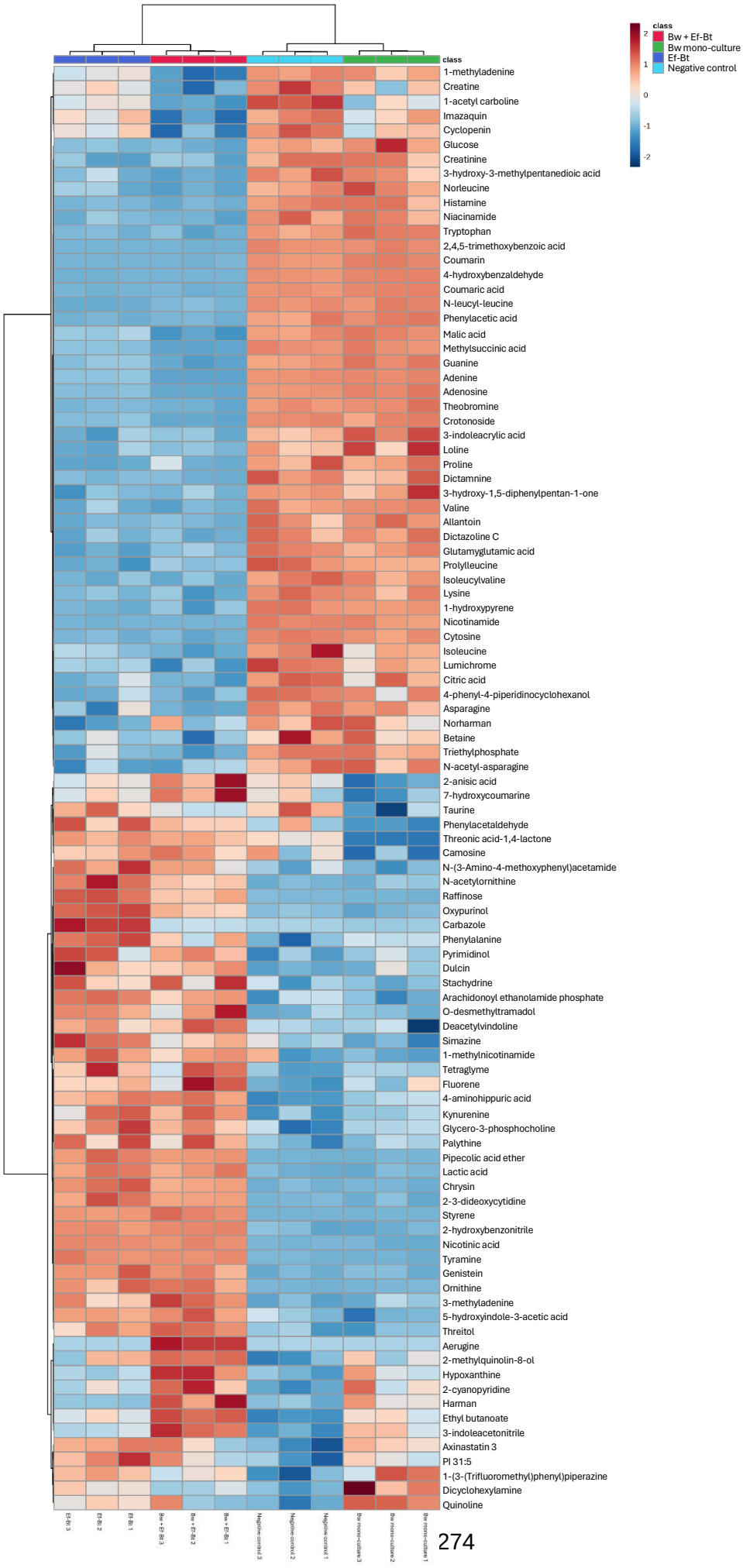

class
Bw + Ef-Bt
Bw mono-culture
Ef-Bt
Negative control
2
1
0
-1
-2
1-methyladenine
Creatine
1-acetyl carboline
Imazaquin
Cyclopenin
Glucose
Creatinine
3-hydroxy-3-methylpentanedioic acid
Norleucine
Histamine
Niacinamide
Tryptophan
2,4,5-trimethoxybenzoic acid
Coumarin
4-hydroxybenzaldehyde
Coumaric acid
N-leucyl-leucine
Phenylacetic acid
Malic acid
Methylsuccinic acid
Guanine
Adenine
Adenosine
Theobromine
Crotonoside
3-indoleacrylic acid
Loline
Proline
Dictamnine
3-hydroxy-1,5-diphenylpentan-1-one
Valine
Allantoin
Dictazoline C
Glutamylglutamic acid
Prolylleucine
Isoleucylvaline
Lysine
1-hydroxypyrene
Nicotinamide
Cytosine
Isoleucine
Lumichrome
Citric acid
4-phenyl-4-piperidinocyclohexanol
Asparagine
Norharman
Betaine
Triethylphosphate
N-acetyl-asparagine
2-anisic acid
7-hydroxycoumarine
Taurine
Phenylacetaldehyde
Threonic acid-1,4-lactone
Camosine
N-(3-Amino-4-methoxyphenyl)acetamide
N-acetylornithine
Raffinose
Oxypurinol
Carbazole
Phenylalanine
Pyrimidinol
Dulcin
Stachydrine
Arachidonoyl ethanolamide phosphate
O-desmethyltramadol
Deacetylvindoline
Simazine
1-methylnicotinamide
Tetraglyme
Fluorene
4-aminohippuric acid
Kynurenine
Glycero-3-phosphocholine
Palythine
Pipecolic acid ether
Lactic acid
Chrysin
2-3-dideoxycytidine
Styrene
2-hydroxybenzonitrile
Nicotinic acid
Tyramine
Genistein
Ornithine
3-methyladenine
5-hydroxyindole-3-acetic acid
Threitol
Aerugine
2-methylquinolin-8-ol
Hypoxanthine
2-cyanopyridine
Harman
Ethyl butanoate
3-indoleacetonitrile
Axinastatin 3
PI 31:5
1-(3-(Trifluoromethyl)phenyl)piperazine
