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Small Molecules from the Human Microbiota

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Abstract

Developments in the use of genomics to guide natural product discovery and a recent emphasis on understanding the molecular mechanisms of microbiota-host interactions have converged on the discovery of natural products from the human microbiome. Here, we review what is known about small molecules produced by the human microbiota. Numerous molecules representing each of the major metabolite classes have been found that have a variety of biological activities, including immune modulation and antibiosis. We discuss technologies that will affect how microbiota-derived molecules are discovered in the future, and consider the challenges inherent in finding specific molecules that are critical for driving microbe-host and microbe-microbe interactions and their biological relevance.

INTRODUCTION

Symbiotic relationships – including mutualism, commensalism, and parasitism – are ubiquitous in nature (1). Some of the best known symbioses are between a microorganism and a multicellular host; in these inter-kingdom relationships, the fitness of the microbe-host system (the holobiont) often relies on a diverse set of molecular interactions between the symbiotic partners (2, 3). Examples include food digestion, nitrogen and carbon fixation, oxidation and reduction of inorganic molecules, and the synthesis of essential amino acids and cofactors (2, 4–6). In light of the critical role of a molecular dialog in maintaining a productive mutualism, the community of researchers studying the symbiosis between humans and their microbiota has begun moving from a focus on ‘who’s there’ to ‘what are they doing’. The accompanying emphasis on molecular mechanism has sparked a concerted hunt for the mediators of microbe-host interactions, including microbiota-derived small molecules.

It is now possible to identify biosynthetic genes in bacterial genome sequences and, in some cases, predict the chemical structure of their small molecule products. This genome mining has led to the discovery of a growing number of molecules, and recently developed algorithms (7–9) have not only automated biosynthetic gene cluster identification, but also have led to the unexpected discovery of numerous biosynthetic gene clusters in genomes of

the human microbiota (10). In addition, a wealth of natural products have been discovered from bacterial and fungal symbionts of insects, nematodes, sponges and ascidians, and plants (11–15). The many known examples of microbe-host mutualisms in which the microbe synthesizes a metabolite important for the ecology of the pair raise an intriguing question: To what extent are mammals, including humans, a part of this paradigm?

In this review, we review what is known about natural products from the human microbiota, examining in depth the diverse chemistries and biological functions of these molecules. We focus predominantly on commensal bacterial species, although we include a few notable examples of small molecules from bacterial pathogens. We then discuss recent insights into the metabolic potential of the human microbiota from computational analyses, and conclude by considering four approaches to identify and discover the function of ‘important’ molecules out of a complex cellular and molecular milieu. Some prominent microbiota-derived metabolite classes are not covered here, including short-chain fatty acids (SCFAs) and trimethylamine-*N*-oxide (TMAO); for an in-depth discussion of their role in microbe-host interactions in the gut, see (16).

THE MENU OF MOLECULES

A wide range of natural products have been isolated from human-associated bacteria. These molecules cover the entire spectrum of chemical classes that have been isolated from terrestrial and aquatic bacterial species and include well-characterized mediators of microbe-host and microbe-microbe interactions. Exploring their chemistry and function provides an entry point for understanding the effects of the microbiota on human health and disease.

Ribosomally synthesized, post-translationally modified peptides (RiPPs)

Most human-associated bacteria live in complex communities and compete with other species for resource-limited niches. RiPP natural products, which are unusually prevalent among the microbiota (10), are often active against a limited set of species closely related to the producer (i.e., narrow-spectrum) and are therefore thought to determine which strain of a bacterial species (or species of a genus) colonizes a niche. RiPPs are divided into numerous subclasses (17), five of which include members that have been isolated from human-associated bacteria: lantibiotics, bacteriocins, microcins, thiazole/oxazole-modified microcins (TOMMs), and thiopeptides (see Outlook section for a discussion about thiopeptides).

Lantibiotics and bacteriocins—Lantibiotics and bacteriocins are the most commonly isolated RiPPs from members of the human microbiota; dozens have been found to date. Lantibiotics are short peptides (<40 amino acids) with chemical crosslinks formed post-translationally by connecting the terminal thiol of a cysteine residue to a dehydrated serine or threonine. The resulting ‘lanthionine’ contains a thioether bond, which is typically more redox-stable than a disulfide. In contrast, bacteriocins are longer peptides (>40 amino acids) and are usually unmodified. Microbiota-derived lantibiotics are predominantly produced by the Firmicutes, and are usually active against a narrow spectrum of Gram-positive bacteria that are closely related to the producing strain.

Some microbiota-derived lantibiotics are produced by commensals (18), including the salivaricins from oral resident *Streptococcus salivarius* (19–22), a cocktail of five lantibiotics from the skin commensal *Staphylococcus epidermidis* (23–27), and ruminococcin A from the gut commensals *Ruminococcus gnavus* and *Clostridium nexile* (Table 1) (28, 29). Each of these molecules inhibits pathogens that are closely related to the producer. Lantibiotics have also been isolated from human pathogens: staphylococcin Au-26 (also known as Bsa) from *Staphylococcus aureus* (30, 31), SA-FF22 from *Streptococcus pyogenes* (32, 33), and the two-component lantibiotic cytolysin from *Enterococcus faecalis* (34) exert antibacterial activity against a range of common human commensals, indicating that lantibiotic production are used by commensals and pathogens to compete and establish resilient colonization.

Microcins and thiazole/oxazole modified microcins (TOMMs)—Microcins are prototypical narrow-spectrum antibacterials. They contain a wide range of unusual post-translational modifications including, the conversion of cysteine and serine residues to thiazoles and oxazoles (microcin B17), the addition of adenosine monophosphate (microcin C7) or a siderophore to the C-terminus (microcin E492, Figure 2) and internal amide crosslinking forming a lasso-like topology (microcin J25) (35–38). As they derive exclusively from enterobacteria and have potent (typically nM) antibacterial activity against close relatives of the producer (35), their role in the Gram-negative microbiota is analogous to that of lantibiotics in the Gram-positive microbiota. Most microcins have been isolated from *E. coli* strains and are widely distributed in both commensal and pathogenic enterobacteria (35, 39–41).

TOMMs are similar to microcin B17 in their biosynthesis and post-translational modifications, but encompass a larger family of natural products from both Gram-positive and Gram-negative bacteria (17, 42). The best-studied example is streptolysin S from the human pathogen *Streptococcus pyogenes* (43). Despite intensive efforts for almost a century, the precise chemical structure and mechanism of action of streptolysin S have not been fully determined (43). However, streptolysin S contains multiple oxazole and thiazole residues that are required for its hemolytic activity (44, 45). Related biosynthetic gene clusters from other human pathogens and commensals have been characterized (43), including listeriolysin S from *Listeria monocytogenes* (46) and clostridiolysin S from *Clostridium botulinum* and *Clostridium sporogenes* (47).

