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# Small molecule metabolites at the host-microbiota interface

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## Abstract

The trillions of bacteria that constitutively colonize the human gut collectively generate thousands of unique small molecules. These microbial metabolites can accumulate both locally and systemically and potentially influence nearly all aspects of mammalian biology, including immunity, metabolism, and even mood and behavior. Here, we briefly summarize recent work identifying bioactive microbiota metabolites, the means through which they are synthesized, and their effects on host physiology. Rather than offering an exhaustive list of all known bioactive microbial small molecules, we select a few examples from each key class of metabolites to illustrate the diverse impacts of microbiota-derived compounds on the host. In addition, we attempt to address the microbial logic behind specific biotransformations. Finally, we outline current and emerging strategies for identifying previously undiscovered bioactive microbiota metabolites that may shape human health and disease.

## Introduction

Recent interest in the microbiome field has shifted from identifying associations between microbial taxa and human health towards mechanistic studies of the specific impacts of defined microbes and microbial products. One prominent mechanism by which the microbiome influences both local and systemic physiology is through the production of thousands of unique bioactive small molecules. Indeed, up to 50% of all serum metabolites are either produced by or modulated by commensal microbes. In this review, we will provide an overview of recent studies illuminating the roles of diverse microbial metabolites in shaping mammalian biology, with a general focus on immunological phenotypes. We will not exhaustively detail all classes of microbial metabolites or all known effects on the immune system as these topics have been covered previously by others (1, 2). Instead, we hope to provide illustrative examples from throughout the field for three broad classes of microbial metabolites defined based on the origins of their core building blocks (diet vs. host vs. de novo synthesis) and to provide proof-of-concept examples for specific microbial metabolite-mediated impacts on immunity. Throughout this review, we will also

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attempt to tackle the less well-discussed subject of why bacteria initially evolved the capacity to perform specific biotransformations. In this vein, we will classify microbial biotransformations into four non-mutually exclusive categories: attainment of nutrients, signalling (either to other microbes or the host), detoxification, and competition (Figure 1). Finally, we will outline existing and emerging strategies for identifying new bioactive compounds that are hidden among the vast array of tens of thousands of uncharacterized microbiota metabolites.

## Biotransformations of Exogenously Consumed Compounds

### Dietary fiber and the Short Chain Fatty Acids

Dietary fiber refers to plant-derived carbohydrates that cannot be metabolized by host enzymes, including complex polysaccharides, lignans, and resistant starches. As dietary fiber cannot be absorbed or metabolized into absorbable components by host enzymes, it passes unaltered through the small intestine to the large intestine where it is fermented by resident microbes that encode specialized machinery to import and process dietary carbohydrates. In non-Westernized populations, members of the *Prevotella* genus are the major consumers of ingested fiber, whereas *Faecalibacterium prausnitzii, Clostridium leptum, Eubacterium rectale,* and *Roseburia* spp. are more predominant in the West (3, 4). Fermentation restores NAD+ consumed during glycolysis of monosaccharides liberated from fiber, and generally leads to the production of short chain fatty acids (SCFAs), which are the 2–4 carbon carboxylic acids: acetate, propionate, and butyrate. SCFA producers preferentially resort to fermentation despite their capacity for respiration, which may be partially explained by biogeography and mutualism. An appreciable portion of gut microbes have been observed to have respiratory machinery, but no biosynthetic capability for quinones (intermediary electron carriers). It is speculated that these microbes must rely on quinones synthesized by other microbes to carry out respiration (5). Accordingly, some SCFA-producers will switch from fermentation to respiration in oxygen rich environments (6, 7).

SCFAs can accumulate at up to 100 mM concentrations in the colonic lumen (8) and have been implicated in shaping diverse aspects of host biology. (See (9) and (10) for a comprehensive review of the many effects of SCFA on host immunity and beyond.) As just a few examples, colonocytes preferentially utilize butyrate as an energy source, and SCFA are critical regulators of both innate and adaptive immune responses through both receptor-mediated and non-receptor-mediated mechanisms. For instance, SCFA drive regulatory T cell differentiation and production of anti-inflammatory cytokines by macrophages through histone deacetylase inhibition (11–13) and regulate diverse immune responses through the engagement of G protein-coupled receptors (GPCRs) including GPR41, GPR43, and GPR109a (9, 14–17).

In addition to the SCFAs, the metabolic intermediate succinate is also produced during bacterial fermentation of dietary fibers (18–20). Microbiota-derived succinate can potentially modulate the immune response via diverse mechanisms, including engagement of the succinate receptor GPR91 (SUCNR1), which is expressed on multiple immune cell types (21). For example, succinate can directly activate tuft cells in the small intestine via

GPR91 and elicits a multifaceted type 2 immune response through engagement of the tuft cell-IL-25-ILC2 circuit (22, 23).

