The lettuce-associated microbiota*

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This Compendium would be incomplete without a section that highlights our current understanding of the microbial communities, or microbiota, that naturally associate with lettuce (*Lactuca sativa*). The term 'microbiota' is used here to mean the microbial biota –bacteria, fungi, yeast, oomycetes, even viruses– that can be found on and in the above-/below-ground parts of lettuce plants. Included in the lettuce microbiota (or 'microbiome') are the pathogens that are featured in this Compendium, as well as all other microorganisms, more specifically those for which pathogenic potential has not (yet) been demonstrated and can be classified as commensals (living off the plant but causing no harm), as well as those that confer a known or unknown benefit to the plant (often in exchange for some service by the plant). The technology to describe these microbiota has advanced rapidly in recent years, in particular the types of technologies that are DNA-based and do not require culturing of the microorganisms. Costs for these types of analyses have gone down, so that more data can now be collected cheaper, which has really lowered the threshold for exploring the lettuce microbiota in depth: what are these microorganisms, how do they interact with each other and their host, and how do these interactions impact the health of lettuce and of those who consume it? An overview will be given of the types of microorganisms that are found on lettuce, their co-occurrence with each other, their functions insofar known, and the various factors that influence the structure of microbiota, including the plant itself and the environment it grows in. It should be remembered that this overview is based on a small set of pioneering studies on the topic of lettuce microbiota and that many of the insights shared here will require future scrutiny. Still, several key principles are already emerging, as will be discussed at the end of this section.

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1. Population size, structure, and function of lettuce microbiota

Many protocols exist for describing the size, structure, and function of microbial communities that associate with lettuce (and other plants). Most start out with the washing of leaf or root material in a suitable buffer to isolate microorganisms from the plant surfaces, followed by transfer of these microorganisms to nutrient broth or agar (culture-dependent approaches) or extraction of microbial DNA, RNA, or protein (culture-independent approaches). Sometimes, washing is replaced by surface swabbing (e.g. for leaves) or by maceration of the plant tissue (e.g. to analyze the endophytic microbial communities, i.e. those that reside inside the plant tissue, including viruses). Important to note is that culture-dependent and -independent methods can differ dramatically in their representation of the microbial community, both in terms of size and composition. For example, estimates of bacterial population sizes on leaf surfaces of field-grown lettuce were 10 to 1,000 times smaller based on agar plate counts than on DNA-based measurements of the number of copies of the 16S ribosomal RNA gene (Rastogi et al., 2010). There are several explanations for this difference. The first is that not all bacteria retrievable from lettuce leaves are able to utilize the nutrients provided in the agar plates, and so not all will grow, make a colony, and be included in the count. Secondly, some bacteria might be in a so-called 'viable but not culturable' ('VBNC') state that precludes them from growing at all, regardless of the medium. A third possibility is that some of the single colonies on the agar plate represent outgrowth from more than one bacterial cell, e.g. a bacterial aggregate that was not properly broken up into individual cells after it was washed from the lettuce leaf. Together, these three explanations clearly warn of limitations to culture-dependent approaches, as they tend to underestimate microbial population size and diversity on plants. However, culture-independent approaches are not perfect either: the fact that many bacteria carry more than one copy of the 16S ribosomal RNA gene on their genome can lead to overestimation of population size and misrepresentation of community structure. What all this suggests is that a survey of lettuce microbiota should probably combine both culture-dependent and -independent approaches.

Much of the microbial data collected from lettuce to date suggests that different parts of a lettuce plant, e.g. leaves versus roots, represent very different habitats that feature significantly different microbial communities. While some microbial taxa can be found in both habitats (as true generalists, or as contaminants, for example soil particles on leaves), most taxa appear to be unique to either leaves or roots (Ibekwe and Grieve, 2004; Zwielehner et al., 2008; Erlacher et al., 2014). In the next two subsections, leaf and root communities will be discussed separately, before dedicating sections 2, 3, and

4 to a more detailed description of the various factors that impact the size, structure, and function of microbial communities on lettuce.

