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Plant-microbe interactions in lettuce: implications for both plant and human health

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ABSTRACT

Salad plants such as lettuce (*Lactuca sativa* L.) and arugula (*Eruca sativa* Mill.) belong to the most commonly eaten raw vegetables. The high dietary fiber content, a variety of important vitamins and minerals together with the low calorie balance adds them as healthy food. Despite their beneficial properties, they have also been involved in severe pathogen outbreaks, often caused by *Enterobacteriaceae,* in humans.

As all plants, leafy green vegetables are colonized by a vast diversity of microbes across all plant parts, which benefit their host on assorted ways including the protection from pathogens and diseases, the acquisition and mobilization of nutrients, and the production of plant growth stimulating metabolites or phytohormones. Various biotic and abiotic factors, including the soil and the plant-type, cooperatively shape the microbiome structure.

However, only little is known about correlations in complex plant-microbe interactions and microbial changes which putatively cause foodborne outbreaks. Therefore, novel cultivation independent techniques including next-generation sequencing approaches and visualization methods combined with robust cultivation dependent techniques were applied in this study to address several hypotheses to unravel the structure, stability and plasticity of food plant associated microbiomes.

Enterobacteriaceae were identified as keystone organisms on the foliage and in contrary to reports on foodborne outbreaks, as important members of the lettuce core structure. Enterobacteria highly responded to biotic stress caused by plant-pathogenic fungus *Rhizoctonia solani* and the agricultural pests *Gastropoda*. Severe shifts in the native microbiome caused by *R. solani* were compensated by the use of the commercially available biocontrol product *Bacillus amyloliquefaciens* FZB42. Complementary FISH/CLSM analyses revealed an un-washable lifestyle for enterics in the phyllosphere. Distinctive colonization patterns of *Gamma*- and *Betaproteobacteria* on the rhizoplane of young plantlets where detected. Both formed large colony structures but did not share the space, rather they exclude each other upon contact and coat the root as a dense multispecies biofilm. Root microbiome network analysis of different cultivars resulted in loose network pattern and suggested susceptibility for biological control products, but also for pathogens.

The lettuce and arugula microbiomes were identified as highly diverse ecosystems valuable for both, plant -and due to the raw-eaten aspect- also human health. On the other hand they were also recognized as fragile reservoirs for a long list of opportunistic and emerging pathogens. These results open new opportunities for future targeted studies on pathogen suppression and the biocontrol of lettuce, and thus might be important for both environmental and public health.

Kurzfassung

Salat (*Lactuca sativa* L.) und Rucola (*Eruca sativa* Mill.) haben als roh verzehrte Lebensmittel einen besonderen Stellenwert für eine gesunde und ausgewogene Ernährung. Sie sind reich an Ballaststoffen und liefern wertvolle Vitamine und Mineralstoffe bei einer vergleichsweise geringen Energiebilanz.

Wie alle Pflanzen sind auch Salat und Rucola keine sterilen Organismen, sondern werden auf sämtlichen Pflanzenteilen durch eine Vielzahl von Bakterien, mitunter hochspezifisch, besiedelt. Diese Mikroorganismen spielen eine wichtige Rolle und können eine positive Wirkung auf ihren Wirt besitzen, unter anderem als Wachstumsförderer durch die Produktion von Pflanzenwachstumshormonen. Des Weiteren stellen sie wichtige Nährstoffe zur Verfügung und unterstützen die Wirtspflanze bei der Pathogenabwehr. Die Zusammensetzung dieser Pflanzen-assoziierten Bakteriengemeinschaften hängt von vielen abiotischen und biotischen Faktoren, wie zum Beispiel dem Pflanzentyp und dessen Exsudaten oder der Zusammensetzung des Erdsubstrats, ab.

Über das indigene Mikrobiom von roh zu verzehrenden Nahrungspflanzen war bisher vergleichsweise nur wenig bekannt. Um diese komplexen Zusammenhänge besser zu untersuchen und um die mikrobiellen Gemeinschaften in ihrer Gesamtheit eingehend zu erfassen, wurden vielseitige Arbeitsprotokolle entwickelt. Hierfür wurden mikrobiologische und moderne molekularbiologische Methoden in Kombination mit *in situ* Visualisierungstechniken angewandt.

Es wurden hochdiverse Mikrobiome in den Nahrungspflanzen entdeckt; im Speziellen konnten auf den essbaren oberirdischen Pflanzenteilen Enterobakterien als integraler Bestandteil des mikrobiellen Kerngerüsts identifiziert werden. Zwei untersuchte biotische Störungen, hervorgerufen durch das Pflanzenpathogen *Rhizoctonia solani* und den pflanzenschädigenden Gastropoden, verursachten ausgeprägte Verschiebungen im Mikrobiom. Hier konnte gezeigt werden, dass der Einsatz von dem kommerziell erhältlichen Biokontrollprodukt *Bacillus amyloliquefaciens* diesen Veränderungen entgegenwirken kann und Symptome abschwächt. Visualisierung mithilfe von Fluoreszenz-*in-situ*-Hybridisierung kombiniert mit konfokaler Laser-Scanning-Mikroskopie (FISH/CLSM) lieferte Hinweise, dass Enterobakterien oftmals in die Pflanzen eindringen und dadurch kaum durch übliche Abwaschtechniken entfernbar sind. Starke bakterielle Besiedlung wurde vor allem auf den Wurzeln von

Jungpflanzen beobachtet. *Betaproteobakterien* und *Gammaproteobakterien* bildeten hier große, Biofilm-ähnliche Aggregate, die sich gegenseitig ausgrenzten. Eine Korrelationsanalyse basierend auf mikrobiellen Netzwerken von Rhizosphären unterschiedlicher Salatsorten zeigte, dass die häufigsten Bakterien nicht zwingend in einem modular aufgebauten Zusammenhang stehen. Dadurch lässt sich schlussfolgern, dass es einzelnen Mikroorganismen, wie zum Beispiel Biokontroll-Stämmen, aber auch Pathogenen erleichtert wird, in das Mikrobiom einzudringen und dadurch den Wirt zu besiedeln.

Zusammenfassend konnten umfassende Einblicke in die hoch diversen aber auch fragilen Mikrobiome der Salatpflanzen gewonnen werden, die sowohl für die Gesundheit der Pflanzen aber auch für den Menschen von großer Bedeutung sind. Des Weiteren ergeben sich Anhaltspunkte für zukünftige Studien im biotechnologischen Kontext, für die Biokontrolle in der Agrarwirtschaft und zur Risikoabschätzung für den Konsum von roh verzehrten Nahrungsmitteln.

Thesis Introduction

Plant-microbe interactions and their importance for plant and human health

Due to their generally immobile lifestyle, plants are strongly bound to their local environment in multifarious ways. Geographical adaption and plant-microbe interactions are the result of enduring evolution. The constant evolutionary pressure on the plant (plant genotype) together with the ecosystem-specific abiotic and biotic impact shapes the highly specific and diverse microbiome in plants (Bouffaud et al. 2014). Usually the microbiota are not equally deployed on the plant; moreover these species rich communities specifically change in abundance, function and diversity in particular plant habitats (Berg et al. 2005a; rev. in Berg & Smalla 2009; rev. in Bulgarelli et al. 2013; rev. in Berg et al. 2014a). Such plant-microbe interactions are highly important for the plant's growth and fitness (Berg 2009), and in the case of raw eaten leafy greens or medicinal plants they also affect human health (Berg et al. 2005b, Erlacher et al. 2014, Berg et al. 2014b, Berg et al. 2015). Additional, essential functions of the plant microbiome for the host include disease and pathogen suppression, stress resistance or nutrient acquisition, mobilization and transport (Lugtenberg and Kamilova 2009, Yang et al. 2009).

Roughly divided, the plant consists of three main compartments; the rhizosphere presents the soil-plant interface, the phyllosphere is the aboveground interface interacting with several environmental conditions, and the endosphere refers to the microorganisms colonizing the inner compartments of the plant. The plant can be additionally be subdivided, and depending on the plant type further divided into more microenvironments including seasonal plant features e.g. anthosphere (flower), spermosphere (seeds), caulosphere (bark/stem), calusphere (bud) and the carposphere (fruit) for aboveground and endorhiza (inner root) and mycorrizosphere (root surface attached by mycorrhizal fungi) for root associated habitats (Berg et al. 2014a, Bonfante & Desiro 2015). The rhizosphere was already defined by Hiltner (1904) and he was the first to suggest the potential role and importance of the rhizosphere for plant health and growth. This rhizosphere effect describes the phenomenon of increased microbes richness and functional activity in the close proximity to the plant roots compared to the surround bulk soil (Berg et al., 2005a., Herron et al., 2013, Erlacher et al 2015). The large exposed surface of the

phyllosphere is an extreme habitat and the microbial structure is mainly driven by the plant genotype and environmental conditions. Bacteria living on aboveground parts of the plant need to withstand many fluctuations and extreme conditions even within the course of a single day such as uv exposure, temperature drops and extremes, light changes, etc. (Leveau 2006, Vorholt 2012). Endophytic bacteria are often directly associated with important plant functions and were often linked to beneficial host interactions including modulation of hormones or plant metabolism (Hardoim et al 2008, Mercado-Blanco & Lugtenberg 2014). There are several factors influencing the structure and composition of the specific microbiome.–

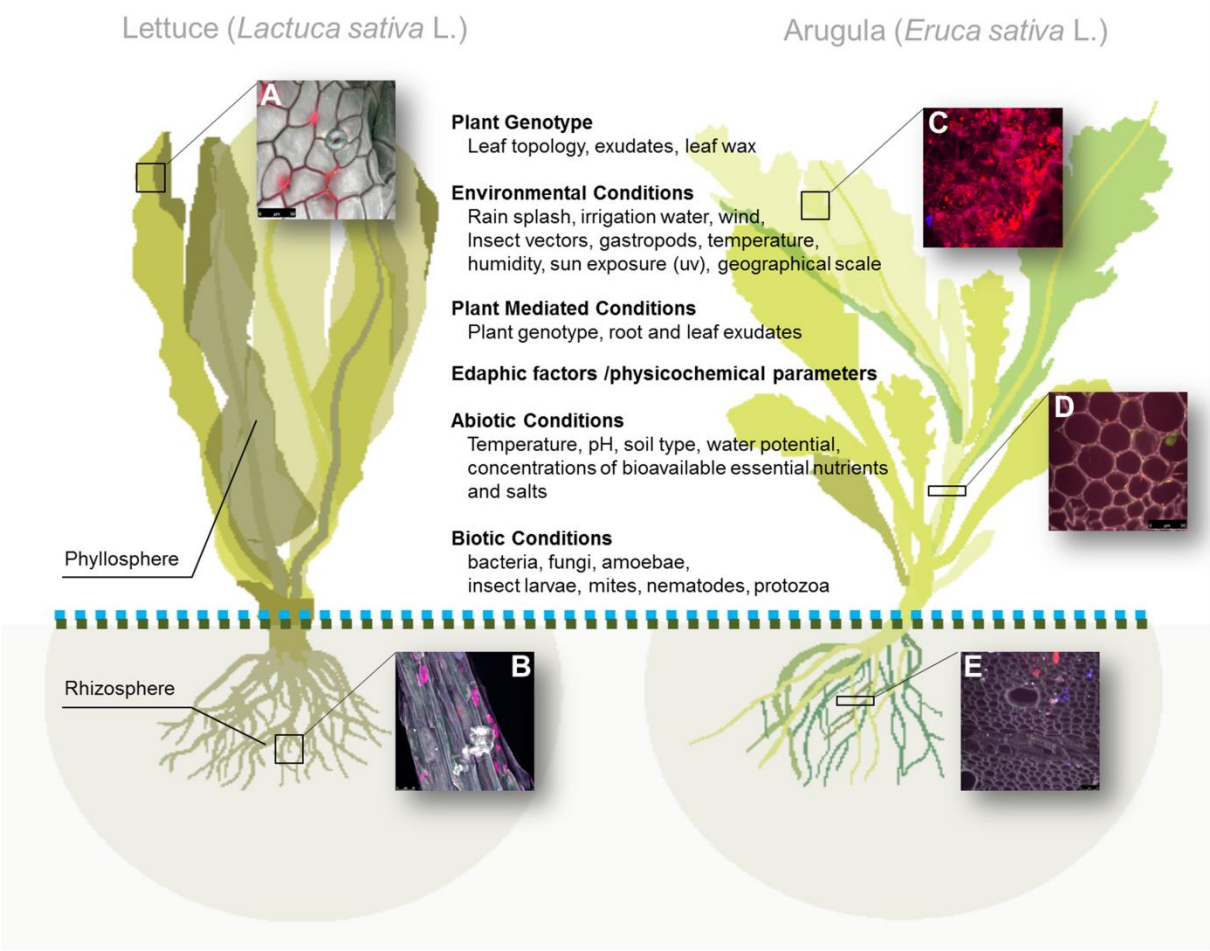

Figure 1 Summary of factors and conditions that shape the plant-microbe ecosystem. Additional CLSM micrographs from exterior (A-C) and interior (cross-sections; D, E) plant tissue allow reconstruction of microbial colonization patterns *in situ*. A. The epicuticula stained with calcofluor. B. Large colonies of *Betaproteobacteria* (pink) on lateral roots of young plantlets. C. Heavy colonization of non-classified bacteria (red) and *Enterobacteriaceae* (blue) in the phyllosphere. D and E. Cross sections of arugula stems and roots showed bacterial cells in niches of plant compartments.

Compared to the well-studied impact of abiotic factors (Fierer et al. 2006 and 2008), biotic factors are less understood, less predictable, and affect the plant and its microbiota often in complex ways through direct (cell-cell interactions, metabolites, cell allocation) or indirect (effect on plant fitness or hormone modulation) interactions (Jousset et al. 2013 and 2014, Erlacher et al. 2014). The major drivers of microbial community structure on plants were summarized in Figure 1.

Lettuce und arugula, between health benefits and health risks

Green salad and is traditionally seen as an important staple food of a balanced, healthy diet and includes several seasonal cultivars and varieties from the taxonomic families *Asteraceae* (lettuce, *Lactuca sativa* L.) and *Brassicaceae* (arugula/rocket, *Eruca sativa* L.). Early cultivation of lettuce has been traced back to the ancient Egyptians, and arugula has been grown as a herb since roman times (Bennett et al. 2002, Zohary *et al.,* 2012). Lettuce production has steadily increased globally over the last years. China is the main driver for this steady increase of production over the last decade, and more than half of the global lettuce production was noticed with 14 Mio t in China (Figure 2, FAOSTAT).

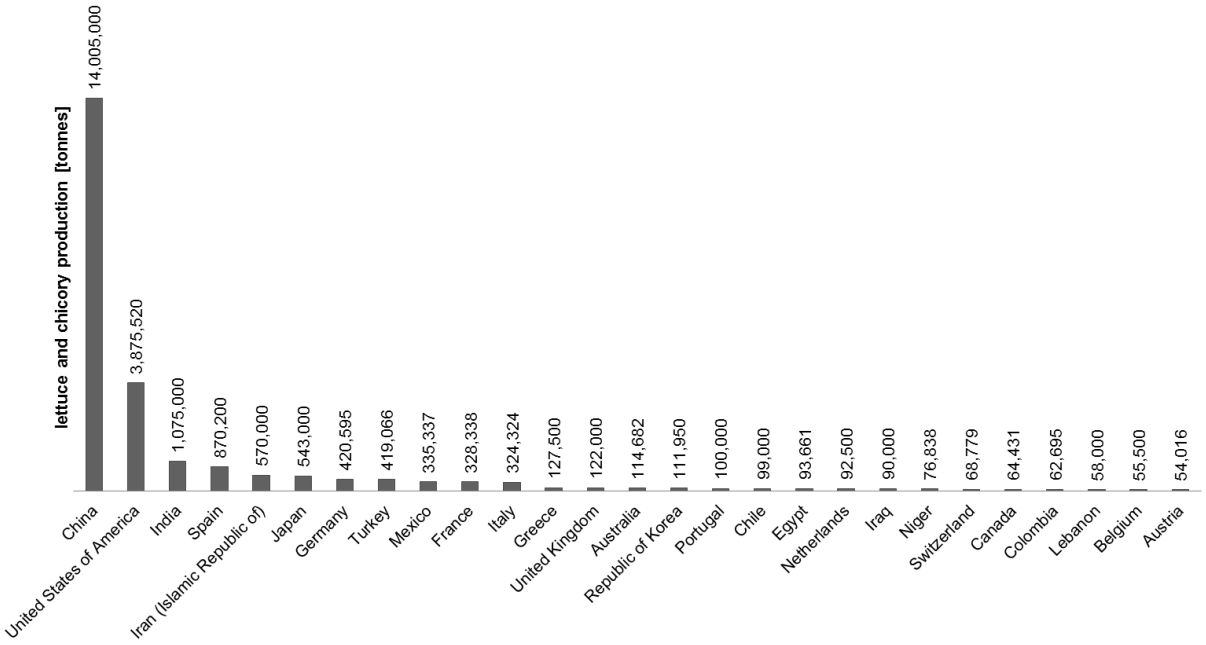

Figure 2 Global lettuce production ranked by top producers in average recent production (2012-2013, FAOSTAT)

As shown by Figure 2, Austria was ranked 27th with an average production of 54,016 tons, although production has slightly declined in the past five years. The nutrient

content of lettuce and arugula contain many of the same valuable nutrients found in other green vegetables, including high water and dietary fiber content, vitamins (A, C), and minerals (Ca, P, Fe, Na, K). The high water and fiber content of lettuce adds to a healthy diet and lettuce varieties with dark green or reddish leaves in the edible portion have considerably more vitamins and minerals (Rubatzky and Yamaguchi, 1997). The specific antimicrobial activity of arugula has beneficial effects on cholesterol LDL/HDL ratios. It has general antioxidative activity and although evidence is controversial, it also has potential as a cancer protective agent (Bennett et al. 2002, Nicolle et al. 2004, Slattery et al. 2000, Chu et al. 2002)

On the other hand lettuce and arugula were frequently reported as causes of severe foodborne disease outbreaks resulting from enterobacterial contamination (Martinez-Vaz et al. 2013). Prominent foodborne disease outbreaks originating from lettuce were reported according the Center of Disease Control and Prevention (CDC, 2013) in the USA in 2011 (*E. coli* O157:H7, 60 cases) and in 2010 (*E. coli* O145, 27 cases), in Iceland and the Netherlands in 2007 (*E. coli* O157:H7, 50 cases) and in Sweden with the highest number of cases in 2005 (*E. coli* O157:H7, 135 cases, rev. in Martinez-Vaz et al. 2014). The major outbreaks illustrate the risk associated with the consumption of lettuce and were well studied. Controversially, less is known about the indigenous microbiome including many beneficial microbes which positively influence both, the plant and human health.

Next generation methods for next generation analyzes in microbial ecology

New powerful tools for molecular and microbial ecology extended the frontiers and enabled new insights into the structure, abundance, and functions of microbe-host interactions. In the past, cultivation-dependent bacterial isolates and cultivation-independent fingerprinting methods were used to decipher functions and occurrence of bacteria in diverse associations (Nichols, 2007; Ritz, 2007). While the first reliable method of DNA sequencing was already developed more than 40 years ago (Wu 1972, Sanger et al. 1975), new methods are orders of magnitude faster and yielding in highly increased data size, and these techniques continue to vastly expand our understanding of the microbial world (Petterson et al. 2009).

DNA sequencing costs continue to decline and enable fast high-sample-throughput sequencing of DNA fragments or even whole metagenomes. The 16S rRNA gene fragments were depicted as valuable and reliable markers for bacterial taxonomic

identification. Consequently, studies of microbe-host interactions including plant-microbe interactions in an extended frame became highly important in microbial ecology. However, as with all methods, next-generation sequencing and other Omics methods also have specific limitations and disadvantages. Robust, comprehensive research approaches benefit greatly from combining the assets of different methods - (Overview, Figure 3). In this instance fluorescence *in situ* hybridization coupled with confocal laser scanning microscopy (FISH/CLSM) as a visualization tool proved invaluable. In comparison to metagenomics, amplicon sequencing and other Omics techniques have the disadvantage that the host, including all microbes, needs to be pre-processed. This also includes the physical displacement of occurring bacteria out of their natural microhabitat or location. Thus localization, visualization and colonization patterns in microbe-hosts systems have also progressed in a parallel manner (Cardinale 2014). Another limitation of next generation sequencing approaches is evident in terms of its application in quantitative methods; hence combination with Real-time PCR (RT-PCR) can improve the quality of results and enhance the understanding, comparison and interpretation not only within a particular habitat but also between microbe-habitat borders. Understanding of single cell or whole microbiome functioning greatly benefitted from advances in Omics methology, however final assessments still rely on culture-dependent approaches, including antagonism testing or *ad planta* assays.

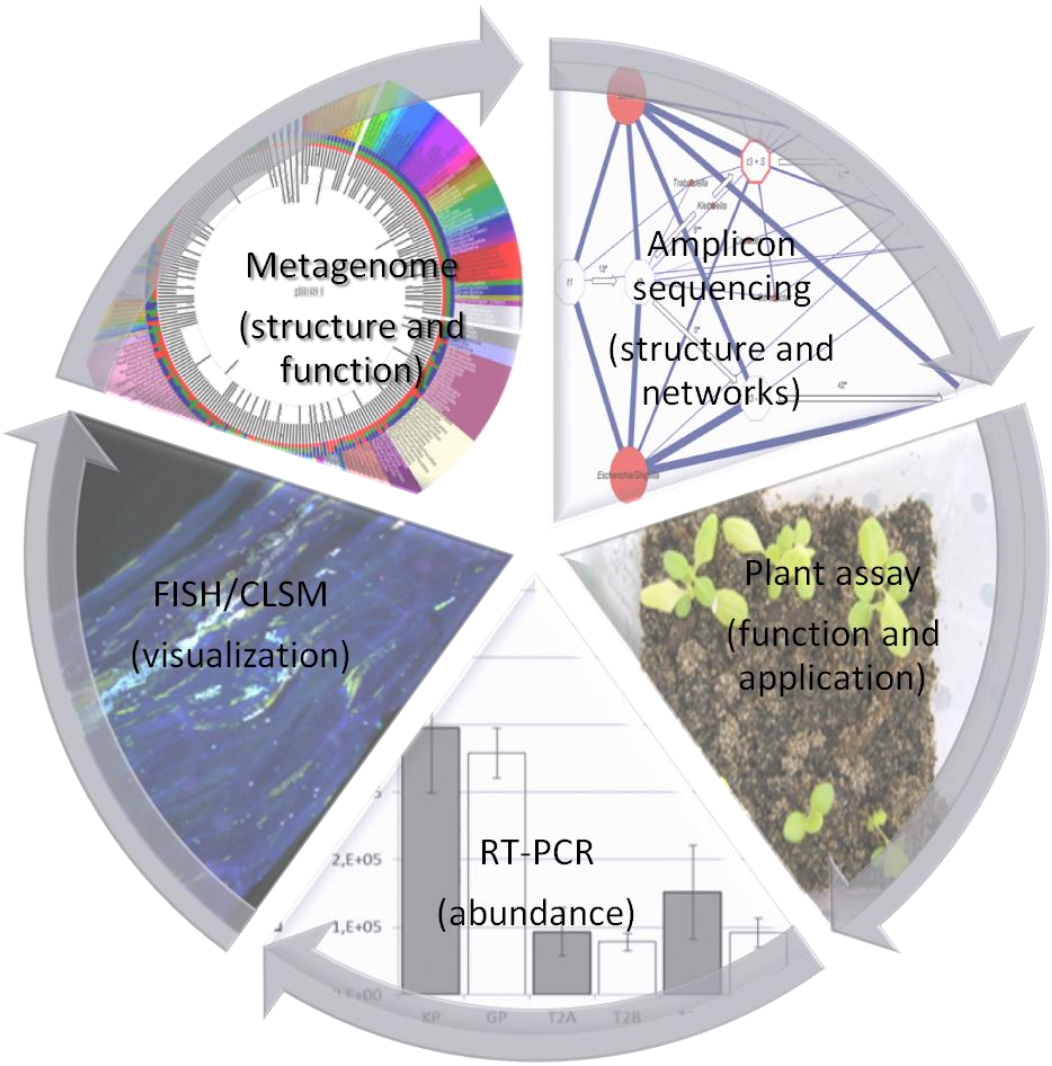

Figure 3 polyphasic workflow scheme demonstrating the integrative techniques utilized in the study.

Objectives and Summary of the work

The fundamental purpose of the study was to decipher the lettuce and arugula microbiome and to evaluate the structure, plasticity and stability for both plant and human health, and therefore several working hypotheses were advanced to address the study.

Manuscript I

Metagenomes of whole habitats allows plant-microbe ecology to be investigated on a much larger scale. Comparison between the phyllosphere, the rhizosphere, and the corresponding bulk soil analyzed by deep sequencing of targeted amplicons and

visualization techniques results in novel insights and explorative data analyses of bacterial structure and functions across distinct habitats. In order to reveal colonization patterns and specific colonization niches, different microenvironments, including seasonal compartments such as the spermosphere or anthosphere, were examined by FISH/CLSM using group-specific and universal bacterial probes of native- and enterobacterial inoculated plants. Results should greatly enhance the understanding of colonization patterns of bacterial groups, and determine heavily colonized plant parts, along with providing an better overall interpretation of results from different fields of research.

Metagenomics revealed a major divergence for the phyllosphere when compared to the rhizosphere and the bulk soil, accompanied by an overall enrichment of Gammaproteobacteria. A rhizosphere effect, (which describes the accumulation of particular taxa and the loss of others in the close vicinity of the roots compared to the bulk soil structure) and high functional abundances were demonstrated for the rhizosphere. Combined metagenomic and amplicon sequencing datasets detected a strong enterobacterial core network within the whole plants including, potential plant and human pathogens together with a preference of enterics to colonize the foliage of arugula.

Publication I

The microbiome of *Lactuca sativa* L. is shaped by many factors (Figure 1). While abiotic factors are tightly bound to the local environment and are well studied, less is known about of biotic factors which influence the plant, the indigenous microbiome, and in consequence also human health in a less predictable way. Therefore, the impact of the plant pathogenic fungus *Rhizoctonia solani* and agricultural pests *Gastropoda* on *Enterobacteriaceae* of the lettuce associated microbiota was assessed.

The impact of biotic disturbances shifted the structure notably, introduced new enterobacterial genera and enriched *Enterobacteriaceae* on lettuce. While the occurrence of particular taxa was only transient during the perturbation event, others such as *Enterobacter* were additionally able to colonize leafy parts of the plant over

the long term. In contrast to the relative low number of foodborne outbreaks in humans, we hypothesized that the natural occurrence and enrichment of *Enterobacteriaceae* including putative opportunistic taxons might represent an important, previously underestimated attribute of lettuce by stimulating the immune system and enriching our commensally gastrointestinal microbiota.

Publication II

Plant protection by microbials is a sustainable way to improve the plant's quality, growth and fitness. Further, antagonistic properties are used to inhibit plant diseases. In the scenario of *R. solani* infected lettuce plants treated with *B. amyloliquefaciens* (Commercially available product FZB42, Abitep) we evaluated the impact on important, and most dominant bacterial class on lettuce leaves, *Gammaproteobacteria*.

Lettuce plants with bottom rot caused by *Rhizoctonia solani* showed significant changes in their microbiome composition accompanied by an enrichment of *Cellvibrio* (rhizosphere) and *Enterobacteriaceae* (phyllosphere). On the other hand, *Alkanindiges* and *Acinetobacter* could be identified as indicators for healthy plants. This, in consequence, might recommend them as promising sources for biocontrol applications. *Bacillus amyloliquefaciens* co-inoculation compensated severe structural changes in the gammaproteobacterial fraction. In addition, a strong habitat specificity was detected in this study for Gammaproteobacteria.

Publication III

Commercially available lettuce cultivars are the result of a long lasting breeding history traced back to the ancient egyptians. A comparative rhizosphere study of eight cultivars with different presumptive age should demonstrate the core structure within the different cultivars and should make it possible to create a model for evolutionary traits.

Rhizosphere analyses across different lettuce cultivars revealed that domestication of lettuce led to increased bacterial diversity. Further network analyses showed an

overall loose network structure, where even highly abundant core taxa are not necessarily involved in highly correlated modules. This finding might partly explain the higher susceptibility of the lettuce microbiota to disturbances and pathogen attack. On the other hand it reveals a valuable open door for successful application of biological control products.

Publication IV

As raw eaten vegetables, all lettuce and arugula cultivars harbor a vast diversity of microbes when consumed. Washing off bacteria widely fails to remove higher bacterial proportions. While most bacteria do not harm healthy human individuals, the situation is unclear for immunocompromised persons. Metagenome analysis of arugula together with complementary FISH/CLSM was applied to decipher possible risks and constraints for the consumption of raw eaten vegetables.

The lettuce or arugula microbiome was identified as potential reservoir of opportunistic and emerging pathogens including *Enterobacteriaceae* as keystone species. In immunocompetent hosts, this vast microbial diversity, together with the presumptive un-washable lifestyle of enterics, might play an important role in maintaining or even enhancing human health. At the same time, according to current data, severe health risks to immunocompromised individuals cannot be denied.

