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## Biochemical features of beneficial microbes: foundations for therapeutic microbiology

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### BACKGROUND

The gastrointestinal tract (GIT) is a diverse and complex ecosystem shaped by continual interactions between host cells, nutrients, and the gut microbiota. The gut microbiome is estimated to contain approximately  $10^{13}$  bacterial cells and is dominated by the major phyla Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia (1, 2). Early colonizers of the GIT include bifidobacteria from the phylum Actinobacteria. These commensal microbes colonize immediately after birth and are speculated to prime the GIT and influence the gut-brain-axis (3–5). The infant microbiota is considered to be relatively unstable. Despite dramatic changes in the microbiome structure during early life, the gut microbiota increases in diversity and stability over the first three years of life (6). Following this initial establishment, the microbiome of children are generally enriched in *Bifidobacterium* spp., *Faecalibacterium* spp., and *Lachnospiraceae* compared to adults (7–9). During adulthood, the gut microbiome is considered to be stable and is dominated by the phyla Firmicutes and Bacteroidetes. While bacterial populations vary between individuals, the fecal microbiota of adults is highly stable through time (6). This stability is maintained until older age (> 65), when the microbiome stability and function begins to decline (10, 11).

A mutualistic relationship exists between the gut microbiota and the host. Commensal microbes metabolize indigestible food components, produce vitamins, prime the immune system, modulate the enteric nervous system (ENS) and potentially the central nervous system (CNS), contribute to intestinal architecture development, protect against colonization of opportunistic pathogens and more for the benefit of the host (12). Conversely, the host generates a stable ecosystem for the gut microbiota, providing nutrients and ecological niches. The gut selects resident commensal microbes based on their capacity to adapt to and colonize within the host.

Select commensal bacteria are able to positively alter the gut microbiome and/or host. Many of these commensal groups harbor strains that are considered to be probiotics. Probiotics are defined as microbes which confer advantages to the host and have not been implicated to confer human disease (World Health Organization) and these well characterized organisms

are known to produce a number of beneficial products which promote host health. Certain strains of *Lactobacillus*, *Bifidobacterium*, *Escherichia coli*, *Bacillus*, and *Propionibacterium* spp. have been classically used as probiotics. The value of probiotics has been supported by multiple clinical trials, which demonstrate improvements for patients receiving probiotics in response to multiple pathologies including diarrhea, inflammatory bowel disease (IBD), allergic reactions, viral infections, cancer, and others. However, a number of commensal bacteria that are not defined as probiotics are also known to produce bioactive metabolites with health promoting effects. These commensals include *Ruminococcus*, *Eubacterium*, *Roseburia*, *Faecalibacterium*, and *Akkermansia* spp. This review seeks to identify major pathways used by commensal bacteria to beneficially modulate the host, with the caveat that the majority of studies that have identified specific mechanisms tend to focus on probiotic strains.

Commensal and beneficial microbes have been hypothesized to promote health in a variety of ways: (1) exclusion of pathogens, (2) immunomodulation, and (3) enhancement of the intestinal barrier (Figure 1). Several mechanisms have been proposed to explain the beneficial effects of observed by key commensal microbes. One mechanism of great interest is the secretion of molecules which are capable of altering both the host and the microbiome. Bacterial metabolites can be generated as intermediate or end products of bacterial metabolism (Figure 2). Secreted products can target the microbiome by acting as signaling molecules for intra-species/strain communication (quorum sensing), altering the intestinal environment, and targeting certain microbes to control microbiota composition (antimicrobials). These molecules include lactic acid, hydrogen peroxide, and bacteriocins (Figure 3). Likewise, bacterial molecules can modify the host. Metabolites can participate in functional complementation to the host metabolic capabilities, immune regulation and improvement of intestinal barrier function. These factors include small molecule metabolites such as histamine, vitamins, short chain fatty acids, polyunsaturated fatty acids, serpins, lactocepins, and secreted proteins (Figure 3). Of note, the metabolic potential of microbes varies greatly among species, and even among strains of the same species. Several studies have found that microbe-driven protective effects depend on the strains used (13–15). These differences could be due in part to the diversity of secreted metabolites. As a result, it is important to characterize the secreted products of individual microbes in order to identify which strains should be used for a specific disorder. Herein we describe the most prominent examples of well characterized secreted products and their documented effects on the microbiome and/or host function.

## Signaling Compounds

### 1. Bacterial-host signaling compounds

**Biogenic Amine Neuromodulators**—Select microbes are known to produce biologically active compounds that are associated with mammalian neurotransmission and behave as neuroactive compounds. These molecules include histamine, gamma-aminobutyric acid (GABA), and tryptophan metabolites. Microbe-generated neuromodulators in the intestinal lumen likely regulate signaling within the enteric nervous system (ENS) and ultimately effect the “gut-brain axis”. Although bacterial products such as

acetate (16) and peptidoglycan (17) have been found in the central nervous system (CNS), these neuroactive compounds primarily affect the enteric part of the peripheral nervous system and are thought to act in a local manner.

Biogenic amines (BAs) are low molecular organic bases generated through decarboxylation of specific free amino acids, reductive amination of aldehydes and ketones, transamination or hydrolytic degradation of nitrogen compounds. BA are ubiquitously present in both pro- and eukaryotes. In bacteria, the most common mechanism of BA synthesis is the microbial decarboxylation of amino acids catalyzed by amino acid decarboxylases (18). Certain bacteria are able to use BA production to harness the proton gradient and generate energy and/or increase the cytoplasmic pH, thereby protecting cells against acid damage (19). BAs are separated into classes based on the number of amino groups present in the structure: monoamines, diamines, and polyamines (18). BAs are known to be key regulators of host health, particularly when acting as hormones or neurotransmitters. BAs can be classified according to their chemical structure as aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine,  $\beta$ -phenylethylamine), or heterocyclic (histamine, tryptamine) (20, 21). Several BAs have been associated with promoting human health.

**Histamine**—Specific gut microbiota members have been reported to produce the biogenic amine histamine. Histamine is produced by decarboxylation of dietary L-histidine. Amino acid decarboxylation and biogenic amine synthesis maintain bacterial intracellular pH homeostasis (22) and can be used to generate energy using proton motive force (23). As a result, bacterial amino acid decarboxylase expression and activity is enhanced in acidic environments, leading to a locally increased pH and pH counter-regulation. Bacterial amino acid decarboxylase expression is also regulated by fermentable carbohydrates, sodium chloride concentration and oxygen saturation. Select gram-negative and gram-positive organisms generate histamine from histidine (24–28). The majority of histamine-producing strains belong to species of the genera *Oenococcus*, *Lactobacillus*, and *Pediococcus* (29, 30). These species harbor the gene encoding histamine decarboxylase (*hdcA*) which converts dietary histidine into histamine. Histamine is known to exert pro-inflammatory and anti-inflammatory effects on immunoregulatory processes (**Figure 5**). The type of response is dependent on the type of histamine receptor (of four known histamine receptors) that is activated. Activation of histamine receptor type 1 ( $H_1R$ ) or 3 ( $H_3R$ ) has been associated with pro-inflammatory effects; in contrast, activation of histamine receptor type 2 ( $H_2R$ ) or 4 ( $H_4R$ ) is associated with anti-inflammatory responses. While a number of species are capable of producing histamine, luminal histamine generated by *L. reuteri* has been documented to have beneficial anti-inflammatory properties. For example, several human-derived strains of *Lactobacillus reuteri* contain the histidine decarboxylase gene cluster (*hdcA*, *hdcB*, *hdcP*, *HisS*) which is required for histamine production. *L. reuteri* ATCC PTA 6475 -generated histamine suppressed the pro-inflammatory cytokine TNF in Toll-like receptor 2 (TLR2)-activated human monocytoïd cells (27). This suppression was driven by activation of the anti-inflammatory  $H_2R$  and downregulation of mitogen-activated protein (MAP) kinase activation. *In vivo*, histamine producing *L. reuteri* strains ameliorate inflammation *in vivo* in a trinitrobenzene sulfonic acid (TNBS)-induced mouse model of acute colitis (26, 31). Likewise histamine from *L. saerimneri* strain 30a (ATCC 33222) was

able to significant lower NFkB activation in human monocytoïd cells and suppress IL-17 and IFN- $\gamma$  secretion in wild-type mice, but not in H2R-deficient animals (32). Histamine has also been demonstrated to alter dendritic cell (DC) responses to microbial ligands (33). Histamine was found to suppress lipopolysaccharide (LPS, TLR-4 ligand) driven pro-inflammatory cytokine secretion (TNF, IL-12, CXCL10) and Pam3Cys (TLR-2 ligand) driven TNF production. Moreover, histamine increases production of the anti-inflammatory cytokine, IL-10. These responses were driven by H<sub>2</sub>R signaling through cyclic AMP. *In vivo* addition of histamine-secreting *L. rhamnosus* was suppressed cytokine (IL-2, IL-4, IL-5, IL-12, TNF- $\alpha$ , and GM-CSF) secretion from Peyer's patches in wildtype, but not in H<sub>2</sub>R-deficient mice (33). These studies indicate that bacterial histamine exerts immunoregulatory effects *in vitro* and *in vivo*.

### Amino acid neurotransmitters

**GABA ( $\gamma$ -aminobutyric acid):** GABA is a four carbon amino acid which functions as an inhibitory neurotransmitter in the mammalian nervous system and mediates diverse functions with the host. GABA can be produced by select bacterial species from the decarboxylation of glutamic acid via glutamate decarboxylase (GAD) (34). GABA production is highest in microbes when the environment is more acidic. Decarboxylation of glutamate consumes a proton and results in the stoichiometric release of GABA. In this manner, GABA production increases the pH of the bacteria cytosol and allows cells to resist acid stress (34). Several microbes have been demonstrated to produce GABA (35–38). LAB strains are considered to be the major group of microbes responsible for GABA production. Some examples of known GABA producing LAB members include *L. paracasei* NFRI 7415, *L. plantarum* C48, *L. paracasei* PF6, *L. brevis* PM17, *L. delbrueckii* subsp. bulgaricus PR1, *L. lactis* PU1, and *B. dentium* to name a few (39–41). GABA is known to elicit a number of beneficial effects on the host. As an inhibitory neurotransmitter GABA lowers the blood pressure *in vivo* in animal models and human subjects (42–44). GABA is also known to have diuretic and antidiabetic consequences (45–47). In the brain, GABA is able to enhance plasma concentration, growth hormones and the protein synthesis (48). GABA intake can regulate sensations of pain and anxiety. Mice fed *L. rhamnosus* JB-1 were shown to have region-dependent alterations in GABA receptor mRNA in the brain when compared with control-fed mice (49). *L. rhamnosus* JB-1 also reduced stress-induced corticosterone and anxiety- and depression-related behavior in treated mice (49). In human subjects, administration of a 30-day course of *L. helveticus* and *B. longum* led to decreased anxiety and depression (50). Other groups have found that daily oral administration of fermented products containing microbial GABA was effective in treating the neurological disorders of sleeplessness, depression and autonomic disorder in female subjects (51). Together, these findings indicate that gut microbes are able to participate in the bidirectional communication of the gut-brain axis via production of neurotransmitters such as GABA. Modulation of the gut-brain axis may prove to be beneficial for stress-related disorders such as anxiety and depression.

