Food For Thought:

How Shared Metabolites Regulate Host Neurology and Alter Pathogenesis in the Human Gut

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Abstract

The human gut microbiome expands the host's metabolic functionality approximately 10-fold and subsequently contributes to host health by providing otherwise inaccessible nutrients. Many metabolic products derived from the activity of this diverse microbiome, along with host metabolism, are bioactive and contribute to functions beyond basic cellular maintenance. These bioactive compounds can regulate enteric pathogens through two-component system signaling and contribute to host neurological disorders like depression and schizophrenia through the gut-brain axis. The control of bioactive metabolites, whether through dietary alterations, microbiome manipulation, or some combination thereof, presents an intriguing and promising approach to not only controlling enteric infections but also as possible biomarkers or treatments for a range of human neurological disorders. Though promising, exogenous fine control of metabolism is difficult and the mechanistic interactions of metabolites with their microbial and human targets remains understudied due to the inherent complexity of metabolism and the notable inter- and intra-individual variation of the gut microbiome.

However challenging, disentangling the potential widespread effects of bioactive metabolites on host neurological health is imperative for the development of future therapeutics and biomarkers. In Chapter 2 we attempted to sort out some of this relationship between shared microbe-host metabolites and human neurological disorders through a deep dive into existing literature. Many metabolic products from tryptophan, an essential amino acids for humans and a central substrate for protein catabolism and energy production, have gained notoriety in this field for their potential role as markers or agents of common neurological disorders. Anthranilate, one intermediate product of the tryptophan-reliant kynurenine pathway, is particularly interesting given current literature supports that this compound may be neuroprotective or neurotoxic depending on context. Further investigation of this compound revealed that common neurological disorders like depression, Schizophrenia, and Alzheimer's may be

linked together by the activity of the anthranilic metabolic pathway. This search additionally exposed the significant role gut microorganisms play in altering the precarious balance of circulating tryptophan-derived metabolites, the variation of which can have serious implications for host health.

Given the clear importance of microbial metabolites to host neurological health, we hypothesized host-derived metabolites could likewise control the activity of microorganisms in the gut. Indeed, a thorough review on the effect of host-derived metabolic products on enteric microorganisms supported the concept of metabolite-driven bi-directional communication and interkingdom signaling. Two-component signaling systems, ubiquitous in prokaryotes, are one mechanism by which bacteria translate the host-produced extracellular small molecules into intracellular responses, such as the induction of virulence factors or repression of extraneous metabolic functions. A follow-up search of the Joint Genome Institute's Integrated Microbial Genomes and Microbiomes (IMG/M) platform revealed genes encoding two-component systems are omnipresent in the metagenomes of human gut microbes, lending support to the importance of better understanding how host-derived substrates influence activity in the gut microbiome for better or worse.

The strong body of literature supporting the integral role of metabolic substrates as contributors to host health and regulators of enteric microorganisms led us to propose the addition of exogenous dietary substrates *in vitro* would alter the metabolic crosstalk between enteric pathogen *Salmonella enterica* sv. Typhimurium and human colonic epithelial cells. *In vitro* work using colonic epithelial cells and *S.* Typhimurium treated with either human milk oligosaccharides (HMO) or mannanoligosaccharides (BioMos®), evaluated using dual RNAseq and untargeted metabolomics, exposed clear and substrate-dependent metabolic shifts in both host and pathogen. Intriguingly, both substrates reduced overall

host-pathogen association, but BioMos® treatment shifted *S.* Typhimurium towards more energetically favorable aerobic respiration while HMO did not induce this same shift, although both treatments did increase the expression of common virulence factors. Also notable was the induction of amino acid metabolism in BioMos® treated *S.* Typhimurium, a likely consequence of the inclusion of compounds beyond mannanoligosaccharides in this commercial prebiotic product. Although reduction in pathogenic association was decreased by both treatments, the distinct metabolic and gene expression profiles of host and pathogen in each condition suggests prebiotic supplements alter gut activity irrespective of the presence of commensal bacteria, their intended targets, and may actually induce virulent activity in dysbiotic gut environments, contrary to the goal of their application.

This work demonstrates the hypotheses that 1) tryptophan-derived metabolites from shared host-microbe metabolism can indicate and/or alter host neurology through circulation of small metabolites; 2) host metabolic products can likewise alter microbial function in the gastrointestinal tract, including but not limited to, regulating virulence factors via interactions with prokaryotic two-component systems; and 3) exogenous addition of dietary prebiotics shifts the shared metabolome of host epithelial cells and pathogenic *S.* Typhimurium, alongside simultaneous regulation of redox related metabolism in both cell types in a prebiotic-dependent manner. Taken together this work validates not only the importance of small metabolites in host health but also the potential for enteric pathogens to manipulate the tightly regulated metabolic relationship between host and gut microbes. Findings here additionally suggest that diet-driven manipulation of gut microbiota (for example the consumption of prebiotics) holds great promise as a next generation therapeutic approach, but widespread and off-target impacts remain a concern for widespread application at this stage in prebiotic research.

Dedication

To all the microscopic and macroscopic beings that made this work possible.

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Chapter 1

What's for dinner? An introduction to host-microbe metabolism and how *Salmonella* manipulates this relationship for pathogenic gain

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Abstract

The metabolism of both the human host and of microbial residents is imperative for growth and function, but these necessary cellular processes may also be fueling pathogenic activity and altering host health beyond the gastrointestinal walls. The shared metabolism between a host and the resident microorganisms helps create a mutualistic relationship in which the host provides dietary substrates, and the microorganisms contribute a breadth of metabolic potential otherwise unavailable to the host. Due to the extensive genetic richness and flexibility, the diverse microbiome membership of the large intestine can break down a wide array of substrates and thus provides essential vitamins and nutrients to the host. Beyond providing extended metabolic potential, the gut microbiota also shares numerous metabolic routes with the host. For example, enzymes needed to produce many tryptophan related metabolites are found in both humans and their associated microbes. Such shared pathways leave room for metabolic interactions but can also lead to competition for joint substrates. This substrate competition is where enteric pathogen *Salmonella enterica* thrives. *S. enterica* is a widespread foodborne pathogen with a potent ability to hijack the gut lumen environment for pathogenic gain. Though there are many mechanisms by which *S. enterica* may promote gut invasion, the control of metabolic substrates is one of the main routes through which this pathogen exerts extensive influence and ensures successful gut colonization. *S. enterica* uses both host and microbial metabolic products to fuel its invasive behaviors and possesses extensive genetic and metabolic breadth to support proliferation in both extra- and intracellular environments. Though a prevalent pathogen with severe clinical implications, the metabolic capabilities of *S. enterica* remain understudied regarding its contribution to infection, but especially in the context of diet and widely available dietary supplements that are nutrients to the microbiome as well as the host. Adding to the complexity of these interactions, but also underscoring the importance of understanding them, is the influence of metabolic substrates on host health at a broader scale. Besides pathogens like *S. enterica,* commensals also contribute to the

metabolome, which is the assortment of small molecules produced via collective host-microbe metabolism. Many of these metabolic products, like the aforementioned tryptophan-derived metabolites, are bioactive and exert control over immune function, inflammation, and cell signaling within the host organism, in addition to regulating microbial processes. Given the significant health ramifications of an altered metabolome and the potential of pathogens like *S. enterica* to trigger changes of the metabolome, a better understanding of the microbial metabolism and its contribution to host health is imperative for improved therapeutics and treatments in the future. This introductory section will cover the basics of shared host and microbial metabolism, with a particular focus on the contribution of metabolic pathways to host health and on how pathogens may influence these metabolic pathways for successful host colonization.

The Gut Microbiome: Microbes, Metabolism, and Host Modulation

Microbial cells account for approximately half of the total cell count in a human body, making us approximately half microbe, half human [1], but the microbiome has many more metabolic functions than humans – making the metabolic capacity of bacteria ~10-fold that of our metabolic capability [2]. The abundance of microbes in and on the human body underscores their significance in modulating host health, contributing to shared host-microbe metabolism, and influencing host physiology. Though there are microbial communities across the body, the most studied microbial population is found in the gastrointestinal tract [3]. This consortia of gastrointestinal microbes, termed the gut microbiome, is taxonomically and functionally diverse as well as highly variable between individuals [4, 5]. The microbiome is mot a static entity either, as gut microbiota diversity and function are notably affected by environmental factors like diet, seasonal shifts, and exposure to xenobiotics, and by other factors like exercise, disease, and age [6-9]. The gut microbiome, however variable between and within individuals, plays an integral role in host function through its invaluable contribution to host health and metabolism [10, 11].

The notable taxonomic diversity of the gut microbiome is surpassed by its genetic diversity and accompanying functional breadth [12, 13]. Many foundational microbiome studies made use of the conserved region of the 16S rRNA genes and DNA sequencing approaches to profile the microbial community and establish the taxonomic make-up and changes of any given sample [14-16]. This marked a significant methodological improvement from earlier culture-based studies, which had inherent limitations for taxa identification from novel environments such as the gut. The labeling of genera in the early days of sequencing-based gut microbiome research provided invaluable insights into the composition of microbes in hosts across a myriad of conditions (i.e., disease states, age, diet, location,

etc.) and allowed for the establishment of capstone gut microbiota knowledge, like commonly present taxa and the variable nature of the microbial community [16, 17]. Taxonomic breakdowns from 16S rRNA genes based studies suggest the guts of healthy adults are typically dominated by members of Bacteroidetes and Firmicutes [17]. Verrucomicrobia, Proteobacteria, and Actinobacteria are also usually found at lower relative abundance in the typical healthy gut microbiome [17]. Identification of microbial constituents through genetic identification has been a major breakthrough for our understanding of the microbial world, including within the gut, but assigning species names alone does not capture the underlying activity of the gut microbiome. Community taxonomy is important, but a thorough understanding of the role microbes play in host health and their metabolism, and their functional interactions, now requires a deeper look into the metabolic activity of the gut community. Sequencing technologies beyond amplicon-based approaches, like shotgun metagenomics and RNAseq (metatranscriptomics), allow for the investigation of not only taxa, but also of genetic potential and metabolic activity for entire microbial populations [18, 19]. Indeed, an increasing number of studies investigating microbial function in the gut are turning to these more revealing sequencing methods to uncover the mechanistic underpinnings of microbe-microbe and host-microbe relationships [20-23].

On a broad functional level, the gut microbiome supports the metabolism of three major macronutrients: carbohydrates, amino acids, and lipids [24]. As a widely studied and abundant microbial product by concentration, short-chain fatty acids (SCFAs), end products of microbial carbohydrate fermentation, are often cited as an important microbiome metabolite for host regulation and health [25]. Three main SCFAs, butyrate, acetate, and propionate, are important sources of energy in the colon and regulate T-cell generation [26]. The gut microbiota possesses an abundance of genes encoding carbohydrate-active enzymes (CAZymes), which are responsible for the degradation, modification and synthesis of oligosaccharides and polysaccharides that commonly result in SCFA [27].

SCFAs collectively regulate glucose metabolism in the host through control of plasma glucose, modulation of related gene expression, and by altering host hormone production [25]. One specific SCFA, butyrate, is important for epithelial proliferation and improves gut barrier function by promoting the expression of tight junction proteins, indicating microbial metabolism in some cases exerts direct control over host health and activity directly in the gut but also in deeper tissues and across the body compartments [28]. .

Like carbohydrate metabolism, amino acid metabolism is a crucial function of the gut microbiota, and the byproducts are important contributors to the host's physiology [27]. Protein degradation in the small intestine, where proteolysis is primarily driven by host enzymes, results in the release of free amino acids. Unabsorbed amino acids and peptides from the small intestine then make their way to the colon for microbial degradation [24]. Protein degradation in the colon by the gut microbiota provides the host with essential free amino acids, aromatic amino acid, and amino acid-derived branched-chain fatty acids, which can then fuel colonocytes in addition to providing bacterial modulation [27]. Amino acid metabolism by the gut microbiota can also yield bioactive metabolites, small molecules able to modulate host and microbe physiology far beyond the gut lumen. Tryptophan, an essential aromatic amino acid, is metabolized in the microbiome via the kynurenine, serotonin, or indole pathways, all of which contain bioactive metabolic products. There is an abundance of literature suggesting products from these pathways, like anthranilate, kynurenic acid, and quinolinic acid, can exacerbate, modulate, or cause neurological diseases in mammals [29-34]. Together this information suggests diet and the gut microbiota together exert remarkable influence on host physiology. Chapter 2 of this dissertation covers in depth the host and microbial production of these neuroactive metabolites and their potential involvement in common neurological disorders.

Lipids, the third major macronutrient, are less well studied in the context of microbiome metabolism as compared to carbohydrates and amino acids, as lipids do not reach the gut microbiota in as great of concentrations as the other two macronutrients [35]. Despite being in lower abundance, lipid access and digestion in the gut microbiome may still influence overall taxonomic composition and functionality [36]. Exploratory studies also suggest the modification of the microbial diversity and function depends heavily on the lipid source (i.e.; milk fat vs fish oil vs palm oil) and even closely related fat sources produce divergent results, both in terms of microbial shifts and host health outcomes [35]. Mouse model studies have also shown the gut microbiome can significantly impact host lipid metabolism through the production of intermediary products [35]. While microbial lipid metabolism may modulate host metabolism and health, there are conflicting studies in the literature, and it is generally accepted that gut microbes in the large intestine access relatively low concentrations of lipids from host digestion.

The gut microbiome greatly expands the metabolic capabilities of the host and contributes essential dietary components like amino acids and vitamins to this synergetic relationship. Microbial diversity and function in the gastrointestinal tract vary across individuals and change with external factors like diet and age. Nevertheless, these microbes are indispensable to host metabolism and represent important host health regulators, both through direct contact with gut-associated lymphoid tissue (GALT) and more indirectly through the production of circulating bioactive metabolites. To better understand how the gut microbiome contributes to host health and how the host modulates the microbiota, it is important to first examine the environment of these microbes, the gastrointestinal tract.

A Breakdown of the Gastrointestinal Tract

The colonic microbiome detailed in the above section is the most well-studied of the gastrointestinal tract microbiome, likely due to the abundance of microbes and the easy access to study material, but the human gastrointestinal tract extends beyond just the colon. The longitudinal transit of dietary substrates through this multi-compartment tract and substrate interactions with host enzymes dictates what food sources microbes encounter. Food enters the digestive system through the mouth, where chewing mechanically disturbs the food and saliva enzymes, such as α-amylase and triacylglycerol lipase, work to further breakdown starches and lipids, respectively [37]. The mouth also has an accompanying oral microbiome, the diversity of which has been shown to shift with diet, age, and disease states, much like the colonic microbiome [38].

The food bolus moves from the mouth, through the esophagus and into the stomach. There has been some debate as to whether the stomach has its own distinct microbiome, or whether the microbes found in gastric samples are remnants of salivary transit and not resident microbes [39]. Increasing evidence supports the existence of a gastric microbiome [40], and dominant gastric phyla include *Prevotella, Streptococcus, Veillonella, Rothia* and *Haemophilus* [39]. The stomach, with its low pH (between pH 1-3) and mechanical mixing, represents a harsh environment primed for the breakdown of proteins into easily digestible intermediates that result in bioactive metabolites prior to reaching the upper digestive tract. Pepsin in the stomach works in concert with the peristaltic actions and low pH to hydrolyze proteins into peptides and amino acids, ready for further digestion and absorption in the small intestine [37]. The bolus that entered the stomach is now an acidic pulpy mixture called chyme that transits through the pyloric opening to the small intestine, where a new ecological niche provides

numerous new selective pressures for microbes that alter the community and metabolic capabilities that enable changes in microbial relative abundance to that of the stomach.

The small intestine, despite its name, is at 6-7 meters the longest section of the gastrointestinal tract [37]. The small intestine is further broken down into three parts, the duodenum, jejunum, and ileum, each with a distinct role in digestion and absorption. In the duodenum, leftover gastric juices and new pancreatic secretions, along with liver-produced bile, mix together to process all three major macronutrients. Starches are broken down through pancreatic amylase, dextrinase, sucrase, maltase, lactase, and amyloglucosidase, and lipids are digested by pancreatic lipases and phospholipase [37]. Proteins, which underwent their first round of digestion in the stomach, are further broken down by trypsin, chymotrypsin, carboxypeptidase, and elastase [37]. The folded structure of the small intestine results in an increased absorption area, which facilitates the uptake of monosaccharides, amino acids, and short chain fatty acids into host circulation for systemic use [37]. The single layer of epithelial cells with microvilli that make up the brush border of the small intestine are the site of terminal host carbohydrate digestion and responsible for the absorption activity in the small intestine [41]. This absorption primarily happens in the jejunum, but some other substances like bile salts, are absorbed in the ileum [37]. Though less well studied than that of the colon, the small intestine does contain an active microbiome. Similar to the gastric microbiome, *Streptococcus* and *Veillonella* are two commonly found phyla in the ileum [42] and *Lactobacillus, Bacteroides,* and *Clostridiales* are also dominant [42]. The microbiota of the small intestine contributes primarily to carbohydrate and lipid digestion and are producers of key nutrients, like vitamin K [42]. Small intestine digestion and absorption depends on host mechanical and enzymatic actions, as well as microbial metabolism, creating a typically cooperative relationship, a relationship that is even more well observed in the subsequent large intestine.

The large intestine, the last part of the tract before excretion, is the site of a diverse and rich microbial community that typically works in conjunction with the previous host digesta to breakdown any remaining substrates that transited through and to provide indispensable vitamins and nutrients. On the host side, the large intestine does not facilitate further host-driven digestion and instead is focused primarily on the absorption of water and electrolytes from remaining indigestible material before removal from the body [37]. The large intestine is comprised of the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. Much like the small intestine, there is a single layer of cells (i.e., enterocytes, goblet cells, enteroendocrine cells, and intestinal epithelial stem cells) that make up the intestinal barrier, though the colon has a much thicker and stratified apical mucosal layer [41]. On the basal side of this epithelial membrane GALT, innervated tissues, stem cells and vasculature can be found, ultimately granting direct access to the host immune system, nervous system, and cardiovascular system from the large intestine [43-45].

The strong relationship between the gastrointestinal tract, dietary substrates, and gut microbiota is a notable contributor to holistic host health. The three-way cross talk between these entities (the host, the microbiota, and the diet) drives systemic metabolism, immune function, and disease states [46]. This tri-directional cross talk is increasingly studied across different contexts (e.g., diet changes, disease influences, environmental factors) utilizing different approaches (e.g., *in vivo, in vitro,* and *in silico*). These studies have already revealed the outsized role of the metabolome as a driver of host neurology (Chapter 2) and as signals for the control of microbial activity in the gut (Chapter 3). The control and manipulation of tri-directional crosstalk is also of great interest in the quest for advanced therapeutics, where dietary substrates are being tested for their health benefits and potential regulation of enteric

infections (Chapter 4). While there is an abundance of literature on host-microbe-diet interactions, the complexity of this relationship and intra-host variation means there is still more to explore.

The Metabolome Exerts Bidirectional Control

One product of the host-microbe relationship discussed above are small metabolites, which grouped together make up the metabolome. These circulating small molecules are effectors of host neurology and modulate bacterial activity. Metabolic products from digestion can act as ligands for host receptors on enterocytes, as seen with tryptophan metabolites and aryl hydrocarbon receptors (AHR) [47], or as signaling molecules for microbes via their two-component systems (TCS), like the CpxA/CpxR TCS and serotonin [48] (covered in Chapter 2 and Chapter 3, respectively). Though they may exert localized control through interactions with gut microbes and cells, many metabolic products from the gut, regardless of their origin (i.e., host vs microbial), can end up in systemic circulation via absorption from the apical side to the basolateral side of the gut and into the surrounding capillaries [49]. Through systemic circulation these metabolites gain access to a litany of host organs, including the brain [49, 50].

The connection between the gut and host neurological components, from various nerve cells to the brain, is termed the 'gut-brain axis'. Though it has long been known that the brain exerts some control over digestion, recent research has supported the idea that the gut also exercises control over neurological activity [51]. The gastrointestinal tract does have a direct connection to host neurological circuitry through the vagal afferent projections in the basolateral side of the gut epithelium and through the specialized enterochromaffin, enteroendocrine, and neuropod cells embedded in the gut epithelium [52]. Though some of this bidirectional control is from direct cell-cell signaling between the two body locales, there is data to suggest small circulating metabolites play a regulating role as well [53-55].

There are many metabolic products derived from digestion that lead a double life as bioactive molecules, able to influence host health through interactions with systemic organs. One notable set of these metabolites and a prime example of this phenomenon are tryptophan-derived products, like anthranilate, kynurenine, picolinic acid, quinolinic acid [53]. Tryptophan metabolites are particularly interesting from the perspective of their role in the gut-brain axis, since many products are produced by both the host and microbe and because tryptophan is an essential amino acid positioned at the center of multiple important metabolic pathways [56]. The gut microbiome collectively contains the genetic capability to perform all major tryptophan pathways and so contributes to the host digestion of this aromatic amino acid [56-61], ultimately driving the balance of metabolic products in ways both useful and detrimental to the host.

One of the three major tryptophan pathways is towards serotonin and melatonin production, both well-known neurotransmitters [56]. The role of the gut microbiome in serotonin is not understood at a detailed level, but studies in germ-free mouse models suggest the microbiome is a key driver of serotonin production [62]. About 90% of serotonin is produced in the gut and dysbiotic microbiota appear to reduce the neurotransmitter's concentration, subsequently reducing the availability of circulating serotonin for host regulation [63]. The ability of the gut microbiota to regulate the production of a neurotransmitter through digestive activity and through indirect regulation of host enterochromaffin cells certainly supports the concept of the microbiome as a regulator of host health and disease. Further work on the tryptophan metabolite kynurenine has also lent credence to the role of microbiome metabolism as host regulator. Kynurenine is the primary route for tryptophan digestion with the terminal product of NAD^+, though there are many other intermediate compounds known to be

neuroactive [31, 64-66]. Both host and microbes utilize the kynurenine pathway and its intermediates, making this yet another shared metabolic pathway that requires delicate balance between host and microbial requirements. Kynurenine metabolites have been implicated in neurological diseases such as bipolar disorder, depression, schizophrenia, and Alzheimer's [34, 57, 64, 66-68]. The ability of some metabolites, like those mentioned above, to regulate host neurological health highlights their foundational importance as bioactive molecules. The capacity of metabolites to exert such outsized influence over host health shifts the paradigm of disease etiology inwards towards the microbiome. Future research may point to the gut microbiome as the root cause of disease or may alternatively find the microbiome to be a useful tool for the amelioration of disease. More than likely, whether the gut microbiome is a disease-causing entity or therapeutic weapon will depend on the disease itself.

Metabolites are intersectional products for the host and its gut microbiome. Both host and microbe can utilize these small molecules as indicators of environmental conditions, as signaling molecules to regulate transcription and most simply as cellular fuel. As central regulators for a myriad of host and microbiome functions, it is important to understand in a more mechanistic way how these small molecules, derived from digestion of the host's diet, contribute to host health and microbial activity. There is great therapeutic potential in metabolic control, but there are still expansive knowledge gaps in both how to tightly regulate shared metabolic pathways and more notably, questions around which pathways we should be regulating. Given this is such a promising area of research, there has already been some attempts at shifting this finely honed metabolic balance through the addition of exogenous dietary components, like prebiotics.

Adding Substrates: Prebiotics and Dietary Supplements

Diet has a marked effect on both the host and microbiome, as illustrated by the many studies looking at microbial shifts and host health in response to dietary alterations [69-71]. As noted above the metabolic products from combined microbial and host digestion of dietary substrates can exert inordinate influence on systemic host health, including neurological function. Considering the outsized role of the microbiome in host health and the ability of dietary products to shift the gut microbiome community and activity, it is not much of a stretch to suggest modulation of the microbiome through dietary supplements for improved health. Indeed, many companies have capitalized on the popularity of the gut microbiome in today's everyday culture and are marketing supplements to the public as microbiome modulators. Dietary supplements are clearly a growing field, as the global market for dietary supplements in 2022 was $155 billion and is projected to grow to over $200 billion by 2027 [72], indicating research on the potential unexpected consequences of these supplements on host health and the microbiota is urgently needed.

Prebiotics are a class of dietary supplements noted to be substrates that produce beneficial outcomes for host health presumably through modulation of the gut microbiome [73]. The term prebiotic as currently defined encompasses a range of substrates including polyunsaturated fatty acids, oligosaccharides (mannan- (MOS), fructo- (FOS), galacto-(GOS), human milk oligosaccharides, phenolics and phytochemicals, and fermentable dietary fiber [73]. These diverse substrates, connected by their suggested health benefit, present a gray area for regulation and research. The U.S. Food and Drug Administration (FDA) released guidance in 2006 declaring prebiotics "biologically based practices" which are to be regulated according to their intended use [74], suggesting any of these substrates could be regulated as a dietary supplement, drug, or food depending on marketing intention. Many prebiotics

found on marketplace shelves gain the food-related designation of “generally recognized as safe” (GRAS) from the FDA, which means there is currently little to no scientific oversight evaluating the claims around efficacy [75]. Considered together the lax regulation structure around prebiotics coupled with the marketing claims of supplement companies, indicates the need for a greater reliance on rigorous scientific studies prior to applying prebiotics in a clinical manner, whether for the attenuation of chronic gastrointestinal issues or acute enteric infections.

The notable public and academic interest in dietary supplements and their potential health benefits has resulted in many studies looking at potential therapeutic applications for these products. The findings appear specific to host and disease status and the research around prebiotics is conflicting. Some studies show positive outcomes after prebiotic addition, some indicate neutral results, and a select few state there may be some potential harm [76-78]. One of the more common diseases-prebiotic combination for study is irritable bowel disease (IBD) and its related disorders, Chron’s, ulcerative colitis (UC), and irritable bowel syndrome (IBS) [78, 79]. IBD is marked by chronic inflammation of the gastrointestinal tract with an unknown etiology. The unknowns around the cause of IBD makes its treatment more about symptom management and therefore places IBD as a prime candidate for prebiotic interventions. Studies using rodent models of IBD have tested various prebiotics including but not limited to inulin, oligofructose, pectic polysaccharides, and rice fiber, all resulting in the successful reduction of symptoms [80-82]. As is typically the case with prebiotic studies, the primary mode of action is suggested to be prebiotic fermentation by commensal microbes, resulting in the production of SCFAs, increased colonization resistance against inflammation causing microbes, and inflammation modulation through immune system interactions in the gut. Select prebiotics have been tested for their efficacy against IBD symptoms in human clinical trials [78]. One systematic review looking at 39 trials for prebiotics, probiotics, and synbiotics (a combination of pre- and probiotics) found mixed results

overall, a stark contrast to the findings in rodent studies [78]. One study looking at FOS for IBD related disorders found no statistically significant benefit, though the results still tended toward a possible reduction in symptoms [83]. A different clinical trial investigating the potential effects of lactulose on IBD symptoms found that this prebiotic addition not only failed to improve patient status compared to the placebo group, but lactulose addition may have increased intestinal permeability [84]. The contrasting findings for prebiotics in the treatment of IBD supports that positive effects of prebiotics are incredibly specific to host and disease. Such mixed study results suggests that more standardized research is needed, starting from *in vitro* studies with reduced models and moving towards rigorous and extensive clinical trials. The abundance of literature supporting the ameliorative properties of prebiotics suggests that they are not a dead end therapeutic but does mean there is a need for more thorough research to capture their effects in the broad range of host-disease contexts.

Some studies suggest that prebiotics may be useful for alleviating symptoms associated with metabolic disorders like obesity [85]. One study in rats showed that a combination of inulin and oligofructose decreased glucagon levels and increased probiotic abundance in diabetic rat [86]. Another study also looking at oligofructose but in diabetic mice showed the prebiotic attenuated chronic inflammation [87]. It's interesting to note that both studies suggested the positive outcomes from prebiotic treatment were due to the increased abundance of bacterial groups thought to be beneficial to gut health, like *Bacteroidetes*, and not from a direct effect on the host gut barrier cells. Some studies suggest that certain prebiotics may also exert direct effects on the host colonic epithelial cells and not just on the microbiota. The addition of HMOs and MOS to differentiated colonic epithelial cells *in vitro* showed interaction with both prebiotics primed the cells for an immune response to enteric pathogen *Listeria monocytogenes* through glycosylation remodeling on the cell surface [88]. Much of the research around prebiotic application across various disease states suggests the beneficial health outcomes are primarily

derived from microbial fermentation, the subsequent metabolites, and promotion of commensal bacteria in the gut. There is some research, however, like the aforementioned study with HMO and MOS that indicate prebiotic supplementation may modulate host cell activity as well through prebiotic-cell surface interactions [88]. This implies off-target positive, as well as potentially negative, effects are possible depending on prebiotic and host context.

Off-target effects are certainly something that need to be further explored in the field. Reasonably, many studies employ pure oligosaccharide or fiber products to evaluate the health effects both *in vivo* and *in vitro* to simplify the experimental design and remove confounding factors. Unfortunately, the use of pure substrates as prebiotics belies the actual product consumed by the public, which often is a by-product of some other production line (like BioMos®, which is derived from brewer's yeast cell wall components). With expanding research, we see that prebiotic origin matters just as much as host context [89]. Additional substrates beyond the primary prebiotic compound, a result of the prebiotic's origin, may unintentionally provide metabolizable substrates for less desirable bacteria, like enteric pathogens.

Enteric Pathogens Perturb the Gut: The Lifecycle of *Salmonella*

As illustrated in previous sections, gut microbiota composition and function can be altered by exogenous factors like diet and age. One additional factor that drastically perturbs the gut microbiota and the gut environment are enteric pathogens. Enteric pathogens are typically ingested through contaminated food or water and cause gastrointestinal infections, which can lead to severe clinical outcomes including death [90]. *Escherichia*, *Shigella*, *Vibrio*, *Campylobacter* and *Salmonella* all contain species that act as enteric pathogens, together causing over 1.7 billion infections globally per year [91]. Enteric pathogens are particularly interesting to study in the context of the shared metabolome and related host health

effects, as they are invaders of the gastrointestinal tract with ready access to shared host-microbe metabolic products and the gut microbiome.

The genus *Salmonella* contains just two species, *Salmonella bongori* and *Salmonella enterica*, with seven subspecies, six of which belong to *Salmonella enterica* [92]. *Salmonella enterica* subsp. *enterica* (Subspecies I) is a well-studied subspecies, capable of infecting a broad species range and accounts for 99% of human *Salmonella* infections [93, 94]. A gram-negative intracellular pathogen, *S. enterica* subsp. *enterica* can associate with and invade multiple host cell types including enterocytes, macrophages, and mesenchymal stem cells [93, 95]. The extensive literature on *S. enterica* subsp. *enterica* paints a comprehensive picture of the pathogen's genetic capabilities, metabolic activity, and general infection cycle, making many serovars of *S. enterica* subsp. *enterica* ideal models for studying the mechanistic underpinnings of host-pathogen metabolic interactions in different contexts.

S. enterica serovars present a particular threat to food safety, as these pathogens can survive in unusually harsh environments, like low pH or low humidity, that typically eliminate other pathogens, [96]. To survive the osmotic stress of a low moisture environment, *S. enterica* increases intracellular K^+ content through the activation of the Kdp membrane transporter, activates the transport of the osmoprotectants proline, glycine-betaine, and ectoine, and increases OmpC porins in the membrane [96]. *S. enterica* can also form protective biofilms in response to starvation stress. Curli fimbriae, a key component of *S. enterica* biofilms, are increased in *S. enterica* in desiccated environments [96].

The ability of *S. enterica* to survive in harsh food environments may be necessary to its survival in gastrointestinal transit. To cause infection, *S. enterica* must reach the host intestinal epithelium. The low gastric pH is typically a first line of defense for the host against foodborne pathogens, but not for *S. enterica*. *S. enterica* overcomes the acidic stress of the stomach through the activation of pH homeostasis response systems, like the induction of lysine and arginine decarboxylase expression, synthesis of acid shock proteins, and through membrane modification [97]. Decarboxylation of lysine and arginine results in the consumption of a proton, thus increasing intracellular pH relative to the extracellular environment, and is one metabolic mechanism *S. enterica* can employ for survival during passage through the stomach. In combination with this metabolic response, *S. enterica* can also induce the production of acid shock proteins like RpoS and Fur to facilitate cellular repair and can remodel the membrane composition to increase robustness through reduced fluidity [97]. *S. enterica* cells that survive the harsh gastric conditions are then carried through into the small intestine, where they can begin their colonization of the host.

Once *S. enterica* enters the small intestine it can adhere to and invade both phagocytic and non-phagocytic cells in the epithelium [98]. To support its existence, proliferation, and pathogenic activity both intra- and extracellularly, *S. enterica* has a wide breadth of genetic and metabolic capabilities that give the pathogen the ability to succeed in a diverse array of environments [99]. One review coined *Salmonella* "The Swiss Army Pathogen", a fitting nickname for a highly flexible pathogen [99]. *S. enterica* in the small intestine can disrupt the integrity of the intestinal epithelium through multiple effectors that include *sopB, sopE, sopE2* and *sipA*, allowing for the spread of *S. enterica* into the accompanying vasculature and to systemic organs [100]. *S. enterica* may also cross the host cell barrier through forced entry into non-phagocytic enterocytes through use of the type III secretion system (T3SS), allowing for transit from the apical to basolateral side of the epithelium through host gut

epithelial cell uptake and escape [101]. Phagocytic cells primed for the uptake of microorganisms, like macrophages and dendritic cells, also engulf *S. enterica*. The genetic and metabolic flexibility of *S. enterica* means it can exist intracellularly in these immune-privileged phagocytic cells without killing the host cell [95, 102]. *S. enterica* can colonize the small intestine and proliferate, causing clinical symptoms like gastroenteritis, but *S. enterica* also continues its transit through the intestinal tract to the large intestine.

S. enterica proliferates in the large intestine much the same way as in the small intestine, though the more robust colonic microbiota presents an additional hurdle to colonization. In the large intestine *S. enterica* must compete with the established microbiota for limited nutrients and must carve out its own ecological niche to survive against the robust commensal population. Fortunately for the pathogen, and unfortunately for the host, *S. enterica* has well-developed mechanisms that perturb the lumen and create an inflamed environment advantageous to pathogenesis [103]. Inflammation, driven by host cytokines in response to pathogen detection by toll-like receptors (TLR), disrupts normal microbiota activity, leading to bacteria inducing stress responses and commensal population depletion [104]. Under normal and healthy host conditions, the production of SCFAs by the gut microbiota acts as an inhibitory mechanism against *S. enterica* colonization [103]. The mildly acidic gut conditions coinciding with SCFA production stresses *S. enterica* and dampens its ability to proliferate, but disruption of the microbiota and their SCFA production opens a new and unoccupied niche for *S. enterica* to colonize [103].

To stage a successful colonization, *S. enterica* must produce ATP in concentrations able to support replication and division. SCFA depletion from reduced gut microbiome fermentation allows *S. enterica*

to maintain a more homeostatic cytosolic pH (i.e., pH 7.2-7.8), subsequently increasing redox potential and supporting energetically favorable aerobic respiration [103]. Interestingly, in addition to modulating commensal membership for pathogenic gain, *S. enterica* also co-opts bacterial metabolites to increase metabolic productivity. *S. enterica* sv. Typhimurium encodes the propionate to pyruvate controlling *prpBCDE* operon, which allows *S.* Typhimurium to use this abundant microbial metabolite (propionate) for utilization as a main carbon source [105]. 1,2-propanediol, a product of microbial fermentation from commensals like *Bacteroides thetaiotaomicron*, has been shown to be advantageous to *S. enterica* growing under low-oxygen and high-osmolarity conditions like those of the large intestine [106]. Literature also supports that *S. enterica* can hijack gut products like tetrathionate and ethanolamine for use as electron acceptors to fuel growth, lending the pathogen an advantage over commensals unable to utilize these products [107, 108]. *S. enterica* is also auxotrophic for all 22 proteinogenic amino acids, conferring an advantage to the pathogen in amino acid depleted environments [109]. Amino acids are tightly regulated by the host as they are foundational to protein synthesis and precursors for central metabolic pathways, like tryptophan for NAD^+ production [110]. Intracellular *S. enterica* can even force amino acid starvation of epithelial cells, leading to the repression of immune signaling pathway mTORC1 and general inhibition of the innate immune system [110]. As detailed, *S. enterica* is a dexterous pathogen able to employ an array of metabolically and genetically-drive tactics to overcome host and microbiome defenses. *S. enterica* is typically consumed via contaminated food or water, travels to the stomach where it employs stress response tactics to overcome the low pH and finally makes it to the small and large intestine, where it deploys its full genetic and metabolic breadth to outcompete the host and commensal microbiome. The ability to not only overcome but instead thrive on the harsh gastric pH, host immune actions, and microbial defense mechanisms, and ultimately cause severe illness, makes *S. enterica* a multifaceted pathogen of great interest to the research community.

Research seeking treatments for *S. enterica* infections investigated the use of multiple therapeutics, including established antibiotic routes and more novel applications of probiotics and prebiotics. Antibiotic therapies have long been applied for the control of salmonellosis, but as with many other modern-day pathogens, resistance to these drugs is increasing in *S. enterica* [111]. Fluoroquinolones, which is a class of antibiotics targeting two bacterial DNA topoisomerases, are the primary class used to treat gastroenteritis causing multidrug-resistant *S. enterica* infections. With continued use, this class of antibiotics has become less effective against *Salmonella* [112]. *Salmonella* was ranked as a high priority pathogen for novel antibiotic research by the World Health Organization (WHO) in 2017 due to the dwindling treatment options [111]. Even though it has been years since this designation, no new antibiotics have been successfully developed for the treatment of *S. enterica* infections, indicating the need to search beyond the realm of antibiotics for new therapies.

Given *S. enterica* is a gut pathogen, it is reasonable to suggest using the host microbiome as a weapon in this fight. Probiotics, microorganisms which confer health benefits to the host when taken in sufficient quantities, are one possible route to more successful *S. enterica* treatments [113-116]. Much like the prebiotics mentioned in an earlier section, probiotics is an amorphous term encompassing numerous different microorganisms that are often shown effective in ameliorating specific disease conditions, but who's efficacy may deviate depending on contexts [117]. In the case of *S. enterica* infections, studies have tried using multiple different microorganisms as prophylactics, on their own post infection, or in tandem with current antibiotic therapies [113, 118, 119]. Much of the literature around probiotic control of *Salmonella* in the gut centers on lowering *Salmonella* carriage in production animals, which can serve as pathogenic reservoirs or who also face their own salmonellosis conditions [113]. The select studies looking at using probiotics to control human *S. enterica* infections have used *Lactobacillus*

rhamnosus GG [120], *Lactobacillus plantarum* [121], *Bifidobacterium thermophilum* RBL67 [122], and multiple yeast types in the ascomycetes grouping [113].