Book Chapter I

Vegetables consumed raw, fruits, and herbs are substantial parts of a balanced diet. However, the plant-associated microbial diversity, also referred to as edible microbiome, has been largely ignored in this context. The importance of the edible microbiome for human health and for biotechnological application was evaluated using two specific examples of plant microbiomes, lettuce and banana.

Eukaryotes and Prokaryotes inevitably depend on each other and form integral interactions. It has been demonstrated that bacterial diversity is inherently correlated with a reduced incidence of pathogen outbreaks in both plants and humans, and therefore enrichment of plant associated diversity might be a valuable method for sustainable and safe agriculture.

Additional Publication I

Frontiers research topic cover page.
FISH/CLSM using group-specific and universal bacterial probes of native- and enterobacterial inoculated plants is an important method for visualizing microbial ecology. Results should greatly enhance the understanding of colonization patterns of bacterial groups, determine heavily colonized plant parts, and provide an improved overall interpretation of results across different areas of research focus.

FISH/CLSM visualization revealed uncommon colonization patterns on the lateral roots of young plantlets where Gamma- and Betaproteobacteria heavily colonize the rhizoplane and form detached biofilm structures. In addition to the scientific scope the micrograph resulted in a visually appealing picture suitable for use as a cover page.

Additional Publication II

It was demonstrated that *Rhizobiales* are the dominant taxa in the lichen symbiosis, but less is known about their role, occurrence or function. We have analyzed the taxonomic structure and assigned functions in a *Lobaria pulmonaria* metagenome. *In silico* analysis of the only available *Rhizobiales* FISH-probe revealed an incomplete detection spectrum of taxa retrieved in the metagenome. Therefore, we designed a novel FISH probe and evaluated it by combining the metagenomic dataset with *in silico* databases to efficiently and specifically target members of Rhizobiales

One third of all bacteria associated with *L. pulmonaria* were assigned to *Rhizobiales* including the families *Methylobacteriaceae*, *Bradyhizobiaceae* and *Rhizobiaceae* as the most dominant taxa. Important functions of *Rhizobiales* supporting the lichen symbiosis were identified, including the production of vitamins and plant hormones, stress protection and nitrogen fixation. Complementary FISH/CLSM analysis revealed that the majority of bacteria preferentially colonize the fungal surfaces, but there was also evidence that *Rhizobiales* are able to invade the interhyphal matrix of the upper cortical layer of the lichen and that in rare occasions bacteria also colonize the interior of fungal hyphae.

Additional Publication III

Pink pigmented facultative methylotrophic bacteria (PPFMs) colonize almost all known plants on their exterior and often fulfill beneficial functions for their host. Very little is known about their occurrence on mosses and lichens. The lichen *Lobaria pulmonaria* and the two closely related moss species *Sphagnum fallax* and *Sphagnum magellanicum* were selected in order to study the diversity and abundance of PPFMs.

Single strand conformation polymorphism (SSCP) of methylotrophs revealed co-occurrence of high abundant genera which were shared by the hosts and rare abundant genera which showed host specificity. *Sphagnum magellanicum* was only rarely colonized by PPFMs in contrast to the lichen *Lobaria pulmonaria* and *Sphagnum fallax*. Acidic pH of *S. magellanicum* was recommended as a natural colonization barrier for PPFMs.

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Manuscript I

In preparation

Bacterial communites and the role of *Enterobacteriaceae* associated with *Eruca sativa*

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Abstract

Arugula (*Eruca sativa* L.) originates from the Mediterranean and is popularly known for its use in traditional Italian cuisine, for its peppery, pungent taste and its health benefits. As with all plants, *E. sativa* is colonized by a vast diversity of microbes throughout all of its tissues. The plant microbiome contributes to both plant and human health, although imbalances or pathogen contamination can lead to foodborne outbreaks. This study has focused on the structure, abundance and functions of the plant associated microbiota on the phyllosphere, rhizosphere and the corresponding bulk soil in a tripartite approach including next generation sequencing of habitat specific metagenomes and additional enterobacterial 16S rRNA gene amplicons combined with fluroescence *in situ* hybridization coupled with confocal laser scanning microscopy (FISH/CLSM) visualization. We found highly variable colonization patterns across different microhabitats with a major divergence for the phyllosphere compared to the soil-derived habitats as shown by habitat distances. The overall enrichment of *Gammaproteobacteria,* including *Enterobacteriaceae,* depletion of *Deltaproteobacteria* and *Alphaproteobacteria* within the aerial plant parts, and a strong rhizosphere effect was observed. The rhizosphere was depicted as a functional hotspot. In contrast, highest alpha diversity indices were found for the bulk soil but with variable functional abundances. A spring embedded network revealed important core taxa of highly enriched *Enterobacteriaceae* including strains related to potential plant and human pathogens. This study provided important insights into an improved understanding of the nature of bacterial life and ecosystem functioning on *Eruca sativa.* In addition, we were able to shed light on the important role of enterics as a core microbiome member of arugula and to unravel sources of pathogen transaction.

Introduction

Arugula (syn. *E. vesicaria* subsp. *sativa* (Miller) Thell., *Brassica eruca* L.), also known as garden rocket, has its origin in the areas surrounding Mediterranean Sea (Zeven et al. 1982) and has been cultivated as an edible herb since roman times (Bennett et al. 2002, Martinez-Sanchez et al. 2006). Presently, arugula is commonly used as a salad vegetable, and is a widely popular annual, usually consumed fresh (leafs or sprouts) with a characteristic pungent, peppery flavor. Arugula´s complex aroma structure was already partially deciphered by Jirovetz et al. in 2002. They were able to identify more than 50 constituents as essential volatiles responsible for the highly characteristic aroma of these salad leaves including mainly isothiocyanates, and butane derivates. Hexane, octane and nonane were additionally identified. Arugula has been known for decades as a traditional pharmacopoeia and ancient literature documents has documented its several therapeutic properties. It contains a number of health promoting agents and features including carotenoids, vitamin C, fibers, flavonoids, glucosinolates and antimicrobial activity (GLs; Warwick & Francis 1994, Bennett et al. 2002, Barillari et al. 2005). Application of the crude juice on cell cultures showed effective inhibition of *Escherichia coli*, *Salmonella typhi* and *Bacillius subtilis (*Abdou et al. 1972). In the latter, Alqasoumi et al. 2009 demonstrated that arugula extract possesses anti-secretory, cytoprotective, and anti-ulcer activities against experimentally-induced gastric lesions. This anti-ulcer effect was hypothesized through prostaglandin-mediated activity and/or through its anti-secretory and antioxidant properties.

All parts of plants are colonized by a vastly diverse microbiota. However, structure, abundance and occurrence of bacteria can be highly variable across different plant habitats.Due to the agricultural aspect and the importance of the soil habitat, the majority of current publications are focused on the rhizosphere. The phyllosphere has been less well studied, it is more unpredictable, and its relative importance is a subject of controversy. Due to its large exposed surface and the vast amount of factors and environmental conditions including precipitation, wind, insect vectors, etc. which can alter the plant physiology but also influence associated bacterial taxa (Vorholt 2012, Rastogi et al. 2013). The phyllosphere also includes additional seasonal microenvironments such as the anthosphere (blossoms), the spermosphere (seeds), and the carposphere (fruit, rev. in Berg et al. 2015). Microbiome research of

such specific microenvironments has largely been ignored in the past, although cryptograms have been the subject of related research (Bragina et al. 2013, Aschenbrenner et al. 2013) and may therefore be proven to play a crucial role in the life cycle and vertical transmission of plants. Microbes contribute greatly to the plant's fitness and growth by accomplishing important functions for their host. Such functions include the induction of stress resistance, additional nutrient acquisition, the production of phyto-hormones and the suppression of pathogens or diseases (Berg 2009, Lugtenberg and Kamilova 2009, Yang et al. 2009).

As with all raw eaten vegetables, arugula has also been reported to be the potential source of pathogen outbreaks (Nygård et al. 2009), caused mainly by *Enterobacteriaceae* which are main health concerns in the production of leafy green vegetables. There are numerous ways for pathogen contamination to occur including insects as vectors, pasture run-off and soilborne, irrigation water or seed derived contamination. There is also the risk that seed or harvested leavy green mixtures could cause cross-contaminations (Brandl 2006, Teplitski et al. 2011, Olaimat et al. 2012, Hou et al. 2013, Moyne et al. 2013). In the past, *Enterobacteriaceae* were mainly associated with the habitat of human and animal intestines, however they have also been recognized as important indigenous members of higher plants, especially in the phyllosphere (Brandl 2006, Teplitski et al 2011, Erlacher et al. 2014, 2015). In our previous work (Erlacher et al. 2015) we demonstrated that the microbiome of *Lactuca sativa* L. is highly sensitive to biotic and abiotic stress and *Enterobacteriaceae* were depicted as particularly strong responders. This high sensitivity to environmental changes might be partly explained by Cardinale et al. 2014 who showed that the core-microbiome network structure across several lettuce cultivars is quite loose and that even high abundant core taxa do not necessarily need to interact in high correlated modules. This could explain the susceptibility of pathogens to become established with relative ease in the microbiome, while simultaneously increasing the potential for the successful use of new beneficials or plant growth promoting rhizobacteria (PGPR).

The occurrence of both, a plant-pathogen (*Rhizoctonia solani*) and the commercial available plant strengthener *B. amyloliquefaciens* on *L. sativa* was investigated in Erlacher et al. 2014. *R. solani* (bottom rot) was shown to be responsible for an overall decrease in the plant's fitness while accompanied by higher abundances of

Enterobacteriaceae including putative pathogenic strains. Co-inoculated plants with the commercially available product FZB42 (Abitep, Berlin) based on *Bacillus amyloliquefaciens* have been shown to successfully increase plant growth and fitness, but also to counteract the increase of *Enterobacteriaceae*.

In this study we investigated the *E. sativa* phyllosphere, rhizosphere and the surrounding bulk soil using HiSeq Illumina whole metagenome sequencing and additional 454 pyrosequencing of 16S rRNA gene amplicons of *Enterobacteriaceae*. Complementary fluroescence *in situ* hybridization coupled with confocal laser scanning microscopy (FISH/CLSM) Microscopy contributed to data evaluation and assisted in revealing the specific colonization patterns.

Materials and Methods

Sampling and DNA extraction

Arugula plants were grown in garden soil (in the latter referred to bulk soil) in the suburban region of Graz (approx. 47°4'13"N 15°28'19"E) The microbial fraction with *Eruca sativa* was extracted separately for the phyllo- and rhizosphere in two consecutive samplings. Additional bulk soil was sampled for the metagenome approach.

First sampling and cell extraction for 454 pyrosequencing of 16S rRNA gene amplicons

In the first sampling we investigated the enterobacterial biome of arugula in a 454 pyrosequencing approach of 16S rRNA gene amplicons. Plants were harvested in their final growth stage of leaf development before flowering. The microbial fractions were extracted separately for the rhizo- and phyllospere and four independent single replicates per habitat consisting of 15–20 leaves or roots were collected and stored separately, placed into sterile plastic bags, and transported to the laboratory. Five g of plant mass per replicate was physically disrupted with a sterile pestle and mortar, resuspended in 10 ml of 0.85% NaCl, transferred in two 2 ml Eppendorf tubes and subsequently centrifuged (16500 g, 20 min, 4°C). The pellet obtained was used for isolation of the total-community DNA with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). For mechanical lysis, the cells were homogenized twice in a FastPrep® FP120 Instrument (Qbiogene, BIO101, Carlsbad, CA, USA) for 30 s at a speed of 5.0 m sec^{-1} and treated according to the manufacturer`s protocol.

Second sampling and cell extraction for the metagenomic und FISH/CLSM approach

Sampling for the metagenome was carried out in early November at their final growth stage. The plants have already formed flowers and seed bodies. Plants were grown in a warm summer continental or hemiboreal climate, and climate profiles (Fig. S1) starting two weeks before sampling show relatively warm temperatures, moderate but fluctuating precipitation-, sunshine- and atmospheric- pressure levels, overall low wind levels and average air humidity.

Arugula foliage, roots with adhering soil, and bulk soil were sampled. Five g of each sample were weighed on a balance, transferred into bagmixer bags together with 10

ml 0.85 % NaCl and mechanically processed twice for 210 s. Samples were placed at interims of 5 min on ice. Homogenized cell suspensions were further transferred into S34 tubes, and centrifuged at 10,000 rpm for 20 minutes. The supernatant was discarded for each approach and the cell pellets were stored at -20 °C. In total we processed 24 × S34 tubes (35 ml/S34) foliage samples from a total 90 bags (cell pellets were stacked three times and the supernatant discarded between each centrifugation round in order to increase the total yield), 8 × S34 tubes (35 ml/S34) rhizosphere samples from a total of 35 bags and 6 × S34 tubes (35 ml/S34) soil samples from a total of 35 bags, respectivley. DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) from six pellets per habitat (foliage, rhizosphere, soil) using 300 mg each of the crude cell pellets and processing as stated in the manufacturer´s protocol. The DNA samples were eluted in 100 µL $H_2O_{ultra\ pure}$ and checked for quality and quantity using nanodrop Photometer. 2 µg of each replicate were pooled for a total 12 µg of metagenomic DNA per habitat.

Metagenomic sequencing and bioinformatic analysis

Metagenomic sequencing was performed with an Illumina HiSeq2000 system (2 × 150 bp) by Eurofins MWG Operon (Ebersberg, Germany) following the Eurofins MWG Operon protocol. The Metagenomic RAST (MG-RAST) server (Meyer et al. 2008) was used for functional and phylogenetic analyses of the metagenomes. The metagenomes (phyllosphere, rhizosphere, bulk soil) were uploaded and initially processed using default parameters. Initial processing included removal of artificial replicate sequences (Gomez-Alvarez et al. 2009), low quality sequences (Cox et al. 2010), short sequences, and sequences containing ambiguous bases. Phylogenetic and functional annotations were trained on the M5NR database and the SEED subsystems with a maximum e-value of 10^{-5}, minimum identity of 60% and minimum alignment length of 15 aa for protein and bp for RNA databases. Each single subsystem within the metagenomes, represents a group of sequences that encode for a specific biological- or structural process or complex (Overbeek et al. 2005).

The principle coordinate analysis (PCoA) tool of the MG-RAST server was used to compare our metagenomes to appropriate public available ones (summarized in Table S1). The PCoA plot was created using hierarchical classification with default parameters and each data set was normalized and scaled according to the algorithm

specified at the MG-RAST server. The distance matrix for the PCoA plot was based on the Bray-Curtis (Bray & Curtis 1957) distance metric.

454 pyrosequencing of *Enterobacteriaceae*

The 16S rRNA gene fragments of *Enterobacteriaceae* were amplified in a dual phase nested PCR approach using Multiplex Identifier (MID) tagged primers. The PCR was conducted with specific primers for enterics, using Entero-F234 and Entero-R1423 (V3-V8) according to the method of Binh et al. 2010 The nested PCR was carried out as described by Heuer et al. 1997 using the primer pair F984 and R1378 modified for multiplex 454 sequencing according to the specification. The products of two independent PCR reactions per sample were pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). Purified PCR products were pooled (200 ng each) and sequenced on a Roche GS FLX+ 454 Titanium platform (Macrogen Korea, Seoul, South Korea). Sequences were analyzed with the QIIME software version 1.8.0 (Caporaso et al. 2010). MID, primer, and adapter sequences were removed, and the sequences were quality- (minimal score: 50) and length filtered (minimal raw fragment length: E1 ≥ 350; E2 & E3 ≥ 430), followed by a denoising step (denoise wrapper and denoiser script). Chimeras and remaining sequences of non-target, plastidal, and mitochondrial origin were removed. OTU tables were created with UCLUST at a 100% cut-off level. Unique reads were classified with the RDP classifier v2.5 and the NCBI refseq database. The datasets were rarified on a naïve Bayesian classifier (Wang et al. 2007) to the appropriate number of least reads, within each experiment to compute alpha and beta diversity indices.

Cell fixation and FISH/CLSM analysis

FISH-CLSM was applied on arugula plant compartments (blossom, root, stem, seed, foliage) to decipher and localize bacterial colonization patterns. After collection, samples were fixed with 3:1 (v/v) 4% paraformaldehyde/phosphate-buffered saline (PBS) for 6 h at 4°C. Partitions of fixed samples were additionally cut with a cryotome. Samples were stained by in-tube FISH as described by Grube et al. 2009. Consequently, samples and cryosections of each habitat were individually transferred into 1.5 ml Eppendorf tubes and rinsed with 1× PBS. Lysozyme (1 mg/ml; Sigma-

Aldrich, St. Louis, MO, USA) treatment was applied and incubated at RT for 10 minutes. After an ethanolic series (50-70-96% EtOH solutions; 3 min each) samples were rinsed and further washed for 3 min with ice-cold 1× PBS. All hybridizations were performed in a two-step hybridization protocol at 43°C for 2 h in a buffer containing 0.9 M NaCl, 0.02 M Tris-HCl, 0.01% sodium dodecyl sulfate, 25% and 30% ultrapure formamide (FA; Invitrogen), and 5.0 ng of each FISH probe μl^{-1} (pH 8). An equimolar mixture of Cy3-labeled EUB338, EUB338-II and EUB338-III probes (Amann *et al.,* 1990; Daims *et al.,* 1999) and EnterobacD (Ootsubo et al. 2002) were used for staining all Bacteria and *Enterobacteriaceae* using 25% FA. Gam42a (Manz et al. 1992) was used to stain *Gammaproteobacteria* at 30% FA, respectively. NONEUB probes (Wallner *et al.,* 1993) labeled to fluorochromes analogous to the positive probes were used as negative controls. The hybridization buffer was replaced by a prewarmed (44°C) washing buffer (20 mM Tris-HCl, 149/102 mM NaCl (25%/30% FA), and 5 mM EDTA (for 25% and 30% FA)) and incubated for 15 min in a water bath (44°C). The hybridization and washing step were repeated sequentially for the utilized FISH-probes in accordance with the specific FA requirements. After eliminating the washing buffer, the sections were again rinsed with ice-cold double-distilled H_2O in order to remove residual salt crystals. FISH stained samples were transferred on optical slides, dried and mounted with SlowFade Gold antifadent (Molecular Probes, Eugene, USA). For visualization, a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) was used. The fluorescent dyes Cy3, Cy5 and ATTO488 labeling the FISH probes were sequentially excited with 532 nm, 635 nm and 488 nm laser beams, respectively. Autofluorescence of plant tissue was excited with a 405 nm laser beam to improve visualization of the host structure. The confocal stacks were acquired with a Leica ACS APO 40× oil CS objective lens (NA, 1.15) and a Leica ACS APO 63× oil CS objective lens (NA, 1.30) and for each field of view, an appropriate number of optical slices were acquired within a Z-step ranging from 0.15 to 0.7 µm. The software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used for imaging and post-processing of the confocal stacks and maximum projections. Adobe Photoshop (Adobe Systems Inc., USA) was used to label the final figures.

Results

Metagenome of microbiomes associated with *Eruca sativa* rhizosphere and phyllosphere, and bulk soil

Sequencing of rhizosphere and phyllosphere (including carposphere and caulosphere) and the surrounding bulk soil on a Illumina HiSeq (2x150 paired-end) platform resulted in 23,464,406 reads and 5,607,531,701 bps/total for the rhizosphere, 28,557,634 reads and 6,690,868,514 pbs/total for the phyllosphere and 19,233,382 reads and 4,567,945,962 pbs/total for the bulk soil metagenome after removing all no-overlapping paired-end reads. The MG-RAST QC pipeline resulted for the metagenomes rhizosphere, phyllosphere and bulk soil, respectively: 271,785/2,023,443/202,072 sequences (1.2%/7.1%/1.1%) failed to pass the QC pipeline. Sequences that passed QC, 90,284/1,346,340/24,931. Sequences (0.4%/4.7%/0.1%) contain ribosomal RNA genes. Of the remainder, 11,428,757/15,793,254/8,400,892 sequences (48.7%/55.3%/43.7%) contained predicted proteins with known functions and 10,959,003/7,875,393/10,064,999 sequences (46.7%/27.6%/52.3%) contained predicted proteins with unknown functions. 714,183/1,423,419/540,424 (3.0%/5.0%/2.8%) of the sequences that passed QC have no rRNA genes or predicted proteins. Based on their best e-value, SEED subsystems were selected as an annotation source for functional analysis of the metagenomes (Overbeek et al. 2005) and taxonomical assignments. The M5NR database was used as an annotation source (Meyer et al. 2008). A rarefaction analysis of the three metagenomes at a maximum e-value of 1e-5, minimum identity of 60% and a minimum alignment length of 15 measured in aa for protein and bp for RNA databases is supplied in the supporting information (Fig. S2). Comparative alpha diversity of all metagenomes (Fig. S3), including those metagenomes where non-overlapping primers were retained as a matrix, showed high species diversity for the bulk soil (631.222 species) and the rhizosphere (611.732 species). In comparison, the phyllosphere metagenome had only about a 1/3 of diversity (214.204 species).

To assess the specificity and to investigate habitat distances of the *E. sativa*-microbiome, the complete metagenome of the rhizosphere, phyllosphere and bulk soil were compared with publicly available metagenomes of similar quality and were obtained from the same habitats (Table S1). Three metagenomes derived from the

oral cavities of humans functioned as outgroup. The PCoA analysis (Fig. 1) revealed a distinct clustering for the phyllosphere and a higher degree of scattering. One agricultural soil metagenome (United States) was closely related to our bulk soil, and also to our rhizosphere metagenome. As demonstrated in Table S1, our metagenomes had the highest amount of sequences and alpha diversity was lowest for the human metagenomes (217-299) and the phyllosphere metagenome of *E. sativa (214)*. In comparison, the alpha diversity of the metagenomes of rice, clover, soybean and *A. thaliana* ranged between 380-580. Rhizosphere and soil samples showed a higher diversity ranging between 621-678 and was only exceeded by the rice rhizosphere (Philippines) metagenome with 844.

Taxonomic assignment (Fig. 2) demonstrate clear differences between the rhizosphere and bulk soil compared to the phyllosphere. Dominant taxa on the phyllosphere are *Protoeobacteria*, *Actinobacteria*, *Bacteriodetes* and *Firmicutes*. Additional abundant taxa in the rhizosphere and bulk soil were *Planctomycetales*, *Acidobacteria*, *Verrucomicrobia*, *Chloroflexi* and Cyanobacteria. *Alphaproteobacteria* were the most abundant class of *Proteobacteria* in the rhizosphere and bulk soil while *Gammaproteobacteria* were the major class in the phyllosphere. Although the main proportion of this proteobacterial assignment accounts for *Pseudomonas* spp. (44%), there is also a high proportion of *Enterobacteriaceae* (7%) enriched in the phyllosphere metagenome of *E. sativa*. Heatmapping of the metagenomes (Fig. S4) allowed insights into the structural differences. Minor differences between the bulk soil and the rhizosphere were typical, however 18 out of 82 annotated taxa showed distinct differences (color shading) in the heatmap. The phyllosphere is more variable with the most noteable increase of *Streptophyta*, *Proteobacteria*, *Bacteriodetes*, *Actionbacteria*, also with increase of fungal assignments such as *Asomycota*, *Basidomycota* and *Blastocladiomycota*. Focusing on enterobacterial abundance (Fig. 3) revealed similar structural pattern for the phyllosphere, the rhizosphere and the bulk soil but at different abundances. *Pectobacterium* was highly enriched in both, the phyllosphere and rhizosphere. In addition *Erwinia* and *Pantoea* were highly enriched only in the phyllosphere.

Functional analysis using SEED subsystems (Fig. 4) permitted the assigning of metagenomic sequences to functional groups with known hypothetical functions with the exception of miscellaneous and clustering based categories. Overall, the most abundant functions represented protein, carbohydrate and amino acids metabolism

and production. A high redundancy within the metagenomes could be shown across the subsystems where the soil metagenome showed an average lowest functional abundance for most mapped functions and the rhizosphere showed the highest. A clear increase according the two tier plant system could be observed for phyllosphere. Subsystems for Iron acquisition and Potassium metabolisms were found in relatively low abundance in the bulk soil, but were enriched in the rhizosphere and were highly abundant in the phyllosphere. Photosynthesis subsystems were found only in the plant metagenomes, and were totally absent in the bulk soil.

Enterobacteriaceae analyzed with the metagenomic approach and 454

Based upon the metagenomic data, we constructed a comparison focusing on the important bacterial family of *Enterobacteriaceae*. The results showed a similar diversity, however there were strongly varying abundances between the metagenomes, with a more than threefold higher abundance in the phyllosphere. The most abundant taxa were *Pantoea* and *Erwinia*, followed by *Pectobacterium*, *Serratia* and *Yersinia*. Pectobacterium was found in similar abundance in the rhizosphere metagenome and was at the same time the dominant genus in the rhizosphere.

454-pyrosequencing of 16S rRNA gene amplicons allowed deeper insight into the indigenous enterobacterial microbiome at OTU based taxonomical levels. After quality filters and the OTU table were built, we constructed an edge-weighted spring embedded network using Cytoscape (Fig. 5), showing OTUs at 100% cut-off level and their connections to the arugula samples plotted in a distance matrix (more connections plots them closer to the center). Following that procedure, we were able to unravel the core structure within the set of samples, interactions and putative key stone species. Across the dataset, 52 distinct denovo OTUs were assigned after removal of chimeric sequences and non enterobacterial hits from the biom file. Further, 14 OTUs were mapped to the enterobacterial core microbiome (OTU present in every sample across phyllosphere and rhizosphere) at 100% samples size. At a 50% fraction of all samples (Fig. S5) 37 OTUs or, in other words, around 71% of all detected OTUs are considered as core, taking the nature of the habitat into account. All high abundant OTUs (OTU52, OTU58, OTU27, OTU11, OTU72 and OTU71) were almost exclusively mapped in the core. For robust assignments, taxonomical assignments were supported by Neighbor Joining trees (Fig. S6-S8) and revealed for

OTU52 = *Erwinia*, OTU58 = *Shigella/Salmonella*, OTU27 = *Shigella/Eschericha*, OTU11 = *Pantoea*, OTU72 = *Erwinia*, OTU71 = *Rahnella*. To compute alpha- and beta diversity indices, each sample was rarified to 3950 reads. Habitat distances (Fig. 6) based on two-sided Student's two-sample t-test revealed tight grouping for the rhizosphere and a higher heterogeneity for the phyllosphere. Distances between all and within all habitats were similar although with higher scattering between habitats.

Cell fixation and FISH/CLSM analysis

FISH/CLSM of diverse plant parts (Fig. S9), including the outer surfaces of the phyllosphere, rhizosphere, spermosphere, anthosphere and their corresponding endospheres (cryo-tome cross sections) showed highly variable colonization patterns of bacteria. Putative Enterobacteria were found in some confocal stacks and were often associated to stomata, plant cell inclusions or some sort of non plant derived substrates with high autofluorescence at 405 nm wave length. While the roots are traditionally heavy colonized by bacteria on the rhizoplane, bacteria were also found in the vascular system or extracellular niches of plant cells as shown by cryo-cut slices of the roots. Similar observations could be made about the stems where small colonies or singles cells attached to cell wall structures of the plant. Only minor bacterial colonization (small cell compartments and single cells) were found for the surface of blossom leafs and pollen. The phylloplane is not frequently heavily colonized, although some spots were found which were extensively colonized by bacteria.

Discussion

Eruca sativa is currently distributed widely around the world and belongs to the group of important vegetables which are eaten raw for its specific taste. Its benefical health effects include its antioxidative and antibacterial activity (Warwick & Francis 1994, Bennett et al. 2002). *Eruca sativa* was traditionally used as a herb or medicinal plant for its health promoting properties. The production of bioactive plant metabolites is one of the main driving factors in medicinal plants and defines the structure of the plant-associated microbiome (Rev. in Köberl et al. 2014). Additionally, Schmidt et al. (2014) demonstrated that appearances of certain taxa and the structure of the microbiome can affect the metabolism of the plant, and *vice versa*. Such microbe-plant interactions are of great importance, since they can supply their host with essential metabolites or according the Dynamic Energy Budget theory (Kooijman 2010), associate with their host and produce new substances and metabolites in syntrophic interactions. Rhizobacteria also enhance the production of glucosinolates (van de Mortel et al. 2012) and the high antioxidative activity from *E. sativa* might be strongly influenced or even completely accomplished by its indigenous microbiome. As explained by the two-step selection model formed by Bulgarelli et al. 2013, the bulk soil microbiota is mainly driven by edaphic factors. Rhizodeposits and cell wall features initiate a structural shift followed by fine tuning of the community profiles on the rhizoplane as a consequence of convergent host genotype-dependent selection. By comparing the bulk soil with the rhizosphere we were able to clearly demonstrate a rhizosphere effect. In particular, the proteobacterial taxa *Alphaproteobacteria* and *Deltaproteobacteria* were partially depleted on the rhizosphere, while Gammaproteobacteria and Betaproteobacteria became more dominant. This microbial shift could be extended to the phyllosphere accompanied by more drastic changes. *Gammaproteobacteria* became highly dominant at the cost of a significant loss of abundance and diversity of *Deltaproteobacteria*, as well as *Beta*- and *Alphaproteobacteria*. The phyllosphere microbiota is driven mainly by environmental- and biotic factors (rev. in Vorholt 2012) but it was also reported that the plant genotype might remain a central factor (Redford et al. 2010). In addition, different lettuce cultivars have been shown to possess their own complete bacterial indigenous core microbiome (Cardinale et al. 2014) and to influence the metabolitic activity and colonization capabilities of enterics (Klerks et al. 2007, Quilliam et al 2012). The comparison to other metagenomes and the habitat distances

demonstrated the higher heterogeneity and more unpredictable patterns for microbial colonization on the phyllosphere compared to the rhizosphere. In contrast to the close distance within the PCoA-plot between bulk soil and rhizosphere, major functional and taxonomic differences were revealed by the analysis of the whole metagenomes.

