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Specialized metabolites from the microbiome in health and disease

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Abstract

The microbiota, and the genes that comprise its microbiome, play key roles in human health. Host-microbe interactions affect immunity, metabolism, development, and behavior, and dysbiosis of gut bacteria contributes to disease. Despite advances in correlating changes in the microbiota with various conditions, specific mechanisms of host-microbiota signaling remain largely elusive. We discuss the synthesis of microbial metabolites, their absorption, and potential physiological effects on the host. We propose that the effects of specialized metabolites may explain present knowledge gaps linking the gut microbiota to biological host mechanisms during initial colonization, and in health and disease.

Introduction

In the human body, microbial cells outnumber eukaryotic cells by as many as ten to one (Savage, 1977), and contribute two orders of magnitude more genes to the hologenome (Gill et al., 2006; Turnbaugh et al., 2007). Their significance to host health and disease is thus unsurprising, although it was long overlooked. The bacterial metagenome contributes to production of primary metabolites and conversion of small molecules into secondary metabolites, also called “specialized metabolites”. Products of bacterial metabolism are believed to modulate human health in many ways (Hooper et al., 2012, Blumberg and Powrie, 2012). Crosstalk between microbial and human metabolites influences processes such as nutrient and xenobiotic metabolism (Maurice et al., 2013), protection against pathogens (Clarke et al., 2010), regulation of the enteric nervous system (Soret et al., 2010), immune regulation (Brestof and Artis, 2013), resistance against colorectal cancer (Hague et al., 1995; Hinnebusch et al., 2002; Lan et al., 2007; Tang et al., 2011; Nicholson et al.,

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2012), complex neurological behavior (Hsiao et al., 2013), and affect lipid and cholesterol levels in serum (Berggren et al., 1996; Delzenne et al., 2002). Metabolic pathways operating in the human body are thus the result of the combined activities of the human genome and microbiome.

Diet is important to the microbiota and metabolome, as food is a major source of precursors for metabolite production. Humanized mice fed a polysaccharide-deficient diet differed in both the microbiome and metabolome from humanized mice on regular chow (Marcobal et al., 2013). Conventionally raised mice fed the same diet were also significantly different from conventional mice fed regular chow or the humanized mice (Marcobal et al., 2013). In humans, both long term dietary patterns (Ridaura et al., 2013; Wu et al., 2011) and rapid, extreme dietary changes (Levy and Borenstein, 2014) are reflected in the microbial communities and metagenome. In particular, amino acid intake correlates with changes to microbial communities. For example, increased amino acid intake increased the relative abundance of Bacteroidetes, and changed the metabolome (Ridaura et al., 2013; Wu et al., 2011). Individuals who ate a primarily animal-based diet with little to no fiber for two days showed decreased levels of acetate and butyrate in the gut compared to those who consumed a plant-based diet over the same period (Levy and Borenstein, 2014).

Energy balance depends on the diet, but also on the microbiome. Kwashiorkor, a severe form of malnutrition, can be transferred from affected people to mice by fecal transplantation (Smith et al., 2013). Specifically, fecal samples from human twins, where one co-twin was malnourished and the other was not, produced substantially different phenotypes in recipient mice. Feeding the mice a traditional Malawi diet led to a loss of 30% of body mass in just 3 weeks in the mice that received microbes from the malnourished co-twin, while mice transplanted from the healthy co-twin maintained normal body weight on this diet. These differences in the microbiome were coupled to differences in amino acid, carbohydrate and fat metabolism, even on the same diet. Dietary responses also differed depending on the microbiome: Ready-to-use Therapeutic Food, a nutrient rich and energy dense dietary supplement, was able to rescue the mice receiving the kwashiorkor microbiome from weight loss, but produced little change in the mice receiving the healthy microbiome (Smith et al., 2013). This study reflects the importance of both microbiome composition and diet in determining the metabolic profile, which, in turn, influences disease outcomes.

At the other metabolic extreme, obesity can also be modulated by the gut microbiome, metabolome, and diet. Ridaura et al transferred microbes from lean and obese co-twins into germ-free mice (2013). The metagenomes of lean co-twins were enriched for genes encoding proteins that ferment complex carbohydrates from plant sources. Co-housing lean and obese mice mitigated the obese phenotype in a diet-dependent fashion. A low-fat, high plant polysaccharide diet promoted weight loss in co-housed obese mice, while a diet high in saturated fat and low in plant materials did not significantly alter the obese phenotype of co-housed animals. Co-housed obese animals fed a low fat diet also differed in metabolite profiles from controls.

Commensal microbes, present at various mucosal surfaces of the mammalian host, play a critical role in processing environmental signals (e.g., diet, xenobiotics) and relaying "messages" to the epithelium and via the circulatory system. These messages are classically thought of as microbial-associated molecular patterns (MAMPS), common to various microorganisms, or as metabolites involved in paracrine or endocrine signaling to the host. Often, these metabolites can be beneficial. For example, yet unknown secreted factors of commensal skin bacteria can induce production of human antimicrobial peptides such as defensins resulting in modulation of human innate immune response (Maurice et al., 2013). An important function of the human microbiome is to supply essential water soluble vitamins including vitamin K, vitamin B12, biotin, folate, thiamine, riboflavin and pyridoxine which are then absorbed by the intestines (Martens et al., 2002; Said, 2011). Several recent reports have now identified specific microbial metabolites beyond those traditionally associated with gut bacteria, and may represent incipient advances in understanding the inextricable chemical link between mammals and their microbiota. Likely, the vast majority of interkingdom molecular communications remain unknown.

Here, we present pathways associated with the production of primary and specialized metabolites, and examine their possible role in pathogenesis. We suggest a framework for studying and understanding the role of specialized metabolites, focusing on their production in the gut, followed by absorption, then circulation to their target sites (Figure 1). Application of this framework would allow better understanding of many pathologies, and potentially reveal new therapeutic targets.

