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Natural Products as Mediators of Disease

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Title: Natural Products as Mediators of Disease

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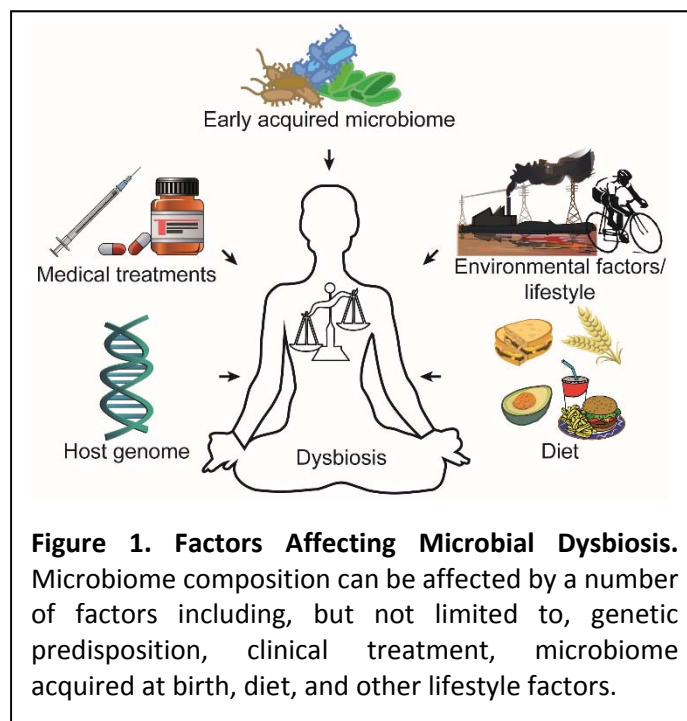
Abstract: Humans are walking microbial ecosystems, each harboring a complex microbiome with the genetic potential to produce a vast array of natural products. Recent sequencing data suggests that our microbial inhabitants are critical for maintaining overall health. Shifts in microbial communities have been correlated to a number of diseases including infections, inflammation, cancer, and neurological disorders. Some of these clinically and diagnostically relevant phenotypes are a result of the presence of small molecules, yet we know remarkably little about their contributions to the health of individuals. Here, we review microbe-derived natural products as mediators of human disease.

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1. INTRODUCTION

Microbial natural products are integral to human health. They are heavily utilized in medicine as antibiotics, antifungals, immunosuppressants, and anticancer agents.¹⁻³ Advances in sequencing technologies and bioinformatics approaches have revealed an untapped reservoir of natural products in hosts with diverse and biosynthetically rich microbial ecosystems, including humans.³⁻⁸ It is estimated that microbial cells outnumber mammalian cells 1.3 to 1 in the human body, with a combined genetic potential that exceeds the number of human genes 100-fold.^{7, 9, 10} Correlation of shifts in microbiome community structure to a number of diseases including irritable bowel syndrome,¹¹⁻¹⁴ obesity,^{15, 16} and arthritis,¹⁷⁻¹⁹ strongly suggests that the functional structure of host-associated ecosystems is incredibly important for maintaining host health.²⁰ However, it should be noted that correlation is not necessarily indicative of causality.

There are two prevailing models for microbial dysbiosis.²¹ In the first model, a shift in community composition due to infection with an outside agent, overgrowth of a commensal, or treatment with antibiotics leads to a weakened host immune response and opportunistic microbes overgrowing their typical niche. The second model suggests that altered host biology and reduced immune function due to injury, genetic predisposition, or chronic conditions can lead to microbial community compositional changes, microbial overgrowth, and altered immune response. Several factors implicated in microbial dysbiosis are highlighted in Figure 1.^{22, 23} Both proposed models suggest microbial interactions including metabolic exchange of natural products may be an important factor in continuous microbial community dysbiosis and, by extension, disease. Natural products provide an ecological advantage for the producing microbe by enabling competition for nutritional resources against competing microbes and the host tissue.



Here, we review the role of select microbe-derived natural products in mediating disease in humans (Figure 2). The comprehensive review of all small molecule virulence factors is beyond the scope of a single article and many high quality reviews have been published demonstrating the importance of quorum sensing,²⁴⁻²⁶ siderophores,^{27, 28} short chain fatty acids (SCFAs),²⁹⁻³¹ and single metabolites such as

(pre-)colibactin.^{32, 33} Therefore, our emphasis is on brief examples of the role of specific metabolites as mediators of disease for the major human organ systems which may be of interest to the natural products community. We further describe emerging technologies, propose potential strategies, and highlight specific challenges for natural product discovery from the human microbiome.

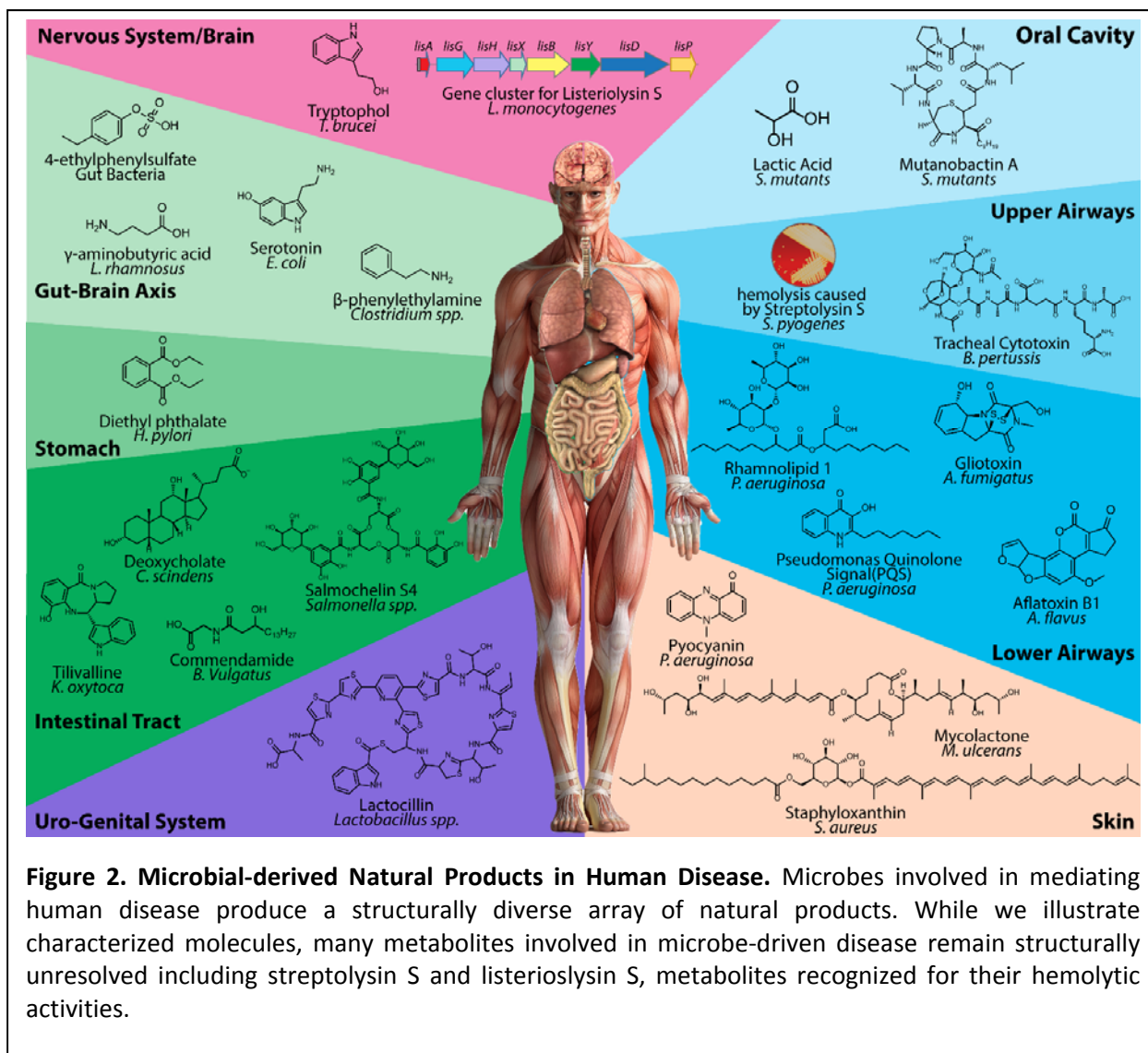


Figure 2. Microbial-derived Natural Products in Human Disease. Microbes involved in mediating human disease produce a structurally diverse array of natural products. While we illustrate characterized molecules, many metabolites involved in microbe-driven disease remain structurally unresolved including streptolysin S and listeriolysin S, metabolites recognized for their hemolytic activities.

2. NATURAL PRODUCTS IN HUMAN DISEASE

The association of microbial diseases with the human host was first discovered in the late 17th century when Antony van Leeuwenhoek visualized his own dental plaque using a microscope. However, the concept that microbes cause some diseases was proposed as early as the mid-16th century. This theory was further substantiated by the work of a number of prominent scientists including Louis Pasteur and Robert Koch through the second half of the 19th century. The discovery of penicillin in 1928 by Alexander Fleming ushered in the antibiotic era for treatment of microbial diseases.³⁴ During this time, researchers recognized that human hosts are inhabited by a large number of microbes, yet only a few were identified as causing disease. This observation led to the hypothesis that these microbes were unique and could be

classified as pathogenic microbes. Classification of pathogenic microbes was established by a microbe's expression of determinants of disease, otherwise referred to as virulence factors. While the definition of virulence factors is broad, one category includes natural products and other small molecules with specialized function.³⁵ The small molecule virulence factors of several common pathogenic microbes have been thoroughly studied. However, specialized metabolite production remains uncharacterized for the majority of human-associated microbes. Recent bioinformatics analyses of specialized metabolite biosynthetic gene clusters (BGCs) applied to metagenomics data from the National Institutes of Health (NIH) Human Microbiome Project (HMP) revealed over 3,000 small molecule BGCs belonging to all natural product molecular families, although very few BGCs were widely distributed.⁵ The number of BGCs detected is likely an underestimate of the true biosynthetic potential of the microbiota due to the stringent search parameters used. While the total number of BGCs detected in human microbiome is smaller than the number of BGCs from other environments,^{5, 36, 37} the biosynthetic potential of the microbiome suggests that natural products play a critical role in maintaining microbial community structure and the microbiome remains an untapped reservoir for natural products research.