Heat-stable enterotoxin—While most RiPPs from the human microbiota are thought to mediate microbe-microbe interactions, heat-stable enterotoxin is a RiPP produced by strains of *E. coli* associated with diarrheal disease and has a well-characterized host target. It is a 14-amino acid peptide stabilized by three internal disulfide bonds (48) and mimics the effect of the host peptide hormones guanylin and uroguanylin by agonizing guanylate cyclase 2C, a transmembrane protein expressed in intestinal epithelial cells with an extracellular ligand binding domain and a cytoplasmic catalytic domain (49). Guanylate cyclase 2C generates cGMP to stimulate electrolyte secretion into the gut lumen. A single amino acid variant of heat-stable enterotoxin was approved by the FDA in 2012 for the treatment of constipation associated with irritable bowel syndrome (linaclotide, Figure 1) (50). The enzyme that

introduces disulfide crosslinks post-translationally into heat-stable enterotoxin is not encoded in its biosynthetic gene cluster, raising the question of whether the endogenous disulfide bond formation (Dsb) system is responsible, and whether other small peptides from enterobacteria undergo similar post-translational processing. Importantly, heat-stable enterotoxin indicates that a modified peptide can survive the proteolytic milieu of the gut lumen and target a host receptor expressed in intestinal epithelial cells, delivering a potent biological activity without absorption into host circulation.

Products of amino acid metabolism

Gut bacteria living in an anaerobic environment require an electron acceptor to drive fermentation or an anaerobic electron transport chain (51). Commonly amino acids are used as electron acceptors, resulting in the production by gut bacteria of high levels (in some cases, >100 mg/day) of reductive amino acid metabolites, such as phenylpropionic acid and phenylacetic acid - molecules that are not found in most other habitats. Importantly, the amounts of these metabolites produced vary widely among individuals and, unlike RiPPs, they are generally permeable and accumulate systemically in the host (52). For example, humans with comparable levels of dietary tryptophan but distinct gut bacterial communities can end up with markedly different profiles of gut metabolites following microbial metabolism. One prominent tryptophan metabolite, indole, is derived from tryptophan by as-yet-unidentified enzyme(s) that are presumably homologs of tryptophanases seen in other bacterial species. In its unmodified form, indole serves as a signalling agent in bacterial communities (53). In addition, following absorption through the intestinal epithelium, indole is 3-hydroxylated and *O*-sulfated in the liver to become indoxyl sulfate, a well-known uremic toxin that is known from germ-free rodent studies to be derived entirely from the GI microbiota (54). Indoxyl sulfate is present at a wide range of concentrations in human urine (10–200 mg/day), likely reflecting differences among individuals in diet and in the level of indole-producing bacterial species in the gut community (55). A second reductive tryptophan metabolite, indolepropionic acid (Figure 2), is found in mouse serum if *Clostridium sporogenes* is present in the gut (52). Although the function of this molecule is unknown, several other *Clostridium* spp. producers have been identified. A third tryptophan metabolite, the decarboxylation product tryptamine, is a biogenic amine neurotransmitter that is synthesized by a variety of gut bacteria (56) and has been linked to signaling in the enteric nervous system (57), one of several findings that reveal a role for the microbiota in the gut-brain axis (58–61). Thus, tryptophan can be diverted to end products with distinct biological activities depending on the composition of the gut community.

The metabolic products of aliphatic amino acids are equally prominent but less well characterized. Notable examples include δ -aminovaleric acid, which derives from arginine, proline, and ornithine, and acts as an electron source for secondary fermenters (62); and α -aminobutyric acid, which derives from threonine or methionine. Notably, the neurotransmitter γ -aminobutyric acid (GABA), the decarboxylation product of glutamate, is both produced and consumed by various species of gut bacteria (62), although its potential role in microbe-host signaling remains unexplored. Many of the less common short-chain fatty acids, including isobutyric, valeric, 2- and 3-methylbutyric, caproic, and isocaproic

acids, are also the products of reductive amino acid metabolism, but it is not known whether their signaling properties differ from those of the better known SCFAs (62) .

Oligosaccharides

Oligosaccharides provide some of the best-characterized examples of how small molecules from the human microbiota can mediate microbe-host interactions. Diffusible oligosaccharides are well known in the natural products community (63, 64) but the best-studied oligosaccharides from the human microbiota are cell-associated. Capsular polysaccharides from *Bacteroides* and *Streptococcus* show that capsular polysaccharides are not simply structural or nonspecifically adhesive, they can have highly specific ligand-receptor interactions that result in immune modulation, similarly to glycolipids (see below).

Species of *Bacteroides*, the most abundant bacterial genus in the human gut, produce an array of capsular polysaccharides, the best characterized of which is polysaccharide A from *Bacteroides fragilis*. Polysaccharide A is an oligomer in which the tetrasaccharide repeating unit consists of four derivatives of galactose: galactofuranose, *N*-acetylgalactosamine (GalNAc), 4,6- pyruvoylgalactose, and 4-amino-6-deoxy-GalNAc. Although the biosynthesis of polysaccharide A has just begun to be explored (65, 66) , its biological activity has been investigated in detail. Polysaccharide A signals through TLR2 to induce regulatory T cells to produce the tolerogenic cytokine IL-10. This signaling event restricts the activity of Th17 cells, promotes *B. fragilis* colonization and suppresses *Helicobacter hepaticus*-induced colitis (67, 68) . Remarkably little is known about the structures and biological activities of the tens to hundreds of other *Bacteroides* capsular polysaccharides, although they are likely to be the most abundant natural products in the human gut (10) .

Cell-associated oligosaccharides can also play a defensive role, as is the case with the richly diverse capsular polysaccharides elaborated by species of *Streptococcus*, including a family of capsular polysaccharides from group B *Streptococcus*, a pathogen (69) . Although the repeating unit varies in size, composition, and connectivity among group B *Streptococcus* serotypes, a common chemical feature is a terminal sialic acid, which blocks phagocytosis by inhibiting the deposition of the complement component C3b (69) .

An area of great promise in the microbiome is the identification of microbially derived ligands for host receptors. In addition to recent studies on the role of short-chain fatty acids and G-protein-coupled receptor 43 (GPR43) in regulatory T-cell function (70) , glycolipids and saccharides are significant candidate ligands. One example is muramyl dipeptide, a glycopeptide fragment of the repeating unit of peptidoglycan, which is a ligand for nucleotide-binding oligomerization domain-containing protein 2 (NOD2) and forms the scaffold for the immune -stimulatory osteosarcoma drug mifamurtide (Table 2) (71, 72) . Additionally, the large number of uncharacterized oligosaccharide biosynthetic loci in the human microbiome are particularly interesting in light of the C-type lectin receptors in the dectin/langerin/DC-SIGN family, which are known to bind oligosaccharides and modulate immune cell function, but for which few convincing ligands have been discovered (73) .