## TMAO

Longitudinal human cohort studies initially implicated trimethylamine N-oxide (TMAO) in the development of cardiovascular disease and follow-up mouse studies suggested a causal role for TMAO in these disorders (24, 25). TMAO is produced exclusively by the microbiota via metabolism of dietary choline and L-carnitine, which are abundant in red meat and shellfish (24, 26, 27). Both choline and L-carnitine are tertiary amines and microbial cleavage yields trimethylamine (TMA), which is oxidized to TMAO by flavin monooxygenases in the liver (28). The impetus for bacterial production of TMA is potentially multifactorial, but likely supports bacterial metabolism and growth. For example, catabolism of L-carnitine produces a 4-carbon intermediate that can enter the TCA cycle (29) and acetaldehyde, which is used in the biosynthesis of Acetyl-CoA (30). Carnitine can also serve as a terminal electron acceptor in anaerobic respiration, although this functionality is repressed by access to superior electron acceptors such as nitrate (31). When carnitine is used as an electron acceptor, gamma-butyrobetaine and not TMA is generated, which suggests that the nutritive status of a microbe may affect TMA levels. Despite the intense interest in TMAO as a therapeutic target, the precise molecular mechanisms by which it promotes cardiovascular disease remain largely unclear (32, 33). However, recent studies suggest that TMAO may contribute to cardiovascular pathology by directly promoting platelet hyperreactivity (34) or by facilitating chronic inflammation (35). For example, TMAO can trigger inflammasome activation in both macrophages and endothelial cells, leading to increased release of proinflammatory cytokines including IL-1β and IL-18 (36, 37).

## Amino acid derivatives

Due to their ubiquity, amino acids are prime candidates for microbial energy balance and derivatization into signalling molecules. As any autotrophic diet contains proteins, diet-derived amino acid substrates are readily available in the mammalian gut lumen. In addition, many bacteria directly synthesize amino-acids from basic building blocks, providing an additional source of endogenous amino acid substrates in the distal gut. Host-derived proteins may provide an additional minor source of lumenal amino acids. Thus, although we have included amino acid-derived compounds under the heading of exogenously consumed compounds, microbiota-derived amino acid metabolites may also be derived from amino acids produced by other microbes, the host, or synthesized de novo by individual microbes themselves (38).

## Biogenic amines

Amino acid-derivatives constitute many of the key mammalian signaling molecules, including the neurotransmitters histamine, dopamine, and serotonin, trace amines such as phenethylamine and tyramine, and hormones such as melatonin and epinephrine. While best known for their roles in regulating diverse physiological processes in mammals, nearly all of these compounds can also be produced by microbes, including select gut microbes (39–42). Given the availability of large amounts of amino acid substrates in the intestinal

lumen, gut microbes can potentially produce high concentrations of amino acid-derivatives, raising the possibility that microbially-derived biogenic amines may influence local or systemic host physiology, including mood and behavior (39). For example, microbially-derived histamine can enhance gut motility (43) and modulate allergic asthma in the lung (44). Diverse immune cell types express myriad receptors for neurotransmitters, trace amines, and hormones, but our understanding of how engagement of these receptors by microbiota-derived metabolites impacts the immune response remains limited (45). In addition to production of neurotransmitters, microbial breakdown of amino acids can also lead to the production of polyamines, such as spermine, which have been shown to exhibit immunomodulatory properties, including regulation of inflammasome activation (46).

### Branched Chain Amino Acids

The branched chain amino acids (BCAA), isoleucine, L-valine and leucine, are essential amino acids that are synthesized from the metabolic precursors pyruvate and threonine (47). Gut microbes can affect BCAA levels in the intestine by acting as both producers and consumers of BCAA (48). BCAA can impact various aspects of mammalian physiology, including the immune response, through their effects on protein synthesis, metabolism, and cell proliferation (49). For example, gut microbes such as *Prevotella copri* and *Bacteroides vulgatus* produce excess BCAA, and microbial BCAA-production is associated with increased systemic BCAA levels and insulin resistance in humans and mice (48).

### Indole and Indole Derivatives

A variety of commensal microbes convert tryptophan into indole or related indole derivatives that can shape diverse immune processes by engaging the aryl hydrocarbon receptor (AhR) or pregnane X receptor (PXR). For example, indole 3-propionic acid can enhance intestinal barrier function through PXR (50), indole-3-aldehyde can induce intestinal IL-22 production and *Lactobacillus*-derived tryptophan derivatives can regulate intraepithelial lymphocyte differentiation through AhR (51, 52), and indole-3-lactic acid can inhibit Th17 differentiation and experimental autoimmune encephalomyelitis through an unknown mechanism (53). Microbial tryptophan metabolism was systematically dissected in *Clostridium sporogenes*, which can convert aromatic amino acids into their respective propionic acid derivatives (54). Targeted disruption of a key enzyme for these biotransformations (fldC) revealed critical roles for *C. sporogenes*-derived indole metabolites in shaping gut physiology and systemic immunity as gnotobiotic mice colonized with fldC-mutant *C. sporogenes* displayed increased gut permeability and increased mucosal and systemic *C. sporogenes*-specific antibody responses (54). Indole may also serve as a potential signalling molecule. Indeed, in many microbial species, indole and indole derivatives regulate biofilm formation and the expression of virulence factors (55, 56) and it has been speculated that indole may function as a quorum-sensing-like regulator (57).

