Deciphering the chemical lexicon of the gut microbiota

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Abstract

The enduring coexistence between gut microbiota and host has led to a symbiotic relationship that benefits both parties. In this complex, multispecies environment, bacteria can communicate through the exchange of chemical molecules to sense and respond to the chemical and physical properties and ecology of the surrounding environment. One of the best studied cell-to-cell communication mechanisms is quorum sensing. Chemical signaling through quorum sensing is involved in regulating bacterial group behaviors, often required for host colonization. Here, we will focus on the latest reports of quorum sensing in the gut microbiota and on group behaviors adopted by microbiota symbionts to efficiently colonize the mammalian gut. Moreover, we will address challenges and approaches to uncover molecule-mediated communication mechanisms, which will allow to unravel the mechanisms that drive the establishment of gut microbiota.

Introduction

The long-lasting interactions between the diverse repertoire of gastrointestinal bacterial species (microbiota) and the host seem to have coevolved into a symbiotic relationship that fulfills several important functions for both players. An imbalanced microbiota (dysbiosis) has been associated with increased susceptibility to infection, inflammatory bowel disease (IBD), diabetes and obesity.[1-3] The microbiota complements the host coding capacity providing a repertoire of additional metabolic functions including the digestion of complex polysaccharides, production of fatty acids, essential amino acids, and vitamin biosynthesis.[4-6]In addition, gut microbes promote the development of the intestinal tract, digestion of dietary components, maturation of the immune system, immunological tolerance to antigens, and protection against pathogens.[1,7,8] Despite inter-individual variation at the species level, there is strong conservation at the highest taxonomic levels within the bacteria colonizing the mammalian gut. The predominant phyla are Bacteroidetes and Firmicutes, with a smaller proportion of Proteobacteria, Verrucomicrobia, and Actinobacteria phyla being present.[9] It is likely that the major molecular processes involved in the functions of these communities are also conserved, but our understanding of the most relevant molecular properties of the microbiota are only now starting to be revealed. While there is no doubt that microbiota species composition is important, and diversity of the microbiota community is clearly associated with host health, species composition provides limited insights into its mechanisms of action and functional properties. The gastrointestinal tract is a very rich environment where microbes interact in a battle for space and nutrients through different mechanisms. Strategies involving microbe-microbe interactions mediated by signaling, metabolic networks, competition for nutritional niches occupancy, production of inhibitory toxic compounds or contact-dependent mechanisms, all seem to play a role in the establishment and resilience properties of the gut microbial community. Deciphering the molecular bases of these mechanisms, beyond description based on species composition, is essential to understand the functional role of the microbiota.

We have learned from microbial culture studies in laboratory settings (*in vitro*) and infection studies in animal models with pathogens, that microbial interactions through diffusible molecules enable bacteria to gather information about the ecology, chemical and physical properties of the environment, and respond by modulating gene expression accordingly.[10-13] Evidence is accumulating that similar processes are also relevant in the regulation of bacterial functions in the dense communities colonizing the mammalian gut.[14-17] Theoretically, all the molecules present in the chemical repertoire of the mammalian gut have the potential to directly influence members of the microbiota community. These molecules can either be directly

channeled into metabolic pathways altering the metabolism of the recipient bacteria or can function as canonical signals inducing responses at the level of gene expression that change the behavior of the bacteria. In both cases, these molecules can have important consequences for the physiology and behaviors of specific populations or bacterial communities. It is not always easy to differentiate between these different types of responses. The distinction between metabolite and signal has deserved much discussion in the past.[18] Here, we will use the term metabolite when referring to intermediates or end-products of metabolism, and signals for molecules which, at least in *in vitro* cultures, have been shown to induce changes in gene expression mediated by specific sensing mechanisms (receptors). Moreover, the response to this signal, at the level of gene expression, should include more than changes involved in the biosynthesis or catabolism of the molecule in question. On the basis of evolutionary and ecological considerations, in theory, signals that mediate cell-to-cell communication should present a benefit for the receiver of the signal to respond, and this response should benefit the sender in return.[18,19] However, that is difficult to prove and evidence for this is often lacking.

In this review, we will discuss challenges and possible approaches to decipher the chemical molecules exchanged among the intestinal bacterial species, as an entry point for the identification of the major microbial mechanisms driving the establishment and resilience of the microbiota. We think that such level of knowledge is essential towards the identification of the key microbiota functions that influence human health and disease.

Bacteria communicate through chemical signals to regulate group behaviors

Bacteria use sophisticated cell-to-cell signaling mechanisms to regulate gene expression on a population-wide scale. Quorum sensing is highly prevalent among these organisms, being one of the best studied cell-to-cell signaling mechanisms in bacteria.[10,11,13,20-22] This intercellular mechanism of communication enables bacteria to communicate via chemical signals and engage in group behaviors. Hundreds of species of bacteria have been shown to use quorum sensing systems to synchronize gene expression of bacterial populations as a function of cell density regulating processes including antibiotic production, virulence gene expression, competence, and biofilm formation.[12,13,22] Quorum sensing is mediated by the production, release, and subsequent detection of small diffusible molecules, known as autoinducers. Upon detection of the autoinducer, signal recognition leads to activation of signal transduction cascades that changes gene expression of the bacterial populations responding to the signal. Often, processes regulated by quorum sensing are essential for enabling bacteria to form communities that can benefit from

group behaviors. Such processes include, for example, degradation of extracellular polymers by the production of lytic enzymes (e.g.: proteases in the human pathogen *Pseudomonas*,[23] plant cell-wall degrading enzymes in plant pathogens like *Erwinia* and *Pectobacterium* spp.[24] or production of secreted compounds required for the formation of biofilm's extracellular matrices.[25,26] These processes encompass the release of sharable compounds and thus are only productive in highly dense populations, as it can happen in the dense mammalian gut microbiota communities. Extracellular degradation of complex polysaccharides is a core function of the members of the microbiota, and evidence that gut microbiota members can form biofilms and/or interact by contact-dependent mechanisms is also emerging. Thus, it is reasonable to expect that gut microbes that need to compete and, sometimes, cooperate in the dense gut environment, will also benefit from having these processes regulated by quorum sensing. While there are examples of very specialized dual-partner microbe-host symbioses, such as the *Vibrio*-squid and the *Rhizobium*-plant symbioses, known to rely on quorum sensing-regulated traits for host colonization,[27,28] the vast majority of microbial-host signaling interactions mediated by quorum sensing systems has been studied mainly in the context of pathogenesis. Thus, the knowledge of the role of chemical crosstalk between symbiotic members of the microbiota and the host, in health and disease, is still very limited.

Here, we will highlight recent advances in the potential role of quorum sensing as means of bacterial communication in the mammalian gut, as well as examples of group behaviors adopted by microbiota symbionts.