Glycolipids and Terpenoids

Perhaps the best known microbiota-derived molecule is lipopolysaccharide (LPS), a glycolipid that is a major component of the outer membrane of Gram-negative bacteria (Figure 2). LPS is the ligand for the innate immune receptor TLR4 and has been reviewed extensively elsewhere (74); here, we focus on two other families of bacterial glycolipids with similar immunomodulatory activities: the *Bacteroides* glycosphingolipid α -galactosylceramide and the mycolic acids of *Mycobacterium* and *Corynebacterium*.

Glycolipids— α -Galactosylceramide is a glycosphingolipid that was originally discovered nearly two decades ago as a natural product from a sponge and was later found to be a potent ligand for CD1d-restricted natural killer T (NKT) cells (75). Even though >1000 papers have been published on synthetic derivatives of this sphingolipid and the identification and stimulation of NKT cells, the source of the ‘native’ ligand for CD1d has remained a mystery (76). Recently, *Bacteroides fragilis*, a common gut commensal, was discovered to produce α -galactosylceramide (77). Colonization of germ-free mice as neonates by wild-type *B. fragilis* suppress NKT cells in the gut and block oxazolone-induced colitis (78). These findings suggest that the ‘native’ ligand for the highly conserved mammalian receptor CD1d might originate in the microbiota rather than the host. This may be true for other ‘orphan’ receptors expressed in immune and epithelial cells.

Mycolic acids are distinctive components of the cell wall of *Mycobacterium* (mostly pathogens) and *Corynebacterium* (both pathogens and skin and oral commensals). They consist of an all-carbon backbone with two lipid tails, one of which can be 40–60 carbons long in *Mycobacterium*, and are present both as free carboxylic acids and as esters of cell-wall polysaccharides. In addition to being important structural components of the capsule (79), glucose monomycolate serves as a ligand for CD1b-restricted T cells, eliciting a specific immune response against infection (80–83). Another glycolipid derivative of mycolic acid, trehalose-6,6-dimycolate, is a potent immune elicitor that binds to the C-type lectin Mincle to induce macrophage activation and a T-cell response characteristic of vaccination (84). Mycolic acid has many chemical modifications, including methylation and cyclopropanation, both of which appear to shield it from immune detection: the MmaA4-catalyzed methylation of mycolic acid by *M. tuberculosis* blocks IL-12 production and prevents detection and elimination by macrophages (85), and an *M. tuberculosis* mutant that is deficient in mycolic acid cyclopropanation is attenuated and hyperinflammatory in a mouse model of infection (86).

Terpenoids—The major microbiota-derived terpenoids are not synthesized *de novo* by the microbiota; they are secondary bile acids, derivatives of the host-derived primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) (Figure 1). CA and CDCA are biosynthesized in the human liver, conjugated to taurine or glycine, and then excreted in bile; although 90% of the bile acid pool is absorbed in the terminal ileum, the remaining 10% enters the large intestine (87). Here, the bile acid pool reaches concentrations of ~1 mM and varies widely in composition among healthy humans. Numerous biochemical transformations of CA and CDCA are performed by gut bacteria, including deconjugation from taurine and glycine, oxidation and subsequent epimerization of the hydroxyl groups at

C3, C7 and C12, dehydration and subsequent reduction of the hydroxyl group at C7, and esterification with ethanol at the C24 carboxylate. Among these, one modification – dehydroxylation at C7 – has been characterized most extensively (87–89).

Several species of Firmicutes (e.g., *Clostridium scindens* and *Clostridium hylemonae*) dehydroxylate CA and CDCA at the C7 position to form deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. The high flux of this biochemical transformation results in DCA and LCA making up nearly two-thirds of the fecal bile acid pool (87). Both DCA and LCA are toxic to human cells and have been implicated in hepatotoxicity and colon cancer (90). 7-Dehydroxylation is carried out by the 8-gene *bai* operon. This gene cluster is thought to perform eight successive chemical transformations in a pathway for which the early oxidative steps have been characterized biochemically, but the later, reductive steps remain speculative (89). Little is known about the biosynthetic genes for other secondary bile acids, although there is preliminary evidence that some bile acid pathways might be collaborative, in the sense that they involve transformations by more than one gut bacterial species.

The carotenoids are terpenoids, exemplified by staphyloxanthin, the golden-coloured pigment for which *Staphylococcus aureus* is named. Staphyloxanthin is composed of a glucose residue that is esterified with both a fatty acid (12-methyltetradecanoate) at the C6'' position and a carotenoid (4,4'-diaponeurosporen-4-oate) at the C1'' position (91). The core structure of staphyloxanthin is assembled by two biosynthetic enzymes: a glycosyltransferase, which esterifies the C1'' position of glucose, and an acyltransferase, which esterifies its C6'' position. The unusual 4,4'-diaponeurosporen-4-oate originates from dehydrosqualene by further dehydrogenation and oxidation steps (92, 93). The conjugated double bonds of staphyloxanthin's carotenoid tail serve as a 'sponge' for oxygen radicals, protecting *S. aureus* against killing by hydrogen peroxide, superoxide and hydroxyl radical, which are produced by host neutrophils and macrophages (94, 95).

Polyketides and Nonribosomal Peptides

Although polyketides (PKs) and nonribosomal peptides (NRPs) are among the largest classes of natural products in soil and aquatic bacteria, relatively few are known from human-associated bacteria. Two recently discovered examples come from the common pathobionts *Staphylococcus aureus* and *Streptococcus mutans* (Table 1 and Figure 1). A conserved NRPS gene cluster in *S. aureus* encodes a family of pyrazinones, which are derivatives of the ubiquitous diketopiperazines (96, 97). Although the role of the pyrazinones in regulating the expression of *S. aureus* virulence factors remains unclear (96, 98), they are unlikely to function exclusively in the context of pathogenesis since they are also produced by the skin commensal *Staphylococcus epidermidis* (97). Isolates of *S. mutans*, the leading cause of dental caries, harbor a genomic island encoding hybrid PKS/NRPS pathways (99). The product of one of these pathways, mutanobactin, contains an unusual 1,4-thiazepan-5-one ring, which originates from the cyclization of a cysteine and a glycine residue. The biological activity of the mutanobactins has not been fully determined, but may involve modulating growth and biofilm formation by the fungal pathogen *Candida albicans* (100–102).