Dicyclohexylamine
Quinoline
Ef-Bt 3
Ef-Bt 2
Ef-Bt 1
Bw + Ef-Bt 3
Bw + Ef-Bt 2
Bw + Ef-Bt 1
Negative control 3
Negative control 2
Negative control 1
Bw mono-culture 3
Bw mono-culture 2
Bw mono-culture 1

**Figure S1: Comparison of the cell-free supernatant metabolome of *B. wadsworthia* mono-culture, Ef-Bt enrichment culture, and Ef-Bt enrichment with *B. wadsworthia* co-culture at 6 h post-inoculation.** Heatmap displaying relative abundance of the top 100 differentially abundant metabolites in the culture conditions.

**Table S1**: Bt strain 1 (QI0072) expression data for sulfur-associated genes that were not differentially expressed between conditions. The table shows TMM-normalised gene expression data obtained via RNA-seq of mono-cultures and co-cultures.

| | | | **Bt strain 1 mono-culture** | | | | | **Bt strain 1 + *B. wadsworthia*** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene ID | Gene product | Pathway catalysed | Rep1 | Rep2 | Rep3 | Rep4 | Rep5 | Rep1 | Rep2 | Rep3 | Rep4 | Rep5 | Rep6 |
| QI0072_1554 | Sulfate adenylyltransferase (Sat) subunit 1 | Sulfate to APS | 7483.7 | 7580.9 | 12009.5 | 9950.2 | 15905.7 | 5844.0 | 7813.6 | 7476.7 | 6595.8 | 6644.5 | 4961.1 |
| QI0072_1555 | Sulfate adenylyltransferase (Sat) subunit 2 | Sulfate to APS | 4080.3 | 3775.9 | 6070.5 | 5608.8 | 8112.1 | 3249.9 | 4057.5 | 3917.8 | 3761.4 | 3442.8 | 2941.8 |
| QI0072_1556 | Adenylylsulfate kinase / APS kinase | APS to PAPS | 2180.8 | 1771.7 | 2815.9 | 2513.1 | 3720.2 | 1507.4 | 1973.8 | 1889.3 | 1700.3 | 1670.5 | 1354.5 |