Fostering chemical communication in multispecies communities: regulated behaviors

In nature, bacteria often exist in multispecies communities, hence, it is expected that interspecies communication plays an important role in natural complex consortia, as found in the gut environment. The only known and widely studied interspecies and inter-phyla quorum sensing system is mediated through the Autoinducer-2 (AI-2) signal molecule. In the 90's, Bassler *et al*. proposed that two signal-response pathways converge to regulate luminescence expression in *Vibrio harveyi*.[29] The second signal was named AI-2 and it was proposed to be involved in interspecies communication. Its role in interspecies communication was proposed due to the observation that cell-free supernatants of different bacterial species could induce bioluminescence in *V. harveyi*, suggesting that different bacterial species, like *Salmonella* and *Escherichia coli*, produce AI-2.[30] While *E. coli* or *Salmonella* are unlikely to coexist with *V. harveyi* in the marine environment, this interspecies signal was also shown to repress virulence in another *Vibrio* species – *V. cholerae* – and cross-species signaling was demonstrated when AI-2 was

delivered by *E. coli*.[31,32] These two bacteria can coexist in the human intestine during *V. cholerae* infection. High AI-2 levels in the gut produced by *E. coli* might signal to *V. cholerae* that too many competitors exist, hence downregulation of virulence and host dispersal could be a more effective strategy for survival. As for *E. coli,* it can perform chemotaxis towards AI-2, leading to the formation of cell aggregates, that enhance bacterial stress resistance and promote biofilm formation.[33] Moreover, secretion of AI-2 by *E. coli* seems to also attract other AI-2 producers, like *Enterococcus faecalis*. This AI-2-dependent chemotaxis results in enhanced biofilm formation of a mixed community (Figure 1A).[34] In other species, AI-2 production has been shown to influence the architecture, volume and species composition of mutualistic biofilms between *Streptococcus oralis* and *Streptococcus gordonii,* two closely related oral bacteria.[35,36] Presumably, such AI-2-induced coaggregation of mixed species benefits the community by making them less susceptible to invasion by other species.

Multiple gut-associated bacteria that encode the AI-2 synthase, LuxS, or that produce AI-2 have been identified, with a large proportion of Firmicutes and Proteobacteria, and some species of Bacteroidetes and Actinobacteria, encoding putative LuxS orthologs.[37,38] The first study investigating the effect of AI-2 manipulation in the mammalian gut revealed that AI-2 can shape the composition of the gut microbiota in mice, by influencing the most abundant phyla in the gut: Bacteroidetes and Firmicutes (Figure 1A). Prolonged treatment with streptomycin in mice drastically altered the proportions of these two major phyla in the gut, by almost entirely depleting Firmicutes, while allowing the expansion of Bacteroidetes (specifically *Bacteroides vulgatus*, recently renamed *Phocaeicola vulgatus*), which completely dominated the mouse microbiota.[39] This antibiotic-induced extinction of Firmicutes offered the possibility of analyzing the effect of manipulating AI-2 levels in the gut by engineering *E. coli* strains to deliver, scavenge, or not interfere with the AI-2 signal. Increasing AI-2 levels suppressed Bacteroidetes expansion and contributed to the increase of Firmicutes in the gut, countering the effect of streptomycin, while still being under treatment. Interestingly, estimates from the genomes available in 2015 indicated that among these two major phyla present in the gut, about 80% of Firmicutes encode the *luxS* gene, while less than 20% of Bacteroidetes are potential producers of AI-2.[39] Therefore, the AI-2 producing *E. coli* in antibiotic-induced dysbiosis seems to favor AI-2 producers, presumably leading to a positive feedback loop, a common phenomenon in quorum sensing-regulated systems. This report showing AI-2 having an impact in the gut at a microbial community level, opened the door to the possibility of using quorum sensing signals to tailor the gut microbiota composition to our benefit. Given the different capabilities for AI-2 production in bacteria belonging to Bacteroidetes and Firmicutes, perturbations to the equilibrium among these phyla in the gut,

which are known to have consequences to host health,[40,41] will also potentially cause altered AI-2 levels and shifts in gene expression profiles and in important bacterial group functions. Interestingly, no effect was observed upon AI-2 scavenging, possibly because Firmicutes depletion by streptomycin already leads to a strong decrease in AI-2 levels and, therefore, there is little left to scavenge. In the future, it would be interesting to analyze if AI-2 scavenging on microbiotas with increased and disproportional levels of Firmicutes, which is characteristic of undernourished people as well as obese patients under western style diet,[42] could counter dysbiosis by decreasing the levels of Firmicutes and promoting the expansion of Bacteroidetes.

As additional members of the Bacteroidetes phylum are being discovered, it is becoming clear that the proportion of members of this phylum with *luxS* homologues might be higher than the previously reported 20%.[39] The distribution of *luxS* homologues in representative members of this phylum, shown in Figure 1B, highlights how AI-2 producers might be more prevalent among members of the recently identified *Muribaculaceae* family, as well as in the *Barnesiellaceae* family.[43] Future experiments will reveal if these microbiota species with *luxS* homologues, and thus with AI-2 production capability, will also respond positively to increasing AI-2 levels in gut colonization after perturbations.

Mechanistic insights into the role of AI-2 in the gut have been focusing on the role of AI-2 produced by specific commensals or pathogens colonizing the intestinal tract, particularly the ones that form biofilms. In *Limosilactobacillus reuteri* (formerly *Lactobacillus reuteri*), *luxS* has been shown to influence adherence and biofilm formation in the mouse gastrointestinal tract, since a *luxS* mutant strain forms thicker biofilms when compared to the wild-type (WT) strain,[44] seemingly by destabilizing their perception of the environment. Moreover, *in vitro* studies with a closely related species showed that *luxS* gene expression increased significantly in *Lactobacillus acidophilus* cells in the mid-exponential phase either after incubation with viable *Listeria monocytogenes* cells or after addition of cell-free culture supernatants of *L. monocytogenes*.[45] This indicates that the increase in *luxS* expression is a response to a secreted compound produced by *L. monocytogenes* cells, that can also regulate AI-2 behaviors, such as the aforementioned adherence capacity, in *L. acidophilus,* which competes with *L. monocytogenes* in the intestinal tract. Additionally, in pathogens like *Clostridioides difficile* and *V. cholerae*, biofilm formation and production of virulence factors seem to be coupled with AI-2 production, although in an inverse manner, with AI-2 acting as an activator or a repressor in *C. difficile* and *V. cholerae,* respectively. AI-2 produced by *C. difficile* is involved in prophage induction and modulation of species interactions, namely by affecting the formation of multispecies biofilms with *Bacteroides fragilis,* which in turn suppresses growth, biofilm formation, and virulence in *C. difficile*.[46] In germ-

free mice co-colonized with *V. cholerae* and an *E. coli* strain expressing the *luxS* gene from *Blautia obeum* (formerly *Ruminococcus obeum*), there was significantly lower *V. cholerae* colonization compared to co-colonization with an *E. coli* serving as a vector control strain.[47] This effect of the AI-2 producing *E. coli* might be through the AI-2-mediated repression of virulence and biofilm formation that may cause *V. cholerae* dispersal and decreased colonization (Figure 1A). In any case, this would hardly be a specific interaction with the AI-2 produced by *B. obeum*, since the native AI-2 production of *E. coli* can exert similar responses *in vitro*.[32] Biofilm formation requires cell aggregation and AI-2 has been shown to be able to act both as a chemoattractant and a chemorepellent molecule in *E. coli* and *Helicobacter pylori*, respectively (Figure 1A)[33,48-51]. *E. coli* self-produced AI-2 attracts bacteria in a chemotaxis-dependent manner towards growing aggregates, which enhances formation of mature biofilms. In a recent study, these *E. coli* aggregates were visualized in intestinal contents.[52] This study provided the first evidence for the benefit of chemotaxis *in vivo* by demonstrating that chemotaxis towards AI-2 is advantageous for *E. coli* gut colonization. The authors proposed that chemotaxis towards AI-2 controls niche occupancy, as the ability to sense AI-2 enabled *E. coli* to occupy specific nutrient niches. The mechanisms of action involved in this process, namely the AI-2 receptors, have already been identified, and will be further discussed below.

Beyond biofilm formation, AI-2 might have a role in niche competition between probiotics and intestinal pathogens. *Bifidobacterium spp.* are known probiotics shown to be protective against entero-hemorrhagic *E. coli* (EHEC) and *Citrobacter rodentium* infections.[53,54] Specifically, in *B*. *breve*, *luxS* was shown to be essential for gastrointestinal colonization of a murine host and for promoting iron acquisition.[55] Iron availability increases the pathogenic potential of several gastrointestinal pathogens, and so, AI-2-mediated iron scavenging by *Bifidobacterium* might interfere with pathogens' colonization of the gut through competition for iron (Figure 1A).