There are mixed results for the efficacy of each probiotic in controlling *S. enterica* growth, but generally probiotics appear to hold some promise as potential adjuvants or therapeutics in their own right. The suggested mode of action for pathogenic control varies by context and strain, but studies propose that probiotics may promote colonization resistance through a more robust microbiome, alter the lumen to be less hospitable to *S. enterica* through increased SCFA production, outcompete *S. enterica* for vital nutrients, positively modulate the host immune system for a more robust response, or through some combination of these factors [113, 122, 123]. It remains to be seen if these positive probiotic effects hold true *in vivo* across different stages of infection and hosts, but there is hope that probiotics might provide some alternatives to antibiotics.

Prebiotics, a sibling product to probiotics, may also prove useful in the fight against *S. enterica* infections. As with probiotics, prebiotics have been studied for their use as prophylactics, active treatments, and adjuvants to current therapies. Paralleling the predominantly production-animal focused probiotic research, much of the literature investigating the efficacy of prebiotics against *S. enterica* are also in the context of production animals like broiler chickens and less focused on human application [124]. This research disparity between production host types is likely due to the difficulty of human applied research, contrastingly easy access to production animals, and the regulation structure of dictating appropriate use of these dietary products in humans versus animals. Prebiotics, regardless of host type, are applied to enteric infections with underlying idea that prebiotics support commensal populations through selective feeding and subsequently this positive population shift leads to improved

colonization resistance and improved host immunity through commensal interactions. Some studies have investigated applying synbiotics to synergistically improve the effects of both [125]. Other studies have investigated the potential of prebiotics on their own. One using porcine intestinal cells as an *in vitro* model for humans tested the effect of β-galactomannan, D-mannose and yeast-derived mannanoligosaccharides on *S. enterica* sv. Typhimurium [126]. IL6 and CXCL8, both involved in the inflammation response to infection, were reduced in cells treated with beta-galactomannan or mannanoligosaccharides but not those treated with D-mannose [126]. This suggests ameliorative effects are structure specific and not applicable to all derivates of an effective substrate (like mannose vs mannanoligosaccharides). Prebiotics themselves are not a novel concept however, their application as targeted therapeutics for enteric control needs further exploration.

Despite the thorough literature on virulence mechanisms and host interactions of *S. enterica* subsp. *enterica*, much remains unknown about how pathogens interact with dietary supplements like prebiotic oligosaccharides. As noted above, dietary supplements and prebiotics are publicly available and touted to the average consumer as important for the maintenance of ideal gut health, but many of these supplements have not been evaluated for their potential effect on pathogenic activity in the gut. Considering the prevalence of *S. enterica* subsp. *enterica* infections and the widespread availability of dietary supplements, it is imperative to understand the potential protective effects such supplements may have towards enteric pathogens, but it is also equally necessary to understand any risks dietary supplements may pose in the context of an ongoing enteric infection.

Conclusion

The human gut directly links the outside world with internal host physiology, making it an area of great interest when it comes to the effect of dietary substrates on microbial function, pathogenic activity, and holistic host health. In the context of host health, the gut microbiome is a community whose contribution to host physiology and disease status cannot be ignored. Indeed, the gut microbiota, in large part through the production of bioactive metabolites, can train the immune system, regulate digestion, control intestinal barrier function, and exert undue influence on host neurology (Chapter 2). The host also employs some control over the microbiota through dietary choices and through the production of metabolites that have a double life as interkingdom signaling molecules. These small molecules, like serotonin and arginine, can regulate microbial activity, from biofilm formation to the expression of virulence factors, through their interaction with widespread two-component signaling cascades (Chapter 3). The bi-directional communication between the host and its resident microbes via metabolites is an expansive area of research, even in the slightly more straightforward context of healthy hosts. Expanding on this research of metabolic signaling, it is important look beyond simply healthy conditions and more deeply investigate what happens to this bi-directional communication in the presence of enteric pathogens. Though there are many studies investigating how the gut microbiome shifts in response to dietary substrates, few focus solely on the interactions of enteric pathogens with such dietary substrates. Prebiotics are one dietary class gaining a lot of momentum in both academic research and the public sector. Considering the public health component, a better understanding of how enteric pathogens and prebiotics interact in the context of a host is pressing and imperative (Chapter 4). Though a daunting task for the field, teasing apart the mechanistic interactions in the host-microbe-metabolite triangle is crucial for improved public health and safety standards, alongside the potential that future therapeutics may come from such a mechanistic approach to prebiotic research.

The strong literature investigating bi-directional control of the metabolome on the host and the gut microbiota warranted a thorough review. In Chapter 2 we synthesized the current literature around how one distinct set of metabolites, derived in part from gut microbial activity, influence the neurological status of the host, including how such metabolites may exacerbate conditions like depression or even be a causal agent in the disease development and progression. This chapter specifically delves into the world of tryptophan-derived metabolites, a group of metabolic products quickly gaining notoriety for their diverse bioactive roles in human physiology. Chapter 3 presents the other side of this relationship, diving into how host-derived metabolic products influence microbial activity in the gut. This chapter, which was originally published as an independent review, investigates what metabolic signals may drive pathogenic behaviors in enteric pathogens through interactions with the ubiquitous two-component signaling systems in microbes. Finally, we sought to test the knowledge gained from both reviews through the application of two distinct dietary prebiotics to an *in vitro* system containing host colonic epithelial cells and enteric pathogen *S. enterica* sv. Typhimurium (Chapter 4). Given dietary components drive both host and microbe metabolism, we hypothesized that the addition of two structurally distinct dietary prebiotic oligosaccharides, HMO and BioMos, would alter host cell and *S.* Typhimurium metabolism in routes specific to each prebiotic, ultimately attenuating virulence. We also hypothesize that, given the distinct chemical structures of each prebiotic, surface interactions of each prebiotic with host and pathogen would alter membrane signaling mechanisms involved in virulence and immune responses in ways unique to each prebiotic. To test these hypotheses, we used dual-RNAseq for evaluation of the host and pathogen cellular activity and untargeted metabolomics to detect distinct metabolite changes resulting from each treatment. All together this work illustrates the importance of the microbe-host shared metabolome in modulating host health and microbial activity, and how the addition of dietary substrates may alter these interactions for better or worse.

Hypothesis: Shared host-microbe metabolism produces small molecules able to exert bi-directional bioactive control and shifts in this shared metabolome result in consequential health outcomes for the host, altered microbial activity in the gut, and additionally influences the virulence of enteric pathogens.

Aim 1- Elucidate the role of microbial metabolism in influencing host neurology through an extensive literature search using the example of tryptophan-derived metabolites and through evaluation of gene co-occurrence for related enzyme encoding genes in common gut microbiota using the STRING database.

Aim 2- Illustrate the bi-directional nature of small metabolite signaling through evaluation of interactions between the prokaryotic two-component signaling system and host-derived small molecules, with specific focus on the control of virulent traits in enteric pathogens by these host compounds. Follow-up work using JGI's IGM/M database to reveal the ubiquity of these two-component systems in common gut microbiota.

Aim 3- Determine the effect of two distinct dietary prebiotics on host-pathogen crosstalk and metabolic regulation using a focused set-up of colonic epithelial cells and *S.* Typhimurium with dual RNAseq and untargeted metabolomics to measure genetic expression and shared metabolism. Specific targets for post-hoc analysis include in modulation of virulence factors and connected host cell surface receptors, responses of *S.* Typhimurium two-component systems, and the regulation of energetically favorable metabolism by structurally distinct prebiotic oligosaccharides.

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Chapter 2

Microbial-Derived Tryptophan Metabolites and their Role in Neurological Disease:

Anthranilic Acid and Anthranilic Acid Derivatives

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Abstract

The gut microbiome provides the host access to otherwise indigestible nutrients, which are often further metabolized by the microbiome into bioactive components. The gut microbiome can also shift the balance of host-produced compounds, which may alter host health. One precursor to bioactive metabolites is the essential aromatic amino acid tryptophan. Tryptophan is mostly shunted into the kynurenine pathway but is also the primary metabolite for serotonin production and the bacterial indole pathway. Balance between tryptophan-derived bioactive metabolites is crucial for neurological homeostasis and metabolic imbalance can trigger or exacerbate neurological diseases. Alzheimer's, depression, and Schizophrenia have been linked to diverging levels of tryptophan-derived anthranilic, kynurenic, and quinolinic acid. Anthranilic acid from collective microbiome metabolism plays a complex but important role in systemic host health. Although anthranilic acid and its metabolic products are of great importance for host-microbe interaction in neurological health, literature examining the mechanistic relationships between microbial production, host regulation, and neurological diseases is scarce and at times conflicting. This narrative review provides an overview of the current understanding of anthranilic acid's role in neurological health and disease, with particular focus on the contribution of the gut microbiome, the gut-brain axis, and the involvement of the three major tryptophan pathways.

Introduction

The metabolome, small molecules produced by the collective metabolism of an organism and its microbiome, is difficult to comprehensively examine, in large part due to the sheer complexity of the interaction between the microbiome and the host metabolic state. This difficulty is especially pronounced in the human gut, where extensive overlap between small molecule metabolism of the host and microbiome exists [1]. Despite this complexity, determining causation and correlation with host health requires distinguishing which molecules are derived from the host's diet, produced by the microbiome, or produced and secreted into the gastrointestinal tract (GT) from the host metabolism. In the broader context of host health, the importance of the host GT activity and resident microbiome contributions cannot be overstated, specifically with the extensive reach of small molecules to distant tissues in the body [2]. For example, the gut and brain are inextricably linked, exerting bi-directional control, with the gut microbiome playing the part of complementary brain in this feedback loop [3]. The microbiome density, diversity, and composition differ across the GT. The human GT consists of many specific sections that have differential metabolic capacity and microbiome members, but for the purpose of the review will be broadly discussed in terms of the small intestine, which has a low-density microbial consortia and the contrasting microbially dense large intestine [4].

The small intestine utilizes digestive juices and enzymes that are secreted by the pancreas and gallbladder to breakdown carbohydrates, lipids, and proteins. The small intestine also contains microbes, such as *Bacteroides* and *Prevotella*, that aide in the synthesis of micronutrients, like vitamin K2 and B12 [4, 5]. The microbiome of the small intestine is exposed to transient conditions that include periodic pH fluctuation from the influx of stomach contents and the microbiome must adapt to the ever-shifting local environment from stomach proximity [6]. This instability may explain why the small intestine harbors only ~10^5 microbial cells/gram, whereas the large intestine supports ~10^{12} microbial

cells/gram [4]. Despite the relatively lower abundance, the small intestine microbiome is an important contributor to host digestion as the small intestine is the primary site of macronutrient digestion and absorption.

Although the small intestine accounts for most macronutrient digestion, the large intestine is an essential contributor to host metabolism and has a differing role in digestion. Host cells of the large intestine, goblet cells and enterocytes, produce mucosa and digestive enzymes for the further breakdown of food matter from the small intestine [7]. The large intestine is also responsible for the absorption of any remaining water and salts not absorbed during transit through the small intestine. In addition to host cell activity, microbes expand the digestive and metabolic functionality of the large intestine by an estimated multiplier of ~150,000 [8], which expands the host's metabolism beyond its genome capacity.

The microbiome of the large intestine, especially of the colon, is arguably more well studied than that of the small intestine, likely due in large part to the more robust study material, typically feces via non-invasive methods. The diverse microbiome of the colon adds metabolic breadth to the GT, providing the host with access to otherwise inaccessible small molecules and nutrients, like the short chain fatty acid (SCFA) butyrate, which is a key contributor to healthy gut epithelial function (e.g. increasing tight junction integrity) [9, 10] and has shown to alter immune function (for details see Kim [11]). In addition to synthesizing nutrients, the gut microbiome can also produce metabolites, ultimately shifting the balance and activity of overall metabolism.

Amino acids are one substrate set that both host and microbe breakdown and divert to key and central metabolic pathways. Glutamine and asparagine, for instance, are utilized by host intestinal cells as energy source [12]. In bacteria, glutamine is also of central importance and serves as a precursor for the synthesis of key nitrogen-compounds and glutamine supplementation has shown to promote and support populations of fiber-degrading bacteria [13-15]. The consequential role of the microbiota in amino acid homeostasis has further been highlighted by studies that showed germ free mice display altered amino acid distribution along the intestinal tract when compared to mice that possessed their natural microbiome [13]. While amino acids are centrally important to protein synthesis and other basic host-microbe functions, amino acids can also regulate host neurological health by serving as precursors to bioactive molecules that circulate throughout the host.

Tryptophan (Trp) is one such amino acid involved in bioactive metabolite production and Trp metabolism is a central pathway shared by the host and its microbiome (Figure 1). Trp is derived from the digestion of dietary protein in the small intestine, where free Trp is absorbed through the intestinal epithelium and enters systemic circulation. Any unabsorbed Trp is transported through the intestinal tract until it reaches the colon where it is utilized by the colonic microbiome [16]. In the colon, Trp plays a major role as primary substrate for microbial metabolism [17], since easily digested carbohydrates have been utilized early on after indigestion and are in short supply in the distal portion of the digestive system. Trp metabolism is not limited to a single genus of microorganism but is found across many gut microbes, including multiple *Lactobacillus, Bifidobacterium, Bacteroides*, and *Clostridium* species [18]. Some of the microbes known to utilize Trp are often associated with host health and are part of the gut-brain axis.

Digestion of Trp results in the well-known neuroactive compounds, serotonin and melatonin, and is a starting point for the NAD^+-producing kynurenine pathway in humans. In addition to the ability to perform parts of the serotonin and kynurenine pathways, microbes have the additional option of producing indole from Trp catabolism. Humans lack this indole pathway and instead rely on exogenous indole, either from dietary sources or microbial metabolism, which is metabolically straight forward but the resulting bioactivity in the microbiome is complex and can alter biofilm formation, virulence, and host interaction [19, 20]. The kynurenine pathway, one of three main Trp metabolic pathways, has garnered increasing interest for its connection to neurological diseases through the production of the bioactive compounds quinolinic, picolinic, kynurenic and anthranilic acid in this pathway [21-28]. The three primary Trp pathways are critically interconnected. Shunting Trp into the kynurenine pathway removes the substrate from the production of indole by microbes or serotonin (in microbes and humans). This 'push-pull' nature of Trp metabolism, along with the notable bioactivity of the many related metabolic products, make understanding the equilibrium of these interconnected pathways important from the perspective of host health.

The role of microbes as drivers of, and not just responders to, bioactive metabolites (and metabolism) is quickly emerging as an important component of host wellbeing that is modulated by the microbiome co-metabolism. At the same time, this newly elucidated role for microbial metabolites is not well understood or defined. This in part due to the complex set of in flux metabolites that, combined with microbiome membership changes, leads to altered metabolic potential. Additionally, host diet, the source of substrates for the microbiome, may also be in flux [29]. Many studies examining the gut microbiome focus primarily on taxonomic diversity, and do not make the link to metabolism and metabolite changes that are inevitably from diet and host metabolism [30, 31]. While a necessary part of the puzzle, bacterial identity alone does not indicate microbiome functionality, which changes the

collective metabolism and metabolome [32-35]. A companion focus on the resultant metabolome is imperative for a holistic understanding of microbiome function, and ultimately, for an understanding of its role in host health. Although there are hundreds of known bioactive metabolites stemming from many dietary substrates, this review will focus first on Trp and then more specifically delve into Trp-derived anthranilic acid and its direct derivatives.

Anthranilate and its derivatives are only one part of the broader Trp puzzle; however, they are an understudied piece with important influence on holistic host health. As will be discussed in more detail later in this review, anthranilate is not often the target metabolite of metabolome/microbiome/host health studies, but a pattern of anthranilate and derivatives is emerging alongside neurological findings. And specifically, as a shared metabolic substrate between host and microbe, a closer look at anthranilate and its derivatives may help fill in some missing pieces around the enigmatic gut-brain and bioactive metabolites connection.

The production, circulation, and interactions of Trp-derived anthranilic acid and subsequent derivatives have profound impacts on host health. Despite the important role of the human microbiome in anthranilic acid production from Trp, no comprehensive review that summarizes the role of microbially-derived anthranilic acid in neurodegenerative diseases exists to date. To place anthranilate in the wider framework of this family of bioactive metabolites, this review contains also contains a brief discussion of the activity of other more widely known neuroactive Trp metabolites. The objective of this review is to highlight the importance of anthranilic acid and its derivatives by outlining the current understanding of anthranilic acid's role in health and disease, followed by a discussion of the microbes that may affect the production and circulation of anthranilic acid.

A gut feeling: the microbiome as a complementary brain

Microbes in the gastrointestinal (GI) tract work synergistically to break down substrates and survive as a complex consortium with expansive metabolic capability that exceeds human metabolism at least 10-fold [36]. Microbes provide nutrients to the host, and it is increasingly clear that the microbiome contributes to host health and behavior through the production of neurologically active compounds, like serotonin, that move from the gut to distant locations throughout the body [37]. Within the gut these metabolites are often beneficial, supporting immune function and promoting tight junctions, but they can also be detrimental, stimulating inflammation and dysbiosis (Table 1) [38-41]. Microbial metabolites find their way out of the gastrointestinal tract and into circulation via both passive and active transport [16] and once in circulation bioactive compounds are free to travel to any number of body locations, including the liver, kidneys, lymph nodes, reproductive tract and even the brain [37, 67, 68].

Crossing through the gut epithelium into systemic circulation is not always necessary for these microbial metabolites to exert control over host neurology. Interactions between luminal gut metabolites and the host nervous system can be local in nature but with wide-reaching in effects [69]. The enteric nervous system, containing sensory afferent and motor efferent nerve fibers, provides a direct link between the brain and physical/chemical gut activity [70]. The Vagus nerve, a part of the enteric nervous system, is central to basic physiological process and innervates among others the cardiovascular system and respiratory system [71]. The bi-directional (with both afferent and efferent fibers) Vagus nerve is a big part of the gut-brain connection and has recently been shown to exert direct control from the gut to the brain via physical contact with enteroendocrine cells in the gut epithelium [72]. In addition to direct interactions with host organs through active circulation, microbial metabolites can signal to the host via the Vagus nerve and the broader enteric nervous system [73]. Beyond the simple function of digesting food for energy production, the gastrointestinal tract is intimately linked with systemic host physiology

through both the uptake and transit of metabolites through the circulatory system and the conveyance of signals through the fibers of the nervous system. This inextricable connection between the host brain and the microbial brain of the gut highlights the importance of metabolic control in host health.

The gut microbiome and metabolome have been correlated with, and in some cases suggested to be, the causative agent in common disorders, such as Irritable bowel disease (IBD). IBD is a broad inflammatory disease of the gastrointestinal tract and one of the first conditions that was cited as a link between the gastrointestinal tract and host neurological state [74]. Patients diagnosed with IBD in a Canadian cohort were twice as likely to have mood disorders, such as depression or anxiety, when compared to the control group [74]. The directionality of the link of IBD and mood disorder remained ambiguous for many years, as 80% of the IBD cohort were diagnosed with anxiety two or more years before the IBD diagnosis and so directionality nor causation between the mood disorders and IBD was clearly established [74]. A 2016 review re-evaluated this IBD observation in conjunction with other studies to tease apart the gut-brain connection, ultimately finding that mood disorders such as anxiety, depression, and stress all have tight links to increased inflammatory states, potentially contributing to a physiological state that favors a long-term inflammatory disease [75]. Since this initial work in 2008, additional research has emerged on the connection between different gut disease and depression, as well as on the more specific routes and signals of communication between the physically separate body locales of the gut and brain via microbially derived metabolic products [76]. One example is the bacterial derived metabolite, *p*-cresol, a compound that has been connected to exacerbation of autism spectrum disorders in an expanding body of literature [77-82]. *p*-Cresol is an aromatic derivative of tyrosine produced as a catabolite by many members of the gut microbiota, including some *Bifidobacteriaceae, Enterobacteriaceae, Clostridiaceae*, *Lactobacillaceae, Coriobacteriaceae* and *Bacteroidaceae* [77]. *Clostridia difficile* has displayed a particular growth advantage on *p*-cresol and

people with autism spectrum disorders often have a gut microbiome that is enriched in *Clostridia*, suggesting a direct relationship between microbiome membership, microbial metabolism, and neurological symptoms [77]. More systematic studies in mice support this suggestion of a gut-brain axis link for symptoms that fall into the autism spectrum [77, 79], further suggesting microbial metabolism might result in disorders beyond the gut barrier.

Microbially-derived metabolic products play a dual function, serving both as substrates for the microbes and as interkingdom signaling molecules between the gut microbiome and peripheral host systems [39, 83]. Perhaps one of the most widely recognized example of these is the mood modulating serotonin; a Trp catabolite that acts on multiple brain regions via its specific receptors that are found throughout the circulatory system, but also in the brain [84]. Though known primarily for its activity in the brain, over 90% of serotonin is found in the GT lumen [85] (Figure 1). Other small molecules, such as SCFAs are imperative for healthy gut function, but they are also able to cross the gut epithelium and have been reported to breach the blood brain barrier in small concentrations [86]. SCFAs that make the journey to the blood brain barrier help to restore the barrier's integrity [86]. These small molecules can serve as sources of energy (e.g. succinic acid) for host and multiple members of the microbiome, but their role as signaling molecules with an array of effects should not be ignored due to their wide array of functional activities [87, 88]. Propionic acid, a SCFA produced through fiber and amino acid fermentation by gut microbes in the colon, is transported through the portal vein to the liver, where it can inhibit liver damage and [89] gluconeogenesis [90]. Propionic acid is a microbially derived metabolite with broad implication for host health given its ability to move to the liver and into circulation, where the compound can exert effects across a range of distant sites to the GT. Microbial metabolites that cross the epithelial barrier, or interact with the host nervous system, from the GT, have the potential to act upon several tissue systems ranging from the circulatory system to the brain [91].

The system-wide health effects of microbial metabolites in the host makes the modulation of these pathways a relevant strategy that might alter the outcome of many human diseases. Microbial metabolism can drive the production of bioactive metabolites that have either direct roles in the development and progression of diseases, or that exert more indirect effects on the disease progression. Modulating these microbial metabolite-host system interactions, like those seen with metabolites from Trp catabolism, through microbial engineering, gut microbiome remodeling, or dietary interventions could produce treatment for diseases for which effective treatments are currently not available or are instead extremely invasive.

The tryptophan pathway: microbial-derived metabolic mind control

Trp is a significant contributor to the crosstalk between the gut microbiome and the brain. As the precursor for many important products, like indole, serotonin, and NAD^+, Trp is crucial to basic microbial and human physiology. Trp is an essential amino acid that is introduced to the host primarily through diet, though some gut microbes can synthesize Trp from indole and serine via the Trp synthase enzyme complex (TrpAB) [83, 92]. A study looking at the distribution of amino acid biosynthesis pathways across the publicly accessible genomes of 2,856 human GT microbiome members from over 800 distinct microbial species found Trp biosynthesis was highly variable and individual microbes lacked a complete enzymatic repertoire to produce Trp [92]. There was a group of 38 genomes that were predicted Trp auxotrophs making them unable to produce anthranilate, but interestingly possessed the genes for synthesis of Trp from anthranilate [92]. The authors posited that Trp biosynthesis pathways may show high variation across human gut microbes due to the high energetic cost of producing Trp in comparison to other amino acids, because Trp is encoded by only a single codon, UGG, and is a rarely incorporated amino acid with high molecular weight (204.23 g/mol) [92]. The high variation in Trp biosynthesis across gut microbes pulls focus from basic host use towards the microbial use of this central amino acid and

the metabolites resulting from such use. Trp, regardless of the source, is central to many cellular functions and the general path from Trp to bioactive derivatives is important study for host health and disease impacts.

Trp in the host intestinal tract is freed during protein digestion within the small intestine or via microbial digestion and biosynthesis in the large intestine. Free Trp in the small intestine is absorbed into systemic circulation by the transmembrane broad spectrum neutral amino acid transporter (B^0AT1) that is encoded by the *SLC6A19* gene in intestinal epithelial cells [93]. Interestingly, 19 *SLC6A19* paralogues that encode transporters in the GT have been reported. All paralogues are part of the SLC6 transporter family, a group that is implicated in human diseases and are actively being investigated as therapeutic targets for diseases that include Parkinson's, depression, anxiety, hyperekplexia, and Tourette Syndrome [93, 94]. Once in systemic circulation, albumin-bound Trp is carried to the liver, where it is digested via the kynurenine pathway, or to the brain, where Trp crosses the blood brain barrier with the help of amino acid transporters and is shunted into both the serotonin and kynurenine pathways [95, 96]. Neutral amino acid transporters able to facilitate Trp-derivative absorption exist in the small intestine, but most of the gut's Trp metabolism occurs in the large intestine and is mediated by microbial metabolism [97]. There is some evidence that disease states, such as small intestinal bacterial overgrowth (SIBO), can shift Trp metabolism in the small intestine, driving an increase in locally-produced kynurenine [98], but the basal level of kynurenine production in the small intestine appears to be low under normal *in vivo* conditions [53]. The fraction of Trp that is not digested and absorbed in the small intestine is instead digested by the gut microbiome of the large intestine via the kynurenine, serotonin, and indole pathways [16]. Trp metabolites produced via microbial metabolism in the large intestine are utilized locally or cross the GT epithelium, via passive diffusion (kynurenine and indole) or with the help of transporters (serotonin) [99, 100]. Bioactive molecules stemming from Trp digestion,

whether from digestion in the small intestine or from microbial production in the large intestine, can act on the local gut tissue or exert control over distant host tissues via circulation.

The bioactive products of these pathways, like kynurenic acid, have been associated with neurological conditions. Alzheimer's, Schizophrenia, and depression have been linked to the serotonin, kynurenine and indole pathways, and intermediates of these pathways have been identified as potential causative agents or as potential biomarker for disease states [21, 23]. The microbiome, alongside the host, contributes to fluctuating intestine luminal and circulating serum levels of Trp-derived bioactive compounds, which are critical to the regulation of the gut-brain axis. A more mechanistic understanding of the function of these compounds in disease and of the microbial control of their production is imperative for improved neurology therapeutics. The three major pathways of Trp digestion, microbial metabolism, and the link to neurological diseases will be discussed briefly below and are summarized in Figure 2.

Indole

Exogeneous Trp that makes it through the small intestine and into the large intestine is primarily digested by gut microbes, as colonic epithelial cells do not absorb amino acids in great quantity [101]. Whereas gut microbes can convert Trp into indole via a one-step reaction catalyzed by tryptophanase (TnaA), humans lack the ability to synthesize indole and depend instead on the microbially derived indole. Indole and its directly related compounds, collectively called indoles, include indole-3-acetamine, tryptamine, indole-3-acetylaldehyde, and inole-3-pyruvate, are produced by members of the *Proteobacteria, Firmicutes, Fusobacteria,* and *Bacteroidetes* [102]. Further transformation of indoles results in the production of indole-3-acetate, indole-3-aldehyde, and skatole [102]. Not all microbes can produce indole. Fungi and bacteria that are unable to synthesis indole or its derivatives still have

mechanisms for sensing and utilizing indoles as metabolic precursors or ligands for signaling cascades [103, 104]. *Candida albicans, Staphylococcus aureus,* and *Pseudomonas aeruginosa* all use indoles to induce biofilm formation, and *Salmonella enterica* serovar Typhimurium relies on indole signaling to activate an oxidative stress response to resist antibiotic activity [104]. Indole that is not utilized by other gut microbes and is instead absorbed into circulation can be used in the liver to produce indoxyl sulfate, a toxic metabolite known for its ability to effect drug clearance in the liver through the modulation of drug transporter expression [105]. Indole also acts as an interkingdom signaling molecule between microbes and their host, promoting tight epithelial junctions, and regulating inflammation [39, 106]. Indole can directly induce interferon and interleukin 22, two immune system products that mediate repair of the gut barrier and inflammation, but this induction appears dependent on acute stressors in the gut and is thought to be a spatially regulated response [106]. Indole regulates a variety of bacterial responses within the gut microbiome itself, including biofilm formation and virulence [19, 107-110].

Humans may not be able to produce indole, but they do interact with indoles via the gut epithelium and through systemic circulation. Indole concentrations in feces of healthy individuals range widely, and studies suggest there are many factors contributing to this variance, including microbiome diversity, free exogenous Trp and the availability of other substrates [103, 111]. Dietary Trp is also a significant driver of luminal indole concentration [112]. Once dietary Trp is microbially converted to indole, it can travel through the lumen and gut mucosa via the host's aryl hydrocarbon receptors (AhR) on the gut epithelial cell surface [113]. AhRs are not limited the gastrointestinal tract, instead this cytosolic receptor is broadly distributed in the host [105, 114, 115]. AhRs are most highly expressed in the nervous system, especially the tibial nerve, and in the lungs, but are also expressed across much of the cardiovascular system and in reproductive tissues as well [116]. The breadth of tissues expressing these receptors is due to the crucial role of AhRs throughout the human body. AhRs serve as a mediating signal between

environmental conditions and appropriate host response, allowing for dynamic interplay between internal host physiology and fluctuating environmental cues [117]. As a signaling mechanism, AhRs are a key link between the gut microbiota and host via their binding to numerous ligands [118], but these receptors and indole together display a unique affinity for one another [113]. Interestingly, though also found in mice, AhRs and indole in the mouse gut do not display the same unique binding activity as has been observed in human cells [113]. In humans, once bound with indole, AhRs move intracellularly and, through modifications within the cell, regulate the associated target genes [119].

Though indole is now understood to be a central player in the metabolic crosstalk between host and microbiome, current research primarily focuses on the localized effects in the GT system, neglecting potential effects of indole on distant tissues. Indole can affect host neurology from the gut lumen, via the enteric nervous system, or through direct contact with host tissues via transit in the circulatory system. In the lumen, indole can activate enteroendocrine L-cells, which are specialized and electrically excitable epithelial cells that interface with the gut lumen, secrete peptide hormone glucagon-like peptide1, and are directly innervated by enteric afferents [120]. In addition to stimulating nerve-connected L-cells, indole can also cross the blood brain barrier, granting microbially derived metabolites direct access to host neural circuitry inside the brain [121]. In a recent study using a rat model, the effect of indole derivatives oxindole and isatin on the depressive and anxiety-related actions was examined [122]. Rats injected with an acute high dose of these indole derivatives in the cecum displayed lower motor activity and increased anxious behaviors [122]. To test the effect of a less extreme condition, gnotobiotic rats were inoculated with indole-producing *E. coli* to generate more constant and medial indole levels [122]. Rats with indole-producing bacteria were found to display more anxiety behaviors as compared to their uninoculated counterparts [122], suggesting that even modest concentrations of elevated indole promote anxiety.

Somewhat contrasting to the behavioral effects observed by Jaglin et al. (2018), Wei et al (2021) reported that indole promoted neurogenesis of adult brain tissue in mice [123]. Mice treated with indole, both through systemic inoculation and via monocolonization with *E. coli*, showed increased neuronal growth and greater circuitry development than germ free mice without access to indole [123]. Indole's part in reviving neurogenesis in the adult mouse brain, where neural growth is typically on the decline, is linked back to AhR-mediated signaling and the upregulation of *ß-catenin, Neurog2, and VEGF-alpha* in the hippocampus [123]. The results of this study, which investigated both mechanistic and holistic actions, underscore the importance of microbial metabolites in regulating host neurology.

Serotonin

In contrast to indole, serotonin can be synthesized both by gut microbes and by the host in various tissues. Serotonin (5-hydroxytryptamine) and melatonin (N-acetyl-5-methoxytryptamine) are well-known as mood and sleep regulating neurotransmitters. Though a key element in neural activity, only 5% of the body's serotonin is found in the brain, the remaining 95% of serotonin can be found instead in the gastrointestinal tract, primarily in serotonin-producing enterochromaffin cells of the gut barrier [85]. Serotonin is also the precursor of melatonin, which is synthesized in both humans and microbes via a two-step acetylation and a methylation process [124]. Studies with gnotobiotic mice indicated that although gut serotonin is produced mostly by host cells, the microbiome still plays a crucial role in the regulation of serotonin production, both via the consumption or production of metabolic precursors and through control of host activity by signaling cascades [125].

Human production of serotonin in the gut primarily takes place in the epithelial enterochromaffin cells via the activity of the rate limiting Trp hydroxylase enzyme (TPH1). Although the serotonin-producing enterochromaffin cells of germ-free mice are normally developed, these germ-free mice are deficient in serotonin and display a Trp build-up in the large intestine [126]. In conjunction with decreased serotonin production, the expression of serotonin transporter SLC6A4 is increased, likely to compensate for the diminished levels. This same trend of decreased serotonin and increased Trp is not observed in the inherently microbially-sparse small intestine [126]. To explain this microbial-induced serotonin deficit, one study inoculated gnotobiotic mice with altered Schaedler flora, a defined consortia of gut microbes that is widely used with mice models. Interestingly this study found that the deficit remained even in these minimally colonized mice [126]. Colonic serotonin levels did increase back to normal levels after inoculation with spore-forming members of the specific pathogen-free microbiota, mostly clostridial species, via stimulation of *TPH1* expression in enterochromaffin cells [126]. Gnotobiotic mice studies have demonstrated that the microbiome is responsible for modulating around 64% of the colonic serotonin concentration and affected almost half (49%) of the circulating serotonin [126, 127]. It appears that the microbial control of serotonin production by the host is not a one-way mechanism, as gut bacteria have also been shown to modulate their activity in response to colonic serotonin concentrations. Increased luminal serotonin levels stimulate bacterial synthesis of serotonin [126] and serotonin produced in the GT improves the local integrity of the enteric nervous system, which then regulates gut motility and nutrient absorption [128]. Circulating serotonin has been reported to be excluded from crossing the blood brain barrier (BBB) due to its slight polarity and is instead produced in the brain by the raphe nuclei from free Trp, which does cross the BBB [129]. It is noteworthy that BBB permeability is altered by many factors including age, temperature, and pharmaceuticals as extensively reviewed by Zhao, Gan [130]. Pathogens can also alter BBB permeability or take advantage of decreased barrier integrity, as seen with uropathogenic *Escherichia coli* reducing activity in the TGFBRII/Gli-2

signaling pathway that normally works to maintain barrier integrity [131]. Changes in the BBB integrity open the door for normally blocked substrates, like serotonin and other small molecules, to cross this barrier and gain access to the brain. The reported inability of serotonin to cross the BBB suggests that high levels of serotonin produced in the GT has a function outside of regulating behavior via direct interaction with receptors located in the brain. Indeed, serotonin is a potent and ubiquitous neurotransmitter involved in the pulmonary, enteric, immune, and circulatory systems that remains to be studied for the exact linkage from the GT to the brain and the possible behavior changes that this compound can impart [132-134].

Melatonin, like serotonin, can be produced by the host and when secreted by the pineal gland in the brain, melatonin exhibits behavioral control. Host melatonin production in the brain typically follows an exogenous light cycle to sync host behavior to light exposure [135]. Interestingly, it is also produced by enterochromaffin cells in the gut at concentrations 400 times higher than what has been observed for pineal gland production [136]. In the gut melatonin contributes to the relaxation of gut tissue and lower overall motility, effects which oppose those of serotonin [137]. Exogenous melatonin administration alleviated some irritable bowel (IBS) symptoms, potentially due to the relaxation regulation by melatonin [138], but further studies are needed to pinpoint a specific mode of action. Additionally, melatonin supplementation was shown to reduce obesity-related bacterial taxa in mice on a high-fat diet, restoring the microbiota to that resembling the one associated with normal chow mice [139]. Melatonin may also be a potential therapeutic agent in neurological disorders like multiple sclerosis and Huntington's disease [140], but more work in this area is needed to understand the mechanics of this potential metabolite as a therapeutic. The regulation of host and microbe activity by gut-derived serotonin and melatonin is a crucial aspect of the gut-brain connection. Though these bioactive Trp metabolites may be well-known, they are not necessarily well understood. Further evaluation of how

serotonin and melatonin regulation may play into the control of neurological disease will be critical for the development of advanced disease treatments.

One aspect of melatonin that also requires further study is its role as a signaling molecule amongst gut microbes. Multiple studies have reported the use of indole as a mechanism for bacterial communication, allowing both for symbiotic coordination amongst gut bacteria and conveying the existence of competitive environmental conditions [19, 141, 142]. Gut-derived melatonin may also support chrono-related bacterial changes. *Klebsiella* (formerly *Enterobacter*) *aerogenes*, a human commensal organism, shows increased motility with increased luminal melatonin levels, indicating the host secretion may be regulating the microbial activity in a circadian-dependent manner [143]. *In vitro* work provided evidence that *K. aerogenes* regulates key physiological pathways in response to melatonin exposure, including pathways involved in stress response, carbohydrate transport, and metal ion homeostasis [144]. This same study also showed circadian rhythm-dependent synchronization of gene expression across *K. aerogenes* cultures in response to melatonin concentration [144]. Though this same depth of study regarding the gene expression and growth response of a single gut commensal to melatonin has not been done in many other microbes, it stands to reason that the circadian clock and sensitivity to melatonin is not unique to *K. aerogenes*. The exploration of circadian rhythmicity in gut bacteria may provide an even more direct connection between host homeostatic rhythms and microbiome activity, further tightening the link between hosts and their microbes.

Kynurenine

Utilizing approximately 90% of Trp, the kynurenine pathway is a robust multistep metabolic pathway with numerous bioactive intermediates and the terminal product of NAD^+, as well as other small

molecules [145]. Kynurenine metabolism produces both neuroactive and neurotoxic products which creates a 'push/pull' network that depends on other aromatic compound intermediates in the overall pathway. This complex metabolic pathway has garnered interest for its many bioactive metabolites and for its role in neurological diseases [146, 147].

The production of kynurenic acid, a single step metabolite of kynurenine, pulls the pathway flux from the production of neurotoxic quinolinic acid; thereby detoxifying the local niche. Due in part to this metabolic trade off, kynurenic acid is thought to have some neuroprotective properties [148]. In a study looking at metabolic markers of fibromyalgia and chronic fatigue syndrome, the kynurenic acid/quinolinic acid ratio decreased in patients with chronic fatigue syndrome [148] and kynurenic acid/3-hydroxykynurenine ratios decreased in patients with fibromyalgia compared to healthy patients [148]. These findings suggest these ratios could serve as potential biomarkers to evaluate the overall metabolite balance of patients. A different study, investigating kynurenine levels in the blood of dementia patients over a duration of 5 years, found that both unusually high and low levels of circulating kynurenine correlated to negative cognition prognoses [149]. During this work the investigators looked more holistically at the pathway and related metabolic ratios and found that mean kynurenine serum levels did not appear correlated with progressing dementia [149]. Instead, a non-linear relationship was detected where deviation from midline kynurenine concentrations in either direction was associated with more extreme neurological and dementia symptoms [149]. Kynurenine metabolites have also been linked to bipolar disorder, which is a complex neurological disorder that affects approximately 2% of the population [150, 151]. Notably, recent meta-analyses have shown there is a connection between metabolites of the kynurenine pathway and bipolar disorder, but the mechanistic and possibly causative relationship remains less well understood [150, 151]. Though the

causative relationship may not yet be fully elucidated, it is nevertheless clear that metabolites stemming from the kynurenine pathway play key roles as markers of disease or perhaps even causative agents.

