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Phyllosphere Microbiome

Reza Sohrabi,[1,2] Bradley C. Paasch,[1,2] Julian A. Liber,[1] and Sheng Yang He[1,2]

[1]Department of Biology, Duke University, Durham, North Carolina, USA; email: reza.sohrabi@duke.edu, bradley.paasch@duke.edu, julian.liber@duke.edu, shengyang.he@duke.edu

[2]Howard Hughes Medical Institute, Duke University, Durham, North Carolina, USA

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Abstract

The aboveground parts of terrestrial plants are colonized by a variety of microbes that collectively constitute the phyllosphere microbiota. Decades of pioneering work using individual phyllosphere microbes, including commensals and pathogens, have provided foundational knowledge about how individual microbes adapt to the phyllosphere environment and their role in providing biological control against pathogens. Recent studies have revealed a more complete repertoire of phyllosphere microbiota across plant taxa and how plants respond to and regulate the level and composition of phyllosphere microbiota. Importantly, the development of several gnotobiotic systems is allowing causative and mechanistic studies to determine the contributions of microbiota to phyllosphere health and productivity. New insights into how the phyllosphere carries out key biological processes, including photosynthesis, biomass accumulation, reproduction, and defense against biotic and abiotic insults, in either the presence or absence of a normal microbiota could unleash novel plant- and microbiota-based technologies to improve agriculturally relevant traits of crop plants.

Contents

PREFACE: A HISTORICAL PERSPECTIVE

Microbiota: a community of microorganisms

Phyllosphere: used in this review to refer to the total plant-associated microbial habitat comprising aboveground plant tissues

Epiphyte: an organism residing on the external surface of plant tissues

Endophyte: an organism residing within plant tissue

Endosphere: the plant-associated microbial habitat inside plant tissues

Plants host a variety of microorganisms as part of their microbiota. The aerial parts of plants, which are collectively referred to as the phyllosphere, includes leaves, flowers, stems, fruits, and pollens, each with unique morphological and physical properties. The overall biomass of the phyllosphere is estimated to constitute approximately 60% of the total biomass on Earth, making it one of the largest habitats for hosting microbial life (11). Among the different phyllosphere parts, leaves have been the most extensively studied for microbial colonization and provide a habitat for different coexisting microorganisms, including bacteria, fungi, protists (e.g., oomycetes), and viruses (30, 98, 178). The microbiota members residing on the surface of the phyllosphere are called epiphytes, whereas microbiota members that reside inside phyllosphere tissues, either in the intercellular spaces (apoplast) and/or within the plant cell, are called endophytes. The same microbe could be both an epiphyte and an endophyte, occupying both niches. The majority of the members of the phyllosphere microbiota have not been characterized in detail and are often considered to be commensal, meaning that they do not appear to have obvious positive or negative effects on plant health, at least under laboratory conditions tested. However, further studies may reveal that many of the commensal microbiota members have beneficial (or harmful) effects on plant life under other conditions yet to be tested. Bacteria represent major colonizers of leaves and are most extensively studied among phyllosphere microbiota members. Comparative microbiome studies suggest that the complexity of phyllosphere bacterial communities is reduced compared to that of bulk soil, but still there is a high level of species richness observed, suggesting a selective pressure to establish these diverse communities (40). In general, the leaf epiphytic surfaces show higher microbial diversity and load compared to the leaf endosphere compartment. For example, recent studies of *Arabidopsis* leaves suggest that the endophytic bacterial colonization is limited to 10^2 to 10^3 colony-forming units (CFU)/cm^2, with levels an order of magnitude lower for fungal members

(34, 77). In comparison, total bacterial microbiota load (mostly from epiphytes) can be 100-fold higher in *Arabidopsis* leaves (34).

Phyllosphere microbiology research has had a long history. The premetagenomics era of phyllosphere microbiology research greatly benefited from sustained contributions by a number of pioneering researchers who focused on individual phyllosphere bacteria. For example, Lindow, Upper, and coworkers (97, 100) discovered a causal relationship for epiphytic bacteria in ice nucleation and frost damage in plant leaf tissue and developed novel approaches for frost damage control by competitive colonization of plants by genetically engineered bacterial strains lacking the ice nucleation genes (95, 101). Similarly, Lindow et al. (99) found that fruit russeting, which results in loss of fruit quality, can be due to localized exudation of the plant hormone auxin [indole acetic acid (IAA)] by bacterial epiphytes on developing fruits, and it could be eliminated via competition by spraying flowers with non-hormone-producing bacterial strains. Transposon mutagenesis and biosensor-based approaches allowed these and other researchers to expand the understanding of bacterial physiology on leaf surfaces and elucidated processes important for epiphytic bacterial fitness under natural field conditions (62, 72, 90, 96).

The application of early molecular techniques such as denaturing gradient gel electrophoresis to characterize culturable epiphytic leaf bacteria provided a glimpse of the complexity of phyllosphere microbial communities and highlighted the demand for alternative methodologies to capture the diversity of culturable and nonculturable phyllosphere communities (187). The advent of high-throughput (or next-generation) sequencing approaches, such as 454 pyrosequencing and Illumina technologies, changed the landscape of microbial ecology for phyllosphere studies in the 2000s and started a new era of omics-based exploration of microbial communities through 16S ribosomal RNA (rRNA) gene and ribosomal DNA internal transcribed spacer (ITS) amplicon sequencing, shotgun metagenomics, transcriptomics, and whole-genome sequencing (40, 73, 140, 141) (see the sidebar titled Meta-Omics Tools for Microbiota Analysis). The wealth of data generated by high-throughput sequencing technologies has expanded our knowledge of drivers of plant microbial community structural variations and their interactions under different biotic and abiotic contexts, as will be discussed below.

Amplicon sequencing: a method of targeted metagenomics in which specific genomic regions recovered from microbiota samples are amplified and sequenced; for example, 16S and ITS rRNA gene sequencing

Metagenomics: analysis of the entire genomic content recovered directly from a microbiota sample

META-OMICS TOOLS FOR MICROBIOTA ANALYSIS

Most microbiome studies began with taxonomic surveys based on polymerase chain reaction (PCR) amplification of a conserved gene, commonly regions of the 16S rRNA gene for prokaryotes or regions of the 18S rRNA gene for eukaryotes. Taxonomic composition can be determined with varying resolution and some functional information inferred from taxonomy. However, these methods lack direct characterization of the functional attributes of a microbiota. Approaches such as metagenomics and metatranscriptomics allow for insights into the functional potential of a microbiota by sequencing its entire collection of genes or actively expressed genes. New tools are emerging that build upon these traditional meta-omic approaches by incorporating single-cell methodologies, which allow for a higher degree of resolution. For example, a single-cell approach named Microbe-seq was used to determine the genomic information of bacteria across multiple human stool samples and provide understanding on strain-level diversity, which could not be accomplished with standard metagenomic approaches (195). Other emerging methods such as PETRI-seq (22) and MicroSPLiT (85) facilitate single-cell rRNA-seq on Gram-negative and Gram-positive bacteria. How these and other emerging technologies are applied to characterize individual microbial cells within the phyllosphere microbiota will be of great interest moving forward.