Many other studies have been conducted with this molecule and many AI-2-regulated phenotypes, which might be important for bacterial survival in the gut environment, have been described.[56] Furthermore, bacterial communication in the gut might be monitored by the host, which seems to have *learned* to sense and respond to what bacteria are signaling. Another study demonstrated that AI-2 secreted from a nonpathogenic *E. coli* stimulates the transcription of immune-related pathways, such as NF-kB, followed by a negative-feedback response, mainly through NOD-like signaling pathways, which may serve to temper the inflammatory tone.[57] Additionally, it was shown that a molecule that mimics AI-2 is produced by colon, lungs, and cervical mammalian cell lines, in response to bacterial cell factors and tight-junction disruption.[58] The function of this host-produced AI-2 mimic is not yet clear (Figure 1A). Given that some

bacteria can perform chemotaxis towards AI-2, one could speculate that this could be a host strategy to repel or attract specific species to form biofilms near the epithelial barrier.[48] This is only one example of how, by mimicking the autoinducer responsible for interspecies communication, the host might be able to maximize the manipulation of bacterial behavior in its favor, in a multispecies environment like the mammalian gut.

Fostering chemical communication in multispecies communities: mechanistic insights

AI-2 is a product of central metabolism derived from S-adenosylmethionine (SAM), and the biosynthesis pathway for AI-2 is widely conserved, with LuxS homologs being present in more than 500 different bacterial species.[56] However, the same is not true for the AI-2 signal transduction mechanisms, as AI-2 receptors are not as conserved as its synthase. Until recently, only two AI-2 receptors had been identified, LuxP and LsrB. Both receptors are periplasmic-binding proteins that belong to the high-affinity substrate-binding protein family. Nevertheless, sequence similarity between these two receptors is very low, and they act through different regulatory mechanisms. Upon AI-2 binding, LuxP modulates the activity of a membrane-spanning sensor protein, regulating a phosphorylation signal transduction cascade, while LsrB interacts with the membrane components of an ABC transport system (encoded in the LuxS regulated – *lsr* – operon) that internalizes AI-2.[56] Additionally, these receptors bind different chemical forms of AI-2. Whereas LuxP binds the AI-2-borated form, LsrB binds the non-borated form of AI-2. LuxP was first identified in *V. harveyi*[59] and has only been found in the Vibrionales. LsrB, on the other hand, was first identified in *Salmonella* Typhimurium,[60] but it is present in other enteric bacteria and members of the *Rhizobiaceae*, *Clostridiaceae*, and *Bacillaceae* families.[61,62] LsrB and the remaining genes in the *lsr* operon are involved in AI-2 recognition, internalization, and degradation. This behavior at high cell density results in AI-2 elimination from the environment and, consequently, AI-2 can no longer be used to trigger other group behaviors. This signal depletion was proposed to be a quorum sensing interference mechanism,[32] as part of the population can be misled to interpret the environment as low in density, preventing their engagement in complex group behaviors. *lsrB* was also shown to be responsible for the phenomenon described above, by which *E. coli* can swim in response to AI-2 gradients. This AI-2-dependent chemotaxis enabled *lsrB* positive *E. coli* strains to occupy specific nutrient niches in the gut.[52] Therefore, AI-2 might signal to free-living bacterial cells to swim towards the source of AI-2 or AI-2 mimic, recruiting these planktonic cells to form biofilms, while AI-2 internalization can disrupt this recruitment, possibly to avoid increased interspecies niche competition. Similarly, although using the opposite strategy, *H. pylori* is chemorepelled by the detection of AI-2 through

the chemoreceptor TlpB, which is also thought to be involved in the mechanism of nutrient competition avoidance, in the stomach.[51]

Identification of AI-2 receptors homologous to the *Salmonella* LsrB receptor was possible by combining sequencing analysis with biochemical and genetic studies. The demonstration that functional LsrB receptors exist in *Bacillus* and *Clostridium* genera was important to show that gram-positive bacteria belonging to the Firmicutes phylum also sense AI-2, thus confirming that AI-2 can foster signaling across bacteria from different phyla.[62] The following criteria can be used to guide the identification of potential LsrB receptors: I) amino acid sequence identity higher than 36% with LsrB receptors from gram-negative/positive organisms, II) genomic context of the *lsrB* gene (*i. e.*, the organism under study has orthologs for the other key transport proteins encoded by the *lsr* operon), and III) the conservation of amino acid residues in the putative ligand-binding site.[62] As these receptors share sequence and folding similarities with other large families of high-affinity ligand binding proteins (namely sugar-binding periplasmic proteins), elucidation of the protein structure with bound AI-2 and/or measurement of protein-binding affinities are required to confirm the role of these proteins as AI-2 receptors; AI-2 receptors should bind the signal with an affinity in the sub-micromolar range, as previously described.[62]

Even though these criteria have led to the successful identification of LsrB-like AI-2 receptors in bacteria from different phyla (Proteobacteria, Firmicutes, and potentially Spirochaetes and Actinobacteria), there are still many bacteria that respond to AI-2 and have no LsrB or LuxP homologues.[56] For example, phage dispersal, biofilm formation and interbacterial interactions within biofilms, have been shown to be regulated by AI-2 in *E. faecalis*, *Staphylococcus aureus* and *C. difficile*, respectively, but no LsrB or LuxP homologues have been found in these bacteria.[46,63,64] It is possible that unknown AI-2 receptors exist that share an identical fold to LsrB and LuxP, but have a very low homology to the known receptors, because that is what happens with LsrB and LuxP, which only share 11% amino acid sequence identity. Recently, a chemical-AI-2 probe (d-desthiobiotin-AI-2) incorporated in a pull-down protocol was shown to bind the known AI-2 receptors, providing a new tool to potentially unravel new classes of receptors and associated quorum sensing mechanisms.[65] More recently, a new class of AI-2 receptors, which seems to be widespread across different phyla, has been identified in *Pseudomonas*. *Pseudomonas aeruginosa* is an example of a species where AI-2 signaling is implicated in chemotaxis and biofilm formation, even though it does not produce AI-2, nor possesses canonical receptors.[66] Deletions of two transmembrane proteins with a dCache_1 domain abrogated chemotaxis to AI-2 in *P. aeruginosa* and variations in the conserved residues led to decreased affinity to AI-2 of these novel receptors.[66] Transmembrane proteins with

dCache_1 domains are amongst the most abundant extracytoplasmic sensors in bacteria and more than 1500 dCache_1-containing proteins from bacteria and archaea were predicted as potential AI-2-binding receptors.[66] Following this promising finding, searches for dCache domains in *S.* Typhimurium led to the discovery that one of the many diguanylate cyclase enzymes involved in producing cyclic-di-GMP (c-di-GMP), called YeaJ, contains a dCache domain.[67] This protein binds AI-2 with sub-micromolar affinity via the GAPES1 (Gammaproteobacterial Periplasmic Sensor1) domain and AI-2 supplementation promoted increased c-di-GMP levels in a YeaJ-dependent manner. In *S.* Typhimurium, high c-di-GMP represses Type III secretion system (T3SS) effectors. It was shown that AI-2 also repressed T3SS effectors via YeaJ (Figure 1A) and that this leads to decreased mortality in mice infected with *S*. Typhimurium.[67] C-di-GMP is an intercellular second messenger, often involved in repressing virulence and while the intracellular mechanisms involved in this regulation are well understood, the extracellular stimuli controlling c-di-GMP are largely unknown. The identification of an AI-2 receptor controlling the levels of c-di-GMP, provides evidence that bacteria can integrate information on cell density with host-derived cues (which also regulate c-di-GMP) to regulate virulence. These GAPES1-domain containing proteins are also present in pathogenic *E. coli*, like EHEC. It remains to be shown if members of the microbiota also contain these receptors, but as AI-2 is produced by many intestinal resident bacteria, these recent studies suggest that AI-2 produced by the microbiota can modulate traits required for colonization of intestinal pathogens like *S*. Typhimurium and EHEC via these recently identified receptors.