Metagenome, metatranscriptome and metabolites

The microbial metagenome links taxonomic identification to metabolite production. Metagenomic analysis catalogs genes within a microbial environment, and helps provide mechanistic explanations for metabolic changes associated with disease. A systems-level approach comparing the metagenomes of individuals with obesity or inflammatory bowel disease (IBD) to healthy controls suggested that the disease states were associated with loss or gain of enzymes which served to initiate or terminate metabolic pathways (Greenblum et al., 2011). Furthermore, shifts in the microbial metagenome translate to changes in metabolic profiles. Mice transplanted with microbes from obese humans had a reduction in butyrate fermentation genes, along with decreased butyrate levels in the cecum (Ridaura et al., 2013). Horizontal gene transfer between species, and poor species-level resolution in 16S rRNA gene sequencing, can limit the accuracy of direct metabolic predictions (Ploz et al., 2013; Wang et al., 2007), although coarse-grained predictions of metabolism from the metagenome has still been useful in both host-associated and environmental (Larsen et al., 2014) contexts.

Metagenome sequencing also shows how pathogenic organisms adapt to polymicrobial infections in the host. Sequencing *Rothia mucilaginosa* genomes from cystic fibrosis (CF) patients and healthy controls revealed adaption by encoding multiple genes for lactate dehydrogenase, enabling utilization of lactate produced by the host and co-inhabiting pathogens (Lim et al., 2013). Other means of adaption included acquisition of genes for phage lysins, modification of genes responsible for horizontal gene transfer, and genes

speculated to play roles in modulation of biofilm formation, namely CRISPR elements (Lim et al., 2013). Metagenomic prediction could, potentially, be refined through metatranscriptomic and metaproteomic analyses. These methods may provide mechanistic insight into the regulation of enzymes of interest, as the presence of a gene does not guarantee expression of the corresponding protein product. Bacterial protein expression is regulated at the transcriptional and the translational level by many mechanisms (reviewed in Berthoumieux et al., 2013; Quax et al., 2013). Thus expression-level techniques may be especially useful in examining rapid changes in bacterial function (Poretsky et al., 2009; McCarren et al., 2010; Kolmeder et al., 2012). Integrating multi-meta-omic techniques may provide many more direct links between bacteria and their metabolites than are currently known.

Microbial metabolite synthesis acts in concert with host metabolism

The best-studied microbial pathways influencing human health involve production of short chain fatty acids (SCFAs) including propionate, butyrate, and acetate. The end product of microbial fermentation of complex non-digestible polysaccharide in the colon (Cummings, 1983; Brestof and Artis, 2013), SCFAs, and their role in metabolism and immunity, have been appreciated for years (see review by Brestof and Artis, 2013). They contribute to protection from infection and inflammation (Fukuda, et al., 2011, Maslowski, et al., 2009, Kim, et al., 2013), recruitment and maturation of various subsets of immune cells (Arapaia, et al., 2013, Smith et al., 2013, Sina, et al., 2009), and metabolism (Bellahcene et al. 2013), and mediate host-microbe interactions. SCFAs interact with the host in several ways: via specific G-coupled protein receptors (GPR) -41 and -43 (FFAR3 and 2, respectively) (Brown, et al., 2003; Le Poul, et al., 2003), inhibiting histone-deacetylases (HDAC) and thereby altering host gene expression (Waldecker, et al., 2008), and inducing autophagy (Brestof and Artis, 2013).

Propionate production is especially important in human health, promoting satiety, preventing liver lipogenesis, lowering cholesterol, and providing anti-carcinogenic activities (Havenaar, 2011). Pathways leading to bacterial propionate formation in the human gut have been extensively investigated using multiple approaches (PCR amplification by degenerate primers, 16S rRNA gene analysis, tblastn analysis, and microbial physiology), highlighting dominant microbes and microbially-influenced propionate pathways in the human gut (Reichardt, et al., 2014). The succinate pathway may be the most abundant pathway leading to propionate formation in the human gut, as it is present both in Bacteroidetes, the most abundant phylum in the gut, and the less-abundant phylum Negativicutes. The biosynthetic route via the propanediol pathway is found mostly in Lachnospiraceae, and may be the most important pathway utilizing deoxy-sugars such as rhamnose and fucose from host glycans. The acrylate pathway is the least widespread, and *Coprococcus catus* was the first human gut bacteria in which this pathway was identified. Propionate and butyrate pathways mainly operate in phylogenetically distinct groups of anaerobic bacteria (Reichardt, et al., 2014). In general, caution should be used in predicting pathways using *in silico* methods, because the presence of pathways may simply use butyrate or propionate as energy sources rather than produce them (Ridaura et al., 2013; Vital et al., 2014). Further, the importance of assessing the directionality of pathways along with gene abundance was found to be critical in

differentiating between herbivores and carnivores (Muegge et al., 2011). Thus, identifying the directionality may also be crucial for understanding the link between microbes and metabolites. These findings highlight that future studies deciphering roles of specific microbial species in human health via biosynthetic pathways from metagenomes should be performed via multiple approaches (Muegge et al., 2011; Reichardt, et al., 2014).

Bacterial and host enzymes are linked into complex metabolic pathways. For example, the neurotransmitter serotonin (5-hydroxytryptamine) is a specialized metabolite synthesized through the interaction between the host and its microbiome. Approximately 90% of the serotonin in a human is synthesized in the gastrointestinal tract (Berger et al., 2009), and serotonin acts locally to regulate gastrointestinal, cardiac, respiratory, and endocrine functions, as well as crossing the blood-brain barrier (Berger et al., 2009; Nakatani et al., 2008). At the most basic level, serotonin is derived from tryptophan by tryptophan hydroxylases -1 or -2 in a tetrahydrobiopterin-coupled reaction, followed by a decarboxylation by amino acid decarboxylase (Walther et al., 2003; Sainio et al., 1996). Tryptophan is an essential amino acid; it must be obtained from dietary or microbial sources (Young, 1994). Germ-free (GF) mice have lower levels of tryptophan, serotonin, and indoles than conventionally raised or humanized (i.e., colonized with human microbiota) animals (Marcobal et al., 2013). Tryptophan is synthesized from chorismate by members of several bacterial phyla including Proteobacteria, Actinobacteria, and Firmicutes through enzymes whose genes are encoded in complex operons (Yanofsky, 2004). *Escherichia coli* can synthesize chorismate, a tryptophan precursor, which acts as a branch-point for many microbial metabolic pathways (LeBlanc et al., 2013; Dosselaere and Vanderleyden, 2001). Bifidobacteria are also predicted to have the capacity to synthesize chorismate (LeBlanc et al., 2013). Consistent with this key role for bacteria, antibiotic treatment altered tryptophan and indole metabolism in rats (Zheng et al, 2011).