**Polyamines:** Polyamines (PAs) are small aliphatic hydrocarbon molecules with two or more amino groups (-NH<sub>2</sub>) that have a net positive charge at physiological pH (52). PAs and inorganic cations, like magnesium and calcium, play an essential role in maintaining optimal

conformation of negatively charged nucleic acids. Moreover, PAs are essential factors for normal cell growth, cell differentiation, and for the synthesis of DNA, RNA, and proteins (53). The majority of colonic PAs are derived from commensal gut microbiota (54–56). The main PAs are spermidine, putrescine, spermine and cadaverine. These compounds are critical for the growth and multiplication of both prokaryotic and eukaryotic cells (52, 57). In the majority of bacteria, intracellular concentrations of spermidine (1–3 mM) are higher than putrescine (0.1–0.2 mM), with the known exception of *E. coli* which has higher putrescine (10–30 mM) levels (57). Cadaverine is considered to be the least prevalent bacterial PAs (52, 57). In contrast, spermine is primarily produced in the presence of specific dietary sources. Consumption of pectin has been shown to increase the cecal concentrations of multiple PAs, particularly spermine (56). This effect was shown to be gut-microbe dependent as consumption of pectin- or guar-containing diets altered the concentrations and the composition of cecal PAs in conventional rats, but not in germ-free rats (56). The gut members *Bacteroides thetaiotaomicron* and *Fusobacterium varium* are major contributors of spermine and spermidine in the gnotobiotic rat cecum, suggesting that commensal bacteria can modulate PA levels (55).

Commensal bacteria can both produce and be influenced by PAs. PAs are known to possess anti-inflammatory activities, which can benefit the host. PAs can decrease systemic inflammation by inhibiting pro-inflammatory cytokine production in macrophages and intestinal epithelial cells (58, 59). Additionally, PAs function as reactive oxygen species (ROS) scavengers, chemical chaperones, positive regulators of stress genes and antimutagenic agents (60, 61). Spermine possesses anti-inflammatory activity by inhibiting NF $\kappa$ B activation (62) and inflammatory cytokine synthesis (58, 63), and by selectively activating T cell protein-tyrosine phosphatase (TCPTP) (64). In mammals, systemic PA levels decrease with age (65, 66) and decreased PAs have been associated with intestinal barrier dysfunction (67). Supplementation of middle-aged (10-month-old) mice with *B. animalis* subsp. *lactis* LKM512 for 6 months increased fecal spermine concentrations (67). This spermine increase correlated with increased survival, reduced skin ulcers and tumors, improved colonic barrier function, downregulation of inflammation-associated genes and alteration of the gut microbiota composition (67). In addition, supplementation of arginine to the diets of mice and rats increased colonic concentrations of spermine and putrescine (68). A combination of both arginine and *B. animalis* subsp. *lactis* LKM512 further suppressed inflammation, improved longevity, and protection from age-induced memory impairment in mice (68). Several of these findings have been mirrored in patients, as addition of *B. animalis* subsp. *lactis* LKM512 increased intestinal PAs (putrescine, spermidine, spermine and cadaverine) (69, 70) and reduced quantities of biomarkers of acute inflammation in hospitalized elderly patients (70).

Commensal bacteria also benefit from PA production as PAs can protect bacterial cells from ROS (71) and mutagens (61, 72–75). PAs can also modulate gut community dynamics. Supplementation of PAs to a formula diet of neonatal BALB/c mice was found to regulate the concentrations of *Akkermansia muciniphila*, *Lactobacillus*, *Bifidobacterium*, *Bacteroides-Prevotella* and *Clostridium* groups to levels found in the breast-fed group (76). Although PAs can be used by commensal bacteria, PAs can also be used by pathogens. Pathogens utilize PAs to promote toxin activity, bacteriocin production, biofilm formation,

microbial carcinogenesis, and protection from oxidative and acid stress (77–85). While PA production and utilization is not unique to commensals, the highlighted studies provide evidence that PA synthesis by commensal bacteria may serve to modulate the host immune response and improve patient well-being.

**Tryptophan and Indole**—The essential amino acid L-tryptophan is used by the host to synthesize proteins and specialized molecules including the hormone and neurotransmitter serotonin (86). In addition to its use by the host, pathogens such as enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) can also utilize tryptophan as the sole source of carbon and nitrogen (87). As a result, conversion of tryptophan to its metabolites by commensal bacteria may effectively remove tryptophan from the amino acid pool available to pathogens. Luminal tryptophan can undergo bacterial degradation generating indole, indican, and indole acid derivatives (indolyl-3-acetic acid, indolyl-acetyl-glutamine, indolyl-propionic acid, indolyl-lactic acid, indolyl-acrylic acid and indolyl-acryloyl-glycine). Indole represents the main bacterial byproduct of tryptophan. Several microbes are capable of producing indole and indole derivatives including *Bacteroides* spp, *Bifidobacterium* spp, *Clostridium* spp, *E. coli*, *Proteus vulgaris*, *Paracolobactrum coliforme*, and *Achromobacter liquefaciens* (86, 88, 89). Bacterial tryptophanase converts tryptophan into indole, ammonia, and pyruvate. In many different microbes, tryptophanase activity has been shown to be induced by tryptophan and repressible by glucose (89). Protein-rich diets have also been shown to induce bacterial tryptophanase activity (89).

Indole metabolites are noteworthy as they have been shown to provide several benefits to the host. Indole compounds secreted by commensal *E. coli* reduce attachment of pathogenic *E. coli* to epithelial cells (90). Specifically, indole-3-carboxaldehyde (ICA), downregulated production of *E. coli* Locus of Enterocyte Effacement (LEE) virulence factors and inhibited pedestal formation on mammalian cells (91). Oral administration of ICA was found to inhibit virulence and promote survival in a lethal mouse infection model of *C. rodentium* (91). Select indole derivatives also yielded bacteriostatic effects on gram-negative enterobacteria, particularly pathogens such as *Salmonella* and *Shigella* (89).

In addition to bacteriocidal effects of indole metabolites, indole itself may enhance the mucosal barrier by inducing tight junction mediated trans-epithelial resistance and mucin production, and by diminishing TNF-alpha-mediated activation of NF-kB (90). *In vivo*, oral administration of indole-containing capsules to germ free mice resulted in increased quantities of tight junction and adherens junction-associated proteins in colonic epithelial cells (92). Furthermore, germ free mice supplemented with indole-containing capsules yielded greater resistance to dextran sodium sulfate (DSS)-induced colitis (92). Indole compounds secreted by gut bacteria may serve as an important signal for the maintenance of intestinal epithelial homeostasis. Similar to other compounds produced by commensal microbes, indole is also utilized by several pathogens. Indole is known to regulate biofilm formation, virulence, and production of Shiga toxins in pathogenic *E. coli* strains, as well as virulence in the rodent A/E pathogen *C. rodentium*, *Pseudomonas* and *Salmonella* strains (91). However, since select indole metabolites synthesized by commensals inhibit these same species, future work may focus on selecting key commensals to combat pathogens via indole metabolism.

## 2. Bacteria-bacteria communication

**Quorum Sensing Molecules**—In addition to the many routes of bacterial-host communication, bacteria themselves must communicate with each other in order to regulate cooperative activities (93). A number of bacteria release, sense and respond to small diffusible signal molecules, known as quorum sensing molecules, as a method of intra- and inter-species bacterial communication. In this manner, bacteria can behave as a collective unit (94–96). Quorum sensing regulates bacterial symbiosis, formation of spore or fruiting bodies, bacteriocin production, genetic competence, programmed cell death, virulence and biofilm formation (93, 96–99). This behavior is thought to offer significant benefits to bacteria in terms of biofilm community structure, defense against competitors, adaptation to environmental changes and overall host colonization (93, 96, 99, 100). In general, quorum sensing relies on diffusible signaling molecules and sensors or transcriptional activators which work in concert to promote gene expression (93, 96, 99, 101, 102). Quorum sensing can be divided into three classes: (1) LuxI/LuxR–type quorum sensing in gram-negative bacteria (2) oligopeptide-two-component-type quorum sensing in gram-positive bacteria; and (3) luxS-encoded autoinducer 2 (AI-2) quorum sensing in both gram-negative and gram-positive bacteria (93).

In LuxI/LuxR–type quorum sensing, acyl-homoserine lactones (AHL) act as the signaling molecules (103, 104). In this system, AHL synthesis is dependent on a LuxI-like protein and AHLs increase in concentration in proportion to cell density. AHLs can freely diffuse across bacterial cell membranes where they are recognized and bound by the cognate LuxR-like protein, which subsequently binds specific promoter DNA elements and activates target genes. Hundreds of gram-negative bacteria use the LuxI/LuxR-type quorum sensing system, with each species producing a unique AHL. As a result, only similar species are able to recognize and respond to signals from their own kind (96, 99, 103, 105).

In contrast to the LuxI/LuxR system, the oligopeptide-two-component-type quorum sensing system is comprised of three components: a signaling molecule and a two-component signal transduction system (TCSTS). The most common oligopeptide-two-component-type quorum sensing system involves the autoinducer signaling peptide (AIP) and a corresponding TCSTS that specifically detects and responds to AIP (96, 102, 106–108). Unlike diffusible AHL signals, AIP must be transported by a dedicated oligopeptide transporter, typically an ABC transporter, in order to exit the cell (106–108). The AIP signal is then sensed by the two-component signal transduction system, which contains a membrane-associated, histidine kinase protein and a cytoplasmic response regulator protein, which translates the signal via regulation of target gene expression (106–108). Another type of quorum sensing identified in gram-positive streptococci is the ComRS system, which involves a small double-tryptophan signal peptide pheromone, XIP. Similar to AIP, XIP is transported inside the cell via an oligopeptide ABC transport system (Opp/Ami). Inside the cell, XIP interacts with a transcriptional regulator, ComR or ComX, thereby activating competence genes for genetic transformation (109–111). Additionally, *S. mutans* contains ComCDE and ComRS quorum sensing systems which regulate bacteriocin production (109).

Both gram-negative and gram-positive bacteria can utilize the autoinducer 2 (AI-2) quorum sensing system (93, 96, 99). In contrast to the LuxI/LuxR-type and oligopeptide-two-component-type quorum sensing systems which provide for intra-species signaling, AI-2 quorum sensing allows for inter-species communication. As a result, AI-2 has been termed the “universal language” (101, 102). AI-2, a furanosyl borate diester, is produced and recognized by many gram-negative and gram-positive bacteria (96). AI-2 synthesis depends on a luxS encoded synthase; a metabolic enzyme which converts ribosyl-homocysteine into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (112). AI-2 is transported inside the bacteria by the Lsr ABC-type transporter in *Enterobacteriaceae*, *Pasteurella*, *Photorhabdus*, *Haemophilus*, and *Bacillus*. (113). AI-2 is then phosphorylated by LsrK and subsequently binds the transcriptional repressor protein, LsrR. Binding of phosphor-AI-2 to LsrR releases the promoter/operator region of the lsr operon, thereby promoting transcription lsr genes.