This third type of AI-2 receptor seems to be widespread and once again supports the role of AI-2 as a widely used signal. In terms of AI-2-regulated behaviors, chemotaxis is emerging as a common behavior associated with AI-2 sensing both through these novel receptors and LsrB.

AHL-signaling in gram-negative bacteria, an intra-species signal with the potential to enable eavesdropping on other species

Most autoinducers characterized to date are species-specific and thus play a role in intra-species communication. In gram-negative bacteria, the best studied quorum sensing signals are acyl-homoserine lactones (AHLs), which are synthesized by LuxI-homologues using SAM as substrate and are detected by LuxR-homologue receptors. The first LuxI–LuxR pair was described in *Vibrio* spp., but it was rapidly shown to be widespread in Proteobacteria.[20,68,69] Typically, each AHL synthase predominantly produces a single type of AHL, but different bacterial species produce AHLs with different modifications in length and structure of the acyl side chains. These modifications confer the specificity for the corresponding cognate receptor pair,

homologous to the cytoplasmic LuxR receptors.[70,71] Some bacteria produce more than one AHL signal, including the human pathogen *P. aeruginosa*, while others, as the plant pathogens *Erwinia* and *Pectobacterium*, have multiple AHL receptors.[23,24] This level of complexity associated with signaling diversity enables bacteria to use detection of autoinducers to regulate different group behaviors in a sequential manner or even to respond to the presence of other closely related species in the community. *P. aeruginosa* produces two different AHLs, synthesized by the enzymes LasI and RhlI, and possesses two cognate LuxR-homologue receptors (LasR and RhlR). These two systems are organized in series with the LasI/LasR system being the first quorum sensing system to be activated. The LasI/LasR system is responsible for regulating a series of virulence factors and genes required for early stages of biofilm formation and it also activates the second system (RhlI/RhlR), which leads to the production of secondary metabolites and traits required in mature biofilms.[23] Therefore, together the two systems regulate the temporal order of events required for *Pseudomonas* niche colonization. In the case of the plant pathogens *Erwinia* and *Pectobacterium*, quorum sensing regulates the production of cellulases and pectate lyases to degrade the infected plant tissue.[24] Often, multiple species of these pathogens can coexist in infected plants, and rely on AHLs-dependent quorum sensing to induce their main virulence factors.[72] These bacteria possess more than one AHL receptor with different levels of specificity, being capable of detecting and responding to their cognate signal while also *eavesdropping* on similar signals produced by closely related species, which also rely on similar traits for host exploitation.[24,73] Therefore, by relying on multiple quorum sensing systems, and by integrating the information provided by different signals, bacteria can not only respond to self, but also regulate the temporal order of events and the response to others.

Given the importance of AHL signaling in pathogenesis, the potential role of these quorum sensing systems in members of the gut microbiota is being investigated. Several initial approaches to detect AHLs by chemical extraction or the use of LuxR-type biosensors to detect AHLs failed.[74] Recently, an UPLC-MS/MS-based mass spectrometry method that enables the detection of AHLs in intestinal contents from conventionally-raised mice, which were absent in germ-free mice, was developed, demonstrating that AHLs produced by gut commensals can accumulate in the mammalian gut.[75] Importantly, AHLs were also detected in the serum and liver of these mice, due to their ability to diffuse through cell membranes. The fact that AHLs can be detected in host circulation and tissues, leads to the speculation that perhaps the host might also be sensing and responding to these molecules. In fact, there is already evidence that the host can sense and respond to AHLs.[76,77] This also raises the question of which members of the gut microbiota can produce AHLs (Figure 2). Currently, only a couple of species from the gut have

been described to possess potential homologues of the LuxI signal synthase: *Hafnia alvei* and *Acinetobacter baumannii*.[74] It is unrealistic to think that these would be the sole contributors to the AHLs found in the gut, since these are not prevalent members of the gut microbiota. Hence, further work is needed to identify other AHL-producing members of the microbiota now that detection methods are available.

Some gut colonizers of the *Enterobacteriaceae* family, including *E. coli*, *Klebsiella, Salmonella*, and *Enterobacter*, have an AHL receptor called SdiA.[74] SdiA is an orphan LuxR-type receptor, expressed by bacteria that do not produce their own AHLs, as no LuxI homologues were found in these organisms. As a result, this receptor is thought to play a role in enabling bacteria to respond to AHLs produced by other species. Namely, the SdiA receptor from *S*. Typhimurium is able to detect a broad range of AHLs, and thus has been used as a biosensor to detect AHLs in the gut of several animals. However, this *S*. Typhimurium receptor was only found to be active in the gut of mice infected with *Yersinia enterocolitica* (Figure 2).[78] EHEC, which is a deadly pathogen in humans, but a natural member of gastrointestinal tract in cattle, was shown to require the AHL receptor SdiA for colonization of the bovine rumen, where AHLs were also detected.[79] Because in EHEC AHLs repress the virulence locus of enterocyte effacement (LEE) genes through SidA, it was proposed that the SdiA-mediated repression of virulence by the AHLs present in the rumen could be important in promoting adaptation to the commensal lifestyle of EHEC in cattle.[79] In *Klebsiella pneumoniae*, SdiA regulates cell division and the expression of fimbriae and regulate biofilm formation, which are known virulence factors. Interestingly, a *sdiA* mutant produces more AI-2, revealing an interaction between these two signaling mechanisms.[80]

Recent findings support the possibility that additional new chemical classes of LuxR-dependent quorum sensing signals that differ from AHLs are likely to exist, and could be relevant in the mammalian gut.[81,82] Many bacterial species have been described to possess other orphan LuxR, which like the SdiA receptors mentioned above, have no *luxI* genes on their genome. Similarly to SdiA, these other orphan LuxR receptors may be involved in sensing AHL signals, or they might recognize other classes of signals as shown for *Photorhabdus* species. In these bacteria, the LuxR-type receptors are able to detect α-pyrones and dialkylresorcinols, instead of AHLs.[83] Moreover, some orphan LuxR in plant-associated bacteria detect plant-derived molecules,[83] and thus it is also possible that some of these receptors will detect host-associated molecules in gut microbes. Additionally, a recent study identified putative orphan LuxR homologues in human gut microbes and reported differential expression of LuxR genes in different Bacteroides in IBD versus non-IBD cohorts.[81] Overall, these results indicate that not only additional new classes of LuxR-dependent quorum sensing languages are likely to exist but also

demonstrate that many bacterial languages, that we are only now starting to uncover, have evolved.