1.1 Leaves

On lettuce leaves, population sizes of culturable bacteria range anywhere between 10^3 and 10^8 colony-forming units per gram of plant tissue (Hunter et al., 2010; Rastogi et al., 2010; Rastogi et al., 2012; Jackson et al., 2013; Williams et al., 2013). Estimates like these vary greatly between field-grown, laboratory-grown, and store-bought samples (Jackson et al., 2013) and depend on whether bacteria were washed from the leaf surface or extracted from inside the leaf tissue (Jackson et al., 2013). The most commonly found taxa among culturable bacteria from lettuce leaf surfaces are *Pseudomonas*, *Pantoea*, *Arthrobacter*, *Flavobacterium*, *Acinetobacter*, and *Bacillus* (Jackson et al., 2013; Dees et al., 2015; Medina-Martinez et al., 2015). Of these, *Pantoea* is of particular interest: members of this genus belong to the family Enterobacteriaceae and are typical leaf-dwellers, commonly found on the foliage of many plant species besides lettuce, including trees. However, *Pantoea* is also classified, together with its cousin *Erwinia*, as a 'coliform', a term that is often (and unfortunately) misinterpreted. The best-known coliforms are the fecal ones like *Escherichia coli* (*E. coli*): these reside in the gastrointestinal tract of humans and animals, and are therefore good indicator organisms for fecal contamination of drinking water, raw meat, leafy greens and other produce. Fecal coliforms, however, are only a subset of all coliforms; the others, including *Pantoea*, are called non-fecal or environmental coliforms. These are ubiquitous in soil, on plants, and in other natural habitats. Obviously, the distinction between environmental and fecal coliforms is important (and regrettably not always made, see for example the March 2010 issue of Consumer Reports Magazine): a positive outcome of a 'total coliform' test (which does not make the distinction between fecal and environmental) does not necessarily signify a fecal contamination event and will require further testing. Contamination of lettuce with human pathogens such as *E. coli* is a food safety issue and a topic that is discussed in greater detail elsewhere in this Compendium.

Many of the culturable bacteria that are found on lettuce leaves also show up in culture-independent analyses. *Pseudomonas* and Enterobacteriaceae such as *Pantoea* are among the most abundant and consistently identified bacterial taxa by this method (Zwielehner et al., 2008; Hunter et al., 2010; Ibekwe et al., 2010; Rastogi et al., 2012; Leff and Fierer, 2013; Williams et al., 2013; Dees et al., 2015). Other taxa include *Sphingomonas*, *Bacillus*, *Arthrobacter*, *Massilia*, and *Leuconostoc*. The latter belongs to a group of so-called lactic acid bacteria (LAB). Some LABs have been shown to confer health benefits to humans, for example as probiotics. The finding of *Leuconostoc* species on lettuce leaves has therefore led some (Zwielehner et al., 2008) to speculate about the contribution of leaf-associated microbiota to the health benefits of raw lettuce consumption.

It is not uncommon for DNA-based profiles of leaf microbiota from field-grown and store-bought lettuce (Rastogi et al., 2012; Jackson et al., 2013; Leff and Fierer, 2013) to show the presence of bacterial genera (*Pseudomonas*, *Xanthomonas*, and *Pectobacterium*) that include known foliar pathogens. However, most of today's DNA-based methods are not of sufficient resolution to unambiguously confirm, without additional tests (Rastogi et al., 2012), whether they are nonpathogenic representatives of these genera or representatives with true pathogenic potential towards lettuce, such as *Pseudomonas marginalis* pv *marginalis* (marginal leaf blight), *Pseudomonas cichorii* (varnish spot), *Xanthomonas campestris* pv *vitians* (bacterial leaf spot), and *Pectobacterium carotovorum* subsp *carotovorum* (soft rot).

1.2 Roots

Estimates for the size of microbial populations on lettuce roots differ greatly and depend on multiple factors, including soil type and choice of analysis (i.e. culture-dependent or -independent). Population sizes may vary along the length of a single root, and are typically larger in the rhizosphere than in the bulk soil (Maloney et al., 1997). This phenomenon is called the 'rhizosphere effect' and is linked to the exudation of plant compounds into the soil surrounding the roots. A meta-analysis of recent literature on the bacterial biota of lettuce roots reveals consistent association with *Pseudomonas* species, although many other genera have been identified as well (Ibekwe and Grieve, 2004; Erlacher et al., 2014; Krober et al., 2014; Cardinale et al., 2015). Compared to bulk soil, the soil surrounding roots tends to show an enrichment of genera that efficiently utilize these root exudates (Schreiter et al., 2014b). Root endophytic communities (those inside the root tissue) are much less diverse than the ones associated with the root surface (Scherwinski et al., 2008), suggesting that only a fraction of rhizosphere microorganisms are able or allowed to make it into the plant root tissue.