### Phytochemicals

The gut microbiota is constantly exposed to a diverse array of phytochemicals from the diet. As just one example, the lignans are a large group of polyphenols found in many plants and lignan consumption has been associated with lower cancer risk (58). Despite the plenitude of plant lignans, only two biologically active subtypes are prevalent in the mammalian

gut, enterodiol and enterolactone (59), both of which are structurally similar to estrogen. The lignan pinoresinol (PINO) is biotransformed by the gut microbiota to enterolactone via sequential processing by multiple bacterial species and nicely illustrates the role of microbial metabolite exchanges in the production of bioactive metabolites. Select strains of *E. lenta* encode the enzyme ber, which catalyzes the first two reactions of the pathway, PINO to lariciresinol (LAR) to secoisolariciresinol (SECO). After these initial processing steps in *E. lenta*, three separate bacterial taxa, *B. producta*, *Gordonibacter pamelaeae*, and *Lactonifactor longoviformis* were found to sequentially convert SECO to its bioactive end product enterolactone (60).

The microbial benefit of lignan metabolism may be purely energetic in nature as some bacteria can grow on lignans as a sole carbon source (61). However, *in vitro* studies may confound this simple interpretation as supplementation of lignans to stool-derived mixed cultures led to an increase in Proteobacteria (62) even though the main species capable of metabolizing lignans belonged to the Actinobacteria and Firmicutes. Interestingly, lignans exhibit significant antimicrobial activity against select species and also exhibit strong antioxidant properties which could benefit strict anaerobes in the gut (63, 64). Nonetheless, given that enterolactone and enterodiol both have negligible free radical scavenging ability relative to their precursors (65), it seems likely that lignan biotransformation is mainly a means of detoxification, although additional studies are necessary to rigorously test this assumption.

## Pharmaceuticals

Orally ingested drugs may encounter commensal microbes both prior to absorption and during enterohepatic circulation (66). Biotransformations of therapeutic small molecules by the microbiome can potentially 1) reduce the bioavailability of the active form of a drug via conversion to an inert intermediate, 2) convert prodrugs into their active form, or 3) lead to the generation of toxic drug metabolites. While pharmaceutical agents are likely to be entirely foreign to the standard biochemistry of any given gut microbe, microbial life has evolved to encode an arsenal of enzymes devoted to xenobiotic metabolism. Microbial xenobiotic metabolism in the gut generally involves either reduction or hydrolysis. The prevalence of reductive biochemistry may be a consequence of the anaerobic nature of the gut. Xenobiotics may thus serve as alternative substrates for anaerobic respiration, or reactants of oxidoreductases that reduce NADH (67). Hydrolysis may reflect the need to obtain substrates for growth. Recent pioneering studies have revealed the prevalence and diversity of microbial metabolism of medical drugs (68–70).

By systematically evaluating the biotransformations of 271 drugs by 76 bacterial species/strains from the human gut microbiome, Zimmerman et al. revealed a rich landscape of microbial modifications of common pharmaceuticals (68). They found that over two-thirds of assayed drugs were metabolized by at least one strain and that phylogenetically-related taxa often processed drugs with similar structural features. For instance drugs containing an ester or amide group were metabolized mainly by Bacteroidetes species, while nitro- or azo-group containing drugs were metabolized by members of all phyla except Proteobacteria (68). These results suggest that microbial metabolism of medical drugs is the rule and

further highlight the possibility that interindividual variation in gut microbial communities may impact individual responses to medical drugs.

Identifying the ultimate fate of a pharmaceutical in the gut is complicated by microbial metabolite exchanges where the product of one microbial transformation is the substrate for a subsequent biotransformation by another bacterium. For example, levo-dopa, a drug for Parkinson's disease that is converted into dopamine by aromatic L-amino acid decarboxylase after crossing the blood-brain barrier, can be converted by *Eggerthella lenta* into dopamine, which is further metabolized by *Enterococcus faecalis* species into tyramine (71). This pre-processing restricts the bioavailability of L-dopa to the brain and can affect responsiveness to L-dopa treatment. L-dopa is commonly co-administered with carbidopa, an inhibitor of mammalian aromatic L-amino acid decarboxylase, to prevent the premature processing of L-dopa into dopamine, which cannot cross the blood-brain barrier and can cause undesired side-effects in the periphery (72). However, carbidopa is a poor inhibitor of the microbial enzyme that catalyzes L-dopa processing in the gut. Instead, a different drug, (S)-α-Fluoromethyltyronsine can selectively inhibit this microbial enzyme and, in gnotobiotic mice colonized with an L-dopa metabolizing microbiota, co-administration of (S)-α-Fluoromethyltyronsine and L-dopa increased the serum concentration of L-dopa (71). Notably, in these studies, only *E. lenta* strains containing a specific SNP in the enzyme Dadh were capable of decarboxylating L-dopa, which underscores additional challenges in determining the metabolic potential of the microbiome based on marker-genes alone (71).