Four pathogen-derived NRPs and PKs cause disease: cereulide, mycolactone, colibactin, and tilivalline. Cereulide, a dodecadepsipeptide toxin, is responsible for the emetic effects of the food-poisoning pathogen *Bacillus cereus* (103, 104). The ester bonds in cereulide's alternating ester/amide backbone enable the molecule to have high affinity for potassium ions, which causes mitochondrial toxicity and results in uncoupling of oxidative phosphorylation (105, 106).

Mycolactones are PK toxins produced by the causative agent of Buruli ulcer, *Mycobacterium ulcerans* (107, 108). These molecules, which cause the necrosis, ulceration, and immune suppression associated with this disease, are encoded by a >100 kb type I PKS biosynthetic gene cluster that includes two genes >40 kb. Interestingly, a heterogeneous suite of mycolactone derivatives is produced by different strains of *M. ulcerans*, which may explain the variation observed in the virulence of the strains and their geographical distribution among affected countries (107, 109–112).

Colibactin is produced by a subset of enterobacteria, including strains of *E. coli* B2, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and *Citrobacter koseri* (113, 114). Exposure of mammalian cells to colibactin producing *E. coli* and *K. pneumoniae* induces DNA damage *in vitro* and *in vivo*. Surprisingly, the colibactin gene cluster is present in one of the most commonly used probiotic *E. coli* strains (*E. coli* Nissle 1917 or EcN) (114–118). Considerable efforts have been made to study the biosynthesis of colibactin (119–121), and its chemical structure has recently been characterized, revealing a unique spirocyclopropane warhead that crosslinks DNA (122). It is not yet clear what role colibactin plays in the ecology of the interaction between *E. coli* and the host, and how the genotoxic activity of colibactin benefits its producer.

Tilivalline is an NRP toxin produced by colitogenic strains of the pathobiont *Klebsiella oxytoca* (Figure 2) (123). Importantly, tilivalline is essential for the inflammatory pathology characteristic of antibiotic-associated hemorrhagic colitis, and induces apoptosis in cultured human epithelial cells. Although the discovery of tilivalline sheds light on one mechanism of antibiotic-induced colitis, there are likely to be alternative mechanisms, since *K. oxytoca* is present in, at most, 10% of the healthy human population (124).

Much is known about the biosynthesis of NRPS-derived and NRPS-independent siderophores in a broad range of human pathogens, and their role in iron acquisition as an essential component of bacterial pathogenesis, both of which have been reviewed extensively elsewhere (125–127). In contrast, very little is known about the mechanisms by which commensals acquire iron and, to our knowledge, no iron acquisition system has ever been shown to be required for colonization by a commensal.

OUTLOOK

We have used ClusterFinder (7) to identify biosynthetic gene clusters in the human microbiome as a way to assess the metabolic potential of the human microbiota (10). Of >14,000 putative natural product biosynthetic gene clusters identified in human-associated bacterial genomes, 3,118 were present in one or more of the 752 whole-genome shotgun metagenomic sequence samples from the NIH Human Microbiome Project (HMP).

Although each of the major natural product classes is produced by the human microbiota, oligosaccharide and RiPP gene clusters are predominant, underscoring the need to improve analytical chemical techniques to purify and assay these molecules. Nearly all of the gene clusters that were present in >10% of the subjects in the study are uncharacterized, highlighting the potential of studying these genetic elements and the small molecules they encode.

There are two central challenges facing the field: First, from the wealth of microbiota-derived molecules, which ones are the functionally ‘important’ ones? Second, what experimental systems are appropriate for testing the activity of an individual molecule from a complex milieu?

Identifying significant microbiota-derived molecules

Human-associated microbial communities can consist of hundreds of abundant bacterial species and perhaps thousands of molecules at physiologically relevant concentrations. Figuring out which of these molecules drive a phenotype and how they act requires new computational and experimental approaches.

Initial mapping of metagenomic sequence data onto coarse KEGG or COG gene categories provides a way of seeing coarse changes, e.g., a shift from oligosaccharide toward amino acid catabolism in a community. Resolution is not yet high enough to make reliable predictions about specific biosynthetic pathways or products. Methods that predict the gene content of a sample from 16S data can predict pathways that are present or absent in every member of an operational taxonomic unit (OTU) (128), but have limited utility for biosynthetic pathways, which are highly variable even among closely related strains of a bacterial species (129, 130).

Methods are needed that take genomic and metagenomic sequence data as an input and use it to predict, at high resolution, pathways for specific molecules. Multiple algorithms will likely be needed: some for identifying clustered biosynthetic pathways, characteristic of a conventional secondary metabolite, and others for predicting unclustered pathways more commonly found in primary metabolism. The dearth of knowledge about primary metabolic pathways in anaerobes from the gut community is a critical gap in current knowledge, and addressing this problem will be a major achievement.

Nicholson, Holmes and colleagues have pioneered the use of metabolomics to profile microbiota-derived metabolites in a variety of sample types and disease models, developing powerful and widely applicable analytical pipelines (131, 132) (Figure 3a). Using similar approaches, a range of microbiota-derived molecules have been connected to specific bacterial species through the metabolomic profiling of various artificial and disease-associated communities from mice (52, 59, 133, 134). However, although metabolomic profiling is capable of measuring hundreds to thousands of known metabolites in a single run, its application (known as untargeted metabolomics) to discovering molecules of interest is more laborious and generally requires purification and structural characterization of milligram quantities of compound.

Although bioassay-guided fractionation is immensely powerful, it is painstaking and difficult to scale, so it is better suited for unusually important phenotypes of interest, including the search for ligands for orphan G-protein coupled receptors (GPCRs) expressed in the gut (Figure 3b). A derivative of this approach in which microbes, not molecules, are “fractionated” was recently used to identify a cocktail of 17 gut bacterial strains that induce regulatory T cells and attenuate colitis. This activity was further correlated with short-chain fatty acids produced by this anti-inflammatory cocktail of bacteria (135, 136) .

An alternative to bioassay-guided fractionation is the candidate molecule approach (Figure 3C). We used this method for the discovery of the potent thiopeptide antibiotic, lactocillin, from *Lactobacillus gasseri*, a prominent member of the vaginal community (10, 137). Initially, we made a systematic analysis of all biosynthetic gene clusters for small molecules in genomes of human-associated bacteria and identified 13 novel thiopeptide gene clusters, four of which are present in >20% of the HMP samples at one of the body sites. Thiopeptides are a class of antibiotics with potent activity against Gram-positive bacteria that bind to a site on the 50S subunit of the bacterial ribosome. One member of this class, LFF571, is currently in a phase II clinical trial (138). Lactocillin has been purified, structurally characterized, and shown to have low-to-mid nanomolar antibiotic activity against vaginal pathogens but not against vaginal commensals.