Since AI-2 is produced and detected by a number of diverse bacteria, it has been speculated that AI-2 facilitates interspecies communication and social interactions. This compound has been postulated to be particularly relevant in the setting of biofilms. Biofilms are considered to be structured microbial communities organized within an extracellular matrix which harness symbiotic interactions for mutual benefit of the collective (94, 95). Microbes within biofilms have diverse characteristics compared with their free-living counterparts, including enhanced resistance to antibiotics and host immune responses (93, 95, 114). Biofilms are important for both virulent pathogens and complex commensal communities within the GIT (115). For commensal microbes within the GIT, colonization of the intestinal mucus layer is a critical step in establishing ecological niches. The intestinal epithelium is covered by a continuous layer of mucus secreted by goblet cells, chiefly composed of the mucin protein MUC2 (116, 117). Mucus provides a viscoelastic gel barrier that prevents luminal contents and microbiota from interacting directly with the epithelium and immune cells of the lamina propria. The mucin proteins are heavily O-glycosylated and these glycans can serve as adhesion sites for commensal bacteria (118–123). Several studies have shown that intestinal communities can be visualized as biofilms of sessile microorganisms within the mucus layer (116, 124–126). High bacterial cell density within biofilms favors a mode of communication using diffusible small molecules as a mechanism for regulating biofilm formation and for social interactions (97, 127). Several studies have shown that AI-2 signaling is important for the proper development of multi-species biofilms in natural ecosystems (128–130). This signaling pathway has been particularly well documented for the oral cavity. In mixed cultures of *Actinomyces naeslundii* T14V and *S. oralis* 34, AI-2 is essential for interdigitated biofilm growth where saliva is the primary nutrient source (129). Introduction of a *S. oralis* 34 luxS mutant with *A. naeslundii* T14V diminished mutualistic growth; a defect that could be rescued with the LuxS enzyme product DPD. Other groups have shown luxS -dependent biofilm formation in *S. pneumoniae* (131), clinical isolates of *S. pneumoniae* D39 (132), and *L. reuteri* (133).

In the intestine, AI-2 has also been shown to play a key role in community structure and colonization. Thompson and colleagues found that AI-2 can modulate the structure of the gut microbiota by using *E. coli* to manipulate signal levels (98). In this work, AI-2 influenced bacterial behaviors to restore the balance between the major phyla of the gut



microbiota, Bacteroidetes and Firmicutes, following antibiotic treatment. Although there are only few *in vivo* studies thus far, existing data points to the critical role of quorum sensing in establishment of commensal communities.

## Metabolites that benefit the host

### 1. Short Chain Fatty Acids (SCFA)

Several bacterial species in the gastrointestinal tract generate short chain fatty acids (SCFAs) as an end product of complex carbohydrate fermentation pathways in the intestine. SCFAs are comprised of one to six carbons, with the most abundant and well-characterized SCFAs being acetate, propionate, and butyrate. The composition of SCFAs in the GIT depends on microbial composition and environmental conditions, including pH, hydrogen partial pressure and host diet (134–137). SCFAs can reach local concentrations of approximately 13 mM in the terminal ileum, 130 mM in the cecum and 150 mM in the descending colon, making SCFAs a class of abundant colonic anions (138, 139). Most SCFAs are absorbed by the host in exchange for bicarbonate. As a result, SCFAs gradually diminish in concentrations from the proximal to the distal colon and the luminal pH correspondingly increases from cecum to rectum (138, 140, 141). The pH reduction may alter microbial composition of the gut and prevent overgrowth by pH-sensitive pathogenic bacteria such as certain strains of Enterobacteriaceae and Clostridia (142–146). SCFAs are absorbed by intestinal enterocytes via passive diffusion or carrier-mediated transportation through SMCT1 (SLC5a8) and MCT1 (SLC16a1) transporters (147, 148). SMCT1 acts as a sodium-coupled transporter, while MCT1 is a hydrogen-coupled transporter for SCFAs and related organic acids (147–149). These transporters are found on the apical membranes of intestinal enterocytes, dendritic cells, kidney cells and brain cells. In addition to intestinal absorption, SCFAs can activate several G-protein-coupled cell surface receptors (GPCRs). SCFAs bind to G protein-coupled receptors, such as GPR41 (propionate > butyrate >> acetate), GPR43 (propionate, butyrate, acetate) and GPR109A (butyrate) on intestinal epithelial cells and enteroendocrine cells such as colonic L-cells (150–154). In addition to intestinal cells, GPR41 is also expressed in enteric neuronal cells, adipocytes, renal smooth muscle cells, and pancreatic cells (155, 156) and GPR43 is expressed by granulocytes and some myeloid cells (157–159). GPR109a is also expressed by macrophages, dendritic cells and adipocytes. As a result of the wide distribution of receptors, SCFAs have diverse effects on the host (**Figure 5**). SCFAs can regulate different aspects of host physiology, including enhanced sodium uptake and subsequent water absorption, pH regulation, chloride and mucus secretion, immune regulation, decreased bioavailability of toxic amines and remote effects on neural activity and development (160–162).

**Acetate**—Acetate is the most abundant SCFA and is generated as a fermentation end product produced by enteric bacteria, as well as a product of metabolism of H<sub>2</sub>, CO<sub>2</sub> or formate by acetogenic bacteria (135). *Bifidobacterium longum subsp. longum* and *B. longum subsp. infantis* secreted products suppressed epithelial cell apoptosis, ameliorated intestinal inflammation and inhibited translocation of the *E. coli* O157:H7 Shiga toxin, thereby protecting mice against infection by enterohemorrhagic *E. coli* O157 bacteria (163). This protection correlated with fecal acetate quantities. Non-acetate producing

*Bifidobacteria* strains as well as a *Bifidobacterium longum* mutant with reduced acetate production were unable to mimic the protective effects of the parental strain against *E. coli* O157. Moreover, administration of acetylated starch which increased fecal acetate improved the survival rate. These data indicate that bacterial produced acetate protects the host against lethal infection. In addition to epithelial cell effects, acetate also plays an important role in immune regulation. Acetate induces T regulatory cell proliferation and accumulation (160, 164–166) and inhibits T cell histone deacetylase 6, while increasing tubulin- $\alpha$  acetylation (167). In addition to immune cells, Ishiguro and colleagues have demonstrated that acetate inhibits IL-8 production and increases tubulin- $\alpha$  acetylation within intestinal epithelial cells (IEC) (167). Inoculation of germ-free mice with the acetate producer *Bacteroides thetaiotaomicron* increased goblet cell differentiation and expression of mucus-related genes. These *in vivo* findings were confirmed *in vitro* using the mucus-producing cell line HT29-MTX where acetate up-regulated KLF4, a transcription factor involved in goblet cell differentiation (168). Mucus produced by goblet cells creates a protective barrier that prevents the epithelium and immune system from adhesion and invasion by pathogenic bacteria, microbial antigens, and other damaging agents present in the intestinal lumen (116, 127). Mucin glycans are also important sources of carbohydrate for saccharolytic bacteria and the spatial organization and composition of mucosal communities may be influenced by variations in mucin production and glycan composition (118, 127). As a result, modulation of the mucus layer by microbial compounds such as acetate may serve to maintain proper distance between the microbiota and host and potentially modulates the microbial community structure.

Acetate also inhibits the growth of several pathogens. Acetate alone inhibits the growth of *P. aeruginosa* (169), while acetate in combination with propionate and butyrate inhibits the growth of pathogenic *E. coli* O157 (170), *Proteus mirabilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (169). Acetate and propionate acting via GPR43 participate in anti-inflammatory effects via the modulation of regulatory T cells (Treg) cells (164, 171). *In vivo*, supplementation of acetate in germ-free mice was shown to be sufficient to ameliorate the dextran sulfate sodium (DSS)-driven intestinal inflammation, an effect that was not observed in Gpr43<sup>-/-</sup> mice (171). Interestingly de Vuyst and Leroy demonstrated the importance of acetate as a substrate for the production of butyrate by butyrate-producing bacteria (172), implicating a cross-talk of SCFAs in microbial metabolism.

**Propionate**—Propionate is primarily formed via the succinate pathway by the phyla Firmicutes (135, 173). Propionate is principally metabolized by the liver, while acetate is metabolized by peripheral tissues. Production of acetate by the commensal *Akkermansia muciniphila* modulates mouse gene expression, particularly Fiaf, Gpr43, histone deacetylases (HDACs), and peroxisome proliferator-activated receptor gamma (Ppar $\gamma$ ), which are important regulators of transcription factor regulation, cell cycle control, lipolysis, and satiety (174). *In vitro* propionate inhibits several pathogens including *Salmonella* serovar *Typhimurium* (175–178), *E. coli* O157 (170), *Proteus mirabilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (169). Propionate and acetate generated by microbial species stimulated the cellular function of immune cells, specifically promoting neutrophil chemotaxis (157, 171, 179, 180). In addition, propionate has been utilized for its ability to

release short-term modulators of satiation and satiety, including the anorectic gut hormones peptide YY (PYY) and glucagon like peptide-1 (GLP-1) from intestinal enteroendocrine L cells (181–183) (184, 185). In humans, colonic delivery of inulin-propionate esters increased plasma PYY and GLP-1 and reduced energy intake, resulting in significantly reduced weight gain, intra-abdominal adipose tissue distribution, and intra-hepatocellular lipid content after 24 weeks (184). These data suggest that colonic propionate may be an important microbial metabolite in the context of host body metabolism.

**Butyrate**—Butyrate is generated by microbes of the phylum Firmicutes (including *Faecalibacterium prausnitzii*, *Roseburia* spp., *Eubacterium rectale*, *Eubacterium hallii* and *Anaerostipes* spp.) via the butyryl-CoA:acetate CoA-transferase enzyme or phosphotransbutyrylase and butyrate kinase pathway (135, 186–188). Butyrate is primarily metabolized in the colon as an energy source utilized by intestinal epithelial cells, and this SCFA yields immunoregulatory and cancer protective effects in the gastrointestinal tract. Propionate and butyrate alter immune cell function. Both propionate and butyrate inhibit stimuli-induced expression of adhesion molecules, chemokine production and suppress monocyte/macrophage and neutrophil recruitment (179). Butyrate signaling via GPR109A regulates the differentiation of regulatory Treg and IL-10-producing T cells (162), and suppresses activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and induction of apoptosis (189). *In vivo* production of butyrate by clostridial species induces the differentiation of Treg cells and ameliorates development of colitis (160). Apart from the major effects on butyrate on immune cell populations, this SCFA can also serve as an energy source for colonic enterocytes. Furthermore, activation of GPR43 and GPR109A by butyrate and propionate mediate cancer-protective effects associated with high fiber intake (186, 190). Butyrate also participates in anti-tumorigenic properties by inhibiting proliferation and selectively inducing apoptosis of colorectal cancer (CRC) cells (191–194). Intracellular butyrate and propionate, but not acetate, inhibit the activity of histone deacetylases (HDACs) in colonocytes and immune cells. HDAC inhibition promotes the hyper-acetylation of histones, effectively downregulating pro-inflammatory cytokines, such as interleukin-6 (IL-6) and IL-12 in colonic macrophages (191, 195, 196). Together these studies demonstrate that commensal produced SCFAs can elicit multiple advantages for the host through the stimulation of intestinal mucus production, inhibition of inflammation, modulation of immune cell populations, and inhibition of cancer proliferation.