**Table S2**: *B. wadsworthia* (QI0013) expression data for sulfur-associated genes that were not differentially expressed between conditions. The table shows TMM-normalised gene expression data obtained via RNA-seq of mono-cultures and co-cultures.

| | | | ***B. wadsworthia* mono-culture** | | | | | | ***B. wadsworthia* + Bt strain 1** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gene ID | Gene product | Pathway catalysed | Rep1 | Rep2 | Rep3 | Rep 4 | Rep 5 | Rep 6 | Rep 1 | Rep 2 | Rep 3 | Rep 4 | Rep 5 | Rep 6 |
| QI0013_309 | Adenylylsulfate reductase alpha subunit | Adenosine 5'-phosphate (APS) to sulfite | 69.6 | 69.0 | 133.3 | 121.0 | 105.3 | 101.7 | 53.4 | 67.1 | 75.0 | 45.6 | 63.4 | 30.8 |
| QI0013_310 | Adenylylsulfate reductase beta subunit | | 8.8 | 4.6 | 18.9 | 12.2 | 19.5 | 8.9 | 4.2 | 2.8 | 4.9 | 1.0 | 1.0 | 4.8 |
| QI0013_741 | Adenylylsulfate reductase alpha subunit | | 154.3 | 206.1 | 377.6 | 400.7 | 452.3 | 408.1 | 90.7 | 158.9 | 137.3 | 105.6 | 127.8 | 129.1 |
| QI0013_742 | Adenylylsulfate reductase beta subunit | | 2338.5 | 2603.1 | 3019.5 | 2890.6 | 2822.6 | 2484.0 | 2897.7 | 2326.0 | 2572.6 | 2231.9 | 3041.8 | 2239.0 |
| QI0013_194 | Phosphoadenosine phosphosulfate (PAPS) reductase | PAPS to sulfite | 4.4 | 12.9 | 43.3 | 36.6 | 44.7 | 31.8 | 9.3 | 26.5 | 9.7 | 15.2 | 20.2 | 11.6 |