Another class of molecules ideally suited to the candidate molecule approach is secondary bile acids: bile acids, and the sterol scaffold more generally, are rich in biological activity and there are numerous host receptors for these molecules (87). The levels of secondary bile acids vary widely among individuals, although the bile acid pool in the gut lumen is held at a physiologically relevant concentrations. Although dozens of secondary bile acids are known, very few have been assigned a biological activity or have known biosynthetic genes, making this a promising area for detailed experimental investigation.

Studying individual molecules from a pool

The discoveries of highest impact will come not from simply cataloging new microbiota-derived molecules, but from studying the biological activities of individual molecules in a complex molecular milieu. With the exception of fecal metabolite profiling, few microbiota-derived molecules have been detected in host-derived samples. More sensitive analytical and synthetic techniques (e.g., nanospray desorption electrospray ionization (nanoDESI) mass spectrometry) are needed to verify the production of a molecule in the skin, oral, and vaginal communities. An alternative approach – the detection of RNA transcripts for a particular bacterial gene cluster in metatranscriptomic data – has been used to profile the expression of the cluster in a native sample. This approach is consistent with, but not proof of, small molecule production in the ‘native’ habitat of the microbiome (10).

Although preliminary studies to study the biological effects of target molecules have been carried out for three microbiota-derived RiPPs (139–143), their biological relevance have not yet been tested experimentally. Although colonization studies in germ-free mice have the advantage of simplicity, there are drawbacks: (i) a molecule derived from a mono-colonist could be produced at super-physiological levels, leading to a false signal; (ii) the activity of some molecules, e.g., immune modulators, may require the activity of co-stimulatory signals

from other bacterial species; and (iii) the biological relevance of certain molecules, e.g. antibacterials, may only be observed when other members of the microbiota are present. Faith *et al.* have overcome some of these challenges in studies on regulatory T cells in the gut, by using small subsets of gut bacterial strains to colonize mice (Figure 3d) (144) .

Similar experimental systems have recently been developed for the skin. Conventional mice can be colonized by individual skin commensals and the T-cell response can be tracked over long time periods (145) . By contrast, few experimental systems are available for interrogating the role of small- molecule-mediated interactions in the community structure (146) and dynamics (137) of oral and vaginal communities.

Many molecules of interest are produced by bacterial species that are difficult to manipulate genetically, such as the anaerobic Firmicutes (*Clostridium* and its relatives). Two technologies would be transformative for their study: gene knock out and expression (147) , and synthetic-biology-based approaches to ‘refactor’ biosynthetic gene clusters and express them in a more genetically tractable host (148) .

Conclusion

Much is known about which bacterial species are most abundant in human-associated communities and how they vary among individuals. Yet comparatively little is known about the most abundant bacterially derived or modified small molecules in the gut, despite the fact that these molecules are present at high micromolar concentration, their levels can vary greatly among individuals, and the human host is chronically exposed to them for decades. Certain low- abundance molecules with potent biological activities may also be significant. Against this backdrop, it seems likely that in the near future the suite of microbiota- derived molecules in an individual’s gut community will not be left to chance. Pharmaceutical companies go to great lengths to get a single molecule into the human gut at a comparable concentration. Discovering the most abundant, widely (or variably) distributed, and biologically active molecules produced by the microbiota – and connecting them to the genes that encode them – are critical first steps in understanding which molecules have desired effects and which are deleterious, what receptors they target, and how therapeutic communities of microorganisms can be designed in which the production and non-production of molecules can be genetically specified.

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ONE-PAGE SUMMARY

Background

Two developments in distinct fields are converging to create interest in discovering natural products from the human microbiome. First, the use of genomics to guide natural product discovery has led to the unexpected discovery of numerous biosynthetic gene clusters in genomes of the human microbiota. Second, the microbiome research community is moving from a focus on ‘who’s there’ to ‘what are they doing’, with an accompanying emphasis on understanding microbiota-host interactions at the level of molecular mechanism. This merger has sparked a concerted hunt for the mediators of microbe-host and microbe-microbe interactions, including microbiota-derived small molecules.

Advances

Numerous small molecules are known that are produced by the human microbiota. The microbiota-derived ribosomally synthesized, post-translationally modified peptides (RiPPs) include widely distributed lantibiotics and microcins with narrow-spectrum activity that are presumptive mediators of interactions among closely related species, and *Escherichia coli* heat-stable enterotoxin, a guanylate cyclase 2C agonist from which the recently approved GI motility drug linaclotide was derived. Fewer amino acid metabolites are synthesized by the microbiota, but they are produced at very high levels that vary widely among individuals (e.g., indoxyl sulfate at 10–200 mg/day). Gut bacterial species convert common dietary amino acids into distinct end products, such as tryptophan to indoxyl sulfate, indole propionic acid and tryptamine – indicating that humans with the same diet but different gut colonists can have widely varying gut metabolic profiles. Microbially produced oligosaccharides differ from other natural products because they are cell-associated (i.e., non-diffusible) and because many more biosynthetic loci exist for them than for other small molecule classes. Well-characterized examples, such as *Bacteroides* polysaccharide A, show that oligosaccharides may not simply play a structural role or mediate adhesion; rather, they can be involved in highly specific ligand-receptor interactions that result in immune modulation. Similarly, the (glyco)lipids α -galactosylceramide and mycolic acid can play roles in immune signaling. The most prominent microbiota-derived terpenoids are microbial conversion products of the cholic acid and chenodeoxycholic acid in host bile. These secondary bile acids can reach high concentration (mM) in the gut and vary widely in composition among individuals. Several canonical virulence factors from pathogens are derived from nonribosomal peptides (NRPs) and polyketides (PKs), but less is known about NRPs and PKs from the commensal microbiota. A recent computational effort has identified ~14,000 biosynthetic gene clusters in sequenced genomes from the human microbiota, 3,118 of which were present in one or more of the 752 metagenomic sequence samples from the NIH Human Microbiome Project. Nearly all of the gene clusters that were present in >10% of the samples from the body site of origin are uncharacterized, highlighting the potential for identifying the molecules they encode and studying their biological activities.

Outlook

There are two central challenges facing the field. The first is to distinguish, from among thousands of microbiota-derived molecules, which are the ‘important’ ones, i.e., the ones that drive a key phenotype at physiologically relevant concentrations? Second, which experimental systems are appropriate for testing the activity of an individual molecule from a complex milieu? Meeting these challenges will require the development of new computational and experimental technologies, including a capacity to identify biosynthetic genes and predict the structure and target of their biological activity, and new systems in which germ-free mice are colonized by mock communities that differ only by the presence/absence of a biosynthetic gene cluster.