## 2. Long Chain Fatty Acids

While SCFAs have been thoroughly characterized with respect to intestinal biology and human health, long chain fatty acids (LCFAs) are gaining attention for their health-promoting activities as well (197). Similar to SCFAs, commensal bacteria are also responsible for the composition and concentration of LCFAs and subsequently contribute to LCFA-induced signaling in host cells. LCFAs are produced when dietary poly-unsaturated fatty acids (PUFA), such as linoleic acids, are converted into conjugated linoleic acids (CLA) and trans-fatty acids (198–200). Germ-free (GF) mice without a gut microbiota, lack detectable LCFAs (201), while mice inoculated with the commensal *Bifidobacterium breve* in combination with linoleic acid-supplemented diet resulted in increased CLA (202). Production of CLA by bacteria reduced amounts of hepatic triacylglycerols and inhibited

atherosclerosis (203, 204). Although it remains unclear if LCFAs regulate host immune functions, modified Polyunsaturated fatty acids (PUFAs) are potent agonists for peroxisome proliferator-activated receptor (PPAR) $\gamma$  and PPAR $\alpha$ , which are upregulated by commensal bacteria and implicated in attenuating inflammation (197, 205–208). The role of LCFAs in intestinal homeostasis was also further confirmed *in vivo* in ethanol-fed mice. Exposure of mice to alcohol resulted in an altered gut microbiota and reduced synthesis of LCFAs (209). Relative abundances of *Lactobacillus spp.*, known metabolizers of saturated LCFAs, were reduced in the feces of humans with active alcohol abuse (209). The authors hypothesized that targeted approaches to restore LCFA levels might reduce ethanol-induced liver injury and restore an intact community of gut microbiota.

### 3. Vitamins

Bacteria residing in mammals are able to produce vitamins which directly benefit the host (210). Metagenomic analyses of the human microbiota from the distal colon revealed the existence of diverse Clustered Orthologous Groups (COGs) which are involved in vitamin synthesis (211). Vitamins are essential organic micronutrients which are critical for cellular function and may not be synthesized by the host. Vitamins exist as precursors of intracellular coenzymes. The majority of vitamins are produced by bacteria via the 2-methyl-D-erythritol 4-phosphate pathway. Thirteen essential vitamins for human health include the water-soluble vitamins thiamine (B1), riboflavin (B2), niacin (B3), pyridoxine (B6), pantothenic acid (B5), biotin (B7 or H), folate (B11–B9 or M) and cobalamin (B12) and C and the fat-soluble vitamins A, D, E and K (212). Vitamins generated by gut microbes are primarily absorbed in the colon, while dietary vitamins are absorbed mostly in the small intestine (213, 214). Data suggest that colonocytes absorb thiamine, folates, biotin, riboflavin, pantothenic acid, and menaquinones (213, 214). Vitamins provide essential nutrients to the host and directly influence the development and function of immune cells (215–218). In this manner, vitamins may work to promote host growth and immune homeostasis (**Figure 5**).

#### Water-Soluble Vitamins

**Riboflavin (Vitamin B2):** Riboflavin, or vitamin B2, is a known component for cellular metabolism. Riboflavin serves as a precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (219). FMN and FAD serve as hydrogen carriers in cellular redox reactions, making B2 critical for host metabolism. B2 can exist in several active forms including riboflavin (7,8-dimethyl-10-(1'-d-ribityl) isoalloxazine), riboflavin-5'-phosphate (FMN) and riboflavin-5'-adenosyldiphosphate (FAD). Riboflavin can be produced by both gram-positive and gram-negative bacteria and is well characterized in *Bacillus subtilis* and *Escherichia coli* (220, 221). LAB strains including *L. plantarum* and *L. lactis* have also been identified as B2 producers (219, 222–225). B2 can be produced from the precursor guanosine triphosphate (GTP) and D-ribulose 5-phosphate via seven enzymatic steps (220). Enhanced production of B2 has been shown in species that are capable of simultaneously expressing four biosynthetic genes (ribG, ribH, ribB and ribA) (223, 224). Deficiency in B2 levels leads to ariboflavinosis, which is associated with hyperemia, edema of oral and mucous membranes, cheilosis and glossitis (226). In rats supplementation of a fermented milk drink containing a genetically modified B2-producing *L. lactis* strain was effective in reversing ariboflavinosis in a riboflavin-deficiency model

(223). In humans daily consumption of a probiotic yogurt containing *Streptococcus thermophilus*, *L. bulgaricus* and *L. casei* subsp. *casei* for 2 weeks contributed to the total intake of vitamin B2, as reflected by increased blood concentrations of plasma-free riboflavin in healthy women (227). These effects were ameliorated when subjects returned to their previous diet, implicating select supplemented probiotic bacteria in the enhanced production of vitamin B2 (227).

**Folates ((B11–B9 or M):** Folates are hydrophilic anionic molecules which are produced by a number of bacteria. The generic term folate is typically used to include all bacterial derived folate derivatives. Folates are involved in essential cellular metabolism functions including DNA replication, DNA repair, DNA methylation, and nucleotide synthesis. Deficiency in folate has been linked to a large spectrum of disorders including colorectal cancer, osteoporosis, coronary heart disease, Alzheimer’s disease, among others (228, 229). Although folate is primarily absorbed in the duodenum and jejunum, folate compounds generated by the mammalian microbiome in the colon represent a major source of host folate. Bacteria generate mono- and poly-glutamylated folate, a form of folate which is easily absorbed by mammalian cells (230, 231). Multiple LAB strains such as *Lactobacillus reuteri*, *L. acidophilus*, *L. plantarum*, *L. bulgaricus*, *Lact. lactis*, *Bifidobacterium adolescentis*, *Bifidobacterium animalis* and *Bifidobacterium longum* synthesize folate (228, 232–238). However not all LAB strains are capable of generating folate. For example *L. gasseri* (236), *L. salivarius* (239), and *L. johnsonii* (240) do not contain folate biosynthesis genes and do not produce folate. Bacterial production of folate in the presence of existing folate appears to be species dependent; with select species producing folate solely in low folate conditions and others continually producing folate (241). Bacteria produce folate using the precursor 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (DHPPP) and para-aminobenzoic acid (pABA, vitamin B10) (25, 242). All bacteria capable of producing folate contain the folC or homologous genes (235, 238). Additional genes involved in folate production include folKE genes which encode 6-hydroxymethyl-dihydropterinpyrophosphokinase (folK) and GTP cyclohydrolase (folE) or pABA (243). Folate production relies on the combination of folate and pABA biosynthesis. Deletion of the pABA genes in *L. lactis* and *L. reuteri* resulted in loss of folate production and inhibition of growth in the absence of purine nucleobases/nucleosides (243). Genetic manipulation of folate genes increased folate production in a number of species including *L. lactis*, *L. gasseri* and *L. reuteri* (233, 236, 237, 243). *In vivo* addition of *L. lactis* overexpressing the folC, folKE or folC-folKE genes improved folate status in a rat folate deficiency model (228). Moreover, production of a 5,10-methenyltetrahydrofolic acid polyglutamates via the folC2 gene by *L. reuteri* ATCC PTA 6475 was found to suppress TNF production by activated human monocytes and partake in the anti-inflammatory effect of *L. reuteri* in a TNBS-induced mouse model of acute colitis (231). These studies demonstrate that select folate producing microbes can be utilized to improve folate status and modulate the immune system in animal models.

**Vitamin B12:** Vitamin B12, also known as cobalamin, exists in its natural form as 5'-deoxyadenosylcobalamin (coenzyme B12), methylcobalamin or pseudocobalamin, and is a corrin ring or corrinoid compound. B12 is required for the metabolism of nucleic acids,

amino acids and fatty acids (244) and is primarily produced by anaerobic bacteria (245–247). Few bacteria are capable of producing vitamin B12 (248, 249). Typically specialized bacteria found in food source animals produce vitamin B12. As a result, humans must absorb the coenzyme from animal sources such as meats, fish and eggs. However, select lactobacilli have been demonstrated to produce vitamin B12. *L. reuteri* CRL1098 was found to produce a cobalamin-like compound, a form of B12 (250). *L. reuteri* DSM 20016 (237), JCM1112 (237) and CRL 1324 and 1327(251) and *L. coryniformis* (252) produced a cobalamin-type compound. These *L. reuteri* species contain an extensive cobalamin biosynthesis cluster, which is associated with the anaerobic catabolism of glycerol (or 1,2-propanediol) (253, 254). Vitamin B12 deficiency is associated with numerous hematopoietic, neurological and cardiovascular pathologies. Pernicious anemia, a severe form of B12 deficiency, is the result of poor production of a gastric glycoprotein called intrinsic factor that facilitates the absorption of vitamin B12 in the small intestine (255). *In vivo* in a mouse model of vitamin B12-deficient, supplementation of *L. reuteri* CRL 1098 was able to reverse vitamin B12 deficiency (256). Gut microbes may be important in maintaining adequate body concentrations of B complex vitamins, including vitamin B12.

### Fat Soluble Vitamins

**Vitamin K:** Vitamin K comprise a number of series of fat-soluble compounds which share a 2-methyl-1, 4-naphthoquinone nucleus and differ side chain structure at the 3-position. Vitamin K can be produced by both plants and microbes. In plants, vitamin K exists as phyloquinone (vitamin K1), which has a phytyl side chain. In contrast, bacteria generate a family of compounds known as menaquinones (vitamin K2). These compounds contain side chains based on repeating unsaturated 5-carbon (prenyl) units and are designated menaquinone-n (MK-n) according to the number (n) of prenyl units. Vitamin K acts as an essential cofactor for the formation of  $\gamma$ -carboxyglutamic acid (Gla) residues in proteins which bind calcium ions. As a result, vitamin K serves a prominent role in bone formation, tissue calcification, kidney function, and blood clotting to name a few (257, 258). Vitamin K deficiency has been implicated in osteoporosis- driven bone fracture and intracranial haemorrhage in newborns. The gut microbiota synthesize large amounts of menaquinone K2, one of the forms of Vitamin K (259). Quantitative measurements at different sites of the human intestine have demonstrated that most of these menaquinones are present in the distal colon (259). Specific microbes are capable of generating menaquinone K2, one of the forms of Vitamin K. Genera from *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Leuconostoc* and *Streptococcus* are known producers of K2 (260, 261). Other major menaquinone forms are produced by *Bacteroides* (MK-10, MK-11), *Enterobacter* (MK-8), *Veillonella* (MK-7) and *Eubacterium lentum* (MK-6). Vitamin K is predominantly absorbed in the terminal ileum of the intestine; a site where menaquinone-producing bacteria colonize as well. Collectively the data acquired from all vitamin studies point to the selection of multivitamin-producing bacteria to compensate for common vitamin-deficiencies and for promotion of gut homeostasis.

## 4. Outer Membrane Vesicles (OMVs)

Several bacteria are capable of releasing outer membrane vesicles (OMVs) which range in size from 20 to 300 nm in gram-negative bacteria (262) to <20 nm MVs in Gram-positive

bacteria (263, 264). OMV production is considered to be a common feature of gram-negative bacteria. OMVs are generated by membrane remodeling which occurs when the OM bulges and encapsulates periplasmic components (265, 266). As a result, OMVs contain a number of soluble proteins entrapped in the OMV periplasm and multiple proteins on the external surface. Secreted OMVs can disseminate compounds to distant sites. OMVs have yielded a wide range of biological functions, from delivery of enzymes, transport of toxins, transmission of communication signals, nutrient acquisition, to induction of commensal tolerance. One beneficial role of OMVs on host homeostasis is the ability of OMVs to modulate innate and adaptive immune systems (267) (**Figure 5**). *Bacteroides fragilis* OMV delivery of polysaccharide capsular antigen (PSA) yielded multiple immunomodulatory effects. Mono-colonization of germ-free mice with *B. fragilis* was shown to modulate CD4+ T cell homeostasis and cytokine production (3). T cell modulation occurs in a PSA-dependent manner (3). OMV delivered PSA stimulated TLR2 on Tregs and directly modulated dendritic cells (DCs) (268, 269). OMVs were internalized by DCs and this interaction promoted tolerogenic DCs which produced IL-10 and stimulated regulatory Tregs. Furthermore, PSA production ameliorated intestinal inflammation (270), CNS inflammation (271, 272) and neurodegeneration (273). These studies demonstrate that OMV delivery of PSA is capable of promoting an anti-inflammatory profile which leads to tolerance and suppression of mucosal inflammation.