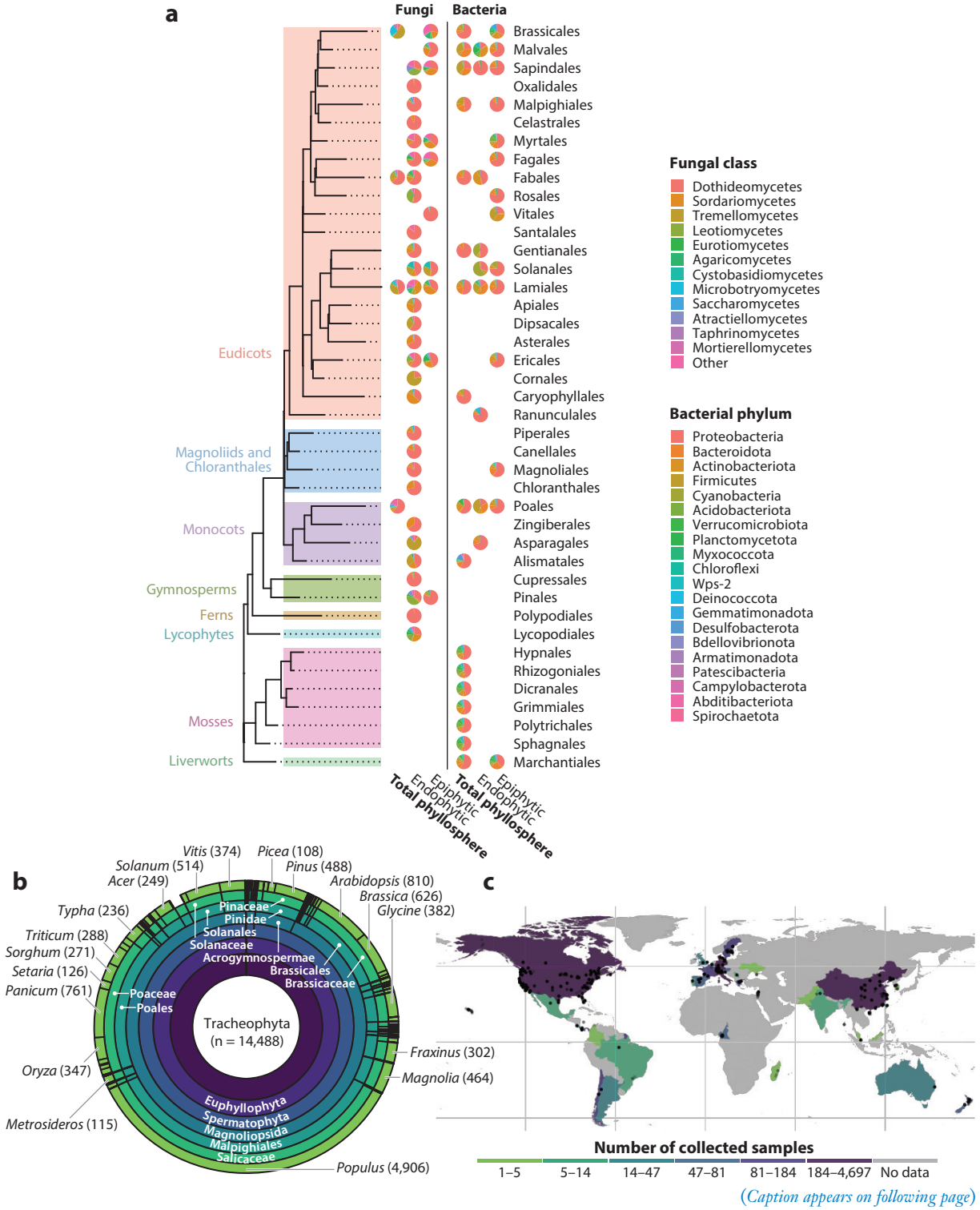
PHYLLOSPHERE MICROBIOTA ACROSS PLANT TAXA

The Phyllosphere of Seed Plants

To date, the majority of phyllosphere microbiota surveys have been conducted in seed plants, especially angiosperms (**Figure 1**). Despite their great taxonomical diversity in nature, seed plant phyllosphere bacterial communities are dominated by relatively few phyla, including Pseudomonadota, Actinomycetota, Bacteroidota, and Bacillota. In several eudicot plant species examined, the phyllosphere microbiotas are dominated by bacterial members from only a few genera, most notably *Sphingomonas* (Alphaproteobacteria), *Methylobacterium* (Alphaproteobacteria), and *Pseudomonas* (Gammaproteobacteria) (40, 178). Similar bacterial genera are present in the phyllosphere of monocots such as rice, miscanthus, and switchgrass crop plants (55, 81). Multiple bacterial families in Alphaproteobacteria, including Sphingomonadaceae, Beijerinckiaceae, and Acetobacteraceae, are found in high abundance in the phyllosphere microbiome of tree species representing angiosperms and gymnosperms in temperate and subtropical forests (87, 92). In particular, the ubiquitous detection of *Sphingomonas* and *Methylobacterium* as members of leaf phyllosphere microbiotas using 16S profiling approaches is linked to their adaptation to low amounts of available nutrients on leaf surfaces (40). Several studies have reported the persistency and co-occurrence of various microbial taxa within the leaf core microbiome across various plant genotypes and in different geographical locations and growing seasons (7, 23, 55, 76, 86, 87, 92). These core taxa reported in different studies include *Sphingomonas*, *Methylobacterium*, *Pseudomonas*, *Pantoea*, and *Variovorax* species.

Phyllosphere fungal communities are consistently composed of filamentous or dimorphic (i.e., yeast or filamentous, depending on conditions or life stage) taxa from the classes Leotiomycetes, Dothideomycetes, and Sordariomycetes, detected from sorghum (52), pine (149), poplar (10), maize (117), soybean (105), maple and hickory (93), *Arabidopsis* (7), and clover (179). Additionally, basidiomycete yeasts in the classes Tremellomycetes and Microbotryomycetes are often detected in these communities. Oomycetes are less often surveyed in amplicon-based microbiome surveys but have been known to include Peronosporales, Albuginales, and Pythiales in *Arabidopsis* (4, 179). Although viruses are widespread in soil and aquatic environments (133) and viral pathogens have been studied extensively, our knowledge of plant viral microbiomes (viromes) is still in its infancy (155). Recently, the abundance and composition of the epiphytic bacteriophage population in the phyllosphere of wheat were studied using a viral metagenomics approach (49). Although viral DNA material was limited in quantity in wheat phyllosphere for the metagenomics analysis, scaling up the sample material resulted in the identification of hundreds of different species-ranked virus groups, many of which were previously uncharacterized novel viral genomes ranging from 2 to 350 kb. Interestingly, phages have been shown to modulate the leaf microbiota community structure (e.g., pseudomonads) in tomato phyllosphere (122). These studies highlight the potential and importance of this neglected component of plant microbiota in regulating structure and function of microbial communities for future phyllosphere microbiota research.

Flowers of seed plants are also colonized by both bacterial and fungal communities (170). Microbial taxa found in flowers include bacterial taxa such as *Pseudomonas*, *Sphingomonas*, *Erwinia*, *Xanthomonas*, and *Streptomyces* (74, 154) and fungal taxa such as *Aspergillus*, *Penicillium*, *Aureobasidium*, *Rhodotorula*, and *Cryptococcus* (123, 139). Various animal visitor–associated microbes are found on flowers, which could have diverse effects on pollinators and other flower visitors (170). During fruit formation, a differential colonization of fruit parts is reported. For example, different parts of apple fruit (stem end, peel, and mesocarp) are colonized by distinct fungal and bacterial microbiotas (2, 3). Nevertheless, a global effort to study the apple fruit microbiome resulted in the identification of a core microbiota composed mainly of two bacterial taxa (*Sphingomonas* and



(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Compositional and metadata meta-analysis of phyllosphere meta-amplicon sequencing studies across land plants. (*a*) Composition of phyllosphere communities by host plant order and compartment and (*b*,*c*) metadata analysis of sequenced BioSamples annotated as derived from the phrases "phyllosphere metagenome" or "leaf metagenome," deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) as of Nov 9, 2021. Host plant phylogeny in panel *a* is structured according to One Thousand Plant Transcriptomes Initiative (131). Metadata are plotted based on (*b*) hierarchical taxonomy of the host plant with number of included BioSamples in parentheses and (*c*) country of sample collection, with points indicating collection sites. Phyllosphere microbiomes are only well characterized within a limited number of model and crop organisms, unevenly across compartment types, and from environments predominantly in the Global North. Scripts and data for generating **Figure 1** are available on GitHub (**https://github.com/liberjul/Phyllosphere_microbiome_meta-analysis**).

Methylobacterium) and six fungal taxa (*Aureobasidium*, *Cladosporium*, *Alternaria*, *Filobasidium*, *Vishniacozyma*, and *Sporobolomyces*) (2). The occurrence of pathogens within fruit microbiota can result in pre- and postharvest diseases of various fruits (2).

The Phyllosphere of Non-Seed Plants

In addition to seed plants, bryophytes such as mosses, liverworts, and hornworts have been surveyed for microbiota composition. Despite their low overall plant biomass of around 1%, cryptogamic groundcovers (including bryophytes and lichens) account for roughly 7% of terrestrial net primary production and, remarkably, 46% of biological nitrogen fixation (45). The microbiomes of these plants, similar to those of others, are functional. For example, mosses associate with diazotrophic bacteria, which provide biologically fixed nitrogen and can increase the growth of the moss (19). While many of these diazotrophs are Cyanobacteria, especially *Nostoc*, bacteria in the Hyphomicrobiales (notably *Methyloferula*, an obligate methylotroph) are overrepresented in the transcript reads of the nitrogen fixation gene *nifH* in *Sphagnum* peat bogs (82). In both *Sphagnum* and *Racomitrium lanuginosum* (an abundant terrestrial mat-forming moss) bacterial communities are dominated by Pseudomonadota, Acidobacteriota, Actinomycetota, and Cyanobacteria (79, 82). *Takakia*, a moss genus of what may be the earliest diverging land plants, hosts Alpha- and Betaproteobacteria capable of nitrogen fixation, in addition to their associations with ascomycete and glomalean (arbuscular-mycorrhizal) fungi, which form mycorrhizal-like structures (150).

Liverworts and hornworts carry bacterial communities that show nitrogen fixation functions (6) and have variation in composition between habitats (26, 115, 126, 127) and sometimes between species (6, 126). Streptophyte algae, a paraphyletic sister group to land plants (160), were found to have a core bacterial microbiota containing members of these groups in addition to *Opitutus* in the phylum Verrucomicrobiota (80). Similarly, lycophytes, or clubmosses, a vascular group of spore-bearing plants, have a phyllosphere microbiota. ITS sequencing targeting fungi found leaf endophytic communities in the commercially grown *Huperzia serrata* in Hunan, China, with high levels of *Phyllosticta*, *Cladosporium*, and *Cladophialophora* species. (46). A culture-based approach identified similar fungal taxa associated with the endosphere of multiple clubmosses collected in New York, United States (136). Notably, Dothideomycetes, such as *Phyllosticta* and *Cladosporium*, are also found to be endophytes of seed plants (93, 189). While the evolutionary relationship between lycophyte hosts and their associated phyllosphere microbiota has not been investigated, some degree of correlation has been observed in lycophyte root communities between host genetic distance and fungal (but not bacterial) microbiome similarity (16).