**Table S3**: Amino acid sequences of AprA and AprB genes from different organisms and *B. wadsworthia* (QI0013).

| Organism | Gene product | Amino acid sequence |
|---|---|---|
| *Desulfovibrio gigas* DSM 1382 | Adenylylsulfate reductase subunit alpha (AprA) | MPKIPSKETPRGVAIAEPIIVEHSVDLLMVGGGMGNCGAAFEAVRWADKYAPEAKILLVDKASLE RSGAVAQGLSAINTYLGDNNADDYVRMVRTDLMGLVREDLIYDLGRHVDDSVHLFEEWGLPVW IKDEHGHNLDGAQAKAAGKSLRNGDKPVRSGRWQIMINGESYKVIVAEAAKNALGQDRIIERIFIV KLLLDKNTPNRIAGAVGFNLRANEVHIFKANAMVVACGGAVNVYRPRSVGEGMGRAWYPVWN AGSTYTMCAQVGAEMTMMENRFVPARFKDGYGPVGAWFLLFKAKATNCKGEDYCATNRAMLK PYEERGYAKGHVIPTCLRNHMMLREMREGRGPIYMDTKTALQTSFATMSPAQQKHLEAEAWED FLDMCVGQANLWAATNCAPEERGSEIMPTEPYLLGSHSGCCGIWASGPDEAWVPEDYKVRAA NGKVYNRMTTVEGLWTCADGVGASGHKFSSGSHAEGRIVGKQMVRWYLDHKDFKPEFVETAE ELKTLIYRPYYNYEKGKGASTCPVVNPEYISPKNFMMRLIKCTDEYGGGVGTYYNTSKALLDTGF WLMEMLEEDSLKLAARDLHELLRCWENYHRLWTVRLHMQHIAFREESRYPGFYYRADFLGLDD SKWKCFVNSKYDPAKKETKIFKKPYYQIIPTDA |
| | Adenylylsulfate reductase subunit beta (AprB) | MPTFVDPSKCDGCKGGEKTACMYICPNDLMILDPEEMKAFNQEPEACWECYSCIKICPQGAITA RPYADFAPMGGTCIPLRGSEDIMWTIKFRNGSVKRFKFPIRTTPEGSIKPFEGKPEAGDLENELLF TETALTVPQVALGQKAQIADAETSQCWFDLPCEGGNR |
| *Desulfovibrio alaskensis* G20 | Adenylylsulfate reductase subunit alpha (AprA) | MPMIPVKEQVKGVAIAEPTIKEHDVDILLVGGGMGACGTAFEAVRWADKYAPELKILLIDKAALER SGAVAQGLSAINTYLGDNSADDYVRMVRTDLMGLVREDLIFDLGRHVDDSVHLFEEWGLPCWIK DENGHNLDGAAAKAAGKSLRNGDAPVRSGRWQMMINGESYKNIVAEAAKNALGEDRIMERIFIV KLLLDANEENRIAGAVGFSLRENVVHIFRTNAMLVACGGAVNVYKPRSTGEGMGRAWYPVWNA GSTYTMCAQVGAEMTMMENRFVPARFKDGYGPVGAWFLLFKAKATNFRGEDYCVTNRAMLKP YEDRGYAKGHVIPTCLRNHMMLREMREGRGPIYMDTKTALQTTFETMTPAQQKHLESEAWEDF LDMCVGQANLWACMNIQPEEVGSEIMPTEPYLLGSHSGCCGIWTSGPDEEWVPEDYKVRAKN GKVYNRMTTVEGLWTCADGVGASGHKFSSGSHAEGRICGKQMVRWCIDHKDYKPAIAEKGED LAKLIYRPYYNYLAGKDASTDPVVNPEYITPKNFMMRLVKCTDEYGGGVGTYYTTSQAALDTGF HLLDMLEEDSLKLAARDLHELLRCWENYHRLWTVRLHMQHIAFREESRYPGFYYRADFMGLDD SKWKCFVNSRYDVKAGTTKVFKKPYYQIVPA |