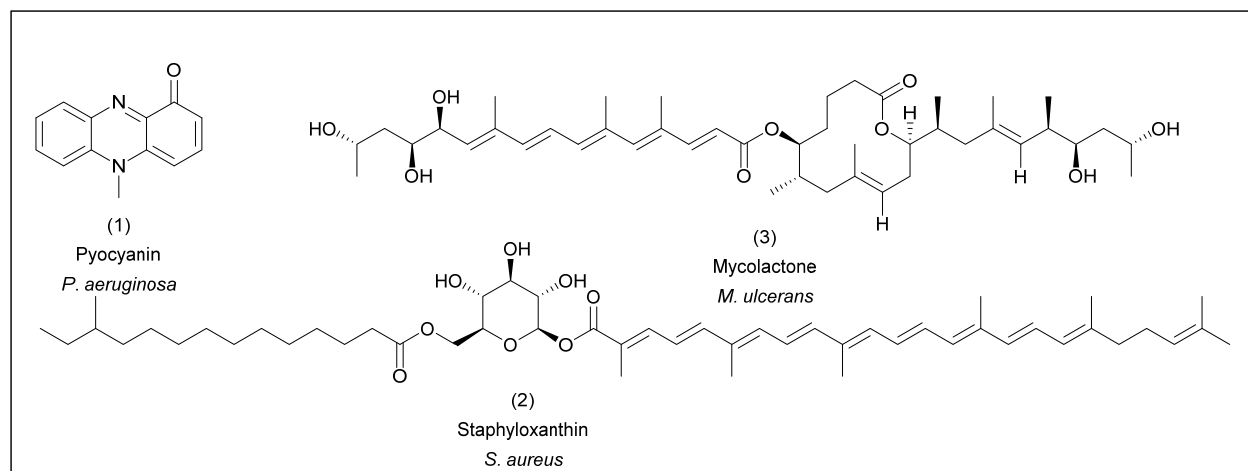
2.1 SKIN

The skin is our largest organ with a surface area between 1.5 and 2 square meters. It is also our most exposed organ, providing the interface between our immune system and the external environment including its associated microbiota. The resident skin microbial communities are highly variable across the body and between individuals, with colonization driven by a number of host and environmental factors.³⁸⁻⁴⁰ Most of the approximately 1,000 microbial species inhabiting our skin are harmless or beneficial, although several species have been implicated in dermatological diseases and chronic wounds.^{21, 41, 42} The fungal genera *Malassezia*, the bacterium *Propionibacterium acnes*, and the opportunistic pathogen *Staphylococcus aureus* have been correlated to dermatological diseases such as seborrheic dermatitis, acne vulgaris, and atopic dermatitis (AD), respectively.^{21, 41, 42} It has been proposed that *Malassezia* ssp. produce lipases which release fatty acids, thereby promoting epidermal hyperproliferation and inflammation.^{43, 44} However, the role of other fungal natural products in skin disease remains largely uninvestigated. Similarly, the bacterial skin commensal *P. acnes* has been shown to degrade and utilize host lipids resulting in the characteristic inflammation associated with acne vulgaris.⁴⁵⁻⁴⁹ *P. acnes* strains have been shown to produce propionate, tyramine, histamine, structurally-uncharacterized thiocillins, and putative natural products which have been described in only the most general terms as heat stable neutrophil chemotactic factors.⁵⁰⁻⁵⁴ *S. aureus*, a well-studied opportunistic pathogen, is associated with the development of AD. However, no specific link between *S. aureus* virulence factors and AD flare-ups has been identified.^{55, 56}

Opportunistic infections of the skin affect a wide population, often occurring as chronic wounds in diabetic, elderly, and immobile people or from severe burns.^{57, 58} While the microbes involved in chronic infections do not cause the initial wound, they are associated with slower healing and persistent inflammation. Culture-based analyses revealed that chronic wound infections arise from a patient's commensal microbiota (skin, gastro-intestinal, or respiratory) or are nosocomial in nature.⁵⁹⁻⁶¹ Bacteria are the best characterized drivers of most chronic infections, although infections can also occur due to viruses, fungi, and yeast.⁶² Only a few natural products have been associated with microbes responsible for chronic infections, despite the wide array of microbial species detected in or isolated from chronic wounds.

Of the microbes investigated for their role in chronic wounds, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *S. aureus*, and *Mycobacterium ulcerans* have been partially characterized. *S. pyogenes* (also referred to as group A streptococci or GAS) is a commensal skin and oral microbe that is implicated in a variety of skin diseases and opportunistic infections.⁶³ One of the key natural products produced by GAS is streptolysin S (SLS), a potent 2.7 kDa cytotoxic peptide of the bacteriocin family of

antimicrobial peptides responsible for the lysis of erythrocytes, leukocytes, platelets, and subcellular organelles.⁶⁴⁻⁶⁶ Interestingly, SLS is only cytotoxic in the presence of carrier molecules or when associated with the bacterial cell surface. The SLS BGC was recently identified and homologous BGCs were found in other streptococci species and Gram-positive pathogens including *Listeria monocytogenes*, *Clostridium botulinum*, and *S. aureus*. The structures of SLS and related peptides remain elusive. Compared to *S. pyogenes*, the specialized metabolite production by *P. aeruginosa* has been more thoroughly investigated.^{25, 67-71} However, only the phenazine pyocyanin (**1**, PYO) has been studied in the context of chronic wounds and was found to impair wound repair mechanisms.⁷² Molecules produced by *S. aureus* during skin infections include staphyloxanthin (**2**)⁷³⁻⁷⁵ and the phenol soluble modulins (PSMs) including delta-toxin.⁷⁶⁻⁷⁸ Staphyloxanthin is a carotenoid pigment proposed to scavenge reactive oxygen species, allowing *S. aureus* to circumvent the immune response.⁷³ PSMs are a family of alpha-helical peptides with concentration-dependent activity.^{79, 80} At low nanomolar concentrations, several PSMs induce cytokine release, while at micromolar levels they induce neutrophil lysis. PSMs have also been postulated to allow *S. aureus* to evade immune system detection by reducing dendritic cell mediated differentiation of T-helper cells. Higher concentrations of PSMs by community associated methicillin resistant *S. aureus* (MRSA) were shown to be critical for neutrophil lysis and full virulence.⁷⁸ Intriguingly, treatment of *S. aureus* with antibiotics leads to the production of PSM structural variations via truncation of the peptide and amino acid substitutions.⁷⁷ Production concentrations of PSMs are much lower from the *S. aureus* related commensal species *Staphylococcus epidermidis*, explaining its reduced virulence.⁸¹ It has been suggested that some strains of *S. epidermidis* may inhibit potential pathogens by enhancing production of human antimicrobial peptides.⁸²⁻⁸⁴ Lastly, Buruli ulcers are the third most common mycobacteriosis in humans after tuberculosis and leprosy. The mycolactone (**3**) polyketides (PKs) produced by *M. ulcerans* have been implicated in ulcer development.⁸⁵ Mycolactone production has been associated with strain-specific virulence phenotypes.⁸⁶ The production and biological roles of structurally distinct mycolactones from pathogenic *Mycobacterium* species have been thoroughly reviewed.⁸⁷⁻⁸⁹ *In vivo* testing of mycolactones A and B in guinea pigs resulted in lesions similar to the Buruli ulcers developed by humans infected with *M. ulcerans*. Despite the severity of Buruli ulcers, the extensive skins lesions are not accompanied by pain. The mechanism of painlessness of the severe mycolactone-mediated lesions has recently been characterized at the molecular level.^{90, 91} Mycolactones induce type 2 angiotensin II based signaling which results in hyperpolarization of neurons and analgesia.



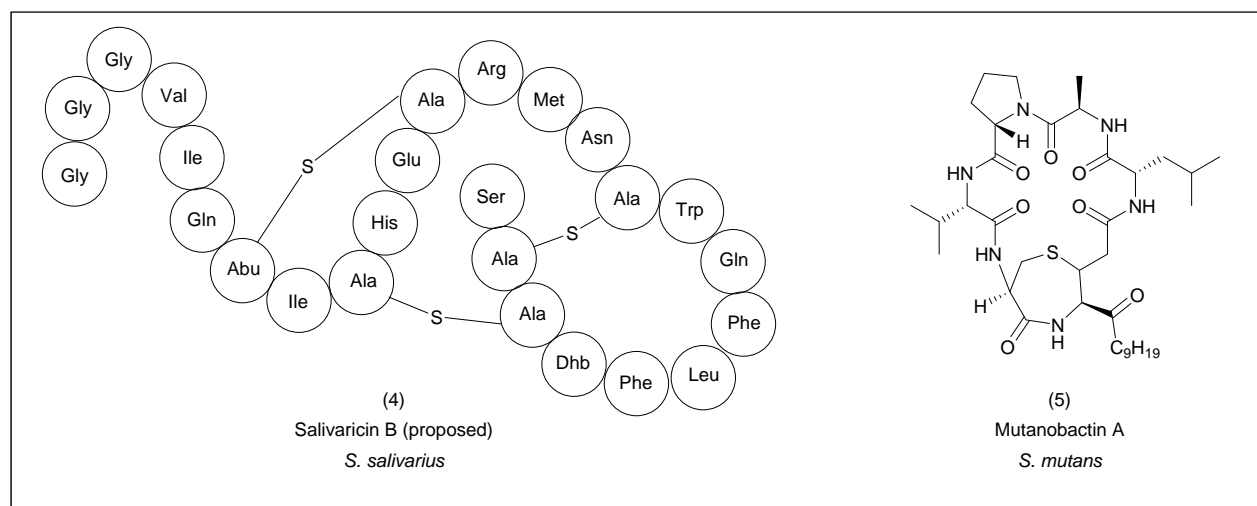
2.2 RESPIRATORY TRACT

The respiratory tract is divided into the upper and lower airways. The upper airways consist of the nose, nasal cavity, mouth, throat (pharynx), and voice box (larynx). The lower airways are composed of

the trachea and lungs including the bronchi, bronchioles, and alveoli. A number of respiratory diseases are the result of infection by a single microbial species including pertussis, scarlet fever, strep throat, and diphtheria. Many of these diseases have been successfully prevented through the administration of vaccines. Other microbe-driven diseases include pneumonia, sinusitis, and lung infections of patients suffering from diseases such as cystic fibrosis (CF) or chronic obstructive pulmonary disorder (COPD). Shifts in microbial community structure have been implicated in respiratory tract infections.^{92, 93} The microbial interactions and cause of community shifts in respiratory diseases remain under-investigated at the molecular level. In general, this is likely due to the poor accessibility of such samples when compared to the gut or the skin.