MOLECULAR TRAITS FOR EPIPHYTIC AND ENDOPHYTIC LIFESTYLES

The leaf surface is a harsh environment where the inhabiting microbes are subjected to a range of physicochemical stresses including high light and ultraviolet (UV) radiation, fluctuating

temperature and moisture, and limited nutrients (178). Therefore, several strategies are used by phyllosphere microbial inhabitants to survive and alleviate detrimental effects of the environment under these abiotic stress conditions and to successfully colonize the phyllosphere. Many phyllosphere microorganisms produce extracellular polysaccharides (EPSs) that allow cell aggregation and accumulate intracellular osmoprotectants such as proline, choline, and soluble sugars, which collectively contribute to protection against desiccation and osmotic stress and improve bacterial fitness compared to single-cell bacteria (33, 98, 120). To deal with detrimental effects of high doses of UV radiation at the leaf surface, bacteria such as *Methylobacterium*, *Pseudomonas*, and *Sphingomonas* combat UV-induced oxidative stress through either pigmentation or activation of antioxidant enzymes, such as catalase and superoxide dismutase (98, 182). Phyllosphere yeasts, including *Aureobasidium pullulans*, similarly defend against the stressful phyllosphere environment by producing EPSs as well as melanin (102). In order to survive the nutrient-poor environment of the leaf surface, several niche-driven biochemical and metabolic adaptations in epiphytes are reported. For example, metaproteogenomic approaches uncovered that increases in the abundance of methylotrophic metabolism proteins are required for consumption of methanol as a source of carbon in *Methylobacterium* species (40). The endosphere compartment, compared to the leaf surface, poses a different set of challenges for microbial colonization. Compared to plant surfaces, the endosphere likely has more abundant nutrients and could protect microbes from external fluctuations in atmospheric changes, including UV radiation and moisture. Yet, compared to epiphytes, endophytes are in closer contact with the plant immune surveillance mechanisms and defense compounds, which could restrain their multiplication potential.

Many phytopathogenic bacteria have an epiphytic, nonpathogenic phase that is followed by an endophytic, pathogenic phase. For example, *Pseudomonas syringae* pv. *syringae* B728a, a bean pathogen, is normally found as an epiphytic microbiota member. Transcriptome analysis of B728a revealed a substantial difference in global gene expression patterns between the epiphytic phase and the endophytic phase of this pathogen in bean leaves. On the leaf surface, an increase in the expression of genes required for motility, chemosensing, absorption of phosphate, acquisition of sulfur compounds, and indole metabolism was observed (191). By contrast, apoplastic colonization results in an increased expression of genes required for the metabolism and transport of γ-aminobutyric acid and biosynthesis of specialized metabolites and phytotoxins during the pathogenic growth phase.

A recent study compared both early- and steady-state transcriptome profiles of a nonpathogenic mutant (disarmed for the type III secretion system and coronatine toxin production) of *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) with those of two endophytic commensal members of leaf microbiota, *Achromobacter xylosoxidans* Col-0–50 and *Pandoraea* sp. Col-0–28 (172) at 6 h, 24 h, and 168 h after being inoculated into *Arabidopsis* leaves. Results suggest that the transcriptomes of the commensal strains are similar to that of the nonpathogenic mutant of *Pst* DC3000, with primary metabolic genes suppressed compared to the corresponding bacterial transcriptomes in culture media. At later time points, the transcriptomes exhibit features of a stationary phase–like status, and a shift in bacterial physiological processes toward production of osmoprotectants and specialized metabolites presumably facilitated an adaptation to the apoplast environment (172). These observations are similar to another recent study that compared early-stage transcriptomes of nine phylogenetically diverse commensal *Arabidopsis* phyllosphere bacteria at 6 h after inoculation into *Arabidopsis* leaves. Again, it was found that the transcriptomes of commensals exhibit similar features of a nonpathogenic mutant of *Pst* DC3000, showing suppression of genes involved in general metabolism in contrast to the virulent pathogen *Pst* DC3000. Interestingly, when transcriptomic comparisons were made between bacteria grown in liquid media versus in leaves, enhanced expression was observed for genes that were previously reported to be plant associated (91),

which may be related to bacterial adaptation to the phyllosphere environment (129). However, enrichment of plant-associated genes was not observed when comparisons were made between bacteria grown in agar media versus bacteria grown inside leaves, raising the possibility that solid agar may mimic some of the cues for the upregulation of plant-associated genes inside the leaf apoplast (172).

In summary, emerging transcriptome analyses have begun to reveal adaptive features of phyllosphere commensal and pathogenic strains to epiphytic and endophytic leaf compartments. Although these transcriptional studies are providing the first insights into understanding bacterial genes differentially expressed in planta, additional functional analyses of genes associated with the identified transcriptomic features are required to better understand the transcriptional and metabolic adaptation of bacteria to the surface and the endosphere of the phyllosphere.

Rhizosphere: the plant-associated microbial habitat comprising the zone of soil immediately adjacent to and influenced by plant roots

Gnotobiotic: describes a plant or organism in which all other associated organisms are known

Synthetic community (SynCom): a collection of two or more microorganisms created artificially by combining desired species in a controlled manner

THE RELATIONSHIP BETWEEN PHYLLOSPHERE, SOIL, ROOT, AND ANIMAL MICROBIOTAS

Phyllosphere microbiota can be derived from multiple sources, including soil, seeds, and air, at different stages of plant growth and development (14, 58, 70, 112) (**Figure 2**). A study comparing microbiotas in above- and belowground grapevine plant compartments showed that the rhizosphere-associated bacterial community was largely different from the phyllosphere bacterial community. However, leaves, flowers, and fruits were colonized by a higher proportion of bacterial members that were more similar to taxa present in soil rather than each other (192). This indicates that soil is a common inoculum source for phyllosphere colonization, and many members of the leaf microbiota are probably derived from soil during either germination or relocalization postgermination. Indeed, for *Streptomyces* bacteria colonizing roots, endophytic translocation to stem and flower via vascular bundle has been reported (78). However, other microbial sources also influence microbiota composition in various parts of the phyllosphere (discussed below).

Despite the fact that aboveground tissues are exposed to a very different environment than belowground tissues (the rhizosphere), studies have shown that there can be substantial overlap in microbiota. For example, Bai and coworkers (9) used a large collection of bacterial strains derived from roots and shoots to perform controlled gnotobiotic colonization experiments. Plant colonization with synthetic communities (SynComs) of bacteria including both rhizosphere and phyllosphere isolates resulted in the assembly of an overlapping community similar to the natural community colonizing their cognate host organs under a gnotobiotic system. This result suggests that specialization and adaptation for colonizing different compartments (i.e., phyllosphere versus rhizosphere) of plants are in play to facilitate niche-specific bacterial community assembly. Genomic analysis of root-, shoot-, and soil-derived bacterial community members suggested an enrichment of carbohydrate metabolism in shoot- and soil-derived strains, whereas root-derived strains showed an enrichment of xenobiotic biodegradation and catabolism (9). These genomic features may be in line with the availability of various nutrients in the rhizosphere via root exudates and the scarcity of available carbon sources in soil and potentially the leaf environment.

Within the phyllosphere, natural events such as wind, rain, and insect visitors could introduce and redistribute microbiota across different phyllosphere compartments such as stems, pollen, nectar, flowers, and fruits. In particular, the plant phyllosphere surface could be considered a continuum across which epiphytic microbiota members could be distributed. In pollen microbiomes, pollination style has a major effect on microbial community structure and diversity, specifically in insect-pollinated plant species (113). Although flowers before opening contain minor bacterial and fungal communities (154, 176), animal visitors, such as hummingbirds, pollinating insects, and florivores during subsequent flower development, are reported to change the composition of

nectar and flower microbial communities by introducing animal-associated yeasts, bacteria, and viruses (170).