## 5. Serpin

Serpins are eukaryotic-type serine protease inhibitors. These molecules are synthesized by several commensal bacteria. Serpins are relatively large molecules consisting of approximately 330–500 amino acids (274). Currently more than 70 serpin structures have been identified. These complex structures act as stoichiometric suicide inactivators and inhibit eukaryotic elastase-like serine proteases. Serpins are known to regulate a wide range of signaling pathways in eukaryotes. Select serpins have been shown to suppress inflammatory responses by inhibiting elastase activity (275) (**Figure 5**). Several bifidobacterial species and subspecies (*B. breve*, *B. longum subsp. infantis*, and *B. longum subsp. longum*, *B. dentium*) are capable of producing serpins (276). *B. longum* NCC2705 was shown to secrete a serpin which binds and inactivates human neutrophil elastase, a product secreted by neutrophils during active inflammation (277). Moreover, bifidobacterial serpin-like proteins have been shown to reduce intestinal inflammation in a murine colitis model (278). Based on these findings, production of serpins which inhibit neutrophil elastases may be beneficial for reducing intestinal damage in the setting of overt inflammation.

## 6. Lactocepin

Lactocepins are bacterial enzymes which can degrade targeted bacteria of different genera via damage to prokaryotic cell membranes and induction of pro-inflammatory modulators (**Figure 5**). Lactocepins can be cell-wall associated or secreted, and the target specificity is strain specific (279–281). Lactocepins are mainly expressed by lactococci and lactobacilli. These enzymes are encoded by prtP, prtB, and/or prtH. The prtP genes are well documented for their caseinolytic properties. *Lactobacillus paracasei* secreted prtP-encoded lactocepins, and this compound selectively degraded the lymphocyte recruiting chemokine IP-10 and

other pro-inflammatory chemokines such as I-TAC and Eotaxin *in vitro* (282). Importantly, prtP-encoded lactocepin selectively degraded IP-10 in inflamed intestinal tissue and had no adverse effects on IEC barrier function *in vivo* (282). This resulted in significantly reduced lymphocyte recruitment after intraperitoneal injection in an ileitis model. Another *Lactobacillus* strain *L. casei* was found to secrete lactocepin which degraded host IP-10 (283). In a murine colitis model (T cell transferred Rag2<sup>-/-</sup> mice), supplementation of a *L. casei* prtP-disruption mutant resulted in more IP-10, T cell infiltration and inflammation in cecal tissue compared to the isogenic wild-type strain. Supplementation of the probiotic VSL#3, which contains *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, normalized intestinal levels of IP-10 in a murine colitis model and reduced inflammation in patients with IBD (283). These studies support the role of lactocepin secreted by commensal microbes as an effective treatment for chemokine-mediated diseases like IBD.

## 7. Other Secreted proteins known to enhance host health

In addition to all the secreted products highlighted by this review, select commensal bacteria generate putative proteins which modulate the host (**Figure 5**). *L. rhamnosus* GG and *L. casei* secrete two proteins designated p40 and p75 (284, 285). Lactobacilli produced p40 and p75 were found to inhibit tumor necrosis factor (TNF)-induced apoptosis in the intestinal epithelium. The proteins signal via the anti-apoptotic Akt kinase in a phosphoinositide 3-kinase-dependent manner likely via epidermal growth factor receptor (EGFR) activation (284, 286). The p40 and p75 proteins also enhance tight junctions and attenuate intestinal barrier disruption via protein kinase C, and extracellular signal-regulated kinase 1 (ERK). Furthermore, p40 was shown to transactivate the EGF receptor (EGFR), activating the downstream target Akt and stimulating mucus Muc2 gene expression and mucin production in the human goblet cell line LS174T and wild type mice. Intestinal mucus is a critical component of the healthy intestinal barrier, particularly in the setting of infection and inflammation (122, 287–289). Stimulation of intestinal mucus by commensal bacteria likely serves to enhance barrier function and promote homeostasis. Other commensal bacteria produce putative proteins with immunoregulatory features. *Faecalibacterium prausnitzii* is known to produce a 15 kDa protein, termed MAM, with anti-inflammatory properties (290, 291). MAM inhibits the NF- $\kappa$ B pathway in intestinal epithelial cells and prevents colitis *in vivo*. As subgroups of Crohn's disease are associated with reduced relative abundances of *F. prausnitzii*, microbial or protein (e.g. MAM) supplementation have been hypothesized to alleviate intestinal inflammation in future clinical trials. These studies highlight the need to define secreted commensal products of the mammalian microbiome.

## Antimicrobial Compounds

### 1. Lactic Acid

Commensal microbes may produce organic acids, such as lactic acid, that may reduce local pH and suppress the growth and survival of neighboring microbes. A group of microbes known as Lactic Acid Bacteria (LAB) are able to produce relatively large amounts of lactic acid as a major catabolic end product of glucose fermentation. In general LAB consists of non-spore forming Gram-positive bacteria with a DNA base composition of less than 53 mol



% G+C(292). LAB members include *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Enterococcus*, *Streptococcus*, *Pediococcus*, *Carnobacterium*, *Aerococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella*. The generation of lactic acid results in the recycling of electron acceptors for ATP generation by bacterial species. In addition to this role in the bacteria, lactic acid production benefits the host by reducing local pH and suppressing colonization and proliferation of potential pathogens. Lactic acid is readily miscible with water due to its low hydrophobicity and low acid dissociation constant (pKa). Lactic acid is effective against gram-negative bacteria and to a lesser extent effect against gram-positive bacteria (293, 294). As a liposoluble organic acid, lactic acid in its undissociated form can penetrate the bacterial cytoplasmic membrane (295). In gram-negative bacteria lactic acid transverses the outer membrane (OM) via water-filled porins and penetrates the cytoplasmic membrane. Additionally, in gram-negative bacteria, lactic acid can act as a potent OM-disintegrating agent, as evidenced by LPS release and sensitization of bacteria to detergents or lysozyme (294). Lactic acid-induced changes in cell membrane permeability can hinder substrate transport and promote further entrance of lactic acid into the cytoplasm, thereby effectively lowering the intracellular pH (294–296) (Figure 4). Intracellular acidity can suppress NADH oxidation, altering the membrane electron transport system and transmembrane proton motive force (296). Malfunction of the electron transport system can lead to oxidative stress and generation of free radicals that damage DNA and proteins. These free radicals can then damage intracellular components as well as the extracellular membrane (297). Collectively these mechanisms result in cell death to susceptible bacteria. Lactic acid in sufficient quantities may inhibit the growth of a wide range of bacterial species (245, 298, 299). Addition of pure lactic acid *in vitro* has both inhibitory and biocidal effects against several pathogens including gram-negative *Escherichia coli*, *Proteus mirabilis*, *Salmonella enteritidis*, *Pseudomonas aeruginosa*, gram-positive *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Bacillus cereus* and *Bacillus megaterium* and minimal fungicidal activity against the yeast species *Rhodotorula sp.*, *Saccharomyces cerevisiae* and *Candida albicans* (300). Generation of lactic acid by commensal bacteria also inhibits the growth a several pathogens including *H. pylori*, *B. subtilis*, *B. cereus*, *S. epidermidis*, *E. coli* CB6, *Klebsiella* CB2, *S. pyogenes*, *S. epidermidis*, *P. aeruginosa*, *S. paratyphi*, and *S. typhimurium* (299, 301–306)(307)(308). In these studies, lactic acid alters *in vivo* pH, inhibits pathogen urease activity, inhibits pathogen growth and acts as a bactericidal agent. Additionally L-lactic acid suppresses immune cell mediated pro-inflammatory responses (309). Modulation of the immune system and alteration in local GIT pH has been speculated to selectively manipulate the gut microbiota composition (120, 121, 310), which may further promote colonization resistance and inhibition of pathogens. In addition to the antimicrobial effects of lactic acid itself, several studies have demonstrated that unidentified bacterial substances, bacteriocins and hydrogen peroxide act in concert with lactic acid to inhibit the growth of pathogens (301, 304, 308).

## 2. Hydrogen Peroxide

Bacterial produced hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is known to act synergistically with L-lactic acid (311)(Figure 4). H<sub>2</sub>O<sub>2</sub> produced by certain microbes can damage bacterial nucleic acids by creating breaks in the carbon phosphate backbone of DNA, releasing nucleotides and

preventing chromosomal replication (312, 313). Additionally, hydroxyl radicals, which can be produced from the dissociation of H<sub>2</sub>O<sub>2</sub>, can attack the methyl group of thymine, resulting in damaged DNA (314, 315). Anaerobic bacteria are more sensitive to H<sub>2</sub>O<sub>2</sub> as they do not produce catalase which can break down H<sub>2</sub>O<sub>2</sub>. In general, gram-negative bacteria are more sensitive than gram-positive bacteria to H<sub>2</sub>O<sub>2</sub>. However, select gram-negative bacteria have developed a mechanism to deal with H<sub>2</sub>O<sub>2</sub>. These microbes utilize an outer lipopolysaccharide (LPS) layer which traps active molecular oxygen (316). Lactic acid disrupts the outer membrane (OM) of gram-negative bacteria, releasing LPS, and making cells sensitive to H<sub>2</sub>O<sub>2</sub> and antimicrobial agents (294). Several microbes are known to produce H<sub>2</sub>O<sub>2</sub> including lactobacilli and bifidobacteria (317, 318). Many studies have demonstrated that bacterial produced H<sub>2</sub>O<sub>2</sub> inhibits the growth of pathogens such as *Staphylococcus aureus*, *Salmonella enterica* serovar *Typhimurium*, *Listeria monocytogenes*, *Enterococcus faecalis*, *E. faecium*, enterotoxigenic *Escherichia coli*, *E. coli* CFT074, *Listeria ivanovii*, *Staphylococcus aureus*, *Yersinia enterocolitica*, *Aeromonas hydrophila*, *Gardnerella vaginalis* DSM494, *Neisseria gonorrhoeae*, *Streptococcus mutans*, *Bacteroides forsythus*, *Campylobacter sputigena*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Wolinella recta* (318–329). The effect of LAB produced H<sub>2</sub>O<sub>2</sub> on pathogen inhibition was found to be significantly enhanced by lactic acid (324), supporting the role of H<sub>2</sub>O<sub>2</sub> and lactic acid in concert, acting to shape microbial communities. However, commensal bacteria are not the sole producers of H<sub>2</sub>O<sub>2</sub>. The pathogen *S. pneumoniae*'s production of H<sub>2</sub>O<sub>2</sub> inhibited growth of viral *Haemophilus influenza* and fellow bacterial pathogens *Moraxella catarrhalis*, *Neisseria meningitidis* and *S. aureus* (330, 331). In this manner, it has been speculated that pathogens use H<sub>2</sub>O<sub>2</sub> production to inhibit competing organisms and secure a niche.