Few studies have been dedicated to the functional (rather than structural) diversity of microbial communities of the lettuce rhizosphere. Most noteworthy is an analysis that involved the DNA sequencing of the entire genome ('metagenome') of the root-associated microbiota (Krober et al., 2014) to provide first insights into the adaptations that allow bacteria and fungi to survive and thrive in the lettuce rhizosphere, including adaptations such as ammonia assimilation, stress resistance, amino acid metabolism, and production of antimicrobial compounds. More targeted approaches towards functional diversity have revealed differences between rhizosphere and bulk soil in the abundance of genes coding for proteases and alkaline phosphatases (Sakurai et al., 2007; Sakurai et al., 2008) and a higher ratio of copiotrophic (growing in high carbon environments) to oligotrophic (growing in low carbon environments) bacteria at the root tip than at the root base (Maloney et al., 1997). The latter is consistent with the exudation of carbon compounds by the lettuce root tips.

2. Plant species, cultivar, age and growth

From studies that compare the microbial community structure of lettuce and other plants grown or collected under similar conditions, it appears that there is such a thing as a lettuce-specific microbiota. Rhizosphere bacterial communities of lettuce are clearly different from those of other plants (zucchini, wheat, pumpkin, sweet pepper, tomato) grown under identical conditions, either in soil or hydroponically (Khalil et al., 2001; Pritchina et al., 2011). Bacterial communities on store-bought lettuce look very dissimilar to those retrieved from other produce types (sprouts, tomatoes, peppers, strawberries, apples, peaches, grapes, mushrooms), including spinach (Leff and Fierer, 2013). Differences were also seen between phyllosphere communities of field-grown lettuce and another leafy green, *Diplotaxis tenuifolia* or perennial wall-rocket (Dees et al., 2015). Even the wild ancestor of lettuce –*Lactuca serriola*– showed different microbial profiles on its leaves (Hunter et al., 2010) and roots (Cardinale et al., 2015) compared to modern *L. sativa* cultivars. Furthermore, leaf microbiota from different lettuce accessions grown under similar conditions revealed cultivar-dependent differences in bacterial diversity (Hunter et al., 2010; Dees et al., 2015). Those differences correlated strongly with leaf characteristics such as morphology, levels of soluble carbohydrates, and phenolic compounds. This is interesting, because many of these characteristics are genetically determined, which led some (Hunter et al., 2010) to suggest that the structure and function of lettuce microbiota could be a target in lettuce breeding. The difficulty (and challenge for the future) is that we know too little still about the correlation between microbial community composition and benefit to the plant to know what microbiota to breed for.

A key point about the plant genotype as a driver of microbial diversity is that while its effect can be demonstrated in carefully designed experiments, it is readily overruled by other factors, including environment (Rastogi et al., 2012). Another point is that plant genotype is not a static driver: in the course of a single growing season or cycle, as the plant grows and matures and different parts of the genotype are expressed, microbial community structures on above- or belowground parts of lettuce may change considerably (Scherwinski et al., 2008; Williams et al., 2013; Schreiter et al., 2014b). On roots, bacterial diversity tends to increase over time (Ibekwe et al., 2010; Krober et al., 2014), possibly due to a richer repertoire of rhizosphere exudates. On leaves, bacterial diversity generally decreases in the period between planting and harvest (Dees et al., 2015), which may be explained by domination of a few phyllosphere-competent bacterial taxa, for example the Enterobacteriaceae, over taxa that depend on immigration from the air rather than growth on the leaf.