The flower microbiome can directly impact fruits and subsequently seed microbiomes, especially seed endophytic communities (119, 125). Among the factors that impact the seed epiphytic microbial community is the mode of seed dispersal. Various seed dispersal methods mediated by wind, water, insects, and animal ingestion followed by defecation or regurgitation can expose

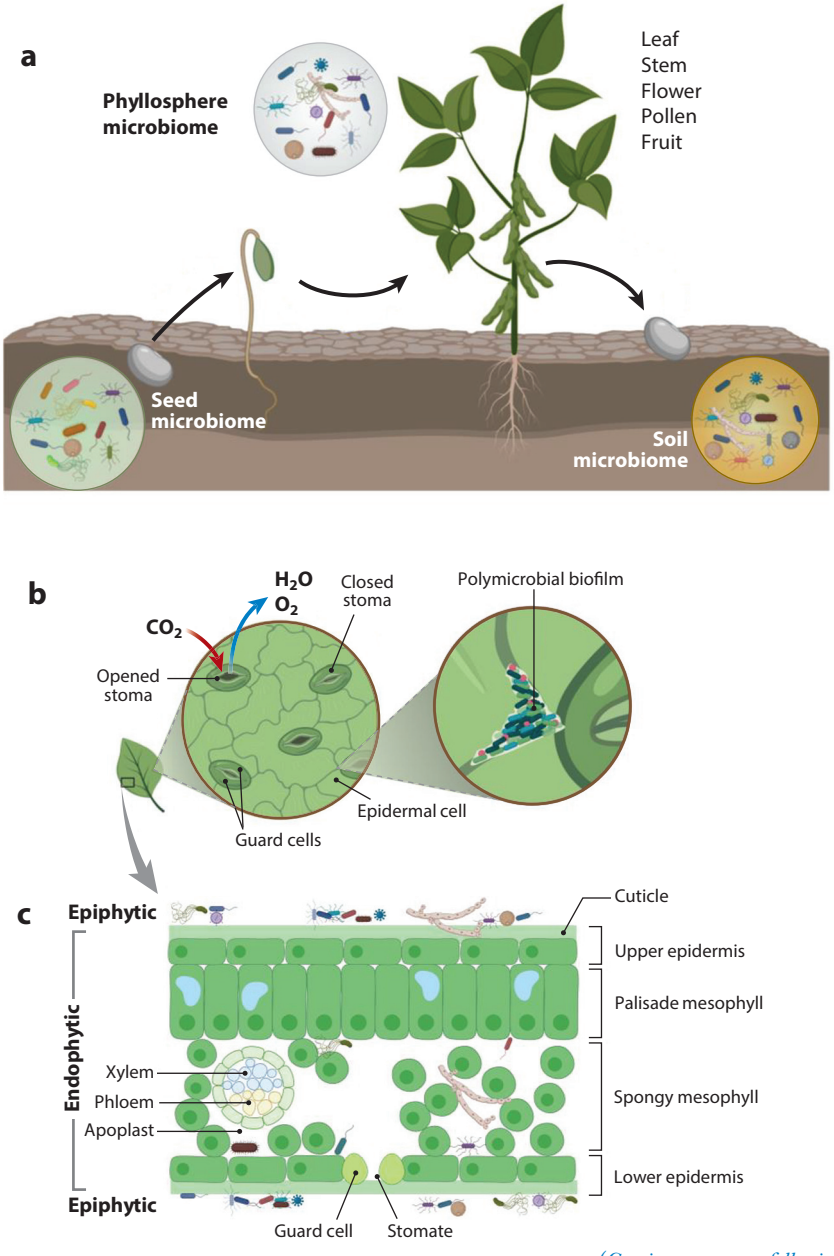


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Figure 2 (*Figure appears on preceding page*)

Various forms of phyllosphere microbiota occupy different phyllosphere niches. (*a*) Colonization of different parts of plants by microbiota during plant growth at different developmental stages is driven by several factors, including geography, seasonal variations, genetics, and plant age. (*b*) A schematic illustration of a zoom-in view of the leaf surface. Stomatal openings are gas-exchange gateways to support plant photosynthesis and growth. A further magnified illustration shows an epiphytic population of microbiota clustered as aggregates in protective biofilms at high numbers containing diverse microbiota. (*c*) A cross-section of leaf shows its different anatomical components. Endophytic microbiota is sparsely distributed at low levels in the air-filled intercellular space within leaves called the apoplast or in the cytosol. Panel *a* was created with Biorender.com, panels *b* and *c* were adapted from the templates "Leaf Surface Structure" and "Evaporation of Eater from the Leaf Surface", respectively, by BioRender.com (2022), retrieved from **https://app.biorender.com/biorender-templates**.

seeds to different microbiota communities (125, 170). Fruit consumption by birds and mammals and passage of intact seeds through the intestinal tracts are especially helpful because they break the seed dormancy and expose the seeds to a microbe- and nutrient-rich environment present in animal fecal matter upon defecation (165). The exposure of plant seeds to a diverse fecal microbiota via either animal-mediated seed dispersal or agricultural application of animal manure to soils has been implicated in enhancing seedling development and protection against selected pathogens (25, 39).

Vertical microbial inheritance is observed in a limited number of microbes, most notably the Clavicipitaceae fungal endophytes of grasses (36). These endophytes produce potent alkaloids, which can improve host defense and increase host fitness (35). However, nongrasses have been observed to be infected by vertically transmitted fungal seed endophytes, including *Alternaria alternata* and *Cladosporium sphaerospermum* (64), while bacterial seed endophytes are known in dozens of plant species (166), with densities as high as $\sim 10^8$ CFU/g in *Brassica napus* (56). Collectively, several environmental and niche-dependent forces impact the overall phyllosphere microbial community structure, highlighting the importance of higher-level trophic interactions in the assembly of plant microbiota (**Figure 2**).

COMMMENSALS VERSUS PATHOGENS IN THE PHYLLOSPHERE

Although commensal microbes dominate phyllosphere microbiota, some microbes that inhabit the phyllosphere are pathogens, including pathogenic bacteria, fungi, viruses, and oomycetes. Despite pathogen species representing only a tiny portion of the overall phyllosphere microbiome, they can cause devastation in natural ecosystems and crop fields. A major difference between commensal and pathogenic microbes is the ability of pathogens to break through the population restriction mechanisms imposed by the plant host, leading to uncontrolled proliferation and a negative impact on host health. This ability is conferred by pathogenicity/virulence genes that commensal members of the phyllosphere microbiota generally lack. Although different types of pathogens can harbor different sets of pathogenicity/virulence genes, research in the past few decades has revealed common themes by which diverse pathogens overproliferate in the phyllosphere. The most important theme is the ability of pathogenic viruses, bacteria, phytoplasmas, fungi, and oomycetes to counteract host immune responses by virulence-associated effector proteins (104, 164, 185). With the exception of intracellular viruses, in which the effector proteins are produced directly inside the plant cell, other pathogens living in the apoplast have evolved specialized protein secretion systems that enable them to deliver effector proteins into the plant cell (104, 164, 181, 185). In addition to suppressing plant immune responses, pathogen effector proteins are also involved in creating nutrient- and water-rich apoplastic microenvironments that are conducive to aggressive proliferation (53, 66, 144, 186). These common themes have been reviewed extensively for model phyllosphere pathogen species (41, 88, 103, 185) and are not repeated here. An important

Vertical microbial inheritance: transmission of a parent's microorganisms to offspring

take-home message is that genetic mutations that affect the production or delivery of these effector proteins often convert virulent pathogens to resemble commensal microbes, in that they are unable to multiply aggressively or cause damage to the phyllosphere. For example, bacterial pathogen mutants defective in the type III secretion systems are nonpathogenic (31), and so are fungal pathogen mutants defective in the effector delivery systems (54, 107).

A question often arises regarding the relationship between commensals and pathogens. In the evolutionary context, pathogens are generally believed to have evolved from their plant-associated commensal ancestral relatives after acquiring a full set of pathogenesis-related traits. In the case of the bacterial pathogen *P. syringae*, for example, a hypothetical path of such evolution has been proposed (185). It appears that at least in the case of the *P. syringae* species, the plant growth-promotion traits, such as auxin production and 1-aminocyclopropane-1-carboxylate (ACC) deamination activity, evolved before pathogenesis-related traits appeared (185). Conversely, as the process of evolution from commensals to pathogens requires acquisition of potentially many traits, one may expect to find that certain commensals could carry some, but not all, pathogenicity/virulence-associated genes. This is indeed the case in genome analyses of microbiota. For example, some rhizosphere and phyllosphere microbiota (bacterial) strains contain well-known pathogenicity/virulence genes, including parts of or full type III protein secretion gene clusters that are characteristic of many apoplastic bacterial pathogens (29, 91). It is not entirely clear, however, whether these microbiota strains are truly evolving toward pathogens or if they represent naturally mutated progenies of ancestral pathogens. In several instances, the transition between pathogenic and mutualistic/commensal microbiota could be accomplished by the acquisition or loss of a virulence plasmid. In bacteria, horizontal gene transfer of a virulence plasmid between *Rhodococcus* isolates can convert a potentially mutualistic strain to a pathogenic strain and vice versa (151). In fungi, horizontal gene transfer of virulence factors, such as ToxA from *Parastagonospora nodorum* transferred to *Pyrenophora tritici-repentis*, can directly lead to the emergence of pathogenic strains derived from commensal leaf inhabitants (50). Hybridization (159), mutations (68), and genomic rearrangements (134) have each been observed to alter pathogenicity-related traits, causing increases in virulence and/or expansion of host range.

It is important to note that the phyllosphere environment also serves as a niche for some human pathogenic bacteria (168). Multiple outbreaks of human illness are linked to consumption of fresh fruits and vegetables contaminated by enteric pathogens such as *Salmonella enterica* and *Escherichia coli* O157:H7 (20). These human pathogenic enterobacteria have the ability to colonize the plant endophytic and epiphytic compartments (20, 152). Apoplastic colonization is reported in a wide range of plant species, including lettuce, tomato, spinach, basil, cilantro, *Nicotiana benthamiana*, and *Arabidopsis thaliana* (27, 38, 57, 84, 145, 184). Upon entering the apoplastic environment, these enteric pathogens remain protected during the sanitation treatments applied to edible crops (153), posing a threat to animal and human hosts. Perhaps apoplastic colonization is unsurprising, since plants are colonized by many other enterobacteria that are found in phyllosphere microbiota, such as *Pectobacterium*, *Erwinia*, *Pantoea*, *Brenneria*, and *Enterobacter* (59). Due to a significant role of fresh phyllosphere tissues in animal and human diets and their potential to act as a reservoir for animal and human pathogens, it is imperative that future research should increasingly pay attention to a causative connection between phyllosphere and animal/human gut microbiomes in the context of the One Health concept (169).