### 3. Bacteriocins

Production of antimicrobial compounds by bacteria provides specific microorganisms with a competitive advantage for colonization. Production of bacteriocins is a nearly universal trait, as it is projected that the majority of bacteria and archaea produce at least one bacteriocin (332–334). The ubiquity of this trait implies that bacteriocins play an important role *in vivo* as colonizing peptides, tools for inhibiting commensal or pathogen niche occupation, or as signaling peptides (334–339). Wide variation exists in the chemical composition and mechanisms of action of different bacteriocins. Bacteriocins can be classified based on the bacteria that secrete them (gram-negative or gram-positive). In general, bacteriocins produced by gram-positive bacteria have antimicrobial effects on other gram-positive bacteria (332). Production of bacteriocins by probiotic bacteria has been speculated to promote colonization and inhibit pathogens (161, 334). In contrast, production of bacteriocins by pathogenic bacteria has been postulated to provide a competitive edge for infection (340). Wide variation exists in the chemical composition and mechanism of action of different bacteriocins. Most bacteriocins target phosphate groups on bacterial cell membranes, deplete the transmembrane potential ( $\psi$ ) and/or the pH gradient and form membrane pores, resulting in membrane disruption and cellular leakage (341–343) (Figure 4). Similar to other compounds such as H<sub>2</sub>O<sub>2</sub>, bacteriocins may yield synergistic effects with lactic acid and exhibit greater antibacterial activities at lower pH values. To simplify our

review, we have chosen to focus on primarily bacteriocins produced by commensal bacteria, as opposed to ones produced by pathogens.

## Bacteriocin Diversity and classification

### Gram-positive Bacteriocins

**Class I: The Lantibiotics:** Lantibiotics are small (<5 kDa) peptides characterized by the unusual amino acids lanthionine (Lan),  $\alpha$ -methylanthionine (MeLan), dehydroalanine, and dehydrobutyrine. Within Class I molecules can be sub-grouped into type A or type B according to their chemical structures and antimicrobial activities (344–346). Type A Lantibiotics exhibit elongated screw-shaped peptides with a net positive charge. The shape and charge of type A molecules facilitate membrane pore formation and membrane depolarization in sensitive species. Type A molecules generally are 2 – 4 kDa in molecular weight. The best characterized of the type A lantibiotics is the *Lactococcus lactis* produced Nisin. Nisin inhibits the growth of a range of gram-positive bacteria including *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus* (347–350). Nisin also prevents spore germination by pathogens *Clostridium botulinum*, *Clostridium sporogenes*, *Bacillus cereus*, and *Bacillus anthracis* (342, 351–354). In vegetative cells, Nisin binds to lipid II on targeted bacterial membranes. Nisin orients parallel to the surface of the target membrane and inserts the C-terminus of peptide into the phospholipids, thereby disrupting cell wall biosynthesis and creating a “wedge-like” pore (355). Pore formation causes a rapid nonspecific amino acid and cation efflux and subsequent cell membrane rupture and cell death (356, 357). In spores, Nisin also utilizes lipid II binding and pore formation in germinated spores during outgrowth, leading to membrane disruption that inhibits spore development into vegetative cells. Type A lantibiotics include lactacin (*Lactococcus lactis* lactacin 3147 (358), *Lactococcus lactis* subsp. *lactis* lactacin 481 (359), lactocin (*Lactobacillus rhamnosus* lactocin 160 (343), *Lactobacillus sake* L45 lactocin S (360)), *Staphylococcus epidermidis* epidermin (361) and *Staphylococcus gallinarum* gallidermin (362). In comparison to type A molecules, type B lantibiotics are smaller (2– 3 kDa) globular peptides with a negative or neutral charge. In contrast to type A lantibiotics, type B peptides exert their antimicrobial activity via cell lysis and inhibition of essential bacterial enzymes. Type B lantibiotics increase membrane permeability and reduce ATP-dependent protein transport and ATP-dependent calcium uptake in sensitive bacterial cells, resulting in cell lysis. Cell lysis was significantly reduced when the type B lantibiotics cinnamycin and duramycin were incubated with the phospholipid phosphatidylethanolamine (363–365). The data indicate that type B lantibiotics interact with phospholipid targets. In addition to membrane effects, type B lantibiotics are also known to inhibit bacterial enzymes, such as phospholipase A and peptidoglycan synthesis. Examples of Type B lantibiotics include *Lactobacillus curvatus* curvacin A, *Streptomyces cinnamoneus* cinnamycin, *Streptomyces* ssp ancovenin (366) and *Streptoverticillium* R2075 duramycins B and C and *Streptomyces griseoluteus* (R2107) duramycins B and C (367).

**Class II:** Class II bacteriocins are small (<10 kDa), heat stable, non-lanthionine containing membrane active peptides. These peptides can be further divided into subgroups based on sequence and function: a, b, c, d and e. The subgroup class IIa comprises pediocin-like peptides containing an N-terminal consensus sequence -Tyr-Gly-Asn-Gly-Val-Xaa-Cys.

Class IIa bacteriocins are produced by food-associated bacterial strains and have garnered attention due to their anti-*Listeria* activity (368, 369). Similar to Class I molecules, class IIa bacteriocins kill target cells by permeabilizing the cell membrane (341, 370). Class II molecules bind to the target membrane using the cationic N-terminal beta sheet domain of the peptide, while the C-terminal regions form a hairpin-like domain which penetrates into the target cell membrane. This penetration results in leakage of cytoplasmic components through the membrane, resulting in cell death. Bacteriocins belonging to the class IIa family include *L. lactis* lactococcin MMFII, *Bifidobacterium bifidum* NCFB bifidocin B, *B. longum* subsp. *infantis* bifidin I, *Pediococcus acidilactici* pediocin PA-1, *Carnobacterium piscicola* carnobacteriocin B2, *C. divergens* divercin V41, *L. sakei* sakacin P, *L. sakei* sakacin A, *Enterococcus faecium* enterocin A, *E. faecium* enterocin P, *Leuconostoc gelidum* leucocin A, *L. mesenteroides* mesentericin Y105 (281, 371–379) and *Pediococcus pentosaceus* K23-2 (380). Class IIb contains bacteriocins that require two separate peptides for activity. Similar to Class IIa they act as pore-forming peptides (381). Class IIb peptides include *L. lactis* lactococcin G and M, *L. salivarius* UCC118 Abp118, *L. johnsonii* lactacin F, and *L. plantarum* plantaricin A, S, E, F, and JK (339, 358, 382–389). The class IIc peptides have a wide range of effects on membrane permeability and cell wall formation. One of the well documented class IIc molecules is *E. faecalis* bacteriocin AS-48 (390). Bacteriocin AS-48 consists of five alpha helices enclosing a hydrophobic core, creating a globular structure which creates membrane pores in susceptible gram-positive or gram-negative bacteria. Other examples include *L. acidophilus* acidocin B, *C. piscicola* carnobacteriocin A, *C. divergens* divergin A and *E. faecium* enterocin P and B (391–394). Class II d bacteriocins are linear, non-pediocin-like, single-peptide bacteriocins with similar antimicrobial activity. Class II d molecules include *L. lactis* lactococcin A, *S. epidermidis* epidermicin NI01, and *S. cremoris* diplococin (387, 395, 396). Class II e bacteriocins are comprised of non-ribosomal siderophore-type post-translational modifications at the serine-rich carboxy-terminal region of the peptide. Class II e molecules include *Klebsiella pneumoniae* microcin E492 (397, 398).

**Class III:** Class III bacteriocins are comprised of large molecular weight (>30 kDa) heat labile proteins. This class is further subdivided in two subclasses: subclass IIIa and IIIb. Subclass IIIa, also known as bacteriolysins, encompasses peptides that degrade bacterial cell membranes, resulting in cell lysis and subsequent cell death. Of subclass IIIa the most characterized is *Staphylococcus species* produced lysostaphin. Lysostaphin is a 27 kDa peptide that cleaves cell wall crosslinking pentaglycin bridges, thereby hydrolyzing susceptible staphylococci, including the pathogen *S. aureus* (399–401). Another member of the subclass IIIa group is *E. faecium* enterolysin (402). Subclass IIIb, comprises peptides which disrupt target cell membrane potential, causing ATP efflux and death. In contrast to subclass IIIa, these peptides do not cause cell lysis. Other members of Class III include bacteriocins from subclass IIIb include *L. casei* Caseicin 80, and *L. helveticus* helveticin J and V-1829 (403–405).

**Class IV:** Class IV bacteriocins were recently described and are defined as complex bacteriocins containing lipid or carbohydrate moieties. The class IV bacteriocins are cyclical due to covalent bonding of the first and last amino acids and are considered to be S-linked

glycopeptides with antimicrobial activity. Examples of class IV molecules include *Enterococcus faecalis* subsp. *liquefaciens* S-48 Enterocin AS-48, *E. faecalis* F4-9 Enterocin F4-9, *Bac. subtilis* 168 sublancin (a S-linked glycopeptide) and *L. plantarum* KW30 glycocin F (GccF) (406–409). Peptides from this class exhibit a wide pH range, variable resistance to heat and loss of antimicrobial activity when exposed to proteolytic enzymes (409–411). The cationic charges of enterocin F4-9 and glycocin F were found to be essential for interactions with charged phospholipids in target bacterial cells. These interactions did not induce cell lysis, implying a bacteriostatic effect these bacteriocins. Enterocin F4-9 yielded antimicrobial activity against *E. faecalis* and *E. coli* JM109, but not *Enterococcus faecium* and other *E. coli* members (409). In contrast, glycocin F was found to have activity against lactobacilli (411) while sublancin 168 had activity against gram-positive bacteria, specifically *Bacillus* species, but not *E. coli* JM101 (410). These studies indicate that class IV bacteriocins target unique epitopes.

### Gram-negative Bacteriocins

**Microcins:** Microcins are low molecular weight (<10 kDa) hydrophobic antimicrobial peptides synthesized on bacterial ribosomes. These molecules are primarily produced by gram-negative Enterobacteriaceae (phylum Proteobacteria). Microcins are generally heat, extreme pH, and protease tolerant (412). Microcins exhibit a wide range of structural diversity and antimicrobial mechanisms. Microcins target other bacterial cells via pore formation, DNase or RNase nuclease function, inhibition of protein synthesis or inhibition of DNA replication (413). One of the more deceptive ways microcins exert their antibacterial activity is through a ‘Trojan horse’ strategy whereby microcins imitate essential nutrients (412). Microcins are able to mimic small, high-affinity iron chelating compounds (iron-siderophore complexes). As iron is required for bacterial DNA synthesis, microcins resembling iron-siderophores are able to bind to outer-membrane receptors on target bacteria and translocate into the periplasmic space where they can exert their antimicrobial activity. Despite the large diversity, these molecules are classified in two classes depending on their molecular masses, disulfide bonds and post-translational modifications: class I and II. Class I microcins are plasmid-encoded, low molecular weight (<5 kDa) and are post-translationally modified. Examples of class I microcins include *E. coli* microcin B17, C7–C51, D93, and J25. Class II microcins are larger in molecular weight (5–10 kDa) and produced by and active against *Enterobacteria*. Class II microcins are subdivided into two subclasses, including class IIa and IIb. Class IIa microcins do not undergo post-translational modification while class IIb microcins exhibit post-translational modifications in the form of a salmochelin-like siderophore motif (414). Additionally, class IIa peptides typically contain 1–2 disulfide bond(s). Class IIa molecules include *Escherichia coli* microcins L, V, S, and N, all of which require three different genes to synthesis the molecule. Class IIb microcins are differentiated from class IIa due to their linear structure or lack of post-translational modifications at the C-terminal (413, 415). Examples of class IIb molecules include *E. coli* Nissle 1917 microcin M, *E. coli* Nissle 1917 microcin H47, and *Klebsiella* microcin E492.