## Biotransformations of Compounds Produced by the Host

### Bile acids

Bile acids are amphipathic molecules synthesized from cholesterol in the liver and secreted into the duodenum to aid in the absorption of dietary fat. Primary bile acids synthesized by the host are conjugated with either glycine or taurine. Given their detergent-like nature, bile acids can disrupt the lipid bilayer of cellular membranes and are toxic to many microbes (73). Accordingly, bile acid supplementation significantly alters the gut microbiome (74) and recent studies suggest that bile acid secretion in the neonate may facilitate maturation of the microbiome in early life (75). Commensal microbes have evolved several means of biotransformation and detoxification of bile-acids including, epimerization, deconjugation by Bile Salt Hydrolase (removing the conjugated glycine or taurine), reduction/oxidation, hydroxylation, and dehydroxylation (a multistep pathway which is found almost exclusively in gut anaerobes) (76). Interestingly, microbiota-mediated bile acid metabolism is at least partially host species-specific as human microbiota are incapable of transforming select bile acids from rodents (77). Bacterially-modified bile acids are referred to collectively as secondary bile acids, though this term most commonly describes deconjugated bile acids. Many microbial bile acid modifications reduce their membrane-damaging effects by decreasing hydrophobicity and potential toxicity (78). However, somewhat paradoxically, deconjugation appears to increase the hydrophobicity of bile acids. Given that deconjugation releases free glycine and taurine, which can be catabolized to ammonia and carbon dioxide, bacterial metabolism of primary bile acids via this route may serve primarily as a source of energy and amino acid building blocks for select commensal microbes.

The consequences of bacterial modification of bile acids for the host stem partially from the autoregulation of primary biliary synthesis. Activation of Farnesoid X receptor (FXR), a nuclear receptor expressed in the gut and liver, by primary bile acids suppresses CYP7A1 expression, which reduces the conversion of cholesterol into primary bile acids. Thus, FXR provides autoregulatory control of the bile acid pool. Since secondary bile acids have differential affinity for the FXR receptor, microbial transformation of primary bile acids can alter FXR signalling and therefore bile acid pool size. Indeed,germ-free rodents exhibit increased bile acid pools relative to conventionalized animals due to microbial processing of the FXR-antagonist T-BetaMCA (a mouse-specific bile acid) (79). Bile acids also serve as ligands for multiple GPCRs, including Takeda GPCR 5 (TGR5). Activation of TGR5 in the intestine and pancreas induces secretion of glucagon-like peptide-1 and insulin, regulating circulating blood glucose (80). Fascinatingly, unconjugated bile acids can also cross the blood brain barrier and activation of TGR5 by these compounds in the hypothalamus reduces food intake and increases energy expenditure (80). TGR5 is also the predominant bile acid receptor of liver-resident macrophages, and activation in these cells inhibits inflammation (81). Finally, recent studies have revealed that specific secondary bile acids can regulate the differentiation of intestinal T cells towards Treg or Th17 phenotypes (82, 83). For example, microbial production of the secondary bile acid isoDCA enhanced peripheral Treg generation and abolition of secondary bile-acid production by individual commensals significantly decreased their ability to induce colonic T regs (82, 84). The impact of bile acids on T cells may also depend on the specific composition of the bile acid pool as distinct bile acid species derived from the same precursor control unique aspects of T cell differentiation via distinct molecular mechanisms (48).

## Human Milk Oligosaccharides

Human Milk Oligosaccharides (HMOs) are prebiotic fibers in breastmilk that support the establishment of a healthy microbiome during early development (85). They are a specialized case of dietary fiber as they are the only source of complex carbohydrates available to nursing infants. We have classified HMOs here as ‘host-derived’, but they could also be classified as ‘exogenously consumed’ from the perspective of the infant. HMOs largely pass unaffected to the large intestine where they support the growth of *Bifidobacterium* species (i.e., the bifidogenic effect), which are early colonizers of the human gut and the dominant member of the microbiome in breast-feeding infants. Bifidobacteria are uniquely suited to metabolize HMOs via the bifid shunt pathway (86). Bifidobacteria support healthy immune system development through diverse mechanisms, including the production of acetate and lactate, and are critical mediators of colonization resistance in early life (87).

