Antibiotic Resistomes in Plant Microbiomes

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Abstract

The plant microbiome, the exterior interface between plants and the environment, acts as a biological barrier to prevent invasion by foreign pathogens. Genomic sequencing and "omic" analyses have found that a few bacterial phyla predominate in the aerial components of various plants and have identified the involvement of plant immunological stress responses in the assembly of the belowground habitat. The plant microbiome, the interface between human and environmental microbiomes, represents a major pathway by which humans are exposed to environmental microbes and antibiotic resistomes. In this review, we first highlight the main findings in plant microbiomic research addressing two fundamental questions, who is there and what do they do, and then focus mostly on the dissemination of the resistomes of plant microbiomes by food consumption or direct contact. Microorganisms associated with plants may alter the traits of the human microbiome associated with human health, but this alteration has not received enough attention.

Plant microbiomes - who is there?

Plants do not grow as axenic organisms in nature but are populated by diverse microorganisms, known as the plant microbiome (Bulgarelli *et al.*, 2013, Muller *et al.*, 2016; Penuelas and Terradas, 2014). Microorganisms can generally colonize all plant tissues (Turner *et al.*, 2013), but we will focus on the phyllosphere, the aerial components of plants, and the rhizosphere, the belowground habitat of plant roots. Our knowledge of phyllospheric microbiology has historically lagged behind our knowledge of the rhizosphere (Vorholt, 2012). This situation has changed in recent years with the advances of next-generation sequencing (NGS) technologies and corresponding bioinformatic tools and with increasing affordability (Bai *et al.*, 2015, Vorholt *et al.*, 2017). A large and rapidly expanding body of phylogenetic information about plant microbiomes is becoming available, which will tell us who is there (Delmotte *et al.*, 2009, Bulgarelli *et al.*, 2012, Horton *et al.*, 2014, Edwards *et al.*, 2015). Recent studies have reported that only a few phyla (Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria) are dominant in plant microbiomes across host species such as *Arabidopsis thaliana* (Lundberg *et al*., 2012, Bodenhausen *et al*., 2013), *Brassica chinensis* (Chen *et al.*, 2017), soybean (Delmotte *et al.*, 2009, Mendes *et al.*, 2014), grapevine (Zarraonaindia *et al.*, 2015) and several tree species (Redford *et al.*, 2010). The presence of some microorganisms may be due to stochastic events (Vorholt *et al.*, 2017), but a core of shared taxa at the phylum and lower levels have been identified within plant microbiotas that consistently and reproducibly establish and are

adapted to life in the phyllosphere (Muller *et al.*, 2016). Various studies have also attempted to identify core microbiota across phylogenetically distinct plants or multiple accessions of one plant species or occupying more than one plant compartment (Delmotte *et al.*, 2009, Zarraonaindia *et al.*, 2015, Muller *et al.*, 2016). The evidence suggests a widespread "global" core community across host species, space and time.

The phyllosphere is relatively nutrient poor and subject to extremes of temperature, radiation and moisture (Vorholt, 2012). In contrast, the rhizosphere is a region of rich, largely soil-derived, microbial diversity influenced by the deposition of plant mucilage and root exudates (Kent & Triplett, 2002; Preece and Penuelas, 2016). Except for the core microorganisms established in the phyllosphere, plant roots grow within extremely diverse microbial communities but assemble a taxonomically limited microbiome (Lebeis *et al.*, 2015). For example, the root microbiota of *A. thaliana*, a common model plant for the study of molecular biology and genetics, has substantial compositional consistencies not only across multiple continental soils but also across multiple *Arabidopsis* lineages (Bulgarelli *et al.*, 2012, Lundberg *et al.*, 2012, Beattie, 2015). We have summarized the bacteria in the phyllospheres and rhizospheres of various plant species based on published data (Table 1).

Plant microbiomes - what do they do?