Though current scientific literature has abundant coverage of the widespread implications and potential systemic results of the dysregulation of the kynurenine pathway, a deep dive into the many branches of this pathway is outside the scope of this review. Fortunately, there many excellent reviews on this subject [152-156]. Instead, the remainder of this review will focus solely on one kynurenine metabolite that may hold the key to understanding a subset of neurological disease in the microbe-host context: anthranilic acid.

Anthranilic acid and beyond

Anthranilic acid, a kynurenine metabolite produced by host and microbe alike, is a bioactive compound with potential systemic neurological effects. Though in the past much research has focused on the kynurenine pathway at large, the role of anthranilic acid in the gut-brain axis is emerging as an important piece of the puzzle. Anthranilic acid is a direct metabolic product of kynurenine digestion in humans and microbes and represents an alternate branch to two other immediate products (i.e., 3-hydroxykynurenine and kynurenic acid) [157]. Kynurenine is hydrolyzed to anthranilic acid and L-alanine with the help of kynureninase. With the transfer of an amino group, kynurenine becomes the alternate product kynurenic acid, and the addition of an oxygen by kynurenine-3-monoxygenase turns kynurenine into 3-hydroxykynurenine. Anthranilic acid, through non-specific hydroxylation, becomes 3-hydroxyanthranilic acid, which is the precursor for quinolinic acid, picolinic acid, and the terminal product NAD^+ [157]. The ratio of anthranilic acid to the downstream 3-hydroxyanthranilic acid has been suggested as one potential biomarker for both neurological and physiological disorders [55]. Given its

pivotal position as a diverging branch of the kynurenine pathway, its bioactive properties, and the dual production by host and microbes, anthranilic acid is a key Trp metabolite to study in the context of the gut-brain axis.

Pharmaceutical research has built on the inherent production and utilization of anthranilic acid metabolites *in vivo* for the creation of novel drugs and therapeutics. A 2021 review of anthranilic acid medicinal research highlights the bioactivity of anthranilic acid derivatives [158]. In this review, anthranilic acid is discussed as a pharmacore, which is a basic compound that is biologically active and that provides a starting point for more complex pharmaceuticals. Specific substitutions and additions on the basic anthranilic structure, 2-amino benzoic acid, yielded active therapeutics with a wide array of targets and anthranilic acid analogues are currently in use for the treatment of multiple metabolic disorders and as anti-inflammatories. Analogues created in pharmaceutical research have exhibited anti-viral properties, activity on drug-resistance cancer cells, and some varied effects on basic cellular processes like the hedgehog signaling pathway [158]. Other reviews and research in this area have likewise supported the notion of anthranilic acid as a key contributor to pharmaceutical research, due in large part to the highly active role anthranilic acid already has *in vivo* [159, 160].

As noted above in the context of pharmaceuticals and further outlined in the sections below, anthranilic acid and chemically similar compounds are important in host physiology beyond the localized intestinal tract. Dual production of these compounds by the host and the microbiome increases the complexity of this host-metabolite interaction. A more mechanistic understanding of anthranilic acid as an agent of neurological disease is imperative.

Human production of anthranilic acid and derivatives

The production of anthranilic acid by humans is integral to this review since it supports the concept of co-production between the host and the microbiome of a bioactive compound. Though the gut microbiome contributes many important and biologically relevant metabolites, humans make their own contributions to systemic metabolism. Many host tissues and cell types, including neurons and macrophages, have the enzymatic repertoire to support most or all of the kynurenine pathway [161-163]. There are various drivers of the kynurenine pathway *in vivo* and many factors that exert control over the divergence into the many branches and resultant metabolites.

In vitro studies have shown the kynurenine pathway in multiple human cell types can be induced and specifically altered by interferon-gamma [164]. Macrophages in particular were driven towards the production of 3-hydroxyanthranilic acid from L-tryptophan via interferon-gamma activation [164]. A recent review has further highlighted the interplay of immune markers and regulation of the kynurenine pathway, stating that key to this relationship is the indoleamine 2,3-dioxygenase enzyme (IDO) [165]. IDO appears to participate in crosstalk with markers of inflammation and though IDO plays a secondary role in pushing TRP towards kynurenine and further down kynurenine-metabolites under homeostatic conditions, the onset of inflammation drives IDO activity [165]. Pro-inflammatory cytokines, IL6, TNF-alpha, and IL-4, drive the expression of IDO through shared signaling pathways, like through the activation of AHRs [166]. IDO expression creates an environment that suppresses the immune response through the production of neurotoxic metabolites and depletion of the central immune-mediating amino acid Trp [167]. Multiple studies have also revealed that IDO is highly induced in colonic tissue and mucosa of patients with IBD and Chron's Diseases, both diseases that are marked by chronic inflammatory states in the gut and potential microbial interference, but for which the underlying taxa

and mechanisms are currently unknown [168, 169]. Inflammation is often correlated, if not implicated, with neurological disorders and declining health. The tight partnership between IDO activity and inflammatory markers may provide one piece of the metabolic-neurologic activity relationship puzzle. Tryptophan 2,3-dioxygenase (TDO), like IDO, is a rate-limiting enzyme that controls conversion of Trp into the early metabolites of the kynurenine pathway [18]. Whereas IDO can be found throughout host tissues, it is primarily expressed in the liver, where 90% of kynurenine metabolites are produced [170]. Hormonal cascades in the host, such as those induced by stress, have been shown to drive TDO activity in the liver and cause a resultant increase in circulating kynurenine metabolites [170].

Hepatic circulation and utilization of Trp metabolites is not limited to the kynurenine pathway. Tryptamine, produced from the decarboxylation of Trp by gut microbes, attenuates pro-inflammatory cytokine production in the liver [171]. Skatole is another Trp derivative, specifically a product of the indole pathway, that is produced in the gut by microbes but potentially bioactive in the liver. One study found that patients without liver disease (hepatic encephalopathy) had no detectable serum skatole, but those with signs of liver disease had detectable skatole levels between 0 an 442 nmol/L [172]. This study did not test the directionality of skatole levels and so did not postulate whether skatole was a causative agent of disease or a byproduct [172]. Skatole may be toxic at high concentrations in the body, but a recent study showed skatole may attenuate hyperlipidemic conditions of the liver [173]. Trp metabolites produced by microbes in the gut, like skatole and tryptamine, find their way to the liver through hepatic circulation and once localized, can either attenuate or exacerbate disease conditions. *p*-Cresol, a product of amino acid fermentation and [2] mentioned previously in relation to autism spectrum disorders, is another metabolite that is derived from microbial activity that enters hepatic circulation. Modification of *p*-cresol in the liver gives rise to uremic toxins and can intensify renal disease as well as promote systemic inflammation [2]. Microbial compounds in the gut that make their

way to the liver through hepatic circulation can then be further modified by the host, altering catabolite activity. Though liver activity is integral to understanding the interaction of Trp metabolites and host health, the gut-liver axis is not the direct focus of this review. More in depth coverage of the gut-liver axis and the contributions of gut microbes can be found in other reviews [2, 174, 175].

There are internal controls of metabolic-related enzymatic activity in the liver, the original limiting factor for the kynurenine pathway is ultimately the availability of the primary substrate, Trp. As Trp is an essential amino acid only acquired from diet and microbial metabolism, the availability of Trp in the gut is a necessary starting point for the estimation of circulating secondary metabolites. An 8-week long high fat diet in mice resulted in a measurable perturbation of gut taxa and subsequently significantly altered the gut, serum, and liver metabolome [171]. The gut microbial community has been shown to be a key driver of metabolism and so it cannot be discounted in the discussion around circulating kynurenine metabolites like anthranilic acid. Despite our understanding of the human contribution to circulating anthranilic acid compounds, it is not always possible to disentangle host and microbe metabolism from one another. The inability to fully dissociate the production of anthranilic acid complicates the already challenging problem of tracing this metabolite's biological activity in the host. Just as is it important to contextualize anthranilic acid research with the potential metabolic contributions of the humans being studied, so too is it important to understand microbial contributions to the shared metabolic pool.

Bacterial production of anthranilic acid and derivatives

The gut microbiome has the functional capacity to produce anthranilic acid and downstream metabolites from the breakdown of kynurenine, and ultimately from the original source of Trp. Research on microbial production of anthranilic acid and its derivatives mostly focuses on industrially

applicable microbes, which produce anthranilic acid in large quantities Anthranilic acid, and its derivatives, are used in the pharmaceutical industry for a myriad of applications, including as antimicrobials and as a potential regulator of diabetic symptoms [158]. In the food industry, the anthranilic acid derivative, methyl-anthranilate, is used as a flavor compound to impart a grape essence to candies and other food items [176]. Many microbes, though it may vary by species, have the enzymatic repertoire to perform either all or part of the kynurenine pathway [177]. A search using the OrthoDB database revealed six major bacterial phyla contain organisms with the kynureninase gene (*KYNU*), which catalyzes the conversion of kynurenine to anthranilic acid (Figure 3) [178]. Anthranilic acid to 3-hydroxyanthranilic acid is a non-specific hydroxylation step, but the conversion of 3-hydroxyanthranilic acid to subsequent quinolinic acid relies on the expression of 3-hydroxyanthranilate 3,4-diozygenase (*HAAO*). Five major phyla contain organisms with the *HAAO* gene, including the gut-dominating *Firmicutes* and *Bacteroidetes* (Figure 3) [178]. The microbial production of anthranilic compounds in the gut is an important, though understudied, area of research on Trp-related neuroactive compounds.

Studies that focus on the production of anthranilic acid by gut microbiota many times rely on a more universal approach to this analysis, highlighting the production in the gut by broad functional groupings and not by a specific taxon. The difficulty of teasing apart microbes and their metabolic products in the gut is in large part due to the complex interactions between microbes and the present diversity [177], which is coupled to a very high genomic diversity of microbiome members [179, 180]. One point that is indisputable however, is that the gut microbiota plays a major role in shifting the availability of free Trp within the host. Germ-free mice have altered free Trp levels compared to inoculated mice and show increased kynurenine pathway metabolites [181, 182]. The gut microbiota at large has the capacity to support the kynurenine pathway in the gut. Previous work demonstrated that the genomes of gut

bacteria contain the necessary enzyme homologs to produce anthranilic acid, kynureninase, as well as other enzymes responsible for producing neuroactive metabolites in this pathway [97, 177]. The strain or species that have higher or lower metabolic capacity for Trp turnover is largely missing from the literature in this area.

The ability to produce anthranilic acid or other Trp-related neuroactive metabolites may be species specific. Metabolic production is also context specific, as the host dietary intake and the resulting concentrations of these shared metabolic products shifts the equilibrium for both microbe and host. Human pathogen *Psuedomonas* can produce multiple components of the kynurenine pathway [49]. More relevant to this review, however, is that *P. aeruginosa* produces anthranilate and then uses it as a precursor to several virulence factors [49]. Kurnasov and colleagues characterized the anthranilate pathway in prokaryotes by doing a genome comparison and searching for three enzymes, tryptophan 2,3-dioxygenase, kynurenine formamidase and kynureninase, which together produce a eukaryotic-like anthranilate pathway [43]. Among others, *Burkholderia fungorum*, *Bacillus cereus*, *Ralstonia solanacearum*, and *Ralstonia metallidurans* were found to encode for all three anthranilate-related enzymes [43]. Another study looking at cold stress in pigs and its effect on microbiome diversity and activity identified anthranilic acid as one of the few metabolites that increased under cold stress conditions [183]. *Prevotella* UCG-003 revealed to be the most strongly correlated microbe with this cold-stress induced increase in anthranilic acid [183]. It is well defined that the gut microbiota is an important contributor to the production of neuroactive compounds from Trp.

Neurological outcomes of anthranilic acid

Research on the mechanistic role of anthranilic acid in neurological diseases has been limited up until now. Many studies focus primarily on the use of anthranilic acid and its derivatives as biomarkers [184], but don't necessarily drill down to determine the causative or protective effects of these compounds on host systems. While there is a place for studying these bioactive metabolites from the perspective of diagnostic potential, future therapeutic development will inevitably rest squarely on a mechanistic understanding of metabolic bioactivity. Current literature suggests that anthranilic acid leads a double life; the metabolite appears to exert neuroprotective effects in some cases while displaying neurotoxic effects in others [185, 186]. The potential neurological activity of anthranilic acid *in vivo* is complex and at times conflicting. Anthranilic acid resides at the increasingly explored intersection of diet, microbe, and host neuroactivity, and a more thorough understanding of anthranilic acid's bioactivity is important for future use as a biomarker or therapeutic target.

Anthranilic acid is often seen as part of the neurotoxic branch of the kynurenine pathway, a branch that includes quinolinic acid and hydroxykynurenine [187, 188]. Elevated serum levels of anthranilic acid have been measured in people with Schizophrenia and in those diagnosed with Parkinson's Disease [184, 189, 190]. Oxenkrug and colleagues showed circulating anthranilic acid levels in patients with schizophrenia were two times higher than those of the control population, confirming the results of a previous rat model study [184]. Though the study could not attribute the elevated anthranilic acid to anything specific, such as increased enzymatic activity in the pathway, the authors did affirm that the increase was not correlated with anti-psychotic drug intake [184]. Adding another layer of complexity to this puzzle is the difference seen between the sexes. One study found increased circulating anthranilic acid in schizophrenic subjects was not only related to neurological diagnosis, but also correlated with

being female [189]. Females in the study had plasma anthranilic acid levels 27% greater than those of their male counterparts, all of whom had a schizophrenic diagnosis [189]. It is possible this difference could be explained by sex hormones. For example, estrogen can inhibit some enzymes of the kynurenine pathway, or potentially by the deficiency of these enzymes resulting from other multifaceted signaling cascades that differ between biologically male and female subjects [191, 192]. The impact of estrogen on other signaling pathways has already been shown with another host and microbe derived neurotransmitter, dopamine. Previous work established estrogen effects dopamine activity, typically boosting synthesis and release, in both animal models and follow-up human studies [193]. Gut bacteria, including *E. coli*, some *Bacillus* species, *Klebsiella pneuomoniae*, and *Staphylococcus aureus*, have been reported to produce dopamine from tyrosine [194]. Though primarily thought of as a mammal's molecule, dopamine may be produced by bacteria as a quorum sensing molecule, increasing motility and biofilm formation, or as a growth enhancing factor, through improved iron regulation [194]. The interplay between the hormone estrogen, neurotransmitter dopamine, and microbial production of these bioactive compounds is already established in the literature, so it is not surprising to see emerging literature suggesting a hormone-metabolite cross talk in the context of other microbially-produced bioactive metabolites like kynurenine.

Beyond Schizophrenia, other neurological conditions (e.g., chronic migraines) have been correlated to the levels of circulating anthranilic acid. A study on chronic migraines in humans tracked the serum levels of multiple kynurenine metabolites and found a 339% increase in the concentration of anthranilic acid in those suffering from chronic migraines [195]. In tandem with that increase, the same study showed a 63% decrease in the downstream metabolite 3-hydroxyanthranilic acid [195]. Importantly, the study excluded subjects with neurological comorbidities, suggesting this marked increase in anthranilic acid was specific to migraines and not due to a known underlying cause. It is possible that

the raised anthranilic acid level is a response to neural activity and simply marks the diagnosis of chronic migraines. More interestingly, the authors of this study propose a combination of N-methyl D-aspartate (NMDA) receptor overactivity in migraines and the reduction of neuroprotectant kynurenic acid, which correlates with an increase in anthranilic acid, together contribute to the migraine symptoms [195]. This same research group also demonstrated concurring results in patients with a similar diagnosis of repeated cluster headaches. Those with cluster headaches had a 54% increase in serum anthranilic acid and a 54% decrease in 3-hydroxyanthranilic acid as compared to the healthy control patients [196]. Taken together these studies indicate anthranilic is certainly a part of headache-related diagnoses. It remains to be determined if this metabolite is causative or a responsive biomarker.

Evaluation of depression's metabolic roots has indicated there are correlating Trp metabolite concentrations. In one study on circulating anthranilic acid in patients diagnosed with depression, anthranilic acid was found to be inversely correlated to the severity of depression, though this finding was sex-dependent [197]. As was indicated in the case of Schizophrenia and biological sex, anthranilic acid levels associated with depression appeared more pronounced in women as compared to men in the same study [197]. This study was careful to exclude medication as a factor. Previous work has noted a difference in circulating kynurenine metabolites between medicated and non-medicated subjects [198], making medication an additional confounding factor for gut-brain axis research. A study detailing the onset of depression in patients undergoing treatment for Hepatitis C showed a decrease in Trp availability with a corresponding increase in anthranilic acid circulation [199]. During this 24-week longitudinal study, subjects with increased anthranilic acid levels were significantly more likely to be diagnosed with major depressive disorder [199]. The role of anthranilic acid as a biomarker, or even as a cause, of depression disorders is an ongoing area of research with promising findings for the development of potentially more targeted treatments. There are, however, conflicting studies which

show no correlation or weak correlation between depressive symptoms and anthranilic acid serum concentrations [200]. As human studies are often rife with confounding factors, this conflict amongst studies comes as no surprise. Biological sex [189, 197] and medication [198] can affect the availability of Trp metabolites, and are examples of factors that are not always accounted for but can play significant roles in determining the outcomes of these studies. To obtain meaningful and reproducible results is imperative that mechanistic animal model approaches are employed for a more concrete resolution to the involvement of anthranilic acid in depressive disorders.

Contrasting to predicted neurotoxic properties, studies have suggested that anthranilic acid derivatives may have neuroprotective bioactivity [55, 185]. Decreased 3-hydroxyanthranilic acid levels in stroke patients, along with the corresponding increase in precursor anthranilic acid, had a negative effect on health outcomes for recovering patients [201]. The inverse, increased 3-hydroxyanthranilic acid levels, is correlated with more signs of recovery in patients that suffered from a stroke [201]. 3-hydroxyanthranilic acid is a redox-active Trp metabolite. The increased levels of this compound in circulation may have a potent anti-inflammatory effect, stemming from both this redox activity and through the redirecting of anthranilic acid towards a more beneficial chemical structure [185, 201]. *In vitro* work with 3-hydroxyanthranilic acid has shown this compound to modulate the pro-inflammatory activities of macrophages, ultimately reducing generalized inflammation [202]. Inflammation plays a known part in many neurological disorders, as highlighted by a recent review [203]. Altogether, this indicates the immunomodulatory activities, and specifically inflammation-regulating activities, of anthranilic acid derivatives are key parts of this metabolite-host neurological link and a specific disease or etiology.

Research on Trp metabolites and their relation to neurological outcomes is ongoing and has resulted in an abundance of broad descriptive studies for Trp compound relationships in patients with neurological symptoms. Though examples have been identified for strong correlations between anthranilic acid and multiple disease states, not all neurological conditions are showing the same trend. Studies on multiple sclerosis in children and Huntington's disease showed no or weak correlations with anthranilic acid [204, 205]. A recent meta-analysis investigating the presence of Trp associated compounds across multiple neurological studies showed specific diseases have a weak correlation with anthranilic [206]. Results from human-microbiome studies can sometimes be conflicting, underscoring the complexity of host health and metabolic activity research. However, the observation of Trp and kynurenine metabolic pathway dysregulation as related to host health is well-supported in the literature, but the lack of correlation in cases means this neurological relationship is more complex than a single molecule or single enzymatic reaction. Instead, the relationship for a suite of metabolites to host neurology is likely owed to something more specific regarding the branching and balance (i.e., 'push/pull') of these metabolic pathways that is interconnected between the Trp concentration and the co-metabolism of the microbiome and the host.

The key to understanding this apparent paradox between neuroprotective and neurotoxic activity may be in part that metabolic pathways are not linear. These pathways are dynamic networks with many forces directing the movement of metabolism between the arms of complex interconnecting routes and shunts. Anthranilic acid alters the availability of its own primary precursor, kynurenine, and in so doing changes the compound equilibrium. Anthranilic acid is an inhibitor of 3-hydroxyanthranilic acid oxidase (3-HAO) enzymatic activity, the inhibition of which results in decreased production of quinolinic and picolinic acid from 3-hydroxyantranilic acid [55]. Iron is likewise able to inhibit 2-HAO enzymatic activity [50]. Interestingly anthranilic acid can readily chelate this circulating iron, resulting in a complex

interaction where iron may indirectly increase anthranilic acid concentrations through 3-HAO inhibition and the increased concentration may then sequester that very same iron [50, 55].

In pharmaceutical applications, anthranilic acid and related compounds have exhibited anti-inflammatory properties [158, 207]. In a study on rats modeling rheumatoid arthritis, a positive dose-dependent response to synthetic anthranilic acid derivative N-(3',4'-dimethoxycinnamonyl) anthranilic acid (3,4-DAA) was observed [207]. 3,4-DAA is structurally highly similar with 3-hydroxyanthranilic acid and 3-hydroxykynurenic acid. Arthritic rats received a daily dose of 200, 300, or 400 mg/kg, and all dose group displayed reduced inflammation via immunomodulatory activity [207]. Endogenous anthranilic acid is also suggested to have these same anti-inflammatory properties [55, 208]. 3-hydroxyanthranilic acid also has illustrated anti-inflammatory and antioxidant properties. Such properties are partially derived from the metabolite's induction of hemooxygenase-1, which is involved in the control of inflammation[185]. As metabolomics work is never black and white, focusing on a single metabolite alone does not provide a clear picture of potential bioactivity. Instead, it is necessary to examine the interplay of network flux, protein regulation and gene expression, all in combination with an understanding of the host-microbe interplay.

Anthranilic acid, along with its related compounds, cannot be considered singularly neuroprotective or neurotoxic. Perhaps these Trp-related metabolites should be considered more broadly as neuroactive, leaving space for the host context as a driver of ultimate effect relative to the local niche conditions. In the context of human studies, the directionality of metabolic levels cannot be fully assessed. One study reported that increased 3-hydroxyanthranilic acid levels, along with decreased anthranilic acid, were correlated with more beneficial clinic outcomes in stroke patients [201]. Measured serum

concentrations post disease diagnosis allows for the theory that many of these measured compounds are the clean-up or response-crew rather the instigators of disease conditions. There's also the additional component of host genetics and the predisposition to certain disease states to consider. Host genetics have been shown to predispose certain individuals to the onset of disease, as seen in some stroke cases [209] and with depressive symptoms [210]. Previous studies have also established that genetic variation across hosts is at least partially responsible, in conjunction with host diet and environment, for driving the composition of the gut microbiota [211, 212]. As host genetics play a role in in both disease state and microbial composition, it is important to also consider host genotype in the study of metabolic drivers of disease. Some studies with conflicting metabolic findings may look to host genotype as one possible explanation for subject-specific differences. To tease apart correlation and causality, along with host-specific findings, future research necessitates the use of tightly controlled studies and the additional use of *in vitro* models that utilize a time series design to understand the flux and interconversion between the Trp neuroactive catabolites.

Balancing the microbes and metabolites

Metabolic activity is a balancing act between different metabolic routes rather than a singular linear continuation to a compound in a static situation. Much of the current literature discussing bioactive compounds within the same metabolic network nods to this complexity by using ratios rather than absolute concentrations. The concentration of a single compound within a complex metabolic cascade does not often provide the necessary holistic perspective to determine potential health outcomes. Likewise, a singular focus on microorganisms via taxonomic analysis in this metabolic context is likely inadequate to describe a complete picture of such complex microbial interactions and relative abundance changes, even within the gut. Utilizing metabolic ratios, which highlight the push and pull

nature of metabolism, in place of static concentrations is one key component for an improved study design. This change enables many complexities to be captured in a dynamic niche that leads considering functional rather than taxonomic location to analyzing microbial consortia. This approach could also be very helpful when considering the microbiome changes to understand what microbes, but also what pathways, are enriching or depleting within the community of metabolism. These combined approaches also enable quantitative assessment to determine the ranked importance of metabolic routes as well as taxonomic groups as they change in the population relative abundance.

One part of a functional approach to host-microbe-metabolite interactions is looking at the population distribution of Trp-related metabolic genes in bacteria. Considering the push-pull method to regulate this pathway, it is very likely that specific steps in the pathway will be increased or decreased to change the intermediate concentrations as a flux balance. For example, when shunted towards the kynurenine pathway, Trp is first converted to kynurenine via tryptophan indoleamine 2,3-dioxygenase 1 or 2 (IDO1, IDO2) or via the liver enzyme tryptophan 2,3-dioxygenase (TDO) [177]. Kynurenine can then become kynurenic acid, 3-hydroxykynurenine, or anthranilic acid, which pulls the pathway towards other downstream activities [177]. 3-hydroxykynurenine and anthranilic acid both then feed into the production of 3-hydroxyanthranilic acid, the precursor for picolinic acid, quinolinic acid, and NAD+ [177]. A functional protein association network of human IDO1 (Figure 4) [213] confirms the interconnectedness push-pull nature of the three Trp pathways. A central node of IDO1 shows kynureninase (KYNU), tryptophan 5-monoxygenase (TPH1), and kynurenine formamidase (AFMID) are all in a shared genetic neighborhood (Figure 4A). Gene co-occurrence, as determined using the STRING database [213], reveals a broad distribution of these kynurenine-related enzyme encoding genes across the bacterial kingdom, making the concept of a taxon ratio useful (Figure 4B). Higher resolution down to specific strains will be required to obtain more meaningful insights. Two dominant gut phyla, *Firmicutes*

and *Proteobacteria*, both contain species with genes encoding *KYNU, TPH1, AFMID*. Some *Proteobacteria* also encode *IDO1* but no *Firmicutes* exhibit this ability. A deeper look at *Firmicutes* reveals the distribution of these genes is skewed towards the *Psuedomonadales* order and, interestingly, *Escherichia* lack these four genes. The five *Proteobacteria* orders display a more even distribution of kynurenine enzyme-encoding genes, with *Betaproteobacteria* the only order lacking species encoding *IDO1*. Though the analysis here covers only a small piece of the larger Trp metabolic network, the broad distribution shown here of kynurenine metabolism genes amongst common gut phyla both highlights the complexity of metabolic-related research but also the necessity of a functional approach to such work.

Conclusion

Host neurology and microbial activity can work in union or conflict with each other, and thus the tight regulation of both is essential for optimal host health. A symbiotic partnership between the gut microbiota and host is a beneficial exchange of substrates that results in increased access to nutrients for the host and readily accessible dietary precursors for the microbes. When the balance shifts, however, the host can experience detrimental neurological effects that are either a result of or exacerbated by microbially produced metabolic products.

Trp is one metabolite whose balance must be carefully regulated. Trp is funneled into the indole, kynurenine, or serotonin pathway, all of which contain other bioactive molecules in their ranks. Some Trp-derived compounds can have positive effects on systemic host function, like with serotonin and the immune and circulatory systems. Other Trp-derived compounds, like quinolinic acid, have detrimental effects on host function and have connections to neurological disorders, like dementia. Another

kynurenine-derived bioactive compound produced by both host and microbe is anthranilic acid. As detailed above, anthranilic acid can be useful as biomarker for disorders like Alzheimer's but anthranilic acid may also have a promising future use as a potential therapeutic target for the treatment of devastating and currently incurable neurological diseases. The gut microbiome has the functional capacity to shift the balance of anthranilic acid and subsequent metabolites, to the point of disease exacerbation or onset. Controlling this finely tuned metabolic network, especially in the context of the human microbiome, is a challenge that needs additional investigation before being used more practically as biomarkers or therapeutic targets.

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Tables

Table 1. Tryptophan-derived bioactive metabolites and their functions

Substrate	Metabolite	Known Microbial Producer(s)	Bioactive Function	Cofactors
Tryptophan	Kynurenine	*Pseudomonas aeruginosa* [42], *Ralstonia metallidurans* [43]	Neuronal damage mediator	INF-gamma [44]
	Indole	*Escherichia coli*, *Proteus vulgaris,* Clostridia sp., Bacteroides sp. [45]	Anti-inflammatory, Epithelial tight-junction regulation	Iron [46]
	Serotonin	*Pseudomonas putida* KT2440 [47]	Mood regulation	Vitamin B2 [48]
Kynurenine	Anthranilic Acid	*Pseudomonas aeruginosa* [49], *Burkholderia fungorum*, *Bacillus cereus*, *Ralstonia solanacearum*, and *Ralstonia metallidurans* [43]	Inflammation activation	Iron [50]
	3-Hydroxyanthranilic Acid	Proteobacteria sp., Actinobacteria sp., Firmicutes sp. Bacteroidetes sp., Chloroflexi sp., Cyanobacteria sp., Euryarchaeota sp. [51]	Free radical generation, Apoptotic regulation	Pyridoxal-5'-phosphate [52]
	Kynurenic Acid	*Escherichia coli* [53]	NMDA agonist, Anticonvulsant	
	3-hydroxykynurenine	Proteobacteria sp., Actinobacteria sp., Firmicutes sp. Bacteroidetes sp., Chloroflexi sp., Cyanobacteria sp., Euryarchaeota sp. [51]	Oxidative damage, Apoptotic regulation	
	Quinolinic Acid	*Streptomyces antibioticus, Cyanidium caldarium, Karlingia rosea* [54]	NMDA activator	Anthranilic Acid [55]
	Picolinic Acid	*Burkholderia xenovorans*, [56]	Iron and zinc chelator, Antiviral, Antifungal	Anthranilic Acid [55]
Indole	Indole-3-acetamide	Acidobacteria sp., Bacteroidetes sp., Firmicutes sp. [57]	Antioxidant, Anti-hyperglycemic	
	Tryptamine	*Ruminococcus gnavus* and *Clostridium sporogenes* [58]	Neuroinflammatory modulation	
	Indole-3-acetic Acid	Clostridia sp., Bacteroides sp., Bifidobacterium sp. [59]	Antiangiogenic	
	Indole-3-pyruvic Acid	*Pseudomonas fluorescens* and *Candida tropicalis [60]*	Anti-inflammatory, Anti-angiogenic	
	Indole-3-acetate	*Clostridium sporogenes* [61]	Anti-inflammatory	
	Indole-3-aldehyde	*Lactobacillus* sp. [62]	Epithelial cell cycle regulation, Goblet cell differentiation regulation	
	Indole-3-propionic Acid	*Lactobacillus reuteri*, Akkermansia sp., Clostridia sp., Peptostreptococi sp. [63]	Anti-inflammatory, Neuronal apoptosis regulation	
	Skatole	Clostridia sp. and Bacteroides sp. [64]	Induces colonocyte apoptosis	
Serotonin	Melatonin	*Roseburia hominis* [65]	Circadian rhythm regulation, Antioxidant	Tetrahydrobiopterin and O_2 [66]

Figures

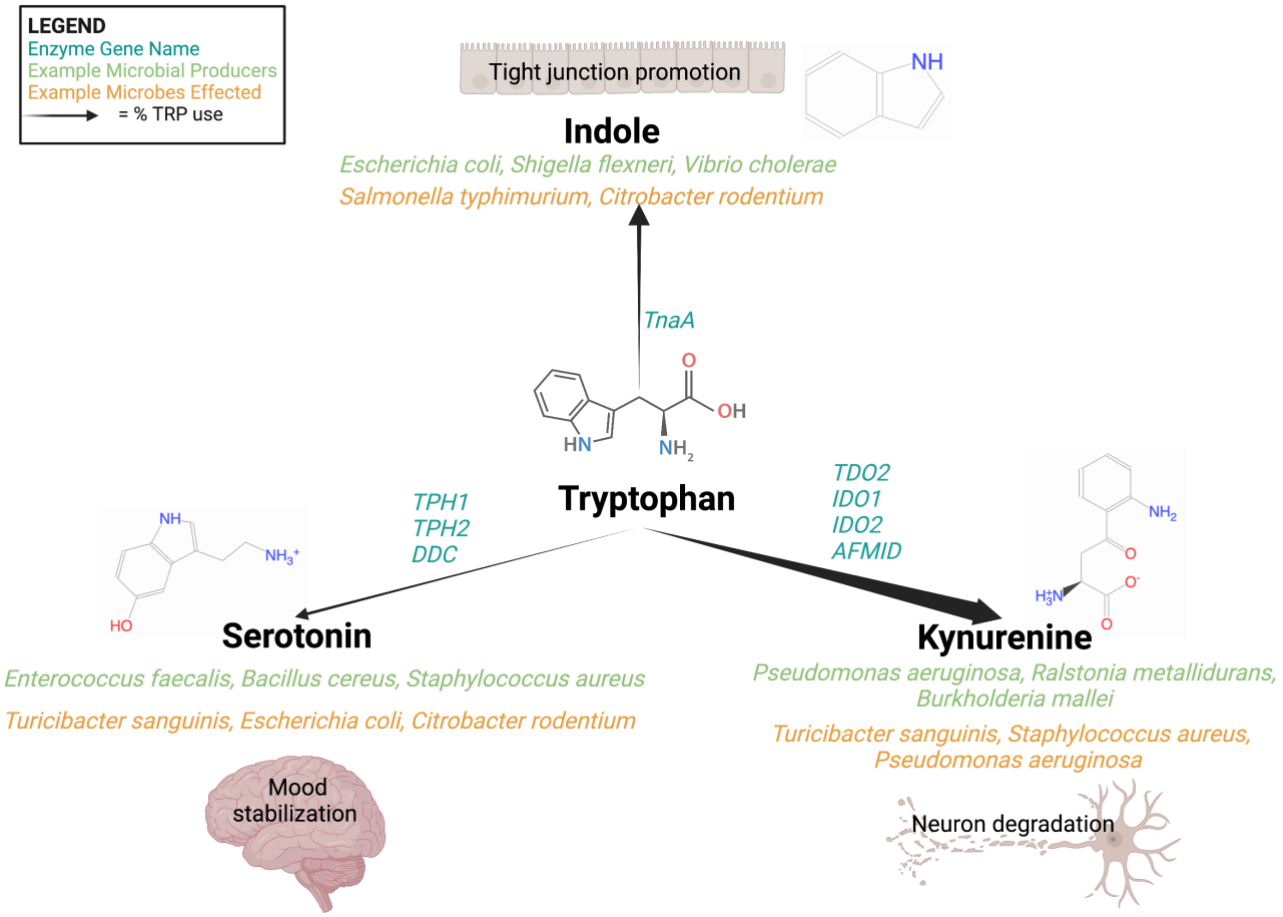

Figure 1. Tryptophan is a central amino acid and is channeled into three pathways: indole, kynurenine, and serotonin. Serotonin and kynurenine are produced by both microbes and mammals via multiple enzymes, listed in teal above. Indole, a tryptophan derivative produced solely by microbes. The majority of tryptophan is moved into the production of kynurenine and related metabolites, with the remaining portion split between indole and serotonin. Example microbial producers of these small molecules are in green while examples of microbes affected by these metabolites are in orange. The broader host effects are graphically represented above.

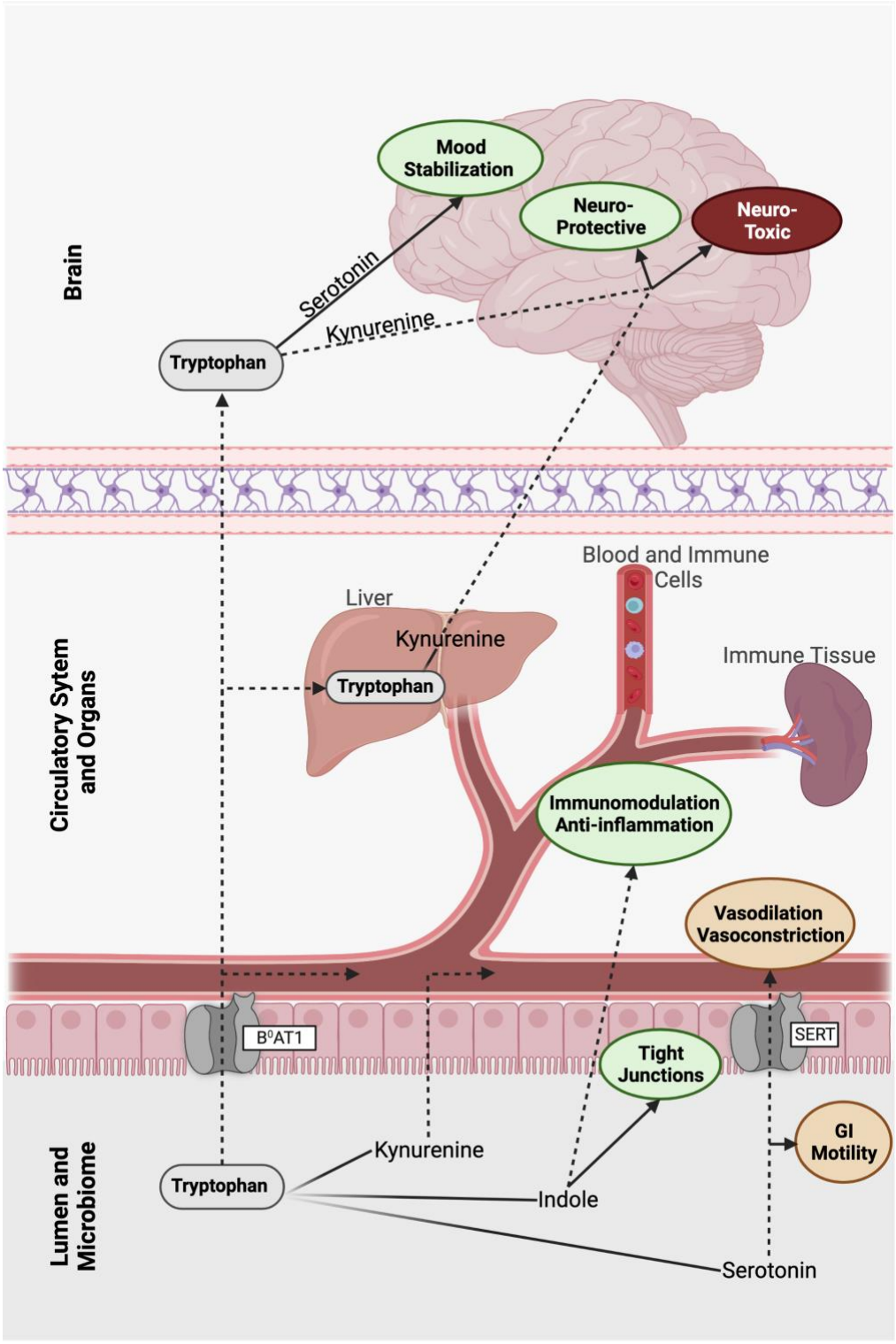

Figure 2. The three major routes of Tryptophan after ingestion, metabolism, and absorption in the human gut. Tryptophan can be absorbed into the blood stream and metabolized via the Kynurenine pathway in the liver, or via the serotonin or kynurenine pathway in the brain. Within the gut, tryptophan can also be funneled towards the kynurenine, indole, or serotonin pathway. These three metabolic pathways contain neuroactive metabolites that effect host physiology and neurology. Fading lines represent metabolism to a primary compound, dotted lines represent metabolism to secondary or downstream metabolites, green text ovals represent positive effects, red represent negative, and orange represent neutral outcomes.