Functional analyses demonstrated the rhizosphere as overall functional hotspot. Subsystems for the potassium and for the iron acquisition metabolism were highly raised in the phyllosphere. This might be explained by the low amount of free iron or potassium in the harsh environment of a leaf surface what puts pressure on both the plant and the associated microbes. Up-regulation and directed colonization by bacteria with this specific metabolic activity might be the consequence. As expected, subsystems for photosynthesis were only detected in the phyllosphere and in lower abundance in the rhizosphere. This finding also clearly demonstrates the distinction between the habitats and can be seen as a quality indicator for the metagenomic datasets.

Our previous studies revealed (Erlacher et al. 2014, 2015) that *Enterobacteriaceae* on lettuce (*Lactuca sativa*) respond particularly well to biotic and abiotic stress and possess the ability to colonize the leafy green in high abundances. Although in contrast to other phyllosphere metagenomes (public metagenomes, Table S1), alpha-diversity indices were significantly lowered. The plant extracts of *E. sativa*, which possess deleterious activity on microbes, might partially explain this observation. However, the exterior of the plant is still colonized by a vast bacterial diversity including many *Enterobacteriaceae*. Congruent to the findings in Erlacher et al (2015), where we analyzed the enterobacterial abundance by using real-time PCR, we have shown the accumulation of *Enterobacteriaceae* in the phyllosphere to be present in approximately threefold higher abundances, as when compared to the rhizosphere. This almost exact overlap is surprising since not only do the techniques utilized differ, but also the lettuce types (*Lactuca sativa* and *Eruca sativa*). The family of *Enterobacteriaceae* comprises many human pathogens but also includes several plant pathogens. Two commercially relevant genera, *Pantoea* and *Erwinia* were also detected in high abundances in both the phyllosphere metagenome and in the amplicon datasets. Not only were they present in high abundances, they also formed the enterobacterial core-biome as putative key species. The other important contributors to the core were associated with human pathogens, but they could not

be unraveled to the full extent by the applied techniques due to the high degree of similiarity in the 16S rRNA genes of the genera; *Escherichia*, *Shigella* and *Salmonella*. As demonstrated in the spring embedded network, the amount of OTUs (referred to enterics at strain level) which have only one or two connections to a sample was quite low (only 8 OTUs with one connection and 9 OTUs with two connections). That observation, combined with markedly low shifts of diversity shown within the metagenomic datasets, provokes the thesis that most *Enterobacteriaceae* were not transmitted accidentally. Moreover, it seems that the soil provides a major reservoir and a high proportion of this enterics can establish and multiply on the whole plant, as was confirmed by microscopy. It has been shown that the leaf age, plant lesions and nitrogen content affect the bacterial communities and promote the multiplication of enterics (Erlacher et al. 2015, Brandl 2008, Brandl & Amundson 2008). We cannot discuss the impact of the leaf age or nitrogen content in this study, however, while we investigated the plant compartments with microscopical techniques it seemed that plant lesions played a lesser role in the assemblage of bacteria when compared to *Lactuca sativa*.

To the best of our knowledge this is the first study which allowed reliable reconstruction of microbial taxa over the soil-plant interphase associated to *Eruca sativa*, and we were additionally able to shed light on the bacterial lifestyle and microbiome functioning.

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Figure legends

Figure 1 Comparison of the *E. sativa* phyllosphere and rhizosphere including the bulk soil (ellipse) and publicly available metagenomes by principal coordinate analysis (PCoA). PCoA plot is based on the Bray–Curtis distance matrix of metagenomes that were assigned to functional subsystems of SEED database and normalized. Single metagenomes are shown by coloured dots (green = phyllosphere, blue = rhizosphere, brown = soil) with MG-RAST identical numbers (IDs). Eigenvalues (k) correspond to variation explained by each principal coordinate, respectively.

Figure 2 Taxonomic structure of the metagenomes for the whole metagenomic data set (upper row) and for Proteobacteria (lower row).

Figure 3 The chart illustrates the enterobacterial abundance in the metagenomes. Only taxa are present with a higher abundance than 100 in at least one metagenome. the chart illustrate the distribution and abundance of taxonomic genera of Enterobacteriaceae, for the annotations. The abundance is based on the percentage of reads with predicted proteins and ribosomal RNA genes annotated to the indicated taxonomic affiliation.

Figure 4 Functional abundance using SEED database of the Metagenome samples.

Figure 5 OTU based Network correlating the OTUs at 100% cut-off level. Cytoscape v. 3.1.0 was used to construct this Edge-weighted Spring Embedded Network. OTUs labled in blue belong to the otu core network at 100% core-similarity. Small node (average size = 10 reads), OTU 52 = 10,028 reads.

Figure 6 Habitat Distances boxplots showing the distance between and within the habitats. The tests of significance were performed using a two-sided Student's two-sample t-test

Figures

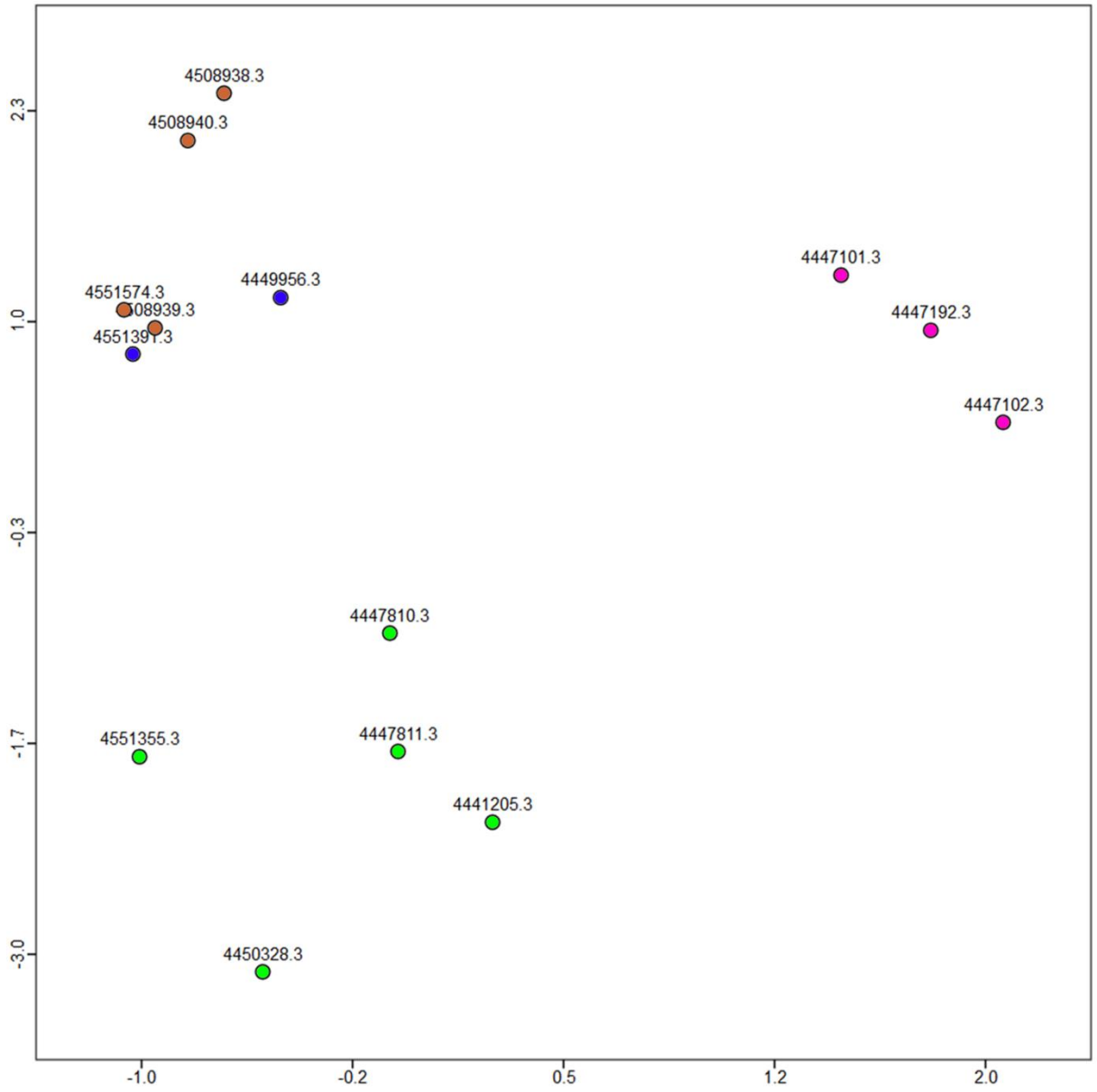


Figure 1

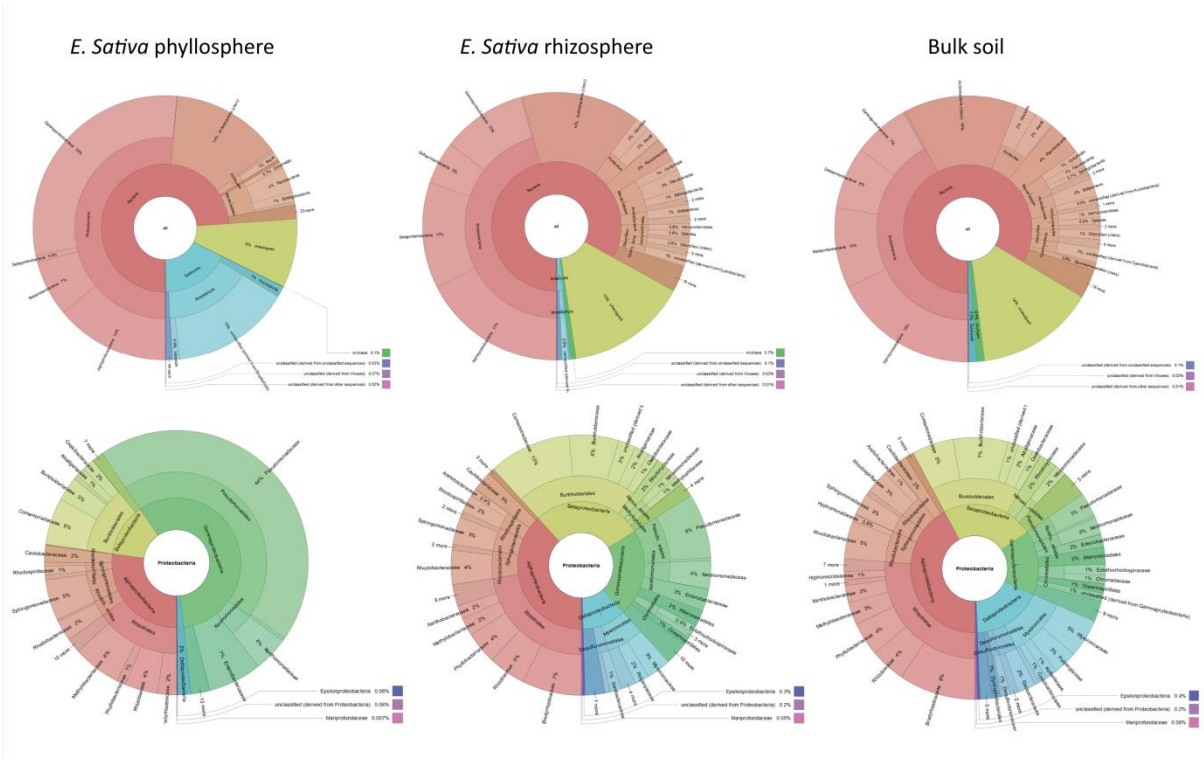
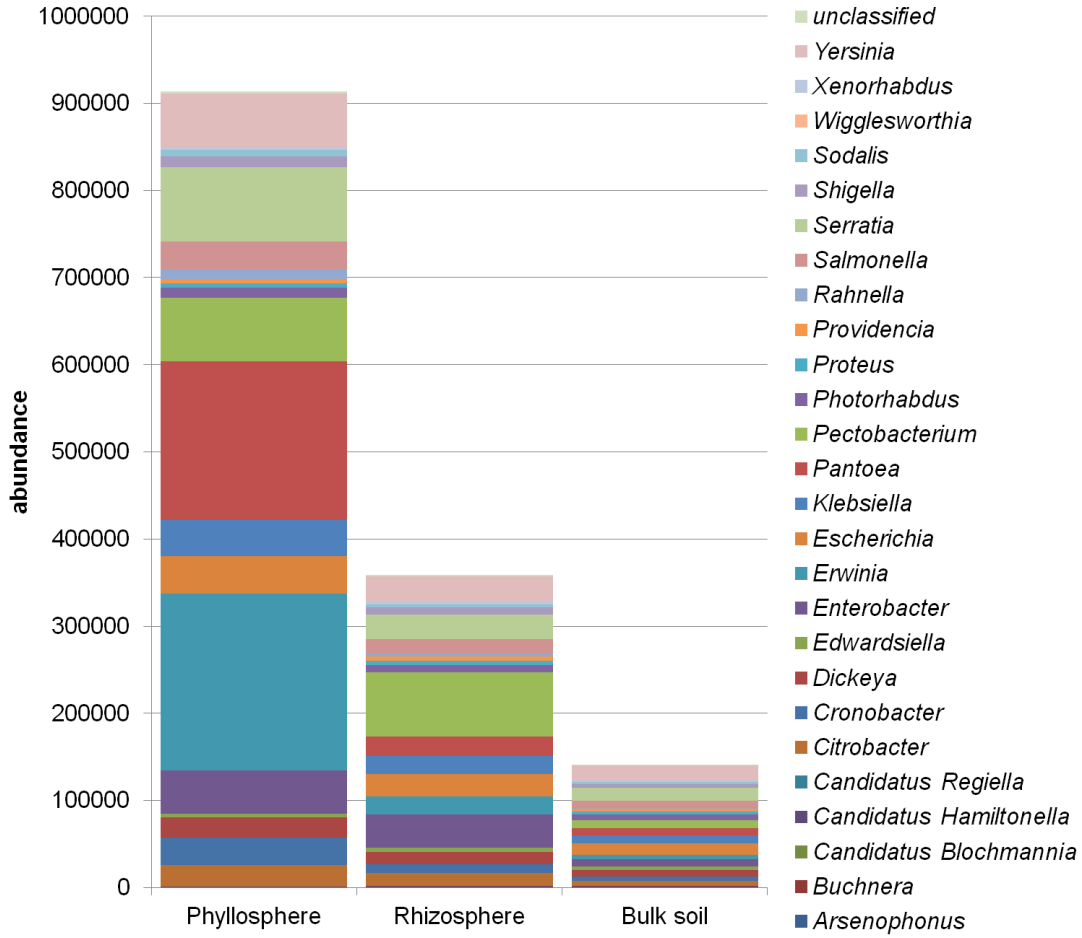


Figure 2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Sodalis
Shigella
Serratia
Salmonella
Rahnella
Providencia
Proteus
Photorhabdus
Pectobacterium
Pantoea
Klebsiella
Escherichia
Erwinia
Enterobacter
Edwardsiella
Dickeya
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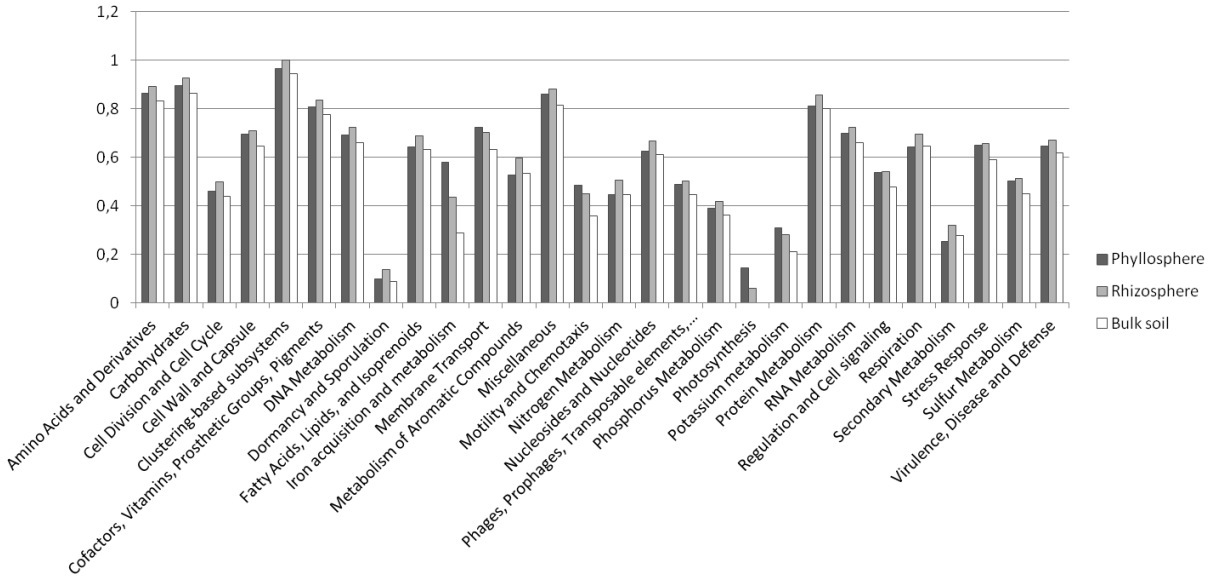
Candidatus Regiella
Candidatus Hamiltonella
Candidatus Blochmannia
Buchnera
Arsenophonus

Figure 3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Carbohydrates
Cell Division and Cell Cycle
Cell Wall and Capsule
Clustering-based subsystems
Cofactors, Vitamins, Prosthetic Groups, Pigments
DNA Metabolism
Dormancy and Sporulation
Fatty Acids, Lipids, and Isoprenoids
Iron acquisition and metabolism
Membrane Transport
Metabolism of Aromatic Compounds
Miscellaneous
Motility and Chemotaxis
Nitrogen Metabolism
Nucleosides and Nucleotides
Phages, Prophages, Transposable elements,...
Phosphorus Metabolism
Photosynthesis
Potassium metabolism
Protein Metabolism
RNA Metabolism
Regulation and Cell signaling
Respiration
Secondary Metabolism
Stress Response
Sulfur Metabolism
Virulence, Disease and Defense
Phyllosphere
Rhizosphere
Bulk soil

Figure 4

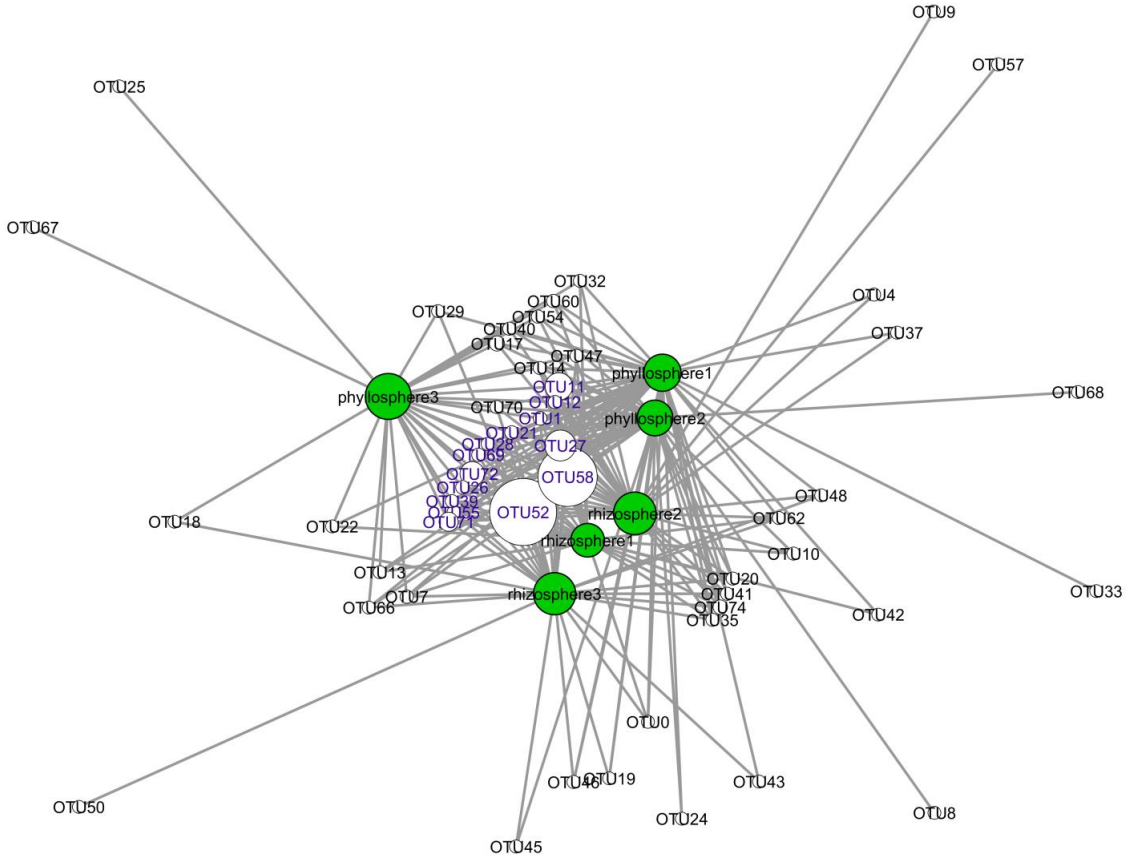
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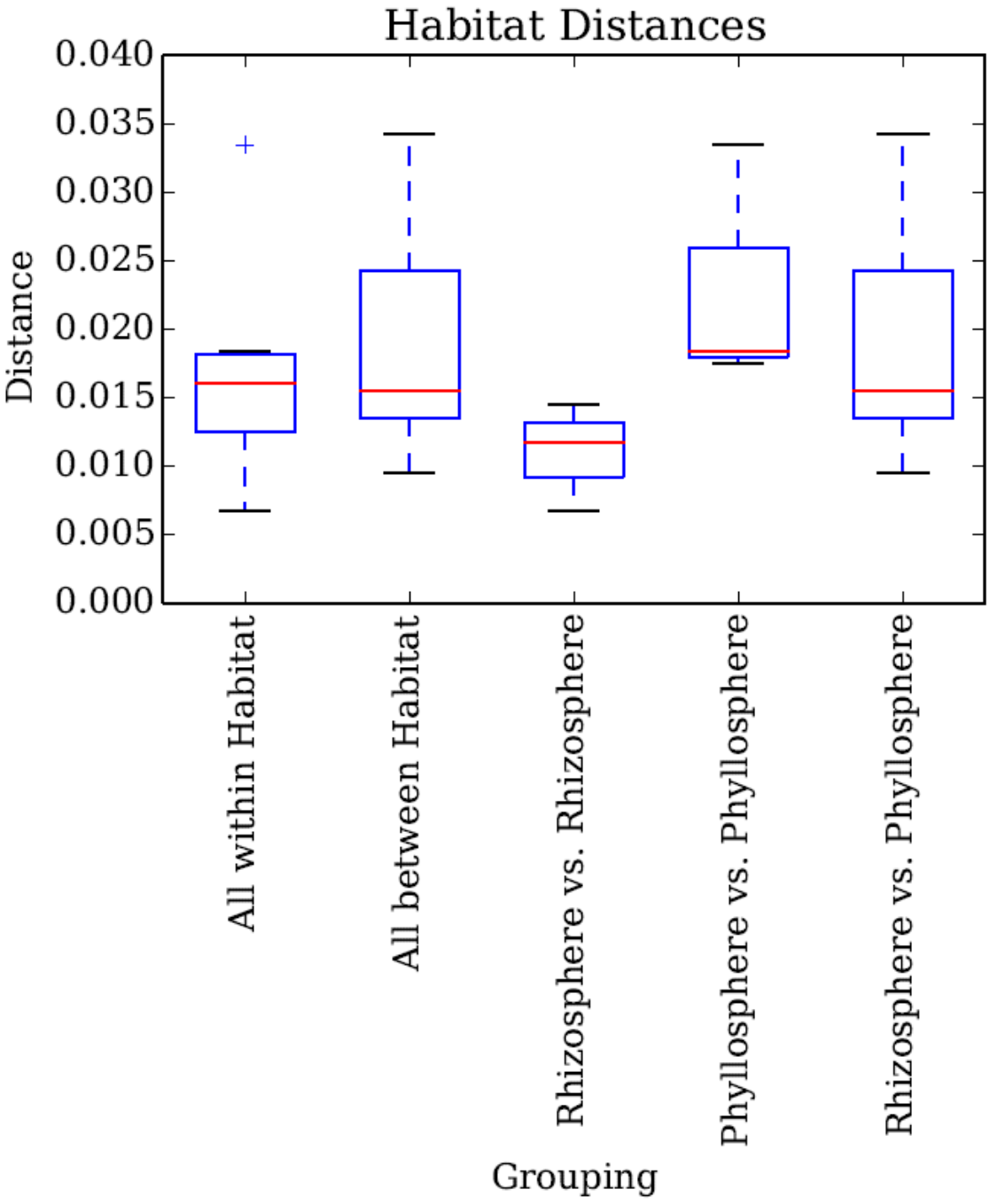
Figure 5


Habitat Distances
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All within Habitat
All between Habitat
Rhizosphere vs. Rhizosphere
Phyllosphere vs. Phyllosphere
Rhizosphere vs. Phyllosphere
Grouping

Figure 6

Supplementary material

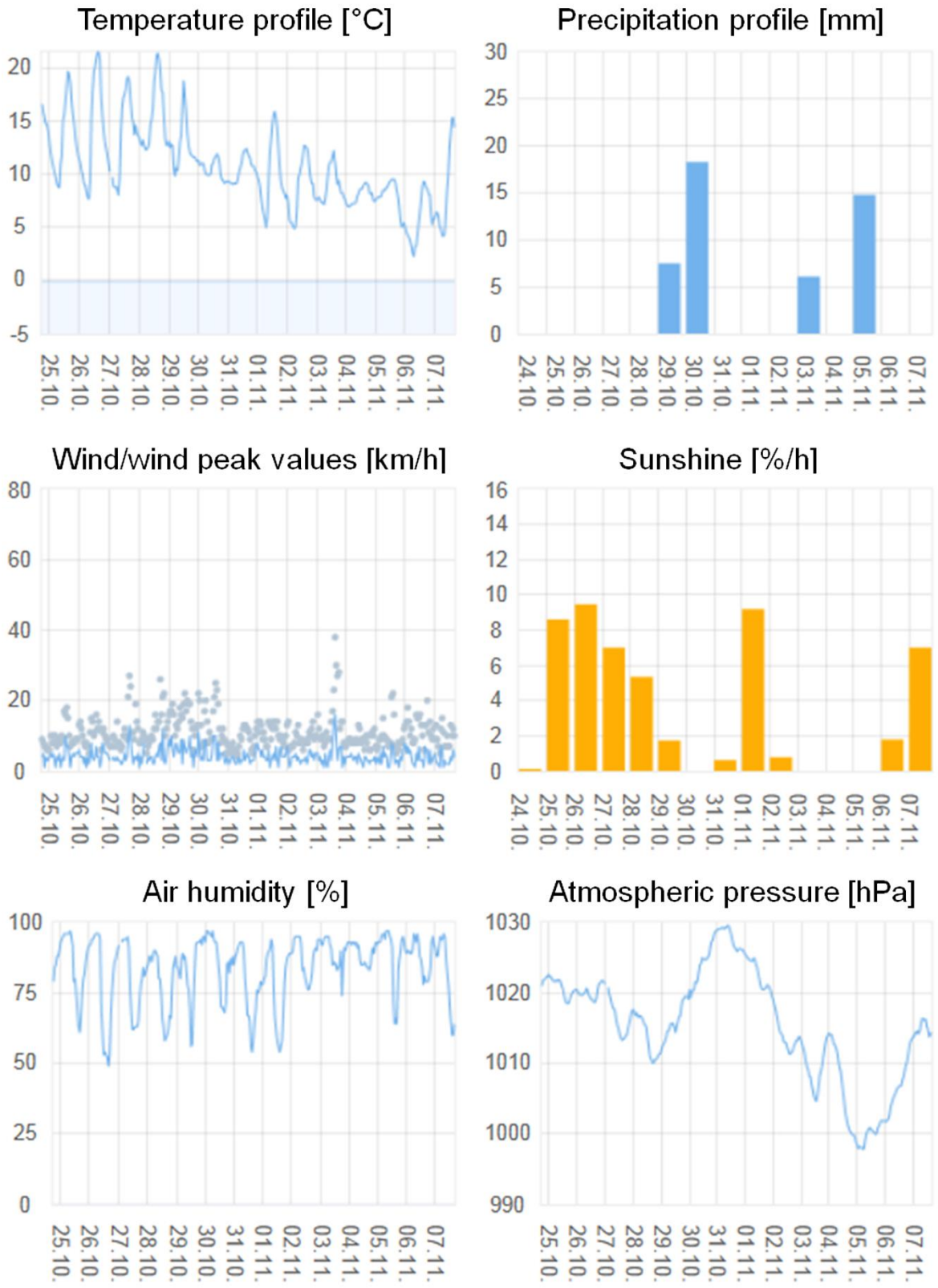

Figure S1 Metadata profile starting 14 days before sampling of plants for the metagenomic approach.