**Colicins:** In contrast to low weight microcins, colicins are high molecular weight (25–80 kDa) proteins with antimicrobial activity. Colicins act by binding to outer membrane receptors, penetrating the cytoplasmic membrane, and then subsequently causing cytotoxic

effects, including cytoplasmic membrane depolarization, DNase activity, RNase activity, or murein synthesis inhibition. Colicins use a two-receptor system to target cells. First colicins bind to outer membrane receptors, such as porins OmpF, FepA, BtuB, Cir and FhuA, which are typically used for entry of specific nutrients into the bacterial cells. Next, colicins are translocated through the outer membrane and enter the cell cytoplasm by either the Tol or Ton system where they can exert their cytotoxic effect (416). Colicins are divided into categories based on their outer membrane translocation system (either Tol or Ton). The Ton and Tol systems are two protein arrangements which *E. coli* uses to transfer energy from the inner membrane to the outer membrane. The systems differ in their component proteins. The Ton system consists of TonB, ExbB, and ExbD, while the Tol system consists of TolA, TolQ, and TolR. In the Ton system, ExbB and ExbD proteins compose the energy harvesting complex which transfers energy to the protein TonB. This arrangement causes a conformational change in TonB which subsequently delivers energy to the outer membrane. In the Tol system, TolQ and TolR proteins compose the energy harvesting complex which transfers energy to TolA, resulting in a conformational change and energy to the outer membrane. The Ton system is involved in transport of compounds across the membrane, while the Tol system is involved in outer membrane maintenance. Group A colicins use the Tol protein system to traverse the outer membrane of sensitive bacteria, while group B colicins use the Ton system. Examples of group A colicins include colicins E1 to E9, colicin A, K, N and examples of group B molecules include colicin 5, 10, B, D, M, V, Ia, Ib (413, 417). Colicins can also be classified based on their mechanism of induced cell death: (1) Pore formation colicins, (2) Nuclease type colicins or (3) Peptidoglycanase type colicins. Pore formation colicins act by creating a pore within the bacterial cytoplasmic membrane, resulting in cytoplasmic content leakage, loss of electrochemical gradients and cell death. Examples of pore-formation colicins include colicin A, B, E1, Ia, Ib, K, and N. Nuclease type colicins utilize DNase, 16S rRNase, and tRNase to non-specifically digest DNA and RNA of targeted bacteria. Examples of these molecules include colicin E2 to E9. Finally peptidoglycanase type colicins are able to digest peptidoglycan precursors. This digestion prevents bacteria from synthesizing peptidoglycan, a key cell wall component, and results in bacterial death (418).

**Bacteriocins *In vivo***—Bacteriocin producing strains have been shown to have an ecological advantage over non-producing strains *in vivo*, pointing to the role of bacteriocins in niche development. For example, *E. coli* BZB1011 strains which produced colicins (A, E1, E2, E7, K, and N) were found to be persistent over time in mice, compared to non-colicin producing isogenic strains (419). Bacteriocins, particularly the lantibiotics from gram-positive bacteria, have been shown to be effective against a number of pathogenic strains *in vivo*. The lantibiotic nisin has been shown to inhibit pathogens *S. pneumoniae* (420), *Staph. aureus* (421–423), and *Listeria monocytogenes* in mice (424). Furthermore lantibiotic B-Ny266 was shown to inhibit *S. aureus* (425) and planosporicin inhibited *S. pyogenes* in mice (426). Class IIa bacteriocin peptides Enterocin CRL35 and divercin V41 were also shown to inhibit *L. monocytogenes* (427, 428) while bacteriocin E50–52 inhibited *Mycobacterium tuberculosis* in mice (429). Intraperitoneal injection of microcin MccJ25 reduced *Salmonella* Newport quantities in the spleen and liver in mice (430). As a result of

these *in vivo* studies, bacteriocins have been considered for the treatment of multi-drug resistant pathogens.

**Bacteriocins as Quorum Sensing Molecules**—For many bacteria, bacteriocin production is regulated by quorum sensing (161, 431, 432). Quorum sensing, as discussed earlier, is a cell-density dependent regulatory system where autoinducing signals facilitates bacterial communication. This system can sense the number of cells of the same species and synchronize the expression of key genes. Bacteriocin peptides are sensed by membrane-located histidine kinases, which transmits a signal via an intracellular response regulator, thereby activating gene transcription of the inducer bacteriocin molecule (433). The two-component signal-transduction mechanism is a key step for transcription activation and production of several bacteriocins or bacteriocin-like peptides. These include the Class I lantibiotics *L. lactis* nisin and *Bacillus subtilis* subtilin, as well as the class II *E. faecium* Enterocin A and *L. salivarius* UCC118 Abp118 (432, 434–438). Thus the bacteriocin peptide itself functions as a pheromone to induce its own production. When bacterial cell density is high, an autoinduction loop is activated and bacteriocins are likewise produced at high concentrations. In this manner, bacteriocins are released to target similar species only when the bacterial levels are high enough to suppress the growth of competitive strains.

Bacteriocins within biofilms are known to play important roles in bacterial competition, ecological fitness and overall community structure (93, 439, 440). In the naturally transformable streptococci, including *S. mutans*, *S. gordonii*, *S. sanguinis* and *S. mitis*, bacteriocin production is tightly regulated by a quorum sensing system that also regulates genetic competence and biofilm formation (93, 439, 440). *S. mutans* has served as a well-studied example of quorum sensing and bacteriocin production. *S. mutans* uses the ComCDE quorum-sensing system to connect to bacteriocin production, stress response, genetic competence and biofilm formation (93, 343, 439, 441–443). *S. mutans* ComC mutants do not produce the signaling molecule CSP, and these biofilms exhibit reduced biomass (93). However, supplementation of cultures with synthetic CSP restores the wild-type biofilm phenotype. *S. mutans* CSP-induced bacteriocin (CipB) can also stimulate cell lysis (444, 445). As a result, bacteriocin production within biofilms is speculated to provide balanced competition and coexistence of multi-organisms within a microbial community (439, 440, 446).

#### 4. Other biogenic substances

In addition to bacteriocins, select microbes are known to produce bacteriocin-like substances (BLS). BLS are used to describe compounds which have antimicrobial properties, but are not ribosomally produced or gene-encoded precursor peptides. Examples of this class of compounds include reuterin and reutericyclin.

**Reuterin**—Select strains of *Lactobacillus reuteri* are able to generate reuterin (3-hydroxypropionaldehyde) during anaerobic fermentation of glycerol (447, 448). During this process glycerol is reduced 1,3-propanediol, an event catalyzed by the coenzyme B12-dependent diol dehydratase, which regenerates NAD<sup>+</sup> from NADH (449–451). Reuterin is water-soluble, active over a wide range of pH values and bioactive against gram-positive and

gram-negative bacteria, viruses and fungi (452). The aldehyde group of reuterin is highly reactive with thiol groups and primary amines. As a result reuterin inhibits bacterial growth by modifying protein thiol groups and inducing oxidative stress in target cells (450). Production of reuterin by *L. reuteri* was found to be enhanced by interactions with *E. coli*, indicating that cross-talk between members of the gut microbiota may regulate reuterin output (450). Reuterin has also been demonstrated to act synergistically with other bacteriocins. Nisin, lacticin 481 or enterocin AS-48 were found to enhance antimicrobial activity of reuterin against the pathogen *L. monocytogenes* (453). However, only nisin was found to increase antimicrobial activity of reuterin against *S. aureus* (453). These studies indicate that synergism between reuterin and bacteriocins are dependent on the bacteriocin and pathogen. Importantly reuterin was found to be produced by *L. reuteri* in mono-associated germ-free mice, indicating that reuterin can be produced *in vivo* in the GIT (454).

**Reutericyclin**—Reutericyclin is a highly hydrophobic, negatively charged compound produced by certain *L. reuteri* strains (455, 456). Reutericyclin ((5R)-1-(2-decenoyl)-2-hydroxy-3-acetyl-5-isobutyl-2-pyrroline-4-one) is a N-acylated tetramic acid (456) and its mode of action resembles that of weak organic acids. Reutericyclin targets the cytoplasmic membrane of sensitive bacteria by acting as a proton-ionophore. This action results in the translocation of protons across the cytoplasmic membrane and subsequent collapse the transmembrane pH (455). Reutericyclin exhibits a broad inhibitory spectrum against gram-positive pathogens including *E. faecalis*, *Listeria innocua*, *S. aureus*, *Bacillus subtilis*, and *B. cereus* (456). Reutericyclin has also been shown to inhibit germination of *Bacillus* spores (456). However gram-negative bacteria are resistant to reutericyclin due to the presence of LPS in their outer membrane. LPS is known to limit access of hydrophobic compounds and the charged polysaccharide moieties of LPS has been proposed to bind reutericyclin, thereby rendering it inactive (455, 457).

## SUMMARY AND CONCLUSIONS

This chapter highlighted multiple well-characterized systems used by commensal bacteria to communicate with other microbes and the mammalian host, to modify microbial communities, and to beneficially modulate the function of the mammalian host. These studies emphasize key roles of bacterial metabolites and secreted products in fine-tuning mammalian biology. Bacteria are capable of secreting multiple and chemically diverse host-modulating factors. This work has focused on commensal bacteria, but a number of these factors can also be used by pathogens. Future studies may begin to define the secretion of products in various environments and under different conditions (i.e. inflammation, altered pH, various dietary nutrients). As we begin to understand health-promoting compounds and metabolites generated by commensal bacteria, we will be better able to identify individual species and strains which can be tailored for specific physiological or biochemical targets of interest. The work herein highlights the importance of selecting well-characterized commensal or probiotic strains with known mechanisms to address specific disease processes. The recent development of the US edition of the Clinical Guide to Probiotic Products reflects the growing need for evidence based selection of probiotics. This online database provides guidelines for probiotic products and corresponding research studies



supporting the use of each specific probiotic (<http://usprobioticguide.com/>). These tools will likely aid clinicians in selecting disease-relevant bacterial species.

Genetic engineering of probiotic strains has also been used as a method to either enhance production of molecules of interest (124). In this system bacteria could be designed with upregulated gene synthesis or to be constitutively active. Using this model, issues of whether a given product is being secreted *in vivo* under various conditions (pH, ion composition, changing osmolarity) could be minimized. Strains have also been engineered with genes from other bacteria to create organisms capable of secreting multiple beneficial factors. Microbial engineering may lead to the production of “super” strains with the ability to release multiple factors targeted for different purposes (i.e. immunomodulation, pathogen exclusion, enhancing the mucus barrier). Additionally genetic engineering has also be used to facilitate probiotic selection and maintenance in the host (124). This method may ensure the transient introduction of strains which are not desired to colonize the host long term. Currently social acceptance of genetically modified (GM) foods or microbes is not universal and thus these types of modifications will likely be closely monitored in the future. An alternate approach could be to isolate and purify valuable metabolites, which themselves could also be used directly as novel therapeutics for specific disorders.