In short, it is likely that, in nature, the phyllosphere microbiota in healthy plants is composed of microbes with a continuous spectrum of plant-impacting traits, including mostly commensals but occasionally pathogen or symbiont species. This then inspires the question of how the plant immune system engages and negotiates with different members of phyllosphere microbiota, particularly the vast number of commensal microbiota, a topic that is discussed below.

One Health concept: recognizes that the health of humans is closely connected to the health of animals, plants, and their shared environment and encourages a collaborative, multisectoral, and transdisciplinary approach to achieving optimal health outcomes

PLANT IMMUNE RESPONSE TO PHYLLOSPHERE MICROBIOTA

In the past three decades, how plants respond to microbes has been extensively studied and reviewed (62, 71, 96, 130). The plant immune system appears to have multiple interconnected functional modules that allow plants to respond to different types of microbes (i.e., symbiotic, commensal, or pathogenic). A basic module relevant to potentially all microbes is activated upon plant detection of conserved microbe-associated molecular patterns (MAMPs) by pattern recognition receptors (PRRs). Activation of PRRs results in signal transduction involving a series of kinase relays, an increase in cytosolic calcium concentration, the activation of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades, and the production of apoplastic reactive oxygen species (ROS) and subsequent global transcriptional and metabolic changes, ultimately leading to MAMP-triggered immunity (MTI) against microbial proliferation (71).

Several studies have probed the extent of plant immune interaction with microbiota via gene expression analysis under various experimental systems (111, 129). A recent study using 38 phylogenetically diverse bacterial members of microbiota derived from the *Arabidopsis* phyllosphere (*At*-LSPHERE) suggested that a majority of the tested bacterial community members trigger expression of a common set of immunity-associated genes in the phyllosphere upon plant colonization (111). This overlapping transcriptional output comprises a set of 24 immune-associated genes called the general nonself response genes. Additionally, the same phyllosphere microbiota strains induced accumulation of tryptophan-derived defense metabolites, suggesting a role for specialized metabolites in plant response to leaf microbiota.

A well-known MAMP is flg22, a bioactive 22–amino acid (aa) peptide derived from bacterial flagellin. Flg22 is recognized by the FLAGELLIN SENSING 2 (FLS2) PRR, a plasma membrane (PM)-localized leucine-rich repeat family receptor kinase. A wide variety of commensals, including members of the bacterial phyla Pseudomonadota, Bacillota, and Actinomycetota, although not Bacteroidota, have the capacity to produce flagella (13, 106). In the phyllosphere, a higher activity level for the *FLS2* promoter is observed in leaf bacterial entry sites such as stomata and hydathodes (13), suggesting that these microbial entry sites may be where the plant immune system is on high alert. Recent studies suggest that some commensals, similar to virulent pathogens, have evolved strategies to evade flagellin-triggered immune response (37, 180). For example, several microbiota members have the ability to abolish or dampen flg22-mediated host responses (37). Additionally, a high degree of variation and diversion from the flagellin-encoding *fliC* gene of *Pseudomonas aeruginosa*, PA22, from which the canonical flg22 epitope is derived, was observed in 627 studied bacterial genomes mostly derived from the phyllosphere and rhizosphere of *Arabidopsis*. These variations can be categorized into 3 clades. While Betaproteobacteria, Gammaproteobacteria, and Sphingomonadales (clade 1) showed 47% overall sequence identity, in Bacillota and Actinomycetota (clade 2), 58% sequence identity was observed. The highest degree of divergence (41% sequence identity) was observed for Alphaproteobacteria, particularly Hyphomicrobiales and Caulobacterales (clade 3). These variations were associated with evading or causing a differential effect on several plant immune responses. In *Arabidopsis* plants colonized with a community of 185 bacterial members harboring all the observed variations, an enrichment in plant colonization with immune-evading flg22 variants in both shoot and root communities was observed, while the immunogenic flg22 peptide-containing strains were depleted (37). These data suggest that evading the flg22-mediated response positively correlates with the ability of community members to dominate host colonization in healthy plants.

In addition to evading PRR-mediated recognition, commensals have evolved other mechanisms to suppress immune activation during the course of plant colonization. Several studies have highlighted the involvement of lowering local environmental pH (190) and secreted proteins of

the bacterial type II secretion system (162) in immune suppression in the rhizosphere. Although these examples are based on root microbiota studies, similar mechanisms might be utilized by phyllosphere microbiota, and therefore their relevance to phyllosphere microbiota colonization needs to be examined in the future. Interestingly, the presence of only immune-suppressive strains has been correlated with loss of the immune protective capacity of the community against pathogens, while plant colonization by the immunogenic community has enhanced plant protection but at the cost of compromised plant growth, presumably due to growth–defense trade-offs (60). Therefore, in the context of a complex community in which different members present varying degrees of immunogenic MAMPs and plants are colonized with commensals with both immune-suppressing and immune-activating potentials, an intricate interplay of microbe–microbe and host–microbe interactions likely define immune homeostasis, a topic that will require more extensive and holistic studies.

FUNCTIONS OF THE PHYLLOSPHERE MICROBIOTA

Microbial colonization of the phyllosphere can influence plant health and productivity at different scales, from individual plants to terrestrial ecosystems (**Figure 3**). Below we highlight studies that implicate phyllosphere microbiota in global carbon and nitrogen cycles, growth promotion, stress tolerance, and biological control.

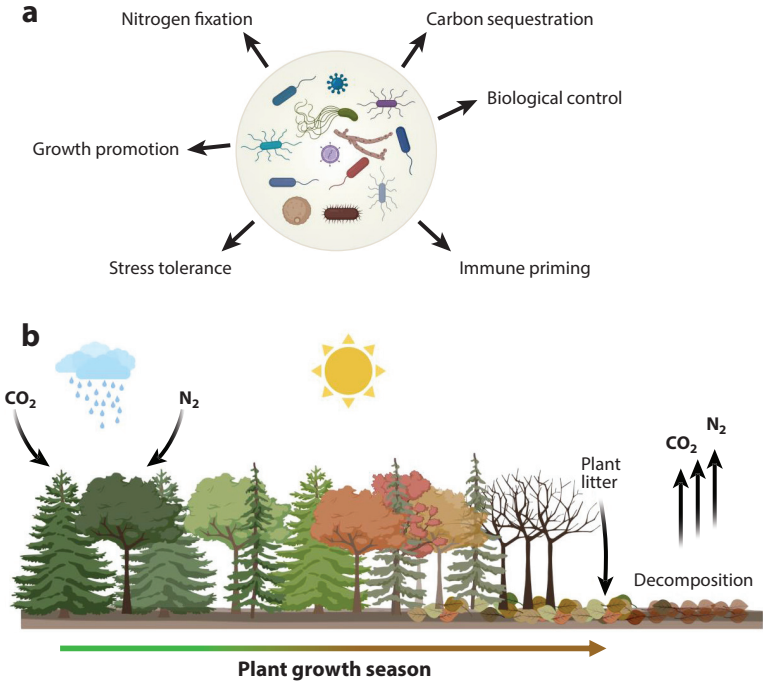

Figure 3

Impact of phyllosphere microbiota colonization on plant biology. (*a*) Different roles of microbiota in promoting plant health are depicted. (*b*) Microbiota influences plant health and productivity at different ecological scales, from individual plants to ecosystems. The process of diazotrophic nitrogen fixation and carbon dioxide sequestration during plant growth and subsequent conversion into plant litter upon senescence followed by decomposition impacts the global cycle of carbon and nitrogen at the ecosystem scale. Figure adapted from images created with BioRender.com.

Ecosystem Carbon Cycles

Axenic: describes a plant or organism that is free from other viable organisms

The phyllosphere can emit diverse volatile compounds, such as terpenes (including isoprene and monoterpenes), flavones, and C1 compounds (methanol, methane, and halogenated methane), into the atmosphere, constituting a major source of biogenic volatile organic compounds (VOCs). The plant-emitted volatiles have significant effects on other organisms and can regulate global climate by impacting the atmospheric chemistry and physics via acting as greenhouse and ozone-depleting gasses (135). The members of the phyllosphere microbiota play an important role in the carbon cycle by metabolizing VOCs as a source of carbon and energy to support their growth (75). For example, epiphytic populations of methylotrophic bacteria in the genus *Methylobacterium*, in particular, have the capability to utilize C1 VOCs such as methanol emitted, at the highest levels, from plant stomata to support their growth (28), which is correlated with their abundant presence surrounding stomata (1). Additionally, methanol emissions reported from axenic *Nicotiana tabacum* seedlings were significantly higher compared to the seedlings colonized by *Methylobacterium*, suggesting methanol consumption by bacteria (1). Collectively, the ability to utilize VOCs provides certain phyllosphere microbiotas the ability to adapt and thereby thrive on the leaf surface environment and contribute to the global carbon cycle and climate regulation. However, it remains to be clearly shown if the production of VOCs is a plant-adaptive trait to recruit specific microbiome members.