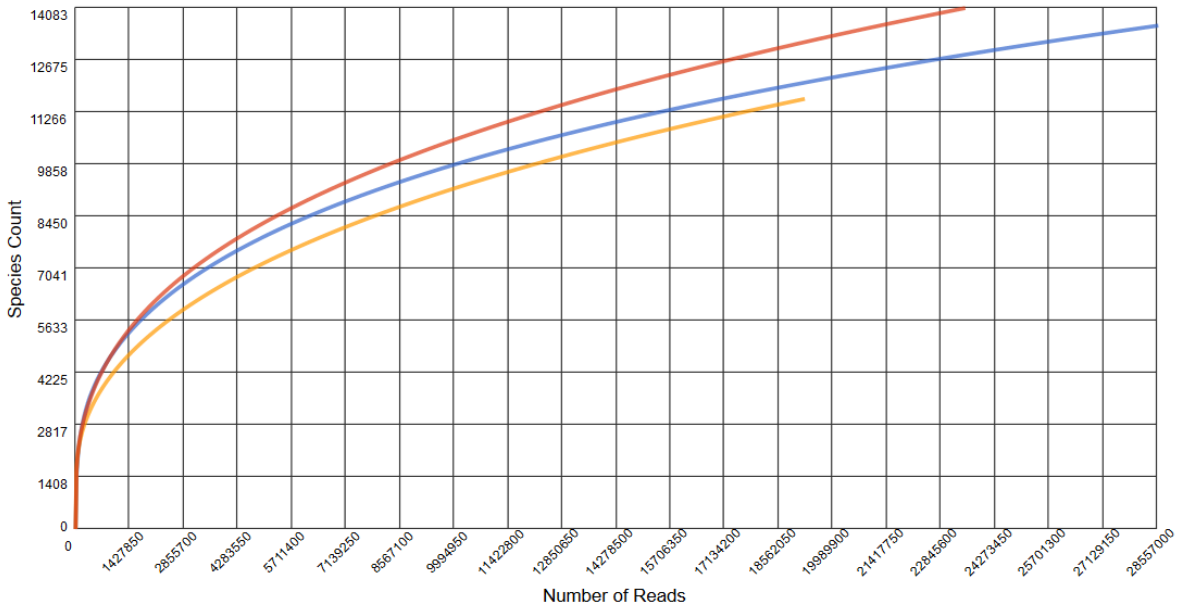

Figure S2 Rarefaction curves. This data was calculated for metagenomes 4551574.3 (yellow), 4551391.3 (red) and 4551355.3 (blue). The data was compared to M5NR using a maximum e-value of 1e-5, a minimum identity of 60%, and a minimum alignment length of 15 measured in aa for protein and bp for RNA databases.

Figure S3 Comparative Alpha Diversity barchart of all metagenome datasets (inclusive retained and non retained). The min, max, and mean (μ) values are displayed with the standard deviation ranges (σ and 2σ) in different shades. The α-diversity of the non retained are shown in green (phyllosphere; 214.204 species), blue (rhizosphere; 611.732 species) and brown (bulk soil; 631.222 species).

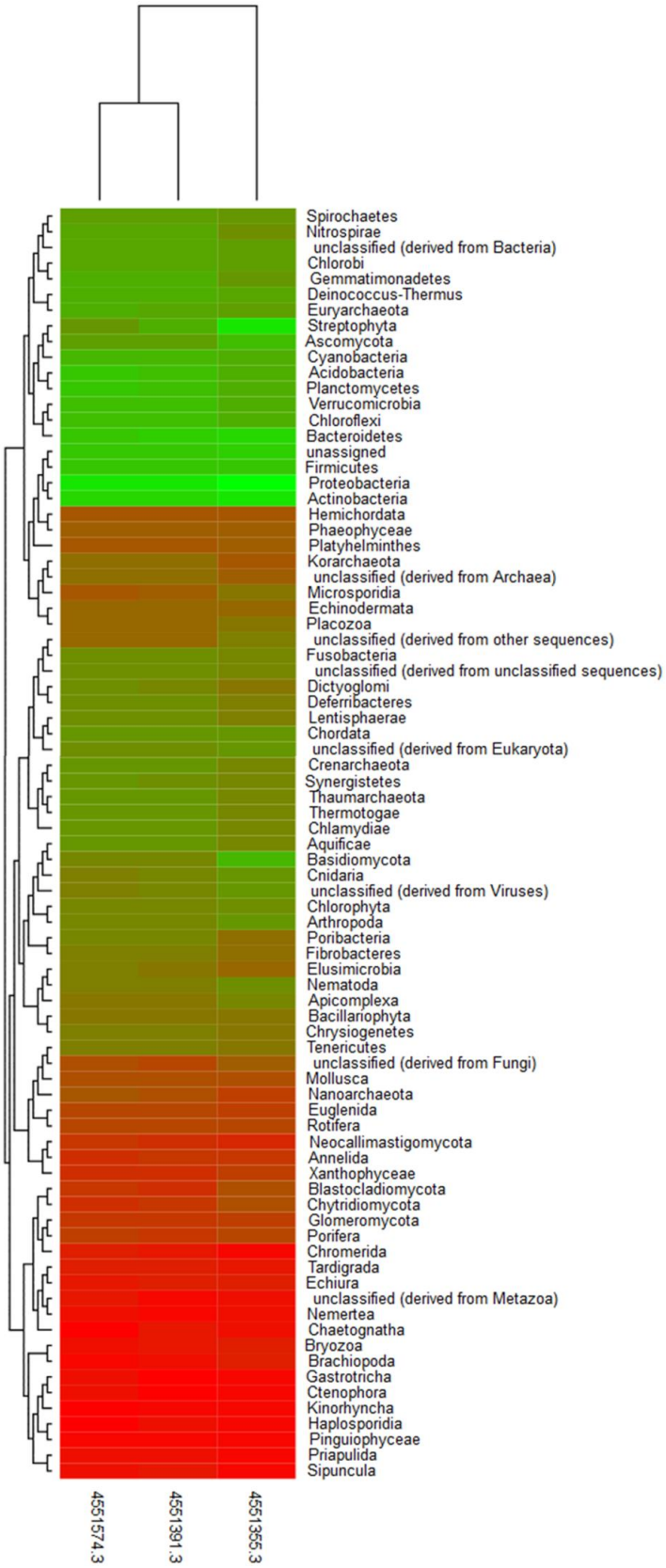

Figure S4 Comparative Heatmap of *E. sativa* phyllosphere, rhizosphere and bulk soil. The Comparison of taxonomical family level is based on the M5NR database at a 90% identity

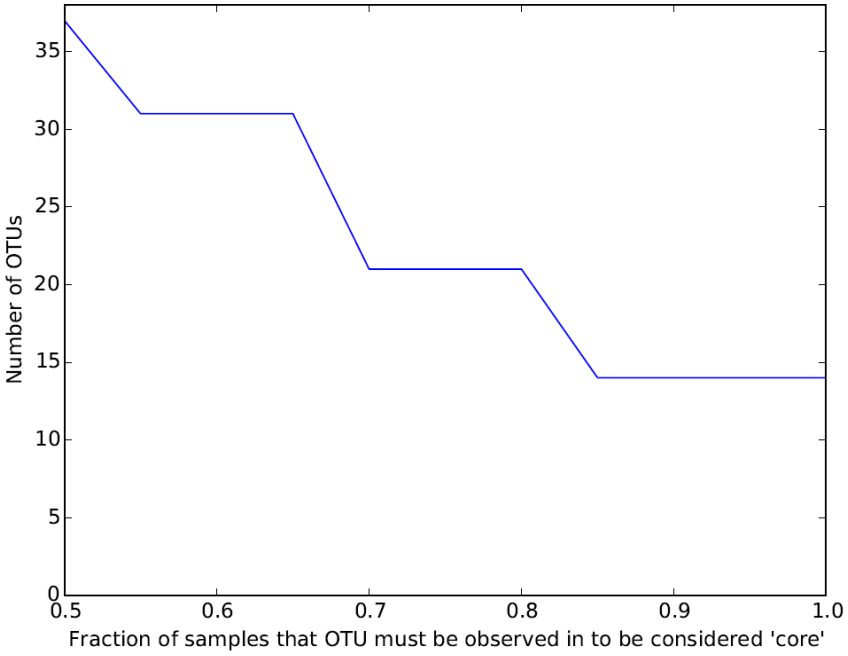

Figure S4 Core OTUs across 50% - 100% Samples

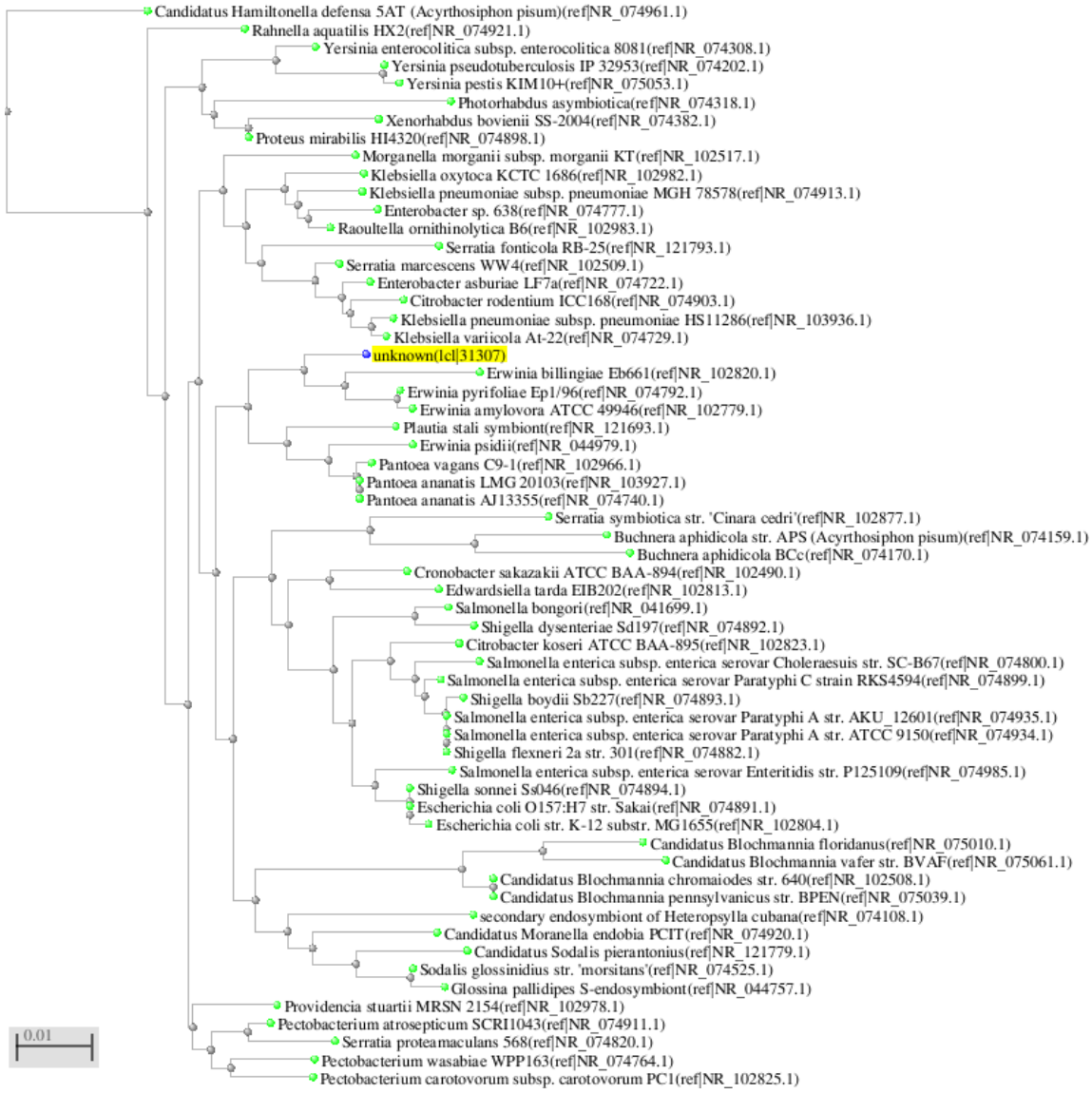

Figure S6 Neighbor Joining tree, Max Seq Ident 0.1 (90% Similarity), RefSeq BLAST, OTU 52

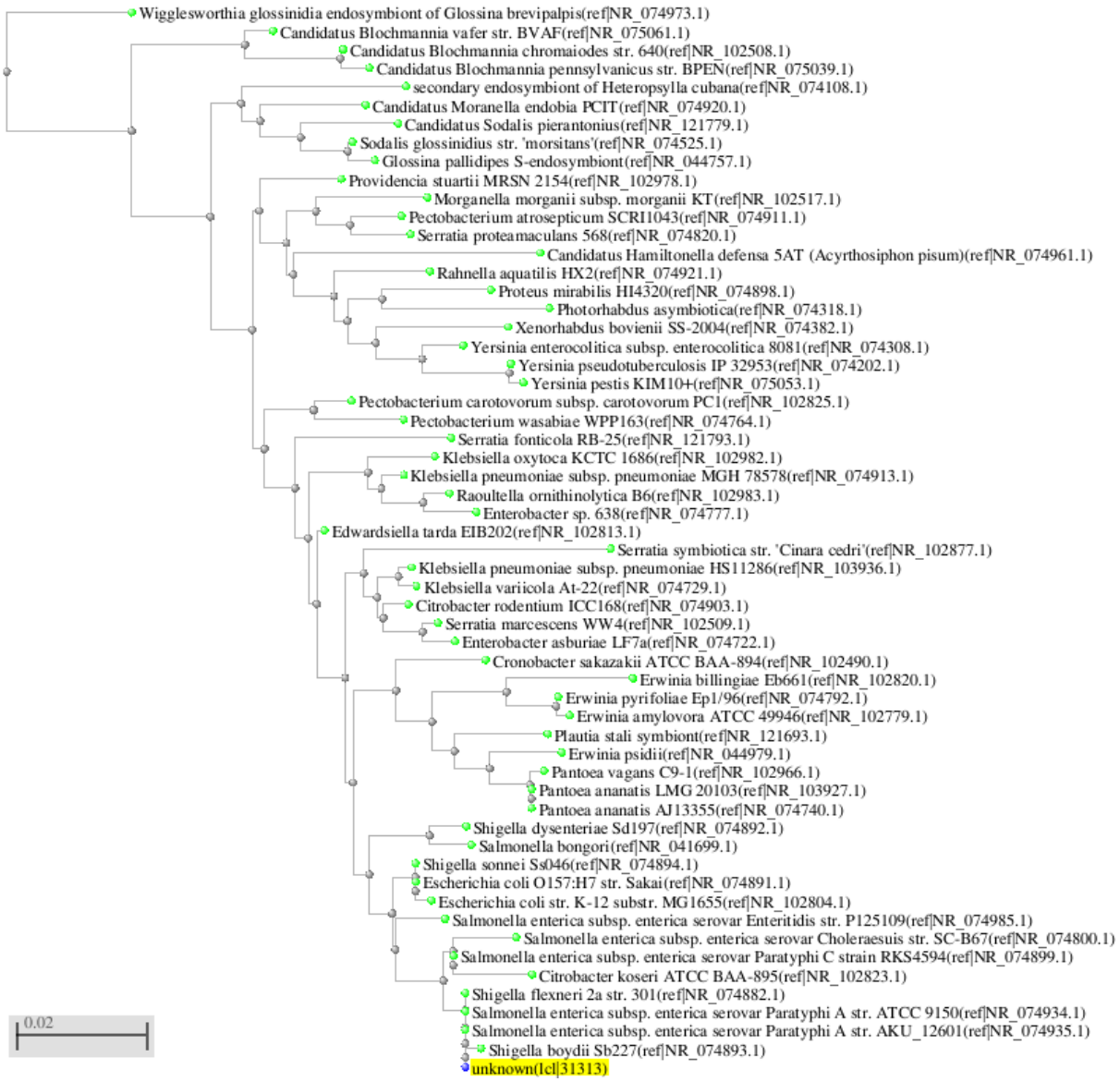

Figure S7 Neighbor Joining tree, Max Seq Ident 0.1 (90% Similarity), RefSeq BLAST, OTU 58

Figure S8 Neighbor Joining tree, Max Seq Ident 0.1 (90% Similarity), RefSeq BLAST, OTU27

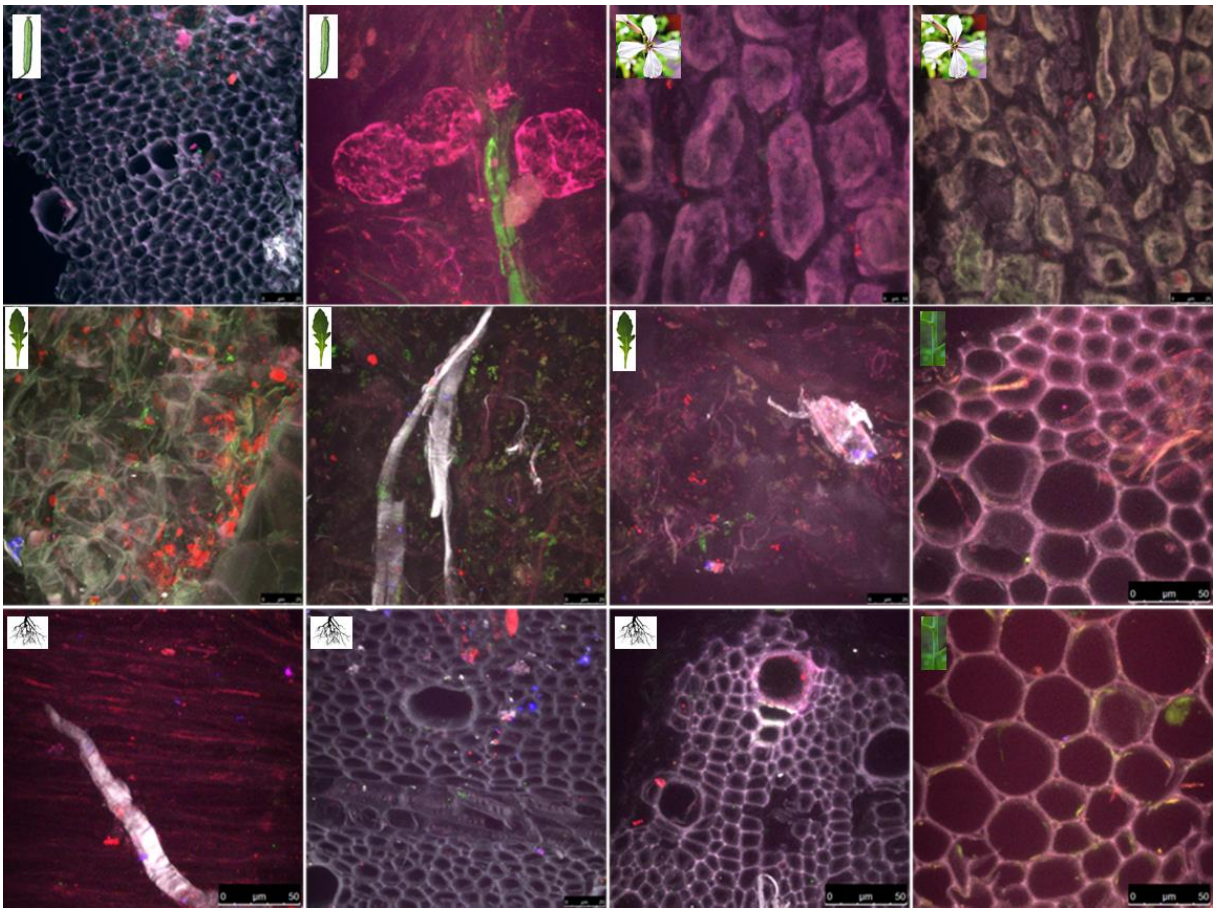

Figure S9 Confocal Z-stack maxium projection images showing different *E. sativa* microenvironments colonized by bacteria unclassified bacteria (red) or *Enterobacteriaceae*. Micrographs in the top left corner of each z-stack represent the associated habitat.

Table S1 Sample description and corresponding MG-RAST metagenome IDs for all compared datasets used in the PCoA plot.

MG RAST ID	Biome	Habitat	Country	# of sequences	Alpha-diversity
4551574.3	**Soil**	**Bulk soil**	**Austria**	**19,031,310**	**631.222**
4551391.3	**Plant-associated**	***E. sativa* rhizosphere**	**Austria**	**22,387,760**	**611.732**
4551355.3	**Plant-associated**	***E. sativa* phyllosphere**	**Austria**	**23,668,647**	**214.204**
4450328.3	Plant-associated	Rice phyllosphere	Philippines	2,213,945	582.844
4447810.3	Plant-associated	*A. thaliana* phyllosphere	Spain	1,098,311	470.804
4447811.3	Plant-associated	Clover phyllosphere	Switzerland	1,028,814	421.794
4441205.3	Plant-associated	Soybean phyllosphere	Switzerland	523,769	381.345
4508938.3	Soil	Agricultural soil	USA (IN)	9,999,775	678.281
4508939.3	Soil	Agricultural soil	USA (IN)	16,923,988	641.538
4508940.3	Soil	Agricultural soil	USA (IN)	17,442,824	621.506
4449956.3	Plant-associated	Rice rhizosphere	Philippines	1,026,982	844.453
4447101.3	Human-associated	Oral cavity	Spain	295,072	299.147
4447192.3	Human-associated	Oral cavity	Spain	204,218	217.793
4447102.3	Human-associated	Oral cavity	Spain	244,881	217.806

Publication I

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Biotic stress shifted structure and abundance of Enterobacteriaceae in the lettuce microbiome

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RESEARCH ARTICLE

Biotic Stress Shifted Structure and Abundance of *Enterobacteriaceae* in the Lettuce Microbiome

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Abstract

Lettuce cultivars are not only amongst the most popular vegetables eaten raw, they are also involved in severe pathogen outbreaks world-wide. While outbreaks caused by *Enterobacteriaceae* species are well-studied, less is known about their occurrence in natural environments as well as the impact of biotic stress. Here, we studied the ecology of the human health-relevant bacterial family *Enterobacteriaceae* and assessed the impact of biotic disturbances by a soil-borne phytopathogenic fungus and *Gastropoda* on their structure and abundance in mesocosm and pot experiments. Using a polyphasic approach including network analyses of 16S rRNA gene amplicon libraries, quantitative PCR and complementary fluorescence *in situ* hybridization (FISH) microscopy we found substantial yet divergent *Enterobacteriaceae* communities. A similar spectrum of 14 genera was identified from rhizo- and phyllospheres but the abundance of *Enterobacteriaceae* was on average 3fold higher in phyllosphere samples. Both stress factors shifted the bacterial community of the leaf habitat, characterized by increases of species abundance and diversity. For the rhizosphere, we observed significant structural shifts of *Enterobacteriaceae* communities but also a high degree of resilience. These results could be confirmed by FISH microscopy but it was difficult to visualize phyllosphere communities. Additional inoculation experiments with *Escherichia coli* as model revealed their presence below the wax layer as well as in the endosphere of leaves. The observed presence influenced by stress factors and the endophytic life style of *Enterobacteriaceae* on lettuce can be an important aspect in relation to human health.

Introduction

Lettuce (*Lactuca sativa* L.) is regarded highly re-esteemed as an important staple of a balanced, healthy diet. Globally, lettuce consumption has increased during the past several years [1], and in 2012 lettuce and chicory production was noticed with 24.9 Mio t (FAOSTAT). Pathogen outbreaks associated with lettuce, caused mainly by members of *Enterobacteriaceae*, are a

major problem for human health world-wide; therefore they are well-studied [1–5]. In the past, *Enterobacteriaceae* are known as inhabitants of the intestines of animals and humans with *Escherichia coli* as long-studied model bacterium, pathogens, such as *E. coli* pathovars, *Yersinia pestis*, *Klebsiella*, *Salmonella* and *Shigella* spp. but also symbionts, suggesting that they are important for human health [4]. Recently, it was reported that *Enterobacteriaceae* are also indigenous members of several plant microbiomes [2,4]. However, less is known about the natural occurrence and ecology of this health-relevant bacterial group on lettuce. As with all plants, lettuce provides habitats for specific microbes as shown by Rastogi et al. [6,7]. High bacterial abundances of 10^5–10^6 CFU g^{-1} fw and diversities with a substantial proportion of *Enterobacteriaceae* were identified in the phyllosphere of pot-grown lettuce [6–8]. Despite these studies, less is known about the variability and stability of the lettuce microbiome under various conditions.

Plants are colonized by specific microbiomes, which contribute to the growth, health, and fitness of their hosts [9,10]. This is due to specific conditions and exudates, as well as defensive mechanisms, and all these factors trigger species- and even cultivar-specific microbiomes [11,12]. Moreover, each individual plant harbors different microbial communities specific to the organs of each individual plant, mainly the phyllosphere (leaves) and rhizosphere (roots) [10]. Although the majority of microorganisms may be characterized as having a positive interaction with their hosts, human and plant pathogens are also part of the plant-associated microbiota [13–15]. Bacteria are key players in plant-associated ecosystems, and therefore it is crucial to identify the structure and function of bacterial communities. It is additionally important to understand the effect of biotic disturbances, which often occur in nature as well as under agricultural conditions, and their consequential structural and functional responses [16]. The basidiomycetous fungus *Rhizoctonia solani* Kühn cannot only cause severe diseases on lettuce with yield losses up to 70%, it can also colonize lettuce plants without or with only slight symptoms [17]. Slugs and snails (*Gastropoda*) are widespread agricultural pests and are also important for the cultivation of lettuce of both commercially and privately grown lettuce [18]. Over the past decade, the application of novel technologies has not only revolutionized our knowledge of the microbiome; it has drastically changed our view on pathogens and diseases [15,19]. For more than a century, pathogens have been defined as causative agents of diseases in accordance with Koch's postulates, which were later added by molecular criteria [19]. In terms of the human microbiome, however, we have learned that pathogen outbreaks are associated with shifts of the whole community including shifts of supporting pathogens [20–22]. In contrast to Chowdhury et al. [23] who applied the 16S rRNA gene-based fingerprinting method terminal restriction fragment length polymorphism (T-RFLP), we were able to confirm the finding for the plant microbiome by deep sequencing of the microbiota. Both the phytopathogenic fungus *R. solani* and its antagonistic counterpart *Bacillus amyloliquefacies* FZB42 shifted the bacterial community in lettuce [24]. Moreover, we found indications that especially the enterobacterial fraction was highly affected by the pathogen [24]. Therefore, we developed the hypothesis that biotic stress induces shifts in the lettuce-associated *Enterobacteriaceae* community.

Our objective was to study *Enterobacteriaceae* communities on lettuce plants and to assess the impact of biotic stresses on them. We evaluated the influence of the pathogenic fungus *Rhizoctonia solani* and *Gastropoda* on the structure and the abundance of *Enterobacteriaceae* in the rhizo- and phyllosphere of lettuce (*Lactuca sativa* L. var. *capitata*) in mesocosm and pot experiments using a combined approach including network analyses of 16S rRNA gene amplicon libraries, quantitative PCR and FISH microscopy.