2.2.1 ORAL CAVITY

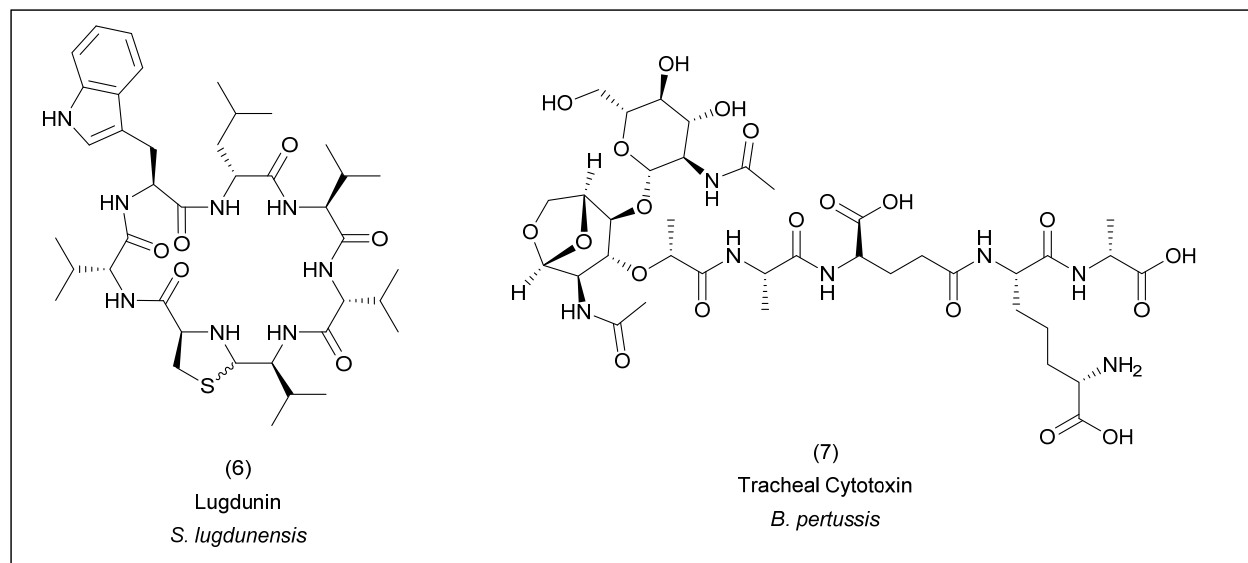
The oral cavity (consisting of the teeth, tongue, cheeks, hard and soft palates, gingival sulcus and tonsils) is inhabited by over 600 bacterial species and 100 fungal species.⁹⁴⁻⁹⁶ Different oral structures and tissues are occupied by distinct microbial communities with community structure affected by diet, dental hygiene, xenobiotics and host genetics.⁹⁷ Microbes (and in some cases, entire communities) isolated from the oral cavity have been implicated in the development of oral diseases including dental caries, periodontitis, tonsillitis, and many others.⁹⁸ Sequencing-based correlative studies have also suggested a role for oral bacteria in a number of systemic diseases including cardiovascular disease, stroke, preterm birth, diabetes, and pneumonia.^{95, 99} Despite efforts to inventory the constituents of the oral microbiome, the role of natural products in the advancement of oral diseases is poorly understood. However, some metabolites have been characterized. For example, the commensal microbe *Streptococcus salivarius* produces the lantibiotic peptides salivaricin A and B which have been shown to inhibit a black pigmented strain of the genus *Prevotella* associated with halitosis *in vitro*.¹⁰⁰⁻¹⁰² A proposed structure of salivaricin B (**4**) has been recently reported.¹⁰³ In dental caries, it has been postulated that demineralization is caused by increased acidity due to lactic acid production by the commensal species *Streptococcus mutans*.¹⁰⁴ Other microbes, such as *Lactobacillus* sp., may also be involved in the development of caries through production of similar acids.¹⁰⁵ In addition to lactic acid, *S. mutans* produces a variety of molecules under the microaerophilic conditions it encounters in the oral cavity including formate, acetate, ethanol, and two different mutanobactins.^{104, 106, 107} Mutanobactin A (**5**), isolated from *S. mutans* UA159, is a hybrid polyketide-nonribosomal peptide (PK-NRP) with dual roles. It allows *S. mutans* to colonize the oral cavity and to compete with the fungal pathogen *Candida albicans* by preventing its transition from an avirulent yeast-like state to its hyphal form.^{106, 107} One of the most prevalent oral diseases is periodontal disease which includes the mild and reversible gingivitis and periodontitis, a chronic disease resulting in



destruction of both connective and bone tissue. It is widely accepted that the progression of periodontal diseases is the result of the complex interaction between the associated microbial community and host response.¹⁰⁸⁻¹¹⁰ The induction of host response has been attributed to a number of bacterial factors including lipopolysaccharides, proteases and other pathogen-associated molecular patterns, SCFAs, and *N*-formyl-methionyl-leucyl-phenylalanine type peptides which function as chemo-attractants for leukocytes.^{111, 112}

2.2.2 UPPER AIRWAYS

The measured microflora of the upper respiratory tract is representative of the oral microbiome and includes common genera such as *Staphylococcus*, *Streptococcus*, *Neisseria*, *Bacillus*, and *Corynebacterium*, among others.¹¹³⁻¹¹⁵ It is postulated that colonization by opportunistic pathogens including species of *Haemophilus*, *Pseudomonas*, *Bordetella*, and *Mycoplasma* first occurs in the upper airways and spreads to the lower respiratory tract.¹¹⁶ In terms of natural products, only a handful of small molecules have been implicated in disease progression in the upper airways. In the nasal cavity, lugdunin (**6**), a NRP antibiotic produced by *Staphylococcus lugdunensis*, was identified from bioactivity-guided *in vitro* inhibition of *S. aureus* and validated in animal models.¹¹⁷ Another example is tracheal cytotoxin (**7**), a disaccharide-tetrapeptide, considered to be integral to the virulence of *Bordetella pertussis*, the causative agent of whooping cough.¹¹⁸⁻¹²³ Tracheal cytotoxin causes extensive damage to ciliated epithelial cells by inducing the host production of interleukin 1 (IL-1) and nitric oxide which leads to diminished ciliary beating, impaired removal of mucus, and the characteristic symptomatic cough.¹²⁴ Another example is the role of a two-peptide bacteriocin, pneumocin MN, produced by emerging *Streptococcus pneumoniae* serotypes.^{125, 126} *S. pneumoniae* is the most common bacterial agent in community-acquired pneumonias and is divided into more than 90 serotypes. It has been demonstrated that the strains of pneumococci inhabiting the upper airway are constantly in flux, as new strains outcompete existing strains.¹²⁷ Vaccination with the 7-valent conjugate vaccine consisting of protein-polysaccharide derivatives of the capsule has led to an incredible decrease in invasive pneumococcal disease (IPD) in children.¹²⁸ At the same time, isolates of non-vaccine serotypes have become more common. It has been proposed that the bacteriocins produced by these serotypes provide a competitive edge against other serotypes.¹²⁵ The bacteriocin-producing serotypes were subsequently included in the 13-valent vaccine. However, both the structure of pneumocin MN and its mechanism of interaction with the host have not been resolved. Lastly, *S. pyogenes*, in addition to being an opportunistic skin pathogen, is the causative agent of streptococcal pharyngitis, commonly known as strep throat. Traditional clinical

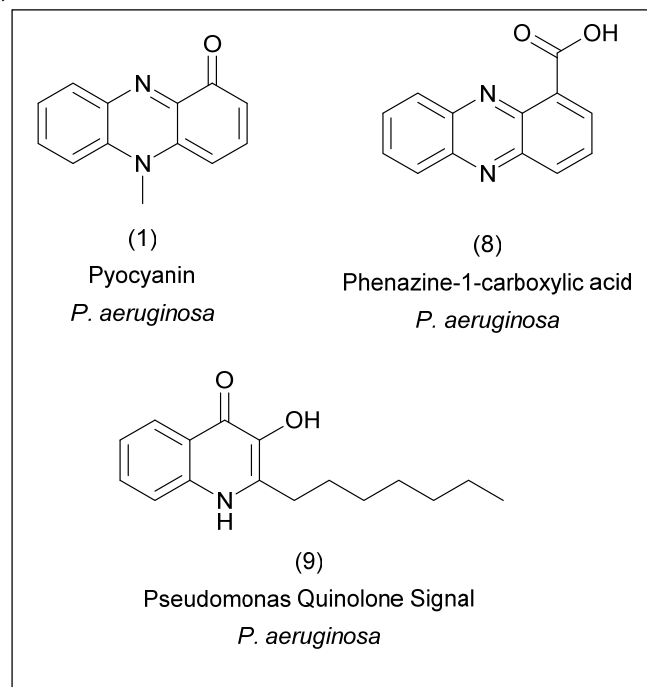


diagnosis of *S. pyogenes* infection is through its characteristic haemolysis pattern after culturing on blood agar plates; a method first described over a century ago.¹²⁹ This haemolysis is primarily due to the bacteriocin SLS which is a contributor to *S. pyogenes* virulence.^{130, 131}

2.2.3 LOWER AIRWAYS

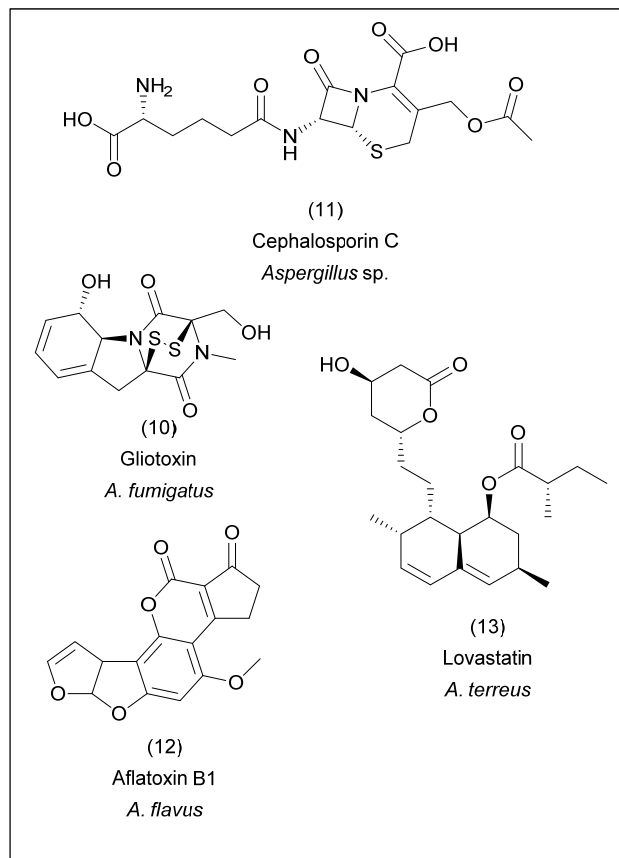
Once thought sterile, the lower respiratory tract is now known to harbor its own microbial community, albeit at much lower abundance than the oral cavity or gut.^{113, 132} The complex anatomical structure of the lung leads to a surface area of 30 times that of human skin with environmental gradients of oxygen, temperature, and pH.¹³³ The overall complexity of the lung represents a very dynamic, heterogeneous environment that is exposed and inhabited, at least transiently, by microbes from the air, oral cavity, and nasal passages. The characterization and investigation of the lung microbiome suffers from possible contamination of the community from microbes inhabiting the oral cavity. However, statistical methods have been utilized to identify organisms that are enriched in the lung.^{114, 134, 135} Healthy lungs are generally inhabited by a variety of species including the genera *Pseudomonas*, *Streptococcus*, *Veillonella*, and *Prevotella*. Clinical features of several lung diseases have been correlated with lung microbiome dysbiosis including asthma,^{136, 137} COPD,^{138, 139} and CF.^{140, 141}