The plant microbiome, as the second genome of the plant, can influence host phenotypes such as growth and tolerance to pathogens, pests and environmental stresses (Mendes *et al.*, 2011, Lau & Lennon, 2012, Beattie, 2015, Muller *et al.*, 2016, Bakker

et al., 2018). The plant-root system, where nutrients are taken up by the host and plants are exposed to surrounding microbes, has been suggested to be functionally analogous to the animal gastrointestinal system (Berendsen *et al.*, 2012, Haney & Ausubel, 2015). An increasing body of evidence also suggests the importance of this root microbiome, which consists of the entire complex of rhizospheric microbes, their genetic elements and their interactions, in increasing plant growth and decreasing susceptibility to diseases caused by pathogenic fungi, bacteria, viruses and nematodes (Kloepper *et al.*, 2004, Yang *et al.*, 2009, Lebeis *et al.*, 2015, Mendes *et al.*, 2018).

Plant microbiomes extend the host phenotypes that affect host reproductive fitness, so plants have likely evolved mechanisms to attract and maintain microbial populations based on their key functional capabilities by innate or induced, stimulatory or inhibitory, activities and may have thus co-evolved with their associated microbes (Panke-Buisse *et al.*, 2015, Theis *et al.*, 2016). In addition to the direct effects on deleterious rhizospheric microbes, many rhizospheric microbiotas elicit beneficial physical or chemical changes to boost the defensive capacity in aboveground plant components, a process referred to as induced systemic resistance (ISR) (van Loon *et al.*, 1998, Bakker *et al.*, 2013). ISR in leaves can be activated by specific rhizospheric microbiotas colonizing roots and is mediated by jasmonic acid and ethylene (Pieterse *et al.*, 2012, Belkhadir *et al.*, 2014, Carvalhais *et al.*, 2015). In contrast, salicylic acid, a key immune regulator in leaves, also drives selection from the available microbial communities to form the root microbiome, indicating that plant immune signaling and root microbiomic assembly are functionally linked and suggesting potential cross-talk between above-

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and belowground plant components (Turner *et al.*, 2013, Lebeis *et al.*, 2015).

Plant microbiomes may also influence ecosystem function and global biogeochemical cycles (Philippot *et al.*, 2013, Turner *et al.*, 2013, Laforest-Lapointe *et al.*, 2017) by their role in extending the host genome and may contribute to the host phenotype (Muller *et al.*, 2016). The diversity of the foliar microbiome has been positively linked to ecosystem productivity, and this link is also maintained after accounting for the contribution of host diversity (Laforest-Lapointe *et al.*, 2017). Phyllospheric microbiome play also a key role in the large amounts of biogenic volatile organic compounds emitted annually by plants to the atmosphere (Penuelas et al 2015, Farre-Armengol et al 2016). Moreover, the soils of agricultural crops can stimulate microbial denitrification and methanogenesis, contributing to emissions of N_2O and methane, respectively (Turner *et al.*, 2013). These gases not only represent a loss of carbon and nitrogen from the system but also exacerbate the greenhouse effect.

Plant microbiomic resistome – movement and human health

The plant microbiome is the interface between the human and environmental microbiomes and represents a major pathway by which humans are exposed to antibiotic-resistant bacteria and resistance genes that are naturally present in the environment. Our knowledge of antibiotic resistomes in nonclinical environments is currently increasing rapidly. Wastewater-treatment plants and operations producing concentrated animal feed are two important reservoirs of antibiotic resistomes associated with humans, and a large body of data is available on the profiles of antibiotic

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resistomes (Baquero *et al.*, 2008, Rizzo *et al.*, 2013, Zhu *et al.*, 2013, Su *et al.*, 2017). The application of sewage sludge and animal manure to agricultural land are common practices for recycling nutrients and increasing crop productivity and also represent a major method for the disposal of waste.

Many studies have investigated the potential spread of resistomes in the environment by agricultural organic fertilization under both laboratory and field conditions using qPCR, high-throughput qPCR (HT-qPCR) and metagenomics. Such data have clearly demonstrated that procedures of organic production facilitate the dissemination of antibiotic resistomes in soil environments (Fahrenfeld *et al.*, 2014, Udikovic-Kolic *et al.*, 2014, Chen *et al.*, 2016), suggesting the need to optimize waste disposal for sustainable agriculture and waste recycling (Burch *et al.*, 2017). These studies, however, have mostly focused on the distribution of antibiotic resistomes in soil or wastewater, with little attention to the subsequent spread of antibiotic resistomes via plant/crop microbiomes.