Based on studies conducted thus far, we have strong evidence to pursue selection of probiotics and examination of specific microbial genes, proteins, and metabolites of interest. In the future we hope to use this knowledge to thoughtfully select commensal strains for a given disease treatment or prevention. Overall, these studies contribute to the rapidly expanding body of evidence demonstrating that secreted microbial metabolites can beneficially influence host physiology. In addition to probiotics, future dietary and medicinal interventions may depend on the foundations of microbial metabolite and protein discovery in the context of microbiome science.

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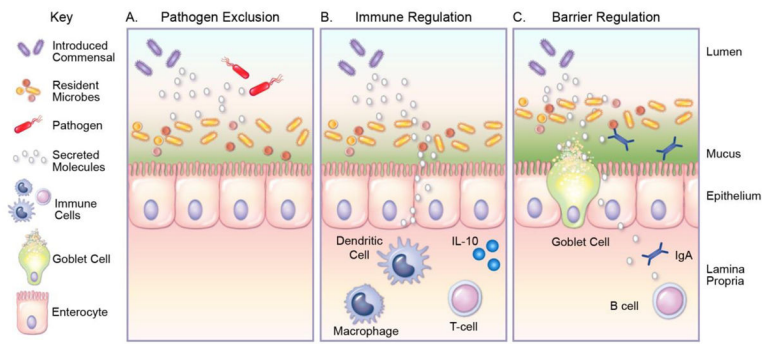
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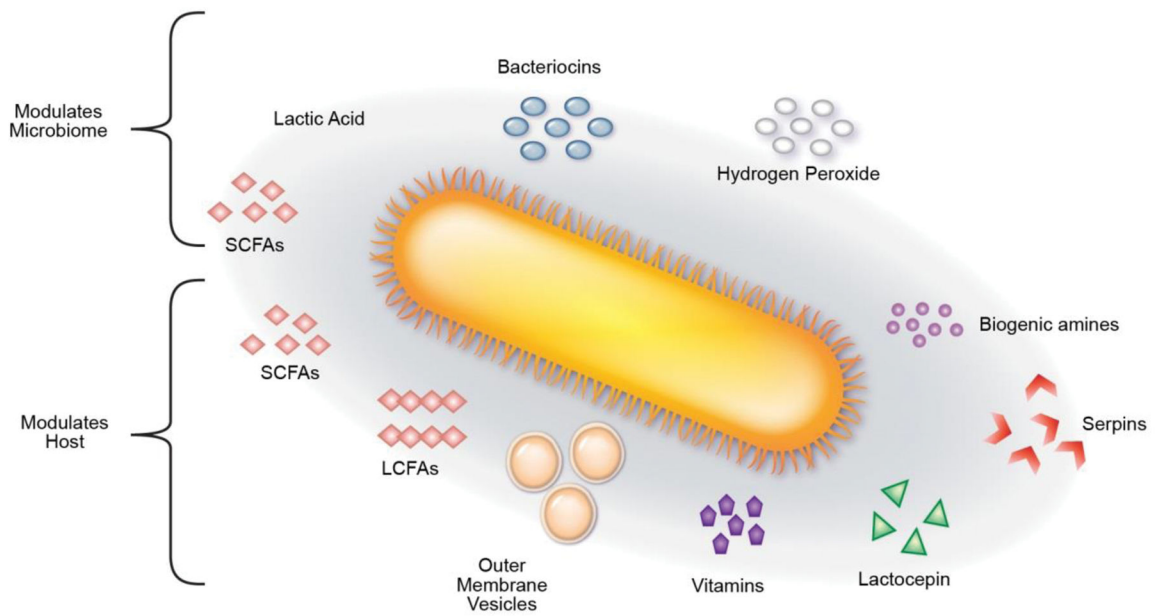


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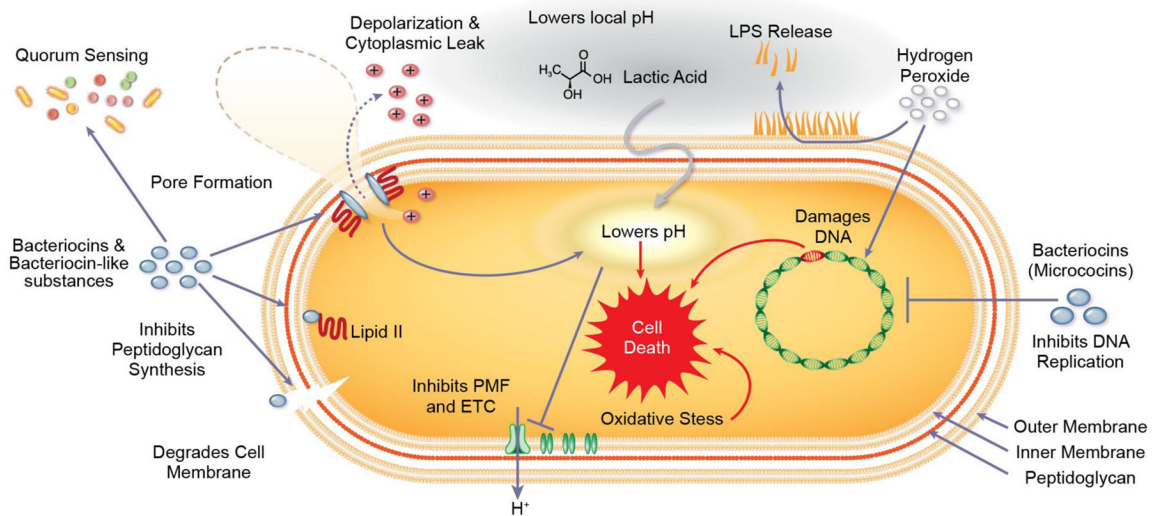
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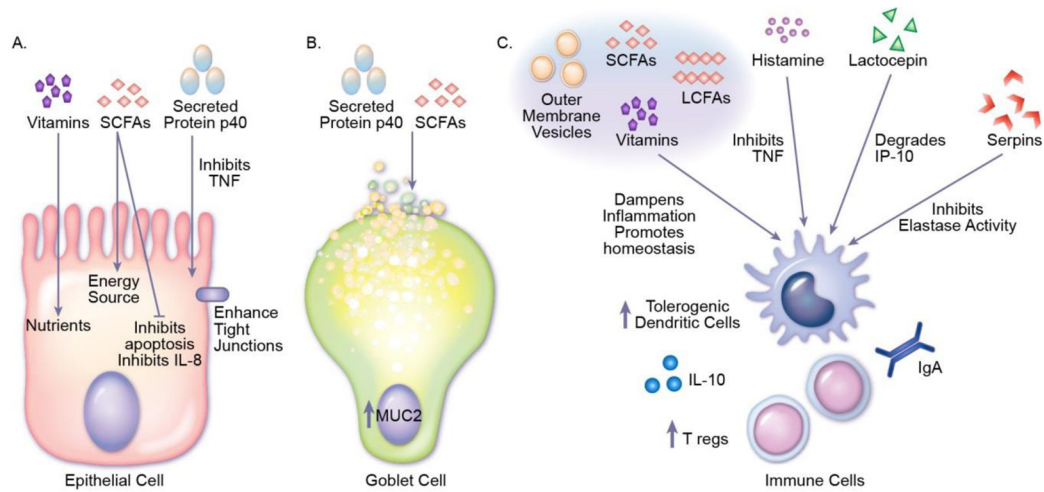
**Figure 1.** Methods utilized by commensal bacteria to beneficially modulate the intestinal environment. **A.** Commensal bacteria are able to secrete molecules which can alter the gut microbiota. By selectively inhibiting resident microbes, commensal bacteria are able to establish an intestinal bacterial niche. Production of anti-microbial factors has also been shown to exclude pathogens. **B.** Select commensal bacteria are also able to secrete compounds which can modulate immune cells such as macrophages, dendritic cells or lymphocytes such as T cells. These compounds decrease intestinal inflammation by dampening pro-inflammatory cytokines and promoting anti-inflammatory factors such as Interleukin (IL)-10. **C.** Finally, commensal bacteria can secrete factors which modulate the functions of the epithelial barrier by enhancing the secretion of the protective mucus layer, upregulating tight junctions and promoting secretion of molecules such as IgA.



**Figure 2.** A depiction of secreted metabolites from commensal bacteria and their interactions with the microbiome or host. Lactic acid, hydrogen peroxide, short chain fatty acids (SCFA), and bacteriocins are all capable of serving as quorum sensing molecules and/or directly modulating the composition of the microbiome. SCFAs, long chain fatty acids (LCFAs), outer membrane vesicles, vitamins, lactocepins, serpins and biogenic amines have all been demonstrated to beneficially modulate the host. Together these bacterial products shape the intestinal environment and host.



**Figure 3.** Schematic representation of the molecular mechanisms of commensal secreted products on a gram-negative bacteria. Bacteriocins are classified based on their structure. Bacteriocins such as nisin bind to a peptidoglycan subunit transporter, thereby preventing cell wall synthesis and resulting in cell death. Furthermore, bacteriocins can initiate pore formation. Pore formation depletes the bacterial transmembrane potential ( $\psi$ ) and/or the pH gradient, resulting in membrane disruption and cellular leakage that leads to rapid cell death. Other bacteriocins are able to directly insert themselves directly or degrade the target membrane, leading to depolarization and death. Bacteriocins have also been shown to serve as quorum sensing molecules for other microbes. Lactic acid decreases local pH and suppresses the growth and survival of pathogens. Additionally, un-dissociated lactic acid can traverse the outer membrane (OM) via water-filled porins and penetrate the cytoplasmic membrane. This shift lowers the intracellular pH and disrupts the transmembrane proton motive force and generates oxidative stress. Hydrogen peroxide and select bacteriocins such as micrococins damage bacterial DNA and inhibit cell growth. Together, these compounds secreted by select members of the microbiota effectively target pathogens.



**Figure 4.**

Mechanisms by which commensal secreted products beneficially modulate the host. **A.** Epithelial cells. Vitamins produced by bacteria provide essential nutrients to the host. Likewise, short chain fatty acids (SCFA) such as butyrate are known to serve as energy sources for intestinal epithelial cells. The SCFA acetate has also been shown to inhibit IL-8 production and increases tubulin- $\alpha$  acetylation. Lactobacilli produced p40 and p75 inhibit tumor necrosis factor (TNF)-induced apoptosis and enhance tight junctions which attenuates intestinal barrier disruption. **B.** Goblet cells. p40 is known to transactivate the EGF receptor (EGFR), activating the downstream target Akt and stimulating Muc2 gene expression and mucin production. Acetate produced by bacteria has also been shown to increase goblet cell differentiation and expression of mucus-related genes. **C.** Immune cells. Vitamins, Outer Membrane Vesicles (OMVs), SCFA and LCFAs are known to directly influence the development and function of immune cells. In general these molecules modulate T cell and dendritic cell homeostasis and cytokine production, promoting production of anti-inflammatory IL-10 and inhibiting pro-inflammatory cytokines such as TNF. Biogenic amines such as histamine have also been shown to suppress pro-inflammatory cytokines such as TNF in immune cells, thereby ameliorating intestinal inflammation. Bacterial enzymes such as lactocepin selectively degrade lymphocyte recruiting chemokine IP-10 and other pro-inflammatory chemokines such as I-TAC and Eotaxin. The protease inhibitor serpin has been shown to suppress inflammatory responses by binding and inactivating neutrophil elastase. Using the highlighted mechanism, commensal bacteria produce signals that reduce intestinal inflammation and promote health.