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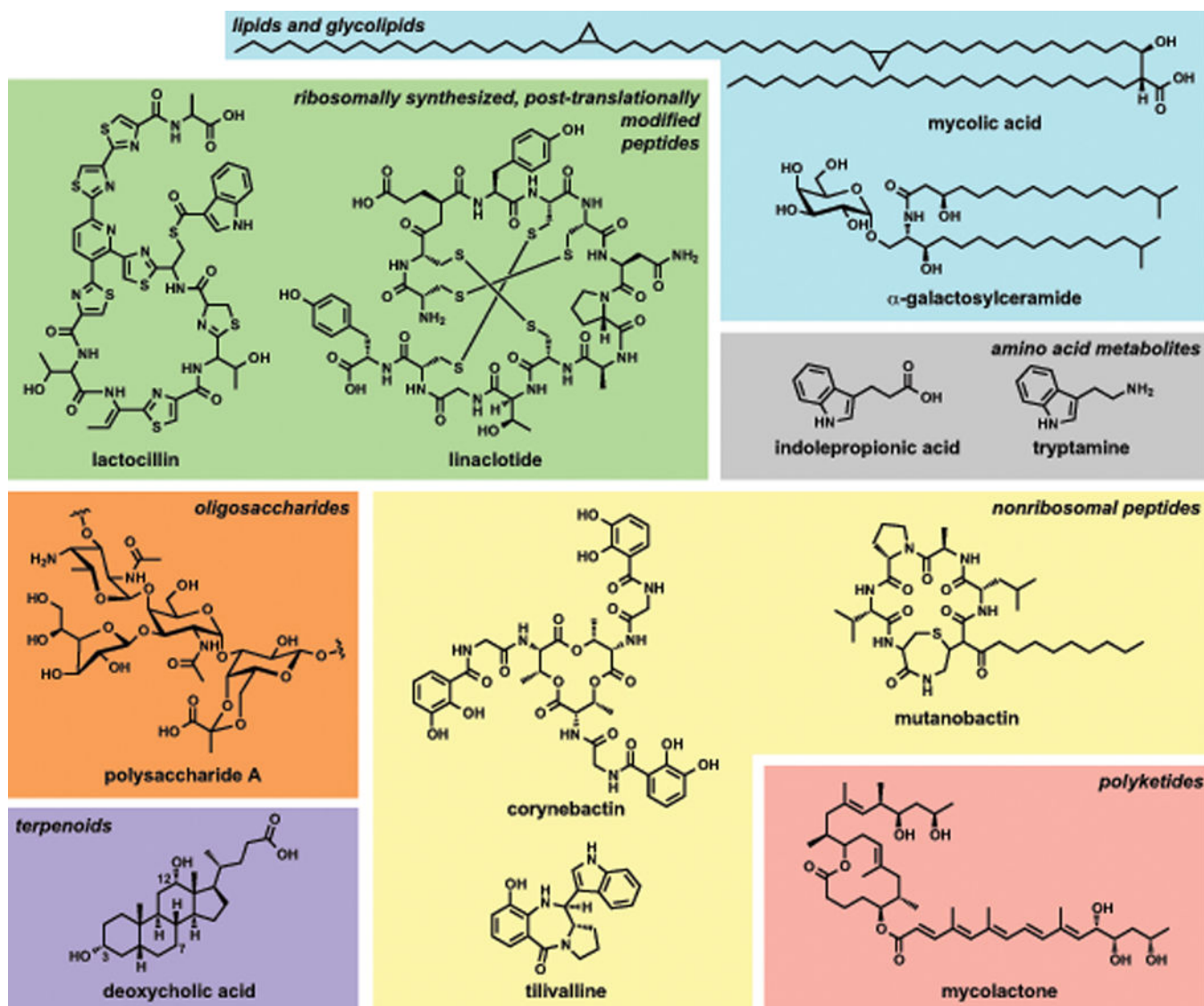


Figure 1. Structurally diverse small molecules from the human microbiota

The diversity of chemical classes produced by the human microbiota rivals that of microorganisms from any ecological niche. Representative molecules are shown for each of the major molecular classes discussed: the RiPPs lactocillin and linaclotide; the amino acid metabolites indolepropionic acid and tryptamine; the oligosaccharide polysaccharide A; the lipids/glycolipids mycolic acid and α -galactosylceramide; the terpenoid deoxycholic acid, in which carbons 3, 7, and 12 of the bile acid scaffold are labeled; the nonribosomal peptides corynebactin, tilivalline, and mutanobactin; and the polyketide mycolactone.