Peptide signaling in the Firmicutes: interactions with commensals and pathogens as promising strategies to fight infections

In gram-positive bacteria belonging to the Firmicutes phylum, quorum sensing signaling and cell-cell communication are most commonly achieved by the activity of post-translationally modified oligopeptide-based autoinducer-peptide signals, also named pheromones. These signals fall mainly into four categories: RNPP regulators, Agr-type, double glycine motifs, and Rgg family.[22,84] Autoinducer-peptides, which often also contain lactone rings, are detected by membrane sensor kinases, and upon signal detection activate transcriptional response regulators to regulate the quorum sensing response. Similarly to the quorum sensing systems mentioned above, these two-component sensory systems have been described to regulate the expression of genes involved in functions that can play a role in gut communities, such as DNA acquisition by competence, conjugation, biofilm formation, or bacteriocin production.[22,84]

Again, the knowledge of the regulatory networks of peptide-based quorum sensing comes mainly from studies on pathogens, but *in vitro* and *in vivo* studies with gut-associated gram-positive bacteria are starting to emerge. Gram-positive bacteria belonging to the Firmicutes phylum with probiotic properties have revealed promising associations between quorum sensing and the regulation of important bacterial functions. Namely, *Lactobacillus plantarum*, a probiotic organism described to be important in restoring the gut wall integrity,[85] has at least four two-component regulatory systems involved in peptide-mediated quorum sensing. This Accessory Gene Regulator (Agr)-family of quorum sensing systems is involved in regulating the production of adhesins and multiple antimicrobial peptides (bacteriocins).[86] Therefore, in *L. plantarum*, quorum sensing is likely to be advantageous for niche competition in the mammalian gut, similarly to the role of quorum sensing-regulated bacteriocins in *Streptococcus pneumoniae*, where the Agr system is crucial for gut colonization.[87] Additionally, the *pln* signal peptide produced by several *L. plantarum* strains disrupt Agr-mediated quorum sensing in *S. aureus*, and thus might have a role in colonization resistance against pathogenic bacteria (Figure 2).[88] A recent study observed that in the gut of rural human populations there was a strong correlation between the presence of *Bacillus subtilis*, which has been widely used as a probiotic, and the absence of *S. aureus*.[89] The production of fengycin lipopeptides by *B. subtilis* was discovered to function as an inhibitor of the Agr-mediated virulence in *S. aureus*, by direct competitive inhibition of the autoinducer-peptide AgrC receptor, promoting this pathogen's decolonization of the mouse gut (Figure 2). This

prompted the investigation on whether *B. subtilis* would have a similar effect on the Fecal Streptococci Regulator (Fsr), an Agr homologous system of the nosocomial pathogen *Enterococcus faecalis*.[90] In *E. faecalis* the Fsr system is not essential for gut colonization, but it is required to promote *E. faecalis* translocation from the mouse gut to the bloodstream. Quorum sensing-mediated inhibition of *E. faecalis*' Fsr by *B. subtilis* fengycin lipopeptide abolished bloodstream translocation and systemic infection in mice (Figure 2). While *E. faecalis* is known to be a common colonizer of healthy individuals and even introduced in consortia to resemble the healthy mouse gut,[91] *Enterococci* spp. have been shown to frequently acquire resistance to antibiotics, particularly to vancomycin,[92] and expand in the gut of patients exposed to high doses of antibiotics. The resulting high levels of gut colonization frequently facilitates vancomycin-resistant *Enterococci* (VRE)-mediated bacteremia in immunocompromised patients where antibiotics are often not as effective.[93] The quorum quenching mechanism identified in *B. subtilis* highlights the potential gain in using quorum sensing systems as therapeutical interventions, both by using natural gut commensal systems or novel and improved probiotics to quench the virulence responses of pathogens.

Pyrazines: a new class of quorum sensing signals with a potential role in interspecies communication in the microbiota

It is well known that members of the gut microbiota can protect against invasion by intestinal pathogens through direct microbe-microbe interactions, a process called colonization resistance. In the last decade great progress has been made towards the understanding of the mechanistic basis of these interactions, which often involve nutrition competition, production of inhibitory metabolites, contact-dependent interactions, or signaling.[1,8,94,95] Before the demonstration that *Bacillus* could interfere with the quorum sensing systems of *Staphylococcus* and *Enterococcus* in the gut, the involvement of quorum sensing signals in the ability of gut microbiota members to provide colonization resistance to pathogens, had been proposed to explain the mechanism by which *B. obeum* could promote colonization resistance to *V. cholerae* in mice.[47] This inhibitory interaction between *B. obeum* and *V. cholerae* was proposed to be mediated by AI-2 produced by *B. obeum*. However, it was not dependent on the *V. cholerae* AI-2 receptor, LuxP, but instead on the transcription factor VqmA.[47,96] VqmA is an orphan LuxR that inhibits biofilm formation in *V. cholerae.*[97,98] Like other orphan LuxRs mentioned above, VqmA does not bind AHLs and was recently shown to be the receptor for a novel quorum sensing signal called 3,5-dimethylpyrazin-2-ol (DPO).[99,100] The DPO-VqmA receptor pair is one among multiple

quorum sensing mechanisms that converge to repress biofilm and virulence at high cell density in *V. cholerae,* being suggested that DPO might be the signal mediating the colonization resistance provided by *B. obeum* against *V. cholerae* (Figure 2).[99,100] DPO is a pyrazine that results from the condensation of aminoacetone (a product of threonine catabolism) with alanine and requires the activity of a threonine dehydrogenase (Tdh). The fact that threonine, the most abundant amino acid in the mucus, is the precursor for DPO provides support for the potential role of this signal in microbe-microbe interactions in the gut. Furthermore, studies in *V. cholerae* revealed that its quorum sensing regulon responds to environmental cues, such as oxygen, with DPO and VqmA being the most relevant quorum sensing pathway in anaerobic conditions, providing additional support for the potential relevance of this signal in the gut.[101] Moreover, a homologue of the VqmA-DPO receptor was also identified in a Vibrio phage (VP882).[102] This phage can respond to bacterial-produced DPO activating the phage lytic program, and consequent cell lysis and phage dissemination. Thus, presumably via the $VqmA_{phage}$ receptor the phage can eavesdrop on host quorum sensing signaling connecting bacterial density to the induction of cell lysis to maximize dissemination.[103]

Tdh homologues are highly conserved in bacteria,[99] and thus it was to be expected that other bacteria produce pyrazine-like signals. Indeed, *E. coli* produces a pyrazine signal, named autoinducer-3 (AI-3), whose structure was unknown for many years.[104,105] In both pathogenic and commensal strains of *E. coli*, AI-3 and other related pyrazines, also require the products of the reaction catalyzed by Tdh, but involve condensation with a second aminoacetone molecule, or with other amino acids rather than alanine, in the final condensation step, yielding different molecules of the pyrazine family. The final condensation step required in the formation of pyrazine AI-3 analogues involves aminoacyl-tRNA synthases, that promote an abortive tRNA synthase reaction, to enable the condensation of the decarboxylation of the Tdh product with the relevant amino acid.[104] Long before the structure of AI-3 was elucidated, its function as a potential quorum sensing signal that activates QseC (quorum sensing *E. coli* regulator C) regulatory cascade was proposed.[106,107] QseC is a membrane sensor kinase regulating a signal transduction cascade via the response regulator (QseB), which together control the expression of an array of quorum sensing-regulated genes. The QseC receptor is widely conserved in γ-Proteobacteria and genes regulated by this system have been extensively studied in many different bacteria, mainly pathogens where it activates virulence.[107] In EHEC and in enteropathogenic *E. coli*, QseC regulates the LEE pathogenicity island, and it also regulates pathogenicity in the murine pathogen *Citrobacter rodentium* (Figure 2). A fascinating feature of this system is that QseC together with another membrane sensor kinase, QseE, can activate bacterial responses (via the response

regulator QseB) to the host neurotransmitters epinephrine and norepinephrine, thus providing an example of inter-kingdom crosstalk between bacteria and host.[107,108]

Given that multiple bacteria have been shown to produce pyrazines and the significant level of conservation of Tdh in bacteria, it is reasonable to expect that many other members of the microbiota produce pyrazine signals. It is possible that pyrazine signals produced by the microbiota will have an impact in modulating the outcome of γ-Proteobacteria pathogenicity, revealing exciting and previously unknown communication pathways in the gut.