Knowledge of plant microbiomic resistomes has largely lagged behind our knowledge of resistomes in their surrounding environments such as soil, water and air (Baquero *et al.*, 2008, Martinez, 2008, Allen *et al.*, 2010, McEachran *et al.*, 2015). Only limited examples are available but nevertheless provide crucial evidence, prompting studies that examine antibiotic resistomes in plant microbiomes. We have summarized the plant microbiomic resistomes based on the current data for food-borne antibiotic-resistant commensal bacteria and resistance genes (**Table 2**). An important finding among these rare examples was that resistome abundance is approximately eight-fold higher in

organically than conventionally produced lettuce (Zhu *et al.*, 2017). The lettuce samples used in this study were collected directly from supermarkets and may be consumed with little processing, so the lettuce poses a substantial risk for the transfer of antibiotic resistance to humans.

Antibiotic-resistance genes (ARGs) assessed by PCR-based methods, however, cannot provide information about the functionality and mobility of the ARGs, so more studies using culture-dependent and metagenomic approaches to investigate a broad range of vegetables are still required to reach broader generalizations. Interestingly, some resistomes were detected in plant microbiomes but not their surrounding environments, including soils, suggesting an intrinsic core resistome in the plant microbiome (Chen *et al.*, 2017). This result is very encouraging, because it is important for deriving the baseline of the resistome before evaluating its dissemination via the plant microbiome. Much of the resistome in the plant microbiome clearly overlapped with the surrounding resistome, indicating that these resistomes could be acquired and that soil resistomes might be a major source for plant resistomes. Direct supporting evidence from additional research, however, is needed.

Scenario 1 via the food chain

Food consumption represents a major route of exposure of the human microbiome to the environmental microbiome; the plant microbiome acts as a bridge connecting these two microbiomes. Recent findings suggested that the daily consumption of food products such as raw leafy vegetables and fruits is likely a major route for the

dissemination of antibiotic resistomes from the food chain to human hosts (Zhang *et al.*, 2011, Verraes *et al.*, 2013). For example, HT-qPCR has identified hundreds of ARGs in the plant microbiome, and organic fertilization has markedly increased the occurrence of ARGs, suggesting that plants can acquire exogenous ARGs (Chen *et al.*, 2017). Plants can also take up antibiotics from soil amended with animal manure (Kumar *et al.*, 2005), an organic fertilizer commonly used in agriculture, which may apply selection pressure on the plant microbiome and contribute to the emergence and spread of antibiotic resistance to the human food chain.

Most of the plant commensal carriers of ARGs are fortunately nonpathogenic (Zhang *et al.*, 2011), but their potential involvement in the spread of ARGs to other bacterial genera, including human pathogens, by the horizontal gene transfer (HGT) of mobile genetic elements (MGEs) is a concern (Rossi *et al.*, 2014). The class I integron-integrase gene (*intI*1) and genes encoding transposases are common on harvest vegetables, indicating the possibility of gene transfer in the phyllosphere (Wang *et al.*, 2015). Phyllospheres and rhizospheres are two key areas for horizontal gene transfer in plant and soil habitats due to the high possibility of cells to cluster, forming biofilms in phyllospheres, and bacterial metabolic rates and the mobility of bacteria and MGEs are high in rhizospheres (van Elsas *et al.*, 2003).

Scenario 2 via food processing, preservation techniques and transportation

Food is often processed and preserved to obtain safe products, extend shelf lives and maximize profits. These processes include drying, heating to reduce water content,

cooling, acidification, modified-atmosphere packaging by decreasing oxygen and increasing carbon dioxide, freezing, mild pasteurization and UV irradiation (Gould, 1996, Rico *et al.*, 2007). Their effects on bacterial flora depend on the combination, type and conditions of the technologies used. Most processing methods can reduce the number of food-borne bacteria and thus the potential risks. For example, freezing and UV irradiation can stress or damage bacterial cells and ultimately kill or inactivate the bacteria (Rico *et al.*, 2007). The dead bacterial cells, however, can be lysed due to cell-wall damage. DNA, including possible ARGs, is consequently released into the environment. The transformation of extracellular DNA plays an important role in HGT and the dissemination of antibiotic resistance in the environment (Levy-Booth *et al.*, 2007, Nielsen *et al.*, 2007).