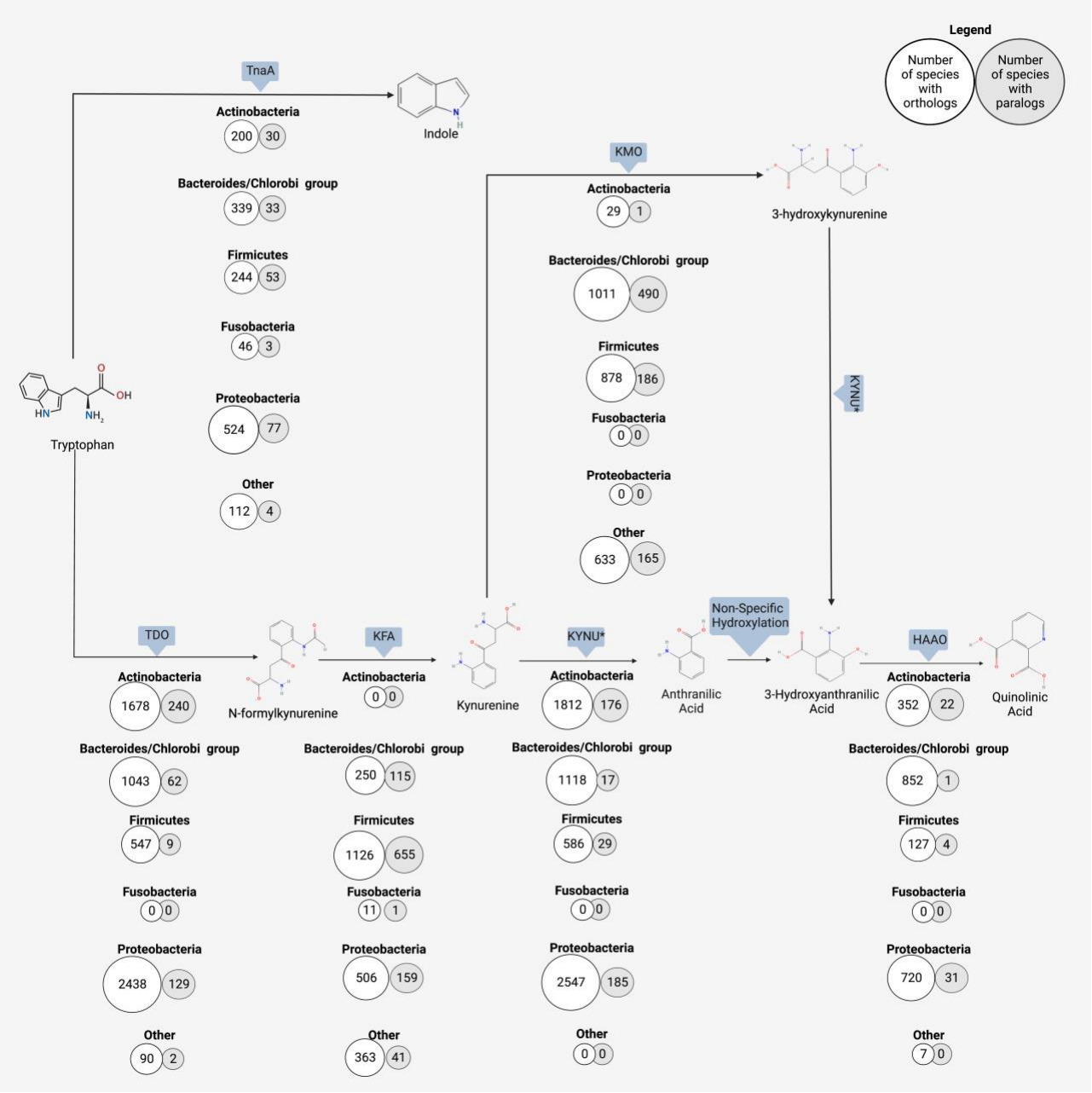

Figure 3. Orthologous and paralogous genes encoding enzymes for the microbial metabolism of tryptophan towards the kynurenine and indole pathways. The number of phyla containing orthologs of the microbial enzyme responsible for each reaction were determined through a search using the OrthoDB database. Enzymes or non-specific reaction types are indicated by rectangular shape above each phyla list. White circles indicate the number of species in the taxa containing orthologs and gray circles represent the number of species containing paralogs in that phyla. Six chosen phyla are shown, all other phyla with hits are grouped into "other". Indole is derived from tryptophan digestion by tryptophanase (TnaA). Down an alternate route from indole, kynurenine can be produced from tryptophan with a two-step process involving tyrptophan 2,3-dioxygenase (TDO) and kynurenine formamidase (KFA). The breakdown of the resulting kynurenine to anthranilic acid by microbes is catalyzed by Kynureninase (KNYU). KYNU also catalyzes the reaction of 3-hydroxykynurenine to 3-hydroxyanthranilic acid; this overlap in activity is denoted by an asterisk (*). Anthranilic acid is converted to 3-hydroxyanthranilic acid via non-specific hydroxylation. 3-hydroxyanthranilic acid can then be converted to multiple downstream metabolites, such as picolinic acid (not shown) or quinolinic acid (shown here). The production of quinolinic acid from 3-hydroxyanthranilic acid is catalyzed by 3-hydroxyanthranilate 3,4-dioxygenase (HAAO).

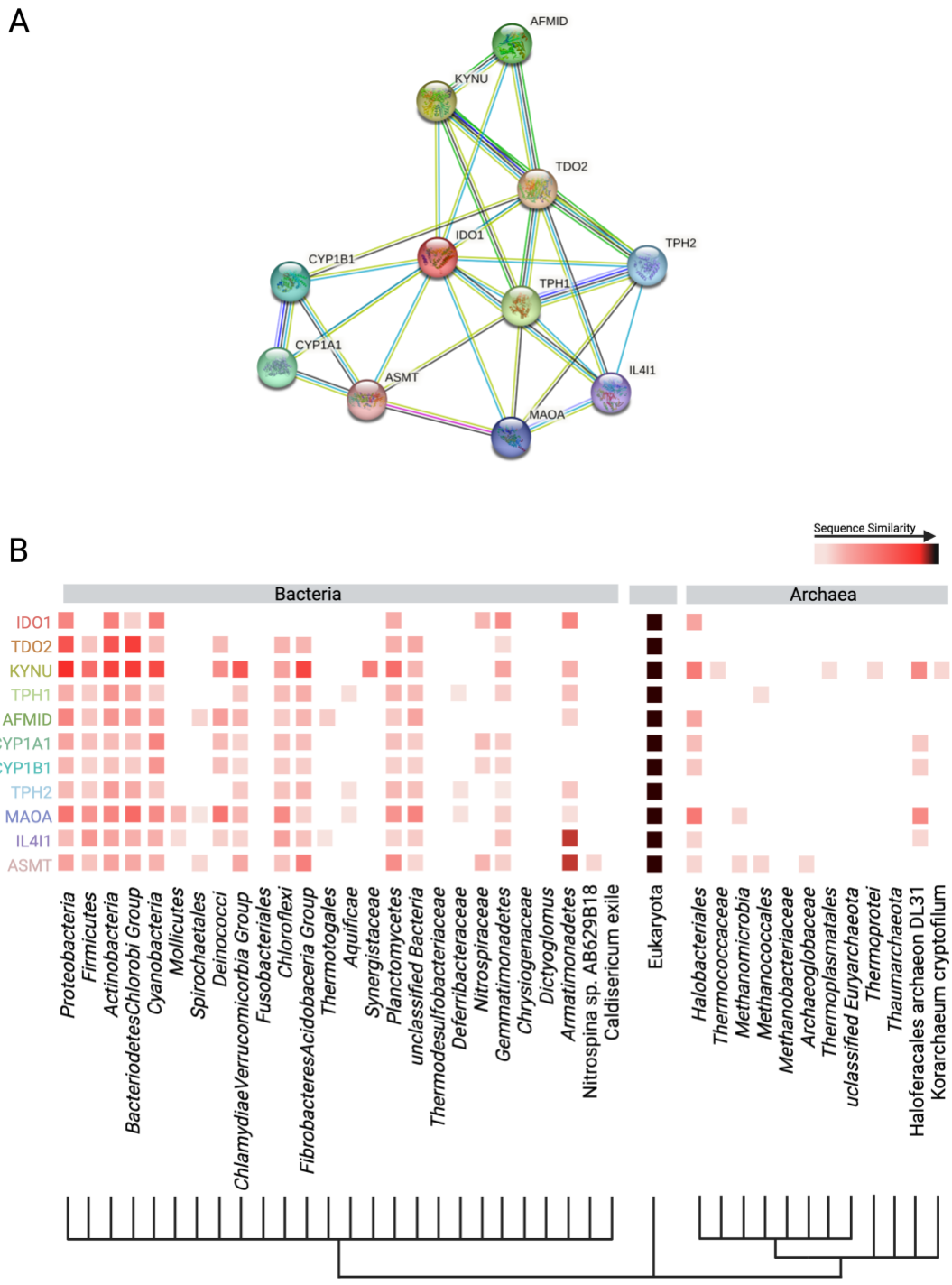

Figure 4. STRING database output from the search for *Escherichia coli* K12 MG1655 indole amine 2,3-dioxygenase 1 (IDO1), one enzyme responsible for the gut conversion of tryptophan to kynurenine. Full protein names are tryptophan 2,3-dioxygenase (TDO2), kynureninase (KYNU), tryptophan hydroxylase (TPH1), kynurenine formamidase (AFMID), human cytochromes P450 1A1 (CYP1A1) P450 1B1 (CYP1B1), tryptophan hydroxylase 2 (TPH2), monoamine oxidase A (MAOA), IL4-induced gene 1 (IL4I1), and acetylserotonin O-methyltransferase (ASMT). **A)** Protein interaction network with IDO1 as the central node. Light blue connections indicate known interaction from curated databases, pink lines indicate the interaction has been experimentally validated. Light green connections indicate the encoding genes are in the same gene neighborhood, red-orange lines indicate gene fusions, and dark blue indicate gene co-occurrence. Black lines indicate co-expression and light blue indicate protein homology. **B)** Gene co-occurrence across Bacteria, Eukarya, and Archaea of IDO1 encoding genes and related protein encoding genes. Color indicates highest sequence similarity within the taxa, with darker reds and black indicating higher similarity.

Chapter 3

Two-Component Systems Regulate Bacterial Virulence in Response to the Host Gastrointestinal Environment and Metabolic Cues

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Abstract

Two-component systems are a ubiquitous signaling mechanism in bacteria that allows for intracellular changes in response to extracellular cues. These regulatory systems in bacteria couple external stimuli to the control of genetic expression via an autophosphorylation cascade that transduces membrane signals to intracellular locations thereby allowing bacteria to rapidly adapt to the changing environmental conditions. Well known to control basic cellular processes, it is evident that two-component systems also exercise control over virulence traits, such as motility, secretion systems, and stress responses that impact the complex cascade of networks that alter virulence traits. In the gastrointestinal system, cues for activation of virulence-related two-component systems include metal ions, host-derived metabolites, and gut conditions. The diversity and origin of these cues suggest that the host can exert control over enteric pathogenicity via regulation in the gastrointestinal system. With the rise in multi-drug resistant pathogens, the potential control of pathogenicity with host cues via two-component systems presents a potential alternative to antimicrobials. Though the signaling mechanism itself is well studied, to date there is no systematic review compiling the host-associated cues of two-component systems and virulence traits. This review highlights the direct link between the host gastrointestinal environment and pathogenicity by focusing on two-component systems that are associated with the genetic expression of virulence traits, and that are activated by host-derived cues. The direct link between the host gastrointestinal environment, metabolites, and pathogenicity established in this review both underscores the importance of host-derived cues on bacterial activity and presents an enticing therapeutic target in the fight against antimicrobial resistant pathogens.

Introduction

Bacteria respond and actively adapt to their surroundings through a variety of well-characterized sensing systems. These sensing mechanisms are essential for adjusting the cellular state in response to shifts in local conditions as these local changes require an immediate response for survival. One response mechanism that has been studied in great detail, and which we will focus on in this review, involves two-component regulatory systems (TCSs). TCS mechanisms are ubiquitous in bacteria and are key regulators involved in a myriad of cellular processes, such as growth, biofilm formation, virulence, and secretion [1-4]. In this system, external stimuli are detected via multi-domain membrane-based proteins that facilitate an intracellular response by transducing the external stimulus into an internal cascade of signals via their corresponding response regulator domain in the cytosol. Phosphoryl transfer between the two proteins subsequently triggers the activation or repression of the corresponding genes that change the cellular response. While the basic structure of TCSs is conserved, the ultimate effect of each system depends on the various molecular building blocks of the specific TCS (e.g., signal molecule, structure of sensor and regulator, and the genes that are activated or repressed). Whereas TCSs are involved in many different cellular responses, this review will focus primarily on TCSs that are involved in controlling virulence among enteric bacteria that reside in various locations within the digestive system. Though the characteristic response cascade of TCSs is well studied, to date there is no systematic review of host-derived activators of these signaling cascades in host-associated pathogenic bacteria.

This curated presentation of a focused subset of these signaling systems draws a direct link between host metabolic and gastrointestinal activity and the control of virulence in the gut, highlighting the possibility of TCSs as therapeutic targets for pathogenic control. This review concentrates particularly on TCSs related to bacterial virulence and that are regulated by conditions in the gastrointestinal tract,

metabolic products, or key host-microbe nutrients (Table 1). Virulence-related TCSs for which the activation signal is not known or is not a host-derived cue will not be discussed here. Although the experimental verification of TCS activation by specific signals is understudied and therefore in some cases ill-defined, this review provides a valuable summary of some of the existing bacterial TCSs and highlights their prevalence and role in modulating the expression of genes that directly regulate virulence of enteric pathogens.

Host-Microbe Interactions at the Gastrointestinal Interface

The digestive tract, and especially the colon, is an ecosystem with high microbial density and activity. For bacterial survival in these highly competitive ecosystems, a rapid, continuous and well-tuned response is needed to accurately adapt to changing, environmental, nutrient and metabolite concentrations. With the high microbial density and exposure to nutrients from the host diet, the gut microbiome contains not only commensals but also a diverse set of opportunistic pathogens, foodborne pathogens, and host adapted pathogens, making tight control of the microbiome composition via metabolism shifts a requirement to avoid dysfunction [5]. One mechanism for the regulation of microbial growth is achieved by sensing metabolites in the immediate surroundings and many of the metabolites that regulate the microbial response can also affect host health directly and indirectly. Indole, for example, is a bacterial product derived from tryptophan that is well known for its ability to control many aspects of bacterial physiology [6], and in the host indole promotes the formation of tight junctions in gut epithelial cells [7]. Though indole exerts a protective effect in the gut, other microbial metabolites derived from the same precursor, tryptophan, can affect host behavior via interactions in the circulatory system and brain [8-10]. Since bacterial metabolites can affect host systems beyond the gut barrier, it is not surprising that metabolic products along with related systemic changes in the host

can also affect the transcriptional regulation of bacterial genes, and therefore bacterial growth and pathogenicity directly in the gut. One integral link in these host activity-pathogenic activity interactions are the TCSs sensitive to host conditions and cues.

Two-component systems and virulence

Two-component systems share a common overarching transduction method to translate extracellular signals into intracellular actions via phosphorylation. During the initial step of this cascade, a membrane-bound histidine kinase (HK) senses changes in extracellular conditions (e.g., pH, osmolarity, metal ions, nutrients, metabolites) and autophosphorylates at the histidine residue using an ATP molecule (Figure 1). This change at the HK is relayed to a cognate response regulator (RR) via subsequent phosphorylation of an aspartate residue of the RR (Figure 1). The activated intracellular RR then elicits the corresponding cellular action, typically the induction or repression of a gene or sets of genes that are involved in complex pathways (Figure 1). Response regulators in the TCS cascade can change the cellular response via direct transcriptional regulation, but the role of RRs is diverse and in some cases they also exercise control over the activation of other TCSs or are involved in other protein-protein interactions [11]. Two-component systems across prokaryotes share core signaling properties and often high sequence similarity [11], but small changes in the receiver or effector domains of these signaling proteins results in distinct activity and response, giving rise to the observed TCS variety amongst prokaryotes. Given their ubiquity and integral role in controlling bacterial activity, the commonalities of TCSs have been extensively reviewed and more details regarding the mechanisms of these systems can be found in other reviews [4, 12, 13].

Signaling systems, like the TCS, that display a multi-step relay that amplifies extracellular signals have been observed across different phylogenetic groups of bacteria. They were observed for the first time in *Escherichia coli* four decades ago [14] and there is now strong evidence of TCS related proteins in all kingdoms of life, except in animals [15]. TCSs regulate many bacterial activities, ranging from basic growth control to chemotaxis to pathogenic capability. Given the wide range of processes that TCSs control, it is unsurprising that single cells can possess multiple different histidine kinase/receptor protein sets. *E. coli*, for example, is known to have at least 29 TCS sets (HK/RR pairs), while *Salmonella enterica* serovar *Typhi* has 30 HK/RR pairs, and *Pseudomonas aeruginosa* has at least 64 genes that encode distinct response regulators and 63 genes for different HKs, the most TCS pairs that have been reported in one bacteria to date [16-18]. The prevalence of these signaling systems coupled with continuously increasing whole bacterial genomes has led to the development of methods for predicting the target genes of TCSs in prokaryotes [19]. Predictive methods, coupled with experimental confirmation, have expanded our overall understanding of TCSs and continually introduce potentially novel mechanisms for the control bacterial activity. The ubiquity and continued expansion of known TCSs together indicate that these systems may offer new targets to control bacterial infections and ultimately, perhaps, become an antibiotic alternative to control infection [20].

TCSs facilitate the tight control of basic cellular processes, and while many of the known TCSs can be critical to cellular survival, none may be more relevant to medical applications than those that regulate virulence [21]. As with many cellular processes, individual TCSs are not the sole determinant for virulence. Instead, a combination of environmental triggers, differing HK sensor domains, and a vast network of response regulators often work simultaneously to promote or repress virulent traits. The bacterially dense and diverse human gut microbiome is rich in known and yet to be discovered TCSs [22]. Pathogens, whether opportunistic full-time residents of the human gut or transient pathogens,

rely on exogenous stimuli that indicate ideal conditions for deployment of virulence traits (e.g., flagellar movement, biofilm formation, secretion systems, and metal sequestration) during the onset of disease initiation [23-25]. Coordinated expression of virulence genes is essential for the adaptive advantage of the pathogen, since upregulation of these virulence genes in suboptimal conditions leads to inefficient use of cellular machinery, while downregulation of these genes when conditions are optimal would equally represent an evolutionary disadvantage for the pathogen's persistence [26, 27]. The balanced regulation of virulence genes requires sensitive and strong signaling cascades, which is something that TCSs, especially those that are sensitive to multiple cues, are capable of doing.

Biomolecules (e.g., amino acids) and cofactors (e.g., metal ions) play a role in virulence regulation via interaction with TCSs and metabolite-TCS interactions have been described for a variety of organisms in the gut, including *Pseudomonas aeruginosa* and *Escherichia coli* [16, 28]. The interplay of host and microbe in the context of microbial pathogenesis and metabolite-TCS interactions presents an interesting but vastly understudied area that brings about an opportunity to modulate virulence using non-antibiotic approaches in the complex gut microbiome. Recognition that small molecules are primary drivers of the complex host-microbe interplay further supports the concept that the actions of both the microbiome structure and the host metabolism are important in promoting and maintaining microbiome diversity and ultimately host health.

Host Metabolic Products and Conditions as TCS Triggers

Metal Ions

Metal ions are imperative to basic functions for host and microbes but can also be toxic once they exceed critical concentrations, making metal consumption a delicate balancing act. Metal requirements

bring a particularly daunting challenge when balancing dietary metal acquisition to sustain basic processes like iron-dependent hemoglobin production and to support the gut microbes' metabolism, while avoiding accumulating metals at toxic levels or leaving extra metal ions circulating for pathogen utilization via siderophores [29]. Presented with pathogenic challenges, the human immune system will undergo nutritional immunity, a metal sequestering event prompted by inflammation [30]. In opposition to host protections, and as co-evolving interactors with the host, microbes have evolved their own mechanisms for metal sequestration, co-opting, and utilization. The first step in the activation of these systems is the activation of a metal-responsive HK sensor. Metal responsive TCSs (e.g., CoIRS, CusRS, PhoPQ, CsrSR) are extremely diverse among organisms and can lead to varying phenotypic changes and impact on virulence. A brief description of how metals interact with different TCSs is described below.

Iron is well known as both a participant in diverse cellular processes and as a regulator of immunity and virulence. The BasRS system in *E. coli* is iron sensitive, promoting lipopolysaccharide (LPS) modifications via activation of the stress response and inducing polymyxin resistance [31]. BasRS is also involved in the bacterial response to metal toxicity, activating the production of membrane metal transporters [31]. This same TCS in *E. coli* responds to mild acidic conditions by inducing the *aceF* and *aceE* genes, which are both part of the pyruvate-dehydrogenase operon, ultimately resulting in increased acetate metabolism [31]. It should be noted that the BasRS TCS in *E. coli* is homologous to the PmrAB system, later discussed in the context of *Salmonella enterica* serovar Typhimurium and LPS remodeling.

Zinc is another metal, like iron, known to be important in the regulation of the immune system and pathogenic activity. The ColRS two component system, found in *Pseudomonas aeruginosa* and

Pseudomonas putida, responds to the presence of Zn^{2+} and induces changes in LPS [32, 33]. *P. aeruginosa*, a nosocomial pathogen, is an adaptive and hardy drug resistant pathogen that relies on its LPS to survive and evade host defenses [34, 35]. ColRS contributes to this LPS-related adaptability, as activation of this TCS by Zn^{2+} promotes changes to the lipid A structure, the common target of antibiotics, contributing to *P. aeruginosa* survival and evasion of treatments [32]. $EptA_{pa}$ is pEtN transferase that can modify the 4' phosphate group of lipid A in *P. aeruginosa* and is under transcriptional control, at least in part, by the ColRS TCS [32]. In the presence of exogenous Zn^{2+}, the ColS HK activates the ColR response regulator which then upregulates $eptA_{pa}$ and leads to the remodeled and more resistant lipid A structure [32]. In conjunction with the upregulation of the pEtN transferase protein, the production of a different modifying protein, aminoarabinose transferase (ArnT), is decreased as a result of the ColRS cascade [32]. The upregulation of one transferase and simultaneous downregulation of another by the same TCS signal indicates both that the *P. aeruginosa* maintains tight control of LPS modifications in response to environmental conditions and that the ColRS system exerts multifaceted control over what appear to be singular endpoints, like the remodeling of LPS.

Just as zinc is sequestered by the host and co-opted as a cue by microbes, copper (Cu) is also part of the immune system's antimicrobial arsenal, shuttled to the sites of invasion to cause bactericidal metal toxicity [30]. As with other metals, some bacteria have evolved mechanisms to overcome the host's immune system response to sequester metals. In the case of Cu, *E. coli* and *Klebsiella pneumoniae* evolved the CusRS system [22, 36]. The CusS HK senses Cu^{+} and Ag^{+} and relays that to the internal CusR response regulator, which in turn activates transcription of the *cusCFBA* gene cluster. This operon encodes for a tripartite efflux pump that allows for the removal of metal ions when the concentration exceeds the cellular threshold. This TCS allows *E. coli* and *K. pneumoniae* to dodge the host's attempt at

bactericidal control and thus allows for further growth and invasion leading to a more favorable environment for the pathogen to cause disease.

Magnesium, much like zinc and copper, is essential to basic functions in both host and microbe. Mg^{2+} serves as a cofactor for over 600 enzymatic reactions, is important in cellular proliferation, and is involved in the excitation-relaxation coupling cycle [37]. Although Mg^{2+} is critical to many basic processes, this metal ion is not well absorbed in the gut with its uptake primarily controlled by the kidney. The lack of active Mg^{2+}transport from the gut lumen makes this mineral more available for bacterial use and it is therefore not surprising that numerous microbial Mg^{2+} transporters and sensing systems exist [38]. *Streptococcus pyogenes* encodes its own Mg^{2+} regulated TCS, CsrSR, which interfaces with up to 15% of the *S. pyogenes* genome, including genes related to phagocyte resistance and increased invasion [39]. CsrSR shares some structural similarity with the TCS PhoPQ, discussed below [39] and it is possible that CsrSR functions similarly to PhoPQ or even resulted from a common evolutionary ancestor. CsrSR co-opts the host immune response to optimize pathogenesis, enhancing resistance to phagocytosis, the primary host defense for group A streptococci via sensing host antimicrobial peptides, such as LL-37 [39]. In contrast, Mg^{2+} appears to decrease this resistant phenotype, perhaps conserving cellular energy for more ideal conditions [39]. In response to low Mg^{2+} concentrations, the CsrSR system in *Streptococcus* is inactive and the capsule genes regulated by CsrSR, *hasABC*, remain available for transcription [40]. The inactivation of CsrSR in response to low Mg^{2+} concentrations is in line with host physiological conditions, as the low ionic concentrations that repress the CsrSR TCS are typical of host extracellular circulating fluids, and so production of the protective hyaluronic capsule, repressed by activation of CsrSR, remains important to pathogenic success [40]. Conversely, higher Mg^{2+} activates the CsrSR cascade and in turn represses the capsule genes (*hasABC*) and other pathogenic secretion product genes (*ska* and *sagA*) [40]. The sensing of Mg^{2+} concentrations

by CsrSR in *P. aeruginosa* confers to the pathogen the ability to determine whether the cell is within a host (low Mg^{2+} concentrations) or in a less-favorable environment for pathogenesis like soil or waste water (higher Mg^{2+} concentrations). This ability to sense environment confers a competitive advantage through the ability to deploy energetically-taxing virulence mechanisms primarily in locations of likely success. The CsrSR TCS is less well characterized than other highly conserved TCS but does appear to contribute to virulence in group A streptococci via the sensing of Mg^{2+} and a host produced antimicrobial peptide.

Mg^{2+} is also tightly connected with virulence in multiple gut-based pathogens, including *Salmonella enterica* serovar Typhimurium [38]. Two systems that use Mg^{2+} as a signal related to virulence, PhoPQ and PmrAB, are highly conserved and appear in many bacterial genera, further highlighting the integral and evolutionary role of magnesium in cellular function [22, 41-46]. Besides Mg2+, Ca^{2+}, Fe^{3+}, low pH and antibiotics can also activate these systems. When experimentally inactivated, the PhoPQ system in *Stenotrophomonas maltophilia* is unable to respond to the typical low Mg^{2+} environmental cue, resulting in increased susceptibility to b-lactam antibiotics [41]. *Salmonella* species with experimentally inactivated PhoPQ system possess decreased virulence, indicating that the PhoPQ TCS is intergral to virulent *Salmonella* phenotypes [44]. Another conserved TCS that is often found in conjunction with PhoPQ, the PmrAB system can cooperatively interact with the PhoPQ TCS. In *S.* Typhimurium, PmrAB activation upregulates the *pmrHFIJKLM* operon, leading to LPS remodeling via 4-aminoarabanose addition [43]. LPS restructuring reduces the binding ability and thus effectiveness of certain positively charged antimicrobial peptides, though the activation of *pmrAB* related genes may also make *S. enterica* serovar Typhimurium more susceptible to other antimicrobial peptides [43]. Though universal protection against antimicrobials is not conferred via a single TCS cascade, TCS-induced LPS alterations can reduce pathogen susceptibility to certain antibiotics, as is the case with the PmrAB TCS in *P.*

aeruginosa and the subsequent decreased vulnerability to colistin When PmrAB and PhoPQ are both present in an organism they interact by transcriptional regulation; with PhoPQ activating the PmrAB TCS via the PhoPQ-regulated *pmrD* gene [43]. *S. enterica* serovar Typhimurium has both these systems and though PmrAB is directly dependent on PhoPQ for activation, the result of these two TCSs are in conflict [43]. More specifically, *S. enterica* serovar Typhimurium PhoPQ knockouts showed decreased resistance to antimicrobials while PmrAB knockouts displayed increased antimicrobial resistance [43]. The resistance phenotype in the case of these two TCSs is due in part to alterations in the rigidity and permeability of the cell membrane, with PhoPQ resulting in a more rigid, less permeable exterior and therefore a more resistant phenotype [43]. The contrasting cellular outcomes between PhoPQ and PmrAB, especially considering the known dependency of PmrAB on PhoPQ activation, highlights the importance of understanding the cellular response to not just singular TCSs but also the results of multiple or interacting TCSs within a single organism. For a bacterium being able to rapidly respond to continuously changing environments, having multiple TCSs work in tandem results in a more fine-tuned response to the presence/absence of external cues like antibiotics, which in turn allows for a quick and customized adaptation to a wide range of conditions via multiple convergent expression pathways.

Amino Acids

Amino acids are key metabolites for host and microbe health, as both require amino acids to support protein catabolism, basic metabolic pathways, and fundamental cellular processes. Just as humans lack the ability to synthesize essential amino acids, some bacterial genomes lack genes required to catabolize certain amino acids [47]. While this tug-of-war over amino acids also makes them crucial to some host defense mechanisms, where the host attempts to sequester amino acids, bacteria have equally evolved mechanisms for efficient amino acid uptake and utilization [47]. TCSs, like GluKR and AauRS, play a role

in the amino acid battle as sensors that use amino acids to regulate the genes and thus phenotype of bacteria [48, 49].

In addition to the role of glutamate in central metabolism, glutamate is well known for its essential role in the glutamate-dependent acid resistance pathway, a bacterial defense mechanism against host acidification [47]. *Streptomyces coelicolor* is a soil-dwelling bacterium and is not known as a prolific human pathogen, but since *S. coelicolor* is a member of the *Streptomyces* genus, it is possible that its TCS cascades may also be found in other *Streptomyces* species that are known human pathogens. The genome of *S. coelicolor* encodes GluRK, a TCS that responds to glutamate [48]. In *S. coelicolor* it appears that circulating glutamate also regulates the glutamate uptake system and synthesis of some antibiotics [48]. Upon phosphorylation via the GluK sensor, GluR activates the glutamate uptake cluster *gluABCD* via direct binding of the response regulator to the promoter region [48]. In addition to the increased production of glutamate transport proteins, previous work on glutamate related TCSs indicate that genes involved in glutamate metabolism may also be directly under the control of this TCS, though that was not confirmed in this study [48]. More directly related to virulence traits, the GluRK TCS promoted the expression of genes encoding several antibiotics like actinorhodin, undecylprodigionsin, and type-1 polyketide [48]. The increased expression of antibiotics upon GluRK activation with glutamate appears to be an independent cascade from that to produce glutamate transporters [48]. The antibiotic regulation is controlled through secondary signals and not through direct binding between the promoter region and GluR [48]. Glutamate is central to bacterial metabolism and exerts control over the virulence-related stress response in many bacteria [47]. The control of antibiotic expression also appears to be peripherally controlled by glutamate in the host-controlled environment, through signaling mechanisms adjacent to the GluRK TCS [48]. Though GluRK is currently only proposed in the conext of *S. coelicolor*, the central role of glutamate in enteric pathogens suggests GluRK or a similar TCS

may also be found in human pathogens as well. To date, studies on the GluRK system are limited and additional work in this area might provide promising new insights into the biological role of this system in enteric pathogens.

As with *S. coelicolor* and the GluRK system, the AauRS complex in the plant pathogen *Pseudomonas syringae* and opportunistic human pathogen *P. putida* may provide an opportunity to shed light on TCSs in *Pseudomonas* species that are human pathogens, as many of these receptor systems appear to be conserved across multiple genera [22, 50, 51].

The AauS histidine kinase senses extracellular aspartate and glutamate, then activates the *aatJ* region via the response regulator AauR, all together upregulating amino acid uptake systems alongside activation of Type 3 Secretion Systems (T3SS). Together this cooperatively increases the virulent phenotype and therefore the risk to host [49]. Interestingly, the relationship between the AauRS system and the T3SS appears to have an evolutionary history, as this combination co-evolves and is conserved across at least 17 organisms [52]. The AauR response regulator, discussed here in the context of the plant pathogen *P. syringae,* can also be found in the metagenome of human microbiomes (Figure 2). The AauR regulator was identified in the genome of members from *Klebsiella, Oscillibacter, Firmicutes* and *Intestinimonas*, indicating that the gut microbiome likely harbors these vital signaling systems in a multitude of organisms for which they are not currently experimentally confirmed. Continued community contribution and careful curation of genetic databases, such as the ones highlighted in Figure 2, is vital for the expansion of TCS knowledge and for the implementation of these systems as therapeutic targets, especially as provided by analysis of the ever-expanding microbiomes that are available.

Amino Acid Metabolites

Just as amino acids play key roles in host-microbe interactions, so do their subsequent metabolic products. As central metabolism substrates, amino acids are responsible, or at least have connections, to almost all major metabolites. Aromatic amino acid metabolites include neurotransmitters (NTs), such as epinephrine, a tyrosine derivative, and serotonin, a tryptophan derivate, which are of particular interest since they have multiple important activities in the host [53, 54]. Not limited to host production or use, neurotransmitters can be produced, utilized, and co-opted by the microbiome, sometimes to the benefit and sometimes to the detriment of the host [55]. In the context of TCSs, neurotransmitters are sensed by bacterial adrenergic receptors, which then relay the external stimulus to the response regulator and corresponding genes, regulating the virulent phenotypes of pathogens, just as done with metals. QseBC and QseFE both use epinephrine as an interkingdom signal for sensing the host condition and subsequently to increase virulence potential within the host gut [56-59]. The QseC sensor is a bacterial adrenergic receptor found across a diverse set of genera and is often linked to virulence, suggesting that the QseC sensor has been conserved over many generations or perhaps co-evolved with the host neurotransmitters [57]. Beyond amino acid derived neurotransmitters, the QseC sensor is responsive to endocannabinoid metabolite, 2-arachidonoylglycerol (2-AG) [60]. The endocannabinoid system is beyond the scope of this review but recent work has linked the QseC sensor and LEE pathogenicity island, both responsive to amino acid derived metabolites, to host-derived endocannabinoid molecules and so will be mentioned briefly in this section. Also discussed in this section is another amino acid derived neurotransmitter, nitric oxide (NO), and its related metabolites, nitrite (NO_2^-) and nitrate (NO_3^-), which exert control over motility and biofilm formation in multiple pathogens [61]. Central to many metabolic routes in both the host and microbe, amino acids serve as precursors to many bioactive molecules. A few of these amino acid derived bioactive molecules are covered here in their relation to the control of virulence through interactions with TCSs.

In most cases, the response to TCS activation by host NTs results in the upregulation of secretion systems, increased motility, drug resistance and biofilm formation [46, 56-59, 62, 63]. The utilization of host NTs as cues to increase pathogenic phenotypes is not surprising, as the NTs signal a state of stress and thus to bacteria signal a weakened host and ideal conditions for pathogenesis. As neurotransmitters of the sympathetic system, the nervous system involved in the stress response of the host, epinephrine and norepinephrine levels are moderately elevated in response to chronic stress and significantly elevated in response to acute stressors [64]. Disease states, whether chronic or acute, are arguably activators of the physiological stress response, a concept supported by the observable increase of circulating epinephrine in patients with Crohn's Disease and Irritable Bowel Syndrome [65]. The QseC sensor uses elevated epinephrine levels to trigger pathogenesis in the gut. The utilization of this stress-related host NT is likely of evolutionary advantage to the pathogen as increased epinephrine levels may correspond in some cases to weakened host defenses.

Serotonin is produced by the host, both by raphe nuclei in the brainstem and by the gut epithelium [66, 67]. Additionally, the microbiome can produce this from the essential aromatic amino acid precursor tryptophan as well as respond to this compound as a ligand. Opposite to the role of epinephrine and norepinephrine, serotonin appears to decrease the pathogenicity potential of enterohemorrhagic *E. coli* (EHEC), as illustrated in mice using the model organism *Citrobacter rodentium* [68]. *C. rodentium* and *E. coli* rely on the locus of enterocyte effacement (LEE) pathogenicity island for the primary virulent apparatus, the T3SS, and the concomitant effectors that are injected into the host cell [68]. Increased luminal serotonin levels cues the CpxA HK to decrease the phosphorylation of the corresponding response regulator CpxR, the inactivation of which causes decreased transcription of the LEE

pathogenicity island [68]. The repression of LEE in turn decreases the virulent phenotype of *C. rodentium* and lowers the pathogenic load in the gastrointestinal system [68]. Conversely, decreased luminal serotonin levels do not actively repress the CpxR regulator, leading to active transcription of the LEE island and expression of the related virulence factors [68]. The CpxRA TCS is also sensitive to indole, another tryptophan derived metabolite and one that is solely produced by bacteria. Indole in the large intestine forms a gradient with high luminal indole levels that decrease towards the epithelial layer [69]. Higher indole levels, like those seen in the lumen, repress the CpxRA signaling cascade, thereby decreasing the expression of virulent traits encoded by the LEE pathogenicity island [69]. In contrast, the low indole concentration observed at the epithelium activates the CpxA HK in the membrane of *C. rodentium* and leads to the phosphorylation of response regulator CpxR [69]. CpxR then promotes the transcription of the T3SS and cofactors, ultimately increasing the virulence of *C. rodentium* when in close proximity to the epithelium [69]. The sensitivity of CpxRA to both indole and serotonin provides flexibility for enteric pathogens encoding this TCS and allows for more tailored responses that are not only targeted towards the host gastrointestinal system, but to specific regions. Serotonin and indole are both tryptophan (Trp) catabolites and both exert some control over the virulent phenotype of pathogens in the gut, indicating that other Trp derived compounds may also display similar bioactive capabilities. Further work to examine the details of Trp catabolite actions between the host and the microbiome is needed for examination of the complex interplay between molecules that both host and microbe produce but utilize very differently.

The QseC sensor and the LEE pathway, both mentioned above in relation to different amino acid derivatives, converge in the context of the host-derived endocannabinoid metabolite, 2-arachidonoylglycerol. Though not an amino acid derivative itself, 2-AG is a host-derived metabolite that is an antagonist for the QseC receptor in EHEC and *C. rodentium* and thus is important to mention in

order to highlight the diversity of cues and responses, even within the context of a single sensor [60]. 2-AG attenuates the virulence of EHEC and *C. rodentium* via modulation of the LEE pathogenicity island, which is described in more detail above [60]. The endocannabinoid system is a known neuromodulatory system that plays a crucial role in controlling the most basic physiological and homeostatic processes [70]. The interaction between endocannabinoid metabolites and the virulence-modulating sensing systems of enteric pathogens is an emerging field and one that should be explored further, especially in light of the central role endocannabinoids play in systemic host physiology and disease [70].