| | Adenylylsulfate reductase subunit beta (AprB) | MYICPNDLMILDPEEMKAYNQEPEACWECYSCVKICPQGAITARPYADFAPMGGTSIPMRSADSI MWTVKFRNGNVKRFKFPIRTTPEGSIKPFEGKPEPGDLENELLFTETELGAPETALGQKFDVAEA DKAVEFKASAV |
| *Desulfovibrio desulfuricans* subsp. *desulfuricans* DSM 642 | Adenylylsulfate reductase subunit alpha (AprA) | MPMIPVKEATKGVAIAEPEVKEHAVDLLIVGGGMGSCGTAFEAVRWGDKHGLKIMLVDKATLER SGAVAQGLSAINTYLGENDADDYVRMVRTDLMGLVREDLIFDVGRHVDDSVHLFEDWGLPCWI KGEDGHNLNGAAAKAAGKSLRKGDAPVRSGRWQIMINGESYKCIVAEAAKNALGEDRIMERIFIV KLLLDKNTPNRIAGAVGFNLRANEVHIFKANTIMVAAGGAVNVYRPRSTGEGMGRAWYPVWNA GSTYTMCAQVGAEMTMMENRFVPARFKDGYGPVGAWFLLFKAKATNSKGEDYCATNRAMLKP YEDRGYAKGHVIPTCLRNHMMLREMREGRGPIYMDTKSALQNTFATLNEEQQKDLESEAWEDF LDMCVGQANLWACTNTAPEERGSEIMPTEPYLLGSHSGCCGIWVSGPDEAWVPEDYKVRAAN GKVYNRMTTVEGLFTCADGVGASGHKFSSGSHAEGRMAGKQMVRWCLDHKDFKPEFAETAE ELKKAVYRPFYNFEEGKAASTDPVVNPNYITPKNFMMRLVKCTDEYGGGVSTYYTTSKALLDTG FNLLAMMEEDSFKLAARDLHELLRCWENYHRLWTVRLHMQHISFREESRYPGFYYRADFMGLD DDAWKCFVNSKYNPATGETKIFKKPYYQIIPD |
| | Adenylylsulfate reductase subunit beta (AprB) | MPTFVDPSKCDGCKGGEKTACMYICPNDLMILDSEEMKAYNQEPDACWECYSCVKICPQGAIT ARPYADFAPMGGTCIPMRSADSIMWTVKFRNGNVKRFKFPIRTTPEGSIKPFEGHPEGANIEDEL LFTETALVAPKTALGKKFDVKDANKTFTCMEHGR |
| *Bilophila wadsworthia* (QI0013) | Adenylylsulfate reductase subunit alpha (AprA) QI0013_741 | MANIAARRMHPFHPKQLSDIEMQVVTCDLLIIGGGNAGCFVATEAAKLDPSLKVVIMEKAEIMRS GACSAGMDAINTYIPKGKTPEDLVRWSRAQVGGGPLREDLALSNAKELNESVEDLERWGLPILR DGDGNVRYRGKWDISIHGEQLKPIMAEKALESGADVYNRVAGTGLLMHGGRCVGAMGLGVRD GKFYVFRAKATVMATGGAGTLYKSYTADSTDSGAQIWMCPYCVGSGYAMGFRQGAELTSLEQ RWVATRTKDFCGPVDTISVGYGAPIVNAKGERVMSRYAALGGDAAPRYIRANAPMEEWLAGRG PCYCDTTHLTPETSKAMMEDYLNERPSFVLFLASRGQDVTKEPIEIYGSDPYILGGHTGGGFWV DMRRMTTVPGLFAAGETAGGNPNKFVGGCCAEGKLAARGAVAYMEGLDLPPLDEAQVKGEME RVYAPLLSRDEEGIRPVEMKERLQRLMDEYAGGISQFYRTNEERLDYALRHIAVLQSQFRYLRAT DSHELMQAMETIDRVDVAEAVVHHLKARKETRWAGWQTRSDYPERDDAHFDCFIESRRDPAT GEVSTFTRPYEQIIPGDRHTA |
| | Adenylylsulfate reductase subunit beta (AprB) QI0013_742 | MPPKVDTRKCTGCAGSAESCCERACPGDLMAVSPENGKAYCRATNECWDCMSCVKACPYGA LETRIPYQLGYYKATLRPIMGKDSITWKCRDIHGRESVYKYVNRLR |