Short chain fatty acid and indole production are among the better-characterized microbial metabolic pathways. They also highlight different strategies in metabolic synthesis. SCFAs are fermented from a variety of different sugars via distinct pathways that do not intersect (Reichardt, et al., 2014). Serotonin and tryptophan biosynthesis are evolutionarily conserved (Radwanski and Last, 1995), and reflect the interaction of host and microbial symbionts in the production of specialized metabolites. These examples may provide a framework for the characterization of novel metabolic pathways: confirming metagenomic prediction using integrated approaches including microbial genomics, microbial community analyses, and microbial physiology (Reichardt, et al., 2014).

Bioavailability of microbial metabolites

The mucosal epithelium regulates passage of essential nutrients into circulation. The epithelium is composed of a single layer of cells, dominated by enterocytes, that separate the gut lumen from the underlying lamina propria. Tight-junction proteins securely bind neighboring epithelial cells at the apical side and regulate molecular transport (see reviews by Marchiando et al., 2010, Peterson et al., 2014). Two modes of transport through the epithelium are possible: transcellular (through cells) and paracellular (between cells). The different nature of different specialized microbial metabolites, from small polar molecules to

larger peptides, affects their ability to withstand the intestinal environment, as well as their capacity to cross the intestinal epithelium into the circulation (Figure 2).

Pharmacokinetic concepts help describe the bioavailability of microbial metabolites produced in the intestines. In the healthy gut, a metabolite (or a drug) may be absorbed into circulation by passive or active mechanisms. Based on their lipophilicity, size, and degree of ionization (intestinal pH varies from 6.6 in the proximal small intestine to 7.5 in the terminal ileum, and 6.4 in the cecum to an average of 7 in the colon (Evans et al., 1988)), metabolites may be absorbed by passive diffusion down a concentration gradient. However, more hydrophilic metabolites cannot penetrate membranes easily. Some hydrophilic molecules reversibly bind carriers that travel down concentration gradients, then cross the epithelium via facilitated passive diffusion. Metabolites (or drugs) that structurally resemble substrates that are shuttled through the epithelial barrier, such as amino acids, sugars, and vitamins, may be actively transported by the corresponding transporters. Lastly, pinocytosis, the ingestion of fluid into cells, may allow metabolite uptake. One example of transcellular transport by facilitated diffusion is glucose, which is taken up on the apical side of the lumen by a Na^+/Glucose symporter and transported from the basolateral side by the glucose transporter-2 (GLUT2). SCFAs, on the other hand, may be transported into epithelial cells and possibly through the epithelial barrier either actively by monocarboxylate transporters (e.g., SLC5A8, SLC16A1) or by diffusion (Ganapathy et al., 2013).

Some metabolites that cannot be absorbed by transcellular transport (whether active or passive) are taken up by paracellular transport. This type of transport is regulated by tight-junction proteins. These proteins, primarily claudins, exclude molecules based on size and charge (see review by Van Itallie and Anderson, 2006). Microbial metabolites may be actively transported or diffuse through the healthy epithelial barrier, or conversely, cross the barrier through paracellular transport when the epithelial barrier is breached. “Leaky gut”, namely gut barrier dysfunction, is the result of dysbiosis and inflammation (Marchiando et al., 2010). Interleukin-6 (IL-6), interferon-γ (IFNγ) and IL-1β-induced barrier dysfunction (Suzuki et al., 2011; Al-Sadi et al., 2010) is a known characteristic of IBD (Hollander et al., 1986). Conversely, several probiotics have been shown to decrease gut barrier permeability (Scaldaferri et al., 2012), and SCFAs alone were sufficient to explain this effect (Elamin et al., 2013).

The gut lumen is a challenging environment for metabolites such as peptides and small molecules. Before crossing the epithelial barrier, a metabolite may be degraded or taken up by microbes in the gut, or may undergo various modifications. The rate of these processes and the rate of a metabolite’s absorption to circulation interact to determine its bioavailability. Subsequently, metabolites, as do drugs, may undergo detoxification by the liver and kidney, and be excreted from circulation. Xenobiotic metabolism may also alter the physiology and gene expression of the host. Microbial metabolism of drugs is exploited to convert prodrugs to active ingredients, and is also known to be associated with drug-induced toxicity (Li and Jia, 2013, Maurice, 2013). Alternatively, microbial metabolism of xenobiotics may inactivate drugs. Digoxin, a cardiac drug, is metabolized by *Eggerthella lenta*, a gut bacterium (Haiser et al., 2013). Interestingly, dietary protein inhibits digoxin

metabolism by this bacterium *in vivo*, and increases digoxin concentration in serum and urine.

The circulatory system is structured such that any blood flowing from the gut flows to the liver via the portal vein, through a network of liver sinusoids, and out of the hepatic veins to the vena cava and circulation. The liver filters any particulate matter and bacteria that enter through the intestines. The route of absorption differs for different metabolites; soluble metabolites and nutrients are absorbed and enter the circulation through the portal vein, but insoluble, fat-based metabolites are most commonly absorbed into the lymphatic system and into the bloodstream through the thoracic duct (Hall and Guyton, 2010).

Taken together, the pharmacokinetic properties of a metabolite, from production and survival in the gut through absorption and excretion, define its window of opportunity to act in driving health or disease in the mammalian host.