Arginine, like the aforementioned amino acids tyrosine and tryptophan, is a key precursor to bioactive metabolites that serve as cues for virulence related TCSs in enteric pathogens. Nitric oxide is a neuroactive molecule derived from arginine that is responsible for the regulation of host physiology, as with its role in peripheral vasodilation, and has emerging roles in the modulation of neurological disorders like Schizophrenia [71, 72]. Bioactive NO can be produced from exogenous and endogenous arginine via nitric oxide synthase (NOS) activity, or from nitrite (NO_2^-) and nitrate (NO_3^-) [73]. Nitrite and nitrate, together with nitric oxide make up the nitrate-nitrite-NO pathway, a dynamic pathway involved in regulation of the cardiovascular system, the modulation of metabolic functions, and the control of inflammation [74]. Nitrite and nitrate are bioactive molecules, able to regulate microbial metabolic and pathogenic activities via the NarLX and NarPQ TCSs, and can be derived from leafy greens in the diet or from the breakdown of arginine [61, 74].

As a review by Rocha et al. highlights, the broad effects of nitrate and nitrite on the gut microbiome at large remain somewhat obscured by the complexity of tracking the source and the route of these multitasking substrates throughout the host [75]. Some research indicates that nitrate does alter the

composition and activity of the gut microbiota in disease states, modulating the nitrate-nitrite-NO pathway, improving dysbiotic conditions [76]. Though the mechanisms of generalized control of gut microbiome activity and diversity by nitrates remains somewhat unclear, the direct interaction of nitrate and nitrite with specific gut pathogens is well understood to be regulated by the TCSs NarLX and NarPQ [77]. NarLX and NarPQ, both involved in nitrate/nitrite sensing, communicate via crosstalk between sensor NarQ and response regulator NarL [78, 79]. In *P. aeruginosa*, NarLX knockouts display increased biofilm formation in conjunction with decreased motility [77]. Mutants with only response regulator NarL attenuated but functioning NarX sensors displayed opposing activities, with decreased biofilm formation and increased swarming [77]. Both NarLX effects can likely be attributed to the control of rhamnolipid production, a key glycolipid that acts as a biosurfactant and is an antibiofilm compound [77]. Nitrate metabolism in *P. aeruginosa* is central to the pathogen's success in the gut, as nitrate can be used as a terminal electron receptor for respiration and the regulatory glycolipid, rhamnolipid, is derived from nitrogen metabolism [77]. *E. coli* uses nitrate and nitrite in a similar way to *P. aeruginosa* and relies on the NarLX TCS to indicate environmental nitrogen conditions to trigger targeted cell responses [78, 80, 81]. *E. coli* additionally uses the NarPQ TCS to sense nitrate in the local environment [78]. The two TCSs interact with NarQ activating regulators NarP and NarX, and NarL activation serves as an inhibiting signal for NarQ-NarX crosstalk [78]. A recent study connected NarL activation by nitrate to mitigatation of the production of CgsD, a protein that contributes to the development of biofilms in *E. coli* [82]. The link between NarL and biofilm formation is apparent in multiple pathogens and suggests a clear bacterial signaling and activity cascade: the activation of NarL by nitrate is a positive indication to the cell of sufficient exogenous nitrogen sources for pathogenic growth, and therefore the protection biofilm formation provides is not necessary for survival in these nitrogen sufficient environments. Similar biofilm control by the NarLX/NarPQ TCSs can be seen in human pathogen *Burkholderia pseudomallei* [61, 83]. As nitrate and nitrite control and metabolism is central to many cellular

processes, it is not surprising that many symbiotic and pathogenic bacteria have one or both of the previously described nitrogen-sensing TCSs or a homolog of these TCSs [84-86], though more detailed mechanistic research is necessary to link these nitrogen related TCSs directly to the modulation of virulence in more pathogens.

Environmental Changes and Other Signals

The gastrointestinal environment is a tightly regulated ecosystem with a network of epithelial transporters, sensors, and feedback loops for the management of nutrient reabsorption, acidity, and immune responses. The gastrointestinal environment and its associated microbiome, while under tight control, displays temporal (via diet and host metabolism), longitudinal (across compartments), and spatial (from lumen to mucosa) succession. As with specific metabolic products, some histidine kinase receptors can sense broader signals, such as fluctuating pH and osmolality. EvgAS in *E. coli* responds to an acidified environment, with moderate regulation at pH~5.5 and increased response at pH2.5 [87]. Activation of the EvgS HK in acidic conditions upregulates the *E. coli* survival-activating promoter AR2 via the EvgA response regulator, leading to increased acid resistance via the stringent response [87]. As with many TCS systems, it remains unclear whether EvgAS senses the pH drop exclusively, or whether there is a combination of factors that activate this sensor in cooperation with pH changes and other stress conditions. EvgAS is a conserved system across *E. coli* genomes and its behavior varies in response to stress signals, with some evidence that EvgAS does not act alone to activate the stress response to alter the TCS activation [88]. For example, the well conserved metal-sensing *phoPQ* operon encodes for a stress sensing TCS, and PhoPQ appears to act in tandem with EvgAS, perhaps even through direct signaling between these two TCSs [88]. Further complicating the regulatory cooperation, EvgAS is inhibited by indole [87]. In the presence of indole, the AR2 promoter is downregulated, subsequently silencing the stress response in *E. coli* [87]. The opposing effect of indole on the EvgAS

system is partially explained by indole's role as an intracellular pH regulator and as indole's alternate activity as a bacterial signaling molecule that changes many phenotypic traits important to persistence and infection. Downregulation of the metabolically and energetically taxing cellular stress system in the presence of increased bacterial neighbors may be optimizing *E. coli*'s growth patterns in complex environmental conditions. The multiple activities of the Trp-derived catabolites as ligands and multi-functional modulators complicates the regulatory dynamics of the TCS and the highly flexible cellular response.

TrxSR is one of 13 TCSs identified in *Streptococcus* and one of three TCSs known to regulate virulent phenotypes. Much like EvgAS in *E. coli*, TrxSR responds to an acidified extracellular environment, and promotes the multiple gene activator (*mga*) virulence regulon, ultimately promoting quorum sensing and biofilm formation [89]. One of the other three virulence TCSs in *Streptococcus* is HK/RR11, an additional regulator of biofilm formation in the presence of low pH [90]. It is important to note that the activation of some TCSs depends on more complex stress-response cascades. Such cascades involve a network of signaling molecules and sensors to start the autophosphorylation that are not directly relevant to host derived molecules or are reliant on molecules that are produced by the microbiome as well [91]. These TCSs with more complex signaling, though related to pH, are outside the scope of this review and thus not discussed here.

Osmolarity is another gut condition that has many contributing factors. Osmotic stress is linked dysbiotic conditions in the gut, such as inflammatory bowel disease and Crohn's disease, and osmotic stress can be a marker for the onset of a pathogenic infection, like that seen with *Listeria monocytogenes* infection [92-94]. Osmolarity of the gut is thought to be utilized by some bacterial TCSs

as an indication of conditions favorable for infection, triggering the onset of virulent phenotypes [95-97]. The EnvZ-OmpR complex is a well-characterized and widespread TCS known to respond to osmotic conditions. In *E. coli* the TCS is active in medium osmotic conditions and in turn controls the production of the OmpC and OmpF porins, ultimately controlling intracellular access to metabolites [98]. In multiple *Yersinia* species the OmpR response regulator regulates virulence traits including antibiotic resistance and motility [99]. *Y. pestis*, like many pathogens, has sophisticated iron uptake and regulation mechanisms in order to both evade the host's immune sequestration of iron and to ensure adequate sources of this central metal ion (see section 4.1 for more on metal ions). OmpR has been identified as one response regulator that directly induces production of the iron-regulating HemR1 protein via binding to the related Phem-1 promoter region [99]. The activation of genes involved in the production of heme receptors as a response to external conditions is invaluable for pathogenic success, as the sense and response mechanisms allows for tighter control and a more attuned virulent response. Though the role of the OmpR regulator in many *Yersenia* species is known, additional work is necessary to confirm the full TCS cascade and to specify the specific environmental cues that activate or repress this cascade.

Conclusion

The ubiquity of TCSs throughout the genomes of individual members of the gut microbiome community members, combined with the ability of TCS to respond to host cues to regulate virulence, emphasizes the importance of these signaling systems in the context host-microbe interactions. Not only do these systems modulate bacterial stress and metabolism, TCSs also modulate biofilm and virulence traits through diverse mechanisms, making them interesting targets to control bacterial behavior. Perhaps the unique control offered by TCSs makes them opportune target for future research aimed at understanding how to modulate bacterial virulence without the use of antibiotics. Metabolic products are known to have roles beyond their direct involvement in basic metabolism, like with tryptophan-

derived serotonin, serving as metabolic substrates, host neurotransmitters, and bacterial signaling molecules. The role of metabolic products as signaling molecules for influencing bacterial phenotypes is not novel, but it is an underutilized therapeutic option, especially with the rise in multidrug resistant pathogens. Host-associated molecules like metal ions, amino acids, and amino acid metabolites and host gastrointestinal conditions like pH contribute to the regulation of virulence traits in the gut microbiome via their role as signals for TCSs. Despite their ubiquity, metabolite activated TCSs remain an understudied area, with very few experimentally confirmed whole cascades, which define the entire pathway from signal to sensor to effect. This lack of evidence, in part, stems from the cooperativity and complexity of the initiating ligands that induce overlapping responses. The area of TCS-host metabolite cross talk is a rich one that requires more investigation to clearly define the specific opportunities to use these systems as alternatives to antimicrobial use. Given the multifaceted activities and diverse bioactivity of metabolites produced by both the host and the microbiome, and the established importance of the gut microbiome, it is worth further exploring the expanded role of metabolites and regulators of gastrointestinal conditions as regulators of the microbiome and as contributors to host health.

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Tables

Table 1. Two-component receptor systems (TCS) sorted by signal type. TCS represented by the histidine kinase (HK) and response regulator (RR) pairs. Result of TCS may be due to either repression or activation post-signal response. Systems marked with * are highly conserved TCSs across genera.

	Signal	HK-RR Pair	Result	Genus
Metal Ions	Zn^{2+}	ColS-ColR	LPS modification	*Pseudomonas [32]*
	Cu^{+}/Ag^{+}	CusS-CusR	Tripartite efflux pumps	*Escherichia [36], Klebsiella [22]*
	Mg^{2+}	CsrR-CsrS	Activation of virulence repertoire	*Streptococcus [39]*
	Mg^{2+}, Ca^{2+}	PhoQ-PhoP*	LPS modification, Low Mg^{2+} adaptation, Antimicrobial resistance, PmrAB cross talk	*Escherichia [22], Salmonella [44], Yersinia [45], Stenotrophomonas [41]*
	Mg^{2+}, Fe^{3+}, Zn^{2+}	PmrB-PmrA* BasS-BasR	Stress response, Antimicrobial resistance, PhoPQ cross talk	*Escherichia [22], Pseudomonas [46], Salmonella [43]*
Amino Acids	Glutamate	GluK-GluR	Antibiotic synthesis	*Streptomyces [49]*
	Glutamate, Aspartate	AauS-AauR	Swarming, Motility, Biofilm formation	*Pseudomonas [50, 51]*
Amino Acid Derived Molecules	Serotonin (Via Tryptophan)	CpxA-CpxR	Type 3 Secretion System	*Citrobacter [69], Escherichia [69]*
	Epinephrine/Norepinephrine (Via Tyrosine)	QseC-QseB	Biofilm formation, Flagellar motility	*Escherichia [60], Haemophilus [63], Salmonella [57]*
	Epinephrine (Via Tyrosine)	QseE-QseF	Type 3 Secretion System	*Escherichia [59]*
	Nitrate/Nitrite (Via Arginine)	NarX-NarL NarQ-NarP	Motility, Biofilm formation, Nitrate Sensing and Reduction	*Burkholderia [84], Escherichia [81], Pseudomonas [78], Salmonella [85]*
Environment & Other	Acidic pH	EvgS-EvgA	Multidrug Transporter	*Escherichia [88]*
	Acidic pH	TrxR-TrxS	Biofilm formation	*Streptococcus [90]*
	Acidic pH	HK11-RR11	Biofilm formation	*Streptococcus [91]*
	Osmolarity	EnvZ-OmpR	Intracellular iron regulation	*Escherichia [99], Klebsiella [22], Pseudomonas [22], Yersinia [100]*

Figures

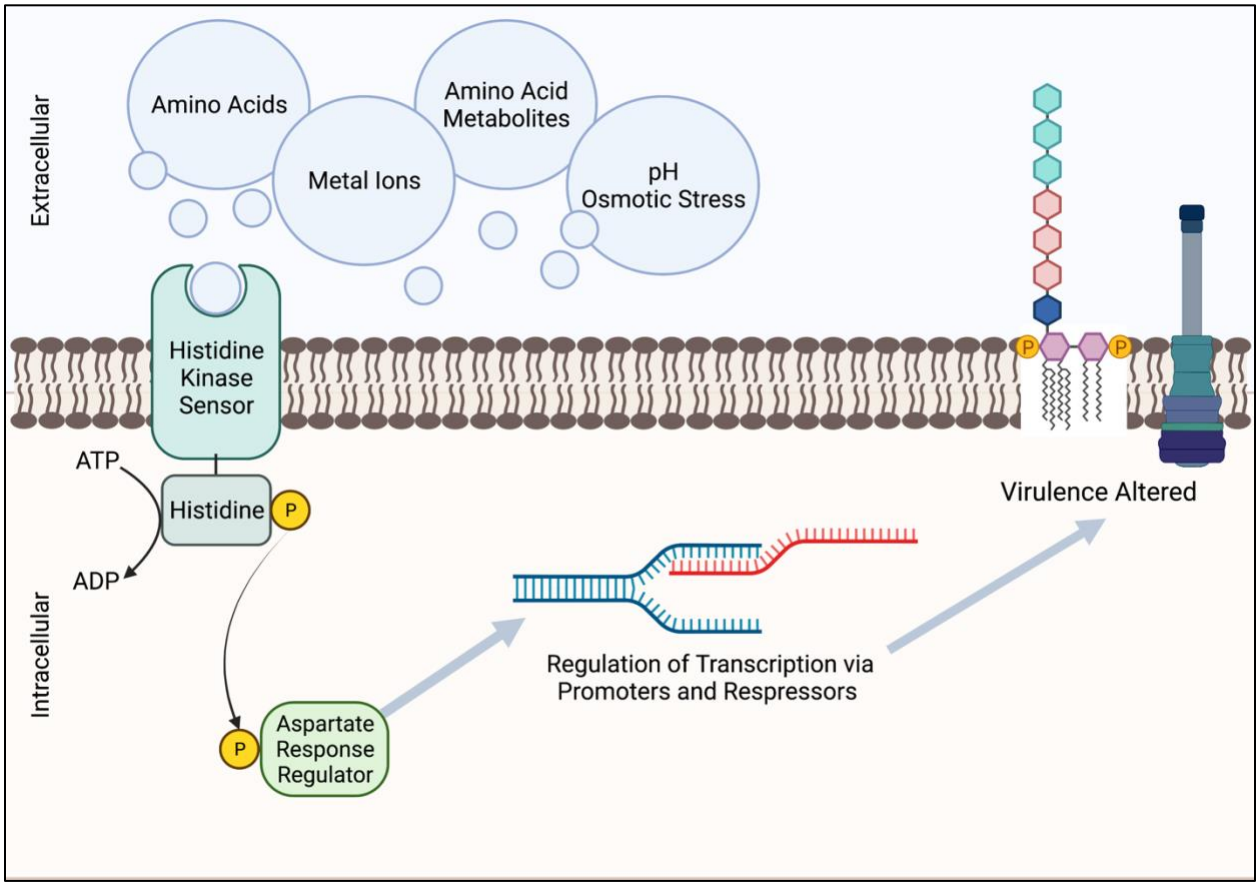

Figure 1. Two-component receptors systems (TCS) related to virulence can be modulated by host-derived metabolic products. Signaling molecules are sensed via the multidomain surface membrane protein histidine kinase (HK), which is then undergoes autophosphorylation. The phosphoryl group is then transferred to an intracellular aspartate residue response regulator (RR). Transcription of the corresponding genes is then repressed or activated, dependent upon the originating TCS and signal. Virulence factors controlled in part by TCSs include LPS modifications for decreased host detection and upregulation of type 3 secretion systems (T3SS).

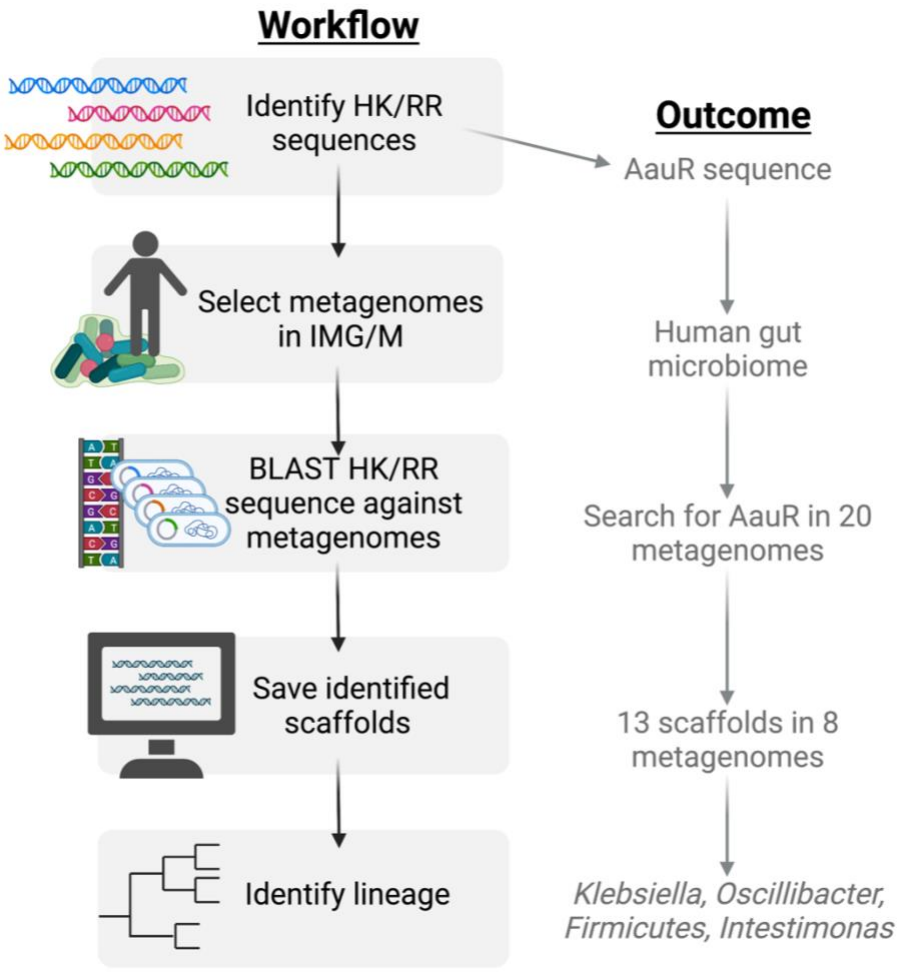

Figure 2. How to search for two-component systems in ecosystems via metagenome-specific BLAST. Known histidine kinase and response regulator sequences can be found via multiple genetic databases, including NCBI GenBank. Publicly available host and ecosystem-specific assembled metagenomes can be found on the Joint Genome Institute's Integrated Microbial Genomes and Microbiomes (IMG/M) platform. BLAST searches with imported HK/RR sequences restricted to specific metagenomes can be done via the IMG/M platform. Search hits in the form of metagenome scaffolds can be saved and analyzed for lineage and further analysis. This platform was used to search for the AauR response regulator in the human microbiome. 20 different human microbiome metagenomes were used as the search parameters, out of which 8 returned hits for the AauR regulator. The results were further narrowed into 4 different taxa: Klebsiella, Oscillibacter, Firmicutes, and Intestinimonas.

Chapter 4

Cellular Respiration and Amino Acid Metabolism Is Altered by Structurally Diverse Dietary Oligosaccharides in *Salmonella* During Epithelial Association

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Abstract

Dietary prebiotic oligosaccharides, complex carbohydrates that support beneficial bacteria, are ubiquitous on marketplace shelves and in people's diets. Though widely accessible and consumed, little is known about how different prebiotics alter enteric infections in a dysbiotic gut. Here we show two structurally different prebiotic oligosaccharides, human milk oligosaccharides (HMO) and mannanoligosaccharides (MOS), alter the metabolism of colonic epithelial cells and pathogenic *Salmonella enterica* sv. Typhimurium in ways specific to each prebiotic during infection. Initially, HMO and MOS addition decreased *S.* Typhimurium association with epithelial cells. However, gene expression analysis revealed significantly induced expression of Specific Pathogenicity Island (SPI) 1 (adj P < 2.0e-6) and 2 (adj P < 3.0e-5) with HMO treatment opposed to increased fimbriae expression (adj P < 3.0e-3) in MOS treatment. Both host and pathogen metabolism were likewise altered by prebiotic treatment. MOS treatment induced the expression of genes related to amino acid metabolism in both the host cells and in *S.* Typhimurium, a metabolic shift that was not observed in the HMO treated cells. MOS also altered respiratory metabolism in *S.* Typhimurium more closely aligned to those observed *in vivo* during gut inflammation, which is divergent from colonization-type expression in HMO treatment. Alteration of virulence observed in this study was found to be prebiotic specific and dose dependent, indicating that some dietary substrates likely alter specific pathogens to change their virulence potential in unanticipated ways that lead to multiple outcomes to potentiate or attenuate enteric infections.

Introduction

Dietary additives that are reported to selectively feed beneficial gut bacteria, termed prebiotics, are commonplace and advertised to the consumers as a panacea for a myriad of health benefits. The US prebiotic market alone is currently worth more than $6 Billion and is growing rapidly [1]. Prebiotic oligosaccharides, complex carbohydrates consisting of between three and 10 monosaccharides, are one prebiotic grouping thought to provide beneficial health effects through their roles as privileged substrates for probiotic bacteria [2, 3] and through modulation of the intestinal barrier [4-6].

Though classified together under a single label, prebiotic oligosaccharides are structurally diverse and very different in the overall monosaccharide composition. Fructooligosaccharides (FOS), galactooligosaccharides (GOS), and mannanoligosaccharides (MOS) are all commercially available functional oligosaccharides derived from different source material and with diverse structures [7, 8]. Not yet commercialized for broad consumption, but still considered prebiotic oligosaccharides, are human milk oligosaccharides (HMO). HMOs are a combination of structurally diverse oligosaccharides produced by the mother in breast milk and consumed by the infant and destined for microbial digestion in the large intestine [9]. The structural and functional diversity of prebiotic oligosaccharides increases the complexity around disentangling substrate-microbe-host interactions and their related health outcomes [10]. Though there has been some research into the mechanisms underpinning positive health outcomes, much remains unknown about the effect of structurally diverse prebiotic oligosaccharides on members of the gut microbiota. Much of the research has focused on the beneficial aspects of prebiotics and their use in combination with probiotic bacteria [11, 12]. In contrast, relatively little research has focused on the impact of prebiotics on important enteric pathogens, including *Salmonella enterica* sv. Typhimurium.

S. Typhimurium is the most prevalent enteric pathogen in humans and is responsible for over 80 million cases of foodborne illness and 155,000 deaths per year globally [13]. Dietary prebiotics present one path towards the mitigation of this pervasive enteric pathogen, but further investigation is required before any can be regularly applied clinically. In previous studies, prebiotics have been employed for the *in vitro* and *in vivo* control of enteric pathogens such as *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica* sv. Enteritidis with limited success [14-18]. The modulation of enteric pathogens by prebiotic substrates is in part suggested to be driven via prebiotic-pathogen binding, precluding pathogen binding to host receptors. Certain prebiotics like HMOs and GOS contain glycan structures similar to those found on the gut epithelial cell surface and used by pathogens for host adherence [19]. Enteropathogenic *E. coli* incubated with GOS prior to host introduction showed significantly decreased host association, but GOS was unable to displace already adhered *E.* coli, suggesting prebiotic-pathogen binding prevented initial adherence to host cells [14]. Similar decoy mechanisms have been shown with *Campylobacter jejuni* and α-1-2-fucosylated glycans, a component of HMO [20]. Prebiotics have also been shown to modulate the immune system irrespective of commensal microbes through interactions with host Toll-Like Receptors (TLR) and G-protein coupled receptors (GPCR) [21, 22]. More generalized protective effects of prebiotics have been attributed to the production of short chain fatty acids (SCFAs), which are small molecules from commensal microbial sources in the gut that support a healthy gut barrier, regulate energy metabolism, and modulate inflammation [23, 24]. The currently understood mode of action for prebiotics suggests the existence of healthy gut microbiota and epithelia is required, indicating gut dysbiosis may impede the function of prebiotics and suggests prebiotics exist as prophylactics, rather than a treatment for enteric infections.

Utilizing a focused system to examine differentiated Caco2 cells during infection with *S.* Typhimurium without any additional microbiota, we showed two structurally different prebiotic oligosaccharides,

HMO and commercially available MOS (Biomos®) differentially drive host-pathogen metabolic crosstalk around two major energy-producing routes that altered redox balance in the host-pathogen system and modulated host-pathogen metabolic interactions. This also led to changes in related amino acid metabolism, mitigating the pathogen's colonization of host cells via prebiotic-specific mechanisms. Though pathogen-host association was attenuated by prebiotic treatments, virulence factor expression in *S.* Typhimurium was induced in both prebiotic conditions that did not result in higher infection conditions. Interestingly, the enzymes needed to digest the host glycan were repressed, which may account for the lack of invasion. These observations indicate that a complex relationship exists between prebiotics and pathogens. Dietary supplements in this study altered pathogenic activity in previously unexpected ways, illustrating the impact diet can have on enteric pathogens and highlighting the potential for such supplements to exert off target effects with significant impact to host health.

Results

Prebiotic pre-treatment of caco2 cells decreases Salmonella association

Pre-treatment of Caco2 cells with structurally different prebiotic oligosaccharides altered the combined invasion and adhesion activity of *S. enterica* serovar Typhimurium LT2 in a dose-dependent manner (Figure 1). *S.* Typhimurium LT2 was capable of infecting Caco2 gut epithelial cells, evidenced by successful pathogenic activity in the control condition with no prebiotic pre-treatment. Pre-treatment of the Caco2 cells at all tested concentrations with either BioMos® or HMO reduced the association of *S.* Typhimurium LT2 with the differentiated host cells. When added at 0.1% (w/v), BioMos® showed a 59% reduction (p-value < 0.02) in adhesion and invasion while HMO showed a lesser effect at 28% association reduction (non-significant). At 0.5% Biomos® reduced LT2 association by 54% (p-value < 0.05) and HMO by 59% (p-value < 0.05). The max tested concentration, 1% prebiotic, had a 44%

reduction (non-significant) in association in the Biomos® treatment while at this same concentration, HMO addition resulted in an 82% decrease in LT2 association (p-value < 0.02). Increased levels of Biomos® decreased efficacy of the pre-treatment while increased HMO levels increased efficacy.

S. Typhimurium 14028 is 98% genetically identical to *S.* Typhimurium LT2 [25], so the dose dependent response to prebiotic treatment seen in LT2 was predicted to carry over to *S.* Typhimurium 14028 activity. *S.* Typhimurium 14028, being more pathogenic than the lab strain LT2, was used to evaluate the response of host-pathogen interactions to prebiotic treatment via metatranscriptomics and metabolomics.

Expression of Salmonella virulence factors and Caco2 receptors altered by prebiotics

The reduction in association to host cells of *Salmonella* in both prebiotic conditions led us to investigate if the modulation of known virulence factors in *S.* Typhimurium 14028 and pathogen-sensing Toll-Like Receptors (TLRs) in the Caco2 cells could explain the adhesion and invasion activity. Thirty-five out of the 44 virulence genes included in this analysis of virulence genes were induced in the HMO condition, while only 16 of the 44 were induced by Biomos® treatment. Genes related to Specific Pathogenicity Island 1 (SPI-1) and 2 (SPI-2) were all significantly induced (between $-\log_{10}P = 6.7$ and 17.6, adjP < 3.0e-5) in the HMO treatment but were unchanged or repressed in the BioMos® treatment (Figure 1). SPI-1 and SPI-2 are both important gene cassettes that drive virulent behavior, with SPI-1 supporting initial invasion and adhesion of host cells and SPI-2 aiding in vacuole escape and systemic spread [26], so their exogenous regulation can potentiate virulence in specific host contexts. SPI-1 genes *sipA*, *sipB, sipC, sipD, prgK, hilA, invA* and *invG* were all unchanged by BioMos® treatment, whereas SPI-2 genes *sseG, sseD, sseC* and *sifA* were repressed and *sseF, sseE, sseB, sifB,* and *sifA* were induced with BioMos®

addition (Appendix Figure 1), suggesting invasion into the epithelial cell was unchanged and escape from the vacuole increased between the treatments.

Fimbriae, structures important for adhesion prior to invasion [27], likewise displayed mixed expression patterns between the prebiotic treatments. HMO treatment of *S.* Typhimurium showed induction of *fimA, fimF, fimY* and *fimZ*. BioMos® treatment repressed *fimF* and *fimC,* but significantly induced *fimY* ($log_2FC = 6.5$, $- log_{10}P = 11.5$), which encodes a mannose-binding type one fimbriae [28]. Expression of virulence genes is in part controlled by two-component systems (TCS), which consist of a membrane-bound receptors and internal response regulators that modulate transcription based on the external feedback. Given their role in virulence, the expression of 13 TCSs across prebiotic treatments was evaluated (Table 1). Generally, TCS-related transcripts in *S.* Typhimurium under the Biomos® treatment were either not found or were repressed. In contrast, HMO treated *S.* typhimurium showed genetic modulation of all 13 TCSs. Most were repressed compared to expression in *S.* Typhimurium without HMO, though the expression of stress-response TCS, *rpoS*/*rssB* [29], was induced, as was the *arcA* receptor from arcA/arcB TCS.

The expression patterns of virulence factors alone did not support the observed reduction in association activity with both prebiotics, so expansion of analysis to expression of enzymes necessary to access the host surface, glycosyl hydrolases, which have been suggested to be part of emerging virulence factors that enable the bacterial cell to gain direct access to the host cell membrane [30] prior to involvement of the T3SS. The combination of these observations highlighted glycosyl hydrolases are important for adhesion activity in *S.* Typhimurium and may be important during exposure to prebiotics, so those same genes were examined in this study to expand the perspective of virulence mechanisms. Glycosyl

hydrolases of *S.* Typhimurium under the HMO condition were repressed - *bcsC, bglX, nagZ, malS, nanH, mltB, glgB, bglA, glgX,* and *STM0907* (Table 2). Out of the aforementioned genes, Biomos® treated *S.* Typhimurium repressed *nagZ* (Log_2FC = -1.44, $-log_{10}P$ = 2.6), only showed a minor repression of *glgX* (Log_2FC = 0.956, $-log_{10}P$ = 2.1), barely induced *glgB* (Log_2FC = 1.04, $-log_{10}P$ = 2.1), while all others were not found in the Biomos®-treated *S.* Typhimurium transcripts. These combined observations suggest that HMO treatment repressed the enzymes needed to digest the host glycan thereby reducing the ability of the bacterium to associate with the epithelial cell membrane that would reduce association and likely invasion, as observed in the association assays (Figure 1).

Host surface receptor expression differentiation with prebiotics

Host epithelial Caco2 cells had a clear difference in the expression of multiple transmembrane receptors specific to prebiotic pretreatment, but none of these differentially regulated surface receptors alone explain the difference in the association activity of *S.* Typhimurium (Appendix Figure 2). To better understand how prebiotics modulated the interaction of the host Caco2 cells and pathogen in this model, we looked at the expression of TLRs, which are integral receptors for mounting an immune response [31]. In host cells, both prebiotic pretreatments resulted in the same TLR expression pattern (Appendix Figure 1). *TLR2* was the only membrane receptor that was induced with prebiotic pretreatment, and previous work has shown yeast component zymosan and HMO structure 3-fucosylactose both induce host *TLR2* expression [32, 33]. *TLR1, 3, 4, 5* and *6* were all similarly repressed in both HMO and Biomos® conditions. Expression of integral TLR signaling protein, MYD88, was induced in both prebiotic conditions, indicating the Caco2 cells in both treatments were interacting with *S.* Typhimurium (Figure 1). *TRAF6*, which is downstream of *MYD88* and a regulator of pro-inflammatory cytokine transcription, was also induced in both prebiotic conditions. The data here showed prebiotic-

specific regulation of transmembrane receptors and immunomodulating TLRs, supporting host-prebiotic interactions through surface mediated contact can regulate intracellular activity. The regulation of expression for transmembrane receptors implies such prebiotic interactions may also be regulating other intracellular functions that rely on extrinsically controlled signaling cascades, like metabolic activity.

Prebiotic pre-treatment of Caco2 cells modulates the expression of metabolic pathways

The incubation of Caco2 cells with prebiotics prior to infection altered the expression of metabolic pathways, as compared to Caco2 cells infected without any prebiotic pretreatment. Both HMO and Biomos® significantly altered expression of the same 3745 genes as compared to no prebiotic, but Biomos® treatment significantly modulated expression of an additional 3533 unique Caco2 genes compared to HMO treatment ($-Log_{10} P > -1.3$) (Figure 2). HMO treatment changed the expression of only 191 unique genes when compared to Biomos®. Both HMO and Biomos® treated, then infected, Caco2 cells had significant enrichment of transcripts related to multiple cholesterol biosynthesis pathways, which are a known response to *S.* Typhimurium infection [34], as well as the upregulation of genes related to ketogenesis and oxidative phosphorylation. Overall, HMO and Biomos® treated and *S.* Typhimurium infected Caco2 cells shared enriched expression of the same 11 metabolic pathways, while HMO treated cells had an addition 10 pathways and Biomos® cells had 15 uniquely enriched pathways.

Caco2 cells pretreated with HMO and infected with *S.* Typhimurium induced expression of epoxysqualene biosynthesis, spermine biosynthesis, and mevalonate pathway I, which are all involved in cholesterol biosynthesis and regulation. Genes related to broad level lipid metabolism are induced in the presence of HMO amid an ongoing infection with *S.* Typhimurium. The differential regulation of

spermine, a polyamine derived from arginine and ornithine, in the presence of HMO and *Salmonella* challenge is particularly notable as the production of polyamines by the host have been previously shown to fuel pathogenic activity in *S.* typhimurium [35, 36]. Additionally, the degradation of the important amino acid Tryptophan was modulated by HMO treatment, but was not significantly altered in Biomos® treated cells. In HMO treated cells, 20% of genes for the tryptophan degradation pathway were repressed, 57% were induced, and 23% were not found in the data. In contrast with the general upregulation of metabolic pathways seen in HMO treated Caco2s, Biomos® treated cells displayed more mixed regulation, with the majority of significant pathways displaying a combination of repressed and induced genes. Notably the lipid-related ceramide biosynthesis and protein citrullination pathways have significantly altered expression in Biomos® treated cells, though ceramide biosynthesis is primarily induced while protein citrullination was repressed. Treatment of Caco2 cells with prebiotics followed by inoculation of *S.* Typhimurium resulted in the induction of metabolic pathways related to cholesterol metabolism in both treatments, but also resulted in distinct expression profiles of pathways unique to each prebiotic.

Pathway enrichment analysis for metabolites from the control, HMO, and Biomos® treatments compared across 60 mins of incubation revealed both prebiotic treatments resulted in a greater number of significantly (adj $P < 0.05$) enriched pathways as compared to host cells without any prebiotic addition (Figure 2). Caco2 cells in the control condition had metabolism of xenobiotics by cytochrome P450 as the most significantly enriched pathway ($-\text{Log}_{10} P > 1.3$), followed by glycerophospholipid metabolism, tryptophan metabolism, glutathione metabolism and primary bile acid biosynthesis. Both prebiotic metabolic enrichment comparisons also had glutathione metabolism and tryptophan metabolism as an enriched pathway across 60 mins. The top five pathways for HMO treated Caco2 cells were porphyrin and chlorophyll metabolism, glycine, serine, and threonine metabolism, cysteine and methionine

metabolism, tryptophan metabolism, and purine metabolism. In Biomos® treated Caco2 cells the top five pathways were tryptophan metabolism, phenylalanine, tyrosine, and tryptophan biosynthesis, phenylalanine metabolism, fatty acid degradation, and fatty acid elongation. Notably both prebiotic treatments resulted in the enrichment of multiple pathways related to amino acid metabolism, though not all enriched amino acid pathways in HMO passed the cut-off for significance but still ranked among the top 25 pathways for that treatment. Interestingly, the metabolic profile of HMO treated Caco2 cells without *S.* Typhimurium added supports the gene expression data for HMO treated and infected Caco2 cells, particularly with respect to tryptophan and glutathione metabolism. Though Biomos® treated Caco2 cells also revealed tryptophan metabolites were enriched across 60 mins, tryptophan metabolism did not appear as a top expressed genetic pathway in infected cells treated with the same substrate.

The significant enrichment of different metabolic pathways in Caco2 cells treated with either HMO or Biomos® then infected indicates the prebiotics in this study were able to exert an effect on the host cell without the presence of normal gut flora. The clearly observable influence of prebiotic treatments on Caco2 gene expression and the influence of *S.* Typhimurium on diverging metabolic enrichment profiles and gene expression in Biomos® led to the investigation of whether such prebiotic-specific effects could be seen for *S.* Typhimurium gene expression in this *in vitro* system.

Prebiotic treatment of Caco2 cells drives divergent metabolic gene expression in S. Typhimurium 14028

S. Typhimurium 14028 was added to the Caco2-prebiotic mixture 15 mins post prebiotic addition and differentially expressed metabolic pathways were determined for each prebiotic in comparison to *S.* Typhimurium with non-treated Caco2 cells. As seen with the Caco2 cells, significant changes in *S.* Typhimurium gene expression were observed in both HMO and Biomos® treatments, but unlike the host

cells, Biomos® treatment did not result in the unique expression of any of the bacterial genes (Figure 3). Both prebiotic treatments significantly (adj $P < 0.05$) altered the expression of the same 2107 genes, although HMO altered an additional unique 2713 genes compared to Biomos®. Visualization of all significantly altered genes resulted in a distinct and opposing expression pattern across the two prebiotic treatments, where most genes that are induced in one treatment are repressed in the other (Figure 3). This observation demonstrates the oppositional effect of the prebiotic treatments on *Salmonella*. The stark difference suggests that bacterial metabolism and virulence will be directly related to the type of prebiotic treatment when consumed in the diet that is not predictable and may lead to potentiation of virulence gene expression but repression of the glycan digestion enzymes that effectively reduces association in a healthy epithelial layer. However, this potentiation for hypervirulence may lead to unexpected virulence in an inflamed tissue where there is no need to digest the host glycan to gain access to the membrane. If so, additional change to the metabolic capability of the system may cooperate to change infection status.