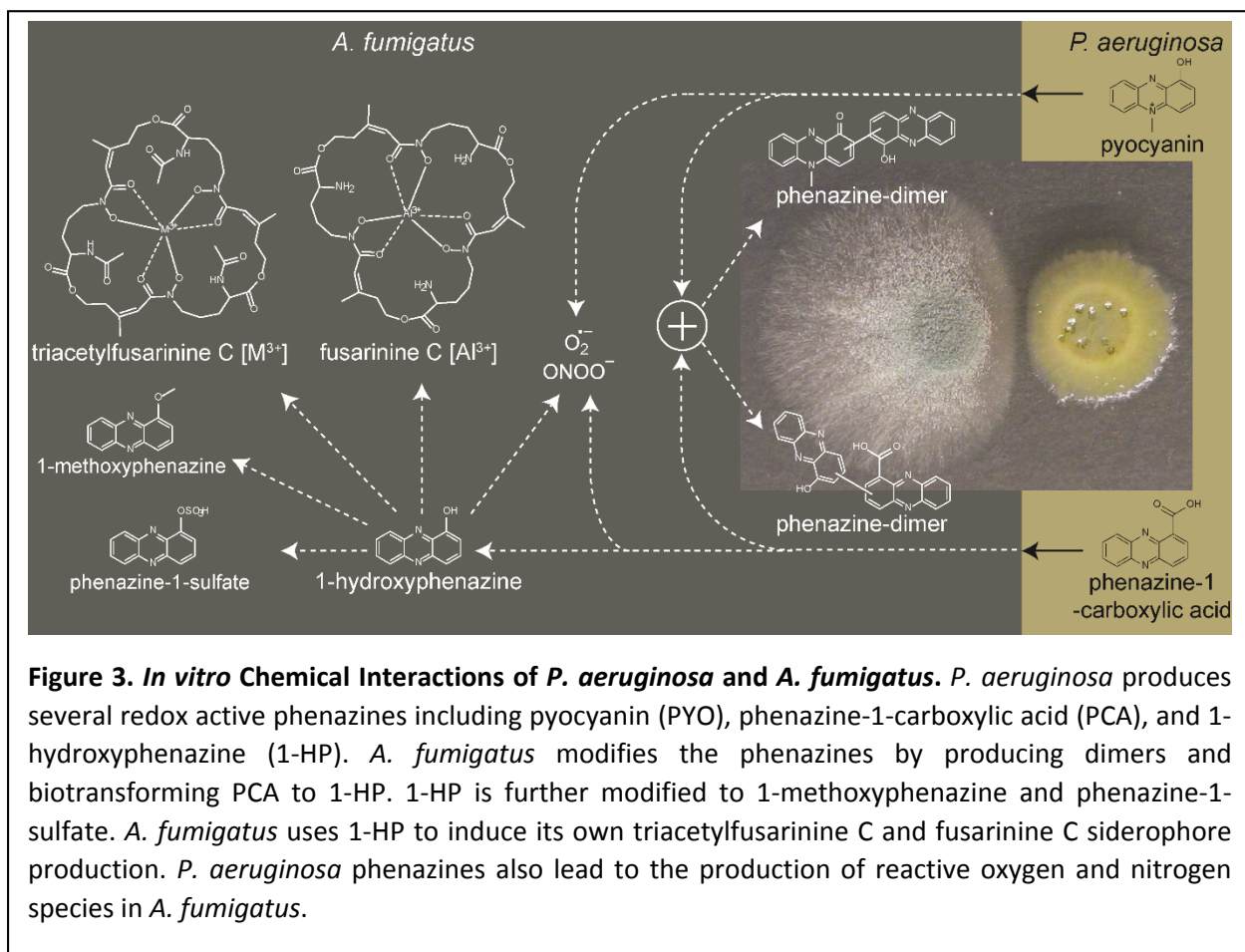
While a number of pulmonary pathogens have been identified, only the most prevalent have been characterized at the chemical level. For example, *P. aeruginosa* has the genetic capacity to produce a suite of virulence factors including homoserine lactones, quinolones, phenazines, rhamnolipids, and siderophores.^{25, 66-70} The molecules produced by *P. aeruginosa* have been shown to interact with host tissue by modulating host gene expression, producing free radicals, directly damaging epithelial cells, and scavenging iron.¹⁴²⁻¹⁴⁵ The detection of the redox active phenazines PYO (**1**) and phenazine-1-carboxylate (**8**, PCA), which are involved in anaerobic respiration, have been shown to negatively correlate with lung function.¹⁴⁶ Interestingly, a number of *P. aeruginosa* metabolites have been detected in serum of CF patients, suggesting that they are trafficked via the circulatory system to other organs and potentially cause unknown off-organ effects.¹⁴⁷ A recent study compared the repertoire of *P. aeruginosa* quinolones produced in laboratory cultures to sputum samples from CF patients.¹⁴⁸ The predominant culture produced quinolone, pseudomonas quinolone signal (**9**, PQS), was not detected in sputum samples.



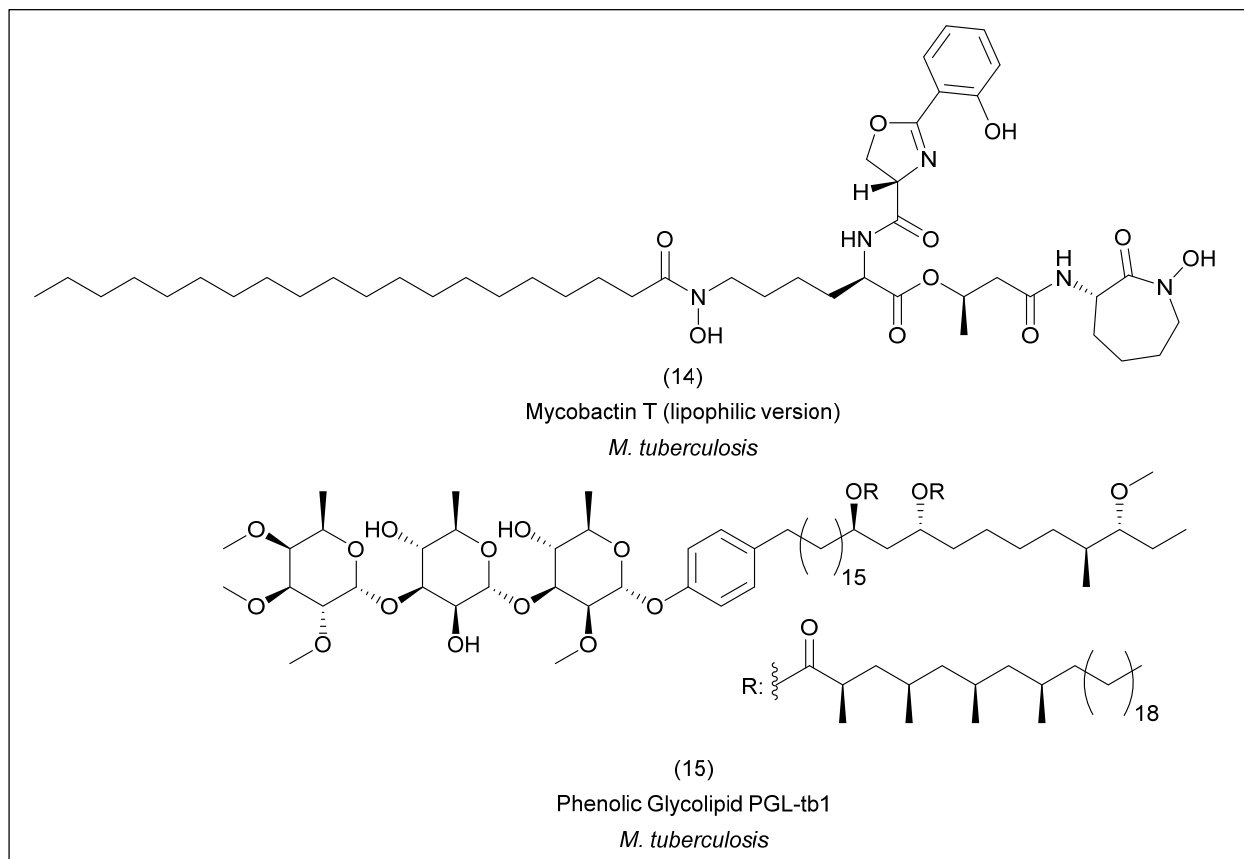
Aspergillus species represent one of the most prevalent fungi responsible for infections of patients with pulmonary disorders. *Aspergillus* species are known to produce a wide variety of natural products including gliotoxin (10), cephalosporin (11), aflatoxin B1 (12), and lovastatin (13).^{149, 150} While cephalosporin and lovastatin have been harnessed for medicinal purposes, aflatoxin B and gliotoxin are both toxins. Produced by *A. parasiticus* and *A. flavus*, the difuranocoumarin compound aflatoxin B is one of the most potent carcinogens.¹⁵¹⁻¹⁵⁴ Although aflatoxin B has been most extensively studied in liver tumorigenesis, the respiratory system is also a target of this mycotoxin. Studies have shown that aflatoxin B is bioactivated by cytochrome P450 monooxygenases and biotransformed into DNA binding metabolites by cytosolic enzymes. Gliotoxin is produced by *A. fumigatus*, an opportunistic pathogen affecting patients with pulmonary diseases which often leads to clinical complications.¹⁵⁵ Gliotoxin is a member of the epidithiodioxopiperazine family and is critical for *A. fumigatus* pathogenicity by inducing apoptosis, preventing NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) activation, and inhibiting angiogenesis; a partial molecular mechanism of action has been described.¹⁵⁶⁻¹⁵⁸ Despite intensive efforts to elucidate the natural products of *Aspergillus* species, the impact of these compounds during infections has seen limited attention.

In CF patients, co-occurrence of *P. aeruginosa* and *A. fumigatus* correlates with decreased lung function, suggesting that they may interact on the chemical level.¹⁵⁹⁻¹⁶¹ *In vitro*, *A. fumigatus* biotransforms the antifungal phenazines produced by *P. aeruginosa* to induce production of its own siderophores, providing *A. fumigatus* with an ecological advantage in the competition for environmental metals (Figure 3).¹⁶² Furthermore, phenazines were shown to induce morphological changes and formation of reactive oxygen and nitrogen species in *A. fumigatus* (Figure 3).^{163, 164} Interestingly, inhibition of *A. fumigatus* biofilm formation was more pronounced when grown in mixed cultures with CF-associated *P. aeruginosa* isolates compared to non-CF isolates.¹⁶⁵ While these findings have yet to be substantiated *in vivo*, they indicate that microbe-microbe interactions may significantly alter host-microbe interactions.





Pulmonary tuberculosis is rapidly re-emerging as a global health concern.¹⁶⁶ Multi-drug resistant (MDR) strains of *Mycobacterium tuberculosis* have been identified.¹⁶⁷ While treatments for MDR tuberculosis are limited, *M. tuberculosis* natural products provide promising targets for drug development. For example, the ability of *M. tuberculosis* to grow inside macrophages has been partially attributed to small molecules.^{168, 169} The production of the PK-NRP mycobactin siderophores (**14**),¹⁷⁰⁻¹⁷² PK phenolic glycolipids (**15**),^{173, 174} and dimycocerosate esters is required for virulence.¹⁷⁵⁻¹⁷⁸ Additionally, a cyclopropyl glycolipid modification of the cell wall has been shown to be critical for induction of host hyperinflammation during initial infection.¹⁷⁹

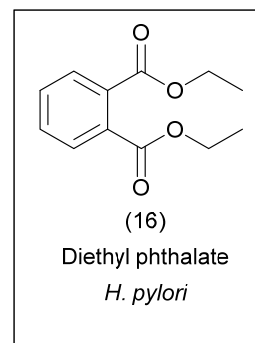


2.3 GASTRO-INTESTINAL TRACT

The gastro-intestinal (GI) tract represents the densest microbial ecosystem in the human host.^{180, 181} Composed of the stomach and intestines, the composition of the microbial community of the GI tract is dependent upon anatomical location with community densities ranging from 10^3 cells/mL at the outlet of the stomach to approximately 10^{12} cells/mL in the colon. The members of the GI microbiota play important and beneficial roles in digestion, vitamin biosynthesis, immune regulation, and pathogen exclusion.¹⁸² Disease can result when GI microbial community homeostasis is disturbed due to stress, infection, poor nutrition, use of antibiotics, or other external factors.¹⁸³ A number of studies have correlated compositional shifts in the microbial community of fecal samples to diseases such as obesity, inflammatory bowel disease (IBD), and Crohn's disease.^{8, 184, 185} The study of the chemical interchange occurring between the gut microbiota and the host is a rapidly advancing field with a specific focus on understanding the role of the healthy microbiota in managing pathogenic microbes and the modulation of host response and behavior by microbial metabolites.