Ecosystem Nitrogen Cycles

In addition to contributing to the global carbon cycle, the asymbiotic fixation of atmospheric nitrogen gas by leaf-associated diazotrophic microbiota members such as Cyanobacteria was initially documented in tropical forests where plants are predominantly grown in nutrient-poor and acidic soils (15). Further research revealed the presence of additional nitrogen-fixing bacteria from Alpha-, Beta-, and Gammaproteobacteria taxa in the phyllosphere (51). Furthermore, several studies have reported diazotrophic bacteria from temperate and taiga forest plants (32, 142). Similar reports of nitrogen-fixing microbes in the phyllosphere of several important crop plants, such as maize, wheat, rice, and potatoes, among others, have also been documented (see 12 and references therein). Estimates of nitrogen fixation in tropical forests suggest that symbiotic nitrogen fixation contributes only 20% to 50% to the total biogenic nitrogen fixation (1.2 kg N ha^{-1} yr^{-1}) depending on forest age (161), indicating that asymbiotic nitrogen fixation is a major source of biogenic nitrogen fixation. A recent study of nine tree species in an Amazon forest site measured the asymbiotic nitrogen fixation in the phyllosphere, litter, and rhizosphere soil as 0.33, 0.2, and 0.03 kg N ha^{-1} yr^{-1}, respectively, suggesting a significant contribution from the phyllosphere in this process (121). Therefore, atmospheric nitrogen fixation by phyllosphere microbiota members could represent a global process in natural and managed ecosystems, contributing to plant growth and ecosystem functioning.

Growth Promotion and Stress Tolerance

The phyllosphere harbors many growth-promoting microbes, which enhance plant growth through the production and/or modulation of plant growth regulators, including auxins (especially IAA), gibberellic acids, ethylene, cytokinins (CKs), and abscisic acid (ABA) (44). Among the phytohormones, IAA and CK synthesis is attributed to many phyllosphere microbes as either beneficial, mostly under stress conditions, to promote overall plant growth or detrimental, acting as virulence factors of pathogens (24, 158). Although IAA production by phyllosphere microbes has been suggested to have a role in enhancing plant growth and overall plant fitness productivity, the mechanism of its direct action in the phyllosphere without stress conditions

is not well understood (1, 173). However, overproduction of microbial IAA could impair root growth, which, in a complex community, has potentially led to selection for plant colonization with auxin-degrading strains to establish auxin homeostasis for plant benefits (47).

Several phyllosphere microbial members such as methanol-utilizing *Methylobacterium* bacteria can produce CKs, a family of plant hormones involved in promoting cell division and elongation, which can trigger the release of methanol during plant cell wall expansion and thereby promote bacterial colonization and plant growth (94, 128). Additionally, CK production by phyllosphere microorganisms can stimulate the transportation of nitrogen to aerial plant parts, thereby increasing the biomass of aboveground plant parts (65). Ethylene, a volatile hormone synthesized from ACC, triggers senescence in plants under adverse environmental conditions (69). ACC, as an amino acid, can be used as a nitrogen source for microbes that are able to produce ACC deaminase (174). This consumption of the precursor of ethylene suppresses senescence in the host and can enhance agronomically relevant tolerance to ethylene-inducing stresses, including drought, salinity, and waterlogging (124). Application of bacteria with ACC deaminase activity can enhance plant growth under these adverse conditions (8, 148). Similar to the ethylene pathway, the ABA pathway is an important regulator of plant stress tolerance (146). However, ABA production by microbiota tends to be substantially less than the amounts produced in plant tissues (167) and therefore may not contribute significantly to increasing stress tolerance. In many cases, phytohormone manipulation is an impactful means for the microbiota to alter host physiology for growth promotion and/or stress tolerance. Yet, the benefits are often context-dependent; thus, future research is needed to understand how to combine and regulate phytohormone-manipulating traits in a microbial SynCom context to provide benefits across variable environments.

Biological Control

A well-documented functional role of phyllosphere microbiota is biocontrol against pathogens, which, in turn, contributes to plant health. Various microbiota-mediated mechanisms have been reported, including induction of plant host immune response, competitive exclusion, and antibiosis (171). The enhancement of host immunity against pathogen invasion involves several mechanisms, including activation of immunity triggered by microbiota MAMP recognition (reviewed in 138) and the release of quorum-quenching molecules to interfere with bacterial pathogenesis (5, 109). Homoserine lactone–based quorum-sensing is involved in regulating the expression of virulence factors in many plant-associated Gram-negative bacterial pathogens such as *Pseudomonas* species (114, 177). Several beneficial members of microbiota in the phyllosphere with quorum-quenching activities were reported to interfere with pathogen virulence gene expression through degradation of quorum-sensing signals and consequently to suppress disease progression (5). In addition, several bacterial genera with quorum-quenching capacity, such as *Acinetobacter*, *Bacillus*, and *Pseudomonas* species, have been reported from the phyllosphere of tobacco (109).

Direct microbe–microbe interactions between pathogens and commensals via competition for access to colonization sites and resources in the phyllosphere can act as an effective biocontrol strategy. Indeed, a direct competition between *Sphingomonas* strains and plant pathogen *P. syringae* for host-derived carbon sources, such as fructose, glucose, and sucrose, is implicated in limiting pathogen growth in *Arabidopsis* (67). This implies that niche competition can potentially act as an effective mechanism to keep pathogenic members of microbiota at bay. Additionally, antibiosis can act as another effective mechanism for reducing plant pathogen abundance. Phyllosphere bacteria are capable of producing broad-spectrum antibiotics that are effective against a range of bacterial and fungal pathogens (98). Genome mining of the phyllosphere bacterial communities from *At*-LSPHERE has identified a large number of biosynthetic gene clusters with putative functions

in the biosynthesis of diverse classes of specialized bacterial metabolites. These include ribosomally synthesized and posttranslationally modified peptides, nonribosomal peptide synthetases, polyketide synthases, and terpene synthases, which could collectively play an important role in microbe–microbe interactions in the phyllosphere (61). Finally, the type VI bacterial secretion system (T6SS), which is involved in the injection of toxic effector proteins into target cells, is known to play a role in interbacterial killing and competition (63). The T6SS in *Pseudomonas putida* effectively mediates the killing of the plant pathogenic bacterium *Xanthomonas campestris* in vitro and protects plant leaves against this pathogen in vivo (21). Recently, a partial contribution to plant protection against *P. syringae* by the T6SS of a *Rhizobium* strain from the *At*-LSPHERE collection was reported (175). Collectively, these findings highlight the importance of direct microbe–microbe interactions in the phyllosphere in microbiota regulation and plant health.

Microbiota homeostasis: the process by which the abundance, composition, or function of a community of microorganisms is kept at a stable equilibrium

Eubiosis: a state of microbiota homeostasis associated with the maintenance of normal host processes typical to healthy plants

In line with earlier studies, the biocontrol mechanisms described above mostly emerged from studies where a single strain was evaluated for its beneficial functions under controlled experimental conditions. However, under natural or field conditions, a complex community of microbiota colonizes the phyllosphere with diverse beneficial and inhibitory effects on plant health and disease protection (175, 178). Under these conditions, new variables such as microbe–microbe interaction with already established communities, effects of environment on hosts, and effects of hosts' developmental stage could all impact the outcome of beneficial functions of the single isolates in a complex matrix. This area needs more research in order to develop truly robust phyllosphere biocontrol agents to effectively control pathogens in crop fields and natural ecosystems.

PLANT GENETIC CONTROL OF MICROBIOTA HOMEOSTASIS

The broad conservation of phyllosphere microbiota suggests that plants must have evolved mechanisms to select and maintain the abundance, composition, and function of phyllosphere microbiota to achieve homeostasis. Microbiota homeostasis likely results from a combination of host–microbe, microbe–microbe, and environmental interactions (**Figure 4**). Here, we use the term eubiosis to refer to the state of microbiota homeostasis associated with the maintenance of normal host processes typical of healthy plants under ideal conditions. The extent to which eubiosis is necessary for maintaining the overall health of a host is a fundamental question that remains to be answered. Indeed, the importance of microbiota homeostasis in the phyllosphere had not been clear until recent studies determined that when plant mutants defective in certain pathways displayed altered microbiota levels and composition they showed serious tissue damage (34, 137). This discovery has been made possible, in part, by the recent development of gnotobiotic plant growth systems with controllable biotic components, which allow for causative studies (67, 83, 118, 193) (see the sidebar titled Gnotobiotic Plant Growth Systems).

GNOTOBIOTIC PLANT GROWTH SYSTEMS

Gnotobiotic plant growth systems are one type of emerging tool that researchers have utilized to determine the causality of the function of a plant microbiota and its individual members. A number of these gnotobiotic plant growth systems have been introduced recently, with a notable difference between them being the type of substrate used for plant growth [discussed in detail by Kremer et al. (83) and Ma et al. (110)]. Broadly, these systems are designed to sequester plant growth under biotically controlled conditions and prevent contamination of plant tissues by environmental microorganisms. This allows for the generation of axenic germ-free plants as well as plants inoculated with a microbial community in parallel. Gnotobiotic plant growth is sometimes paired with the use of rationally designed SynComs (see the sidebar titled Synthetic Microbial Community).