3. Impact of location, climate, and season

It is rather straightforward to assume that factors like location, climate, and time of year all have an effect on the microbial communities that associate with the roots and leaves of lettuce plants. It is not quite so simple, however, to experimentally determine the impact of each of these factors individually, as it is not always easy to uncouple them from each other: different locations may have different climates, and different times of year (spring, summer, fall, and winter) are likely to feature different weather patterns even at the same location. That makes it hard to interpret observed impacts of season (summer vs autumn) or year (e.g. same field sampled in subsequent years) on microbial community composition (Zwielehner et al., 2008; Rastogi et al., 2012; Williams et al., 2013). Another consideration is that location, climate, and time of year can impact leaves and roots differently in terms of 1) inoculum (e.g. soil or air serving as sources of microorganisms to colonize the plant), 2) inoculation (e.g. weather phenomena such as wind that deliver airborne microbes to the foliage), and 3) incubation (e.g. temperature or moisture content of air and soil, which impacts the growth of microorganisms on the plant surface). These points should be kept in mind when interpreting data on lettuce microbiota and the impact of location, climate, and season.

Clear differences have been shown between rhizosphere microbiota from plants grown on different soil types, either in the field (Scherwinski et al., 2008) or in the greenhouse (Ibekwe and Grieve, 2004). Differences in community composition are also apparent among bulk soil samples from fields in different locations (Schreiter et al., 2014a; Schreiter et al., 2014b) which suggests that lettuce roots get colonized by microorganisms that are only a subset of whatever the soil in which the plants are growing is able to offer. This soil-dependent location effect evokes the idea of 'microbial terroir': a local, indigenous microbial soil community, with a unique composition (and possibly function) that impacts plant health, yield, and quality, preferably in a positive way. A 'location effect' has also been demonstrated for lettuce leaves, even when the plants belong to the same lettuce cultivar (Rastogi et al., 2012). This suggests a rather strong impact of location, further evidenced by the finding that lettuce plants grown in the lab carry remarkably smaller and less diverse bacterial communities on their leaves than plants from the field (Williams and Marco, 2014). This phyllosphere 'location effect' may have different underlying causes, one of which is that different environments (e.g. the lab versus the field) represent different sources of airborne bacteria that serve as inoculum for what can be found on the leaves. A good illustration of the atmosphere as a source of foliar microorganisms is the dramatic change in bacterial community composition when a dust storm hits a lettuce field (Rastogi et al., 2012). Other weather phenomena were shown to differentially impact members of the lettuce leaf microbiota (Medina-Martinez et al., 2015). For example, temperature impacted both *Pseudomonas* and coliforms such as *Pantoea*, but radiation, rainfall and wind impacted *Pseudomonas* more than it did *Pantoea*, while relative humidity had a greater effect on *Pantoea* than on *Pseudomonas*. Experimentally controlled exposure to UV radiation decreased bacterial population sizes on lettuce leaves, confirming that UV radiation can greatly impact leaf microbiota (Paul et al., 2012). These examples also show that leaf microbiota are amenable to practical manipulation.

4. Impact of pathogens and management practices

There is considerable interest in the question whether a relationship exists between microbial community composition and the presence or activity of plant (lettuce) pathogens: if such a relationship could be established, it might be practically useful. For example, depending on the direction and timing of the relationship, microbiota-based metrics might be used for the early detection or even prediction of pathogen outbreaks or for developing management strategies that aim to supplement the microbiota with microorganisms that minimize pathogen establishment. Already, several studies have confirmed the impact of a soil pathogen (the fungus *Rhizoctonia solani*) on the microbial community structures of lettuce roots (Adesina et al., 2009; Chowdhury et al., 2013) as well as lettuce leaves (Erlacher et al., 2014). In another study, the foliar presence of *Xanthomonas campestris* pv. *vitians* (*Xcv*), causal agent of bacterial leaf spot of lettuce, correlated positively with the relative abundance of bacteria from the genus *Alkanindiges*, but negatively with *Bacillus*, *Erwinia* and *Pantoea* (Rastogi et al., 2012). The cause-and-effect of this relationship remains unclear and untested still. Perhaps the presence and/or activity of *Xcv* changes the leaf surface community: a similar observation was made after artificial inoculation of field-grown lettuce heads with *E. coli* which led to a significant shift in the microbial community on the leaves, for reasons unknown (Williams et al., 2013). Another explanation for the observed relationship between *Xcv* and other bacterial taxa is that somehow lettuce leaves are more susceptible to *Xcv* establishment if they carry *Alkanindiges* and less susceptible if they carry *Bacillus*, *Erwinia* and *Pantoea*. In this scenario, one might hypothesize that lettuce-derived isolates of *Bacillus*, *Erwinia* and *Pantoea* make good candidates as biocontrol agents of *Xcv* and bacterial leaf spot of lettuce.