Materials and Methods

Experimental design and sampling

We investigated the impact of *R. solani* AG1-IB on the lettuce-associated microbiota in the first experiment (E1). The phytopathogenic fungus *R. solani* was applied to young seedlings of *L. sativa* var. *capitata* in randomized block systems and was grown further under growth chamber conditions (20/15°C, light 16/8 h (day/night). Lettuce was planted at two-leaf stage into pots (500 ml) filled with the same substrate sand mixture used for the seedling trays at a 1:1 ratio (v/v) (Fruhstorfer Einheitserde Typ P, Vechta, Germany; chemical analysis (mg l^{-1}): N = 120, P = 120, K = 170, Mg = 120, S = 100, KCl = 1, organic substance = 167, peat = 309, pH 5.9). The pots were watered daily and fertilized (0.2% Wuxal TOP N, Wilhelm Haug GmbH and Co. KG, Düsseldorf, Germany) weekly. The sampling was carried out four weeks after inoculation with *R. solani*. In the treatments with pathogen inoculation, the substrate mixture was inoculated with ten *R. solani*-infested barley kernels and incubated at 25°C for one week until planting of lettuce into the pots. The microenvironments phyllosphere (leaves of each plant) and rhizosphere (roots of each plant including adhering soil) were separated. Six independent replicates were investigated for each microhabitat as well as for healthy plants (C_P/R) and plants showing severe bottom rot symptoms caused by *R. solani* indicated by brown lesions on petioles and the outer leaves (RS_P/R). The pot experiments were done at the Leibniz Institute of Vegetable and Ornamental Crops (Grossbeeren, Germany).

In the second experiment (E2), we developed an approach to study the impact of *Gastropoda* exposition on the lettuce (*L. sativa* var. *capitata*) microbiome in steady state glass tank mesocosms. The whole experimental procedure was conducted following customized methods to ensure no microbial carryover during transplantation, sample harvest or watering. Two glass tanks (50x30x30 cm) with a constitutional volume of 45 l were filled to one quarter capacity with identical homogenized organic potting soil (Dehner Bio Aussaat- und Kräutererde, Rain Germany; chemical analysis (mg l^{-1}): N = 150, P = 180, K = 750, Mg = 150, S = 220, organic substance = 85%, pH 5.8). Twenty-two seedlings (*Lactuca sativa* var. *capitata*) were purchased at a local organic market, four plants were further separated (phyllosphere/rhizosphere) and processed for DNA extraction (C_T0), the remaining 18 plantlets were equally deployed in the two mesocosms. Lastly, the tanks were covered using a metal frame, coated with a mosquito mesh. The second sampling (C_T1) of four replicates (two plants/mesocosm) was conducted 13 days post planting. The sampling was carried out to determine effects and adaptions of the microbiota within the lettuce growth and establishment phase. The third sampling (C/G_T2) was carried out in the same way to the second sampling, five days later. Between the sampling C_T1 and C/G_T2 we enclosed 12 gastropods, members of *Limacidae* and *Arionidae* and snails (*Cepaea* and *Helicidae*) from the local environment to one mesocosm (G). The last sampling (C/G_T3) was performed six weeks after C/G_T2, to investigate sustainable changes in the microbial structure (gastropods were removed after G_T2).

From each experiment, four independent replicates consisting of 15–20 leaves or roots were collected and stored separately, placed into sterile plastic bags, and transported to the laboratory.

Metagenomic DNA extraction

The microbial fraction associated with lettuce plants was extracted separately for the rhizo- and phyllospere [25]. From the plant material, 5 g of randomly cut tissues were physically disrupted with a sterile pestle and mortar and resuspended in 10 ml of 0.85% NaCl, and 2 ml of suspension were subsequently centrifuged (16,500 g, 20 min, 4°C). The obtained pellet was

used for isolation of the total community DNA with the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). For mechanical lysis, the cells were homogenized twice in a FastPrep FP120 Instrument (Qbiogene, BIO101, Carlsbad, CA, USA) for 30s at a speed of 5.0 m sec^{-1} and treated according to the manufacturer's protocol. Two technical DNA replicates from sampling were pooled for further processing.

Microbial fingerprints by SSCP analysis of the 16S rRNA genes (PCR-SSCP)

In order to gain a first insight into structural changes and occurrence patterns of plants exposed to gastropods, fingerprinting by Single Strand Conformation Polymorphism (SSCP) [26] according to Rossman et al. [27] was performed. Briefly, bacterial 16S rRNA gene sequences were amplified by PCR using the universal bacterial primer pair Unibac-II-515f and Unibac-II-927rP [28] and for *Gammaproteobacteria* γ-prot 395f and γ-prot 871r [29]. After lambda exonuclease digestion the single stranded amplicons were separated using the TGGE Maxi System (Biometra, Göttingen, Germany) and visualized by silver staining. Evaluation of bacterial community profiles obtained by SSCP was performed by using the GelCompar program version 4.1 (Applied Maths, Kortrijk, Belgium).

454 pyrosequencing of 16S rRNA gene amplicons

The 16S rRNA gene fragments of all *Gammaproteobacteria* (E1) and *Enterobacteriaceae* (E2) were each amplified in a dual phase nested PCR approach using Multiplex Identifier (MID) tagged primers. Gammaproteobacterial 16S rRNA gene fragments were amplified using the V3–V5 primers γ-prot 395f and γ-prot 871rP [29] ensued by primer 515f and MID-modified 871r_454 [24,27]. For the PCR to investigate the enterobacterial diversity, the specific primers Entero-F234 and Entero-R1423 (V3–V8) for *Enterobacteriaceae* according to Binh et al. [30] were used. The nested PCR was carried out as described by Heuer et al. [31] using the primer pair F984 and R1378 modified for multiplex 454 sequencing according to the specification. The products of two independent PCR reactions from two independent samples were pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Purified PCR products (200 ng each) were sequenced on a GS FLX 454 Titanium platform (Macrogen Korea, Seoul, South Korea).

Sequences were analyzed with the QIIME software version 1.7.0 [32]. MID, primer, and adapter sequences were removed, and the sequences were quality (minimal score: 50) and length filtered (minimal raw fragment length: *Gammaproteobacteria* $\geq$ 350; *Enterobacteriaceae* $\geq$ 430), followed by a denoising step (denoise wrapper and QIIME denoiser script). Chimeras and the remaining sequences of non-target, plastidal, and mitochondrial origins were removed. OTU (operational taxonomic unit) tables were created with UCLUST at a 97% cut-off level for the amplified *Gammaproteobacteria* and 98% cut-off level for the amplified enterobacteria, respectively. OTU tables were retrained on the latest green genes release (v13.5) and the *Gammaproteobacteria* OTU table (E1) was subsequently filtered to family level of *Enterobacteriaceae*. The datasets were rarified to the appropriate number of least reads per sample within each experiment to compute alpha and beta diversity indices. Unique reads were classified with the RDP classifier (v2.5) and normalized data were then produced from the relative abundance of taxa present in each sample based on a naïve Bayesian classifier [33]. Output sequences were classified as domain, phylum, family, and genus depending on the depth of reliable classifier assignments. Cytoscape (v2.8) was used to create an integrated model network [34] Statistical tests based on the OTU table were performed with the non-parametric ANOVA Kruskal Wallis test. This test is functionally an expansion of ANOVA to cases where the sample means are

unequal and the distribution is not normal. The nucleotide sequences obtained in this work were submitted to the European Nucleotide Archive (www.ebi.ac.uk/ena) and are available under the accession numbers PRJEB6022 (E1) and PRJEB7591 (E2).

Quantitative PCR

The abundance of *Enterobacteriaceae* was quantified by determining the number of 16S rRNA genes per ng of the community DNA isolated from the respective lettuce samples. The preparation of the standard fragment and the quantitative PCR was carried out following the guidelines given by Rossmann et al. [27]. The 16S rRNA gene of *Serratia plymuthica* HRO-C48 served as reference fragment for the generation of standard curves. The quantitative PCR was conducted with the ROTOR 6000 system (Corbett, Mortlake, Australia). The final reaction volume (10 µl) contained 1 µl of the 1:500 diluted template, 5 µl of 2× Kapa SYBR Fast Mastermix (Kapa Biosystems, Woburn, MA, USA) and 2.5 pmol of the primer DG47f and RW01r [35]. The cycling program was adjusted to an initial denaturation at 95°C for 10 min, followed by 40 cycles of 92°C for 30 s, 52°C for 30 s, and 72°C for 60 s. All reactions were prepared in duplicates and analyzed in two independently repeated runs. Calculated gene counts were normalized to 1 ng μl^{-1} based on the DNA concentration determined with a Nanodrop system 2000c (Thermo Scientific, Wilmington, MA, USA). Spectra which did not reach the required quality were automatically removed by the ROTOR 6000 software. Statistics were conducted using SPSS v20 PASW Statistics 18 (SPSS Inc., Chicago, IL, USA).

Fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM)

FISH/CLSM was applied in two distinct approaches to both *R. solani* diseased (bottom rot) lettuce (rhizosphere and phyllosphere) and commercially available lettuce (*L. sativa* var. *capitata*) inoculated with *Escherichia coli* K12; 1.6×10^9 cells ml^{-1} on 20 mm seized plant foliage discs. Root and leaf samples from both approaches were fixed with 4% paraformaldehyde (PFA) for 6 h, washed 3 times with ice-cold PBS and then stored at -20°C in 1:1 (v/v) 96% Ethanol:PBS.

FISH was performed using class specific probes following the protocol by Cardinale et al. [36]. EUB338MIX (Cy3-labeled) was used for staining overall bacterial communities. *Gammaproteobacteria* were probed with GAM42a (Cy5-labeled), *Alphaproteobacteria* with ALF968 (Cy5-labeled), and *Betaproteobacteria* with probe BET42a (ATTO488-labeled) [36,37]. All FISH probes were purchased from genXpress GmbH (Wiener Neudorf, Austria). Briefly, after pre-treatment with lysozyme, the labeled FISH probes were applied to confirm the presence and localization of the taxa detected by pyrosequencing. The FISH stained samples were further mounted with SlowFade Gold Antifadent (Molecular Probes, Eugene, OR, USA) and stored at 4°C over night until observation with a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with solid state and UV lasers. For each field of view, an appropriate number of optical slices were acquired with a Z-step of 0.15–0.5 µm ("confocal stacks") and the software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used for post-processing, 3D rendering and creation of isosurface-spot models.

Results

Enterobacteriaceae on lettuce

Altogether, 454 pyrosequencing of 16S rRNA gene amplicons revealed 14 distinct enterobacterial genera in the rhizosphere and phyllosphere of pot- and mesocosm-grown lettuce plants (Fig. 1). The enterobacterial microbiome contained taxa belonging to *Escherichia/Shigella*,

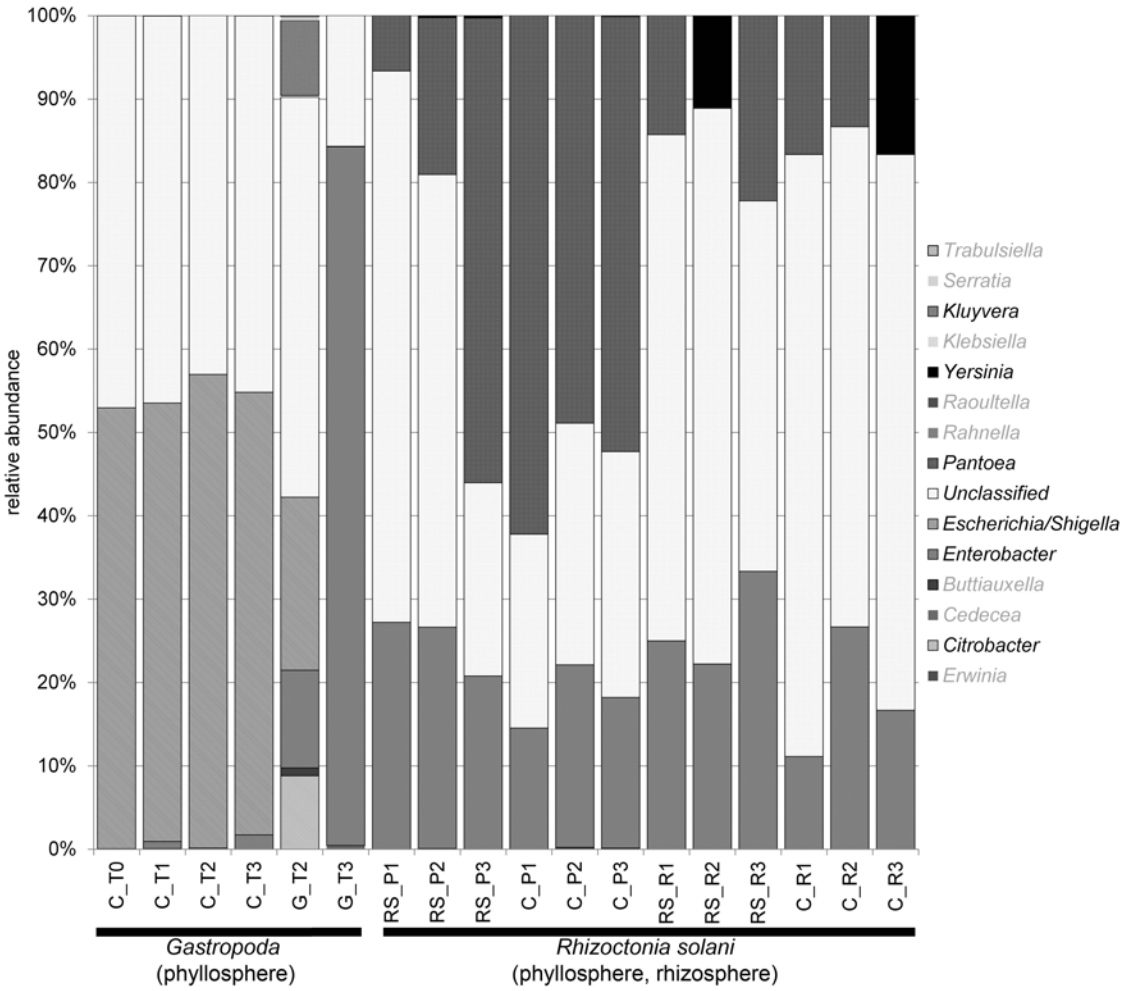

Fig 1. Taxonomic classification and relative abundance of *Enterobacteriaceae* associated with *L. sativa* var. *capitata* of phyllo- and rhizosphere samples. Genera below 1% abundance in any sample were shaded in grey (taxon legend). (Treatments; C = Control/untreated, G = *Gastropoda*, RS = *R. solani*; time of samplings T0–T3; P1–3 phyllosphere (replicates), R1–3 rhizosphere (replicates))

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Pantoea, *Enterobacter* and *Enterobacteriaceae* not classified at the genus level. Although these were identified as the most abundant taxa across the whole dataset, *Escherichia*/*Shigella* was only allocated to the mesocosm samples and was absent in the pot-grown samples. In contrast, we found *Pantoea* only in samples associated to the pot-grown plants from the first experiment. *Enterobacter* was the only identified taxa found in every sample and represents a core taxon. A comparison between the rhizosphere and the phyllosphere revealed that there were no significant structural differences, however the abundance of *Enterobacteriaceae* was statistically significantly higher ($p<0.05$) in a 3:1 ratio on phyllosphere samples determined with quantitative PCR. Interestingly, the occurrence of *Yersinia* was related to an absence of *Pantoea* in the rhizosphere.

Impact of the fungal pathogen *Rhizoctonia solani* on the lettuce microbiome

R. solani inoculated lettuce plants (RS_P) with symptoms of bottom rot showed significant drifts in their associated microbial communities, and most notably a statistically significant

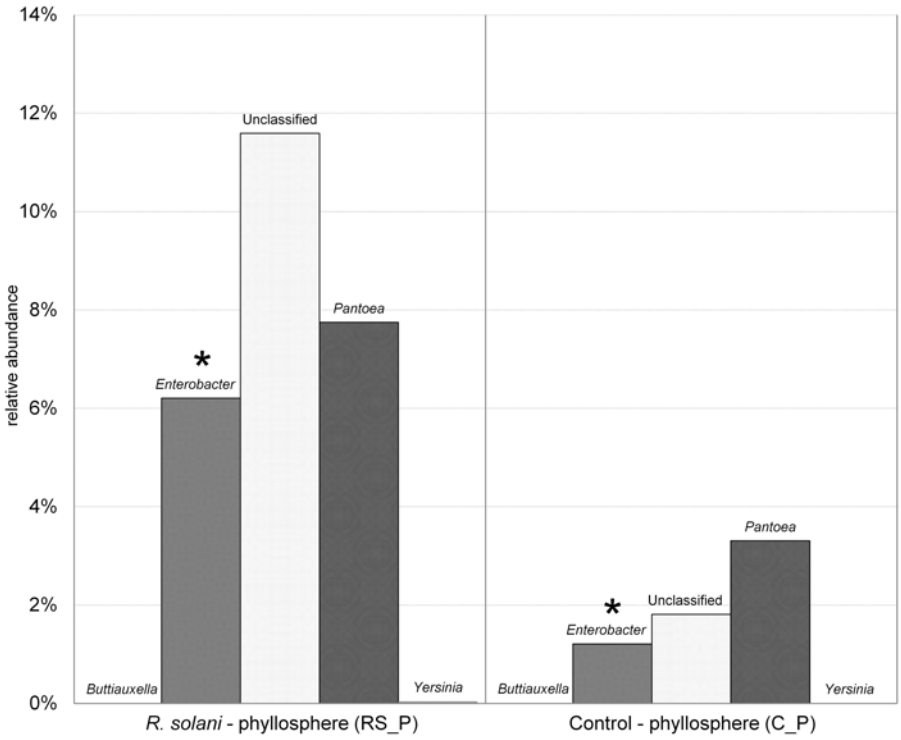

Fig 2. Amplicon sequencing of *Gammaproteobacteria* revealed the relative abundance of *Enterobacteriaceae* associated with *L. sativa* var. *capitata* phyllosphere samples of the healthy control (C_P) and plants showing symptoms of bottom rot caused by *R. solani* (RS_P). Asterisks: significant differences ($p < 0.05$) between C_P and RS_P.

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increase from about 1% to 6% in the genus *Enterobacter* in the phyllosphere (Fig. 2). Interestingly, the structure was not significantly altered, however the genus *Enterobacter* was only found in low abundances in healthy plants (C_P). It increased significantly, likely in correlation with the spread of the pathogen on the host. A similar but less significant effect was found for *Pantoea* and other *Enterobacteriaceae* not classified at genus level (Fig. 2).

Impact of *Gastropoda* on the lettuce microbiome

The stability of the lettuce-associated gammaproteobacterial (SSCP) and *Enterobacteriaceae* (454 pyrosequencing) community in response to biological disturbances from *Gastropoda* was followed using a mesocosm approach at four sampling times (C = Control, G = *Gastropoda*): C_T1 = start point (initial microbiota), C_T2 = adapted microbiota C/G_T3 = after five day lasting exposure to gastropods and C/G_T4 = 42 days after the exposure. Microbial fingerprints (S1 Fig.) based on separation by single strand conformation polymorphism using *Gammaproteobacteria* specific and universal primers revealed a highly stable community structure in the rhizosphere with either no, or only minor effects caused by *Gastropoda* (S1 Fig.). This was observed not only within a set of replicates at a certain time point, but also between the artificial mesocosms and between the respective samplings for both investigated taxonomic levels. However, significant structural differences and changes in the abundance levels could be found for the phyllosphere. After introduction of *Gastropoda*, new unique bands, some highly dominant, were found (G_T2). While some bands appeared transient, others were found in the same or even higher dominance 43 days after removing the *Gastropoda* from the mesocosms.

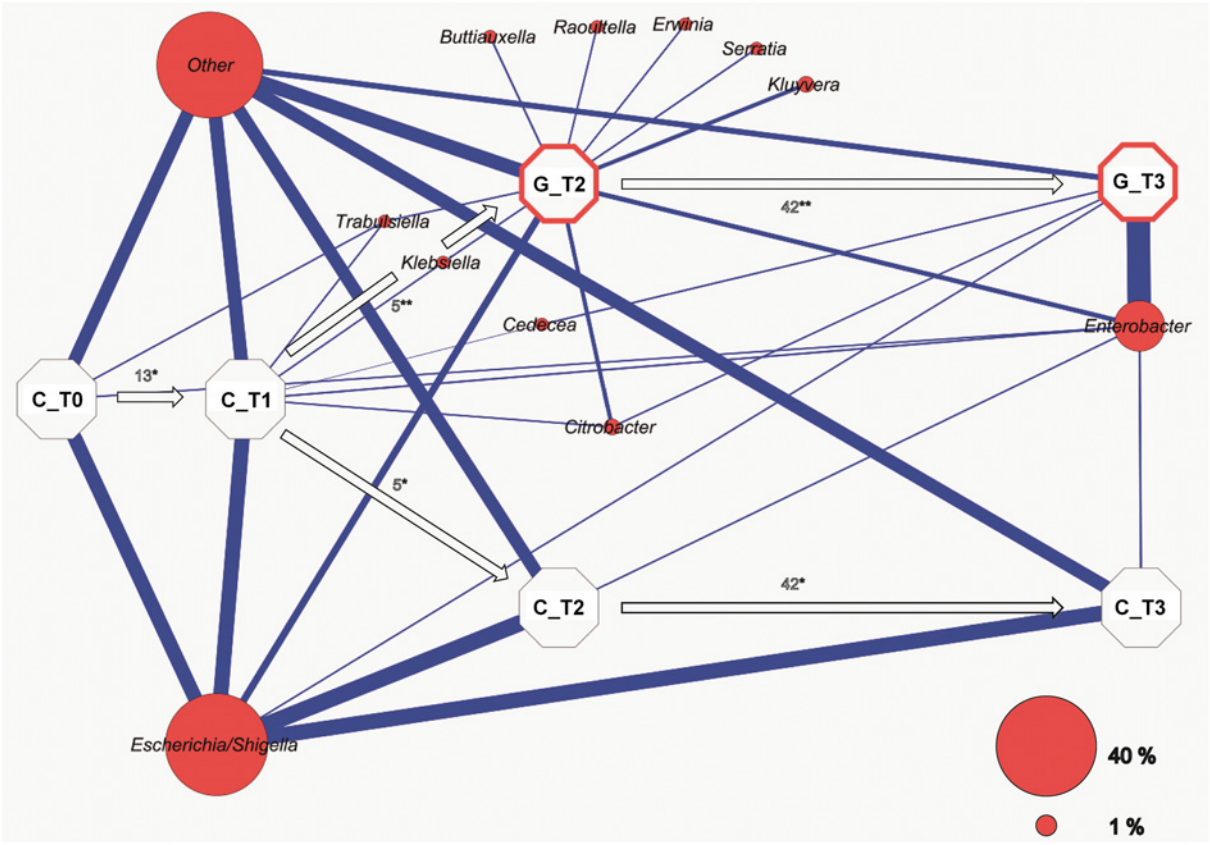

Fig 3. Network analysis showing the experimental process over time based on enterobacterial amplicons as resulted from 454 pyrosequencing. Each octagon represents a particular juncture (Treatment; C = Control/untreated, G = *Gastropoda*, time of sampling; T0-T3) with associated weighted average taxa (red). Edges (blue connection lines) indicate occurrence and abundance of each OTU at the connected time point. Numbers above the arrows indicate the elapsed time between the particular samplings (days). * = Steady state mesocosm ** = Mesocosm with/past snails and slugs contamination.

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454 pyrosequencing of 16S rRNA gene amplicons allowed deeper insight into the indigenous microbiome at OTU based taxonomical levels. As shown in the correlation network (Fig. 3), the structure of enterics remained steady throughout the experiment at the unaffected control mesocosm. Untreated samples were dominated by *Escherichia*/*Shigella* with a relative average abundance of 52% throughout the time. The introduction of gastropods radically decreased the abundance of *Escherichia*, while *Enterobacter* increased and accumulated to a relative abundance of 84% against the total taxonomic affiliations. However, new genera (*Kluyvera*, *Erwinia*, *Serratia*, *Buttiauxella* and *Raoultella*) were also introduced, but could not be permanently established in the phyllosphere. The network once again clearly demonstrates the impact of slugs and snails primarily on the genus *Enterobacter*.

Quantitative PCR supported the results of 16S rRNA amplicon sequencing of gene fragments. Significantly higher levels of enterobacteria were found when a comparison of the samples from the *R. solani* pot experiments (RS_P, C_P) with the samples of the *Gastropoda* mesocosms (C_T2 and T3, G_T2 and T3) was made (Fig. 4). Higher abundances of *Enterobacteriaceae* were found on lettuce samples showing bottom rot disease. Similar observations could be made analyzing the mesocosm experiments. Plants grown in the mesocosm after the biotic disturbance showed also an increase of enterobacterial taxa when compared to the control. The enterobacterial abundance of the root was 1.50×10^5 gene copies ng^{-1} compared to 3.77×10^5 gene copies ng^{-1} community DNA retrieved of the foliage.

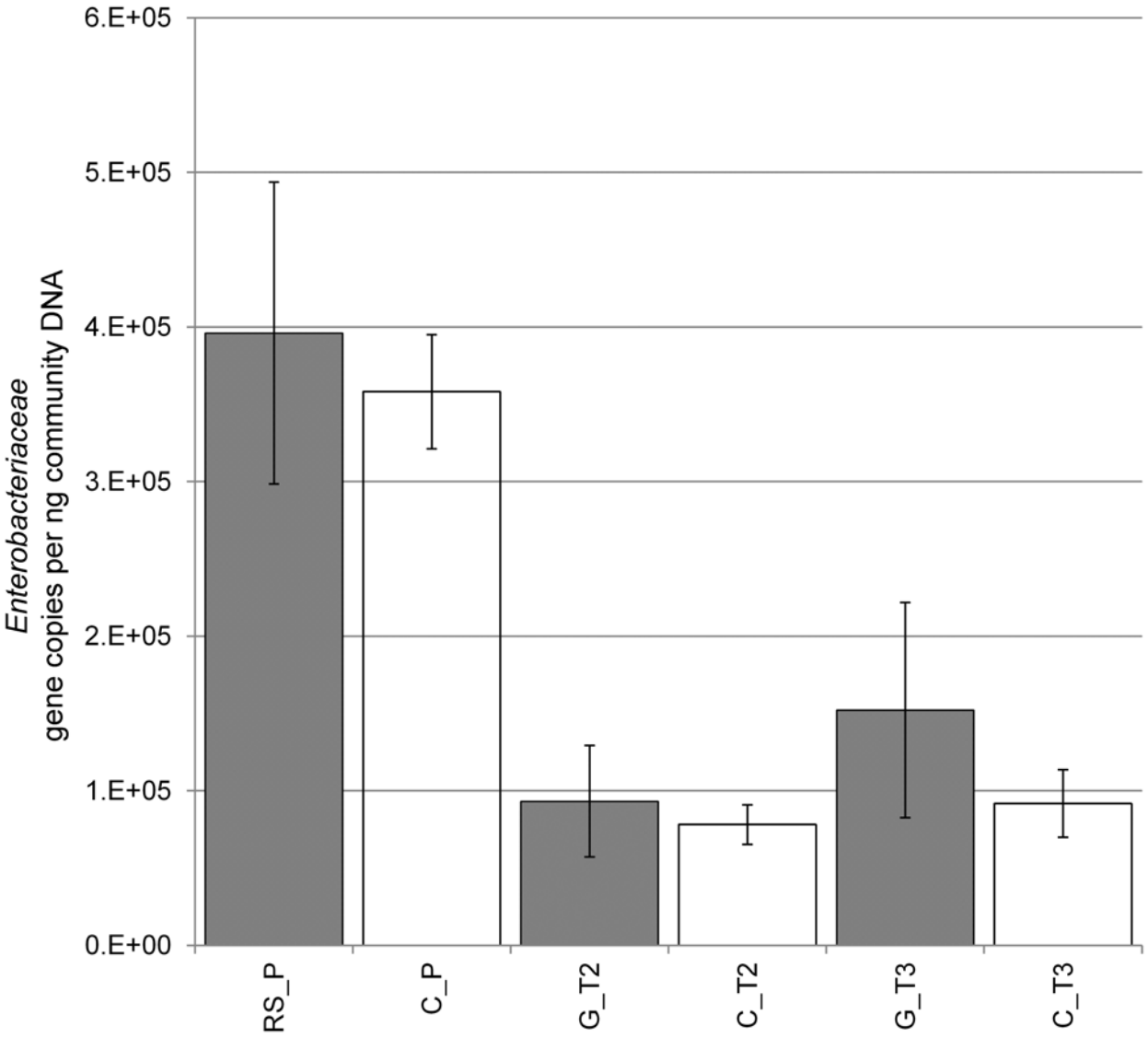

Fig 4. Abundance of *Enterobacteriaceae* per ng community DNA isolated from 5 g of the respective samples of the foliage. Error bars represent the confidence interval under $p < 0.05$. (Confidence error of the mean based on RS_P = n 18, C_P = n 19, G_T2 = n 7, C_T2 = n 7, G_T3 = n 8, C_T3 = n 8). (Treatments; C = Control/untreated, G = *Gastropoda*, RS = *R. solani*; time of samplings T2–T3)

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Lettuce leaves as niches for bacteria visualized by microscopy

All samples from the first experiment were microscopically analyzed to validate our sequencing results and to reveal colonization patterns on lettuce. In all experiments, the lettuce roots were found densely colonized by bacterial colonies which were taxonomically differentiated using specific FISH probes (Fig. 5). The root surface was completely colonized by bacteria. *Gamma-* and *Betaproteobacteria* formed dominant colonies on the lateral roots of young lettuce plantlets, and after disturbances the increase of *Enterobactericeae* could be confirmed microscopically. However, it was difficult to visualize the phyllosphere bacteria on the leaf surface as only a few colonies could be detected, usually concentrated in the surrounding of stomata (Fig. 5B). This observation was directly in contrast to the diversity shown at molecular level. To find the reason for these contradicting results, we analyzed leaves, which were treated with