More specific analysis of gene regulation related to metabolism in *S.* Typhimurium revealed the enrichment of 50 metabolic pathways in the Biomos® treatment and 14 pathways in HMO (Figure 3). HMO and Biomos® treatment did not have any shared enriched pathways in *S.* Typhimurium across the two treatments, revealing distinct reprogramming of metabolism in the pathogen resulting from prebiotic addition. Biomos® treatment resulted in the significant enrichment of multiple amino acid related metabolic pathways including L-tryptophan biosynthesis ($-\text{Log}_{10} P = 1.31$), L-leucine biosynthesis ($-\text{Log}_{10} P = 1.74$), and L-arginine degradation ($-\text{Log}_{10} P = 2.00$). The most significantly enriched pathways in the Biomos® treatment are those related to cellular respiration and energy production. The top four most significant metabolic pathways from the Biomos® treated *S.* Typhimurium are succinate to cytochrome *bd* oxidase electron transfer ($-\text{Log}_{10} P = 3.54$), superpathway of glyoxylate bypass and TCA (-

Log_{10} P = 3.74), succinate to cytochrome *bo* oxidase electron transfer (-Log_{10} P = 4.02), and the TCA cycle (-Log_{10} P = 4.75). *S.* Typhimurium from the HMO condition did not show enriched expression for any of these aforementioned metabolic pathways and instead had enriched expression of genes related to CDP-diaglycerol biosynthesis pathways (-Log_{10} P > 1.31) and multiple glycerol degradation pathways (-Log_{10} P > 1.48). The addition of *S.* Typhimurium to prebiotic pretreated Caco2 cells significantly altered the expression of central metabolic pathways in the pathogen, indicating the addition of these two dietary substrates to host-pathogen systems can affect the metabolic activity of this pathogen in divergent ways.

Metabolic profiles reveal distinct shifts in shared host-pathogen metabolism after prebiotic treatment

The differential expression of genes related to key metabolic pathways in both host and pathogen from prebiotic treatment is supported by the measured metabolites. Untargeted metabolic profiles were determined for each condition (pathogen alone, pathogen and HMO or Biomos®, and HMO or Biomos® alone) between two timepoints, 15 mins post prebiotic addition and immediately after pathogen inoculation (Time 0) and 60 mins post pathogen inoculation (Time 60) with four biological replicates for each combination. K means clustering with a cluster value of two showed distinct profiles across both time and treatment type (Appendix Figure 3). Prebiotic type, pathogen inclusion, and sampling time all had marked effects on the metabolic profiles, reflected by the distinct clustering of replicates by treatment combination (Appendix Figure 4). Caco2 cells with *S.* Typhimurium but without any prebiotic treatment clearly formed two distinct groups. After 15 mins of prebiotic incubation (Time 0) there were distinct clusters by prebiotic type, indicating metabolic profiles of the Caco2 cells were rapidly changed by prebiotic incubation irrespective of pathogen presence. *S.* Typhimurium was initially added at 15 min post prebiotic incubation (Time 0), so unsurprisingly the *S.* Typhimurium added prebiotic replicates at

Time 0 were closely correlated to their matching prebiotic no pathogen replicates. More detailed comparison of these HMO vs BioMos® metabolic profiles at Time 0 showed lipids (adj P < 0.02) were increased in the BioMos® treatments as compared to HMO, as were some amino acids like L-kynurenine (adj P = 1.57e-6). The distinct metabolic profiles by prebiotic type observed at Time 0 may be in minor part due to the addition of exogenous substrates, but combined with the expression data it is clear Caco2 cells alter expression of metabolic pathways in conjunction with prebiotic addition, indicating these noted shifts are not all due to prebiotics addition.

The effect of prebiotic addition on the overall metabolic profile was lessened at Time 60 and more affected by the presence of the pathogen. HMO and BioMos® treated host cells without *S.* Typhimurium formed their own clusters at Time 60, apart from the samples that included *S.* Typhimurium, while Time 60 samples with *S.* Typhimurium formed a cluster set apart from all others regardless of prebiotic treatment type. Though the metabolic profiles of HMO and BioMos® treated cells with *S.* Typhimurium are not separately clustered on the correlation plot by full profiles, a direct comparison of these two treatments showed notable regulation of iron compounds in the BioMos® condition, including precorrin-4 (adj P = 0.02), cobalt-precorrin-2 (adj P = 0.02), $FMNH_2$ (adj P = 0.02), and uroporphyrinogen-III (adj P = 0.02). Though not statistically significant but still notable for the later discussion of respiratory pathways, N_2-succinylglutamate (adj P = 0.2) and pyrimidine-rings (adj P = 0.2) were both among the top 50 features identified in the comparison of BioMos® plus pathogen to HMO plus pathogen at Time 60. The *S.* Typhimurium Time 60 grouping implies a distinctive effect of *Salmonella* on shared metabolism, which overwhelmed the initial prebiotic effect seen for the Time 0 samples, but higher resolution of metabolic products indicated there was still notable differences in the metabolism by prebiotic type.

Across all conditions and time points, 252 metabolites were significant ($-\log_{10}P > 1.3$) and 64 were not significant by a Kruskal Wallis Test. The significant difference for a majority of metabolites (252/316) across all conditions indicates metabolic fluctuation both over time and by treatment (Appendix Figure 5). All three treatments (*Salmonella* alone, Biomos® and *Salmonella*, HMO and *Salmonella*) at time 60 formed distinctly different clusters and that were also very different to the prebiotics alone at the same time point. Findings from the correlation plot suggest the presence of *Salmonella* distinctly alters the metabolic profile of the combined host-pathogen metabolome, which supports differing expression patterns of the pathogen in either prebiotic treatment. The additional difference between time 60 metabolomes in the Biomos® and HMO treatments without *Salmonella* affirm the Caco2 cells respond to prebiotic treatment and remodel expression of metabolic pathways irrespective of the presence of microbes. The distinct metabolic profiles and large fraction of significantly different metabolites indicate prebiotic pretreatment of Caco2 cells has an evident effect on host and pathogen metabolism.

Cellular respiration pathways in both host and pathogen are differentially regulated in a prebiotic-dependent manner

The notable metabolic fluctuations and common theme of differentially regulated energy-metabolism related pathways in both the host and pathogen led to the deeper investigation of three major intertwined metabolic routes: the TCA cycle, glycolysis, and oxidative phosphorylation. Caco2 cells displayed different expression patterns for select genes across these three energy-producing metabolic pathways by prebiotic treatment (Figure 4). However, the effect of prebiotic treatment on the expression of genes related to the TCA cycle, glycolysis, and oxidative phosphorylation was much more pronounced in *S.* Typhimurium (Figure 4).

The TCA cycle in Caco2 cells was similarly expressed in both prebiotic treatments, though Biomos® treated cells showed an induction of all major reactions in the cycle while HMO treated cells had no differential expression for the steps from citrate to isocitrate and from fumarate to malate compared to untreated cells. Relatively unchanged in Caco2 cells, in *S.* Typhimurium the TCA cycle is contrastingly different between the two prebiotic conditions (Figure 4). *S.* Typhimurium mixed with HMO treated cells displayed an incomplete anaerobic TCA cycle where rather than cycling there is a split into oxidative and reductive branches. The repression of the glyoxylate shunt and the repressed reactions from 2-oxoglutarate to succinate seen in the HMO condition indicate TCA regulation in these *S.* Typhimurium cells is following the pattern of early gut introduction and colonization [37]. *S.* Typhimurium combined with Biomos® treated Caco2 cells displayed expression of a more complete TCA cycle, with repression of 3 major reactions (isocitrate to oxoglutarate, succinate to fumarate, and malate to oxaloacetate) that also serve as entry points for external substrates to continue fueling this cycle. This would result in shifting TCA metabolism toward the production of succinate, which is a metabolite with diverse downstream uses including as an electron acceptor to complete the oxidative steps of the bifurcated *S.* Typhimurium TCA cycle [37, 38].

Differential regulation of the TCA cycle, and in particular expression of enzymes related to the oxidative TCA cycle seen in the BioMos® treated cells, is supported by the enriched amino acid metabolism observed in *S.* Typhimurium. *S.* Typhimurium in the BioMos® condition displayed enriched genetic expression related to biosynthesis of the branched chain amino acid leucine (Figure 3). Arginine and histidine degradation were also enriched in BioMos®, as was tryptophan biosynthesis. All four amino acids, arginine, histidine, leucine, and tryptophan are precursors for substrates in the TCA cycle and their metabolism contributes to TCA cycle activity, either through entry as pyruvate, acetyl-CoA, or α-ketoglutarate. The combined findings of a complete oxidative TCA cycle in BioMos® treated *S.*

Typhimurium in conjunction with the enrichment of TCA-contributing amino acids suggests complex metabolic regulation by prebiotic treatment and that the regulated pathways do not necessarily confer a host advantage but rather potentiate virulence in the gut environment. Amino acid synthesis in part relies on precursors from another central energy-producing pathway, the pentose phosphate pathway, so the regulation of this pathway was also examined in more detail.

Two other major energy-producing metabolic pathways that run in parallel, glycolysis and the pentose phosphate pathway, display this same pattern of pathway regulation as unique to each prebiotic. As seen with the TCA cycle, Caco2 cells had similar patterns of expression across the prebiotic treatments for genes involved in glycolysis, but *S.* Typhimurium expression was distinctly regulated by treatment. Unlike the TCA cycle and glycolysis, regulation of the pentose phosphate pathway (PPP) in the Caco2 cells differed by prebiotic treatment. The PPP is typically upregulated in host cells during pathogenesis as a means of creating reactive oxygen species (ROS) from NADPH oxidase ultimately decreasing inflammation through the production of anti-inflammatory cytokines [39]. Likewise, *Salmonella* utilizes the PPP production of NADPH to control redox balance for survival in the host [40]. The finding of differential expression of PPP genes by prebiotic treatment coupled to the known importance of PPP regulation in host-pathogen interactions encouraged a deeper evaluation of the expression patterns of this pathway by treatment and cell type.

Detailed mapping of the pathway revealed repression of the reactions involving transketolase in Biomos® treated Caco2 cells, which drives the latter half of the PPP. This repression of the PPP in Biomos® treated Caco2 cells is not seen in *S.* Typhimurium, which instead induced expression of multiple reactions in the PPP, including transaldolase (*STM14_RS00600,* $-\log_{10}P = 11.5$), which drove the PPP

forward towards the production of fructose-6-phopsphate. Contrastingly, HMO treated *S.* Typhimurium displayed repression of the PPP pathway in every step beyond the production of D-ribulose 5-phosphate. Whereas transaldolase was induced in the Biomos® condition, it was repressed in the HMO condition, as was transketolase (*STM14_RS12910*, *STM14_RS12915, STM14_RS16440*) ($-\log_{10}P > 2.4$). While BioMos® induced multiple parts of the oxidative and non-oxidative branches of the PPP in *S.* Typhimurium, HMO generally repressed the non-oxidative branch of the pathway. In Caco2 cells BioMos® repressed the non-oxidative branch and HMO induced this branch, revealing opposing regulation by BioMos® and HMO distinct to cell type, host or pathogen.

The final energy producing pathway investigated in this study likewise followed the same trajectory as the two prior paths. Oxidative phosphorylation in both the pathogen and in the Caco2 cells showed distinct modulation of genetic expression related to prebiotic treatment. A broad look at gene expression related to oxidative phosphorylation in Caco2 cells revealed a subset of genes related to complex I of the mitochondrial respiratory chain (*NDUFA3, NDUFA7, NDUFB10, NDUFS6,* and *NDUFS8*) that were repressed in the Biomos® condition (Figure 4). Further predictive mapping of the respiratory chain showed repression of Complex IV in both HMO and Biomos® treated cells, but additional repression of Complex I in Biomos®. Notably both prebiotic conditions altered the expression related to the electron transport chain, indicating a level of prebiotic control over this central energy route. Findings in *S.* Typhimurium mirror this observation of electron transport modulation by prebiotics. In *S.* Typhimurium from the Biomos® condition, multiple electron transfer pathways were significantly enriched (Figure 3), these same pathways were not significantly altered by HMO.

Discussion

Prebiotics applied to our focused *in vitro* model altered gene expression and metabolism for both host Caco2 cells and pathogen *S.* Typhimurium in ways specific to each prebiotic. This observation amends

the current paradigm that prebiotics exert protective effects through catabolism by commensal gut microbiota [41] and mitigate infection through competitive exclusion [42]. The pretreatment set-up of colonic epithelial cells performed here supported infection in this case was not attenuated through a prebiotic decoy or receptor-blocking mechanism, as has been suggested in other studies [14, 43]. *S.* Typhimurium still adhered to the epithelial cell surface, but the rate of invasion and subsequently, successful colonization, was significantly decreased ($p < 0.05$). Together these results indicate host-protective effects of the dietary prebiotics tested in this study stemmed from a complex orchestration of changes in expression of bacterial virulence factors, membrane receptors, and metabolic activity by the host and pathogen in a prebiotic-specific manner rather than an inert physical blockade. Previous work from our lab illustrated the same prebiotics used here remodeled the epithelial cell surface and modulated the expression of receptors, priming the colonic epithelial cells for response to infection with *L. monocytogenes* [15, 30, 44-46]. These previous observations in conjunction with those presented here support the bioactivity of prebiotic supplements in cells other than the commensals at which they are aimed, ultimately affecting host epithelial cells and enteric pathogens.

Though both were able to reduce host-association at 1% addition, the path by which HMO and Biomos® drove expression changes in both host Caco2 and pathogen cells were prebiotic-specific. The positive correlation between the tested dose and reduced pathogen association in HMO treatment was not reflected in the Biomos® treatment, as increasing Biomos® concentration decreased the host-protective effects. Intriguingly, both prebiotics induced the expression of different virulence-modulating two-component systems (TCS) and other well-documented virulence factors like SPI-1 and SPI-2. Two-component systems are translators for exogenous conditions and allow microbes to tightly regulate and quickly adjust cellular responses to match environmental conditions, both mechanisms key to pathogenic success [47].

All the TCSs looked at in this study are important for cellular regulation, but one interesting finding is the repression of membrane receptor CpxA and induction of sensor ArcA in the HMO condition. The CpxAR and ArcBA TCSs cross-regulate each other, with membrane CpxA able to phosphorylate ArcA [48]. ArcA controls multiple genes, including those related to fermentative metabolism and free radical formation, and more interestingly, ArcA activity leads to cell death [48]. In the HMO condition, *S.* Typhimurium shows repression of the membrane component CpxA but induction of the ArcA regulator, potentially indicating *S.* Typhimurium in this condition were pushed towards cell death but ultimately at this timepoint began to repress the external sensor contributing to this cell death pathway. It is possible there was early *S.* Typhimurium cell death with HMO treatment, but over the period of infection this pathogenic attenuation was depressed and overridden through other pathogenic mechanisms.

Despite the induction of multiple virulence factors, regulation of *S.* Typhimurium's glycosyl hydrolase enzymes and Caco2 receptor remodeling prevented successful membrane access and host invasion. To stage an effective invasion, *S.* Typhimurium must gain access to the colonic epithelial cell membrane, which requires first drilling through a complex mucosal layer made of glycoproteins and oligosaccharides [30]. In a healthy gut this mucosa provides a literal layer of protection from enteric infection [49], but *S.* Typhimurium possesses multiple glycosyl hydrolases capable of digesting this layer of glycoproteins thus granting the pathogen access to the epithelial cell surface [30]. The reduced expression of these enzymes in the HMO treatment suggests that although key virulence factors like SPI-1 were induced, these cellular mechanisms were not able to contribute to active pathogenesis as *S.* Typhimurium was not able to physically access to the necessary host receptors.

Interestingly the host was also modulating associative potential and immunological responses via the repression of multiple TLRs, which are expressed in epithelial cells and sense pathogen associated molecular patterns (PAMPs) [31]. TLR2 was the only TLR that was induced, and notably induction was similar in both HMO and Biomos® treatments. Previous work noted that increased TLR2 expression exacerbates *S.* Typhimurium infection through negative regulation of nitric oxide synthase expression and a reduction in epithelial barrier integrity [50]. Expression of TLR-related signaling molecule MYD88 was also induced in both prebiotic conditions. Though this experiment was performed in Caco2 cells, it is notable that previous studies using mesenchymal stems cells, in which *Salmonella* can intracellularly persist and transit through the body [51], found *MYD88* expression was induced in these tri-lineage cells [52]. The induction of *MYD88* in mesenchymal stem cell by the Type III Secretion System (T3SS) resulted in the systemic spread of the pathogen through the host [52], illustrating the exploitation of a host immune response for pathogenic gain and is notable given this same signaling molecule was induced here for Caco2 cells in both prebiotic conditions.

In addition to the modulation of cell surface interactions, both prebiotics also regulated metabolic activity in the host and the pathogen. Substrates with the prebiotic label are thought to be privileged for fermentation primarily by commensal bacteria and less accessible to the host and enteric pathogens [53], so the effective metabolic control by prebiotics of two non-commensals observed here is an unexpected finding, analysis of both the metatranscriptome and metabolome indicated energy-producing pathways and related amino acid metabolism were differentially altered in both host epithelial cells and *S.* Tyhpimurium according to prebiotic treatment. The control of energy-producing redox reactions by *S.* Typhimurium in the gut is important for successful luminal colonization [54] and the diverse metabolic abilities of *Salmonella* allow for adaptation of these pathways to numerous host environments, both extracellular and intracellular [26]. Regulation of these central reactions by

prebiotics in this study indicates dietary substrates exert exogenous control of pathogenic metabolism and suggests host diet plays a role in both attenuating and exacerbating enteric infections.

HMO and Biomos® were able to reduce adherence of *S.* Typhimurium to colonic epithelial cells, but this diet-induced reduction was not completely explained by the repression of virulence factor expression or host receptor blocking. The modulation of energetic pathways, and thus energy balance, in both host and pathogen by dietary substrates HMO and Biomos® provides one possible explanation for this attenuated virulence. Predictably, the metabolic composition of samples across time and treatment type (control, prebiotic type, and/or pathogen addition) differed. What was notable about these profiles however was the distinct clustering of Biomos® treated host and pathogen cells across time. Uninfected Biomos® treated at 60 mins of incubation clearly clustered together in a group and apart from all other 60 mins samples, regardless of pathogen presence or prebiotic condition, indicating Biomos® distinctly drives Caco2 metabolism divergent from HMO, and *Salmonella* infection overwhelms the metabolic profile in this treatment. This pathogen-driven metabolome in the Biomos® condition suggests the protective effects of Biomos® are transient in the human gut and pathogenic addition may be successful depending on infectious dose and gut condition. It should be noted that Biomos® is currently used in livestock and primarily studied in the context of animal health, in which Biomos® has been successful for promoting animal health [55]. These findings of less successful mitigation in the human gut context suggests species differences may drive prebiotic efficacy and warrants further research.

This metabolic divergence is further supported by transcriptomic data, which indicate the presence of Biomos® in the media drives metabolic adaptation like that seen in inflamed guts [37]; whereas HMO

treatment did not show this same effect. The forward push of the TCA cycle reactions, along with the repression of the PPP, and induction of ubiquinone-related metabolism to fuel the mitochondrial electron transport chain in Biomos® treated *S.* Typhimurium suggests Biomos® encourages aerobic respiration [56] and improved energetic production for the pathogen. The TCA cycle in BioMos® treated *S.* Typhimurium displayed a push towards the production of succinate and repression of the downstream production of fumarate, an intriguing finding since succinate is a central compound in driving pathogenesis of multiple enteric organisms [37, 57]. Previous work in *Clostridium difficile* coupled with commensal *Bacteroides thetaiotaomicron* revealed the increased production of succinate by *B. thetaiotaomicron* after antibiotic disturbance supported the proliferation and pathogenesis of *C. dificile* in mice [57]. In this *C. difficile* study, succinate was found to be utilized as a substrate for the regeneration of NAD+ [57] and succinate has likewise been shown to support increased gut colonization and pathogenesis by *S.* Typhimurium [37]. The finding here that BioMos®, but not HMO, drives a more complete oxidative and energetically favorable TCA cycle indicates prebiotics may potentiate or attenuate virulence through the regulation of central energy-regulating metabolic pathways in ways specific to each prebiotic substrate. The control of redox metabolism in conjunction with amino acid metabolism in the gut by *S.* Typhimurium is a key driver of pathogenesis and the switch to aerobic respiration pathways in the Biomos® condition suggests virulence-favoring conditions for *S.* Typhimurium [58].

At the same time, while Biomos® treatment moderated host cell metabolism related to immunological responses, such as the reduction calcium transport and expression of multiple lipid-related pathways [59], the addition of *Salmonella* appeared to overwhelm the host protective responses. This takeover may be explained by the prebiotic composition. As Biomos® is a commercial product derived from the cell walls of *Saccharomyces cerevisiae*, it is not pure extracted MOS but instead contains additional

substrates derived from the yeast including metal ions and amino acids [55]. *S.* Typhimurium expression showed significant enrichment of amino acid related pathways, which may be a result of these other substrates and not MOS itself, highlighting the off-target effects of commercial prebiotic products may depend on oligosaccharide purity and product composition.

The enrichment of amino acid metabolism in *S.* Typhimurium is notable in the context of the respiration pathway modulation because amino acids derived compounds, such as fumarate, can act as alternate electron acceptors and contribute to energetically favorable aerobic respiration [60, 61]. Arginine metabolism was also enriched in *Salmonella* in the Biomos® condition, which is of note given arginine serves as a key substrate for proline production and proline is known to modulate oxidative stress in *Salmonella* over the course of infection [62]. The regulation of arginine, leucine, and histidine is also notable given the complete oxidative TCA cycle observed in the BioMos® treated cells, but not in the HMO treated ones. Amino acid regulation is a key component of nutritional immunity between hosts and pathogens [63]. The enrichment of amino acid metabolic pathways able to fuel the TCA cycle through the oxidative branch and towards more efficient ATP production in the BioMos® treatment is a notable finding, as this appears to support pathogenic activity rather than attenuate it.

One more important amino acid regulated in both host and pathogen by Biomos® treatment was L-tryptophan, which is an essential amino acid for humans, a central substrate for many bioactive molecules and a key precursor for nicotinamide adenine dinucleotide (NAD+) [64-66]. L-tryptophan biosynthesis was upregulated in Biomos® treated *Salmonella* and generalized tryptophan metabolism was a significantly enriched metabolite pathway in Caco2 cells. L-kynurenine, a direct production of tryptophan degradation, was an enriched metabolite in BioMos® treatment as compared to HMO. L-

kynurenine is known to be a neuroactive metabolite with broad neurological effects, both protective and detrimental, in humans [67]. This additional finding of Tryptophan regulation by both host and pathogen in conjunction with the more specific measurements of metabolites from this metabolic pathway gives credence to prebiotics potentiating health effects beyond the gut barrier, though more research is needed on these potential systemic effects. Additionally, tryptophan's pivotal role as a key substrate for redox coenzyme NAD+ and as a precursor to bioactive molecules affecting both host and pathogen makes the enrichment of tryptophan metabolism in *S.* Typhimurium with Biomos® noteworthy and something to consider when testing prebiotic efficacy in pathogenic contexts.

HMO treatment did not exhibit the same enrichment of amino acid metabolism in *Salmonella*, likely because the HMO used in this study was a purified non-commercial product with minimal if any additional substrates beyond the complex oligosaccharides. HMO treatment better prevented *S.* Typhimurium association with host epithelial cells and primarily resulted in anaerobic respiration in the pathogen. Somewhat confoundingly expression data for *S.* Typhimurium showed more uniquely expressed genes in HMO (2713) and no uniquely expressed genes in Biomos®. The complexity of the HMO oligosaccharides in contrast with the simpler MOS structure and accompanying yeast cell wall parts may provide an explanation for this finding. Indeed, HMO treatment did not result in the enrichment of many metabolic pathways for *Salmonella* and alterations in host cell expression were minimal. The repression of energy production pathways in *Salmonella* with HMO treatment, known host cell surface remodeling by HMO [15], along with previously understood positive immunomodulatory functions of this prebiotic [68] supports that HMO in healthy gut condition may attenuate enteric infection by *Salmonella.* Pathogenic attenuation by dietary substrates appears highly substrate and host specific, as illustrated by one clinical trial applying FOS to patients with Crohn's Disease, which produced no clinical benefit to participants [69]. This previous work provides some *in*

vivo evidence that prebiotics in an inflamed gut may not always produce the expected ameliorative properties.

Observations from this work indicate dietary substrates may on the surface attenuate virulence but underneath can drive expression of pathogenic-related genes and metabolic pathways, that potentiates virulence in compromised tissues, which is a cautionary finding for the clinical application of dietary interventions. The additional finding here that substrate type influences both the host and pathogenic response points to the complexity of utilizing dietary interventions for the control of pathogens. These results indicate the beneficial effects of dietary prebiotics may be contingent on their addition to an already healthy gut environment, and rescue of a dysbiotic or inflamed gut from infection is specific to only select oligosaccharides. In the context of pathogens, the modulation of invasion by the prebiotic oligosaccharides seen in this study shows promise for the use of dietary substrates as prophylactics, but the diverging effects on pathogenic energy metabolism, which may increase virulent traits, supports the ongoing need for substrate-specific studies across both healthy and dysbiotic gut environments.

Methods

A graphical representation of the experimental set up can be found in Appendix Figure 6.

Oligosaccharides

HMO was isolated and given as a gift to the Weimer lab by Dr. Daniela Barile (UC Davis, CA, USA) [70]. Biomos® is a yeast-derived and commercially available product from Alltech Inc. (Nicholasville, KY, USA). Brief descriptions of each oligosaccharide mixture can be found in Table 3. Both oligosaccharides were made into a 1% working concentration in high glucose DMEM (HyClone Laboratories, Logan, UT, USA).

Bacterial Strain and Growth Conditions

Bacterial cells were grown as previously described [30, 45, 71-73]. Briefly, *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain LT2 was grown for the prebiotic adhesion and invasion panel and *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain 14028s was used for an additional adhesion and invasion assay as well as all following experiments. All *Salmonella* were grown in LB (Difco, BD, Thermo Scientific, Rockford, IL, USA) at 37˚C, shaking at 220rpm for 14-16 hours prior to each use.

Human Cell Line and Growth Conditions

Human colonic carcinoma (Caco2) cell lines were obtained from ATCC (HTB-37) and grown as described previously [15]. Briefly, Caco2 cells were thawed from liquid nitrogen stocks stored in DMEM with 10% DMSO then grown in DMEM with 10 mM MOPS (Sigma, St. Louis, MO, USA), 10 mM TES (Sigma, St. Louis, MO, USA), 15 mM HEPES (Sigma, St. Louis, MO, USA), 2 mM NaH_2PO_4 (Sigma, St. Louis, MO, USA), 20% fetal bovine serum (HyClone Laboratories), 1% glutamax (Thermo Scientific, Rockford, IL, USA), 1% PenStrep (Thermo Scientific, Rockford, IL, USA) and 1% non-essential amino acids (Thermo Scientific, Rockford, IL, USA). Culture medium was renewed every three days. Caco2 cells were seeded at 10,000 cells/cm^2 into 96-well plates 14 days prior to use and differentiated for 12 to 15 days for gentamicin protection assays.

In Vitro Colonic Cell Infection Assays

Colonic cell infection assays were also performed according to Chen at al [15] and Arabyan et al. [30], which adapted methods from Shah et al. [74, 75]. Oligosaccharides were suspended at 1% (w/v) in serum-free DMEM was added to differentiated Caco2 cells and allowed to incubate for 15 mins. Following this, stationary phase *S.* Typhimurium (n=3 biological replicates; multiplicity of infection=1000) was added to the pretreated Caco2 cells and incubated for 60 mins. PBS buffer (pH=7.2) was used to wash the cells and 50mL Warnex buffer (AES Chemunex Canada, Inca, Montrealm QC, Canada) was applied to lyse the cells according to the manufacturer's directions. Deactivation of Warner lysis was done with a 15 mins incubation at 95˚C. Samples were then diluted 1:10 in nuclease-free water and stored at -20˚C for qPCR quantification. Quantification of *S.* Typhimurium LT2 cells was done via qPCR using primers previously validated by Arabyan et al [30], F: 5'- ACG CGG 313 TAT CAT CAA AGT GG - 3'; R: 5' - ATC GGG TGG ATC AGG GTA AC - 3'. Significant differences in association across control and treatment were estimated using one-way ANOVA with Tukey test and graphed in GraphPad Prism V9 (GraphPad Software Inc, La Jolla, CA, USA).

Metabolomics

Two different analytical setups for liquid chromatography coupled to mass spectrometry (LCMS) were used in order to survey a wider variety of non-volatile compounds. Samples were split between two capped LC vials, then were stored at -20˚C prior to analysis. Non-volatile compounds from the culture supernatant were analyzed via LC-MS using a hydrophilic interaction chromatography (HILIC) column for hydrophilic and polar molecules and a reversed-phase (RP) column for nonpolar molecules according to the method by Borras et al. [76] and Aksenov et al. [77]. All samples were analyzed both via HILIC and RP on an Agilent 1290 series ultrahigh-performance LC system with an Agilent 6230 time-of-flight (TOF)

mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). An Agilent Jet Stream (AJS) nebulizer was used working in positive mode (+) and acquiring a mass range between 50 and 1700 Thomson (m/z) at 4 spectra/sec and high-resolution mode. Sheath gas temperature was 350˚C, gas flow was 11 L/min, and fragmentor voltage was set at 120V. A model 6545 quadrupole TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used for final MS/MS compound identification. Water, acetonitrile, and 10% acetonitrile suspension mix were analyzed as blanks alongside the samples, which were all analyzed via injection of 5µL aliquots with samples housed in an autosampler maintained at 4˚C. All LCMS analysis was performed with four biological replicates.

HILIC samples were analyzed on an Acuity UPLC bridged ethyl hybrid amide column (130Å, 1.7µm, 2.1mm x 100mm; Water, Milford, MA, USA). Water (A) and 90% acetonitrile in water (B) were used as mobile phases, both at pH 5 with ammonium acetate and acetic acid buffer. A linear gradient from 0% A to 10% A was applied post-injection in 20 mins with a flow rate of 0.3mL/min. The flow rate was then reduced to 0.2mL/min and phase A was increased to 95% in 10mins, for a total analysis time of 41mins. HILIC quality controls were a Water 1806006963 HILIC QC (Waters, Milford, MA, USA) and a custom-made QC. The custom QC consisted of 5µM carnitine, lysine, adenylputricine, aminocaproic acid, ornithine, tigonelline, alaninol, acetylcarnitine, 1-(2-pyramidyl)piperazine, methoxychalcone, cholecalciferol, 13-docosenamide and oleamide.

RP samples were analyzed on a Poroshell 120 EC-C18 column (2.7µm, 3.0mm x 50mm; Agilent Technologies, Wilmington, DE, USA) at 30˚C. A mix of 1% phase A (60% acetonitrile in water) and 99% phase B (10% acetonitrile in isopropanol), both containing 10mM of ammonium formate and formic acid, was used as the initial mobile phase. The total analysis time was 24mins with a flow rate at

0.3mL/min, which consisted of phase B gradient reaching 30% post injection in 4mins, then rising to 48% B in 1min, 82% B in 17mins, and 99% B in 1min. The quality control was a standard solution Waters 6963 RP QC (Waters, Milford, MA, USA) and was injected along with the samples.

Agilent Mass Hunter Qualitative Analysis B.05.00SP1 software was used to examine the total ion LCMS chromatograms. The "Find by Molecular Feature" algorithm was used within a mass range from 50 to 1700Da for peak deconvolution. Molecular feature abundance was evaluated through integration of the extracted compound chromatograms (ECC) of the corresponding ions and then exported to .cef format. Mass Profiler Professional 12.1 software was used for peak alignment with a mass window of 40ppm, 26mDa, and a retention time shift of 0.5 and 1min for HILIC and RP, respectively. A peak table was made containing retention time in mins, molecular mass, and the intensity values (peak area) for each sample.

Resulting metabolic data was analyzed using MetaboAnalyst 5.0 [78]. Comparisons between treatments and across timepoints were made using both the statistical analysis [one factor] function and enrichment analysis modules. Samples were normalized by median, then all data was $\log_{10}$ transformed and scaled by mean-centering and standard deviation. Metabolite set enrichment analysis (MSEA) was performed using the KEGG database for reference.

RNA Extraction

The 100K Pathogen Genome Project bacterial protocol [79] was used to extract *S*. Typhimurium RNA from the infection assays, while host cells were lysed by passaging cells through a 22-gauge needle [80].

Combined host and pathogen cells were pelted via centrifugation then suspended in Trizol LS Reagent (Cat #10296028, Thermo Fisher Scientific, Waltham, MA, USA). Host and pathogen RNA was extracted from the Trizol LS suspension following manufacturer's instructions. The BioAnalyzer RNA kit (Agilent Technologies Inc., Santa Clara, CA, USA) and Nanodrop (Nanodrop Technologies, Wilmington, DE, USA) were used to confirm RNA purity ($A_{260/230}$ and $A_{260/280}$ ratios ≥ 1.8, ≤ 2.0) and integrity.

RNAseq Library Preparation

RNAseq library preparation was performed exactly as outline in Chen et al. [15]. Briefly, the SuperScript Double-Stranded cDNA Synthesis kit (11917-010; Invitrogen, Carlsbad, CA, USA) was used to synthesize double-stranded cDNA following the manufacturer's instructions. A NanoDrop 2000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) an Agilent 2100 BioAnalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) was used to assay cDNA quality. All sequencing was done with 4 biological replicates in each condition.

The Kapa HyperPlus library preparation kit (kk814, KAPA Biosystems, Boston, MA, USA) with BIOO Scientific NEXTFlex adaptors (514105, BIOO, Austin, TX, USA) were used in the construction of the sequencing library. Library concentration was measured using the KAPA SYBR FAST qPCR kit Master Mix (2x) Universal (kk4903; KAPA Biosystems, Boston, MA, USA) on Bio-Rad CFX96 (Bio-Rad Laboratories, Hercules, CA, USA) and fragment size distribution was assessed using the High Sensitivity kit (Agilent Technologies Inc., Santa Clara, CA, USA). Libraries were indexed at eight libraries per lane and sequenced with PE150 on a HighSeq4000 at the California Institute for Quantitative Biosciences in the Vincent J Coates Genomics Sequencing Lab (Berkeley, CA, USA).

Statistical Analysis for Differential Gene Expression

Sequence processing and analysis was done following the methods in Chen et al. [15]. Raw sequence reads were first trimmed with Trimmomatic [81] then aligned to the Ensembl GRCh38 human genome using HISAT2 [82] with an index downloaded on 07/16/22 (grch38_tran). Alignment was done in paired-end mode with soft clippings permitted and paired-end reads that did not map to the human genome were separated. Reads that did not align to the human genome were subsequently aligned using Bowtie2 [83] to the *Salmonella enterica* genome (GCA_003253385.1_ASM325228v1). Samtools [84] was used to compress alignment files from HISAT2 and Bowtie2 for output to differential expression analysis.

Differential expression analysis was performed in edgeR [85] from gene counts estimated by featureCounts in the Rsubread R package [86]. Human gene counts were produced using the Ensembl GRCh38.86.gtf annotation and *S. enterica* counts were generated from GCA_003253385.1_ASM325228v1.gtf annotation file. Human and *S.* Typhimurium gene count tables were entered separately into edgeR for normalization and differential expression. Genes with counts per million less than one and with expression in fewer than two samples per group were discarded and reads were normalized using the library size. Treatment groups contained pairwise comparisons, so the edgeR exact test was used for differential expression estimation. Significance was defined as adjusted *p*-value (FDR, this was done using a Bonferroni correction) of less than or equal to 0.05. No reads aligned to *Salmonella* from uninfected cells so no differential expression analysis was done for *Salmonella* reads in uninfected cells.

Expression Data for Caco2 Cells

Qiagen's Ingenuity Pathway Analysis software version 01-22-01 (IPA, Qiagen, Redwood City, CA, USA) was used to determine canonical pathways differentially expressed in Caco2 cells by prebiotic treatment. Canonical pathway mapping was performed in IPA and overlayed with experimental data. Expression data for TLRs was plotted using Prism 9 (GraphPad Software, La Jolla, CA, USA). Heatmaps were made using R Version 4.2.2 "Innocent and Trusting" along with the ComplexHeatmap Package version 2.16.0 [87]. All other plots related to expression data not from IPA were made using Prism 9 (GraphPad Software, La Jolla, CA, USA).

Expression data for S. Typhimurium

S. Typhimurium differential expression data was calculated using BioCyc SmartTables (Pathway Tools version 27, SRI International, Menlo Park, CA, USA) [88]. Determination of enriched pathways was done using a two-tailed Fisher's exact test ($p \leq 0.05$) with the Pathway Tools *S.* Typhimurium strain 14028 genome. Enriched pathways from were plotted using Prism 9 (GraphPad Software, La Jolla, CA, USA) and heatmaps were made using R Version 4.2.2 "Innocent and Trusting" along with the ComplexHeatmap Package version 2.16.0 [87].