**Table S4**: Summary of protein-protein comparison results using NCBI BLAST (blastp) to align *B. wadsworthia* (QI0013) AprA and AprB protein sequences to AprAB protein sequences from *D. desulfuricans* subsp. *desulfuricans* DSM 642, *D. gigas* DSM 1382 and *D. alaskensis* G20.

| Query sequence | Subject sequence | **% identity** | alignment length | mismatches | gap opens | q. start | q. end | s. start | s. end | evalue | bit score | % positives |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bilophila_AprA | desulfuricans_AprA | **31.193** | 654 | 357 | 19 | 17 | 584 | 15 | 661 | 6.49e-84 | 265 | 47.09 |
| Bilophila_AprA | gigas_AprA | **30.139** | 647 | 361 | 16 | 26 | 586 | 24 | 665 | 2.82e-81 | 258 | 45.75 |
| Bilophila_AprA | alaskensis_AprA | **30.435** | 644 | 355 | 19 | 28 | 584 | 26 | 663 | 1.20e-78 | 251 | 45.81 |
| Bilophila_AprB | desulfuricans_AprB | **38.095** | 104 | 65 | 0 | 1 | 105 | 1 | 105 | 1.14e-23 | 77.4 | 49.52 |
| Bilophila_AprB | gigas_AprB | **35.577** | 105 | 67 | 0 | 2 | 105 | 1 | 104 | 4.99e-23 | 75.9 | 49.04 |
| Bilophila_AprB | alaskensis_AprB | **38.272** | 81 | 50 | 0 | 25 | 105 | 4 | 84 | 3.75e-18 | 62.4 | 49.38 |

**Table S5**: Assignment of predicted functional protein domains using InterProScan of AprAB genes in *Desulfovibrio desulfuricans* subsp*. desulfuricans* DSM 642 and the putative AprAB genes in *B. wadsworthia* QI0013.