Group behaviors in gut microbiota

Gut commensal bacterial species possess an extensive arsenal of strategies to thrive in the gut, including secretion of enzymes, biofilm formation, and contact-dependent and -independent mechanisms of inhibition of other microbes. Many of these strategies have been extensively characterized in pathogens, where quorum sensing systems are implicated in the regulation of these group behaviors. In contrast, how these processes are regulated in gut symbionts is largely unknow, indicating that novel systems might exist.

Biofilms are ubiquitous in nature and the mammalian gut is no exception.[109] However, the functions of biofilms formed by commensal gut microbiota still remain elusive, as well as its potential manipulation to promote human health. Evidence for the presence of commensal microbiota biofilms in the gut has only recently been documented.[109,110] One of the most prevalent bacteria of the normal human intestinal microbiota is *Bacteroides thetaiotaomicron*, in which biofilm formation is regulated by its capsule.[111] Although there is a lack of robust information about quorum sensing mechanisms in this bacterium, capsular polysaccharide production in other bacteria has been shown to be regulated by quorum sensing systems,[112,113] leading to the possibility that the same regulation might occur in this and other organisms. Recent efforts on the identification of factors involved in regulating biofilms in this organism revealed that bile acids are required to trigger biofilm formation.[114] Integration of host cues with quorum sensing is not uncommon and has been shown, for example, to activate quorum sensing-regulated production of plant cell wall-degrading enzymes in plant pathogens.[115] A similar mechanism of signal integration, with bile acids as gut-relevant cues, might also be present in *B. thetaiotaomicron* for biofilm formation.

Recent studies have focused on adherent bacterial communities, potentially gut biofilms, which typically coat the mucosal/epithelial layers of the intestine, and that can be disrupted by dysbiotic events, such as antibiotic treatments or invasion by pathogens.[109,110] *Giardia duodenalis*, a pathogen responsible for acute diarrheal disease, is among those pathogens described to disrupt

biofilm species composition and biofilm structure of the microbiota and lead to bacterial invasion,[116] potentially causing persistent dysbiosis. Moreover, some studies have hypothesized a link between disruptions of these biofilm-like communities and the dissemination of commensals behaving as opportunistic pathogens (pathobionts).[117] In another case, the commensal species *Paracoccus aminovorans* was shown to engage in the formation of dual-species biofilms with *V. cholerae*, resulting in a better colonization by the pathogen, altering the outcome of the infection.[118]

Importantly, the functional range of commensal biofilms in the gut, and their contributions to host health, remains largely unknown. Biophysical and biogeographical characteristics of biofilms can hint at potential functions, namely protection of the mucosal/epithelial layer against bacterial invasion.[109,110] Also, it is expected that commensal bacteria in biofilms survive better to antibiotic treatments and recover faster after an antibiotic-induced dysbiotic event.[110] Current studies, however, are still preliminary and several conclusions are quite hypothetical. Namely, assumptions that microbiota members form biofilms in the gut are usually based on their ability to form biofilms *in vitro.* In some cases, there is evidence that bacteria form thick compact bacterial layers close to the gut epithelia,[109,110] but, more detailed information is needed to establish biofilms as gut microbial structures, such as *in situ* or *in vivo* detection/visualization of the biofilm structure (e.g., the matrix, a hallmark of biofilm formation) and determination of biofilm-related gene expression profiles. Particularly, further *in vivo* studies will be critical to understand the biological drivers of these important microbial structures in the gut.

Many members of the gut microbiota are essential to degrade non-digestible fibers to fermentation products and secondary metabolites, which are essential for proper host nutrition and immune system maturation. Many of these members belong to the Bacteroidetes phylum, and specifically to the *Bacteroides* genus, which relies on arrays of polysaccharide utilization loci (PULs) to produce lytic enzymes that degrade dietary and mucus-derived polysaccharides.[119-122] Some *Bacteroides* species are also able to assemble polysaccharides into the surface forming capsules, and have been shown to use fucosylated glycans in their capsular polysaccharides and glycoproteins, allowing them to compete and persist within the gut ecosystem.[123,124] It has also been shown that high densities of *B. thetaiotaomicron* in monocolonized mice triggers fucosylation of the ileum,[123] supposedly via bacterial-secreted signals. As already mentioned above, in *Erwinia* and *Pectobacterium* species, extracellular saccharolytic enzymes are tightly regulated by quorum sensing,[24,73] but how microbiota members regulate PULs and polysaccharide assembly machineries as a function of density, is currently unknown.

Type 6 secretion systems (T6SS) are among the most studied contact-dependent mechanisms of antibacterial weaponry, where a needle-like structure punctures a neighboring bacterial cell, injecting effector proteins.[125,126] To survive an attack from this complex machinery that is structurally similar to bacteriophages, the targeted bacterial cells need to encode in their genomes the respective immunity protein, since injector cells do not discriminate between self or non-self. T6SS are widely distributed across gram-negative bacteria and are known to be regulated by quorum sensing in pathogens like *V. cholerae*, *P. aeruginosa*, and *Yersinia pseudotuberculosis*.[127,128] In the highly dense gut, T6SS can be very efficient. Genomic analysis suggests that half of the Bacteroidales species possess at least one T6SS, which can be ultimately translated to 25% of all bacterial species present in the gut having T6SS.[129] It is thought that these Bacteroidales species, which are extremely well adapted to the gut environment, use T6SS to reduce local interspecies competition for shared resources.[121,130] It is estimated that there are more than 10^9 T6SS shooting events per minute per gram of gut content,[131] highlighting the importance of this weapon for commensal bacteria to colonize and persist in the intestinal tract, as well as to provide colonization resistance to competitors.[132-134]

Bacteria also have the capacity to produce and secrete antimicrobial proteins to antagonize competitor cells in a contact-independent manner. Some recent examples demonstrate how bacteriocin-producing symbiotic bacteria can displace other species from their gut niches. Microcin production by the probiotic *E. coli* Nissle 1917 can eliminate adherent-invasive *E. coli* (AIEC) and *S*. Typhimurium from inflamed guts,[135] whereas a nisin A variant produced by *Blautia producta* prevents the expansion of VRE in the gut, as well as other gut commensals.[136] Although unknown for these species, this mechanism has been previously linked to quorum sensing in lactic acid bacteria, where production of such antimicrobial peptides are autoinducer-regulated.[86]

While delivery of toxic compounds by contact dependent T6SS, freely diffusible toxins, or antimicrobial molecules are clearly antagonistic interactions, cooperative interactions among microbiota members also exist. *P. vulgatus* and *Bacteroides ovatus* can promote each other's growth through a nutrient-sharing network, with mutual benefit for these two closely related Bacteroidales.[137] Additionally, sharing polysaccharides enzymatic machineries, and other specialized mechanisms through outer membrane vesicles (OMVs), also seems to be a common behavior among *Bacteroides* spp..[121,138] Although the ecological benefit of OMVs remains to be clarified, it has been hypothesized to be another cooperative behavior among members of the microbiota, where OMVs can serve as vehicles for delivering shareable compounds among members of the same or different species.[121]

All these bacterial traits are important for the execution of important functional properties of the microbiota, which are more productive when performed in group. Future work will reveal if, like in pathogens, these traits are also regulated by quorum sensing systems.

Future Outlook

There is increasing evidence for the relevance of small molecules in the densely colonized gut environment. As we summarized in this review, some are well known quorum sensing signals that have been studied in detail for the regulation of group behaviors in bacterial pathogens and are now being discovered to also regulate similar functions in gut bacterial commensals. The increasing number of culturable bacterial species from the gut opens the opportunity for identifying bacteria that produce known signals using reporter strains carrying known receptors. Additionally, it is important to improve sensitive techniques to detect the presence of these molecules in the gut, similarly to what was achieved recently for AHLs. New available tools are aiding in the genetic manipulation of gut bacteria, such as CRISPR-Cas systems. With this, construction of mutants in known synthases is a classic and effective way of identifying quorum sensing-regulated changes in gene expression using transcriptomic analysis.