2.3.1 STOMACH

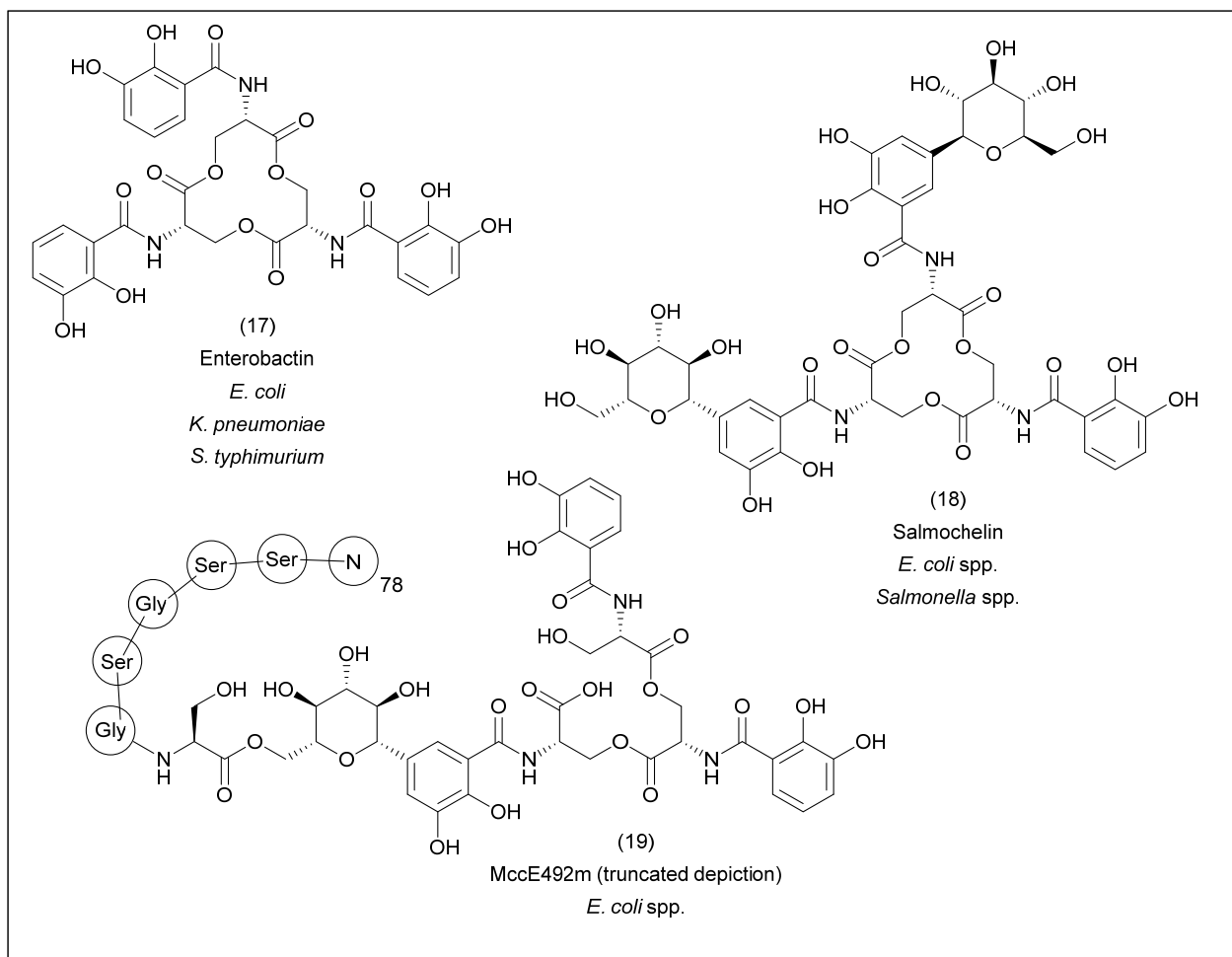
Like the lower respiratory tract, the human stomach was once thought to be sterile. Due to sequencing efforts, we now know that a number of microbes have adapted to exist in this harsh, acidic environment including *Helicobacter*, *Streptococcus*, *Staphylococcus*, *Peptostreptococcus*, *Veillonella*, *Bacteroides*, *Fusobacterium*, *Bacillus*, *Lactobacillus*, *Deinococcus*, and *Neisseria*, among others.^{186, 187} The contribution to the stomach microbiota from transient colonization by microbiota from the oral and nasal cavities is unknown. The most common and well established example of a stomach microflora is *Helicobacter pylori*.¹⁸⁸ *H. pylori* has been associated with a number of diseases including gastritis, hypochlorhydria, duodenal ulcers, and gastric cancer.¹⁸⁹ The discovery of *H. pylori* as a disease-causing agent was awarded the Nobel Prize in Medicine in 2005. *H. pylori* has been postulated to produce several metabolites which interact with the human host including diethyl phthalate (**16**), which induces monocyte migration, *N*-nitrosamines that have been suggested to contribute to gastric cancer (although this is under debate), and a peptidoglycan which induces NF- κ B activation.^{190-192, 193} In general, the role of small molecules in the gastric cavity remains virtually unexplored.



2.3.2 INTESTINAL TRACT

The microbiota of the intestinal tract is currently one of the most intense focal points of microbiome research. We now know that more than 1,000 different species are present in human gut with Bacteroidetes and Firmicutes as the most dominant phyla.¹⁹⁴ Although each location of the intestine is anatomically distinct, exploration of the intestinal microbiota has not been studied at high spatial resolution. The majority of microbiome community studies have focused on characterizing the community composition of fecal content in relation to diseases of the gut. Information about the microbial constituents of the entire intestine has been extrapolated primarily based on analysis of fecal matter. However, this is analogous to identifying the source of a river by studying the ecology of the banks of its delta. It is clear that a more sophisticated, spatially-resolved study of the intestinal tract is critical for the advancement of this field.

Mounting evidence suggests that the gut microbiota strongly influences human health through both primary and secondary metabolism including the modification of bile acids¹⁹⁵⁻¹⁹⁷ and amino acids,¹⁹⁸ carbohydrate fermentation to generate SCFAs,^{29-31, 199} and the production of virulence factors such as siderophores, antimicrobial peptides, and glycolipids.^{200, 201} SCFAs and siderophores are the classical examples of microbial metabolites involved in microbe-host interactions and have been thoroughly reviewed.²⁷⁻³¹ One of the most interesting interactions by intestinal microbiota is the competition for iron. A number of pathogenic microbes including *Escherichia coli*, *Klebsiella pneumoniae*, and *Salmonella typhimurium* produce structurally related siderophores. *E. coli*-produced enterobactin (**17**) is quickly rendered ineffective through sequestration by the mammalian protein siderocalin and is partitioned into host lipid bilayers due to its hydrophobicity.²⁸ To circumvent these limitations, certain pathogenic strains of *E. coli* and *Salmonella* have adapted NRP siderophore biosynthesis by incorporating genes for glycosylation of the enterobactin structure resulting in the production of the salmochelins (**18**). In addition to siderophores, these same organisms produce ribosomally-synthesized and post-translationally modified peptides (RiPPs) which have been shown to have antibiotic properties. For example, probiotic strains of *E. coli* produce microcins which exert antibacterial activity against closely related enteropathogenic species.^{202, 203} Some of these microcins are modified at the C-terminus with linearized monoglycosylated enterobactin (**19**) which target siderophore transporters of competing microbes resulting in a “Trojan horse” type antibiotic.²⁰⁴⁻²⁰⁶



Perhaps most illustrative of the opportunities and challenges associated with characterizing microbiome-derived natural products is the emerging structural elucidation of the metabolite colibactin. Colibactin is a structurally uncharacterized PK-NRP that is thought to arise from a prodrug called precolibactin which has also not been fully structurally elucidated.^{32, 33, 207} Over ten years ago, the colibactin encoding hybrid PKS-NRPS BGC (*clb*) was identified owing to the correlation of its presence in *E. coli* strains to the induction of double stranded DNA breaks in human cell lines.²⁰⁸ This BGC was found to be widely distributed among the B2 phylogenetic group comprised of both commensal and extra-intestinal pathogenic strains. 55 intestinal pathogenic strains of *E. coli*, 97 extra-intestinal pathogenic strains, and 32 fecal strains from healthy individuals were analyzed by PCR-based analyses for the presence of the *clb* BGC. Although absent in intestinal strains, *clb* was identified in 53% and 34% of the extra-intestinal and fecal strains, respectively. The potential link between colibactin and colitis-associated colorectal cancer was first shown in 2012.²⁰⁹ In this study, changes in colonic microbiota of mice models was shown to be associated with inflammation of the colon. This led to the hypothesis that in addition to host-associated inflammation, bacterial factors associated with *clb* gene locus may be important for development of colitis-associated colorectal cancer. In patient samples, 20.8% of the healthy cohort (non-IBD and non-colorectal cancer volunteers), 40% of patients with IBD, and 66.7% patients with colorectal cancer were shown to harbor *E. coli* containing *clb* gene cluster. Thus, it has been proposed that colitis-induced inflammation of the colon creates an environment that promotes carcinogenesis by both host and microbe-associated factors. The intriguing biological activity associated with the presence of the *clb* BGC spurred interest in characterizing the product of gene cluster by various groups. The elucidation of the structure of colibactin has been hindered by both the complexity of the *clb* gene cluster and likely

instability of its product colibactin. Therefore, extensive efforts have been undertaken to structurally characterize the pro-molecule, resulting in various proposed structures of precolibactin (Figure 4A). Several potentially important structural elements in different proposed precolibactins include an *N*-acylated-D-asparagine prodrug conferring moiety which is hypothesized to be cleaved from the active colibactin by a periplasmic protease,^{210, 211} a cyclopropane containing ring system suggested to be the warhead group responsible for crosslinking DNA,²¹²⁻²¹⁷ and the presence of a thiazole, thiazolanyl-thiazole (predicted structure), or bithiazole motif possibly involved in non-covalent interactions with DNA.²¹⁴⁻²¹⁷ A recent study described two additional precolibactins, precolibactin-629 and precolibactin-886. Precolibactin-866 results from the incorporation of an aminomalonyl unit which yields a unique macrocycle.²¹⁸ The discovery of precolibactin-866 led to a proposed biosynthetic scheme as a plausible explanation for the varied chemical framework of isolated precolibactins. In this scheme, the type II thioesterase ClbQ mediates early off-loading of biosynthetic intermediates yielding the described precolibactins.