Specialized microbial metabolites that mediate disease

The microbiome plays an important role in health and disease (previously reviewed by Kee and Mazmanian, 2010; Clemente et al., 2012). A shift in balance between a healthy and diseased microbiome leads to pathologic and metabolic conditions including obesity, colon cancer, and IBD. Host-microbial interactions are in part mediated by the immune response (Round and Mazmanian, 2009), and possibly via specialized metabolites (Ursell et al., 2014). The gut microbiota is a virtual endocrine organ (Clarke et al., 2014) that produces myriad secondary metabolites. Some of these metabolites have positive effects on the mammalian host, as in the case of SCFAs and antibiotics. Although dysbiosis of the host-associated microbiota has been implicated in various conditions (Kee and Mazmanian, 2010; Clemente et al., 2012), the mechanisms by which specific microbes, or microbial communities, induce disease remain largely unknown. It is possible that specialized metabolites may mediate many physiological states of well being and illness (Figure 3).

Microbial metabolites and cardiovascular disease

Gut microbes contribute to atherosclerosis from dietary consumption of red meat, providing a compelling example of microbial metabolism leading to disease in the human host (Wang et al., 2011; Koeth et al., 2013). Comparative levels of choline, trimethylamine N-oxide (TMAO), and betaine, three metabolites of dietary phosphatidylcholine, predicted cardiovascular disease risk in a clinical cohort, and promote atherosclerosis in mice. Concentrations of these metabolites also correlated with microbial activity in the gut – administration of antibiotics suppressed their production, and conventionalization with mouse gut microbes rescued the phenotype (Wang et al., 2011). Trimethylamine (TMA), the precursor of TMAO, is also the product of microbial metabolism of L-carnitine, a compound abundant in red meat (Koeth et al., 2013). Interestingly, vegans and vegetarians consuming L-carnitine produced much less TMAO than omnivores. Specific gut bacterial taxa also correlated with plasma TMAO levels. Finally, supplementing mouse diets with L-carnitine markedly increased plasma levels of TMA and TMAO, and accelerated atherosclerosis. The mechanism by which TMAO accelerates atherosclerosis is unknown, but it may contribute to forward cholesterol transport via upregulation of macrophage scavenger receptors. These

studies demonstrate how a metabolite made by the gut bacterial community enters circulation and contributes to disease. Moreover, 'western' (high-fat) diets have been associated with intestinal permeability (Martinez-Medina et al., 2014), which would facilitate the absorption of microbial metabolites into the circulatory system.

Microbial metabolites and cancer

Gut microbes also play a role in carcinogenesis, especially in colorectal cancer (see reviews by Schwabe and Jobin, 2013 and Sears and Garrett, 2014). Both the whole gut microbial community and specific bacteria at specific times, play a role in initiating or exacerbating cancer (Kostic et al., 2012; Kostic et al., 2013; Zackular et al., 2013; Ahn et al., 201; Belcheva et al., 2014). *Fusobacterium nucleatum* is enriched in human colonic adenomas relative to surrounding tissue, suggesting that it may play a role in early initiation of colorectal cancer (Kostic et al., 2012; Kostic et al., 2013). Further supporting this idea, *F. nucleatum* colonization promoted and exacerbated tumorigenesis in the gut of $APC^{min/+}$ mice, although the mechanism of pathogenesis remains unknown.

Microbes might promote cancer by producing metabolites that increase disease susceptibility and incidence. Risk factors for colorectal cancer include high alcohol consumption and low folate intake. Homann et al (2000) fed rats normal diets, and administered high ethanol with or without antibiotics. Ethanol led to increased acetaldehyde and decreased folate in the colon. This effect was prevented by antibiotic treatment, suggesting that ethanol degradation by gut microbes might play a role in inducing colon cancer. Similarly, liver cancer likely involves a complex interaction between obesity, gut microbes, and their metabolites. In a model for liver cancer where neonate mice were injected with a chemical carcinogen (7,12-Dimethylbenz-alpha-anthracene), genetic- or diet-induced obesity led to changes in the gut microbiome of treated mice, and increased levels of the metabolite deoxycholic acid (DCA), a known DNA damaging agent. The induction of hepatocellular cancer could be prevented by treatment with a cocktail of four antibiotics, or by vancomycin alone. DCA levels were significantly increased by high-fat diet (or in *ob/ob* mice), compared to wild type animals, and addition of DCA to antibiotic-treated mice rescued the tumor phenotype in these mice (Yoshimoto et al., 2013). These studies reveal a chemical link between gut microbes and cancer, and also suggest a potential new treatment modality for disease by targeting microbial processes during carcinogenesis.

Microbial metabolites and cystic fibrosis

In cystic fibrosis (CF), the airway is abnormally colonized by polymicrobial communities that vary among patients. These communities are affected by the conditions and nutrients available in CF lungs. Different microbial communities differ in physiology and metabolite production: even the same microbe may produce different metabolites in the context of different communities. For example, an increase in 2,3-butanedione concentrations was observed in four of seven CF patients when compared to healthy and ambient air controls (Whiteson et al., 2014); the three patients with normal levels were undergoing antibiotic treatments. Similarly, 2,3-butanedione levels decreased when the other patients also underwent antibiotic treatment, suggesting that microbes are involved in the production of this compound. *Streptococcus* spp. was the dominant species carrying genes encoding 2,3-

butanedione biosynthesis, whereas genes for utilizing butanedione pathway products were encoded by *Pseudomonas aeruginosa* and *Rothia mucilaginosa*. Co-culture experiments suggested that *P. aeruginosa*, upon consumption of 2,3-butanedione produced by *Streptococcus* spp, enhanced the production of redox carriers, namely phenazines. Phenazines likely increase reactive oxygen species and provide additional electron acceptors to the colonizing microbial community of the CF lung as oxygen is depleted (Whiteson et al., 2014).