How about unknown signals? As discussed above, many microbiota members perform bacterial group behaviors that are likely regulated by quorum sensing signals, but have no known synthases or receptors. For the density dependent traits with known genes involved, a possible approach is the construction of promoter-reporter fusions to study the expression of these genes in response to cell density, to extracellular cell-free conditioned media obtained from cultures at different densities, or to signals from the mammalian gut. When possible, establishment of easy to measure phenotypic methods which can be used for high throughput screening (e.g.: colorimetric assays to measure degradation of compounds by lytic enzymes, as previously developed for plant pathogens, or the crystal violet assay for biofilm formation) would be advisable. Once reporters or phenotypes that respond to cell density and external signals are identified, transcriptomic analysis of these cultures comparing activated with non-activated reporter conditions can be used to select for genes with a potential role in biosynthesis of small molecules (potential signal synthases) or with homology to signal binding domains (signal recognition), which are also expected to be more expressed, as genes involved in quorum mechanisms are often transcriptionally activated by a positive feedback loop (Figure 3). Alternatively, libraries of mutants, which start to be available for model gut commensals such as several *Bacteroides* spp, can be screened for these phenotypic assays (Figure 3).[120,139-141]

644 Mutants impaired in group behaviors that can be complemented with cell-free supernatants of the
645 wild-type ancestral strains, are mutants in genes potentially involved in pathways for signal
646 biosynthesis, while mutants that can no longer be activated by the extracellular supplementation
647 of signals, *i. e.*, are signal blind, are potential hits for signal receptors. Ultimately, these studies
648 need to be coupled with biochemical and analytical approaches for the identification of the
649 chemical structures of the signals and validation of the receptors.
650 Recently, computational methods have been extensively used to deal with complex
651 microbial communities and machine learning approaches have been used to mine the gut
652 microbiome for predicted quorum sensing systems (Figure 3). This newly created database of
653 possible quorum sensing systems in gut bacteria still requires experimental verification, but it
654 offers an exciting new tool for users to explore.[142]
655 Quorum sensing signaling has been shown to regulate a myriad of bacterial community
656 behaviors everywhere in nature. Several of these behaviors are essential for the bacterial lifestyle
657 in a multitude of environments, being important for both cooperation and competition, and equally
658 for symbionts and pathogens. This acquired knowledge prompts the scientific community studying
659 microbial interactions, in particular in the gut microbiota, to pay more attention to the quorum
660 sensing basis of community behaviors, as well as the quorum sensing-based potential of
661 manipulation to enhance commensal communities and/or weaken pathogens' intrusion in such
662 commensal communities. Recent scientific advances in multi-omics and bacterial genetic
663 manipulation will allow us to fill the current gaps regarding the expected, yet unknown,
664 mechanisms of quorum sensing reigning in several members of the gut microbiota, but also to
665 manipulate the already known signals to study their influence in the numerous behaviors that
666 drive stability, robustness, and protection of this complex and important community.
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668
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Figure legends

Figure 1. AI-2 regulated phenotypes in the gut.

(A) Many gut microbiota members have been described to produce and sense AI-2 in the gut. Under antibiotic-induced dysbiosis, AI-2 delivered by *E. coli* to the gut altered the microbiota composition by promoting the expansion of Firmicutes and reduction of Bacteroidetes, by vastly unknown mechanisms. However, many AI-2-dependent group behaviors have been described in other gut commensals and pathogens, impacting their colonization. AI-2 promotes chemotaxis leading to single or mixed-species aggregation in *E*. *coli* and *E*. *faecalis*. In addition, AI-2 induces phage dispersal in *E*. *faecalis*. Bacterial aggregation can lead to biofilm formation, which can also be induced or repressed by AI-2, such as in *E*. *coli* and *V*. *cholerae*, respectively. AI-2 can foster bacterial competition for essential gut nutrients. *B*. *breve* scavenges iron uptake in the presence of AI-2, resisting the gut colonization by pathogens, like EHEC and *C*. *rodentium*. Host-microbe interactions can also be affected by AI-2, as seen by the inhibition of *S*. Typhimurium T3SS, and consequently reduced virulence. The host might be an active player in regulating AI-2-dependent bacterial group behaviors by producing an AI-2 mimic molecule.

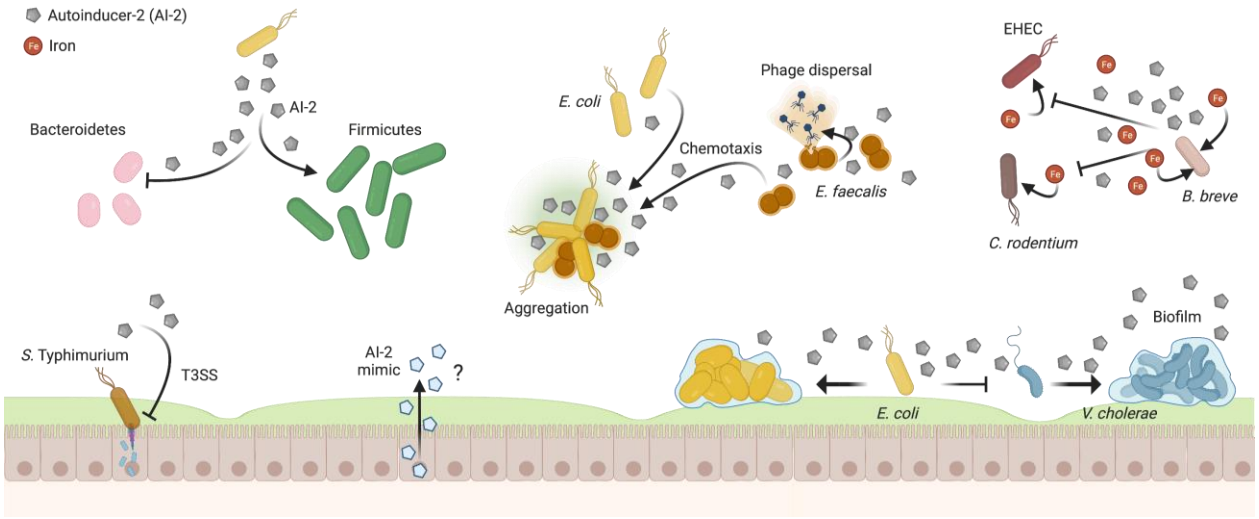
(B) Phylogenetic tree based on the 16S rRNA gene of representative culturable isolates from Bacteroidetes phylum. In blue are Bacteroidetes that possess predicted *luxS* gene (AI-2 synthase); in black are Bacteroidetes that do not have LuxS homologues. Unlike with the highly represented *Bacteroidaceae* family, in which most members lack LuxS homologues, in the more recently identified *Barnesiellaceae* and *Muribaculaceae* families, LuxS homologues seem to be more prevalent.

Figure 2. Other quorum sensing languages. The detection of AHL molecules in the intestines of conventionally raised mice, unlike in germ-free mice, indicates production by gut microbiota members, albeit yet unidentified. The pathogen *Y*. *enterocolitica* has been identified as an AHL producer in the gut that activates *S*. Typhimurium AHL receptor SdiA. Probiotics like *B*. *subtilis* and *L*. *plantarum* are able to inhibit the peptide-based quorum sensing systems of *S*. *aureus*, promoting its decolonization. *B*. *subtilis* also abrogates translocation by *E*. *faecalis* through quorum quenching peptide signals. Two other quorum sensing molecules, the pyrazines DPO and AI-3, have been described to be produced by both gut microbiota members and pathogens. DPO produced by gut bacterial species, like *B*. *obeum*, has the potential to inhibit biofilm formation and toxin production in *V*. *cholerae*. The AI-3 molecule is produced by γ-Proteobacteria species, regulating virulence in several pathogenic *E*. *coli* and *C*. *rodentium*.