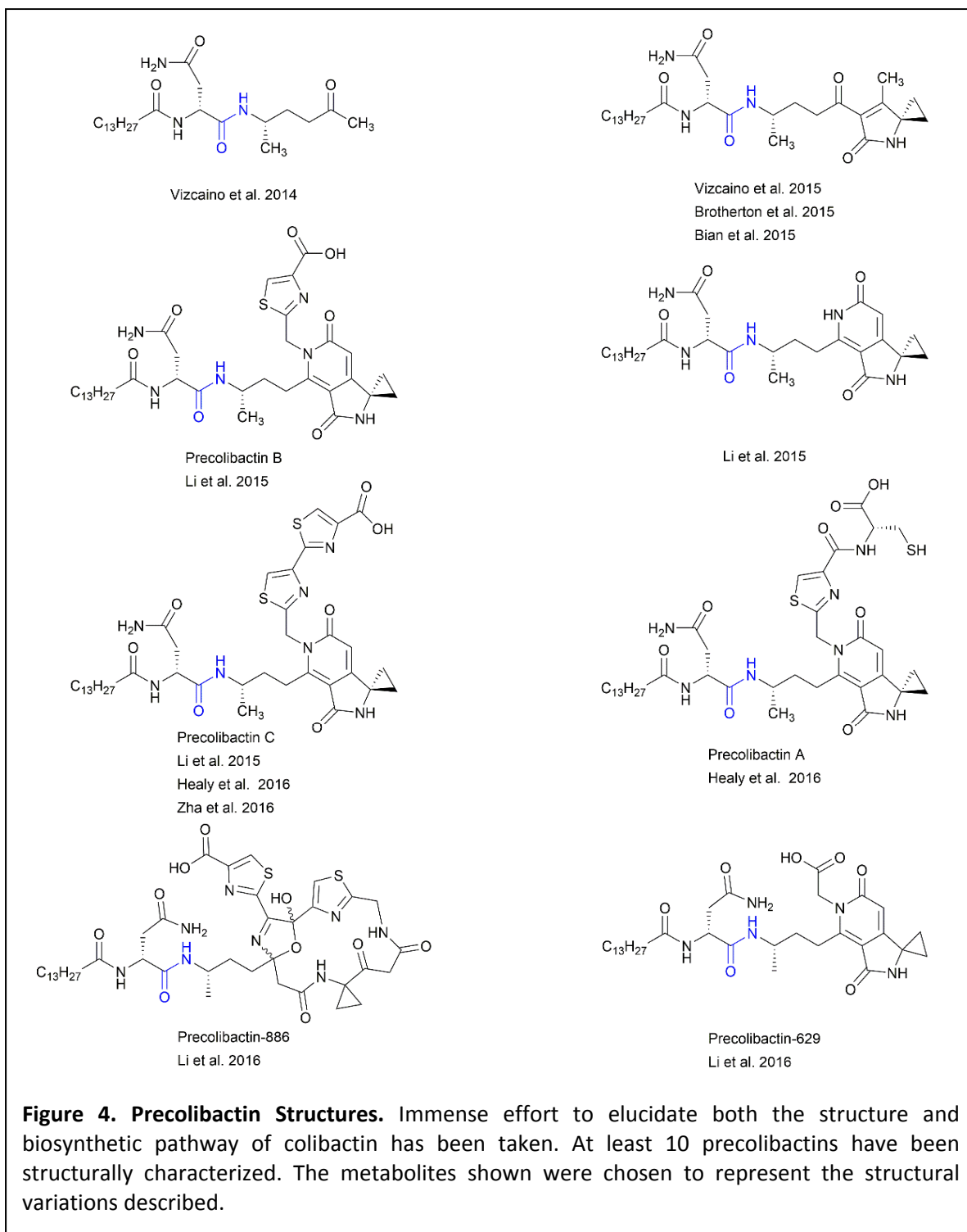
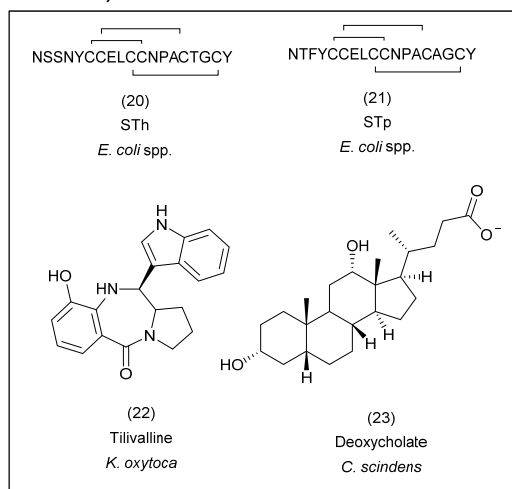


Figure 4. Precolibactin Structures. Immense effort to elucidate both the structure and biosynthetic pathway of colibactin has been taken. At least 10 precolibactins have been structurally characterized. The metabolites shown were chosen to represent the structural variations described.

In contrast to colibactin, the *E. coli* heat-stable enterotoxins STh **(20)** and STp **(21)**, 19 and 18-amino acid RiPPs, respectively, have been suggested to protect against colorectal cancer by suppressing proliferation of cancer cells by increasing intracellular cyclic guanosine monophosphate (cGMP) through a guanylyl cyclase C-mediated signaling cascade.^{219, 220} These enterotoxins interact with the same signaling cascade in intestinal epithelial cells, leading to diarrheal disease. The BGCs for structurally similar peptides have also been identified in *K. pneumoniae*, *Yersinia enterocolitica*, and *Vibrio cholerae*.²²¹⁻²²³ Other metabolites have also been associated with GI diseases. For example, *Klebsiella*-mediated, antibiotic-associated hemorrhagic colitis has been linked to the NRP tilivalline **(22)**.^{224, 225} Tilivalline was first isolated from *Klebsiella oxytoca* in the early 1980s and was shown to have toxicity against mouse leukemia L1210 cells.^{226, 227} More recently, the pathological symptoms of tilivalline-mediated damage in the colon were more pronounced in animals treated with antibiotics and infected with *K. oxytoca* as compared to control animals. This work implicated tilivalline in antibiotic-mediated colitis. Tilivalline has been shown to induce caspase-3-dependent apoptosis in cultured human epithelial cells, which has also been observed in the pathology of antibiotic-associated hemorrhagic colitis.^{224, 225}



A promising application of microbiome research to treat GI disorders is the utilization of fecal transplants for *Clostridium difficile* infections.²²⁸⁻²³⁰ *C. difficile* infections of the colon are thought to arise from alteration of the commensal gut community by treatment with antibiotics.^{231, 232} It has been hypothesized that the susceptibility of patients to *C. difficile* infections post-antibiotic treatment is due to clearance of beneficial microbes capable of preventing *C. difficile* colonization. Recent studies have shed light on potential mechanisms, including the possible involvement of secondary bile acids produced by commensal microbes.²³³⁻²³⁵ Through correlative analysis of microbial abundance and resistance to infection in both mice and humans, *Clostridium scindens* was identified and validated as an inhibitory bacterium of *C. difficile*.²³³ This activity was later associated to the presence of bile acid inducible (*bai*) operon of genes required for secondary bile acid production by *C. scindens*. While primary bile acids are required for the germination of *C. difficile* spores, secondary bile acids have been shown to act as growth inhibitors of vegetative cells both *in vitro* and in mouse models of *C. difficile* infection.^{236, 237} It is hypothesized that although the host-derived primary bile acid taurocholate promotes germination of *C. difficile* spores, the secondary bile acid deoxycholate **(23)** produced by the commensal bacterium *C. scindens* inhibits growth of *C. difficile* vegetative cells, thereby preventing colonization. It is postulated that when commensals such as *C. scindens* are killed by antibiotic treatment, secondary bile acids are not available to prevent growth of vegetative *C. difficile* cells which then leads to infection. Further work will be required to validate these findings and explore the use of secondary bile acids as potential therapeutic treatments.

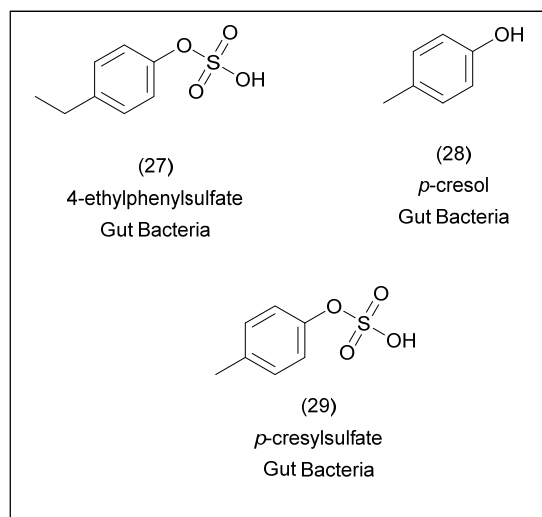
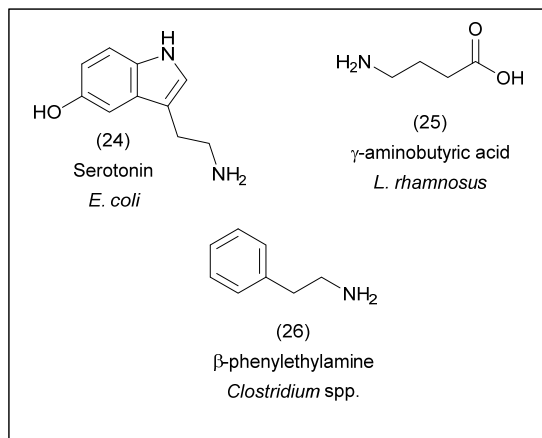
2.3.3 THE GUT-BRAIN AXIS

One of the most intriguing areas of microbiome research is the role of microbial metabolites, particularly from the gut microbiome, in mediating mental health and neurological diseases ranging from clinical depression to autism.²³⁸⁻²⁴¹ This bidirectional communication system between the gastro-intestinal tract and the central nervous system (CNS) is commonly referred to as the gut-brain axis.²³⁸ Amino acid