A recent study based on metagenomes and metatranscriptomes of microbial communities in sputum of CF-patients suggested a gradient of different communities, and hence of which biochemical pathways were operational, based on oxygen availability (Quinn et al., 2014). Of particular interest is the shift of pathways corresponding to microaerophilic respiration by *Pseudomonas* spp. to denitrification by *Pseudomonas* and *Rothia* spp., and anaerobic respiration via reduction of various sulphur species on depletion of oxygen. Other anaerobic pathways corresponded to acetate, butyrate and butanediol fermentation. Furthermore, enrichment in amino acid catabolism correlated with elevated ammonia concentration, previously observed in the sputum of CF patients. Interestingly, genes encoding folate biosynthesis pathways were abundant in samples from the CF population, but absent in healthy controls. The CF microbial community may thus evolve in response to the administration of sulphonamide drugs, which target folate biosynthesis. These findings demonstrate how to evaluate likely physiological roles and biochemical pathways of a microbial community and its adaptation to disease environments.

Microbial metabolites and behavior

The gut microbiome-brain axis is an active area of research (Bravo et al., 2012; Borre et al., 2014; De Palma et al., 2014), and has received much attention recently. Observations that brain development and behavior are influenced by the gut microbiota (Diaz Heijtz et al., 2011; Neufeld et al., 2011; Gareau et al., 2011; Bravo et al., 2011 and others), and that this impact may be transmitted from the enteric nervous system to the central nervous system via the vagus nerve (e.g.; Bravo et al., 2011), make gut metabolites immediate suspects for mediating gut microbiota-brain interactions.

Some bacteria can produce neuroactive metabolites, ranging from serotonin and gamma-aminobutyric acid (GABA), to dopamine and norepinephrine, to acetylcholine and histamine (see Lyte, 2013; Roshchina 2010; Wall et al., 2014). GABA, for example, is an inhibitory neurotransmitter that regulates and participates in various functions in the central nervous system in mammals, and is involved in anxiety and depression. Barrett et al (2012) reported the production of GABA through metabolism of monosodium glutamate by *Bifidobacteria* and *lactobacillus* spp. isolated from the human gut. However, GABA from the gut cannot cross an unbreached blood-brain barrier (Van Gelder and Elliott, 1958). Another connection is that probiotic treatment with *Lactobacillus rhamnosus* increases GABA receptor expression in the hippocampus, and reduces anxiety- and depression-related behaviors in a mouse model (Bravo et al., 2011).

No complete bacterium-metabolite-target pathway has yet been definitively linked to behavioral changes. However, differences in microbial communities are often correlated

with changes in the metabolite profile, and perhaps behavior (e.g.; Daniel et al., 2014; Hsiao et al., 2013). In a maternal immune activation (MIA) mouse model for autism spectrum disorders (ASD), both fecal bacterial communities and serum metabolomic profiles are different between animals displaying altered versus normal behavior (Hsiao et al., 2013). MIA mice also exhibit autistic-like behavioral phenotypes (increased anxiety, increased repetitive behavior, decreased sociability, and decreased vocalization), and increased gut barrier dysfunction. Interestingly, administration of a probiotic, *Bacteroides fragilis* NTCC 9343, corrected some of the behavioral deficits and restored levels of some serum metabolites. One of these metabolites, namely 4-ethylphenylsulfate (4-EPS), was sufficient to induce anxiety-like behaviors in mice when injected into the circulation, and one probiotic, *B. fragilis*, which contributes to a gut microbial community that produces less 4-EPS, could also ameliorate some of the negative effects of potentially neurotoxic metabolites.

The beneficial role of SCFAs in gut health has long been appreciated, but they also play a role in host behavior. For example, acetate crosses the blood-brain barrier, where it is taken up and activates hypothalamic neurons (Frost et al., 2014). Intraperitoneal administration of acetate suppressed appetite and led to weight loss. However, although butyrate and acetate were mostly beneficial, propionate may be pathogenic.

Comparisons between a propionic acid-based animal model of ASD with acquired mitochondrial disorder and a cohort of 213 children with autism specific disorder suggested abnormalities in acyl carnitines via the mitochondrial tricarboxylic acid cycle (Frye et al., 2013). When injected intraventricularly in rats, propionate induces behavior with certain features of autism (MacFabe et al., 2007).

The role of microbial communities in health and disease has been studied extensively in the past decade, but the microbial mechanisms driving many of these processes, and their targets in the mammalian host are yet unknown. Specialized microbial metabolites originating from the oral, urogenital tract, gastrointestinal tract, and skin microbiota may be the next frontier in elucidating molecular mechanisms of pathogenesis and symbiosis (Figure 1, Figure 3).

Specialized metabolites from microbial isolates

Chemical crosstalk, represented by molecular signaling between host and associated microbiota, is among the most fascinating new areas in the realm of the human microbiome. The bioactive molecules produced by the microbiota are likely an important factor governing the establishment of specific microbial communities in humans. These molecules have been mostly studied *in vitro* in pure cultures or co-cultures, enabling investigations of their biological significance. Workflows focused on directly investigating these natural products from human or animal samples urgently need to be developed.

Some isolated examples showing what might be possible in a unified framework include the following. The quorum-sensing pentapeptide, competence and sporulating factor (CSF), from *Bacillus subtilis* helps maintain intestinal homeostasis when taken up by the organic cation transporter-2. After uptake by the transporter, CSF induces expression of heat shock protein 27 and survival pathways such as p38 MAP kinase and protein kinase B (Akt)

pathways in cultured human colonic epithelial cells (Fujiya et al., 2007). Similarly, bacterial peptidic pheromones called competence stimulating peptides produced by human-derived *Streptococcus spp*. in co-culture experiments regulate uptake of environmental DNA and prevent hyphal formation by the oral cavity coinhabitant *Candida albicans* (Jarosz et al., 2009). Other signaling molecules such as homoserine lactones mediate interactions between *P. aeruginosa* and *C. albicans*, co-inhabitants of the CF lung community (Hogan et al., 2004). Biotransformation of CF-associated *P. aeruginosa* phenazines by *Aspergillus fumigatus* has effects on growth and production of siderophores by *A. fumigatus* (Moree et al., 2012). Thus, quorum sensing molecules likely dictate the status of the microbiota and the resulting dynamics.