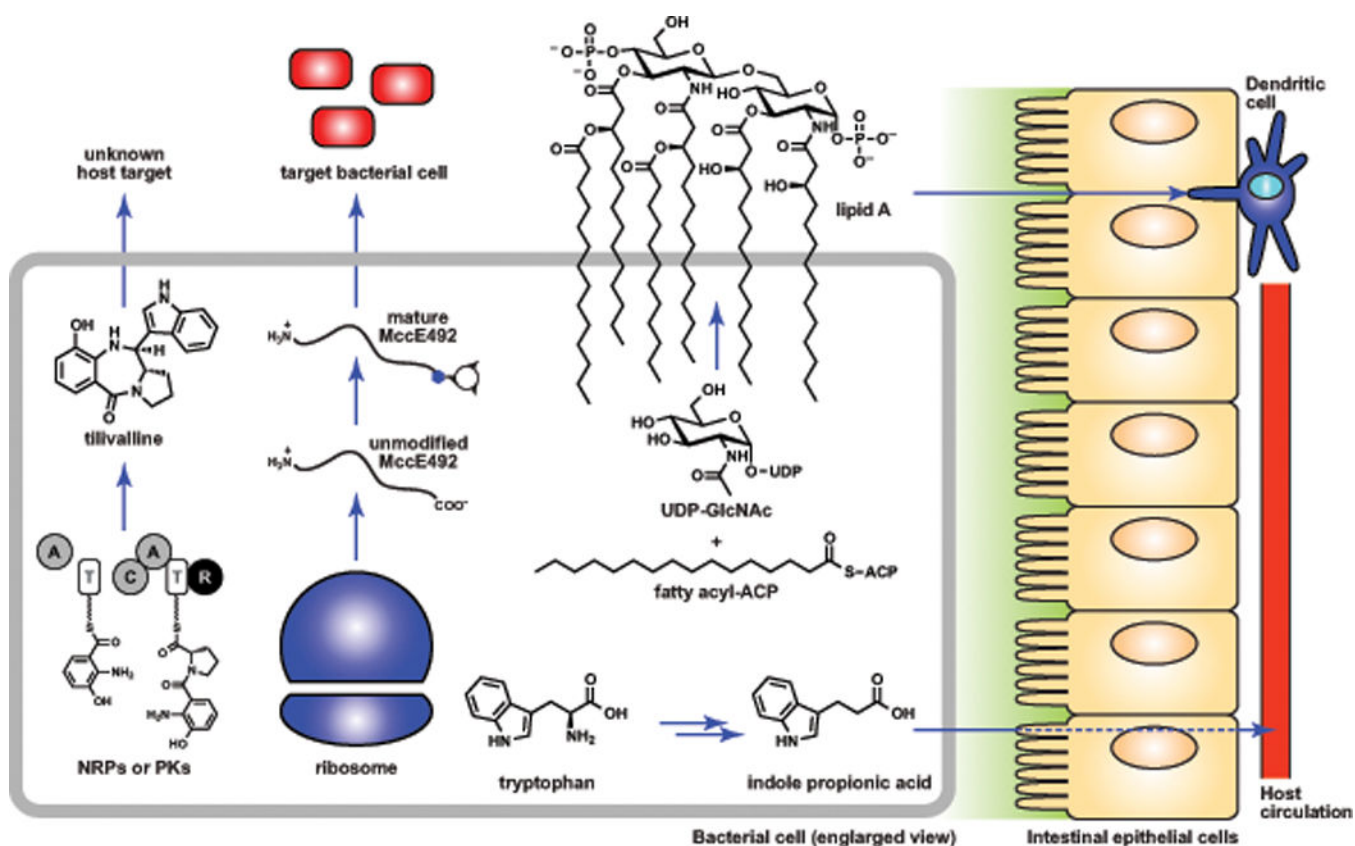


Figure 2. Small-molecule mediated microbe-host and microbe-microbe interactions

The microbiota produce a range of small molecules from various classes with distinct targets. Four examples are shown: the nonribosomal peptide tilivalline, whose host target is unknown; the ribosomally synthesized and post-translationally modified peptide microcin E492 (MccE492), a narrow spectrum antibacterial; lipid A, the glycolipid core of lipopolysaccharide, which targets TLR4 in host immune cells; and indole propionic acid, a reductive metabolite of tryptophan that enters host circulation but whose biological activity is poorly understood. These metabolites are each produced by different species of the microbiota, but are shown here in a single cell for schematic purposes. The following are abbreviations for domains in the nonribosomal peptide synthetase that produces tilivalline: A = adenylation domain, T = thiolation domain, C = condensation domain, R = terminal reductase domain.

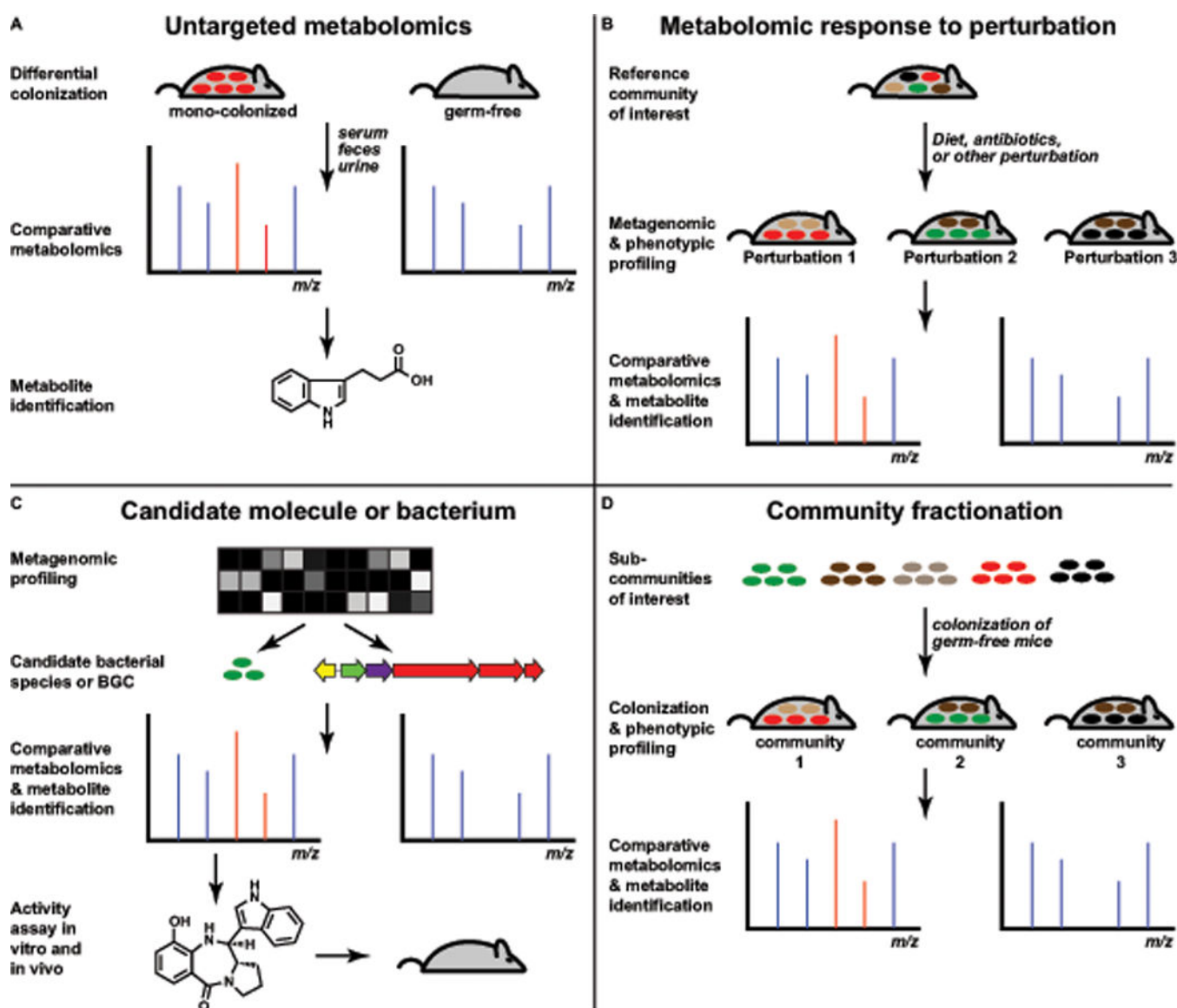


Figure 3. Approaches to discovering small molecules from the microbiota

(A) Samples from germ-free and colonized mice can be analyzed by untargeted metabolomics to identify molecules that are present in a microbiota-dependent fashion. (B) A mouse harboring a reference gut community can be subjected to antibiotic treatment, a dietary shift, or another perturbation. Comparative metabolomics can be used to identify microbiota-derived molecules whose abundance changes as a consequence of the perturbation. (C) Candidate bacterial gene clusters or bacterial species can be selected by metagenomic profiling (e.g., for gene clusters or species that are widely distributed, or differ in abundance between cases and controls). Comparative metabolomics can then be used to identify molecules produced by a gene cluster or bacterial species of interest. (D) Subsets of bacteria from a fractionated complex community or designed synthetic communities can be used to colonize mice in order to identify specific bacterial species whose presence correlates with the production of a molecule of interest.