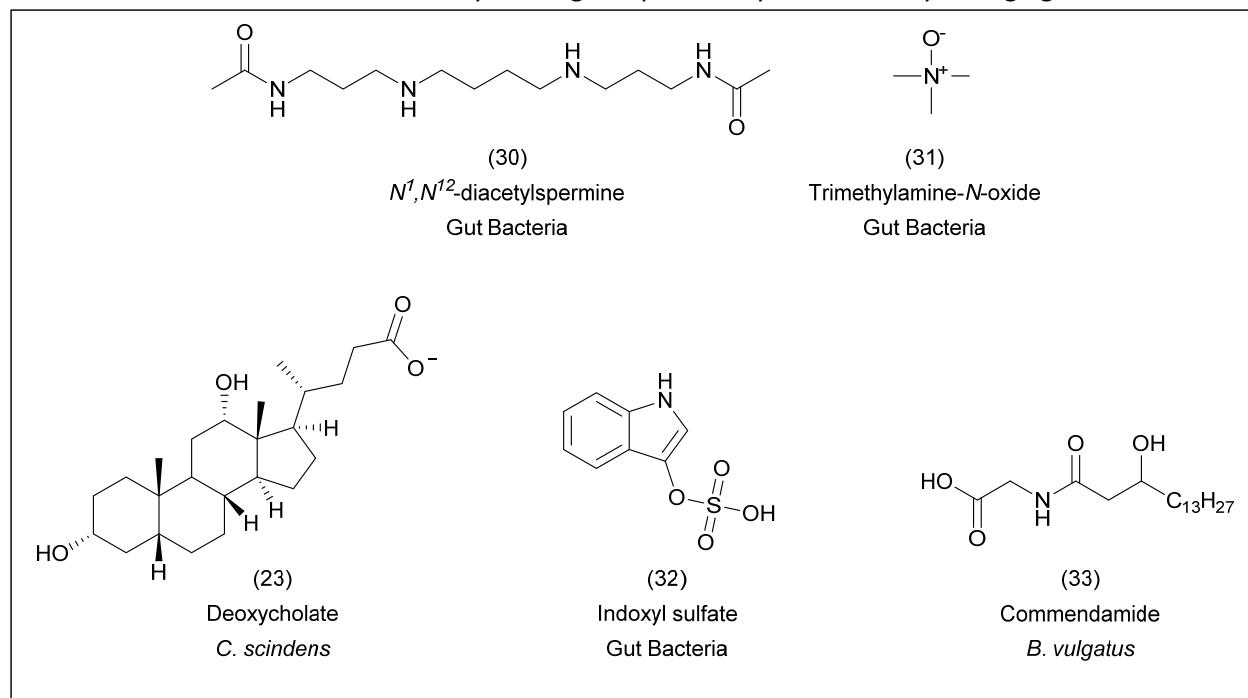
metabolism by gut microbiota generates neurotransmitters such as serotonin **(24)**,²⁴² γ -aminobutyric acid **(25)**²⁴³ and β -phenylethylamine **(26)**.^{244, 245} In healthy individuals, host metabolism is positively influenced by microbe produced SCFAs which promote energy homeostasis, regulate immune response, and affect peripheral nervous system function. SCFAs have been extensively reviewed.^{29-31, 246, 247}

A number of possible links between the gut microbiota and disease have been identified along the gut-brain axis. Although controversial, autism spectrum disorders (ASD) is among the most notable. ASD is generally characterized by stereotypic behaviors such as impairment of communication and social anxieties. Intriguingly, ASD is often accompanied by various disorders of the GI tract including, most commonly, chronic constipation, diarrhea, and abdominal pain.²⁴⁸⁻²⁵⁰ The prevalence of GI disorders in people suffering from ASD has been correlated to more severe autism-related behaviors, several of which have been recapitulated in mouse models.²⁵¹ In ASD-model mice, high serum levels of the small aromatic molecule 4-ethylphenylsulfate **(27, 4EPS)** were observed when compared to control mice.²⁵² Subsequent injection of 4EPS into healthy mice was sufficient to induce behaviors similar to the ASD-model mice. In pediatric patients suffering from ASD, elevated levels of urinary *p*-cresol **(28)** and its conjugate derivative *p*-cresylsulfate **(29)**, a metabolite structurally related to 4EPS, were measured.^{253, 254} These metabolites are primarily produced by the gut microbiota through fermentation of amino acids in the large intestine. In the murine models, autism-related behaviors were corrected by treatment with *Bacteroides fragilis*.²⁵² Mice treated with *B. fragilis* had similar levels of 4EPS when compared to healthy control mice. This suggests that members of the healthy microbiota, and their metabolites, may potentially be used as probiotic therapies to mitigate autism-related behaviors.

Active research programs are investigating direct links between microbial metabolism in the gut and modulation of the immune system.²⁵⁵ It is anticipated that these research areas will shed light on how the human gut microbiome affects distant organ systems. While we have focused on a specific example of gut metabolites affecting the nervous system, other microbe-derived molecules such as *N*¹,*N*¹²-diacetylspermine **(30)**, trimethylamine-*N*-oxide **(31)**, deoxycholate **(23)**, and indoxyl sulfate **(32)** have been implicated in colorectal cancer, cardiovascular disease, hepatocellular carcinoma, and chronic kidney disease, respectively.²⁵⁶⁻²⁵⁹ Untargeted metabolomics of plasma extracts from germ-free and conventional mice suggests that the gut microbiota significantly affects the level of 10% of all metabolites detected in systemic circulation.²⁶⁰ Bioactivity-guided approaches have also been utilized to identify microbial metabolites which may have interesting biological roles in host-microbe interactions. For example, in an effort to identify bacterial effectors which act as environmental stimuli to activate the human transcriptional factor NF- κ B, functional metagenomics workflows were applied. Metagenomic DNA was cloned into a *E. coli* based cosmid library from stool samples of three phenotypically distinct patients including a healthy volunteer, a patient with ulcerative colitis, and a patient with Crohn's

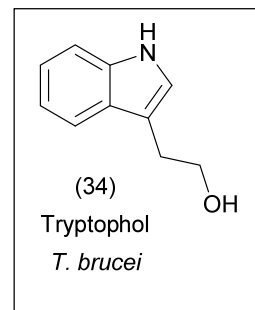


disease.²⁶¹ The sterile spent culture broth of each clone was screened against a human fluorescent reporter cell line for activation of NF- κ B. Of 26 effector genes identified, an effector cosmid encoding for commendamide (**33**, *N*-acyl-3-hydroxypalmitoyl-glycine) was recovered from all three patients. Commendamide and its minor analogues (with variation in length and saturation of the acyl chain) are structurally similar to mammalian *N*-acyl-amides that play a role as signaling molecules through activation of G-protein-coupled receptors (GPCRs). *In vitro* screening of commendamide for activation of a library of 242 GPCRs revealed activation of GPCR132/G2A receptor. G2A-knock out mice were previously shown to be susceptible to atherosclerosis and developed a late-onset stage autoimmune disorder.²⁶² The functional role of commendamide in affecting host immunity requires further testing in appropriate *in vivo* models. It is also important to note that microbial metabolite production in organs other than the GI tract may also have effects elsewhere in the body. For instance, secondary metabolites from *P. aeruginosa* chronic lung infections have been measured in the plasma of CF patients.¹⁴⁷ Unraveling the systemic roles of microbial metabolites is an extremely exciting and potentially revolutionary emerging field of research.



2.4 NERVOUS SYSTEM

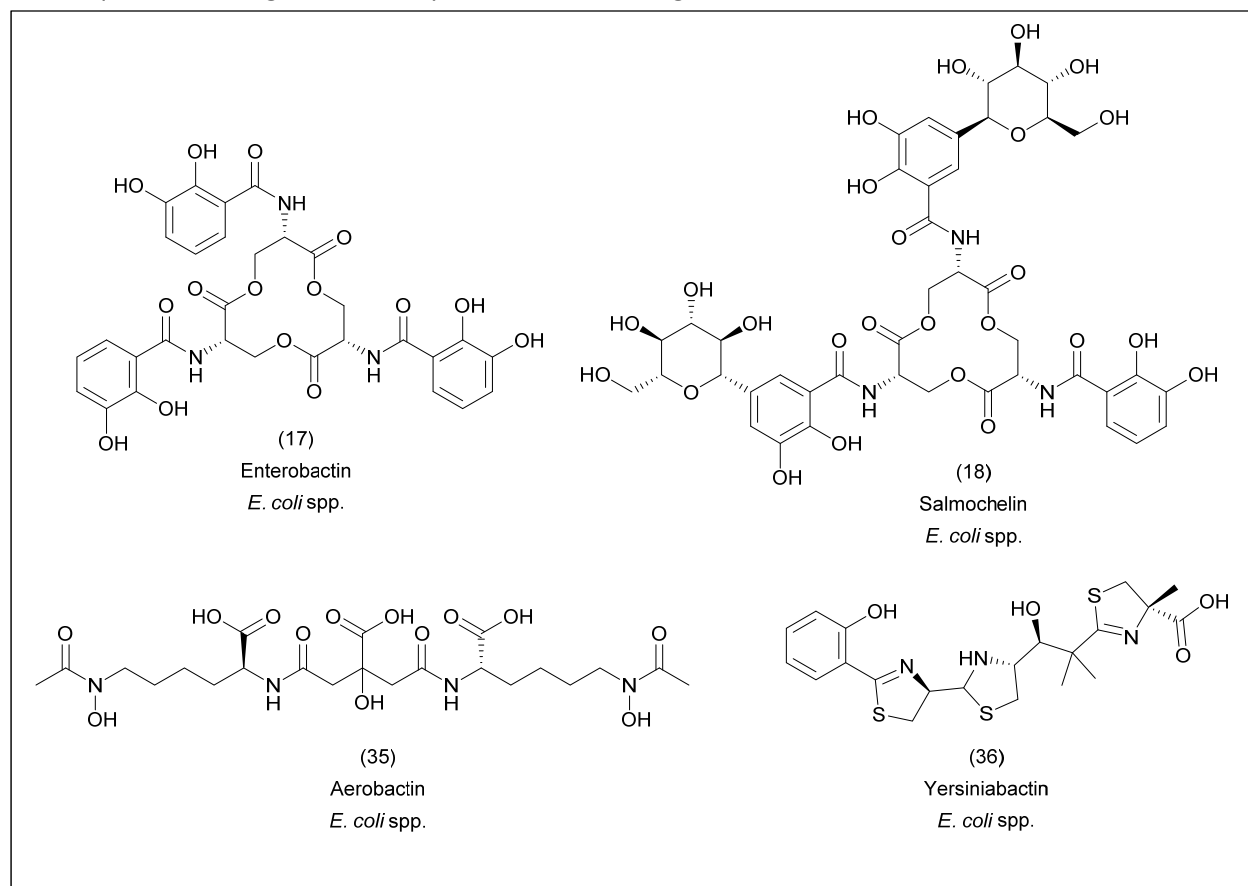
The CNS is comprised of the brain and spinal cord. The CNS is protected by the blood brain barrier, which is lined by endothelial cells that prevent the passage of microbes and large hydrophilic molecules. However, some parasites and microbes (including *Trypanosoma brucei* and *L. monocytogenes*, respectively) express proteins that allow them to cross this barrier which is critical to their infections of the CNS.²⁶³⁻²⁶⁹ Infection of the CNS with *T. brucei* causes the disease trypanosomiasis. It is hypothesized that tryptophan-derived 3-indole ethanol (**34**, tryptophol) is responsible for the induction of sleep from which trypanosomiasis derives its common name of sleeping sickness.²⁷⁰⁻²⁷² Listeriosis is a fatal disease where ingestion of the gram-positive bacterium *L. monocytogenes* by immunocompromised individuals leads to infection of the CNS, resulting in meningitis and eventual death.²⁷³ The hemolytic activity of *L. monocytogenes* is closely correlated to its pathogenicity. Until recently, the hemolytic activity of *L. monocytogenes* was wholly attributed to the protein toxin listeriolysin O.²⁷⁴ *In silico* analysis of the *L.*



monocytogenes genome for the SLS BGC revealed the closely related peptide listeriolysin S.²⁷⁵ However, like SLS, the structure of listeriolysin S remains uncharacterized.