Although quorum sensing molecules have been largely explored in the context of their important roles in host-microbe or microbe-microbe interactions, ribosomally and non-ribosomally synthesized peptides and polyketide classes of specialized metabolites derived from the human microbiota have not yet been extensively studied. The biosynthetic pathways encoding these specialized metabolites are widely present in the genomes of microbial species, including bacterial species in the human gut (Cimermancic et al., 2014). Roles of these specialized metabolites identified in microbial isolates include transition of *C. albicans* from yeast to mycelium by a hybrid nonribosomal peptide-polyketide peptide mutanobactin A produced by oral bacterium *Streptococcus mutans* (Joyner et al., 2010). *E. coli* harboring the *pks* gene locus for biosynthesis of colibactin, a putative hybrid nonribosomal peptide-polyketide genotoxin, increases tumor growth in colorectal cells by enhancing the emergence of senescent cells when compared with pks^{-} (deletion) strains (Cougnoux et al., 2014). These senescent cells expressed higher levels of growth factors leading to proliferation of non-infected cells and hence colorectal tumor promotion. A highly conserved biosynthetic gene cluster for burkholderic acid, a polyketide, was found in the genomes of the human pathogens *Burkholderia mallei* and *Burkholderia pseudomallei* (Franke et al., 2012). These pathogens are considered biowarfare agents because they can cause high mortality, and are rich in biosynthetic pathways encoding specialized metabolites (Nierman et al., 2004; Vial et al., 2007). The *Burkholderia cepacia* complex (Bcc) has been demonstrated to produce a large number of secondary metabolites (Vial et al., 2007). When Bcc infections occur in CF patients, the sputum was shown to display reduced diversity of the polymicrobial infection. These findings encouraged *in vitro* studies, and screening of the Bcc for antimicrobial properties (Lipuma, 2010). These investigations lead to the discovery of enacyclins, a polyene class of antibiotics, that displayed potent activity against CF pathogens.

The ribosomally synthesized lantibiotics salivaricins, such as salivaricin A2, salivaricin B, and salivaricin D among others have been isolated from oral bacterium *Streptococcus salivarius* from healthy individuals. *S. salivarius* has been suggested to have beneficial properties (Hyink et al., 2007; Birri et al., 2012) and whether these peptides mediates some of the probiotic effects is unknown. Streptolysin S from *Streptococcus pyogenes* is another example of ribosomally produced peptide that enhanced virulence and occurrence of necrotic lesions in animal models (Betschel et al., 1998; Lee et al., 2008). Using comparative genomic analysis, biosynthetic pathways similar to the one utilized for

streptolysin S production was found in the genomes of major food-borne pathogens and lead to the *characterization* of the biotoxin clostridiolysin S from *Clostridium botulinum* and its cousin *C. sporogenes*, a soil bacterium that can colonize hosts (Gonzalez et al., 2010; Wikoff et al., 2009). Pyroglutamic dipeptides produced by member of the gastrointestinal tract *Lactobacillus plantarum* have been shown to reduce production of pro-inflammatory cytokine IFNγ (Zvanych et al., 2014). *Ascaris suum* nematode peptidic molecule, cecropin P1, isolated from pig intestines and found in various other insects as well as cysteine-rich *A. suum* antibacterial factor peptides isolated from the human pathogen, *Ascaris lumbricoides*, have been shown to possess antimicrobial activity and may help these pathogens colonize the intestines of associated hosts (Lee et al., 1989, Andersson et al., 2003). Furthermore, *in silico* analyses of genome sequences revealed various biosynthetic pathways for peptidic specialized metabolites such as thiopeptides in human commensal vaginal bacteria *Lactobacillus gasseri* and human pathogen *Streptococcus pneumonia* (Li et al., 2012). Whether or not members of the microbiota use this biosynthetic potential to colonize humans and affect health remains an open question. Human commensal and pathogenic microbes also harbor biosynthetic gene clusters for aryl polyenes and caratenoids that may confer protection against oxidative stress (Cimermancic et al., 2014). A tri-terpenoid cartenoid associated with human pathogen *Staphylococcus aureus*, staphyloxanthin, enhances survival under oxidative stress and prevents killing by neutrophils (Liu et al., 2005; Clauditz et al., 2006).

Thus, various specialized metabolites are associated with the human microbiome. Although *in silico* DNA sequence-based and *in vitro* methods have been exploited to investigate metabolites that the human microbiota may produce, and to gain insights into the biosynthetic potential and metabolic functions of these organisms, direct identification of such molecules in the complex environment of the associated host has not yet been accomplished. Such *in silico* methods and experiments performed in pure cultures or co-culture experiments do not represent the true metabolic capacity of the microbiome *in situ*. Thus, future efforts to develop strategies for metabolic profiling that focus specifically on natural products in fecal matter, biological fluids such as serum, urine, and intact diseased host tissues are needed. In this regard, imaging mass spectrometry can be especially valuable in direct molecular analysis of gut tissue sections (Rath et al., 2012), enabling spatial investigation of host-microbiome interactions. Molecular networking is an effective strategy for differentiating metabolites of microbial and human origin (Garg et al., 2014) from the complex environment of a CF-associated human lung. Since most specialized metabolites have associated biosynthetic pathways that are unique to an organism or a class of organisms, a combination of metagenome and metatranscriptome sequencing (Quinn et al., 2014), and high resolution mass spectrometry-based molecular networking analysis combined with statistical approaches will allow the investigation of specialized metabolite associated crosstalk between a microbiome and its host. Although limited to small volatile molecules, gas chromatography-mass spectrometry is also useful in connecting metabolites and their origin to physiologically relevant diseased states (Pleil et al., 2014, Amann et al., 2014). One potential challenge for future integration of such methodology resides in developing platforms and algorithms for co-analysis of large volumes of high density “Big data” generated on different mass spectrometry based analytical platforms.