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Tables

Table 1. Expression of glycosyl hydrolase enzymes by *S.* Typhimurium

Common Gene Name	Gene ID	HMO (Log_2FC)	HMO ($-Log_{10}P$)	BioMos (Log_2FC)	BioMos ($-Log_{10}P$)	Characterization
bcsC	STM14_RS19140	-1.54	3.2			endoglucanase
bglX	STM14_RS12040	-1.52	4.8			beta-glucosidase
nagZ	STM14_RS06550	-0.78	2.7	-1.44	2.6	beta-hexosaminidase
malS	STM14_RS19390	-0.75	1.6			alpha-amylase
nanH	STM0928	-0.74	1.3			nueriminidase
mltB	STM14_RS15190	-0.10	0.2			murein-transglycosylase B
glgB	STM14_RS08670	0.13	0.2	1.04	2.1	malto-oligosyltrehalose trehalohydrolase
bglA	STM14_RS16310	0.78	2.6			sialidase
glgX	STM14_RS08660	0.837	3.3	0.95	2.1	glycogen debranching enzyme
STM0907	STM14_RS11050	0.83	1.5			chitinase

Table 2. Expression of two-component systems by *S.* Typhimurium, broken down by sensor and regulator expression

TCS Pair	Component	Gene ID	HMO (Log_2FC)	HMO ($-Log_{10}P$)	BioMos (Log_2FC)	BioMos ($-Log_{10}P$)
ArcB/ArcA	Sensor	*STM14_RS17695*	-0.82	2.10		
	Regulator	*STM14_RS17695*	2.37	12.54		
BaeS/BaeR	Sensor	*STM14_RS22535*	-1.27	3.49		
	Regulator	*STM14_RS22540*	-0.50	1.20		
BarA/UvrY	Sensor	*STM14_RS15835*	-0.53	1.37		
	Regulator	*STM14_RS10645*	1.38	4.27		
CpxA/CpxR	Sensor	*STM14_RS21360*	-1.30	2.51		
	Regulator	*STM14_RS21365*	-0.56	1.19		
CreC/CreB	Sensor	*STM14_RS24035*	-1.03	2.16		
	Regulator	*STM14_RS24030*	-0.74	1.37		
DcuS/DcuR	Sensor	*STM14_RS22600*	-0.04	0.06		
	Regulator	*STM14_RS22595*	0.92	2.38		
EnvZ/OmpR	Sensor	*STM14_RS18570*	-1.07	2.79		
	Regulator	*STM14_RS18575*				
GlrK/GlrR	Sensor	*STM14_RS14045*	-0.91	3.09		
	Regulator	*STM14_RS14035*	-0.93	2.05		
KdbD/KdpE	Sensor	*STM14_RS04130*	-1.10	1.42	-1.77	3.76
	Regulator	*STM14_RS04125*	-0.98	2.07	-1.82	3.76
NarX/NarL	Sensor	*STM14_RS09720*	-0.58	1.20	0.85	1.76
	Regulator	*STM14_RS09725*	-0.15	0.21	1.40	1.76
PhoQ/PhoP	Sensor	*STM14_RS06660*	0.69	1.79	-1.42	2.62
	Regulator	*STM14_RS06665*	1.26	3.95	-1.46	2.61
RstB/RstA	Sensor	*STM14_RS08210*	-1.07	2.76	-1.39	2.17
	Regulator	*STM14_RS08230*	0.42	0.52	-1.11	2.16
TtrS/TtrR	Sensor	*STM14_RS07785*	-1.10	2.33	-1.21	2.28
	Regulator	*STM14_RS07790*	-0.51	0.87	-1.49	2.28

Table 3. Source and content of oligosaccharides used in this experiment

Prebiotic	Main Oligosaccharide Structure(s)	Prebiotic Source
BioMos®	Mannan-oligosaccharides	Proprietary *Saccharomyces cerevisiae* derived product
HMO	Five monosaccharides (Glucose, Galactose, N-acetylglucosamine, Fucose, Sialic Acid) combined into complex oligosaccharides	Extraction from human breast milk

Figures

Figure 1. **Adhesion and invasion activity of *Salmonella* sp. Typhimurium in response to prebiotic pre-treatment.** (A) Prebiotic pre-treatment of Caco2 cells reduces combined invasion and adhesion of Salmonella LT2 in an *in-vitro* setting. 0% prebiotic addition (white bars) are used as the control for comparison of increasing (0.1, 0.5, and 1%) prebiotic treatment concentrations. BioMos and HMO were added at the noted concentrations to differentiated Caco2 cells for 15 min, followed by co-incubation of *S.* Typhimurium LT2 for 60 mins. Invasion and adhesion was measured with a gentamicin protection assay. * p-value < 0.05, ** p-value < 0.02. (B) Expression of virulence factors in *S.* Typhimurium was evaluated for each prebiotic treatment, using *S.* Typhimurium without prebiotics as the control. Green up arrows represent an upregulation of genes related to each factor while red down arrows represent repression. Specific gene expression information can be found in supplemental figure 1. (C) TLR gene expression in response to prebiotic treatment was evaluated in the Caco2 cells. The TLR expression pathway from IPA was overlayed with both HMO and BioMos treated Caco2 expression profiles, with no prebiotic Caco2s as the control for both cases. Teal represents decreased measurement for the gene while red represents increased measurement. Blue and orange genes represent predicted inhibition or activation, respectively. Inset graphs display the log2 fold-change comparison between HMO (1) and BioMos (2).

Figure 2. **Regulation of gene expression and metabolism of Caco2 cells in response to prebiotic pretreatment.** (A) HMO and BioMos treatment of Caco2 cells resulted in 3745 shared significantly expressed genes, 191 additional unique genes in HMO treated cells and 3533 in BioMos treated cells. (B-C) IPA was used to evaluate the expression patterns of the most significant (-log10P > 1.3) metabolic pathways in both prebiotic treatments. Bars represent the percentage of each pathway differentially expressed in each condition, red sections being upregulation, blue downregulation, and white as the percentage of genes found to be unsignificant in the treatment for that pathway. Right hand side numbers represent the total genes in each pathway. (D-F) The metabolic profiles of prebiotic treated Caco2 cells were analyzed using Metabolite Set Enrichment Analysis (MSEA) in MetaboAnalyst. Time 0 with no prebiotic (D), HMO (E), or BioMos (F) was used as the control comparison for the same condition at Time 60. MSEA graph display the enriched pathways at Time 60 for each prebiotic condition, with the dot size representing the portion of the pathway enriched in the dataset and color as P-value.

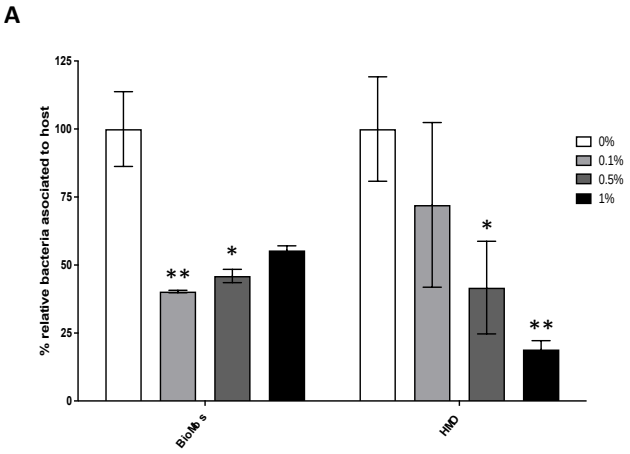
Figure 3. **Regulation of gene expression in *S.* Typhimurium in response to addition to Caco2 cells with prebiotic pretreatment.** All expression data was collected after 60 minutes of co-incubation with Caco2 cells, with or without prebiotic treatment and in four biological replicates. (A) Enriched metabolic pathway in *S.* Typhimurium added to Caco2 cells pretreated with either BioMos (orange) or HMO (purple). Enrichment determined using BioCyc with a cut of of –log10P > 1.3 for significance and *S.* Typhimurium added to Caco2 cells and no prebiotic treatment as control condition. (B) Broad evaluation of *S.* Typhimurium gene expression in response to either prebiotic condition using log2 fold change data, significance cut off of –log10P >1.3 and Euclidean distance clustering for genes. (C) 2107 genes were significantly expressed in both the HMO and BioMos treated *S.* Typhimurium, while an

additional 2713 unique genes were significantly expressed in the HMO condition and 0 were uniquely expressed in BioMos *S.* Typhimurium.

Figure 4. **Effect of prebiotic treatment on three major energy-producing metabolic pathways across both infected Caco2 cells and *S.* Typhimurium.** Expression of genes related to the TCA cycle, glycolysis and oxidative phosphorylation in repone to prebiotic treatment was evaluated for both Caco2 cells (top) and *S.* Typhimurium (bottom). Caco2 expression of these metabolic respiration-related genes was evaluated using *S.* Typhimurium infected Caco2 cells without any prebiotic treatment added as the control for both HMO and BioMos. *S.* Typhimurium expression was determined using *S.* Typhimurium added to Caco2 cells without any prebiotics as control. All heatmaps used Euclidean distance clustering, log2 fold change expression data, and only significant genes with a cutoff of -log10P > 1.3. Underlying data for the genetic regulation of the TCA cycle (right) was mapped using IPA for the Caco2 cells and BioCyc for *S.* Typhimurium. All genes represented in orange and blue in the diagrams were significantly expressed compared to the control with a cutoff of –log10P > 1.3.

Figure 5. **The pentose phosphate pathway is differentially regulated by prebiotic treatment in both Caco2 cells and *S.* Typhimurium.** Genetic regulation of the pentose phosphate pathway was evaluated in both *S.* Tyhpimurium (left within each panel) and Caco2 cells (right within each panel) across HMO addition (left) and BioMos addition (right). Expression is represented as log2 fold change with no prebiotic add matched host/pathogen cells as control and all regulated genes were significant at –log10P > 1.3. Increased expression is represented by yellow to red and decreased expression by blue to purple. Black text and teal arrows indicate those genes were non-significant or not annotated in the dataset.

Figure 6. **Prebiotic treatment of Caco2 cells challenged with *S.* Typhimurium alters oxidative phosphorylation and expression patterns in the electron transport chain.** The canonical oxidative phosphorylation pathway in IPA was overlayed with expression data from Caco2 cells treated with either HMO (top) or BioMos (bottom) and exposed to *S.* Typhimurium for 60 minutes. Red shading in the complexes indicates induction of genes responsible for that complex's activity and teal shading indicates repression of that complex. Orange shading on the products indicates a predicted increase in concentration due to the expression pattern.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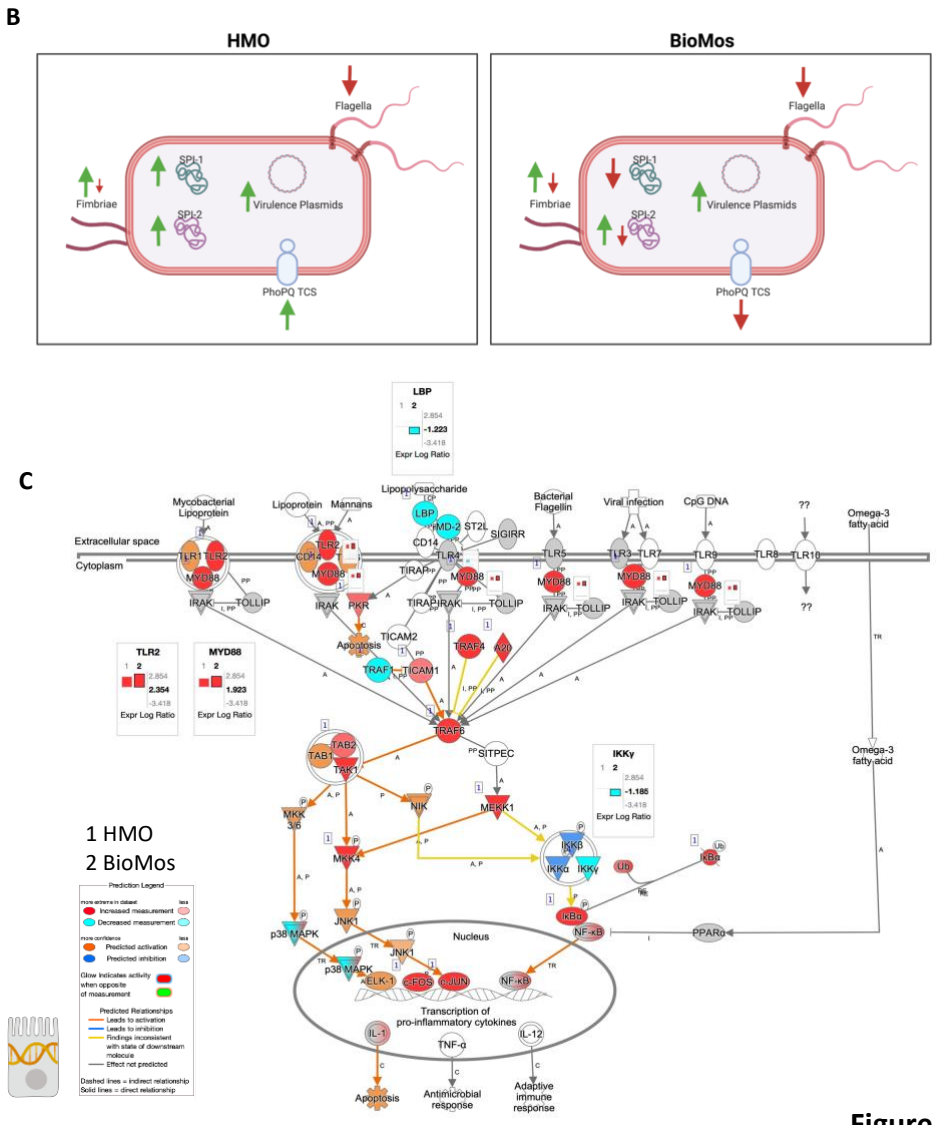
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Lipoprotein
Mannans
Bacterial Flagellin
Viral infection
CpG DNA
Omega-3 fatty acid
Extracellular space
Cytoplasm
TLR2
MYD88
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IKKγ
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2 BioMos
Nucleus
Transcription of pro-inflammatory cytokines
Apoptosis
Antimicrobial response
Adaptive immune response

Figure 1.

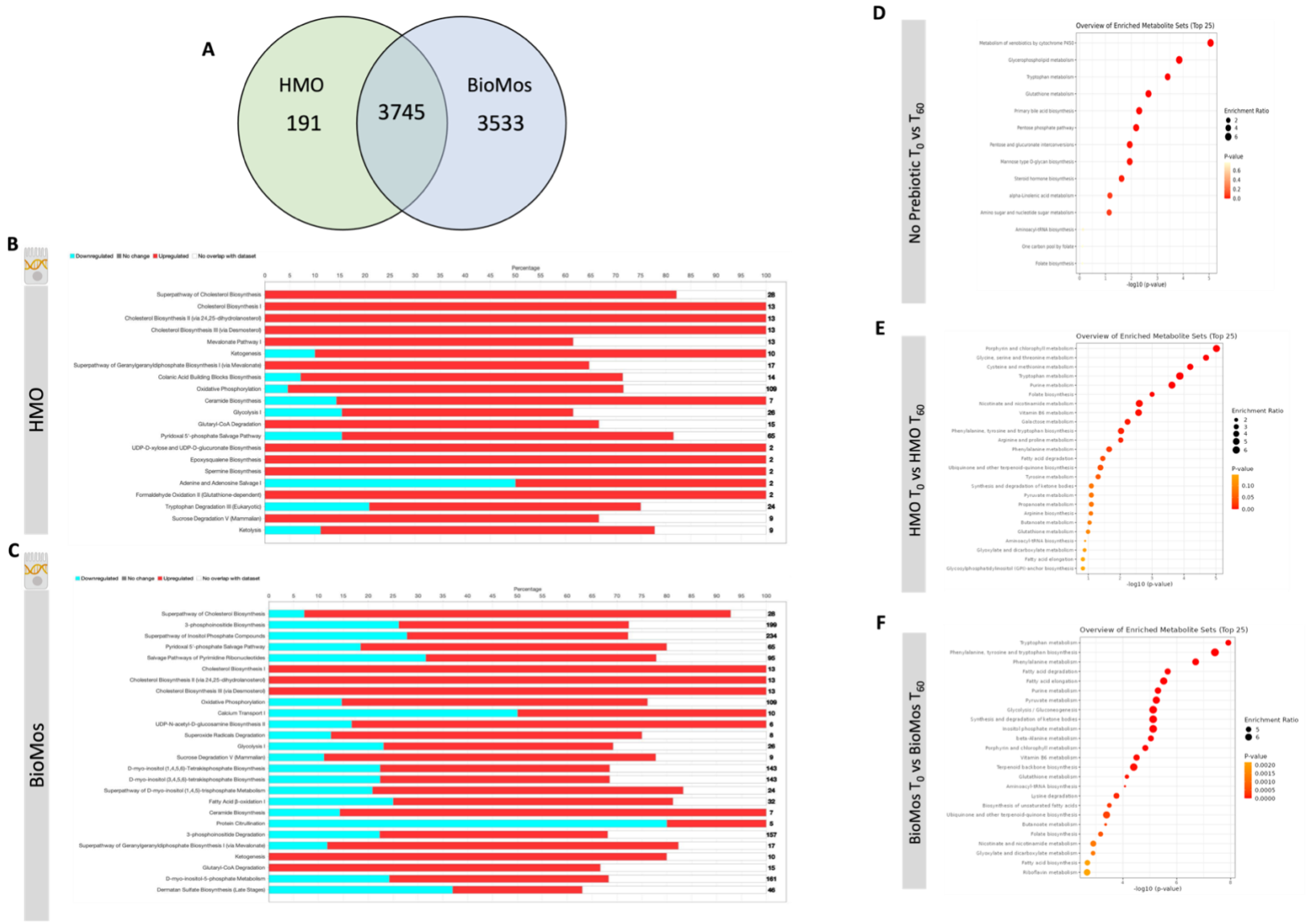

A
HMO
191
3745
BioMos
3533
B
HMO
Downregulated No change Upregulated No overlap with dataset
Percentage
Superpathway of Cholesterol Biosynthesis 28
Cholesterol Biosynthesis I 13
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol) 13
Cholesterol Biosynthesis III (via Desmosterol) 13
Mevalonate Pathway I 13
Ketogenesis 10
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate) 17
Colanic Acid Building Blocks Biosynthesis 14
Oxidative Phosphorylation 109
Ceramide Biosynthesis 7
Glycolysis I 26
Glutaryl-CoA Degradation 15
Pyridoxal 5'-phosphate Salvage Pathway 65
UDP-D-xylose and UDP-D-glucuronate Biosynthesis 2
Epoxysqualene Biosynthesis 2
Spermine Biosynthesis 2
Adenine and Adenosine Salvage I 2
Formaldehyde Oxidation II (Glutathione-dependent) 2
Tryptophan Degradation III (Eukaryotic) 24
Sucrose Degradation V (Mammalian) 9
Ketolysis 9
C
BioMos
Downregulated No change Upregulated No overlap with dataset
Percentage
Superpathway of Cholesterol Biosynthesis 28
3-phosphoinositide Biosynthesis 199
Superpathway of Inositol Phosphate Compounds 234
Pyridoxal 5'-phosphate Salvage Pathway 65
Salvage Pathways of Pyrimidine Ribonucleotides 86
Cholesterol Biosynthesis I 13
Cholesterol Biosynthesis II (via 24,25-dihydrolanosterol) 13
Cholesterol Biosynthesis III (via Desmosterol) 13
Oxidative Phosphorylation 109
Calcium Transport I 10
UDP-N-acetyl-D-glucosamine Biosynthesis II 6
Superoxide Radicals Degradation 8
Glycolysis I 26
Sucrose Degradation V (Mammalian) 9
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis 143
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis 143
Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism 24
Fatty Acid β-oxidation I 32
Ceramide Biosynthesis 7
Protein Citrullination 5
3-phosphoinositide Degradation 157
Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate) 17
Ketogenesis 10
Glutaryl-CoA Degradation 15
D-myo-inositol-5-phosphate Metabolism 161
Dermatan Sulfate Biosynthesis (Late Stages) 46
D
No Prebiotic T0 vs T60
Overview of Enriched Metabolite Sets (Top 25)
Enrichment Ratio
P-value
-log10 (p-value)
E
HMO T0 vs HMO T60
Overview of Enriched Metabolite Sets (Top 25)
Enrichment Ratio
P-value
-log10 (p-value)
F
BioMos T0 vs BioMos T60
Overview of Enriched Metabolite Sets (Top 25)
Enrichment Ratio
P-value
-log10 (p-value)

Figure 2.

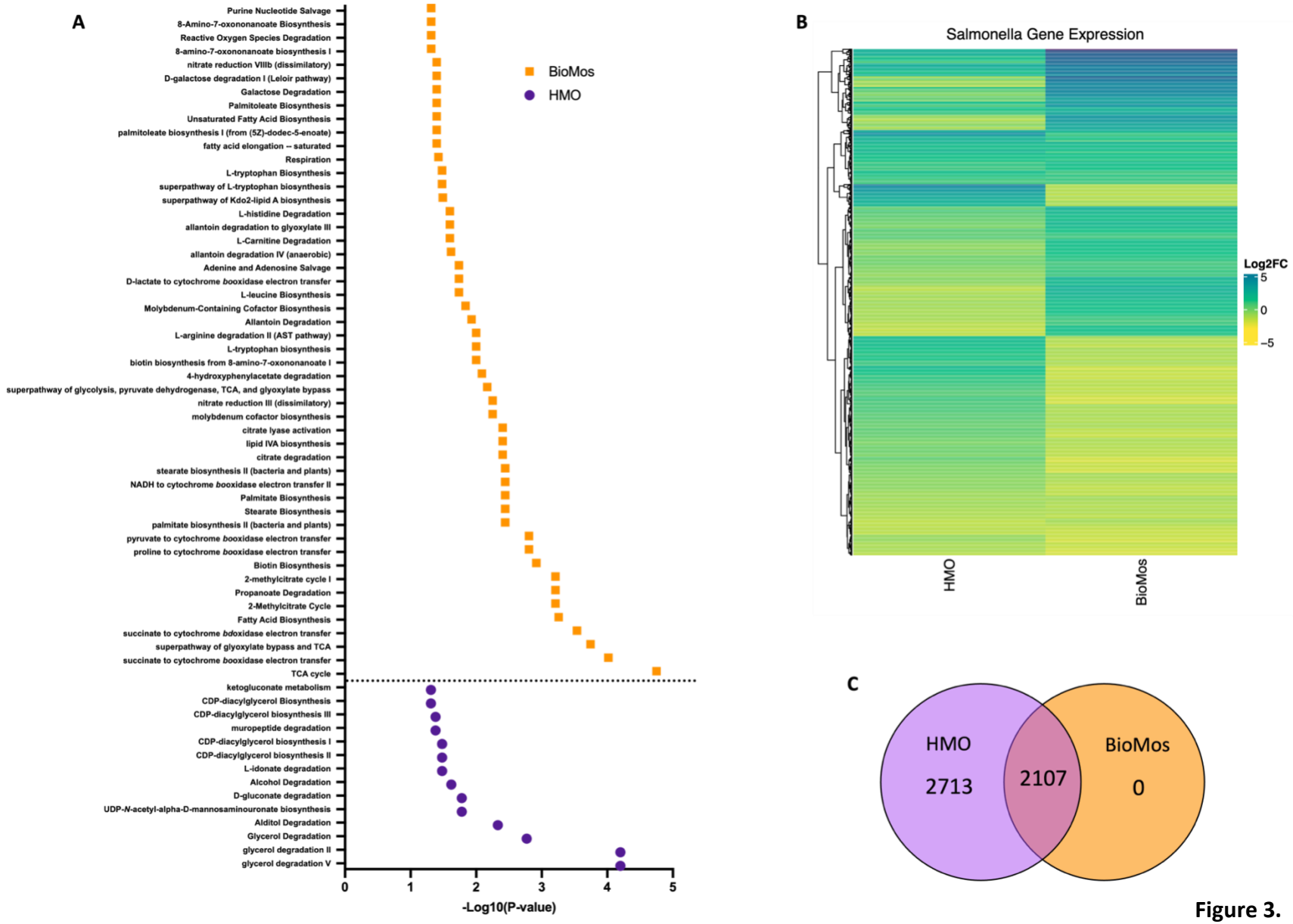

A
Purine Nucleotide Salvage
8-Amino-7-oxononanoate Biosynthesis
Reactive Oxygen Species Degradation
8-amino-7-oxononanoate biosynthesis I
nitrate reduction VIIIb (dissimilatory)
D-galactose degradation I (Leloir pathway)
Galactose Degradation
Palmitoleate Biosynthesis
Unsaturated Fatty Acid Biosynthesis
palmitoleate biosynthesis I (from (5Z)-dodec-5-enoate)
fatty acid elongation -- saturated
Respiration
L-tryptophan Biosynthesis
superpathway of L-tryptophan biosynthesis
superpathway of Kdo2-lipid A biosynthesis
L-histidine Degradation
allantoin degradation to glyoxylate III
L-Carnitine Degradation
allantoin degradation IV (anaerobic)
Adenine and Adenosine Salvage
D-lactate to cytochrome booxidase electron transfer
L-leucine Biosynthesis
Molybdenum-Containing Cofactor Biosynthesis
Allantoin Degradation
L-arginine degradation II (AST pathway)
L-tryptophan biosynthesis
biotin biosynthesis from 8-amino-7-oxononanoate I
4-hydroxyphenylacetate degradation
superpathway of glycolysis, pyruvate dehydrogenase, TCA, and glyoxylate bypass
nitrate reduction III (dissimilatory)
molybdenum cofactor biosynthesis
citrate lyase activation
lipid IVA biosynthesis
citrate degradation
stearate biosynthesis II (bacteria and plants)
NADH to cytochrome booxidase electron transfer II
Palmitate Biosynthesis
Stearate Biosynthesis
palmitate biosynthesis II (bacteria and plants)
pyruvate to cytochrome booxidase electron transfer
proline to cytochrome booxidase electron transfer
Biotin Biosynthesis
2-methylcitrate cycle I
Propanoate Degradation
2-Methylcitrate Cycle
Fatty Acid Biosynthesis
succinate to cytochrome bdoxidase electron transfer
superpathway of glyoxylate bypass and TCA
succinate to cytochrome booxidase electron transfer
TCA cycle
ketogluconate metabolism
CDP-diacylglycerol Biosynthesis
CDP-diacylglycerol biosynthesis III
muropeptide degradation
CDP-diacylglycerol biosynthesis I
CDP-diacylglycerol biosynthesis II
L-idonate degradation
Alcohol Degradation
D-gluconate degradation
UDP-N-acetyl-alpha-D-mannosaminouronate biosynthesis
Alditol Degradation
Glycerol Degradation
glycerol degradation II
glycerol degradation V
BioMos
HMO
0
1
2
3
4
5
-Log10(P-value)
B
Salmonella Gene Expression
Log2FC
5
0
−5
HMO
BioMos
C
HMO
2713
2107
BioMos
0

Figure 3.

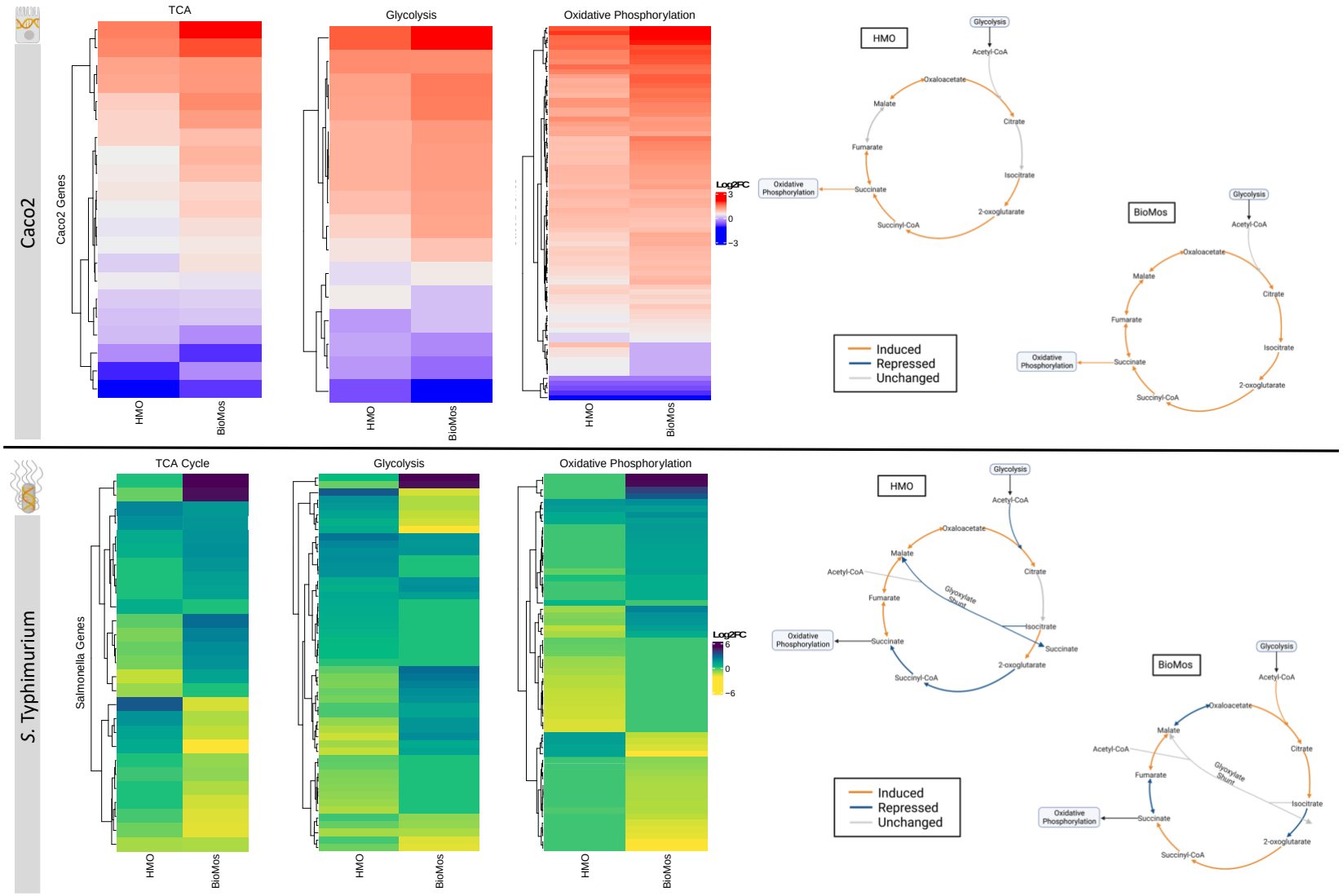

Caco2
TCA
Glycolysis
Oxidative Phosphorylation
Caco2 Genes
HMO
BioMos
Log2FC
3
0
−3
HMO
BioMos
Glycolysis
Acetyl-CoA
Oxaloacetate
Citrate
Isocitrate
2-oxoglutarate
Succinyl-CoA
Succinate
Fumarate
Malate
Oxidative Phosphorylation
Induced
Repressed
Unchanged
S. Typhimurium
TCA Cycle
Glycolysis
Oxidative Phosphorylation
Salmonella Genes
HMO
BioMos
Log2FC
6
0
−6
Glycolysis
Acetyl-CoA
Oxaloacetate
Citrate
Isocitrate
2-oxoglutarate
Succinyl-CoA
Succinate
Fumarate
Malate
Acetyl-CoA
Glyoxylate Shunt
Oxidative Phosphorylation
Induced
Repressed
Unchanged

Figure 4.

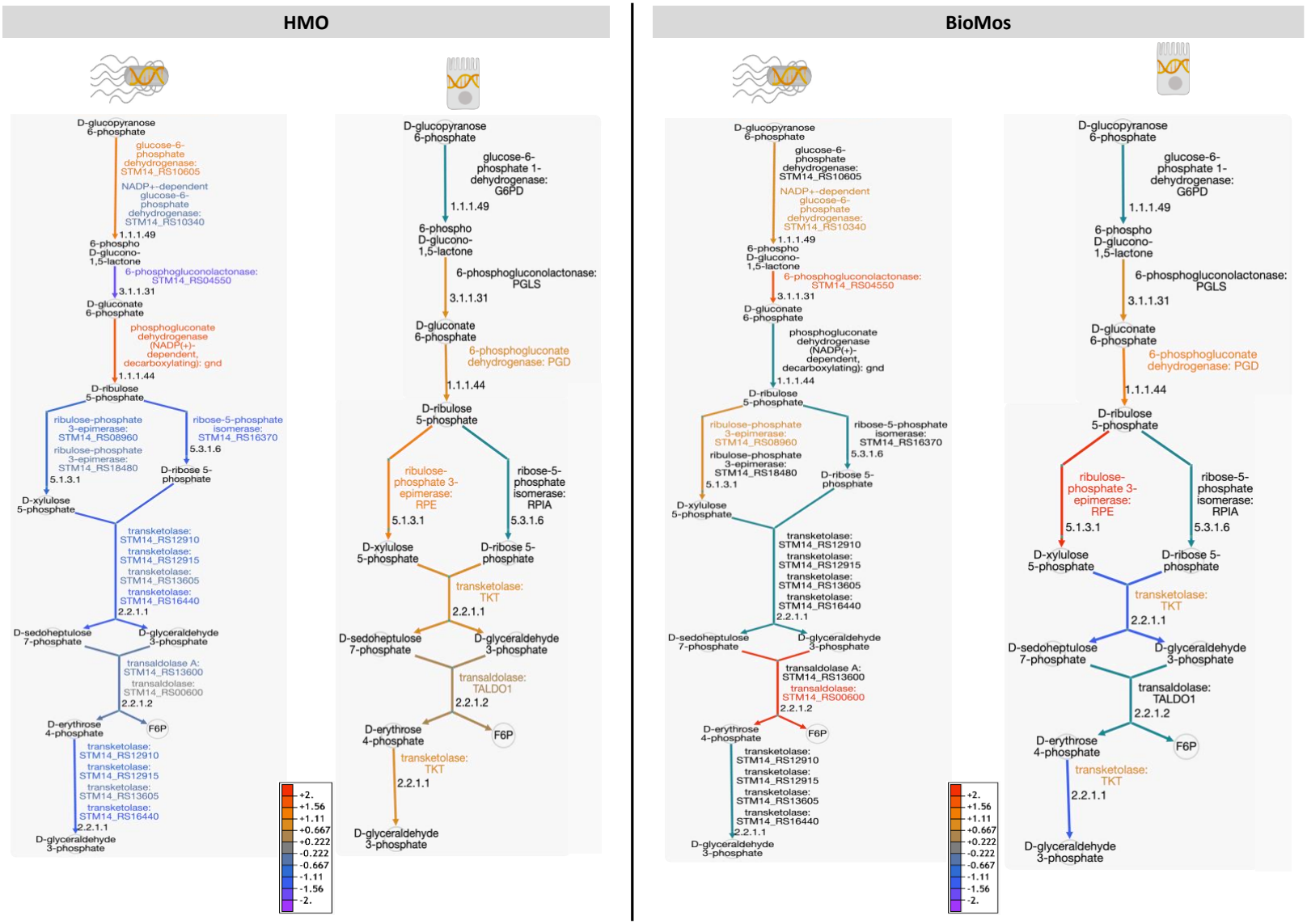

HMO
BioMos
D-glucopyranose 6-phosphate
glucose-6-phosphate dehydrogenase: STM14_RS10605
NADP+-dependent glucose-6-phosphate dehydrogenase: STM14_RS10340
1.1.1.49
6-phospho D-glucono-1,5-lactone
6-phosphogluconolactonase: STM14_RS04550
3.1.1.31
D-gluconate 6-phosphate
phosphogluconate dehydrogenase (NADP(+)-dependent, decarboxylating): gnd
1.1.1.44
D-ribulose 5-phosphate
ribulose-phosphate 3-epimerase: STM14_RS08960
ribulose-phosphate 3-epimerase: STM14_RS18480
5.1.3.1
ribose-5-phosphate isomerase: STM14_RS16370
5.3.1.6
D-ribose 5-phosphate
D-xylulose 5-phosphate
transketolase: STM14_RS12910
transketolase: STM14_RS12915
transketolase: STM14_RS13605
transketolase: STM14_RS16440
2.2.1.1
D-sedoheptulose 7-phosphate
D-glyceraldehyde 3-phosphate
transaldolase A: STM14_RS13600
transaldolase: STM14_RS00600
2.2.1.2
D-erythrose 4-phosphate
F6P
transketolase: STM14_RS12910
transketolase: STM14_RS12915
transketolase: STM14_RS13605
transketolase: STM14_RS16440
2.2.1.1
D-glyceraldehyde 3-phosphate
glucose-6-phosphate 1-dehydrogenase: G6PD
6-phosphogluconolactonase: PGLS
6-phosphogluconate dehydrogenase: PGD
ribulose-phosphate 3-epimerase: RPE
ribose-5-phosphate isomerase: RPIA
transketolase: TKT
transaldolase: TALDO1
+2.
+1.56
+1.11
+0.667
+0.222
-0.222
-0.667
-1.11
-1.56
-2.

Figure 5.

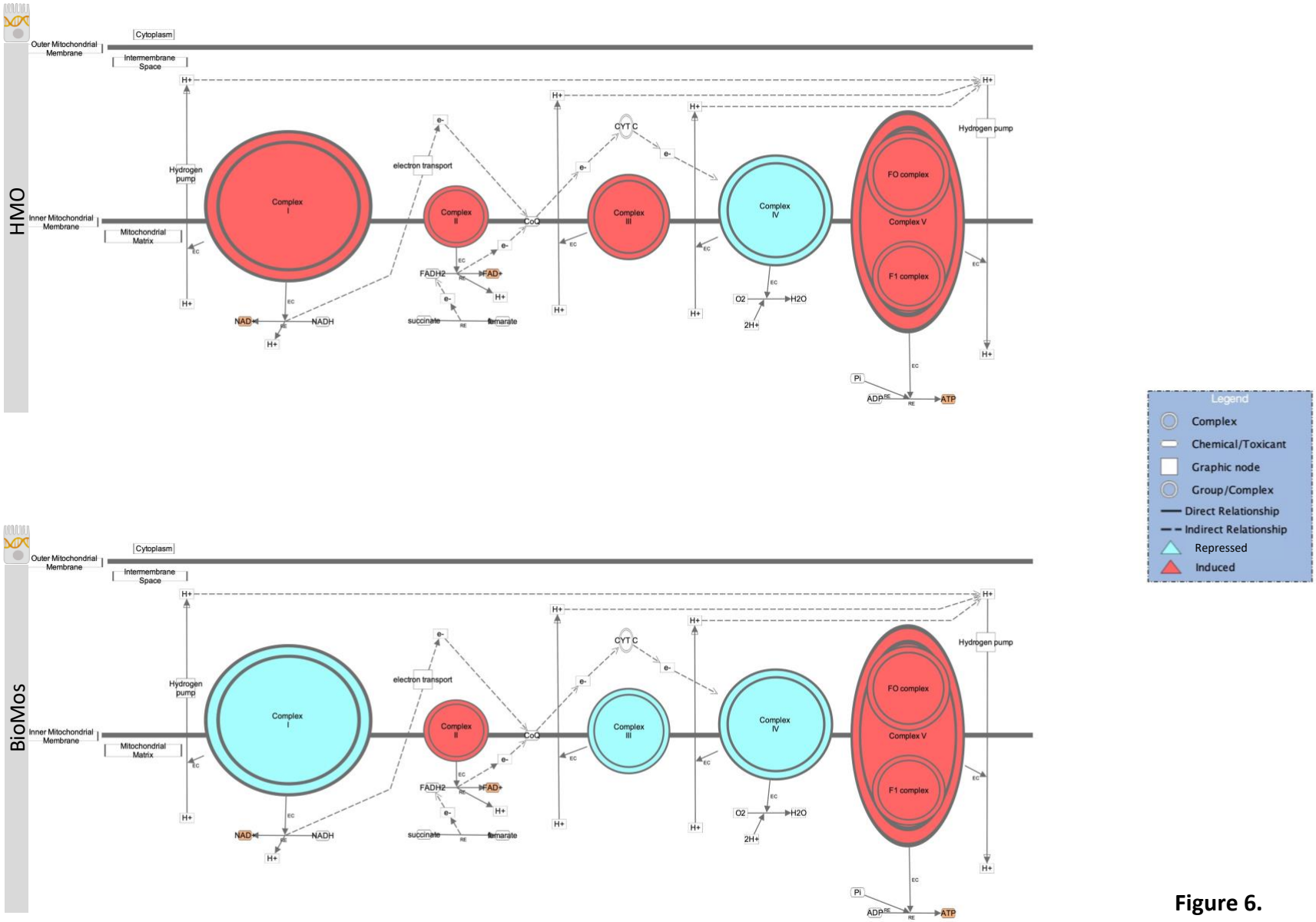

HMO
BioMos
Cytoplasm
Outer Mitochondrial Membrane
Intermembrane Space
Inner Mitochondrial Membrane
Mitochondrial Matrix
Hydrogen pump
electron transport
Complex I
Complex II
Complex III
Complex IV
Complex V
FO complex
F1 complex
CoQ
CYT C
FADH2
FAD
NAD
NADH
succinate
fumarate
O2
H2O
2H+
Pi
ADP
ATP
H+
e-
EC
RE
Legend
Complex
Chemical/Toxicant
Graphic node
Group/Complex
Direct Relationship
Indirect Relationship
Repressed
Induced

Figure 6.