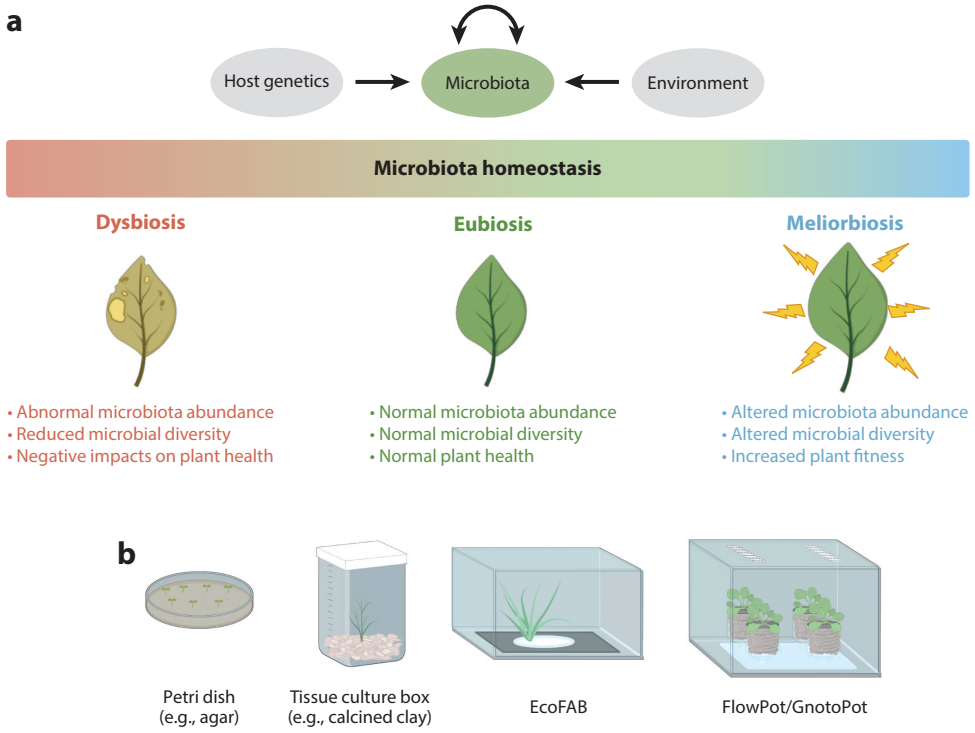

Figure 4

Microbiota homeostasis in the phyllosphere. (*a*) Microbiota homeostasis is the process by which the abundance, composition, or function of a microbiota is kept at equilibrium through interactions between the host, microbes, and environment. The resulting state of microbiota homeostasis exists on a spectrum and can be defined based on the overall effect on plant health and resilience. (*Center*) Eubiosis is associated with typical host functions under ideal conditions, whereas (*left*) dysbiosis is associated with disease and/or negative impacts on plant health as a result of an altered microbiota. (*Right*) Meliorbiosis is associated with a shift in eubiotic homeostasis during periods of stress accompanied with increased plant fitness, as indicated by the orange resilience symbols. (*b*) Various gnotobiotic systems, including those based on agar plates, tissue culture vessels with calcined clay or other plant growth substrates, EcoFAB devices, and FlowPots/GnotoPots, have facilitated novel discoveries in plant microbiota research. Figure adapted from images created with BioRender.com.

In one study, *Arabidopsis* quadruple mutants [*min7 fls2 efr cerk1* (*mfec*) and *min7 bak1-5 bkk1-1 cerk1* (*mbbc*)] were found to be compromised in their ability to regulate the level and composition of endophytic bacterial microbiota and exhibited spontaneous tissue damage under high humidity (34). These mutants are defective in two pathways: the PRR signaling pathway (caused by the triple PRR gene mutation *fls2 efr cerk1* or the triple coreceptor gene mutation *bak1-5 bkk1-1 cerk1*) and a vesicular trafficking pathway associated with the adenosine diphosphate ribosylation factor–guanine nucleotide exchange factor (ARF-GEF) protein HOPM1 INTERACTOR 7 (MIN7) involved in modulating the aqueous apoplastic microenvironment (34) (**Figure 5**). Notably, construction of a bacterial SynCom (see the sidebar titled Synthetic Microbial Community) reflecting the altered bacterial community in leaves of the *mfec* and *mbbc* mutants and transplantation of the resulting dysbiotic SynComs into otherwise healthy wild-type plants resulted in tissue damage (34). This study therefore established the causality of the dysbiotic SynComs in producing harmful effects in the phyllosphere. Additionally, a single point mutation (*cad1*^{S205F}) in the *Arabidopsis CONSTITUTIVE ACTIVE DEFENSE 1* (*CAD1*) gene, which codes for a membrane

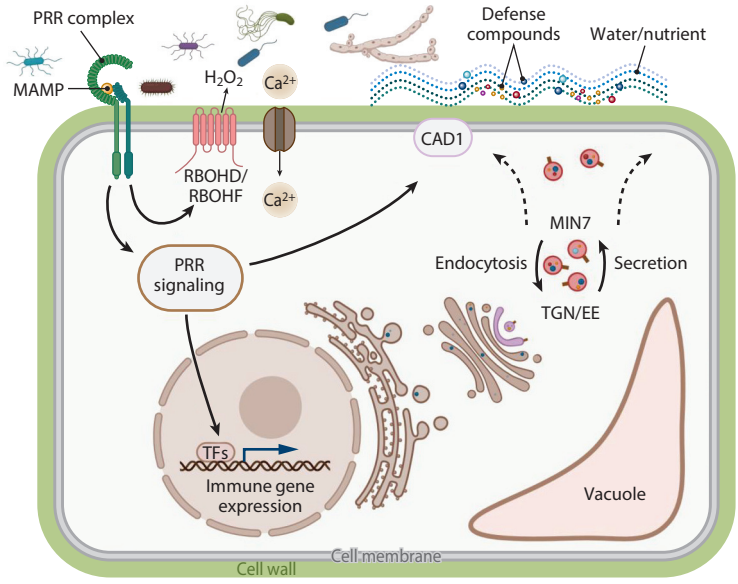

Figure 5

A summary of plant pathways modulating microbiome homeostasis in the phyllosphere. Upon MAMP recognition by PRRs and formation of PRR–coreceptor complexes, an immune signaling cascade is triggered, including activation of RBOHD to trigger extracellular ROS (mainly H_2O_2) production and generation of a calcium burst to further amplify downstream plant immune responses. Immune signaling inputs result in activation of gene expression and biosynthesis of immune-related metabolites (not depicted here). The MIN7 ARF-GEF is localized to the TGN/EE, which is important for regulation of the apoplastic microenvironment. CAD1 is a PM-associated MACPF-family protein. The level of CAD1 increases upon PRR signaling and is reduced in the *Arabidopsis min7* mutants. A dual defect in PTI and MIN7 pathways or genetic mutations affecting CAD1 or RBOHD/RBOHF alter the composition and level of the phyllosphere microbiota community, leading to dysbiosis. Abbreviations: ARF-GEF, adenosine diphosphate ribosylation factor-guanine nucleotide exchange factor; CAD1, CONSTITUTIVE ACTIVE DEFENSE 1; MACPF, membrane attack complex/perforin; MAMP, microbe-associated molecular pattern; MIN7, HOPM1 INTERACTOR 7 (MIN7); PM, plasma membrane; PRR, pattern recognition receptor; PTI, pattern-triggered immunity; RBOHD, respiratory burst oxidase homolog D; RBOHF, respiratory burst oxidase homolog F; ROS, reactive oxygen species; TF, transcription factor; TGN/EE, *trans*-Golgi network/early endosome. Figure adapted from Structural Overview of a Plant Cell by BioRender.com (2022), retrieved from **https://app.biorender.com/biorender-templates**.

attack complex/perforin (MACPF)-family protein, caused dysbiosis similar to that of the *mfec* and *mbbc* quadruple mutations (34). PRR signaling, MIN7, and CAD1 appear to be functionally connected at some level, as PRR signaling activation or the *min7* mutation was found to affect the abundance of the CAD1 protein in *Arabidopsis* leaves (34). In a separate study by Pfeilmeier and colleagues (137), *Arabidopsis* mutants defective in nicotinamide adenine dinucleotide phosphate (NADPH) oxidases belonging to respiratory burst oxidase homolog (RBOH) family, RBOHD and RBOHF, which generate apoplastic ROS, were found to harbor an altered phyllosphere bacterial community and spontaneous disease-like symptoms, while axenic plants did not. Together, these studies provide evidence that leaf dysbiosis can be causative to negative impacts on overall plant health and begin to reveal components of a putative plant genetic network, including PRR signaling, MIN7-associated vesicle trafficking, PM-associated CAD1, and apoplastic ROS-generating NADPH oxidases, in controlling microbiota homeostasis and preventing dysbiosis in the phyllosphere (**Figure 5**).