**Table S5**: Assignment of predicted functional protein domains using InterProScan of AprAB genes in *Desulfovibrio desulfuricans* subsp*. desulfuricans* DSM 642 and the putative AprAB genes in *B. wadsworthia* QI0013.

| | **(bp)** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ***Desulfovibrio desulfuricans subsp. desulfuricans* DSM 642** | AprA, 662 bp | NCBIfam | TIGR02061 | JCVI: adenylyl-sulfate reductase subunit alpha | 26 | 660 | IPR011803 | Adenylylsulphate reductase, alpha subunit |
| | | Pfam | PF00890 | FAD binding domain | 26 | 279 | IPR003953 | FAD-dependent oxidoreductase 2, FAD binding domain |
| | | SUPERFAMILY | SSF51905 | FAD/NAD(P)-binding domain | 15 | 512 | IPR036188 | FAD/NAD(P)-binding domain superfamily |
| | | Gene3D | G3DSA:3.90.700.10 | Succinate dehydrogenase/fumarate reductase flavoprotein, catalytic domain | 296 | 426 | IPR027477 | Succinate dehydrogenase/fumarate reductase flavoprotein, catalytic domain superfamily |
| | | Gene3D | G3DSA:3.50.50.60 | - | 22 | 660 | IPR036188 | FAD/NAD(P)-binding domain superfamily |
| | | SUPERFAMILY | SSF56425 | Succinate dehydrogenase/fumarate reductase flavoprotein, catalytic domain | 272 | 419 | IPR027477 | Succinate dehydrogenase/fumarate reductase flavoprotein, catalytic domain superfamily |
| | | PANTHER | PTHR11632 | SUCCINATE DEHYDROGENASE 2 FLAVOPROTEIN SUBUNIT | 14 | 655 | IPR030664 | FAD-dependent oxidoreductase SdhA/FrdA/AprA |
| | | SUPERFAMILY | SSF46977 | Succinate dehydrogenase/fumarate reductase flavoprotein C-terminal domain | 525 | 660 | IPR037099 | Fumarate reductase/succinate dehydrogenase flavoprotein-like, C-terminal domain superfamily |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | PIRSF | PIRSF000171 | SDHA_APRA_LASPO | 15 | 662 | - | - |
| ***Desulfovibrio desulfuricans subsp. desulfuricans* DSM 642** | AprB, 162 bp | PANTHER | PTHR43687 | ADENYLYLSULFATE REDUCTASE, BETA SUBUNIT | 4 | 110 | - | - |
| | | ProSiteProfiles | PS51379 | 4Fe-4S ferredoxin-type iron-sulfur binding domain profile. | 38 | 67 | IPR017896 | 4Fe-4S ferredoxin-type, iron-sulphur binding domain |
| | | Pfam | PF12139 | Adenosine-5'-phosphosulfate reductase beta subunit | 65 | 135 | IPR022738 | Adenylylsulphate reductase, beta subunit, C-terminal |
| | | Pfam | PF13187 | 4Fe-4S dicluster domain | 9 | 62 | IPR017896 | 4Fe-4S ferredoxin-type, iron-sulphur binding domain |
| | | ProSiteProfiles | PS51379 | 4Fe-4S ferredoxin-type iron-sulfur binding domain profile. | 1 | 35 | IPR017896 | 4Fe-4S ferredoxin-type, iron-sulphur binding domain |
| | | NCBIfam | TIGR02060 | JCVI: adenylyl-sulfate reductase subunit beta | 1 | 132 | IPR011802 | Adenylylsulphate reductase, beta subunit |
| | | Gene3D | G3DSA:3.30.70.20 | - | 1 | 67 | - | - |
| | | SUPERFAMILY | SSF54862 | 4Fe-4S ferredoxins | 3 | 125 | - | - |
| | | Gene3D | G3DSA:6.20.260.10 | - | 68 | 161 | IPR038465 | APS reductase, beta subunit, C-terminal domain superfamily |
| | | FunFam | G3DSA:3.30.70.20:FF:000038 | Adenylylsulfate reductase subunit beta | 1 | 67 | - | - |
| | | ProSitePatterns | PS00198 | 4Fe-4S ferredoxin-type iron-sulfur binding region signature. | 47 | 58 | IPR017900 | 4Fe-4S ferredoxin, iron-sulphur binding, conserved site |
| ***B. wadsworthia* QI0013** | AprA, 590 bp | PANTHER | PTHR11632 | SUCCINATE DEHYDROGENASE 2 FLAVOPROTEIN SUBUNIT | 21 | 580 | IPR030664 | FAD-dependent oxidoreductase SdhA/FrdA/AprA |
| | | PRINTS | PR00368 | FAD-dependent pyridine nucleotide reductase signature | 29 | 48 | - | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | PRINTS | PR00368 | FAD-dependent pyridine nucleotide reductase signature | 376 | 398 | - | - |
| | | Pfam | PF00890 | FAD binding domain | 28 | 400 | IPR003953 | FAD-dependent oxidoreductase 2, FAD binding domain |
| | | Gene3D | G3DSA:1.20.58.100 | - | 453 | 567 | - | - |
| | | Pfam | PF02910 | Fumarate reductase flavoprotein C-term | 463 | 572 | IPR015939 | Fumarate reductase/succinate dehydrogenase flavoprotein-like, C-terminal |
| | | SUPERFAMILY | SSF56425 | Succinate dehydrogenase/fumarate reductase flavoprotein, catalytic domain | 252 | 375 | IPR027477 | Succinate dehydrogenase/fumarate reductase flavoprotein, catalytic domain superfamily |
| | | SUPERFAMILY | SSF51905 | FAD/NAD(P)-binding domain | 21 | 431 | IPR036188 | FAD/NAD(P)-binding domain superfamily |
| | | PIRSF | PIRSF000171 | SDHA_APRA_LASPO | 17 | 588 | - | - |
| | | SUPERFAMILY | SSF46977 | Succinate dehydrogenase/fumarate reductase flavoprotein C-terminal domain | 452 | 583 | IPR037099 | Fumarate reductase/succinate dehydrogenase flavoprotein-like, C-terminal domain superfamily |
| | | Gene3D | G3DSA:3.90.700.10 | Succinate dehydrogenase/fumarate reductase flavoprotein, catalytic domain | 262 | 374 | IPR027477 | Succinate dehydrogenase/fumarate reductase flavoprotein, catalytic domain superfamily |
| | | Gene3D | G3DSA:3.50.50.60 | - | 26 | 571 | IPR036188 | FAD/NAD(P)-binding domain superfamily |
| ***B. wadsworthia* QI0013** | AprB, 107 bp | Pfam | PF00037 | 4Fe-4S binding domain | 45 | 63 | IPR017896 | 4Fe-4S ferredoxin-type, iron-sulphur binding domain |
| | | SUPERFAMILY | SSF54862 | 4Fe-4S ferredoxins | 3 | 67 | - | - |
| | | Gene3D | G3DSA:3.30.70.20 | - | 1 | 67 | - | - |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | ProSitePatterns | PS00198 | 4Fe-4S ferredoxin-type iron-sulfur binding region signature. | 47 | 58 | IPR017900 | 4Fe-4S ferredoxin, iron-sulphur binding, conserved site |
| | | ProSiteProfiles | PS51379 | 4Fe-4S ferredoxin-type iron-sulfur binding domain profile. | 1 | 35 | IPR017896 | 4Fe-4S ferredoxin-type, iron-sulphur binding domain |
| | | ProSiteProfiles | PS51379 | 4Fe-4S ferredoxin-type iron-sulfur binding domain profile. | 37 | 67 | IPR017896 | 4Fe-4S ferredoxin-type, iron-sulphur binding domain |