## ‘Neurotransmitters’ as a Microbial Food Source

The gut harbors more than 90% of the serotonin in the body. Enteroendocrine cells and enteric neurons are major sources of gut neurotransmitters, and recent studies have revealed that specific commensal microbes critically modulate intestinal neurotransmitter production (42). GF mice and mice colonized with altered Schaedler flora exhibit reduced concentrations of colonic serotonin. However, colonization of germ-free mice with spore forming microbes, predominantly Clostridia species, or administration of

associated microbial metabolites restored normal gut serotonin levels by stimulating colonic enterochromaffin cells (88). Artificially increasing colonic serotonin, either through gavage or by genetic ablation of the enteric serotonin transporter SERT, also increased the abundance of spore-formers including *Turicibacter sanguinis*. *T. sanguinis* itself was found to encode a protein similar to human SERT, which conferred the ability to uptake serotonin, and treatment of *T. sanguinis* with serotonin induced the downregulation of genes related to cell differentiation, morphogenesis and sporulation, as well as the upregulation of transporters (89). Taken together, these data reveal a narrative whereby serotonin serves as a signal that the microbe is in an environment conducive to vegetative growth (i.e., the gut lumen). Having exited the spore, the microbe then induces further host production of serotonin presumably for use as an energy source. However, because this endogenous factor has its own signalling role in the host, microbial induction of this factor also leads to altered host physiology.

Serotonin is not the only neurotransmitter that can serve as a microbial growth factor. For example, bacteria are also known to consume γ-aminobutyric acid (GABA) (90), largely through the GABA shunt whereby GABA is converted to succinate which subsequently enters the TCA cycle (91). More recently, GABA was found to be an essential growth factor for a previously uncultivatable human gut microbe of a first-in genus member of the *Ruminococcaceae* family (92).

## Small Molecules Synthesized De Novo

### Quorum Sensing Molecules

Quorum sensing (QS) molecules enable microbes to regulate their behavior based on population size. QS molecules are often subject to autoregulation, whereby a QS compound induces increased QS biosynthesis. Among the classical autoinducers (AIs), AI-1, has generally eluded detection in the human gut (93), while both AI-2 and AI-3 have been detected (94). The dearth of AI-1 could potentially be explained by the expression of the paraoxonases, PON1/PON2/PON3 by gut epithelium (95), which can efficiently cleave Acyl homoserine lactone (AI-1) and disrupt QS in pathogens such as *P. aeruginosa* (96). Interestingly, the host may have also evolved the capacity to listen in on QS-mediated microbial chatter. For example, AI-2 induces IL-8 secretion in HCT-8 cells (97) and AI-3 analogs have been shown to increase macrophage IL-8 secretion (98). The synthetic process for AI-3 was unknown until it was recently discovered that “abortive” tRNA synthetase reactions rather than enzymatic transformations mediate AI-3 production (98). Quorum sensing can also alter the course of re-establishment of the gut microbiota following antibiotic treatment. In mice treated with streptomycin, the presence of an AI-2 producer will shift the composition of a recovered community towards a greater abundance of Firmicutes (99). Within the densely-packed multispecies context of the gut, quorum sensing may also mediate interspecies signalling in addition to intraspecies signalling. For example, *Ruminococcus obeum*, synthesizes AI-2 in response to *V. cholera* infection, which moderates expression of *V. cholera* colonization factors (100).

### Secondary Metabolites

Aside from modifications to molecules ingested or produced by the host, microbes can produce a wide array of complex small molecules from smaller modular components. These secondary metabolites can be broadly classified as polyketides (PKs), non-ribosomally synthesized peptides (NRPs), ribosomally synthesized, posttranslationally modified peptides (RiPPs), terpenes, NRP synthetase-independent siderophores, and saccharides. The genes responsible for the biosynthesis of these compounds typically co-localize on the genome in what are termed biosynthetic gene clusters (BGCs). Notably, the addition of subunits to a nascent secondary metabolite encoded by a BGC generally proceeds in gene order (101–103).

RiPPs are the most widely distributed BGCs in the human microbiome, while PKs and NRPs are less prevalent and abundant. Many BGC-derived natural products from the microbiome exhibit narrow-spectrum antimicrobial activities and may mediate bacterial competition within complex host-associated microbial communities. For example, Lactocillin, a RiPP derived from *Lactobacillus gasser*i inhibits Gram-positive urogenital pathogens, with no commensurate activity against Gram-positive urogenital commensals (104). The NRP Lugdunin from *Staphylococcus lugdunensis, specifically* antagonizes *S. aureus* (105). And, the RiPP Thuricin specifically antagonizes *C. difficile* while sparing most other members of the gut microbiota (106). Although many previously-characterized secondary metabolites are antimicrobials, BGC-derived compounds may also exhibit immunomodulatory activities. For example, the RiPP commendamide activates GPR132/G2A, a GPCR implicated in autoimmune disease and cardiovascular disease (107) and a family of NRPs from the gut microbiome inhibits host cathepsins, potentially as a means of avoiding antigen processing and presentation (108).

## Experimental approaches to identify bioactive microbiota metabolites

The enormous biochemical potential of the microbiome and the resulting complexity of the microbiota metabolome imply that the majority of bacterially-derived chemicals that shape human health remain to be discovered. Here, we will briefly outline existing and emerging strategies for discovering and characterizing novel bioactive microbiota metabolites (see also (2) and (109) for comprehensive reviews on this topic). These approaches can be categorized into three general frameworks outlined in Figure 2.