Dysbiosis: a state of microbiota homeostasis associated with negative impacts on host health

SYNTHETIC MICROBIAL COMMUNITY

Synthetic microbial communities (SynComs) are constructed using culturable members of a natural microbiota based on taxonomical clades, functional properties, and/or other nature-mimicking criteria appropriate for a specific study. SynComs with defined microbiota composition derived from field soil, for instance, allow researchers to apply reductionist approaches in characterizing microbiota impact on soil properties. Together with gnotobiotic plant growth systems (see the sidebar titled Gnotobiotic Plant Growth Systems), designer SynComs have played a crucial role in a number of recent discoveries, including demonstrating the causal role of a plant microbiota in dysbiosis (34); showing the biocontrol activities within a microbiota by commensals (43); and dissecting an interplay between nutrient status, availability, and host colonization (48), among a growing list of other notable findings.

Although dysbiosis in plants has thus far largely been studied in the context of bacterial communities (34, 77, 137), Wolinksa and colleagues (183) demonstrated that fungi too are capable of causing dysbiosis in *Arabidopsis*, in this case in the rhizosphere, which can be countered by the plant tryptophan metabolism pathways combined with bacterial commensals. These emerging studies showing a critical role of plant immunity and other cellular pathways in preventing harmful dysbiosis raise new questions. For example, one might imagine that an alteration of plant immunity and other microbiota-relevant plant pathways under abiotic or biotic stresses could shift the level and composition of phyllosphere microbial communities toward potentially damaging microbiota members. Indeed, Berens and coworkers (18) showed that, when plants were grown under high salt stress, which compromises plant immunity, a change in microbiota community structure was observed. Understanding how plants control eubiotic microbiota and prevent dysbiosis under challenging abiotic conditions represents an exciting area that requires future investigation, as such studies could lead to innovative solutions to improve phyllosphere health and resilience.

It should be noted that deviation of the microbial community from eubiosis can sometimes be associated with positive effects (known as meliorbiosis) (132) (**Figure 4**), particularly during periods of stress (17, 42, 147). For example, feeding on aboveground tissues of pepper plants by aphid (89) and whitefly (188) resulted in restructuring the rhizosphere microbiota, which was associated with enhanced resistance to belowground pathogens. One popular explanation for this phenomenon, which is often referred to as the cry-for-help hypothesis, is that plants actively recruit beneficial microorganisms by selectively altering exudate profiles during times of stress (143). Recently, a mechanism for apparent meliorbiosis in the rhizosphere has been described in *Arabidopsis* (157). Some fungal and nematode pathogens are known to secrete a type of peptide that mimics host plant rapid alkalinization factors (RALFs). These pathogen-derived peptides initiate signaling through the host receptor kinase FERONIA (FER) and subvert jasmonate hormone-mediated plant defense to benefit pathogen infections (116, 163, 194). Song et al. (157) found that peptide signaling through FER alters the basal concentration of ROS on the root surface in a manner dependent on the small GTPase ROP2 to regulate the relative abundance of beneficial pseudomonads in the rhizosphere, acting as a form of pathogen-inducible biocontrol (157). Whether similar mechanisms exist to selectively induce meliorbiosis in the phyllosphere remains an open question and an area for future interest.

Meliorbiosis: a state of microbiota homeostasis associated with positive impacts on host health under stressful conditions

CONCLUSION AND FUTURE PERSPECTIVES

With a long history of studying mostly individual microbial strains, research on phyllosphere microbiota in the past decade has shifted to community-level understanding, owing to the adoption

of omics technologies, model plant species (including *A. thaliana*) and reductionist approaches including gnotobiotic plant growth systems and SynComs. Compared to a decade ago, we now have much better knowledge about which microbes are present in the phyllosphere across plant taxa and an increasing number of advanced tools and resources to investigate the collective functions of phyllosphere-inhabiting microbes at the community level. In celebrating these advances, the field of phyllosphere microbiota research is now at a crossroad regarding the next phase. What are the most critical questions to address next? How can the fundamental knowledge learned from model plants, such as *A. thaliana*, be translated to crop fields and natural ecosystems? Can we develop better microbiota-based solutions to improve plant performance and resilience to abiotic and biotic stresses? Comprehensive inventories of phyllosphere microbiotas across plant lineages, plant developmental stages, and geographic regions will likely remain a high priority in the coming decade. Such surveys will deepen our appreciation of microbiota as an integral part of the Earth's vast phyllosphere. Future inventory studies, however, should increase efforts to analyze microbial taxa beyond bacteria to dramatically expand the knowledge of nonbacterial phyllosphere microbiota members. A concerted effort should also be made in these studies to include high-quality, descriptive metadata, which will allow the larger research community to use these inventories in their own studies.

We predict that a key advancement in the coming decade will be in the area of functional and mechanistic understanding of the phyllosphere microbiota at the community level. Indeed, in several plant species there is strong indication that phyllosphere microbiota research is already transitioning from surveys to mechanistic and causative studies. This transition will likely intensify in the coming years. To facilitate this transition, construction and sharing of well-characterized SynComs are an essential step and will be required in order to facilitate community-wide efforts, including the identification of key phyllosphere trait-impacting strains/SynComs as well as microbiota-derived metabolites that impact phyllosphere traits. Similarly, in the coming years, we can expect increased research progress to identify plant genes that control phyllosphere microbiota homeostasis. Identification of phyllosphere-impacting microbiota features and plant genes should form a new knowledge base for the development of next-generation microbiota-enabled technologies that could robustly improve plant growth, productivity, and resilience under different environmental conditions.

Further understanding of phyllosphere microbiota structure and function will require development and/or adoption of new technologies. For example, 16S rRNA gene–based profiling methods need to be further optimized to more efficiently profile low-abundance microbial taxa in the endophytic compartment of the phyllosphere. This is because the great abundance of host mitochondrial and chloroplastic DNA often overwhelms conventional 16S RNA gene profiling of endophytic bacteria, which are usually very low quantity. A recently developed method for cleaving the host-derived 16S rDNA amplicons using the CRISPR-Cas9 system is a potentially promising improvement in tackling this challenge (156). A method for the simultaneous measurement of microbial load and determination of community composition should facilitate cost-effective functional microbiota studies (108). Other technologies, including microbiota-based biosensors and community-wide single-cell transcriptome profiling, are needed to break away from low-resolution bulk-tissue analyses and to bring our understanding of the phyllosphere microbiota to a higher resolution that reflects more closely the physiology and behavior of individual microbiota members in situ.

Another area of microbiota research to watch in the coming decade is the interplay between microbiota and pathogens in the phyllosphere. Conventional studies of phyllosphere pathogenesis have largely ignored the existence of endogenous microbiota due to lack of proper gnotobiotic plant growth systems to compare disease progression in the absence and presence of microbiota

colonization. Do pathogens act alone in causing diseases, or does pathogen invasion reshape microbiota to facilitate its pathogenesis and/or persistence in the phyllosphere? In the context of emerging infectious diseases, does genetic exchange occur between pathogens and commensal microbiota? If it does, is such exchange affected by climate change that could lead to novel pathogens in a warming climate? Finally, does the phyllosphere microbiota play a role in plant adaptation to climate change? Can we develop microbiota-based solutions to increase the resilience of the phyllosphere to increasingly frequent abiotic stresses associated with a warming planet? Clearly, many new questions have emerged as a result of phyllosphere microbiota studies in the past decade, and many fundamental principles remain to be discovered to fully understand the collective impact of microbiota on the ability of Earth's vast phyllosphere to carry out some of the most fundamental biological processes, including photosynthesis, biomass accumulation, and ecosystem-impacting carbon/nitrogen cycling.

SUMMARY POINTS

1. The phyllosphere provides a vast terrestrial habitat for microbial colonization. Although many microbes live on the surfaces of the phyllosphere as epiphytes, some microbes live inside the phyllosphere as endophytes.
2. Decades of studies of individual phyllosphere microbiota strains have provided a wealth of information regarding how individual microbes colonize the phyllosphere and how microbes compete with each other in the phyllosphere as a basis for biological control.
3. Recent studies have focused on generating metagenomic data to gain a systems-level understanding of the composition and dynamics of the phyllosphere microbiota in diverse plant taxa and under different environmental conditions.
4. Model plants such as *Arabidopsis thaliana* are being used to study the interplay between plants and phyllosphere microbiota at the molecular level, including plant responses to phyllosphere microbiota and how plants regulate microbiota homeostasis in the phyllosphere.
5. A major future research direction is to conduct causative and mechanistic studies to decipher how the phyllosphere microbiota impacts plant health and modulates key biological properties of the phyllosphere. This requires the development of nature-mimicking synthetic microbial communities and gnotobiotic systems.
6. Characterization of bacteria in endosphere-specific compartments by 16S ribosomal RNA gene amplicon sequencing can be problematic due to low microbial biomass and contamination from plant-derived plastid and mitochondrial sequences. Improvement of profiling methods are needed.
7. The phyllosphere microbiota is connected to animal/human gut microbiota. A closer examination of the impact of the phyllosphere microbiota on animal/human gut health is needed in the context of the One Health concept.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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