The movement of microorganisms has historically mainly been due to natural factors, such as air and water currents or even natural events (Zhu *et al.*, 2017). Globalization has dramatically affected these dynamics, with cheap and highly efficacious long-range transport systems allowing many transnational corporations to manufacture food products in regions with less expensive labor, using ingredients from around the world and then transporting them worldwide (Saker *et al.*, 2004). Bacteria associated with plants cannot be completely eliminated during production, storage, processing and packaging, so they are inevitably transported with their host material by global trading, which has profound impacts on the evolution and dissemination of antibiotic resistomes and may cause unpredictable infections. For example, outbreaks of *Salmonella poona* infections in the USA associated with eating melons imported from Mexico have been

linked to unhygienic irrigation and packaging at source farms (Saker *et al.*, 2004). The situation is exacerbated by the cultivation of non-indigenous crops in some low-income regions to meet market demands, and these crops may be more susceptible to indigenous pathogens. For example, Guatemalan raspberries became contaminated with the protozoan *Cyclospora*, causing outbreaks of gastroenteritis in the USA and Canada (Saker *et al.*, 2004). The development and implementation of national and international guidelines for assessing the biological risk of microbiomes associated with food is a strategic priority for alleviating these threats.

Concluding remarks and outlook

Summary

1. The two fundamental questions about plant microbiomes, who is there and what do they do, are becoming increasingly well defined. The phyllosphere has historically been less intensively studied than the rhizosphere, but this situation is beginning to change. The available data suggest that the plant microbiome is dominated by bacteria and bacterial communities on above- and belowground organs, has a defined taxonomic structure and is consistently composed of a few phyla, mainly Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes. The bacterial communities in plant microbiomes have relatively low diversity but have profound consequences on host health and functional traits and even on ecosystem productivity and global climate change.

2. We advocate that more attention should be paid to the antibiotic resistome in plant microbiomes due to the public health threat posed by antibiotic resistance and

because the plant microbiomes can act as a bridge connecting the human and environmental microbiomes. Our current understanding of antibiotic resistomes associated with plants is still in its infancy, but experimental evidence suggests that the food chain is the main route by which human microbiomes are exposed to the antibiotic resistomes in plant microbiomes. Globalization may unpredictably disseminate antibiotic resistomes associated with plants, but we believe that different kinds of experiments and surveys and international and interdisciplinary collaborations will be needed to obtain an integrated understanding of plant antibiotic resistomes.