The first pipeline is exemplified by the “correlation-first” approach whereby comparative metabolomics analyses of samples from humans or mice with divergent phenotypes is used to identify potentially causal metabolites of interest (Figure 2A). These metabolites can then be interrogated for relevant bioactivities either *in vitro* or *in vivo* (e.g., cytokine induction (107) or Th17 polarization (110)). Two prominent successes based on this approach are the discovery of the role of TMAO in cardiovascular disease (24) and SCFAs in Treg differentiation (12). However, a major limitation of correlation-based comparative metabolomics approaches is that they are typically restricted to the discovery of previously known metabolites as deconvolving and resolving unknown MS peaks remains a major challenge. Related approaches such as assessments of previously-characterized families of small molecule metabolites (e.g., secondary bile acids), have led to similar successes

(83), but suffer from the same general limitation. Thus, additional approaches have been developed to enable the unbiased discovery of truly novel bioactive microbiota metabolites.

The most prominent approach for identifying previously undiscovered bioactive compounds is ‘functional metagenomics’ (Figure 2B). Here, sheared metagenomic DNA is cloned into a surrogate host (usually *E. coli*) and large libraries of clones are subjected to high-throughput bioactivity assays. This approach has been particularly successful for isolating natural products synthesized by cryptic BGCs (104, 107, 108, 111, 112) in part because the products of BGCs are often created using simple building blocks present in all microbes including *E. coli*. Two advantages of this approach are that it does not require cultivation of the microbial metabolite producer and that the clones of interest greatly facilitate both chemical identification and genetic dissection of the relevant BGC. The clustered nature and conserved motifs of BGCs makes them ideally suited for *in silico* discovery (113–116). From here, it is occasionally possible to proceed directly to chemical synthesis based on computational predictions of potential chemical structures, dispensing with genetics entirely (117, 118), or to leverage recent developments in synthetic biology to reconstruct complete biosynthetic pathways using cell-free systems (119).

Finally, high-throughput bioactivity screening of chemical extracts from *in vitro* cultures of commensal microbes has also led to the discovery of novel bioactive microbiota metabolites, as well as their specific microbial sources in the gut (Figure 2C). For example, recent efforts to screen microbial metabolomes against nearly all GPCRs have revealed a rich array of GPCR-active commensal microbes and metabolites (43, 120). In these studies, microbial monocultures were screened against individual receptors using engineered reporter assays. However, similar approaches can be applied to any phenotype of interest that can be assessed *in vitro* (from simplified reporter-assays to remodeling of tissue organoids) using microbial metabolomes of any complexity (from monocultures to complete gut microbial communities). Continued improvements in anaerobic culturomics, tissue organoids, and organ-on-a chip models will undoubtedly facilitate further insights into the bioactive microbiota metabolome in the coming years.

## Conclusions

The past two decades of technological advances in microbiome science have ushered in a new golden era of molecular-level dissection of the reciprocal interactions between mammalian hosts and their associated microbiomes. However, many barriers still remain before we can realize the full fundamental and therapeutic potential of the microbiome. For example, the effects of specific metabolites on microbial physiology have been characterized almost exclusively in model organisms grown in axenic culture, and the impacts of specific microbes and metabolites on host biology are most often examined in exquisitely controlled, but admittedly highly artificial, gnotobiotic mouse models. Realizing the next era of microbiome science will require a movement beyond proof-of-concept studies and collections of anecdotes towards a broader and more generalizable understanding of the fundamental principles that dictate beneficial and detrimental interactions between indigenous microbes and their mammalian hosts.

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## Abbreviations used in this article

| | |
|---|---|
| **SCFA** | Short Chain Fatty Acid |
| **HMO** | Human Milk Oligosaccharide |
| **BCAA** | Branched-chain Amino Acid |
| **GPCR** | G protein-coupled receptors |
| **TMAO** | Trimethylamine N-oxide |
| **AhR** | Aryl hydrocarbon receptor |
| **PXR** | Pregnane X Receptor |
| **FXR** | Farnesoid X Receptor |
| **QS** | Quorum sensing |
| **AI** | Autoinducer |
| **BGC** | Biosynthetic gene cluster |
| **GF** | Germ Free |