Conclusion

With the development of a three-pronged strategy combining metagenomics, metatranscriptomics and metabolomics, we are now entering the new era where we can begin to address the physiological role of the human microbiome in health and disease in relation to end products of primary metabolism and secondary metabolites, with recently emerging emphasis on microbial natural products, also termed specialized metabolites in this Perspective. Although a small set of such metabolites have been shown to modulate host physiology, the vast majority of such metabolites in humans (suggested to be present by metagenome sequencing) have not been investigated. We propose that the identification of specialized metabolites, their biotransformations by the microbiome and the host, transport, and their origin via metatranscriptomics, and metabolomics in combination with metagenomics will open new avenues to investigate underlying mechanisms by which the human microbiome influences health and microbial community development. Subsequent *in vivo* studies may further substantiate the role of these metabolites and provide insights into such mechanisms. Finally, a deeper understanding of the complex chemical crosstalk between the gut microbiota and humans may advance potentially revolutionary therapies for immune, metabolic, and neurologic disorders.

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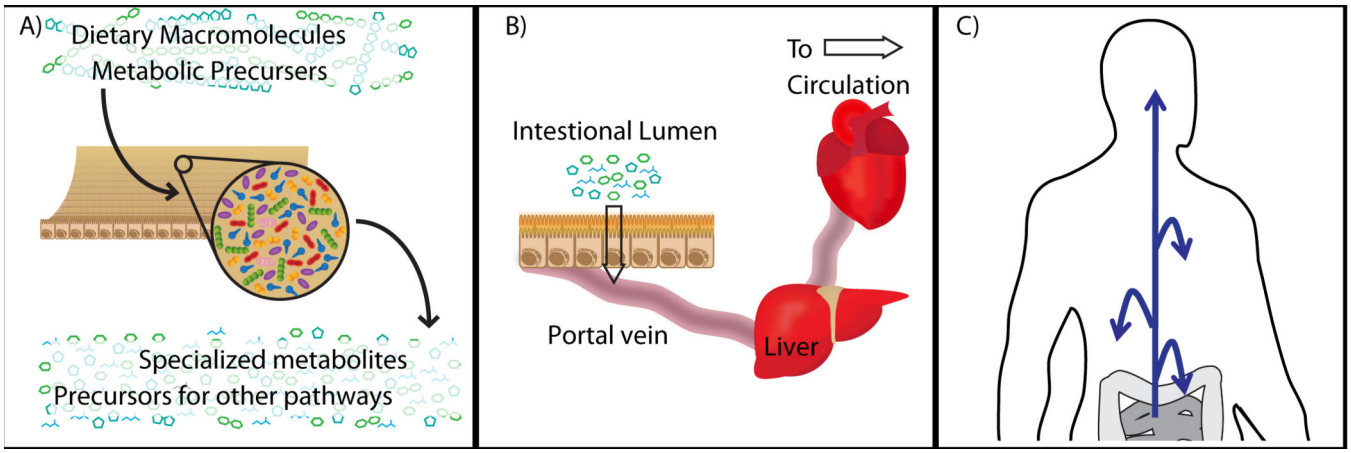

Figure 1. The production-absorption-target site pathway of metabolites

A product of microbial metabolism from dietary or metabolic precursors (left panel), specialized metabolites are absorbed through epithelial barriers. A major site for absorption of specialized metabolites is the intestine where metabolites cross the intestinal epithelium and enter the circulation through the portal vein, via the liver (middle panel). Once in the circulation, metabolites travel to various target sites (organs, tissue, cells, etc) in the mammalian host (right panel).