Acknowledgements
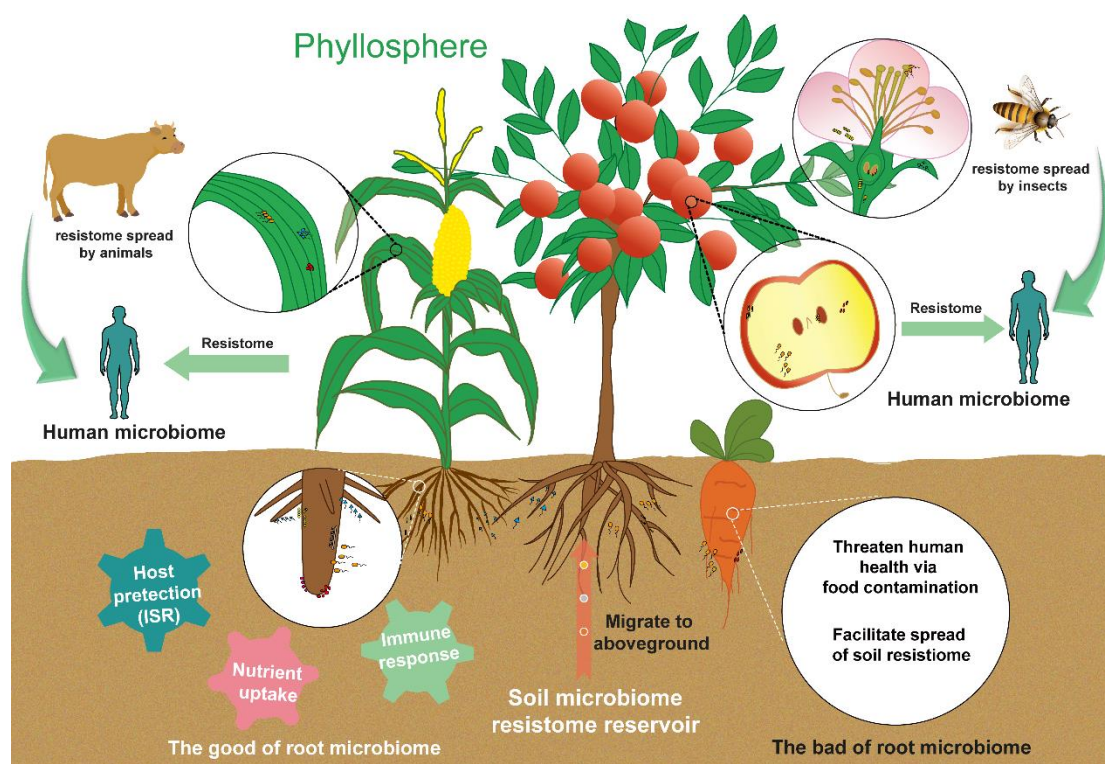
Figures and tables

Figure 1 Schematic overview of the plant microbiome. The aboveground components of plants, collectively known as the phyllosphere, represent an inherently open and variable habitat dominated by leaves. Antibiotic resistomes associated with phyllospheres can influence human microbiomes by direct consumption and contact or by the food chain. The belowground components of plant roots are collectively known as the rhizosphere. The good aspects of the rhizospheric microbiome include nutrient acquisition, tolerance to abiotic stress, protection against viruses and other pathogens via induced systemic resistance (ISR) and activation of the plant immune system in response to foliar pathogenic attack (immune response). The bad aspects of the rhizospheric microbiome include possible facilitation of the spread of antibiotic resistomes and risks to human health from contaminated food.
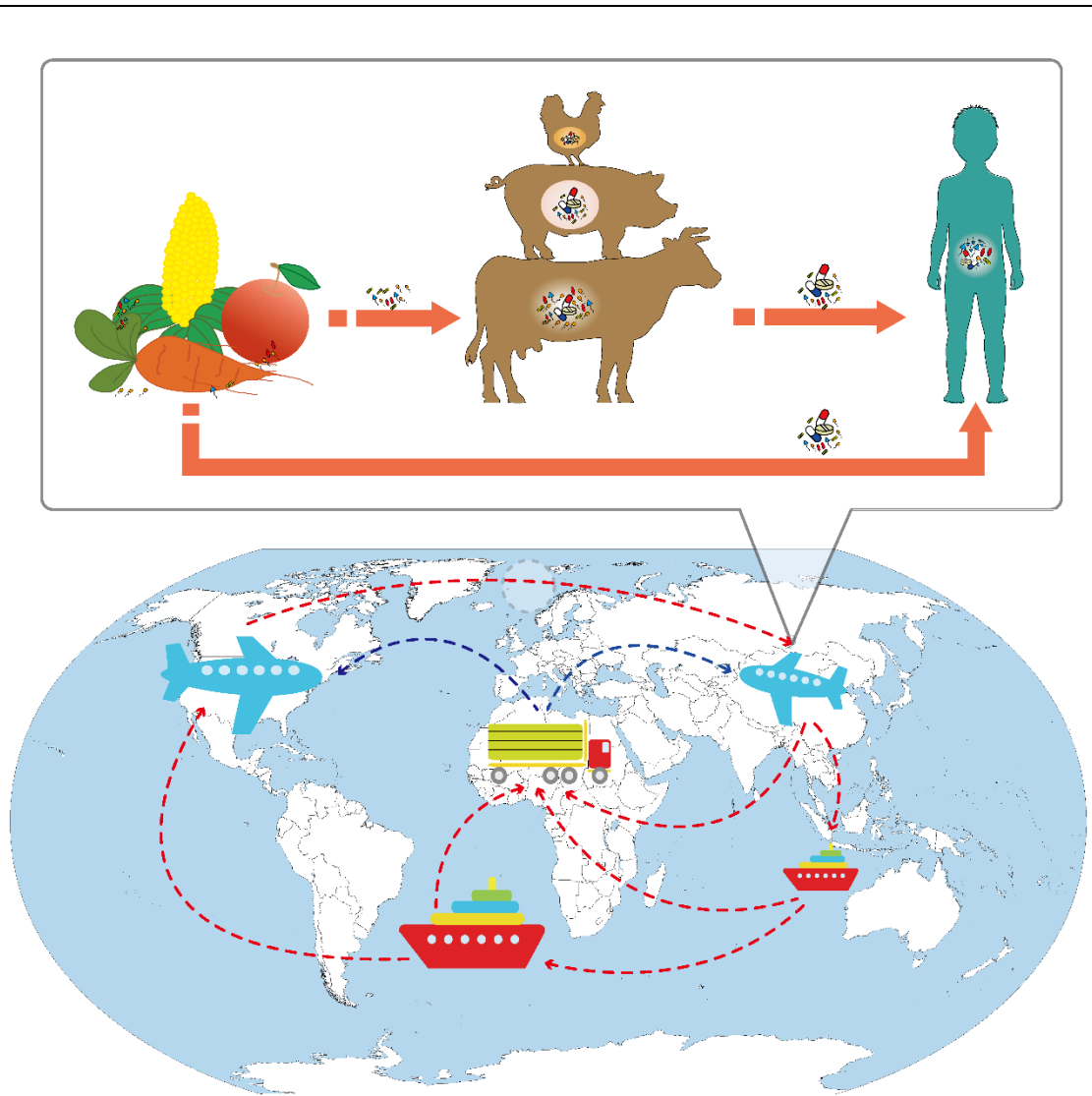
Figure 2 Scenario of the plant microbiomic antibiotic resistome. Direct contact and food chains represent major pathways by which human microbiomes are exposed to environmental resistomes. Globalization may exacerbate the dissemination of resistomes worldwide via plant resistomes.