2.5 URO-GENITAL SYSTEM

The uro-genital system consists of the urinary and genital tracts. Like several other organ systems, the urinary tract was assumed to be a sterile environment. However, recent sequencing studies indicate that it harbors a unique microbiota.²⁷⁶⁻²⁷⁹ While the urinary tract is normally colonized by *Lactobacillus* and *Streptococcus* species, a shift in community composition has been implicated in urinary tract infections (UTIs) through correlative analysis. UTIs are primarily associated with infection by the Gram-negative bacterium *E. coli*, but can also be caused by both Gram-negative and -positive bacteria.²⁸⁰⁻²⁸³ The role of microbial small molecules in most UTIs remains largely uninvestigated. However, siderophore production may be an important role in *E. coli* driven UTIs.²⁸⁴⁻²⁸⁷ UTI causing *E. coli* strains were widely believed to originate from the GI tract. In several studies, researchers compared siderophore production between strains isolated from the urinary tract, fecal samples, and extra-urinary sites.^{285, 287} Two studies revealed different results for the most abundant siderophores produced by *E. coli* strains isolated from UTIs. In one study, using phenotypic assays, the proportion of aerobactin (**35**) producers was significantly higher in UTI and extra-urinary isolates.²⁸⁷ In a second study, mass spectrometry (MS) based analysis showed that both UTI and rectal strains of *E. coli* produce enterobactin (**17**) with a preferential expression of yersiniabactin (**36**) and salmochelin (**18**) in UTI isolates.²⁸⁵ Therefore, the quantitative contribution of iron acquisition during infection requires further investigation both *in vitro* and *in vivo*.



The lower genital tract of both males and females is inhabited by a dynamic microbial community. Normal vaginal flora is dominated by several *Lactobacillus* species, but community structure varies with

metabolism, biotransformation of human metabolites and xenobiotics, and well-characterized secondary metabolites including SCFAs.²⁹¹⁻²⁹⁵ The detection of microbial natural products in human samples is hindered by the presence of highly abundant host metabolites. Therefore, the development of robust, reproducible methods for metabolite extraction and analyte separation on small sample amounts to improve the detection of microbial metabolites is critical to the success of microbiome-based natural product research. It is important to note that metabolomics, like sequencing workflows, suffers from a lack of standardization of analytical and bioinformatics methods which makes harnessing available data and cross-comparison between datasets extremely difficult. Modern tools and resources for metabolomics analysis were recently reviewed.²⁹⁶

Untargeted methods provide detection of a large number of metabolites, but prioritizing key metabolites is a formidable challenge.²⁹⁷ One method to identify compounds of interest is applying self-organizing maps (SOMs). SOMs aid in the identification of metabolites that differentiate samples using multivariate statistics to pinpoint the most abundant changes between case and control for purification and structural elucidation.²⁹⁸⁻³⁰⁰ Correlation detection strategies can also be applied to untargeted metabolomics analysis in order to correlate the presence of metabolites with genotypic and phenotypic data, although these methods are not yet widely used in guiding workflows for metabolite characterization.³⁰¹⁻³⁰⁵ For example, co-occurrence methods have been utilized to reveal relationships between the microbiome and metabolome of the human intestinal mucosa,³⁰⁶ while correlations of spatial distributions of the skin microbiome and metabolome highlighted the role of skin microbes in the degradation of beauty products.³⁸ However, integration of multi-omics datasets is not trivial and has been primarily utilized for microbial community profiling.^{307, 308}

Metabolomics analysis can also be combined with bioactivity-guided and candidate molecule workflows. Bioactivity-guided screening utilizes a collection of microbes, extracts, or cosmid libraries (generated from metagenomics DNA) to test for *in vitro* activity against human pathogens or activation of human signaling pathways.^{117, 261} Identification of a metabolite of interest is achieved by utilizing analytical methods including differential metabolomics,² imaging mass spectrometry (IMS),¹⁶² or by disrupting the biosynthetic genes through random mutagenesis combined with high-throughput phenotypic screening for loss of function.²⁶¹ Data can be integrated to group molecules of similar bioactivity to generate compound activity maps.^{309, 310} This method yields previously uncharacterized natural products from complex samples and probable biological functions.

Orthogonal to bioactivity-guided methods, candidate molecule workflows harness characterized biosynthetic pathways to mine (meta)genomic data to identify putative BGCs and direct metabolite purification workflows.⁵ The Minimal Information about a Biosynthetic Gene cluster (MIBig) consortia aims to develop a public repository of specialized metabolite BGCs to maintain community knowledge of molecule biosynthesis to aid in the analysis of sequencing data from any microbial system, including humans.³¹¹ Growing out of antecedent tools for comparative analysis of genetic information such as BLAST,³¹² HMMER,³¹³ and algorithms for the detection of specific classes of gene cluster families (many of which were recently reviewed³¹⁴), ClusterFinder has been successfully used to identify putative gene clusters from microbiome data and led to the characterization of metabolites.^{36, 315} Part of the antiSMASH platform,³¹⁶⁻³¹⁸ ClusterFinder is a more general probabilistic algorithm used to identify BGCs of broad structural classes ranging from polysaccharides to PKs and peptides. AntiSMASH 3.0 also has algorithms to compare orphan gene clusters to a manually curated database of characterized gene clusters (ClusterBlast) and to predict substrate specificity of active sites with some metabolite structural prediction, thus reducing the manual curation necessary to link metabolites and their BGCs.³¹⁸⁻³²⁰ Candidate molecule methods can be combined with bioactivity data to correlate the presence of a specific BGC to bioactivity as shown with the identification of the *clb* and *bai* gene clusters,^{32, 233} with other -omics datasets as was performed for transcriptional analysis of the metabolite lactocillin in the oral cavity,⁵ or with tandem mass spectrometry (MS/MS) in order to link metabolites to gene clusters using

peptidogenomics³²¹ and glycogenomics³²². Peptidogenomics has recently been automated through the Pep2Path platform.³²³

One of the major challenges facing natural products researchers is the annotation of metabolites. This often occurs because structural information of characterized metabolites, including high resolution MS data and MS/MS data, is buried within manuscripts and, in a growing number of cases, large supplemental information documents. To bypass these issues and coordinate global metabolite assignments, users can contribute to the Global Natural Products Social Molecular Networking (GNPS, gnps.ucsd.edu).³²⁴ GNPS is a public, online platform for analysis of MS/MS data, where similarities in fragmentation patterns function as proxy for structural similarities. This concept can be harnessed by users to rapidly dereplicate their data against spectra generated from characterized metabolites in both public and user-curated MS/MS databases and to generate molecular networks which can be used to visualize the chemical space of samples. Judicious application of the mapping features available in GNPS allows researchers to quickly pinpoint conditions where a metabolite of interest is produced and identify the producing organism. A step-by-step procedure for secondary metabolic pathway targeted metabolomics, including molecular networking, utilized for the elucidation of a preliminary structure of a precolibactin has recently been published.^{216, 325} Other methods including CSI:FingerID combine fragmentation tree analysis and machine learning to search structural databases such as PubChem and ChemSpider to aid in metabolite identification.^{326, 327}

3.2 CULTIVATION, METABOLITE PRODUCTION & VALIDATION

Despite recent success characterizing single microbiome-derived metabolites such as lugdunin and lactocillin,^{5, 117} it is likely that many compounds of interest are under-investigated due to limitations of traditional *in vitro* culturing techniques. Cultivating the uncultivable will allow researchers the opportunity to scale for natural product isolation as well as test interesting hypotheses. A distinct advantage of model systems to induce metabolite production is their inherent reproducibility. However, even if a member of the microbiota can be cultured in a model system, it does not necessitate that the metabolite of interest will be produced. The selective pressures and nutritional availability within a microbiome environment are distinct from the environment of a microbe cultivated in model systems. Therefore, regardless of how similar the community structure may be as measured by sequencing-based analysis, the metabolic output from a model system may not reflect that from the original sample. It is incredibly important to validate the metabolite produced in a culture system is identical to the metabolite originally detected. This validation is most easily accomplished by comparing high resolution MS data, MS/MS spectra, and retention time of the purified compound to the original samples under identical acquisition parameters.

Conventional cultivation of members of the microbiota is identical to the steps used in isolation of microbes from marine and terrestrial environments including agar based culturing of mixed communities to identify single colonies of interest, re-streaking isolates to purity, typing by 16S and archiving.³²⁸ The isolation of microbes from the microbiota is enhanced by the development of environment specific media.³²⁹ For example, YCFA medium and gut microbiota medium (GMM) have been developed for isolation of human gut microbes.^{330, 331} Sequencing-based comparisons were used to validate that the isolates recovered had similar phylogenetic diversity as measured for the community.³²⁸ In CF research, this type of workflow was coined culture enriched molecular profiling and was used to increase metagenomics coverage, but has been also utilized for the gut and can be easily harnessed for microbial metabolite isolation.³³²⁻³³⁴ Extensions of conventional cultivation include culturing single cells in multi-well plate format to create personalized microbiota collections,³³¹ parallel cultivation in hundreds of different conditions,³³⁵ microscale apparatuses,³³⁶⁻³³⁸ culturing with “helper” organisms,^{339, 340} and using sequencing information to access the nutritional needs of isolates to optimize growth conditions.³²⁹ Efforts are also underway to recapitulate entire microbiome communities. A recent review nicely

summarizes the available *in vivo*, *in vitro*, and *ex vivo* models under development to investigate the microbiome,³⁴¹ although newer techniques are continuously being developed.³⁴² While primarily intended to investigate community structure, many of the *in vitro* systems have the potential to be harnessed for natural products research.

Beyond cultivation, molecular biology methods have the potential to enhance the discovery of microbiome-derived natural products. Any method currently applied to natural product discovery has applicability to the microbiome. For instance, similar to how BGCs have been cloned from environmental DNA, microbiome BGCs can be cloned from metagenomics DNA and expressed in bacterial or fungal hosts.²⁶¹ Additionally, rational genetic modification to remove, replace, or enhance regulators within BGCs hypothesized to produce metabolites of interest can be used to increase production yields and to circumvent arduous purification processes from complex cultures as was recently illustrated during the characterization of lugdunin.^{117, 343}

4. CONCLUSIONS AND PERSPECTIVES

One of the greatest challenges and opportunities for natural products researchers interested in microbiome work is characterizing the biological roles for microbiome-derived natural products.³⁴⁴ Although there is a great interest in elucidating the roles of such molecules *in vivo*, in general, members of the microbiota are under-investigated at the chemical level. From a discovery perspective, the human microbiome (including both disease-causing microbes and those that enhance human health) is one of the next frontiers for natural products. Analyses of sequencing data strongly suggest that microbial community dysbiosis may cause a wide range of human diseases including some autoimmune and neurological disorders. With increasing public and industrial interest in probiotic microbes and metabolites, it is apparent that the discoveries of metabolites unique to healthy individuals will lead to new therapeutics for chronic microbe-driven diseases. As the few examples highlighted in this review show, researchers have begun to explore the role of microbial natural products from bacteria in human disease. However, this avenue remains largely unexplored for fungal diseases. The available workflows for microbiome analyses exist on a continuum from simplistic *in vitro* co-culture to complex *in vivo* animal models.^{191, 331} While each workflow and analysis has limitations which should be considered during experimental set-up and addressed in manuscripts, every method contributes to our understanding of the microbiome. These experiments, together with bioinformatics-based multi-omics and human cell culture-based studies, will elucidate the roles of specific metabolites in biological context. As cultivation techniques, analytical methods, and bioinformatics approaches driven by large datasets improve and become integrated with each other, we as a community can start to unravel the role of microbiome-derived natural products in human health and disease.

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