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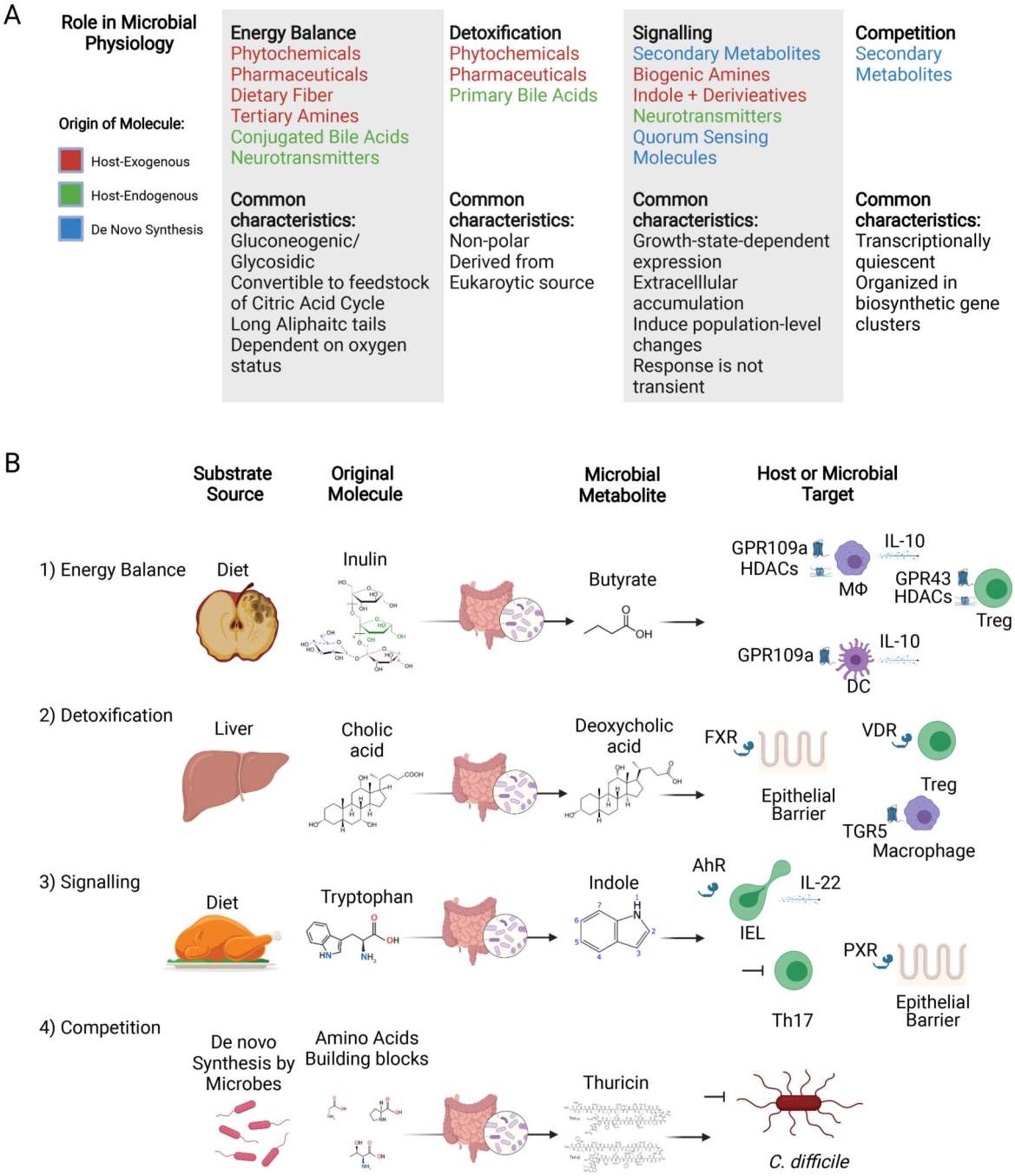


**Figure 1. Examples of microbial metabolites that impact host immunity categorized by their role in microbial physiology.**

**(A)** Classes of small molecules, arranged by their role in microbial physiology. Note: the text for each chemical is color-coded based on the origin of the building blocks for that compound.

**(B)** For each category, we provide one example of a microbial biotransformation relevant to mammalian biology, including the substrate source, molecular origins, microbial metabolite, and host or bacterial targets. *(1)* Microbial fermentation of the dietary fiber inulin results

in production of the SCFA butyrate, which has diverse impacts on the immune system. For example, butyrate induces an anti-inflammatory state in macrophages and dendritic cells via HDAC inhibition and engagement of GPR109a, and enhances Treg differentiation via HDAC inhibition and engagement of GPR43. *(2)* Primary bile acids (e.g., cholic acid) are dehydroxylated by gut microbes to produce secondary bile acids (e.g., deoxycholic acid). Secondary bile acids such as deoxycholic acid can enhance gut barrier function via FXR in epithelial cells, enhance Treg differentiation via the Vitamin D receptor, and suppress macrophage activation by engaging TGR5. *(3)* Dietary tryptamine is metabolized by gut microbes to produce indole and indole derivatives, which can enhance gut barrier function via PXR in epithelial cells, induce IL-22 production by IELs via AhR, and inhibit Th17 differentiation via an unknown mechanism. *(4)* Thuricin, a RiPP is synthesized de novo by commensal microbes and specifically inhibits the growth of the opportunistic pathogen *C. difficile*. Created with BioRender.com.