Host species	Phyllosphere	Rhizosphere	Dominant phyla	Methodology	Reference
Espeletia	●		Proteobacteria	Illumina sequencing of 16S rRNA gene amplicons	Ruiz-Perez *et al.* (2016)
			Actinobacteria		
		●	Proteobacteria		
			Acidobacteria		
Arabidopsis thaliana		●	Acidobacteria	Pyrosequencing of 16S rRNA gene amplicons	Bulgarelli *et al.* (2012)
			Planctomycetes		
			Proteobacteria		
		●	Acidobacteria		Lundberg *et al.* (2012)
			Actinobacteria		
			Bacteroidetes		
			Proteobacteria		
Hordeum vulgare (barley)		●	Proteobacteria	Illumina shotgun sequencing and pyrosequencing of 16S rRNA gene amplicons	Bulgarelli *et al.* (2015)
			Actinobacteria		
			Bacteroidetes		
Arabidopsis thaliana		●	Proteobacteria	Pyrosequencing of 16S rRNA gene amplicons	Bodenhausen *et al.* (2013), Horton *et al.* (2014)
			Bacteroidetes		
			Actinobacteria		
	●		Proteobacteria	Pyrosequencing of 16S rRNA gene amplicons	Bodenhausen *et al.* (2013)
			Actinobacteria		
			Bacteroidetes		
Glycine max (soybean)	●		Proteobacteria	16S rRNA gene clone libraries	Delmotte *et al.* (2009)
			Actinobacteria		
			Bacteroidetes		
		●	Proteobacteria	454 metagenomic shotgun sequencing	Mendes *et al.* (2014)
			Actinobacteria		
			Firmicutes		
Pinus flexilis Pinus strobiformis	●		Proteobacteria	Pyrosequencing of 16S rRNA gene amplicons	Redford *et al.* (2010)
			Bacteroidetes		
Catalpa speciosa Fraxinus pennsylvanica Fraxinus americana Magnolia soulangeana	●		Bacteroidetes		
			Firmicutes		
			Actinobacteria		
			Proteobacteria		

Table1 Dominant bacterial phyla in rhizospheres and phyllospheres.