Appendix

Appendix Figure 1. **Expression of virulence factors in *S.* Typhimurium and TLRs in Caco2 Cells**. (Left) Virulence factor expression in *S.* Typhimurium mixed with prebiotic treated Caco2 cells was measured as log_2 fold change from *S.* Typhimurium exposed to untreated Caco2 cells. Orange represents *S.* Typhimurium add to the BioMos condition and purple represent the HMO condition *S.* Typhimurium. (Right) Toll-like receptor expression in prebiotic treated then infected Caco2 cells was evaluated by log_2 fold change expression data with non-treated but infected Caco2 cells as control. Green represents BioMos treatment and purple represents HMO treatment.

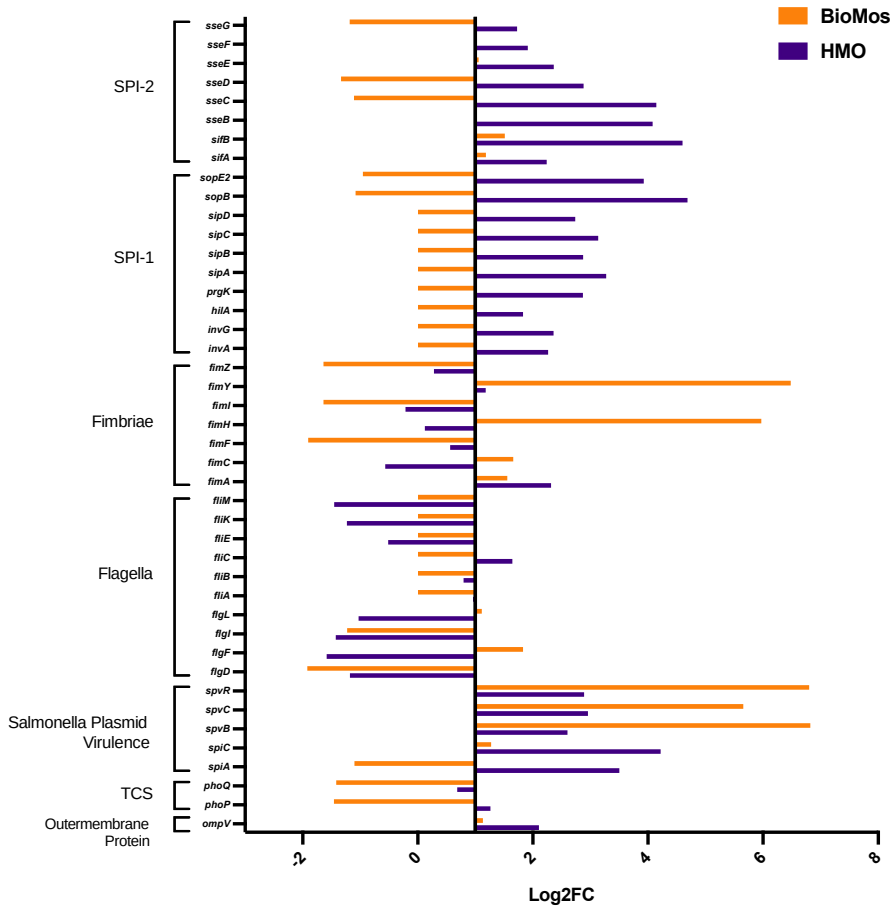
Appendix Figure 2. **Expression of a subset of transmembrane receptors in Caco2 cells diverges by prebiotic treatment.** Transmembrane receptor expression was measured in Caco2 cells pretreated with either HMO or BioMos then infected. Genes were clustered by Euclidian Distance and expression data is log_2 fold change with non-prebiotic treated by infected Caco2 cells.

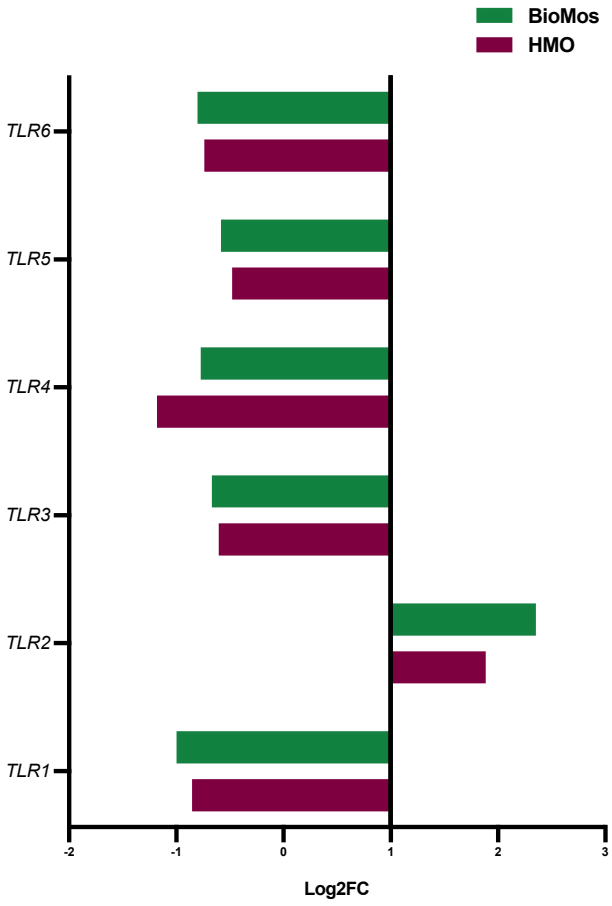
Appendix Figure 3. **Metabolic profiles of prebiotic treatments from infected Caco2 cells cluster by prebiotic type.** K-Means clustering, with cluster setting of 2, of metabolomes from *S.* Typhimurium infection across time and between prebiotic treatment groups across 60 min *S.* Typhimurium co-incubation. T_0 STM v T_{60} STM compares metabolites from Caco2/STM treatment across 0min and 60min time points. BioMos vs HMO compares metabolites across prebiotic pre-treatments at time 0 mins and 60 mins.

Appendix Figure 4. **Metabolic profiles of all conditions reveal distinct differences driven by both prebiotic treatment and *S.* Typhimurium presence/absence.** Correlation plot of untargeted metabolic profiles of all treatment combinations and individual replicates A-D for each combination created using MetaboAnalyst. Pink to red squares indicate positive correlation between metabolic profiles whereas gradients of blue represent negative correlations between profiles across sample type. Colored bars on the right-hand side of the plot indicate sample grouping by prebiotic type, pathogen presence/absence, and time course. Time 0 corresponds to 15 minutes post prebiotic addition and initiation of *S.* Typhimurium addition. Time 60 is 60 minutes post *S.* Typhimurium inclusion or 75 minutes after initial prebiotic addition when a *S.* Typhimurium is not added.

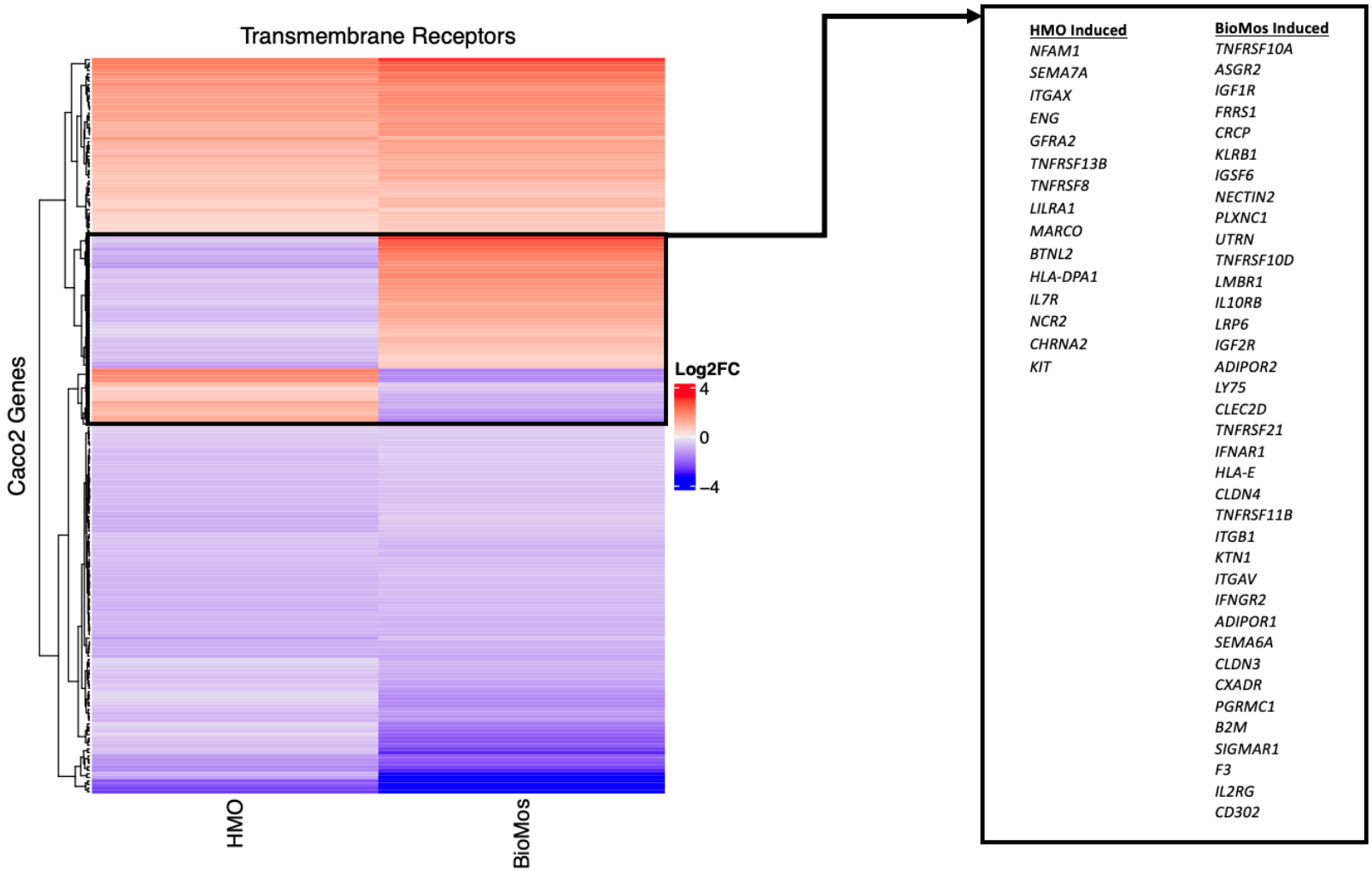
Appendix Figure 5. **Metabolites are significantly altered by treatment.** The metabolic profiles underlying the correlation plot in Appendix Figure 4 were used to search for significance across all treatment combinations. The Kruskal Wallis Test in MetaboAnalyst revealed 252 significant metabolites out of 316 total.

Appendix Figure 6. **Graphical abstract of basic experimental set-up.** Caco2 cells (ATCC HTB-37) were grown and differentiated before being pre-treated with 1% HMO or BioMos for 15 mins. Stationary *S. enterica* sv. Typhimurium 14028 was then added to the cells and co-incubated for 60 mins. Cells and supernatant were collected, washed and stored for RNA-seq and metabolomics follow-up.



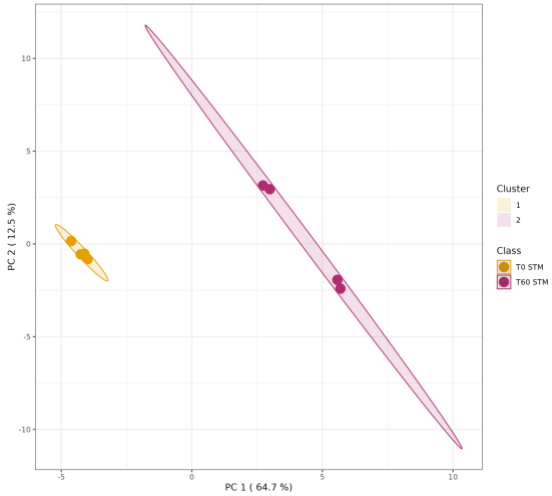


Appendix Figure 1.

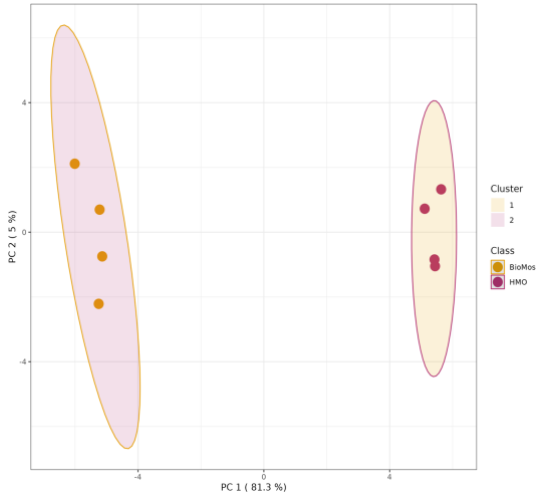


Appendix Figure 2.

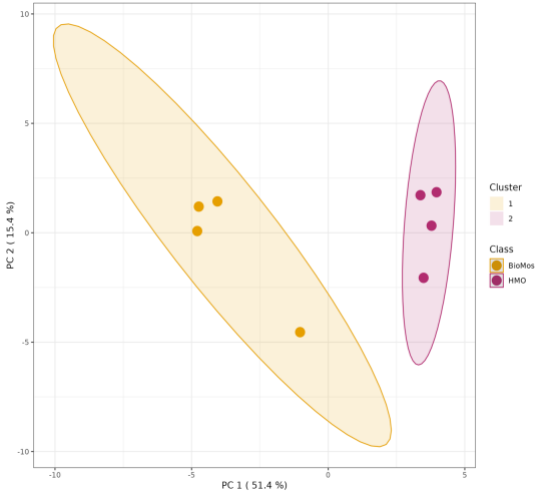
T_0 STM vs T_{60} STM



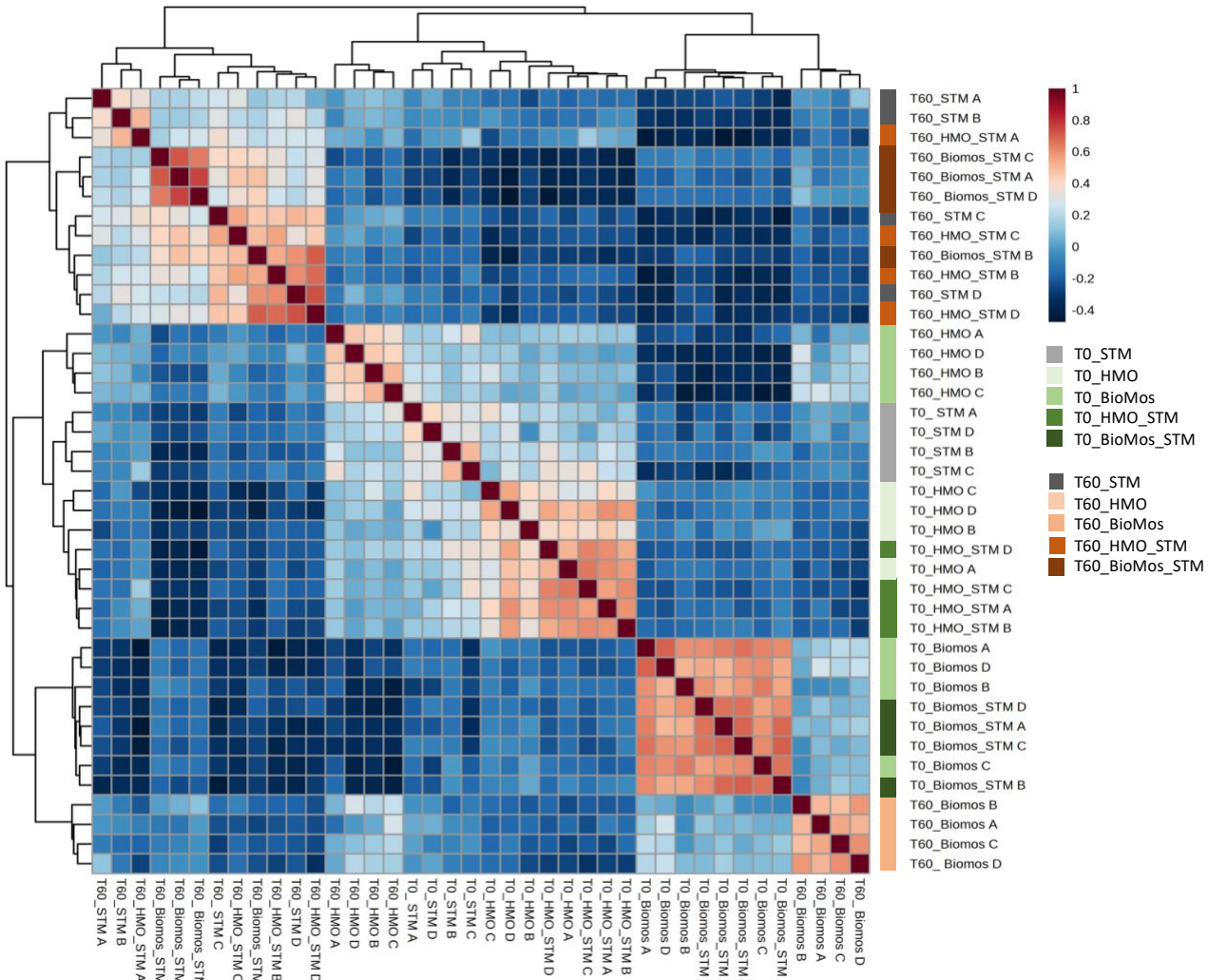
T_0 BioMos vs T_0 HMO



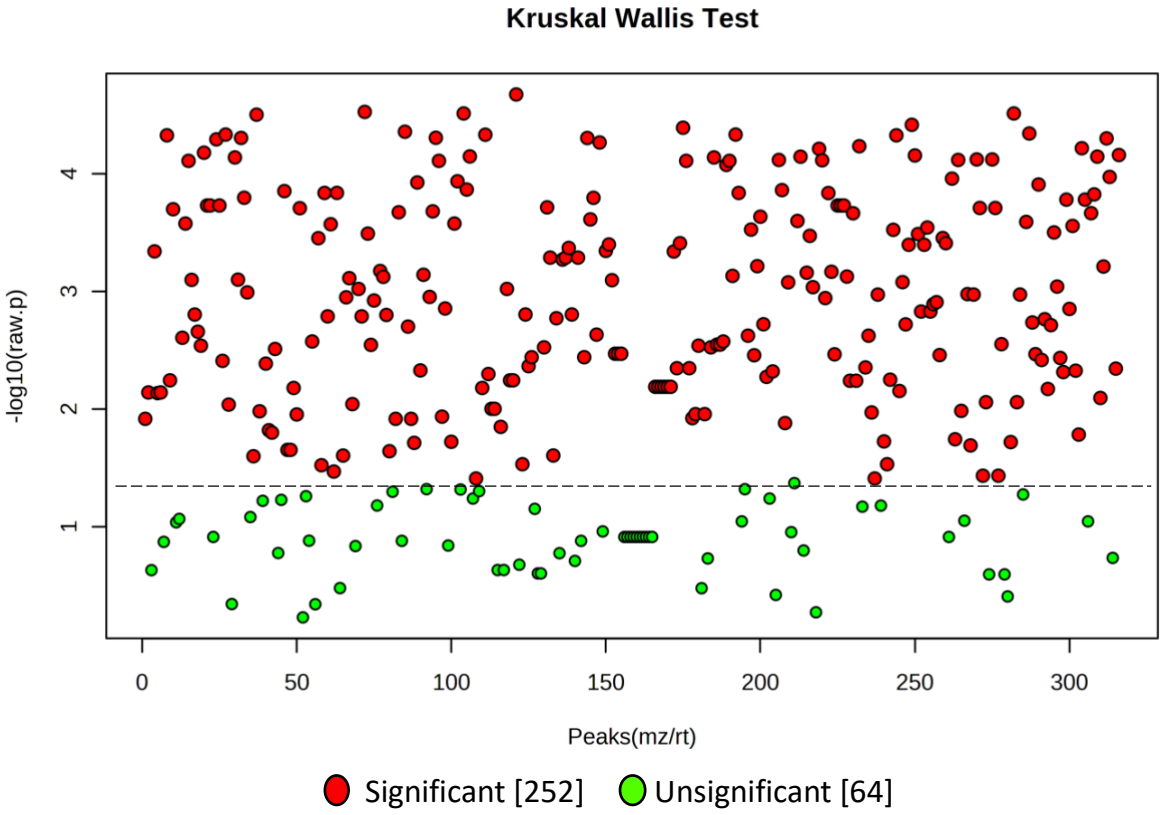
T_{60} BioMos vs T_{60} HMO



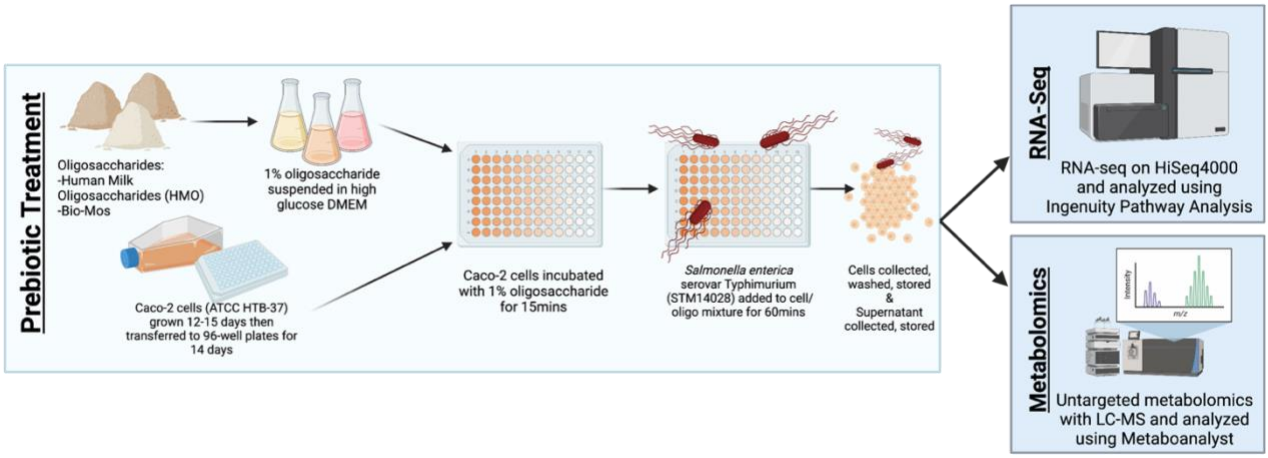
Appendix Figure 3.



Appendix Figure 4.



Appendix Figure 5.



Appendix Figure 6.

Chapter 5

Concluding Remarks

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Abstract

Metabolites are key to host health driving cell function and ultimately host health and disease. Microbial residents of the gastrointestinal system expand the metabolic breadth available to the host and contribute otherwise inaccessible nutrients, like vitamin B_{12}. As transient members of the gastrointestinal tract, pathogens also contribute to this communal metabolic activity, driving shared metabolic pathways in directions that suit the pathogen's needs. *Salmonella enterica* sv. Typhimurium for example is a pathogen that can stage a gut takeover by using metabolic substrates and altering lumen conditions to benefit pathogenic colonization. The complex interplay of the host-pathogen-metabolite triangle renders gaining a mechanistic understanding difficult, but the health implications of this relationship make an improved understanding necessary. The work undertaken in this dissertation examined these complex relationships through a deep dive into the metabolic control of host health and of pathogenic activities. First, there was an investigation of how microbe-produced metabolites from the three major tryptophan pathways (i.e., kynurenine, serotonin, and indole) influence host neurology through the gut-brain axis, as gut pathogens are adept at producing these compounds during carriage and infection. Second, the metabolic story was flipped and the effect of host-produced metabolites on pathogenic behavior via modulation of two-component systems was examined. Finally, these findings were applied via an investigation of the effect of structurally diverse dietary oligosaccharides on pathogenic behavior in *S.* Typhimurium, wherein it was shown that diverse substrates alter host and pathogen metabolism in ways that influence host-pathogen association. Together these findings underscore the importance of metabolic activity in driving host health and disease, as well as highlight the potential role enteric pathogens may play in altering the influential host metabolome that changes both the host and the microbial metabolism – the yin and yang of the association.

Findings

The shared host-microbe metabolome has shown to be an important regulator of host health and microbial activity, and it has become quite evident that small molecules can exert significant influence on microbial signaling cascades and on host neurology [1-4]. Despite the clear importance of the metabolome, the complexity of the triangular host-microbe-metabolite relationship presents great difficulty for developing a better mechanistic understanding. Due to this complexity, there remain many mechanistic questions and the consequences of these interactions remain unknown. Considering both what is known about the importance of the metabolome and the remaining mechanistic knowledge gaps, **we hypothesized that shared host-microbe metabolism produces small molecules that exert bi-directional bioactive control so that shifts in this shared metabolome result in consequential health outcomes for the host, altered microbial activity in the gut, resulting in a cooperative virulence effect between the host and enteric pathogens.** This hypothesis was tested through three specific aims:

Aim 1) Identification of key literature investigating the role of microbial produced tryptophan-derived metabolites in modulating host neurological functions and evaluation of this microbial role through identification of gut microorganisms with key enzymes using known association and informatic protein interaction predictions.

Aim 2) Display of the bi-directional nature of metabolic signaling through evaluation of host metabolic control of the prokaryotic two-component system and identification of gut microbiota with these signaling cascades using informatic assessment and known microbial systems that alter bacterial physiology.

Aim 3) Examination of the interaction between colonic epithelial cells and the enteric pathogen *S.* Typhimurium in the presence of two distinct prebiotic oligosaccharides as a method to change the host/microbe metabolic dialogue via metabolism and physical association.

The host and resident microbes share many metabolic pathways and disentangling these shared pathways and the subsequent effects is challenging due to the microbial variation across individuals, the constant flux of small molecules in host circulation, and the many confounding factors naturally present in human subjects [5, 6]. Regardless of the complexity, the metabolome remains an important contributor to or biomarker of host health. This is supported by the findings highlighted under Aim 1 during which a thorough review of the literature around the role of tryptophan-derived metabolites in neurological diseases was performed. This review revealed that at a broad level there is consensus around tryptophan metabolites being both metabolic substrates and bioactive molecules, though more detailed analysis showed heterogenous and sometimes conflicting results. Human research presents many confounding factors and study designs vary widely, both likely explanations for the conflicting results. The investigation of the gut-brain axis and role of metabolites is a more recent area of research and therefore there are fewer clinical studies, which also contributes to the lack of agreement and minimal mechanistic understanding in this area.

Tryptophan is an essential amino acid in humans and central to basic metabolic pathways in humans and microbes, making tryptophan metabolites shared and potentially competitive substrates between a host and their resident microbes [7, 8]. Anthranilate, kynurenic acid, and quinolinic acid are all tryptophan-derived compounds that either modulate neurological physiology or are biomarkers for neurological conditions in humans [9-11]. As microbial production and consumption of tryptophan metabolites shift the balance of these compounds, the gut microbiome is a key contributor to health outcomes related to these metabolites. Investigation of orthologous and paralogous genes related to microbial tryptophan metabolism through OrthoDB [12] suggested that tryptophan metabolism is widespread throughout microbes commonly found in the gut microbiome. Additional investigation of the gene-cooccurrence for kynurenine-related enzymes (i.e., IDO1, TDO2, KYNU) using the STRING database [13] showed broad

distribution of these enzymes in common gut microbes, concurrent with those results from OrthoDB. Together, the findings from literature database searches that were conducted as part of this dissertation support the importance of tryptophan metabolites in host health, specifically host neurology, and suggests the contribution of the gut microbiota is a key component of interkingdom metabolic signaling.

Small metabolites from shared metabolism also influence the behavior of microorganisms, highlighting the bidirectionality of metabolic signaling. Results from Aim 2 supported this notion of host control over gut microbial actions, and more specifically control over enteric pathogens, through an exploration of host-derived ligands for virulence-associated two-component signaling systems. Two-component signaling systems are integral signaling cascades in prokaryotes, consisting of a membrane embedded histidine kinase sensor and an internal aspartate response regulator, which translate environmental signals into intracellular responses. Under the transcriptional control of these signaling cascades are many significant genes including flagella structures, biofilm regulating proteins, virulence factors, and stress responses [14-18]. Some bacterial genomes have even been shown to encode for over 200 unique cascades [19]. A literature review revealed that many of these signaling cascades respond directly to host-derived metabolic cues, like epinephrine or Mg^{2+} concentrations, thus supporting the notion that the host can exert control over pathogen activities through metabolic regulation [20, 21].

Given that two-component systems are a vital part of prokaryotic metabolic and cellular regulation, and that multiple host-derived metabolites serve as ligands for enteric pathogen systems, we hypothesized that through these signaling cascades the gut microbiome would also be under the same host control. To test this hypothesis, we utilized the Integrated Microbial Genomes and Microbiomes Database (IGM/M) [22] from the Joint Genome Institute (JGI) to look for two-component systems within public

domain human gut microbiota metagenomes. A search of 20 public metagenomes from the gut microbiota revealed the presence of metabolite-controlled two-component systems in common gut taxa including *Firmicutes, Klebsiella,* and *Intestimonas*. The expansive literature investigating the metabolic control of virulence controlling two-component systems in enteric pathogens, alongside the presence of these signaling systems in common gut microbiota, supports that metabolites serve not only as signals for the host but also for gut microbiota. Such exogenous control of microbial transcription implies that control of metabolism across host and microbiome is concomitant with the regulation of host neurology (Aim 1) and microbial function (Aim 2).

Many metabolites serve as both fuel for basic cellular processes and as bioactive signals for host and microbe modulation. This dual role of metabolites indicates the exogenous manipulation of metabolism through the addition of dietary substrates is not necessarily a straightforward process, as the multifaceted bioactive nature of metabolites must be considered alongside their more basic role as cellular fuel. One proposed mechanism for externally driven metabolic manipulation is through the consumption of prebiotics, substrates whose fermentation by beneficial microbes confer a health benefit to the host [23]. Many prebiotics are publicly available and marketed on their proposed benefits for the consumer, but as evidenced by the findings of Aims 1 and 2, the consumption of dietary supplements may have unintended or off-target systemic consequences. In Aim 3 we put this metabolic control to the test by adding two structurally distinct prebiotics into an *in vitro* model containing human colonic epithelial cells and the enteric pathogen *S.* Typhimurium. The use of this focused host-pathogen model without commensal microbes allowed for the development of more mechanistic understanding of pathogen-prebiotic interactions, which supports the more informed use of prebiotics.

In this focused host-pathogen model, two prebiotics were tested for their potential attenuation of pathogen association with host epithelial cells. The addition of both human milk oligosaccharides (HMO) and a commercially available yeast cell wall product (BioMos®) decreased *Salmonella* association with the host epithelial cells. Interestingly, increased BioMos® dosage resulted in increased pathogenic association compared to lower dosages, while the opposite trend was seen with HMO, showing that both prebiotic and dose dependent mechanisms were at play. Further analysis of these results through metatranscriptomics and untargeted metabolomics likewise revealed both prebiotics remodeled the host-pathogen interactions through alterations in gene expression pathways related to virulence, host surface receptors, and shared host-pathogen metabolism. *Salmonella* Typhimurium showed increased expression of virulence factors like specific pathogenicity island 1 and 2, while the colonic epithelial cells showed induction of the pathogen sensing Toll-Like Receptor 2. Also notable was that *Salmonella* incubated with HMO upregulated many virulence-controlling two-component systems which, in combination with the findings of Aim 2, supports that metabolites regulate pathogenicity in the gut. Though virulence factor transcription was clearly affected, likely due to prebiotic signaling and pH shifts, the adhesion and invasion of host cells was still reduced in prebiotic conditions. *S.* Typhimurium uses glycosyl hydrolase (GH) enzymes to degrade the glycocalyx layer of host epithelial cells for membrane access [24], and prebiotic pretreatment repressed the expression of these enzymes. Although multiple common virulence factors were induced, *S.* Typhimurium was unable to deploy these factors at the host membrane due to the reduction in GH activity and the subsequent inability to access the host cell surface. Attenuation of pathogen-host association was observed in this study, but the induction of virulence factors by prebiotic treatment remains a concerning finding, as not all hosts have healthy glycocalyx layers in the colon, suggesting prebiotic treatment in these cases could induce increased pathogenesis.

Beyond the regulation of genes related to physical host-pathogen interactions, the addition of HMO and BioMos® altered the expression and metabolite profile around energy-producing pathways in host and pathogen. *S.* Typhimurium has a sophisticated metabolic repertoire that allows for fine adjustments to improve the chance of survival and colonization in fluctuating host conditions [25]. HMO addition to the focused host-pathogen model revealed genetic regulation of the aforementioned cellular energy and respiration pathways resulted in expression patterns and metabolic profiles more similar to that typically seen in the early stages of colonic lumen colonization. The pathways tended toward anaerobic respiration, which results in ATP concentrations able to fuel basic colonization but not drive increased virulent activity compared to non-prebiotic conditions. In contrast, the addition of BioMos® to this model resulted in *S.* Typhimurium expression patterns more aligned with aerobic respiration and increased ATP production, increasing the available fuel for virulent activity. Notably the metabolic gene expression profiles for BioMos® treated *S.* Typhimurium were more closely aligned to those seen in the active gut colonization phase and suggest trends towards pathogenic success rather than attenuation. The additional finding that *S.* Typhimurium in the BioMos® condition upregulated amino acid related metabolic pathway, also important contributors to pathogenesis, implies commercial prebiotics present the additional risk of non-oligosaccharide components contributing to off-target effects. BioMos® contains amino acids and other components beyond mannanoligosaccharides due to it being a yeast-derive product while the HMO was lab extracted contained minimal extraneous substrates. The prebiotic-specific regulation of virulence factors, host membrane receptors, and metabolic modulation seen in this study suggest prebiotics regulate host-pathogen interactions irrespective of the commensal microbiota and support that prebiotics may in some cases exert protective effects but do so via context and substrate specific mechanisms.

The combined results of Aim 1, 2, and 3 support the hypothesis that shared host-microbe metabolites exert bi-directional control and influence both systemic host health and microbial function and shifts in these shared pathways results in metabolite-dependent alterations of host-pathogen interactions. Findings from Aim 1 showed microbial metabolites from tryptophan digestion affect host neurological function and may either serve as causative agents or biomarkers for diseases such as bipolar disorder, depression, or schizophrenia. Results from Aim 2 demonstrated the interkingdom nature of metabolic signaling by investigating the control of enteric pathogenicity by host-derived metabolic products through the prokaryotic two-component signaling system. Microbes in the gut, whether pathogenic or not, use two-component systems to respond to luminal conditions (e.g., pH, metabolite concentrations, osmolarity) through signal transduction and transcriptional regulation. Furthermore, findings of Aim 2 suggest pathogenic success can be modulated by host control of metabolites in the large intestine. And finally Aim 3 demonstrated the importance of metabolic control in the context of *S.* Typhimurium infection and prebiotic consumption. The ability of HMOs and BioMos® to reduce host-pathogen association was tested in our focused model. Both prebiotics generally reduced host association compared to a control without prebiotic supplementation, though the protective efficacy varied by dosage and by prebiotic type. Further analysis revealed the upregulation of virulence factors in both prebiotic treatments, but the general repression of the GH enzymes necessary to deploy the virulence factors. Both prebiotics also altered energy producing pathways, including the TCA cycle, glycolysis, the pentose phosphate pathway, and oxidative phosphorylation. The regulation of these pathways is a central component of successful *Salmonella* colonization, suggesting this is a critical finding for understanding how prebiotics may exert protective effects, or conversely, how they may support pathogenesis in some gut contexts. Together, findings from this dissertation work support that metabolites are central signaling molecules across host and microbes and are significant contributors to systemic host health and disease outcomes.

Future Work

The manipulation of shared metabolism to promote beneficial host health outcomes is an active and promising area of research [26, 27]. Recent studies in the field indicate prebiotic supplements as a potential path towards positive health outcomes via metabolic shifts in specific contexts [28, 29]. Despite these notable and encouraging results, the direct interaction of prebiotics with enteric pathogens remains understudied. Multiple studies support the control of enteric pathogenesis by prebiotic supplementation [30-32], but there is currently a lack of reductionist models. Such focused *in vitro* host-pathogen models provide insights into how enteric pathogens respond to prebiotics without the healthy buffer of gut commensals, making these models more representative of a gut after antibiotic treatment or of some chronic gastrointestinal illnesses [33, 34]. The work reviewed and performed here indicate focused host-pathogen models [35, 36] as imperative tools for gaining a deeper understanding into the positive effects of prebiotics and also for providing early warning for any potential negative effects which may be overlooked in *in vivo* studies. The indication that both HMO and BioMos® induced the expression of common virulence factors in *S.* Typhimurium, despite the overall observation of reduced host cell invasion, is one such warning sign that not all host contexts are primed for prebiotic treatment. Dysbiosis, microbiome diversity shifts, and chronic inflammation may all play a role in tipping the prebiotic scale towards or away from protective activities.

As many prebiotics are currently publicly available [37, 38], future research should also make use of the focused host-pathogen model used here to investigate whether the consumption of other prebiotic dietary substrates may in fact drive pathogenic behavior in less ideal gut conditions. The next step beyond these focused models would be the controlled addition of keystone commensal microbes [39] to this host-pathogen system to better elucidate how commensal and pathogens compete for prebiotic

substrates in a host context and how such a competition might play out *in vivo*. Together this dissertation work, along with the studies that came before it, suggests that the way forward to protective prebiotics is using a bottom-up research approach wherein we seek to understand the mechanics of prebiotic-pathogen interactions at a deeper level prior to widespread application in yet unknown host contexts.

Impact

Exogenous control of the human-microbe metabolome and the subsequent modulation of host health and microbial activity is as a promising therapeutic route for treating gastrointestinal diseases and neurological diseases like IBD and schizophrenia [40-45]. Though this route holds promise for improved healthcare in the future, much remains unknown about how exactly to exert such fine metabolic control or what metabolites we should even be attempting to alter in the first place. In tandem, the interaction of enteric pathogens with dietary interventions also remains of interest as diet can play a role in attenuating or driving virulence in a pathogenic population [46, 47]. Taken together, the control of metabolism in host and microbe may be a breakthrough for the treatment of drug-resistant or otherwise untreatable diseases, but a cautionary approach must be taken. The work presented here highlighted both sides of this story; that *i)* shared host-microbe metabolites may serve as key markers or causative agents of neurological diseases and thus be therapeutic targets and *ii)* that the addition of dietary substrates to alter host health may contribute to increased virulence of enteric pathogens and thus cause detrimental effects to host health. The development of metabolic interventions for broad clinical application is promising but as suggested by the findings in this dissertation, the complexity of shared host-microbe metabolism necessitates the use of refined mechanistic models for the elucidation of the full range of effects for more conscientious and efficacious applications of metabolic therapies.

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