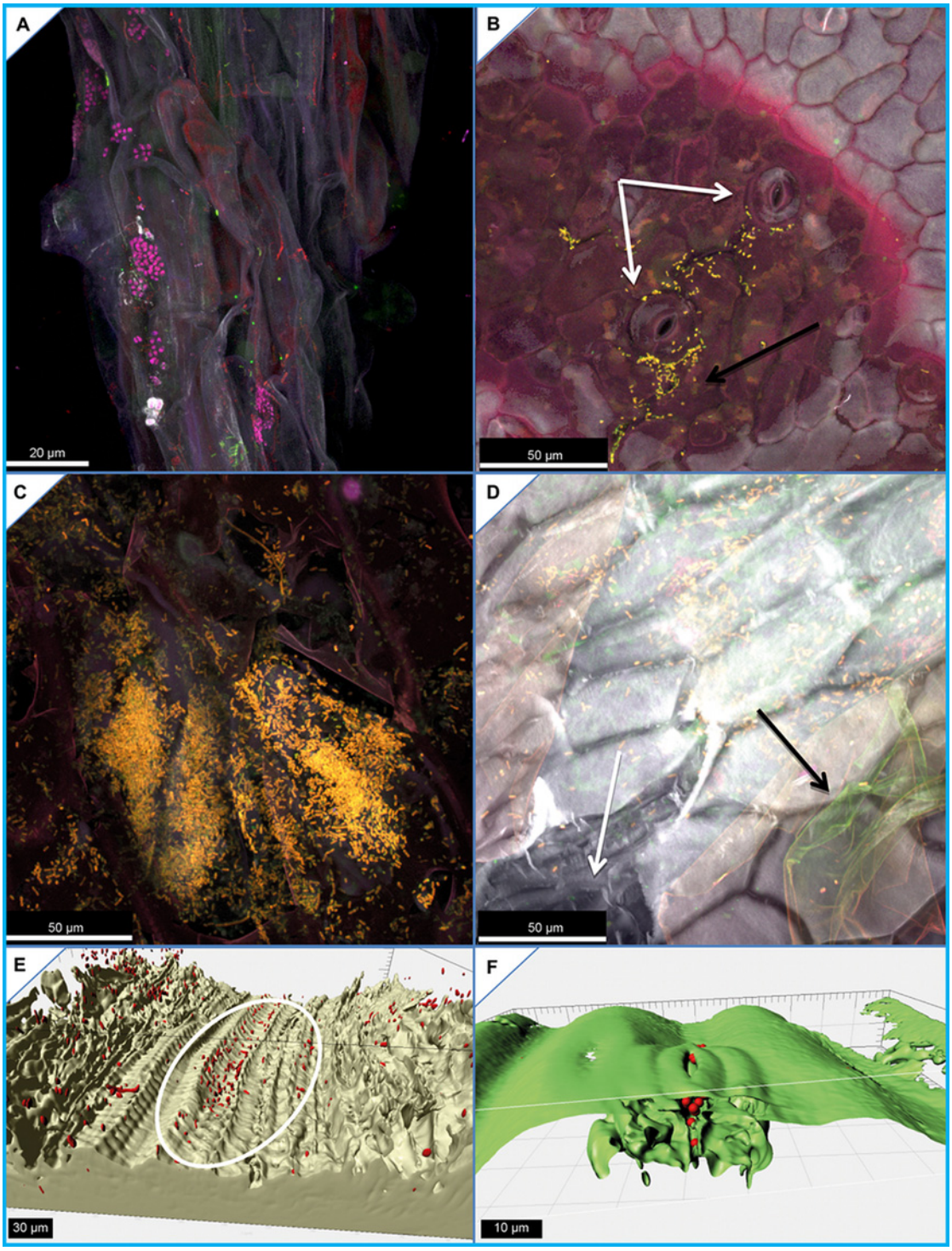

Fig 5. Fluorescence *in situ* hybridization coupled with confocal laser scanning microscopy images. A) *Gammaproteobacteria* (yellow) and *Betaproteobacteria* (pink) form large colonies on the lateral roots of young lettuce plantlets, where they do not share their space, moreover it seems they exclude each other. B) We revealed increased gammaproteobacterial (yellow) colonization close to stomata which could be also detected on *R. solani* infected lettuce. C) Punctiform extensive colonization behavior of *Escherichia coli* K12 (yellow) on inoculated lettuce patches within the inner compartments of the leafy green. D) Physical damage to the leaves affects the epicuticular wax-layer (black arrow) and the cuticle cells (white arrow), and supports endophytic colonization of enterobacteria (yellow). E) Transport of bacteria (red) within a vascular bundle (white marker) and F) trough stoma.

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bacterial cell suspensions of *E. coli* K12. Surprisingly, we observed large colonies and substantial punctiform colonization in endophytic compartments (Fig. 5C). In the inoculation experiment, we could not identify epiphytic bacterial colonization; instead micrographs showed that bacteria colonize the inner leaf compartments through i) bruises or physical damage (Fig. 5D), ii) the endovascular systems (Fig. 5E), and iii) through stomata (Fig. 5F).

Discussion

Our study identified the structure of *Enterobacteriaceae* on and in lettuce leaves and roots and described an impact of biotic stress on their diversity and abundance. The latter result confirmed our hypotheses that biotic disturbances induce shifts in the *Enterobacteriaceae* plant-associated community, and that each pathogen cause significant changes within the bacterial community.

All investigated lettuce plants harbor phyllo- as well as rhizosphere bacteria, which belong to the *Enterobacteriaceae* family. The spectrum was similar to the data published for the lettuce phyllosphere [2,4,5]. Quantitative PCR revealed a higher abundance in the phyllosphere than in the rhizosphere in general as well as an enhancement in response to biotic disturbances. An abundance scatter plot presenting the distribution of the samples revealed that there is not only an increase of abundance of enterobacteria, but also an elevated inhomogeneity across the samples in a disturbed sample pool. Interestingly, samples derived from the pot-grown, healthy lettuce, but also plants from the control mescosm showed much smaller scattering within the comparable samples.

We were able to identify similar *Enterobacteriaceae* species in the rhizosphere and phyllosphere of same lettuce plants and found structural differences between plants grown in mesocosm and in the pot experiments. In general, due to different abiotic conditions in the upper and below-ground parts of plants, these microhabitats are colonized by specific microbial communities and shared only a few ubiquitous species [10,38]. However, the phyllosphere of lettuce is also influenced by the soil microbiome [6].

First, it was difficult to visualize phyllosphere bacterial communities in our microscopic study. Only a few bacterial colonies could be seen on the leaf surface, while the root surface was densely colonized. We assumed that hydrophobicity and/or antimicrobial substances of epicuticular waxes prevent excessive bacterial growth. The leaf surface of a plant, especially its chemical components, constitutes the first line of resistance against herbivores and other pests and pathogens, and also acts as a shield against UV rays. The impact of plant cuticular wax composition was already shown to affect the community composition of phyllosphere bacteria in *Arabidopsis* mutants [39]. After a microscopic survey investigating several respective samples, we found that the bacteria are not equally distributed; moreover they tend to colonize micro-niches on the leaves. To evaluate our results, we inoculated lettuce leaves with a bacterial suspension of *E. coli* K12 that confirmed the native colonization patterns and showed that the inner tissues below the waxes were densely colonized. Our finding is congruent with the observation of Franz et al. [40], who observed the presence of the pathogens in lettuce after thorough surface sterilization. They demonstrated the presence of human pathogens on lettuce, which were unlikely to be removed by the actions of consumer washing and therefore pose a serious threat when occurring in agriculture or home gardening situations.

The biotic stresses studied in this study induced significant shifts in the bacterial community and increased species richness, although they caused only slight (*Rhizoctonia*) or no symptoms (*Gastropoda*) on the lettuce plants. According to the intermediate disturbance hypothesis described in macroecology [41], diversity should be maximized at intermediate levels of disturbance because both competitive K-selected and opportunistic r-selected species can coexist.

This hypothesis has been a matter of controversy in macroecology [42], and cannot be applied in general to microbial communities. It is notable that enterobacteria, which were found in higher abundances after disturbance, belong primarily to r-selected species and are well-known for their opportunistic and fast-growing character. If we consider the impact of fungi and gastropods as intermediate disturbance, then we would see support for an intermediate disturbance hypothesis by the general increase in species richness.

The shift within the *Enterobacteriaceae* family can be of importance for human health due to the connection of plant associated biodiversity and the human immune system. Recently, Hanski et al. [43] showed a correlation between bacterial diversity and atopy, most likely from significant interactions with *Gammaproteobacteria*. Endotoxin derived from Gram-negative bacteria, such as *Enterobacteriaceae*, is known to have allergy-protective and immunomodulatory potential [44]. Therefore, the natural occurrence and enhancement of enterics by biotic stress may have an impact on our immune system and health, which requires further investigation. Moreover, we have shown that enterics tend to invade the plant tissue, although the intact epicuticular wax layer seems to protect the leafy green portion from microbial colonization. We also argue that the high abundance and frequent appearance of opportunistic pathogens is contradictory to the relatively low number and frequency of serious outbreaks. With this knowledge, we suggest the enrichment of enterobacteria might have commensal or even immunomodulatory effects and could act as a "natural vaccine". In particular we hypothesize that the consumption of fresh food such as lettuce may increase general human well-being, and possibly enrich the commensally gastrointestinal microbiota to recurrently stimulate the immune system. The potential priming effect could be an important additional attribute of lettuce as healthy raw food, and in general with regards to nutritional regimens based on consumption of raw plant parts.

Supporting Information

S1 Fig. Band based analysis using dice algorithm of microbial fingerprints showing the structure of all bacteria and *Gammaproteobacteria* of *L. sativa* from the mesocosm experiment.
(TIF)

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Author Contributions

Conceived and designed the experiments: AE GB MG MC. Performed the experiments: AE. Analyzed the data: AE GB. Contributed reagents/materials/analysis tools: MC. Wrote the paper: AE GB MG.

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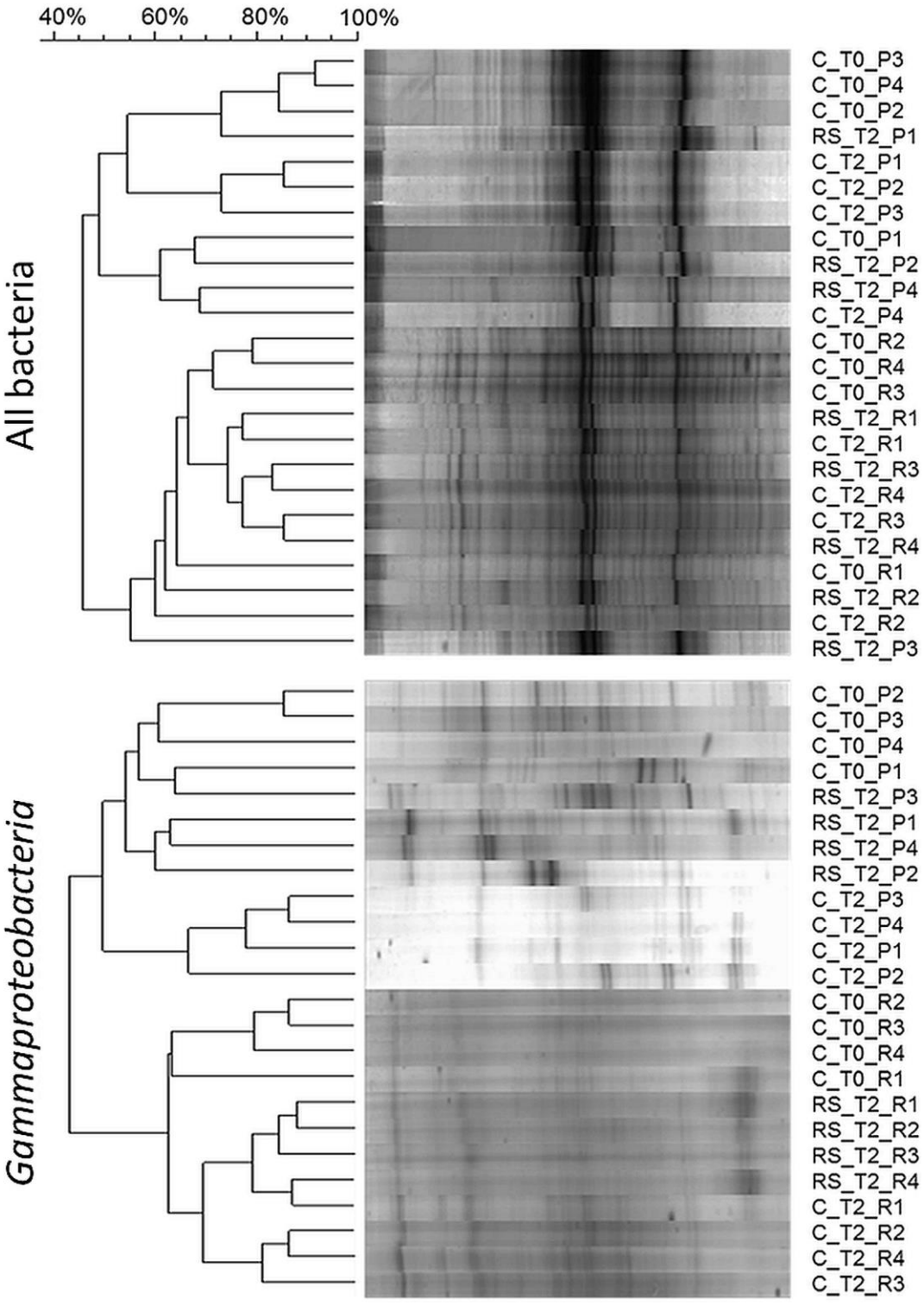
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All bacteria
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C_T0_P1
RS_T2_P2
RS_T2_P4
C_T2_P4
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C_T0_R4
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RS_T2_R1
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RS_T2_P3
Gammaproteobacteria
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Figure S1

Publication II

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The impact of the pathogen *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous lettuce microbiome.

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The impact of the pathogen *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous lettuce microbiome

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Lettuce belongs to the most commonly raw eaten food worldwide and its microbiome plays an important role for both human and plant health. Yet, little is known about the impact of potentially occurring pathogens and beneficial inoculants of the indigenous microorganisms associated with lettuce. To address this question we studied the impact of the phytopathogenic fungus *Rhizoctonia solani* and the biological control agent *Bacillus amyloliquefaciens* FZB42 on the indigenous rhizosphere and phyllosphere community of greenhouse-grown lettuce at two plant stages. The rhizosphere and phyllosphere gammaproteobacterial microbiomes of lettuce plants showed clear differences in their overall and core microbiome composition as well as in corresponding diversity indices. The rhizosphere was dominated by Xanthomonadaceae (48%) and Pseudomonadaceae (37%) with *Rhodanobacter, Pseudoxanthomonas, Dokdonella, Luteimonas, Steroidobacter, Thermomonas* as core inhabitants, while the dominating taxa associated to phyllosphere were Pseudomonadaceae (54%), Moraxellaceae (16%) and Enterobacteriaceae (25%) with *Alkanindiges*, *Pantoea* and a group of Enterobacteriaceae unclassified at genus level. The preferential occurrence of enterics in the phyllosphere was the most significant difference between both habitats. Additional enhancement of enterics on the phyllosphere was observed in bottom rot diseased lettuce plants, while *Acinetobacter* and *Alkanindiges* were identified as indicators of healthy plants. Interestingly, the microbial diversity was enhanced by treatment with both the pathogen, and the co-inoculated biological control agent. The highest impact and bacterial diversity was found by *Rhizoctonia* inoculation, but FZB42 lowered the impact of *Rhizoctonia* on the microbiome. This study shows that the indigenous microbiome shifts as a consequence to pathogen attack but FZB42 can compensate these effects, which supports their role as biocontrol agent and suggests a novel mode of action.

Keywords: lettuce microbiome, Gammaproteobacteria, soil-borne pathogens, 16S rRNA gene pyrosequencing, phyllosphere, rhizosphere, Lactuca sativa

INTRODUCTION

Plants host a broad range of ubiquitous but also highly adapted and specific bacterial communities that colonize their epi- and endophytic compartments (Berg and Smalla, 2009; Berendsen et al., 2012; Bulgarelli et al., 2012). Due to their complexity, specific morphology, and production of secondary metabolites, the structure and function of plant-associated microbial communities are specific in these habitats but also at plant species and cultivar levels (Smalla et al., 2001; Berg et al., 2002; Berg and Smalla, 2009; Raaijmakers et al., 2009). The rhizosphere has already been investigated as a microbial habitat for more than one century (Hartmann et al., 2008), while the phyllosphere microbiome is only partly understood. Recent work suggests that the long-term colonization of phyllosphere is preferred by specific bacteria, while short-time colonization comprises many ubiquitous bacteria (Vorholt, 2012). All plant-associated habitats contain a high proportion of plant-beneficial microorganisms such as antagonists, diazotrophs, and plant growth promoting bacteria (PGPB) but also plant pathogens as well as potential human pathogens (Berg et al., 2005; Mendes et al., 2013). While the modes of action are often understood for single beneficial as well as pathogenic strains and species, less is known about the microbial community impact of single strains. Risk assessment and colonization studies for specific biocontrol agents showed minor and only transient effects on the rhizosphere community (Scherwinski et al., 2007; Adesina et al., 2009; Chowdhury et al., 2013; Schmidt et al., 2014), while impacts of pathogens on the indigenous microbiome are severely underexplored.

Soil-borne plant pathogens cause crucial damage to crops. The phytopathogenic fungus *Rhizoctonia solani* Kühn [teleomorph: *Thanatephorus cucumeris* (A.B. Frank) Donk; basidiomycetes] is

subdivided into anastomosis groups (AGs) according to their hyphal anastomosis reactions (Carling et al., 2002). The fungus causes a wide range of commercially significant plant diseases, such as Brown patch, damping off in seedlings, root rot and belly rot. *R. solani* strains are characterized by a distinct degree of host specificity as well as by different virulence levels to their plant host. *Rhizoctonia* strains occur almost ubiquitously in soils but isolates AG1-IB (Germany), AG2-1 (UK, the Netherlands) and AG4 (the Netherlands, UK, USA) have been isolated from diseased lettuce plants (Grosch et al., 2004). Strains belonging to AG1-IB were responsible for up to 70% yield loss of field-grown lettuce (Davis et al., 1997; Wolf and Verreet, 1999). One strain 7/3/14 of the supposed diploid and heterokaryotic *R. solani* AG1-IB, which was already sequenced, shows a large genome with many unique and unknown features in comparison with other *Rhizoctonia* strains and phylogenetically related fungi (Wibberg et al., 2013). Due to the low degree of host specificity, *Rhizoctonia* strains of different AGs can lower the general fitness of the plant during colonization, which results in higher sensitivity for additional pathogens such as spoilage enterobacteria (Berg et al., 2005). Interestingly, Adesina et al. (2009) could demonstrate direct changes caused by *R. solani* to the fungal and bacterial community patterns using molecular fingerprinting. All *Rhizoctonia* diseases, and subsequent secondary infections, in plants are difficult to control. In the past, only methyl bromide (MeBr) was effectively used. However, this fumigant has been banned for its ozone-depleting and toxic effects (UNEP, 1999). Alternative and environmentally friendly methods to suppress *Rhizoctonia* comprise naturally occurring antagonists such as *Serratia plymuthica* and *Pseudomonas jessenii* (Faltin et al., 2004; Grosch et al., 2005; Scherwinski et al., 2007; Adesina et al., 2009). In addition, *Bacillus amyloliquefaciens* FZB42, a long-time established plant strengthener was successfully applied to suppress *R. solani* on lettuce (Chowdhury et al., 2013). Genome sequencing of FZB42 revealed a high capacity of metabolite production with antimicrobial and antifungal activity, which suggested direct antifungal effects (Chowdhury et al., 2013). However, some reports suggest additional impacts of individual strains on the microbial community (Scherwinski et al., 2007; Schmidt et al., 2012). We therefore hypothesize that both beneficials as well as pathogens can cause significant shifts in the plant-associated microbiome.

The aim of this study was to identify the impact of the phytopathogenic fungus *R. solani* and the biological control agent *B. amyloliquefaciens* FZB42 on the indigenous rhizosphere and phyllosphere community of lettuce, cultivated under controlled conditions in a growth chamber. In our study we focus on the human health relevant group of Gammaproteobacteria, which was studied by analyzing specific amplicon libraries together with corresponding bioinformatic and statistical analysis. Gammaproteobacteria belong to the plant microbiome in general (Brandl, 2006), and are especially a substantial fraction of the lettuce-associated microbiome (Rastogi et al., 2012, 2013). However, they also comprise several species which were frequently identified to cause severe foodborne outbreaks (Teplitski et al., 2011).

MATERIAL AND METHODS

INOCULANTS USED IN THIS STUDY

The effect of *R. solani* and *B. amyloliquefaciens* FZB42 on lettuce growth and health was evaluated in this study. All experiments were performed with the product Rhizovital® 42 liquid (ABiTEP GmbH, Berlin, Germany), which is based on vital spores of FZB42 (Chowdhury et al., 2013). The bottom rot pathogen *R. solani* AG1-IB (isolate 7/3) was obtained from the strain collection of the Leibniz Institute of Vegetable and Ornamental Crops (Großbeeren, Germany) (Grosch et al., 2004).

EXPERIMENTAL DESIGN OF POT EXPERIMENTS

The effect of FZB42 and the pathogen *R. solani* on the microbial community of lettuce was studied by 454-amplicon sequencing analysis. Seeds (cv. Tizian, Syngenta, Bad Salzuflen, Germany) were germinated at 18°C in a seedling tray (92 holes) filled with a non-sterile mixture of quartz sand and substrate [Fruhstorfer Einheitserde Typ P, Vechta, Germany; chemical analysis (mg per l): N = 120, P = 120, K = 170, Mg = 120, S = 100, KCl = 1, organic substance = 167, peat = 309; pH 5.9] at a 1:1 ratio (v/v). The seedlings were further cultivated at 20/15°C until planting in a growth chamber (York, Mannheim, Germany; 16 h/8 h day/night cycle, 500 µmol m^{-2} s^{-1}, 60/80% relative humidity). Lettuce was planted at two-leaf stage into pots (500 ml) filled with the same substrate sand mixture as mentioned above inoculated and non-inoculated with the pathogen *R. solani* AG1-IB and grown at 22/15°C for 4 weeks. In the treatments with pathogen inoculation, the substrate mixture was inoculated with 10 *R. solani*-infested barley kernels and incubated at 25°C for 1 week until planting of lettuce into the pots.

For application of the inoculant FZB42 each lettuce plant was drenched with 20 ml spore solution (10^7 spores ml^{-1}) 3 days before and at planting time respectively. The pots were watered lightly each day to maintain the substrate moisture, and fertilized weekly (0.2% Wuxal TOP N, Wilhelm Haug GmbH & Co. KG, Düsseldorf, Germany). All pot experiments were done at the Leibniz Institute of Vegetable and Ornamental Crops.

An overview about the sampling design is presented in **Table 1**. Here, abbreviations for compartments and treatments used throughout the manuscript were explained: P, phyllosphere; R, rhizosphere; Y, young; M, mature; RS, *R. solani*; C, untreated (control); FZB42RS, FZB42 and *R. solani* co-inoculation; G, healthy; K, diseased.

SAMPLE COLLECTION AND DNA ISOLATION

Sampling was carried out 2 weeks after planting (young plants) for treatments with and without FZB42 application, followed by a second sampling 4 weeks after planting (mature plants) for control and co-inoculated treatments with FZB42 and *R. solani*. The total community DNA was extracted per treatment, and habitat from two young plants and three mature plants (two independent DNA extractions were performed for each plant and the DNA was pooled prior to PCR), according to Bragina et al. (2011). Briefly, 5 g of plant material were physically disrupted with sterile pestle and mortar and resuspended in 10 ml of 0.85% NaCl. Two ml of suspension were centrifuged (16,500 × *g*, 20 min, 4°C) and the obtained pellets were used for isolation of the total-community

Table 1 | Sample design description.

ID	Habitat	Age (weeks after planting)	Treatment	Disease condition	Plant replicates	PCR replicates[b]	Sequencing
PYC	Phyllosphere	Young (2)	Untreated (Control)	not determinable	2	2	MWG, Eurofins
PYRS	Phyllosphere	Young (2)	*R. solani* inoculated	not determinable	2	2	MWG, Eurofins
PYfzb42RS	Phyllosphere	Young (2)	*R. solani* and FZB42 inoculated	not determinable	2	2	MWG, Eurofins
PMG	Phyllosphere	Mature (4)	Untreated (Control)	healthy	3 (6[a])	2	MWG, Eurofins
PMK	Phyllosphere	Mature (4)	*R. solani* and FZB42 inoculated	bottom rot	3 (6[a])	2	MWG, Eurofins
RYC	Rhizosphere	Young (2)	Untreated (Control)	not determinable	2	2	Macrogen, Korea
RYRS	Rhizosphere	Young (2)	*R. solani* inoculated	not determinable	2	2	Macrogen, Korea
RYfzb42RS	Rhizosphere	Young (2)	*R. solani* and FZB42 inoculated	not determinable	2	2	Macrogen, Korea
RMG	Rhizosphere	Mature (4)	Untreated (Control)	healthy	3 (6[a])	2	Macrogen, Korea
RMK	Rhizosphere	Mature (4)	*R. solani* and FZB42 inoculated	bottom rot	3 (6[a])	2	Macrogen, Korea

[a] *Two Independent DNA extractions per plant were pooled prior to PCR.*

[b] *PCR was carried out twice for each DNA sample and each PCR step and pooled prior to sequencing.*

DNA with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). For mechanical lysis, the cells were homogenized twice in a FastPrep® FP120 Instrument (Qbiogene, BIO101, Carlsbad, CA, USA) for 30 s at a speed of 5.0 m s^{-1} and treated according to the manufacturer's protocol.

BARCODED DEEP 454-PYROSEQUENING OF 16S rRNA GENE AMPLICON

The 16S rRNA genes of 24 samples (details are provided in **Table 1**) were amplified (two technical replicates for each sample) in a nested PCR approach with the Gammaproteobacteria primer set 395f (5′-CMA TGC CGC GTG TGT GAA-3′) and 871r (5′-ACT CCC CAG GCG GTC DAC TTA-3′) (Mühling et al., 2008). The PCR reaction mixture (20 µl) contained 5 × Taq-&GO Ready-to-use PCR Mix (MP Biomedicals, Germany), 0.25 µM of each primer, 25 mM $MgCl_2$ and 1 µl of template DNA (96°C, 4 min; 32 cycles of 96°C, 1 min; 57°C, 1 min; 74°C, 1 min; and final elongation at 74°C, 10 min). In a second PCR, 1 µl of the amplicon (1:10 diluted phyllosphere and 1:100 diluted rhizosphere derived PCR products) was used. 16S rRNA gene sequences were amplified by using the forward primer Unibac-II-515f (5′-GTG CCA GCA GCC GC-3′) containing the 454-pyrosequencing adaptors and the reverse primer Gamma871r_454 (5′-CTA TGC GCC TTG CCA GCC CGC TCA GAC TCC CCA GGC GGT CDA CTT A-3′). The reaction mixture for the second PCR (30 µl) contained 5 × Taq-&GO Ready-to-use PCR Mix, 0.25 µM of each primer (96°C, 4 min; 32 cycles of 96°C, 1 min; 66°C, 1 min; 74°C, 1 min; and final elongation at 74°C, 10 min). PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). The technical replicates per sample were pooled and the partial 16S rRNA gene fragments were sequenced using 454 Roche GS FLX (MWG Eurofins, Germany) and 454 Roche GS FLX Titanium (Macrogen Korea, South Korea) pyrosequencer. The nucleotide sequences obtained in this work were submitted to the European Nucleotide Archive (www.ebi.ac.uk/ena) and are available under the accession number PRJEB6022.

DNA SEQUENCE ANALYSIS AND TAXONOMICAL IDENTIFICATION

Sequences were analyzed with the Qiime software version 6.0 (Caporaso et al., 2010). Replicates from sequencing of each treatment and habitat were bioinformatically pooled during the Qiime analysis for data evaluation. MID-, primer and adapter sequences were removed, length filtered (≥350 nt), quality filtered (score: 50), denoised, chloroplast removed and singletons adjusted. The cut-off level was set to 97% sequence identity. Chimeras were detected with Chimera Slayer and then removed. To compute alpha and beta diversity, the dataset was normalized to 5920 reads per sample. Ring-charts were created using the Krona software package version 2.2 (Ondov et al., 2011) and the profile network was constructed using Cytoscape version 3.0.2 (Shannon et al., 2003). Statistical tests based on the operational taxonomic units (OTUs) table were performed with the nonparametric ANOVA Kruskal Wallis test. This test is functionally an expansion of ANOVA to cases where the sample means are unequal and the distribution is not normal.