Figure 3. **Strategies to find new quorum sensing signals, receptors, and systems**. Different approaches have been used to identify unknown producers and receptors of known signals. Reporter strains for specific and known signals have been widely used to identify novel producers, in which mutations in their synthase validates signal-regulated group behaviors. Using sequence and structural similarity of known receptors many homologous receptors were found in different bacteria. Recently, a proof-of-concept pulldown strategy was developed and shown to recover known receptors bound to the signal. Following either strategy, validation of the receptor can be obtained by determining its binding affinity and solving its tridimensional structure while bound to the signal. To identify new quorum sensing systems in bacteria, density-dependent traits can be screened using phenotypic methods and coupled with promotor fusions when the genes that regulated the traits are known. Full transcriptomics of activated versus non-activated cultures will allow to search for genes for signal binding domains and for biosynthesis of small molecules in the activated cultures. Alternatively, phenotypic assays when coupled with mutant library screens allows the detection of defective mutants for the tested trait. When defective mutants can be phenotypically complemented by supernatants from an active culture, it is likely a mutant in signal production. When not phenotypically complemented by supernatants from an active culture, this is most likely a mutant in signal response. Mathematical modelling and machine learning approaches have been recently attempted to identify known quorum sensing systems in complex bacterial communities. This promising new tool still requires experimental validation, but opens the door to discover new quorum sensing-based interactions.

Figure 1 - AI-2 regulated phenotypes in gut bacteria

A



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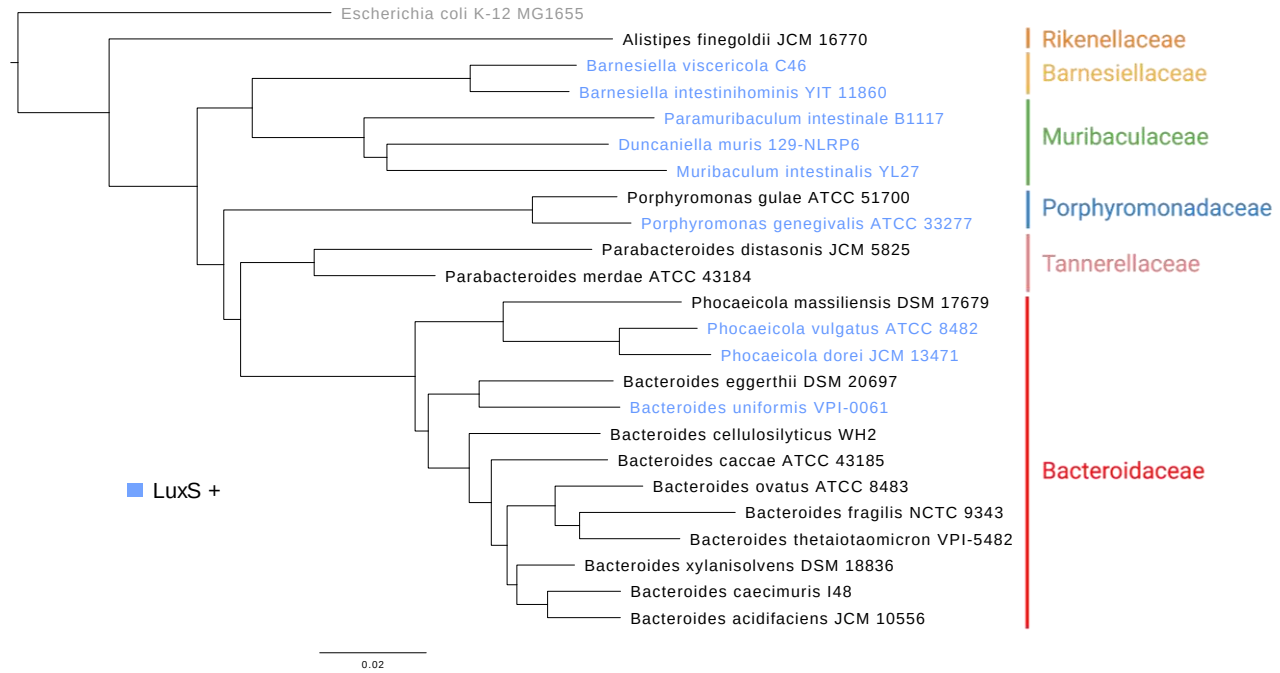

Figure 2 - Other quorum sensing languages

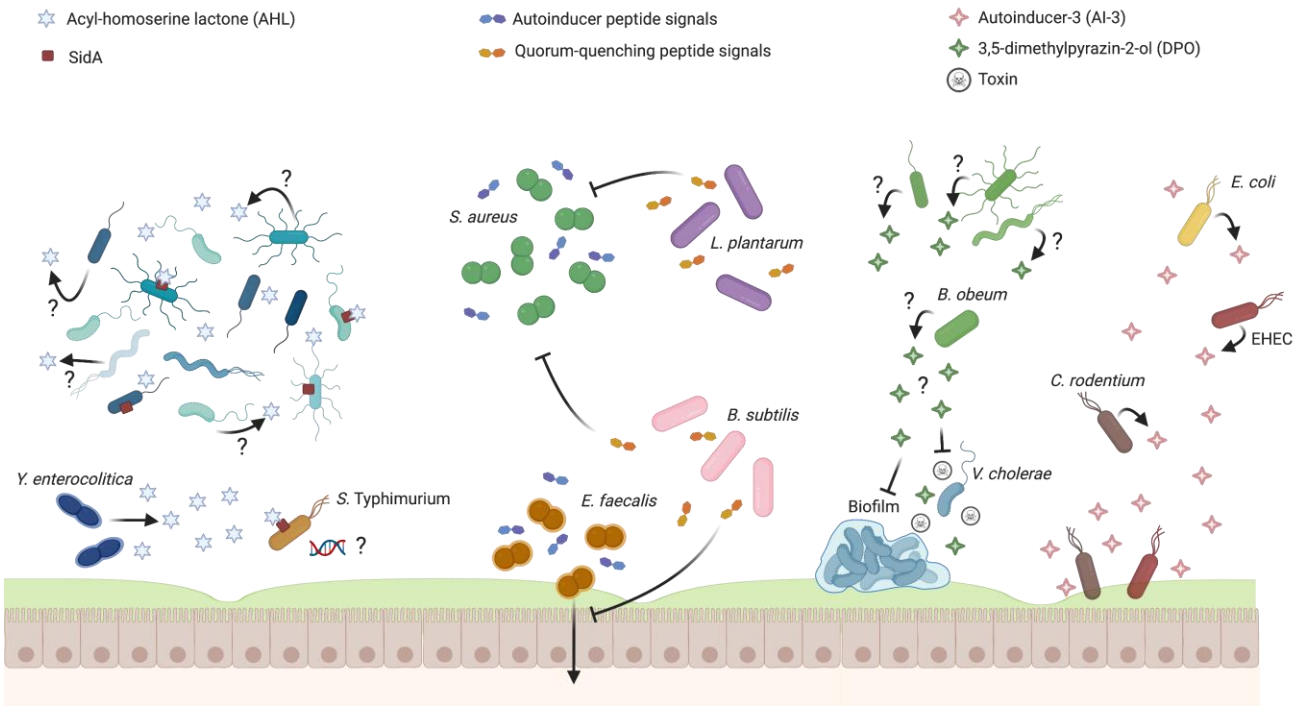

Figure 3 - Strategies to find new Quorum Sensing signals, receptors and systems