Table 1

Selected small molecules from the human microbiota

Class	Compound	Producer (example)	Phylum	Host Site	Known/Predicted Activity
RIPP (thiopeptide)	lactocillin	<i>Lactobacillus gasseri</i>	Firmicutes	Vagina	Antibiotic
RIPP (lantibiotic)	epidermin	<i>Staphylococcus epidermidis</i>	Firmicutes	Skin	Antibiotic
RIPP (lantibiotic)	salivaricin A2 and B	<i>Streptococcus salivarius</i>	Firmicutes	Mouth	Antibiotic
RIPP (lantibiotic)	cytolysin	<i>Enterococcus faecalis</i> *	Firmicutes	Gut	Antibiotic, Cytotoxic
RIPP (lantibiotic)	ruminococcin A	<i>Ruminococcus gnavus</i>	Firmicutes	Gut	Antibiotic
RIPP (lantibiotic)	staphylococcin Au-26 (Bsa)	<i>Staphylococcus aureus</i>	Firmicutes	Skin	Antibiotic
RIPP (lantibiotic)	SA-FF22	<i>Streptococcus pyogenes</i>	Firmicutes	Oral/Skin	Antibiotic
RIPP (bacteriocin)	ruminococcin C	<i>Ruminococcus gnavus</i>	Firmicutes	Gut	Antibiotic
RIPP (microcin)	microcin C7/C51	<i>Escherichia coli</i>	Proteobacteria	Gut	Antibiotic
RIPP (microcin)	microcin B17	<i>Escherichia coli</i>	Proteobacteria	Gut	Antibiotic
RIPP (microcin)	microcin J25	<i>Escherichia coli</i>	Proteobacteria	Gut	Antibiotic
RIPP (microcin)	microcin H47	<i>Escherichia coli</i>	Proteobacteria	Gut	Antibiotic
RIPP (TOMM)	streptolysin S	<i>Streptococcus pyogenes</i>	Firmicutes	Oral/Skin	Cytotoxic
RIPP (TOMM)	clostridiolysin S	<i>Clostridium sporogenes</i>	Firmicutes	Gut	Unknown
RIPP (TOMM)	listeriolysin S	<i>Listeria monocytogenes</i>	Firmicutes	Gut	Unknown
RIPP	heat-stable enterotoxin	<i>Escherichia coli</i>	Proteobacteria	Gut	GI motility (guanylate cyclase 2C)
Amino acid metabolite	indolepropionic acid	<i>Clostridium sporogenes</i>	Firmicutes	Gut	Immunomodulatory
Amino acid metabolite	indole	Unknown	Unknown	Gut	Converted to indoxyl sulfate
Amino acid metabolite	skatole	<i>Clostridium spp.</i>	Firmicutes	Gut	Unknown
Amino acid metabolite	tryptamine	<i>Ruminococcus gnavus</i>	Firmicutes	Gut	Neurotransmitter
Amino acid metabolite	phenyllactic acid	<i>Bifidobacterium spp.</i>	Actinobacteria	Gut	Unknown
Amino acid metabolite	phenethylamine	<i>Lactobacillus spp.</i>	Firmicutes	Gut	Neurotransmitter
Amino acid metabolite	δ -aminovaleric acid	<i>Clostridium spp.</i>	Firmicutes	Gut	Unknown
Amino acid metabolite	GABA	Unknown	Unknown	Gut	Unknown
Amino acid metabolite	α -aminobutyric acid	Unknown	Unknown	Gut	Unknown
Amino acid metabolite	3-aminoisobutyric acid	<i>Clostridium spp.</i>	Firmicutes	Gut	Unknown
Amino acid metabolite	<i>p</i> -cresol	<i>Clostridium spp.</i>	Firmicutes	Gut	Unknown

Class	Compound	Producer (example)	Phylum	Host Site	Known/Predicted Activity
Acid (short-chain)	propionic acid	<i>Bacteroides</i> spp.	Bacteroidetes	Gut	Immunomodulatory (GPR43)
Oligosaccharide	polysaccharide A	<i>Bacteroides fragilis</i>	Bacteroidetes	Gut	Immunomodulatory (TLR2)
Oligosaccharide	capsular polysaccharide	<i>Streptococcus pneumoniae</i>	Firmicutes	Airways	Immunomodulatory
Glycolipid	α -galactosylceramide	<i>Bacteroides fragilis</i>	Bacteroidetes	Gut	Immunomodulatory (CD1d)
Glycolipid	corynomycolic acid	<i>Corynebacterium</i> spp.	Actinobacteria	Skin	Unknown
Glycolipid	mycolic acid	<i>Mycobacterium</i> spp.	Actinobacteria	Airways	Immunomodulatory (CD1b)
Glycopeptide	muramyl di- and tripeptides	<i>Fusobacterium nucleatum</i>	Fusobacteria	Oral	Immunomodulatory (NOD1, NOD2)
Terpenoid	staphyloxanthin	<i>Staphylococcus aureus</i>	Firmicutes	Skin	Unknown (antioxidant?)
Terpenoid	bile acids (e.g., deoxycholic acid)	<i>Clostridium</i> spp.	Firmicutes	Gut	Metabomodulatory (TGR5, FXR, VDR)
NRP	phevalin	<i>Staphylococcus aureus</i>	Firmicutes	Skin	Unknown (virulence inducer?)
NRP	cereulide	<i>Bacillus cereus</i> *	Firmicutes	Gut	Cytotoxic, Immunomodulatory
NRP	yersiniabactin	<i>Yersinia pestis</i> *	Proteobacteria	Bloodstream	Siderophore
NRP	corynebactin	<i>Corynebacterium</i> spp.	Actinobacteria	Skin	Siderophore
NRP	tilivalline	<i>Klebsiella oxytoca</i> *	Proteobacteria	Gut	Cytotoxic
NRP-PK	zwittermicin	<i>Bacillus cereus</i> *	Firmicutes	Gut*	Antimicrobial
NRP-PK	mutanobactin	<i>Streptococcus mutans</i>	Firmicutes	Mouth	Unknown
NRP-PK	colibactin	<i>Escherichia coli</i>	Proteobacteria	Gut	Cytotoxic
PK	mycolactone	<i>Mycobacterium ulcerans</i> *	Actinobacteria	Skin	Immunomodulatory
Porphyrin	coproporphyrin III	<i>Propionibacterium acnes</i>	Actinobacteria	Skin	Unknown
Citrate amide	staphyloferrin B	<i>Staphylococcus aureus</i>	Firmicutes	Skin	Siderophore