RESULTS

THE GAMMAPROTEOBACTERIAL MICROBIOME OF LETTUCE

Two sequential batches of 16S rRNA gene amplicon sequencing resulted in a total of 242,022 reads. After removing chimeras, singletons, and chloroplast sequences, 8233 quality mean reads per sample remained with a median absolute deviation of 1842.5 sequence reads. We analyzed the gammaproteobacterial fraction subjected to different treatments, separately for the phyllosphere and rhizosphere (Figure S1). Twenty-four samples (12 from per habitat) yielded in a total of 4.909 distinct OTUs, 1.102 were statistically different (Nonparametric ANOVA Kruskal Wallis Test, $p \leq 0.05$) between both habitats. The gammaproteobacterial microbiome from whole lettuce plants contained mainly taxa from Pseudomonadales, followed by Xanthomonadales, Enterobacteriales and Legionellales (**Figure 1**). The rhizosphere was dominated by Xanthomonadaceae (48%) and Pseudomonadaceae (37%) while the dominating taxa associated to phyllosphere were Pseudomonadaceae (54%), Moraxellaceae (16%) and Enterobacteriaceae (25%). The genus *Pseudomonas* was almost exclusively assigned to the family Pseudomonadaceae (98%) associated to foliage. The most abundant genus of the root associated microbiome was *Rhodanobacter* (27%) followed by *Pseudomonas* (24%), while 8% of the rhizosphere associated reads could not be taxonomically assigned (**Figure 1**). In addition,

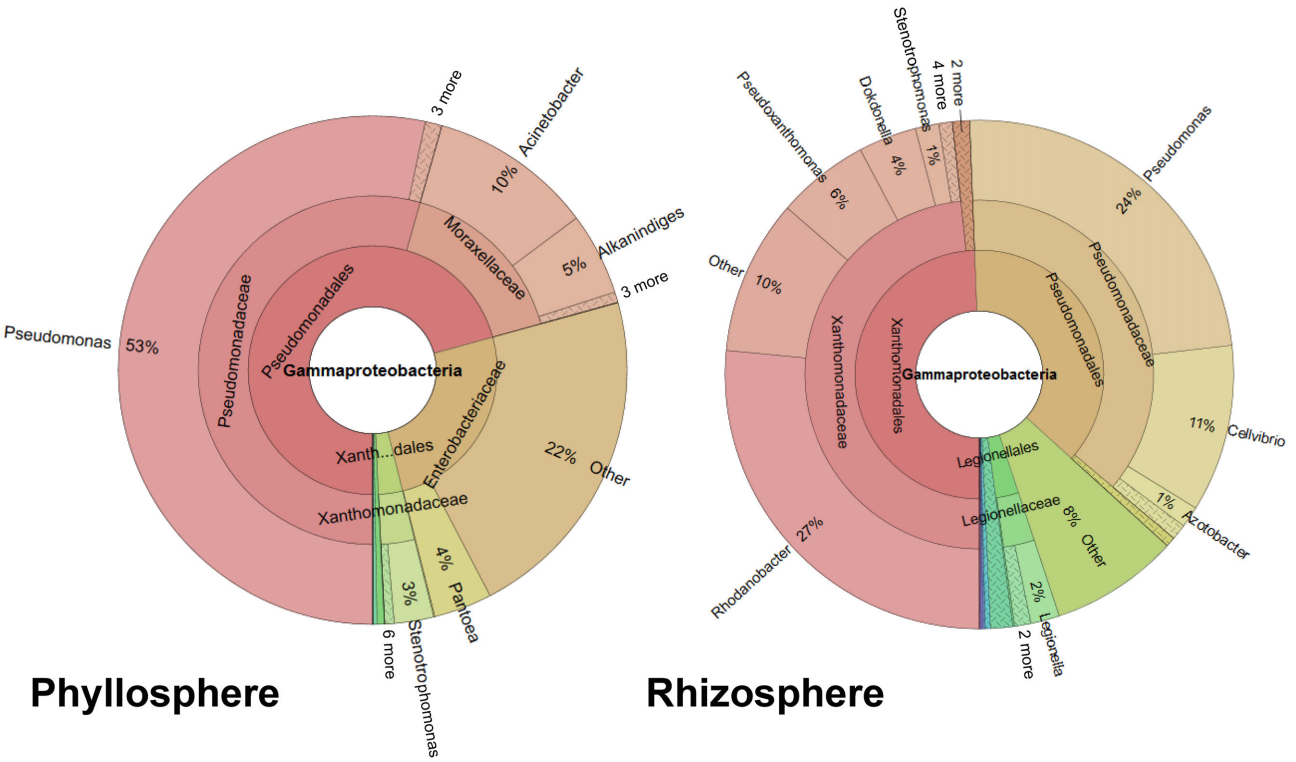

FIGURE 1 | Ring-charts showing the gammaproteobacterial community structure associated with investigated lettuce habitats. The RDP classifier analysis is shown from the mean of 12 samples per habitat. The rings represent different taxonomic ranks (order, family, and genus), and the columns represent distinct taxa. Minor taxa are not specified.

differences were found between both plant development stages as well as between the different treatments (Figure S1). Interestingly, comparing only the phyllosphere samples of mature plants with bottom rot disease against the untreated control, we found 371 distinct OTUs with 99 statistically distinct differences.

THE CORE GAMMAPROTEOBACTERIAL COMMUNITY AND ITS DIVERSITY

A profile clustering network based on all 454-amplicon Gammaproteobacteria libraries was applied to visualize relationships of lettuce-associated bacterial genera and RDP-classified conjoined taxa. The network presented in **Figure 2** can be used to identify the core microbiome of lettuce but also to connect specific taxa with different treatments, plant stages or with healthy and diseased plants. Our study revealed that plants belonging to different growth stages harbor several shared genera (at least 0.1% abundant in all samples compared). From 46 taxonomical assignments, 19 taxa could be assigned to a particular core microbiome structure of lettuce. Only *Alkanindiges*, *Pantoea,* and a lineage of Enterobacteriaceae (not classified at genus level) were exclusively assigned to the phyllosphere; they are indicated as phyllosphere core microbiome (**Figure 2**, surrounded by green color). For the rhizosphere a more diverse core microbiome was identified (red color): core taxa were assigned to *Rhodanobacter, Pseudoxanthomonas, Dokdonella, Luteimonas, Steroidobacter, Thermomonas, Legionella,* and *Haliea*. Additional OTUs were identified at family level as Xanthomonadaceae, Legionellaceae, Pseudomonadales, and Gammaproteobacteria. The highly abundant *Pseudomonas*, *Acinetobacter,* and *Stenotrophomonas* occurred in the core of both habitats (blue color). There are also other taxa found only associated to a specific habitat but did not account to any core group, due to low abundance or not comparable occurrence in the different treatments. In addition, detailed differences in the composition between the particular treatments are displayed by the network structure. For example, *Cellvibrio* was found preferentially in higher abundances in the rhizosphere of *R. solani* treated young plants and decreased in the rhizosphere of mature plants of the same treatment. Comparing all samples which were inoculated with *R. solani* (RS: PMK, PYRS, PYfzb42RS, $n = 7$) with the non-inoculated (C: PMG, PYC, $n = 5$), the highly abundant group of Enterobacteriaceae was dominantly retrieved from phyllosphere samples inoculated with *R. solani* (RS: 34.5% to C: 12.2% mean abundance), while Moraxellaceae including *Acinetobacter* and *Alkanindiges* were more abundant on the foliage of healthy plants (C: 30% to RS: 5.7% mean abundance). Similar gammaproteobacterial patterns could be observed on young plants inoculated only with *R. solani* compared to plantlets inoculated additionally with FZB42.

Beta diversity (pairwise sample dissimilarity) indices based on weighted UniFrac distances revealed clear differences between the habitats, but also between young and mature plants infected by *R. solani* with bottom rot disease compared to non-inoculated healthy young and mature plants (**Figure 3**, Table S1). A general

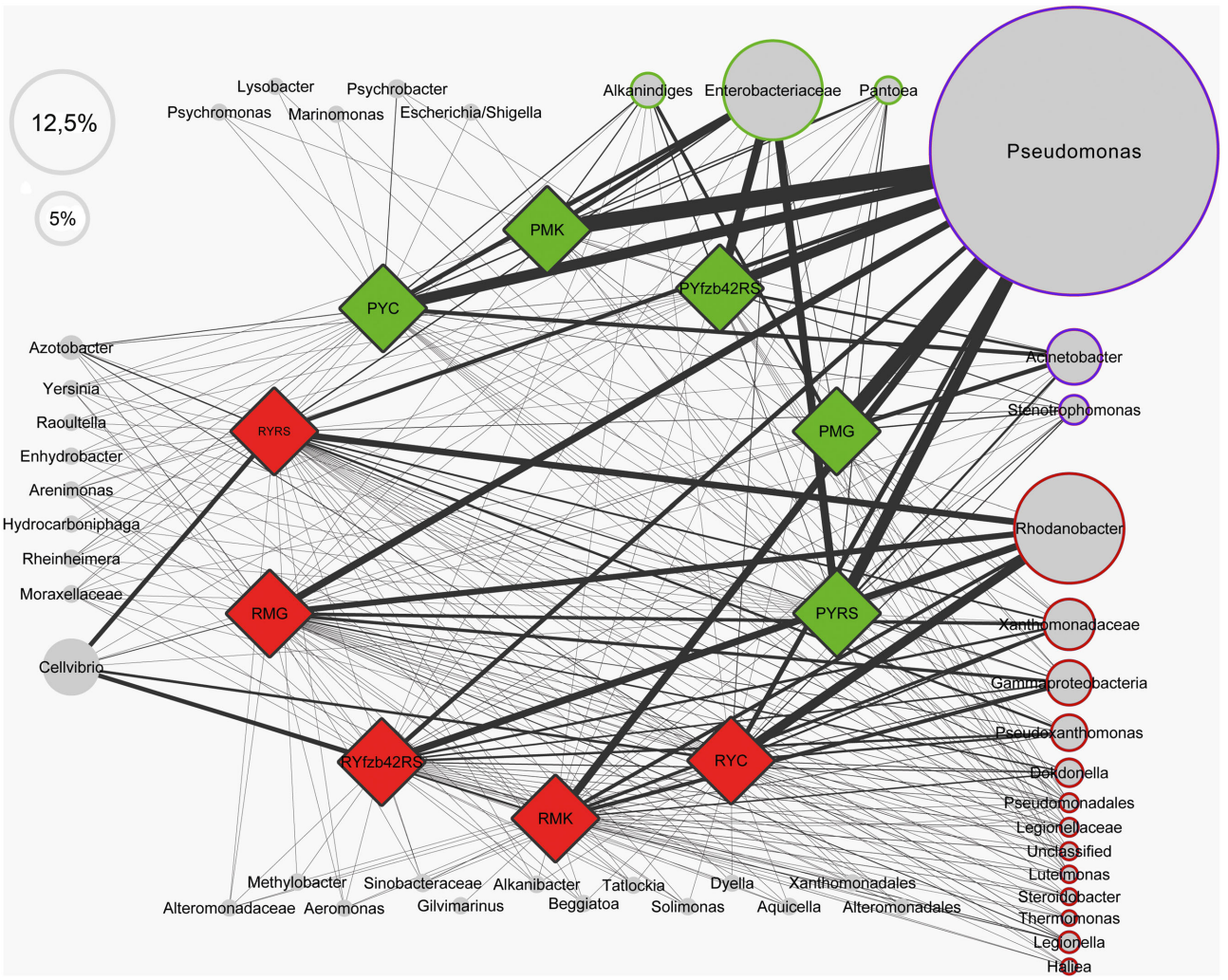

FIGURE 2 | Profile clustering network visualizing the investigated groups of lettuce (var. Tizian) and conjoined taxa. Red diamonds indicate samples derived from the roots and green diamonds from the lettuce foliage, respectively. Nodes represent taxa derived from the RDP classifier and the node sizes correspond to the mean relative abundance of all samples. The abundance of a certain taxa correlated to a particular investigated group is visualized trough the line width of the corresponding connection. The frame color represents the affiliation to the observed core taxa (green—phyllosphere, red—rhizosphere, blue—occurrence in both habitats and all samples, white—not a core taxon). Taxa less abundant than mean ≤0.5% are displayed without size-correlation. Abbreviations: P, phyllosphere; R, rhizosphere; Y, young; M, mature; RS, *R. solani*; C, untreated (control); FZB42RS, FZB42 and *R. solani* co-inoculation; G, healthy; K, diseased.

higher variation was observed among samples derived from the phyllosphere. Alpha diversity indices (Table S1) based on the observed species metric showed a higher diversity of Gammaproteobacteria associated with plants inoculated with *R. solani*, but the co-inoculation with FZB42 seems to reduce this effect (**Figure 4**). This was observed across both investigated habitats, and additionally a slight increase of the overall diversity correlated with the maturity state.

DISCUSSION

Our study gave new insights into the general structure of the lettuce microbiome as well as showed the impact of the plant pathogen *R. solani* AG1-IB and its antagonistic counterpart *B. amyloliquefaciens* FZB42. Sequencing of 16S rRNA gene amplicons provided especially a deeper look into the fraction of often health relevant Gammaproteobacteria in the lettuce-associated microbiome, down to the taxonomic rank of genera. While *R. solani* is a serious lettuce pathogen (Wolf and Verreet, 1999), Chowdhury et al. (2013) demonstrated that FZB42 is an efficient biocontrol agent. FZB42 was able to effectively reduce the disease severity of bottom rot caused by *R. solani* in pot and field experiments. In our study we showed that both microorganisms have not only a significant impact on plant health, they also significantly influence the structure of the plant-associated microbiome.

The rhizosphere and phyllosphere gammaproteobacterial microbiomes of healthy lettuce plants showed significant differences in their overall composition, their core, and diversity

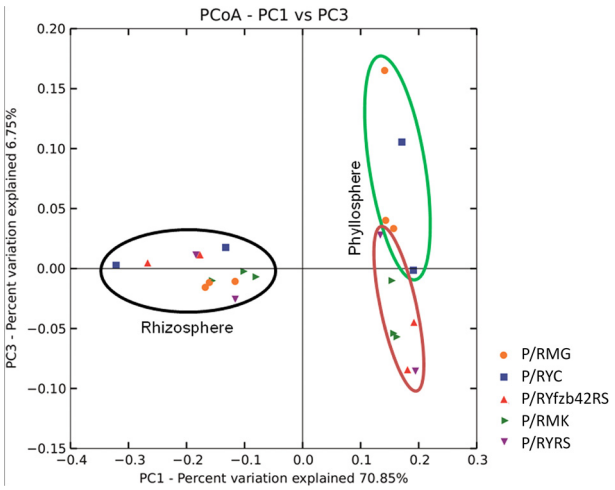

FIGURE 3 | Beta diversity metrics of bacterial 16S rRNA genes reveal distinctly clustered Gammaproteobacteria communities structured between healthy plants and plants affect by *Rhizoctonia solani*. Beta diversity community clustering is observed for phylogenetic beta diversity metrics (weighted UniFrac). In the panel, each point corresponds to a sample from either the lettuce rhizosphere (black) or the phyllosphere (green and red). Red—samples inoculated with *R. solani;* green—untreated control group. The percentage of variation explained by the plotted principal coordinates is indicated on the axes.

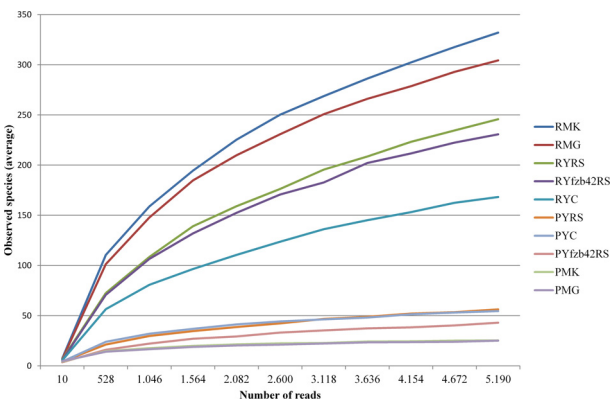

FIGURE 4 | Rarefaction analysis comparing overall diversity of the indigenous microbiota of the investigated lettuce samples (var. Tizian). Prior to rarefaction analysis, rhizosphere, and phyllosphere libraries were pairwise combined corresponding to the particular treatments. Rarefaction curves show saturation of the combined datasets that were clustered at 97% sequence similarity. The curves are supported by 95% confidence intervals. The overall diversity was higher affected in the rhizosphere. In both habitats plants treated with *Rhizoctonia solani* showed higher diversity than plants treated additionally with FZB42 or untreated plants. Abbreviations: P, phyllosphere; R, rhizosphere; Y, young; M, mature; RS, *R. solani*; C, untreated (control); FZB42RS, FZB42 and *R. solani* co-inoculation; G, healthy; K, diseased.

indices. This can be explained by the completely different abiotic conditions in both plant habitats (Raaijmakers et al., 2009; Vorholt, 2012). Here, the most significant and surprising difference we found was the preferential occurrence of enterics in the phyllosphere. Our results are in accordance with the principal findings of Rastogi et al. (2012), who analyzed spatiotemporal variation in bacterial community composition on field-grown lettuce in California. The general composition of phyllosphere bacteria was similar, and Enterobacteriacaea were a substantial fraction also in this study. In general, plant microhabitats are a reservoir for Enterobacteriaceae including potentially human pathogenic bacteria such as human enteric pathogens (Brandl, 2006). Especially after intermediate disturbances such as plant diseases, their abundance was enhanced (Erlacher et al., unpublished data). Due to their impact on human health as either pathogens or immunostimulants, this is an observation that could be of considerable importance for health concerns. Hanski et al. (2012) could show correlation between bacterial diversity and atopy, suggesting significant interactions with Gammaproteobacteria. These authors further showed a positive association between the abundance of *Acinetobacter,* found abundantly in healthy lettuce in our study and Interleukin-10 expression in peripheral blood mononuclear cells in healthy human individuals. Interleukin-10 is an anti-inflammatory cytokine and plays a central role in maintaining immunologic tolerance to harmless substances (Eskdale et al., 1997; Lloyd and Hawrylowicz, 2009).

In general, members of the genus *Pseudomonas* play a prominent role in the lettuce gammaproteobacterial microbiome. They were the dominant inhabitants of the phyllosphere; at family level pseudomonads present 54% and at genus level 53% of the microbiome. Also in the rhizosphere they represent 37% of the OTUs at family level. Altogether, *Pseudomonas* was the most dominant member of the lettuce core microbiome. *Pseudomonas* is a model organism to study beneficial plant-microbe interactions (Haas and Défago, 2005). Interestingly, in our study, *Pseudomonas* was not only related to healthy plants, there was also strong connection to diseased plants. The fact is not surprising because pathogenic pseudomonads are well-known but shows the limitation of the applied method. Using amplicon sequencing we can identify the genus or species but not their functional traits. For functional analysis metagenomic/transcriptomic techniques are required.

Plant-microbe interactions are highly complex and changes in the abundance of individual strains, either pathogens or beneficials can result in non-linear alterations of the entire microbiome composition. Such alterations may lead to negative effects to plants and humans as consumers (Berg et al., 2005). However, microbiome shifts can hardly be predicted and must be traced by thorough screening using culture-independent and sequencing-based approaches. Adesina et al. (2009) used DGGE fingerprint to study microbiome shifts and showed that *R. solani* AG1-IB inoculation severely affected the bacterial and fungal community structure in the rhizosphere of lettuce and that these effects were much less pronounced in the presence of the antagonistic counterpart *P. jessenii* RU47. In our study we used amplicon sequencing, which allowed a deep insight into the composition of Gammaproteobacteria. The human health relevant group of Enterobacteriaceae was affected by the *R. solani*; we found a significant increase in gammaproteobacterial diversity due to the pathogen outbreak. The overall enhancement of diversity after biotic stimulation by a pathogen agrees well with the intermediate

disturbance hypothesis known to ecology from studies of higher plants or coral reefs (Connell, 1978). However, together with FZB42 this increase was less distinct. Until today, the mechanisms described for biocontrol agents focus on direct antagonistic effects against a pathogen or an interaction via the plant's immune system (Doornbos et al., 2012). In this study we showed a selective compensation of the impact of a pathogen on the indigenous plant-associated microbiome by the biocontrol agent, which is an interesting effect of the beneficial aspect of the inoculant.

Biocontrol of plant pathogens is a promising solution for sustainable agriculture. Molecular techniques, which allowed a deeper insight into the crop-associated microbiome, can also be applied to develop new biocontrol strategies (Berg et al., 2013). Using a profile clustering network in our study, *Acinetobacter* and *Alkanindiges* were identified as indicators of healthy lettuce plants. Therefore, they could be promising biocontrol agents. An endophytic *Acinetobacter* strain isolated from healthy stems of the plant *Cinnamomum camphora* was already used as biocontrol strain against fungal diseases (Liu et al., 2007) but nothing is known about any biocontrol activity of *Alkanindiges*. In contrast, in the study of Rastogi et al. (2012), the foliar presence of *Xanthomonas campestris* pv. vitians, which is the causal agent of bacterial leaf spot of lettuce, correlated positively with the relative representation of bacteria from the genus *Alkanindiges*. Here, more research is needed for understanding bacterial networking on plants, which is an essential step toward predictable biocontrol. In addition, beneficial *Pseudomonas* strains could be other interesting candidates for biocontrol because we found a strong connection to healthy lettuce plants. This was shown already successfully for *P. jessenii* RU47 by Adesina et al. (2009). Altogether, new results favorite diverse bacterial cocktails to control plant diseases (Berg et al., 2013); for lettuce they could contain *Acinetobacter, Bacillus*, *Pseudomonas,* and *Serratia* strains as well.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00175/abstract

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SUPPORTING MATERIAL

The impact of the pathogen *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous lettuce microbiome

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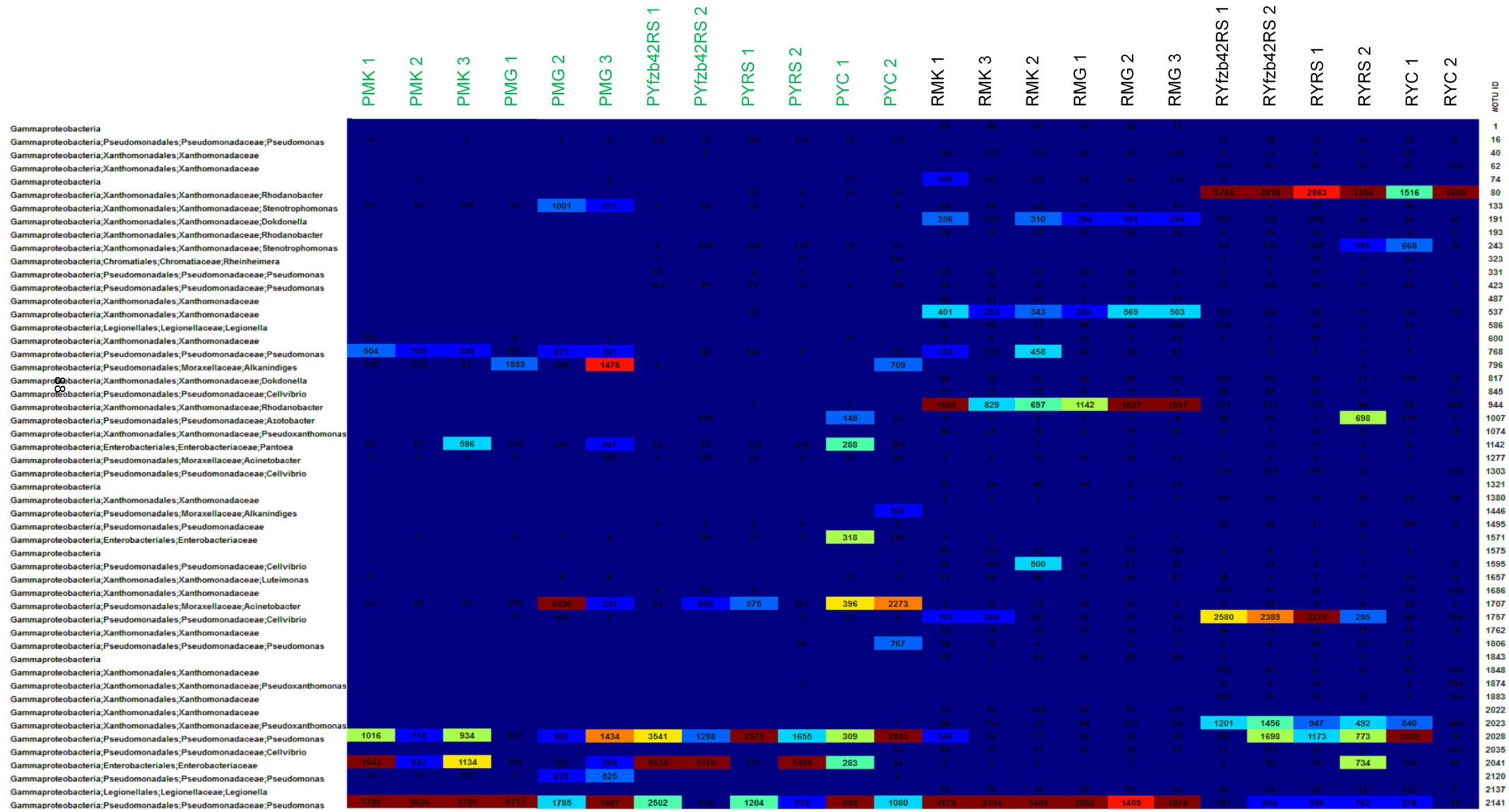
Table S1 Richness estimates and diversity indices obtained.

Treatment	shannon	equitability	dominance	simpson	simpson_reciprocal	simpson_e	PD_whole_tree	chao1	observed_species
PMK 1	2.47	0.51	0.24	0.76	4.17	0.14	2.46	31.00	29
PMK 2	1.81	0.38	0.46	0.54	2.18	0.08	1.99	29.00	28
PMK 3	2.57	0.57	0.22	0.78	4.63	0.20	1.94	26.75	23
PMG 1	1.83	0.38	0.45	0.55	2.22	0.08	3.03	29.50	27
PMG 2	2.77	0.55	0.22	0.78	4.54	0.14	3.22	50.33	32
PMG 3	2.93	0.60	0.17	0.83	5.72	0.19	3.62	37.50	30
PYfzb42RS 1	1.94	0.37	0.33	0.67	3.06	0.08	3.56	42.00	37
PYfzb42RS 2	2.17	0.34	0.35	0.65	2.85	0.04	4.96	81.00	81
PYRS 1	2.37	0.38	0.32	0.68	3.11	0.04	5.35	96.10	73
PYRS 2	2.42	0.39	0.31	0.69	3.17	0.04	5.35	103.08	74
PYC 1	1.78	0.33	0.55	0.45	1.82	0.04	4.07	45.50	42
PYC 2	3.26	0.50	0.16	0.84	6.28	0.07	5.92	133.86	91
RMK 1	5.40	0.64	0.08	0.92	12.59	0.03	24.60	570.80	362
RMK 2	5.01	0.59	0.13	0.87	7.89	0.02	23.72	495.05	362
RMK 3	5.32	0.63	0.08	0.92	13.17	0.04	23.04	545.59	361
RMG 1	4.68	0.56	0.14	0.86	7.00	0.02	22.29	456.27	328
RMG 2	4.96	0.58	0.12	0.88	8.61	0.02	24.76	644.15	369
RMG 3	4.63	0.56	0.13	0.87	7.86	0.03	19.24	391.02	296
RYfzb42RS 1	4.07	0.48	0.17	0.83	5.84	0.02	21.96	511.02	351
RYfzb42RS 2	3.94	0.48	0.14	0.86	6.98	0.02	17.75	379.54	290
RYRS 1	4.03	0.48	0.16	0.84	6.31	0.02	21.33	430.40	340
RYRS 2	4.29	0.53	0.11	0.89	9.04	0.03	18.17	399.12	263
RYC 1	4.02	0.50	0.16	0.84	6.25	0.02	17.58	397.52	270
RYC 2	2.71	0.37	0.40	0.60	2.52	0.02	10.26	220.63	161

Abbreviations: P – phyllosphere. R – rhizosphere; Y – young. M – mature; RS – *R. solani*. C – untreated (control). FZB42RS – FZB42 and *R. solani* co-inoculation; G – healthy. K – diseased

Figure S1 Heatmap based on the relative abundance of obtained OTUs derived from all samples. Only groups ≥200 counts per OTU are displayed. The vertical columns represent the investigated samples. The horizontal axes depict OTUs with the taxonomic affiliation. A color change from cold to warm colors indicates an abundance increase.



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Bacterial networks and co-occurrence relationships in the lettuce root microbiota.