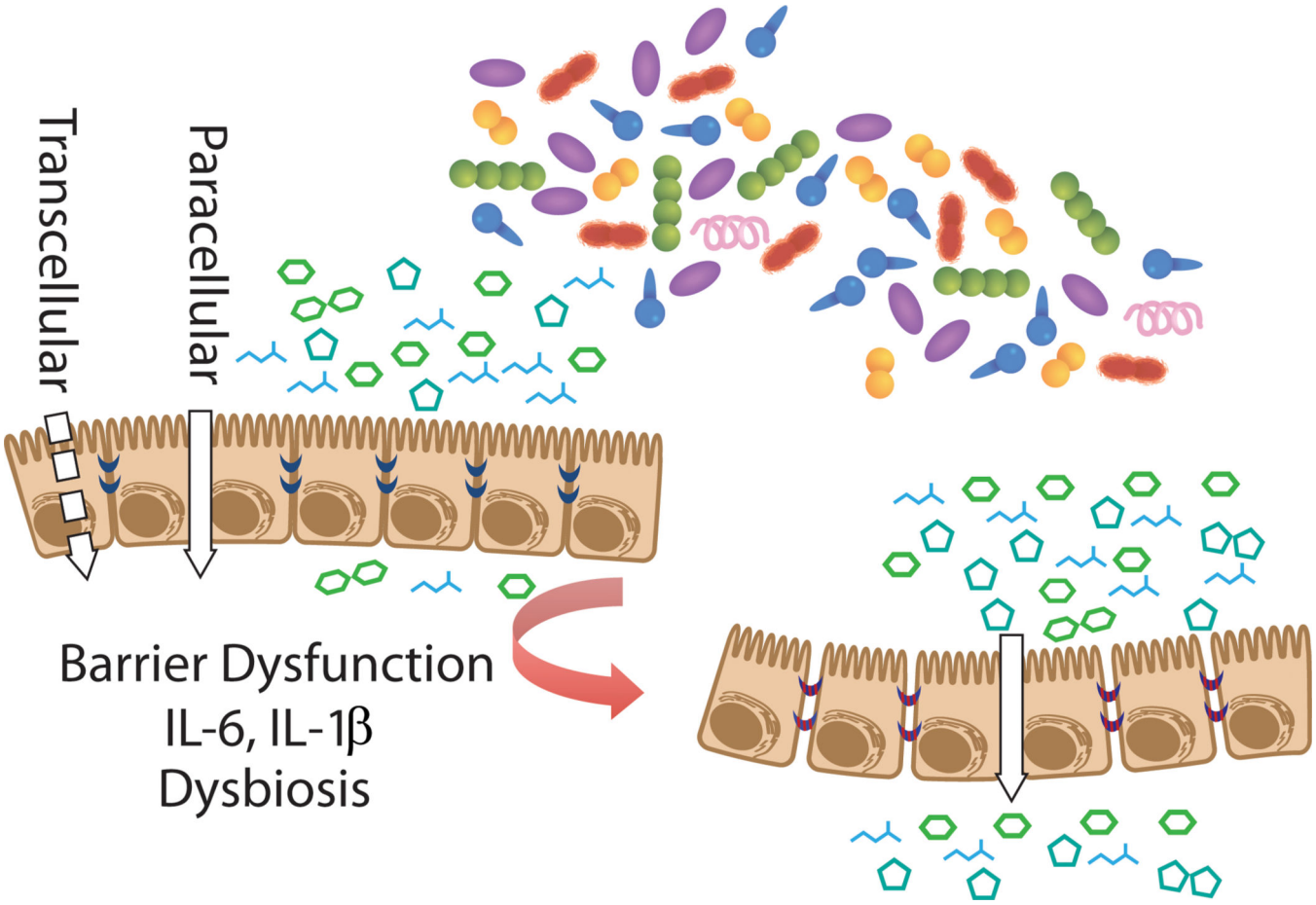

Figure 2. Modes of absorption for specialized metabolites

Metabolites can cross epithelial barriers via various passive and active processes, through cells (transcellular), diffusing through the membrane or using various transporter enzymes. Alternatively, metabolites can cross the barrier between cells (paracellular), a process mediated by tight-junction proteins, namely Claudins. During inflammation, often mediated by cytokines such as IL-6 and IL-1β, the intestinal barrier is disrupted, allowing uncontrolled paracellular passage to metabolites that are excluded by the intact epithelium.

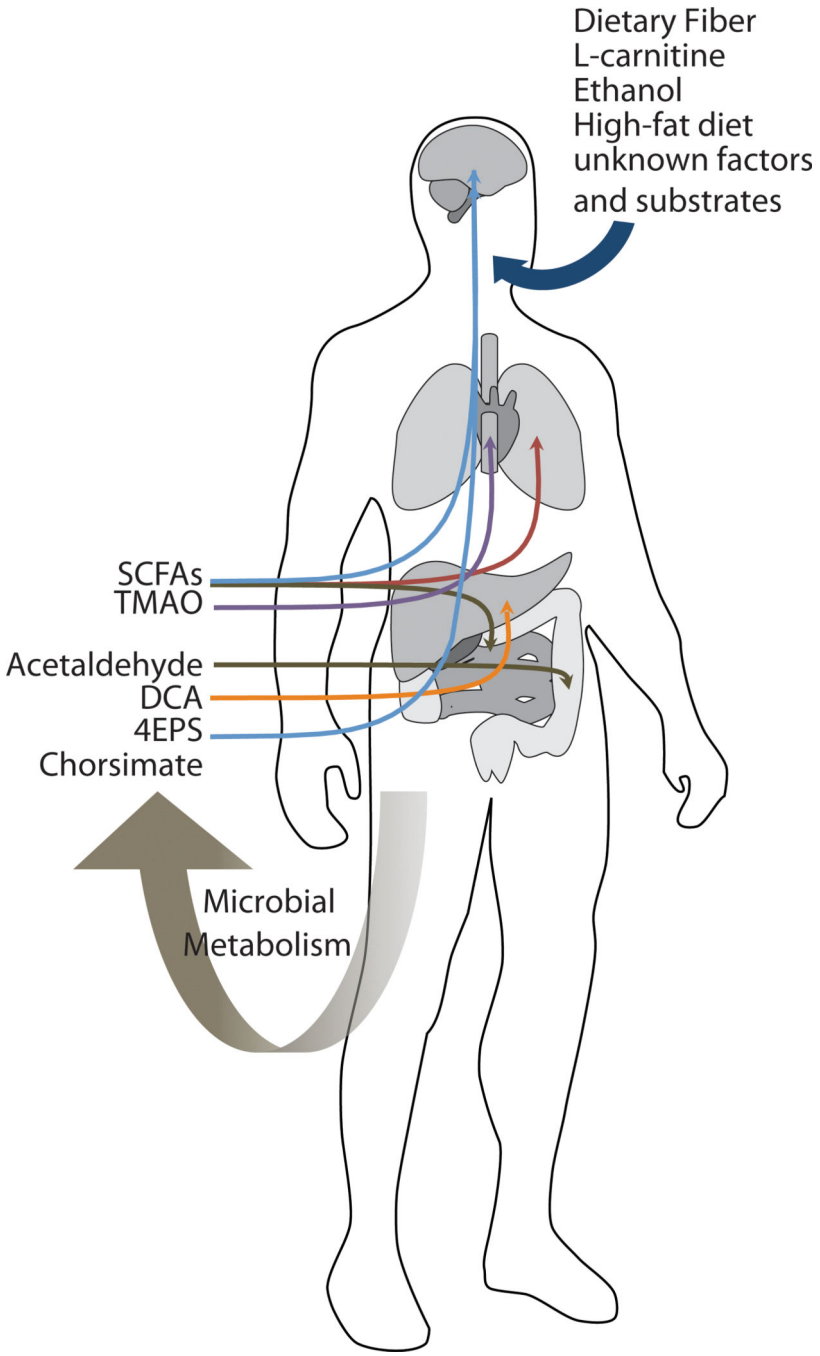

Figure 3. Specialized metabolites, synthesized by the gut microbial community from various precursors, reach target sites and mediate health and disease

SCFA: Short-Chain Fatty-Acids; TMAO:trimethylamine N-oxide; DCA:deoxycholic acid; 4EPS:4-ethylphenyl sulfate.