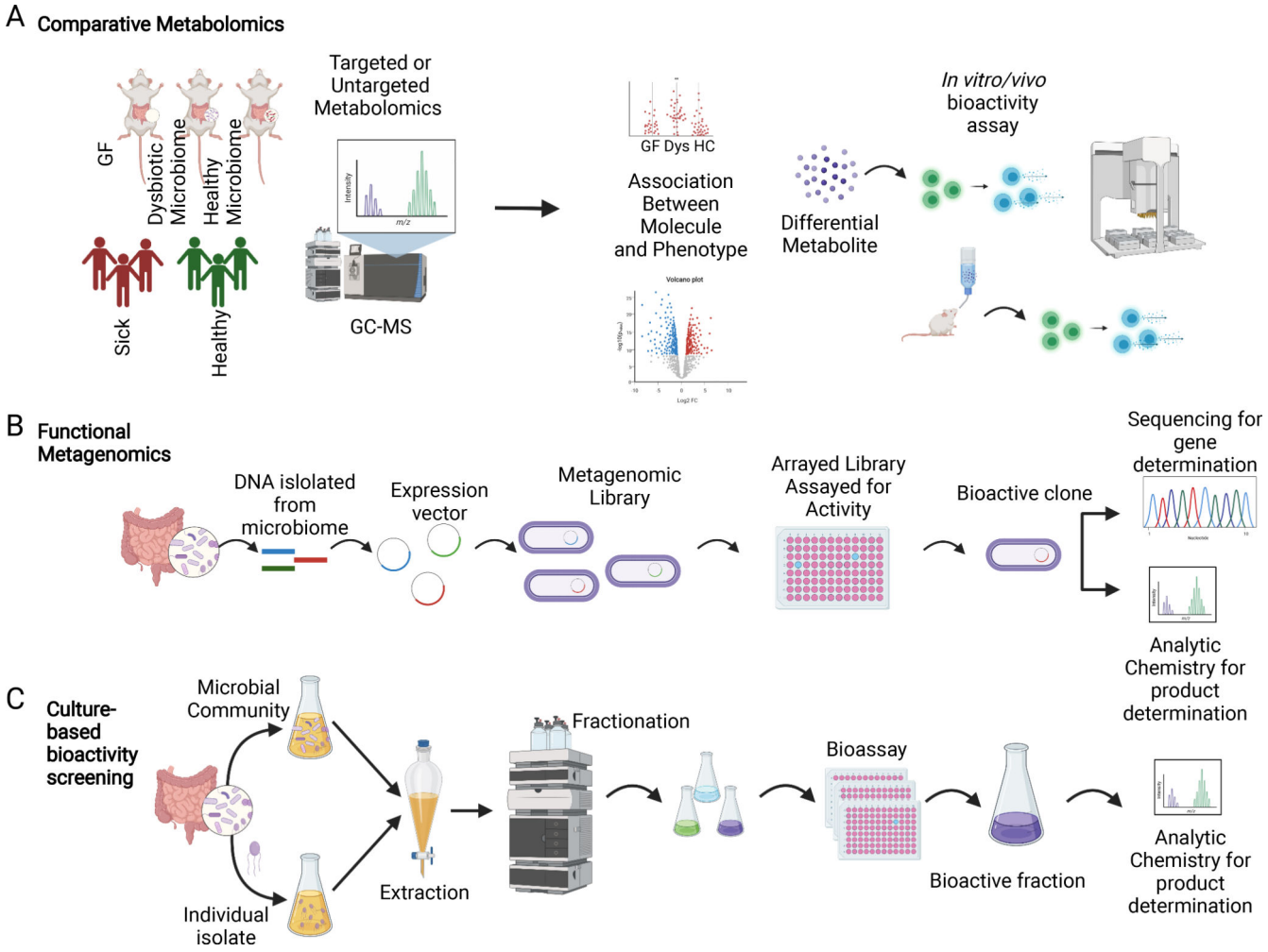


**Figure 2. Experimental approaches for the discovery of novel bioactive microbiota metabolites.**

**(A)** *Comparative Metabolomics.* In comparative metabolomics, metabolomes from groups of mice or humans with distinct phenotypes are assessed via targeted or untargeted metabolomics to generate a list of putative causal metabolites that correlate with a defined phenotype. The potential impacts of these specific metabolites on the host can then be assessed *in vitro* (e.g., using primary cells or reporter cells) or *in vivo* using mouse models. **(B)** *Functional metagenomics.* Metagenomic DNA from a microbiome of interest is fragmented, incorporated into expression vectors, and expressed in a facile recipient to produce large libraries of metagenomic clones, which are typically assayed for bioactivity via high-throughput screening. Bioactive clones are then sequenced to reveal the genes responsible for biosynthesis of active compounds and can be used to facilitate chemical identification. **(C)** *Culture-based bioactivity profiling.* Supernatants from cultures of individual commensal microbes or mixed communities of microbes are subjected to extraction and fractionation. Fractions are then assayed for a bioactivity of interest (e.g., GPCR activation). Bioactive fractions are further interrogated using bioassay-guided natural products discovery approaches to identify and characterize the compound of interest. Created with BioRender.com