Several studies have looked at the impact of biocontrol agents, rather than pathogens, on microbial community composition: in most cases, such impact was deemed negligible (Scherwinski et al., 2008; Adesina et al., 2009; Kohler et al., 2010; Chowdhury et al., 2013; Krober et al., 2014) or minimal, i.e. when two biocontrol agents were applied as a mixture (Grosch et al., 2012). Relatively few studies have so far addressed the impact of disease-reducing or growth-promoting practices on the microbial community composition of lettuce. In a laboratory setting, soil fumigation with methyl bromide and methyl iodide had –surprisingly– no impact on root or leaf microbiology (Ibekwe et al., 2010). In contrast, irrigation type (Williams et al., 2013) and crop rotation (Suzuki et al., 2012) had a clear effect. The latter ties in nicely with the notion that plant genotype is a driver of microbiota composition (see above). Several studies report differences in leaf microbiota between conventionally and organically grown lettuce, although the underlying causes remain unknown (Zwielehner et al., 2008; Leff and Fierer, 2013). Application of organic matter (manure, rice bran, fish meal) increased protease and alkaline phosphatase activity in rhizosphere soil more than application of chemical fertilizer (Sakurai et al., 2007; Sakurai et al., 2008); it was suggested, but not actually measured, that this increase in enzymatic activity helps plant better absorb soil nitrogen and phosphate. Only one study so far actually linked lettuce growth to the lettuce microbiota: plant weight correlated negatively with fungal diversity richness in the soil and positively with the size of *Pseudomonas fluorescens* populations (Bonanomi et al., 2008).

5. Conclusion and outlook

From the modest number of studies published to date on the structure and function of lettuce microbiota (most of the recent ones are featured here), several principles –but also questions– are emerging. It is evident that lettuce harbors a microbial community that is unique compared to that of other plants but that varies significantly along the shoot-to-root axis of a single plant or with its age. A new and interesting insight is that lettuce microbiota are manageable in the same way that lettuce pathogens are: by choosing different cultivars, applying different irrigation methods, or incorporating different soil amendments, microbial communities on leaves and roots can be altered. The big question remains: what, if any, is the impact of such alterations? Are some community structures better than others in providing plants with otherwise inaccessible soil nutrients or protecting them from pathogens or other stresses? Should we move on from just studying structure, and start looking into functional traits of these microorganisms, so as to establish a more mechanistic understanding of how different microbiota might impact plant growth and health? Very few studies so far have systematically tackled these questions, for which answers will be needed if the lettuce microbiota are to be exploited as a 'management tool' for improving lettuce production. Another knowledge gap in need of being addressed is that bacterial communities have received disproportionally more attention than those of fungi, oomycetes, and viruses. Yet, this latter group takes up many more pages in this Compendium than the bacterial pathogens do. How can we know if and how lettuce bacteria interact with or impact lettuce fungi/oomycetes, including those that have the potential to cause disease, without a profile available for both types of communities? A more rounded approach is needed to make sense of pathogen abundance and activity as a function of lettuce microbiota. Perhaps less relevant to the theme of this Compendium, but exciting nevertheless, is the link between lettuce leaf microbiota and consumer health. Much of that discussion is currently focused on the contamination of lettuce or other leafy greens with microorganisms that can make humans sick, such as *E. coli*, *Salmonella* and *Listeria*. However, the other side of that coin is the idea of the 'edible plant microbiota' (Berg et al., 2014), i.e. the microorganisms that associate with raw-consumed food like leafy greens and that might serve as a source of bacteria with beneficial properties, such as probiotic activity and immune stimulation. Some (Leff and Fierer, 2013) already noted that humans are exposed to different types of bacteria depending on the types of produce that they consume and on how that produce was grown, i.e. conventionally versus organically.

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