Plant			Phylum	Method	Reference
Maize		●	**Proteobacteria**	**Pyrosequencing of 16S rRNA gene amplicons**	**Peiffer *et al.* (2013)**
			Bacteroidetes		
			Actinobacteria		
Grapevine	●		Proteobacteria	Illumina sequencing of 16S rRNA gene amplicons	Zarraonaindia *et al.* (2015)
		●	Proteobacteria		
			Acidobacteria		
			Bacteroidetes		
Lettuce	●		Proteobacteria	Pyrosequencing of 16S rRNA gene amplicons	Rastogi *et al.* (2012)
			Firmicutes		
		●	Proteobacteria		Cardinale *et al.* (2015)
			Bacteroidetes		
			Chloroflexi		
Rice		●	Proteobacteria	Illumina sequencing of 16S rRNA gene amplicons	Edwards *et al.* (2015)
			Acidobacteria		
			Chloroflexi		
	●		Proteobacteria	454 metagenomic shotgun sequencing, 16S rRNA gene clone libraries	Knief *et al.* (2012)
			Actinobacteria		
		●	Proteobacteria		
			Chloroflexi		
Edible rape	●		Proteobacteria	Illumina sequencing of 16S rRNA gene amplicons	Chen *et al.* (2017)
			Firmicutes		
		●	Proteobacteria		
			Chloroflexi		
			Actinobacteria		
Sugarcane		●	Proteobacteria	Pyrosequencing of 16S rRNA gene amplicons	Yeoh *et al.* (2016)
			Bacteroidetes		
			Actinobacteria		
Potato		●	Actinobacteria	Pyrosequencing of 16S rRNA gene amplicons	Inceoglu *et al.* (2011)
			Proteobacteria		

Table 2 Recent findings for antibiotic resistomes in plant microbiomes.

Host	Major findings	Methodology	Reference
Lettuce **Tomato** **Radish** **Carrot**	1) All targeted genes were detectable on vegetables in the presence of sewage but not detectable on vegetables in unamended soil. 2) A target was never more abundant on a vegetable grown in the presence than the absence of sludge a year after the application of sewage sludge.	**Culture-dependent method and quantitative PCR**	Marti *et al.* (2014), Rahube *et al.* (2014), Rahube *et al.* (2016)
Lettuce	1) Over a hundred unique ARGs were detected in the phyllosphere and foliar endophyte of market lettuce. 2) Organic lettuce harbored more ARGs than conventionally produced lettuce.	**High-throughput quantitative PCR**	Zhu *et al.* (2017)
Tomato **Radish** **Carrot** **Cucumber** **Pepper** **Lettuce**	1) Soil receiving manure was enriched in ARBs and ARGs, but the abundance of ARBs for vegetable grown in manure-fertilized soil did not correspondingly increase. 2) Numerous ARGs were detected on vegetables grown in control soil. Only a smaller number of ARGs were additionally detected on vegetables grown only in manured	**Culture-dependent method and quantitative PCR**	Marti *et al.* (2013)
Edible rape	1) Detected ARGs	**High-throughput**	Chen *et al.* (2017)

	enriched after application of struvite. 2) Twenty-five ARGs were innate in phyllosphere but not detectable in soil 3) Bacterial taxa shared between rhizosphere and phyllosphere were significantly correlated with ARGs.	**quantitative PCR and Illumina sequencing**	
Lettuce	Biochar amendment can be a practical strategy to protect crops from ARGs, and human pathogenic bacteria (HPB) contamination	**Quantitative PCR and Illumina sequencing**	Ye *et al.* (2016), Duan *et al.* (2017)
Lettuce Endive	1) Five subtypes of ARGs (*sul*I, *tet*G, *tet*C, *tet*A and *tet*M) and a class I integron-integrase gene (intI 1) were detected in both endophytes and phyllospheres. 2) Planting can affect the distribution of ARGs in manure-amended soil	**Quantitative PCR**	Wang *et al.* (2015)

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Figure legend

Figure 1 Schematic overview of plant microbiome. The aboveground parts of plants, collectively called the phyllosphere, represent an inherently open and variable habitat that is dominated by leaves. The phyllosphere-associated antibiotic resistome can influence human microbiome through direct consumption and intact or through food chain. The belowground parts of plants, collectively called the rhizosphere. The good aspects of rhizosphere microbiome including nutrient acquisition, tolerance to abiotic stress, protection against pathogen and viruses via induced systemic resistance (ISR), and activation of the plant immune system in response to foliar pathogen attack (immune response). The bad aspects of rhizosphere microbiome including it may facilities the spread of antibiotic resistome and pose risks to human health through contaminated food.

Figure 2 The scenario of plant microbiome antibiotic resistome. Direct intact and food chain represents a major pathway by which expose human microbiome to nature resistome. While globalization may exacerbate the dissemination of resistome worldwide via plant resistome