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Bacterial networks and co-occurrence relationships in the lettuce root microbiota

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Summary

Lettuce is one of the most common raw foods worldwide, but occasionally also involved in pathogen outbreaks. To understand the correlative structure of the bacterial community as a network, we studied root microbiota of eight ancient and modern *Lactuca sativa* cultivars and the wild ancestor *Lactuca serriola* by pyrosequencing of 16S rRNA gene amplicon libraries. The lettuce microbiota was dominated by Proteobacteria and Bacteriodetes, as well as abundant Chloroflexi and Actinobacteria. Cultivar specificity comprised 12.5% of the species. Diversity indices were not different between lettuce cultivar groups but higher than in *L. serriola*, suggesting that domestication lead to bacterial diversification in lettuce root system. Spearman correlations between operational taxonomic units (OTUs) showed that co-occurrence prevailed over co-exclusion, and complementary fluorescence in situ hybridization-confocal laser scanning microscopy (FISH-CLSM) analyses revealed that this pattern results from both potential interactions and habitat sharing. Predominant taxa, such as *Pseudomonas*, *Flavobacterium* and *Sphingomonadaceae* rather suggested interactions, even though these are not necessarily part of significant modules in the co-occurrence networks. Without any need for complex interactions, single organisms are able to invade into this microbial network and to colonize lettuce plants, a fact that can influence the susceptibility to pathogens. The approach to combine co-occurrence analysis and FISH-CLSM allows reliably reconstructing and interpreting microbial interaction networks.

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Introduction

Plants are associated with diverse microbial communities and invest a substantial amount of energy in providing rhizodeposits to nurture their root microbiota, which is known for its importance on growth and health of their hosts (Mendes *et al.*, 2011; Philippot *et al.*, 2013). Because plants markedly vary in these parameters, specific microbiota were found associated with different host plants (Berg and Smalla, 2009; Bulgarelli *et al.*, 2012). One of the primary questions is to what level the specificity of microbiota is maintained in related plants. Several studies suggest a small but significant effect on the rhizosphere depending on the plant genotype (Smalla *et al.*, 2001; Schweitzer *et al.*, 2008; Weinert *et al.*, 2011; Bulgarelli *et al.*, 2012). Even the phylogenetically oldest land plants on Earth, the bryophytes, show an outstanding degree of plant specificity and diversity across closely related species (Opelt *et al.*, 2007; Bragina *et al.*, 2012), but it is still unclear at what level specific microbiota emerge. In maize, genetic differentiation through crop diversification was identified as a significant factor for plant–microbe interactions (Bouffaud *et al.*, 2012; Peiffer *et al.*, 2013). It still remains to be studied what ecological role these interactions have and what general function microbial diversity could have in the plant habitat (McGrady-Steed *et al.*, 1997). Recently, it was shown that plant-associated microbial diversity at both structural and functional levels is crucial to hamper pathogen invasions (Balint-Kurti *et al.*, 2010; Latz *et al.*, 2012; Van Elsas *et al.*, 2012) but the underlying principles are not well understood. To shed new light on this phenomenon, analysis of co-occurrence networks as developed by Barberán and colleagues (2012) could be utilized. They already helped to identify microbial co-occurrence relationships in the human microbiota (Faust *et al.*, 2012), but have not been applied to complex plant microbiota.

Cultivated lettuce is among the most popular raw-eaten vegetables in health-conscious societies because of its nutritional value. Lettuce-associated outbreaks of pathogens, however, are a recurrent health threat known throughout the world and primarily caused by enterics (Teplitski *et al.*, 2011; Ongeng *et al.*, 2013). Because

bacterial strains originating from soil can extend via the endosphere in the whole lettuce plant, it is therefore particularly important to know about the root microbiota and its implications on lettuce health and safety. Lettuce cultivars originated from different regions of the world and the complex breeding lines are hardly dated with precision (de Vries, 1997). Mediterranean and Chinese cultivars are regarded as the oldest, and tomb wall paintings of garden lettuce appear in numerous Old Kingdom and Middle Kingdom tombs in Egypt showing evidence for cultivation since 2680 BC (Křístková *et al*., 2008; Zohary *et al*., 2012). Today, lettuce cultivars are classified in 'cultivar groups'. Basically, these comprise phenotypes either forming either typically closed heads (convar. *incocta* Helm), or open forms (convar. *sativa* Helm). Closed heads are characteristic, for example, in the cultivar groups crisphead and butterhead lettuce, while open forms include the cultivar groups Cos and stalk lettuce. So far, studies of the lettuce microbiota demonstrated that this morphological difference is more influential on the structure of the phyllosphere microbiota than the lettuce genotype (Hunter *et al*., 2010; Rastogi *et al*., 2012). In contrast, the root-associated microbiota has not yet been studied at this level. We hypothesize that breeding of lettuce resulted in diversified microbiota at belowground level. In addition, we argue that the understanding of the indigenous lettuce network is imperative to limit pathogen outbreaks in the future.

The objective of this study was to investigate the specificity, diversity and interactions of the root microbiota associated with four main *Lactuca sativa* cultivar groups: Cos and stalk (subspecies. *longifolia* and *augustana*, respectively) belonging to the convar *sativa*; crisphead and butterhead (subspp. *crispa* and *capitata*, respectively) belonging to the convar. *incocta*. Additionally, we analysed the wild ancestor *Lactuca serriola*. All plants were intercropped for several years at a unique sampling site provided by the Seed Savers Association 'Arche Noah' (Austria) that preserves crop biodiversity in Europe. We combined 16S rRNA gene amplicon libraries of lettuce root microbiota and corresponding correlation analyses with fluorescence in situ hybridization-confocal laser scanning microscopy (FISH-CLSM) to (i) understand the impact of domestication on lettuce root microbiota, (ii) to assess the effect of host genotype on lettuce microbiota specificity and (iii) to detect potentially interacting bacterial taxa *in situ*. Finally, we discuss our results in relation to health issues.

Results

Structure and diversity of the lettuce root microbiota

A total of 709,021 reads were obtained using pyrosequencing of the 16S rRNA amplicon libraries from 27 root microbiota (24 representing *L. sativa* and three *L. serriola*; Fig. 1). After primer removal and length and quality filtering, 216 466 high-quality reads remained prior to denoising (mean sequence length 296.65 bp, mean GC content 54% ± 2.68; percentage of reads that were annotated at least at phylum level ranged from 98.37%, when calculated with 100% OTU cut-off level, to 99.06%, calculated with 90% OTU cut-off level). Sequences were grouped into OTUs with 90%, 95%, 97% and 100% similarity levels respectively. After removal of both plastid and mitochondrial OTUs, three *L. sativa* samples with a low number of reads were discarded and about 4000 ± 1690 sequences per sample were finally obtained. The properties of the four OTU tables are shown in Fig. S2. The taxonomic assignment of OTUs revealed 38 bacterial phyla, 10 of which exceeded 1% of relative abundance (Fig. S3). The most abundant phylum was Proteobacteria (39.5–40.00%), followed by Bacteroidetes and, surprisingly, Chloroflexi. Gammaproteobacteria was the most abundant class retrieved, irrespective of OTU cut-off level (Fig. S3). Most abundant OTUs (showing relative abundance > 0.5% of the total microbiota) included members of Gammaproteobacteria (Pseudomonadaceae, Xanthomonadaceae and *Cellvibrio*), Betaproteobacteria (Comamonadaceae), Bacteroidetes (*Flavobacterium*, Sphingomonadaceae and Chitinophagaceae), Chloroflexi (Anaerolineae), Acidobacteria-6 and Actinobacteria (*Streptomyces*) (Fig. S4A). These dominant taxa represented a rather high fraction of the microbiota ranging from 31.3% to 74.6% (at 100% and 90% OTU cut-off level, respectively; Fig. S4B).

Shannon, Equitability and Chao 1 indices were highest for the 100% cut-off level OTUs and lower at 97%, 95% and 90% OTU cut-off levels (paired samples *t*-test, $P < 0.001$). Shannon and Equitability indices were significantly higher in *L. sativa* than in *L. serriola* [analysis of variance (ANOVA) $F_{(4,19)} = 8.661$, $P = 0.0004$ and $F_{(4,19)} = 12.861$, $P = 0.00004$, respectively, calculated on the whole microbiota at 97% OTU cut-off level; Fig. 2], indicating that domestication of lettuce lead to the increase of bacterial diversity in the root system. No significant differences were found for Chao1 richness estimator (ANOVA $F_{(4,19)} = 1.338$, $P = 0.294$, calculated on the whole microbiota at 97% OTU cut-off level; Fig. 2). Similar results for both Shannon and Equitability indices were obtained with the other OTU cut-off levels, both on the whole microbiota and after removal of singletons or < 10 reads OTUs (Table S1). Removing singletons had the effect to reveal significant differences between *L. sativa* and *L. serriola* for the Chao 1 richness estimator. Removing the OTUs with < 10 reads resulted in significantly different Chao 1 values between *L. sativa* and *L. serriola* only at 100% and 97% OTU cut-off levels (Table S1). Among *L. sativa* samples, statistically significant differ-

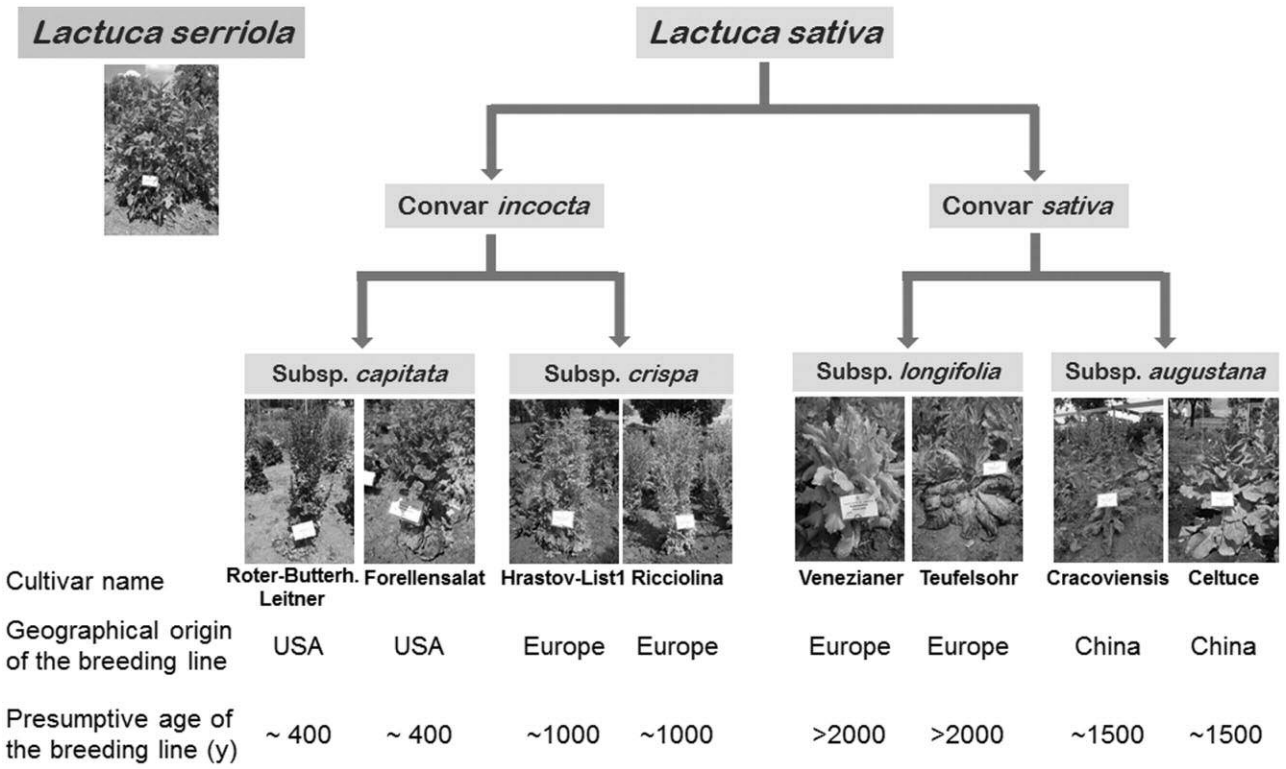

Fig. 1. Sampling scheme. Eight *Lactuca sativa* cultivars and *Lactuca serriola* were collected at the field of the Archae Noah (Schiltern, Austria). Four main cultivar groups (corresponding to subspecies) were sampled. Three replicates per sample were collected.

ences were found for both Shannon and Equitability indices and for Chao1 richness estimator only at cultivar level (Table S1), with the cultivars Hrastov Lst1 (subsp. *crispa*) and Venezianer (subsp. *longifolia*) belonging to the convars *incocta* and *sativa*, respectively, showing the highest diversity (Fig. S4). Since three *L. sativa* samples were removed from the analysis due to the low number or sequence reads (hence three cultivars had only two replicates), differences at cultivar level would require confirmation with more replicates per cultivar.

Root microbiota associated with lettuce were significantly different between subspecies, and more different than between convars (Adonis test on UniFrac weighted pairwise distances, Fig. 3A). UniFrac distances within subspecies were significantly lower than between subspecies (ANOVA $F_{(8,476)} = 3.451$, $P = 0.0007$) (Fig. 3B). Pairwise distances between subspecies were also significantly different: *L. sativa capitata* and *crispa* (belonging to the convar *incocta*) were more different to each other, whereas *longifolia* and *augustana* (belonging to the convar *sativa*) were more similar (Fig. 3B and Fig. S5). These results indicate a prevailing effect of the host genotype over the plant morphology on the structure of the root microbiota. The OTU cut-off level and removal of singletons had a negligible effect on the extent of beta-diversity (Fig. 3A). Moreover, UniFrac distance matrices obtained with different OTU cut-off levels were extremely similar to each other (Mantel test, $r = 0.960–0.973$), similar to the distance matrices obtained with and without singletons at each cut-off level ($r = 0.982–0.990$). Statistical differences between microbiota of lettuce cultivars were comparable or even higher than between those of subspecies (Adonis ≤ 0.001, Fig. S5), pending confirmation by more replicates per cultivar.

Core microbiota and cultivar-specific OTUs

Because beta-diversity was not significantly influenced by the OTU cut-off level (Fig. 3), both shared and cultivar specific fractions of the microbiota were computed only at the 97% OTU cut-off level. Sixty-eight OTUs, representing 48.79% of all reads in the rarefied OTU table, were found in all *L. sativa* microbiota ('core microbiome'), including members of Gammaproteobacteria (15 OTUs), Bacteroidetes (10), Actinobacteria (7), Alphaproteobacteria (7), Acidobacteria (6), Betaproteobacteria (6), Chloroflexi (6), Verrucomicrobia (5), Deltaproteobacteria (2), Saccharibacteria (2), Firmicutes (1) and Planctomycetes (1) (Fig. 4). Five of these shared OTUs, identified as Flammeovirgaceae, Bradyrhizobiaceae, Actinoplanes,

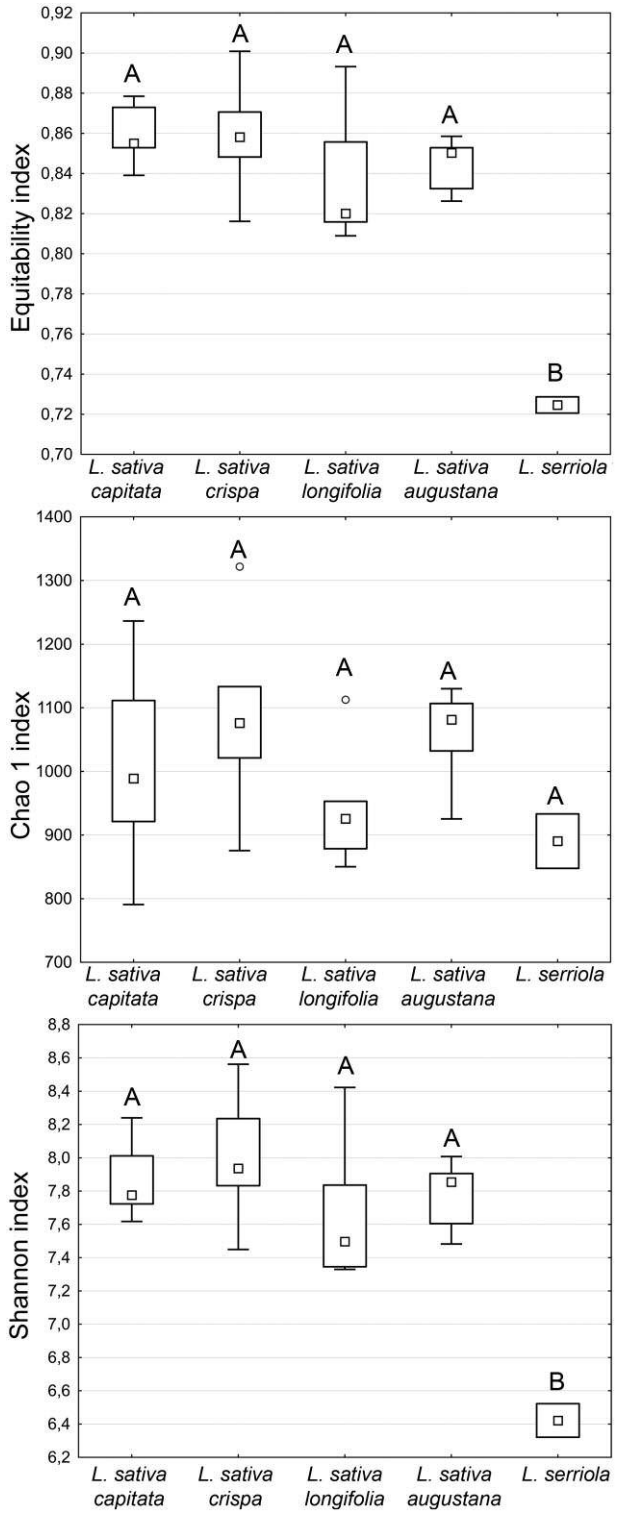

Fig. 2. Alpha-diversity analysis. Comparison of diversity indices between *L. sativa* subspecies and *L. serriola*, calculated on the whole microbiota grouped at 97% similarity level OTUs (including singletons). Different letters indicate significantly different means at $P < 0.05$.

Opitutaceae and Chthoniobacteraceae, respectively, were not detected in *L. serriola*, suggesting that a significant fraction (7.4%) of the shared root microbiota could be highly *L. sativa* specific. These OTUs were homogeneously distributed across the samples.

Seventy-one OTUs showed significantly different relative abundances between lettuce subspecies, as estimated with ANOVA after false discovery rate (FDR) correction (Fig. S6). These OTUs were responsible for significant UniFrac/Adonis differences, and included 12.86% of all reads in the rarefied OTU table. Among such OTUs, members of Planctomycetes (15), Chloroflexi (11), Bacteroidetes (10), Gammaproteobacteria (9), Actinobacteria (4), Betaproteobacteria (4), Verrucomicrobia (4), Acidobacteria (3), Gemmatimonadetes (3), Deltaproteobacteria (2), Alphaproteobacteria (2), Saccharibacteria (2), Elusimicrobia (1) and one unidentified bacterium were found (Fig. S5). The most abundant organisms occurring within such host-specific OTUs were identified as TM7-3 (Saccharibacteria), Acidobacteria-6 (Acidobacteria) and *Cellvibrio* (Gammaproteobacteria; Fig. S6). Interestingly, six shared OTUs showed a significantly different relative abundance between lettuce subspecies (labelled with 'C' in Fig. S6), which indicates a higher affinity of different lettuce cultivars for certain bacteria. These strains included three Gammaproteobacteria, one member of the Alphaproteobacteria, one of the Verrucomicrobia and one belonging to Chloroflexi (Fig. S6).

Correlation analysis of co-occurrence patterns

To reveal potential interactions between bacteria, Spearman correlations between OTUs were calculated based on their occurrence patterns across the 24 *L. sativa* and *L. serriola* samples. The analysis was performed with sufficiently abundant OTUs that were detectable by microscopy (> 0.5% of the total microbiota) because our aim was to detect the colocalization of correlated OTUs *in situ* by FISH-CLSM analyses. A total of 27, 37, 77 and 102 highly significant correlations were found between OTUs at 100%, 97%, 95% and 90% cut-off levels respectively. The decreasing OTU cut-off level revealed a higher number of abundant OTUs (Fig. S4B). The number of correlations per OTU increased, demonstrating that the OTU cut-off level had a strong impact on the analysis (Fig. S7). However, some traits remained unchanged, such as the central position of Gammaproteobacteria and the marginal involvement of Betaproteobacteria. Surprisingly, all correlations found were positive except one between *Streptomyces* and Acidobacteria-6. This negative correlation was present at 95%, 97% and 100% OTU cut-off levels, but not at 90% (Fig. S7). Since it was demonstrated that different organisms (with non-coherent occurrence patterns) can be

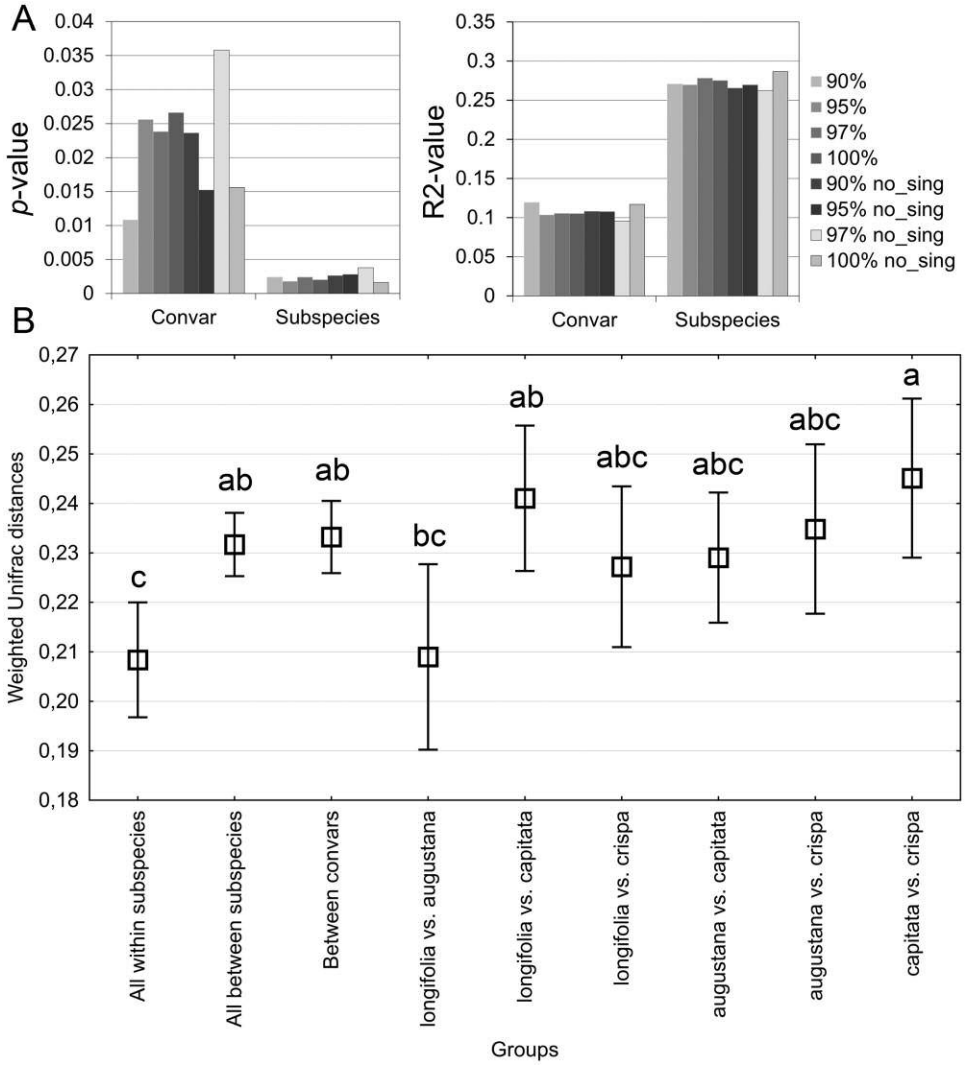

Fig. 3. Beta diversity analysis.
A. Significance of differences (left plot) and fraction of variability explained (right plot) for UniFrac pairwise distances between lettuce convars and between subspecies at different cut-off levels (90 to 100), with and without singletons (no_sing).
B. Pairwise UniFrac distances between groups of both lettuce convars and subspecies. Means with 95% confidence interval are shown. Different letters indicate different means at $P < 0.05$ (Tukey honest significant difference (HSD) test).

grouped into the same OTU even at 99% cut-off level (Patin *et al*., 2013), we avoided potential bias by focusing on the correlations detected with 100% OTU cut-off level (Fig. 5). The correlation network was organized in fully connected subunits (such as Ps-Ps-Fs-Spm and Xa-Xa-An-Spb) (Fig. 5). Correlated out pairs among either Pseudomonadaceae or Xanthomonadaceae (Ps-Ps and Xa-Xa), respectively, could be interpreted as similar organisms (possibly the same species) sharing ecological traits, when they share connections with other OTUs (as suggested by Barberán *et al*., 2012). For example, representative sequences of V4 variable rRNA gene region of two correlated Pseudomonadaceae OTUs (Ps-Ps) showed similarity of 98.35%. The two connected Xanthomonadaceae OTUs (Xa-Xa) were 93.05% similar, and might represent different species but with similar ecology. The two connected Anaerolinae OTUs (An-An) of 86.18% similarity did not share all further connections (Fig. 5), suggesting different species with differential ecological relations in the microbiota. Two OTUs in Sphingomonadaceae of 95.05% similarity were not correlated with each other and share only one connection with *Flavobacterium succinicans* (Fig. 5). We regard these as phylogenetically close species with slightly different ecology. These results demonstrate that phylogenetically close OTUs are often positively correlated, as also shown also by Faust and colleagues (2012) in the human habitat.

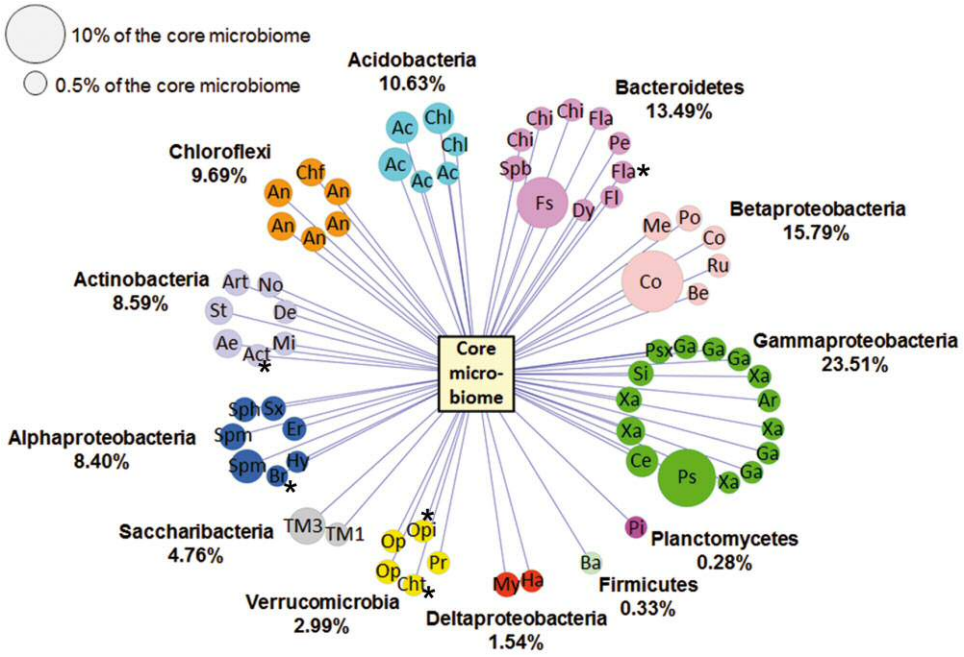

Fig. 4. Core root microbiota of lettuce. Percentage after the phylum/class indicates the respective contribution to the core microbiota. Node labels indicate the taxonomic identification of the OTUs: Ac, Acidobacteria-6; Act, *Actinoplanes*; Ae, *Aeromicrobium*; An, Anaerolineae; Ar, *Arenimonas*; Art, *Arthrobacter*; Ba, Bacillales; Be, Betaproteobacteria; Br, Bradyrhizobiaceae; Ce, *Cellvibrio*; Chf, Chloroflexi; Chi, Chitinophagaceae Chl; Chloracidobacteria, Cht – [Chthoniobacteraceae]; Co, Comamonadaceae; De, *Demequina*; Dy, *Dyadobacter*; Er, Erythrobacteraceae; Fl, *Flavobacterium*; Fla, Flammeovirgaceae A4; Fs, *Flavobacterium succinicans*; Ga, Gammaproteobacteria; Ha, Haliangiaceae; Hy, *Hyphomicrobium*; Me, *Metylotenera*; Mi, Micromonosporaceae; My, Myxococcales; No, Nocardioidaceae; Op, Opitutaceae; Opi, *Opitutus*; Pe, *Pedobacter*; Pi – Pirellulaceae A17, Po; *Polaromonas*, Pr – *Prosthecobacter debontii*; Psx, *Pseudoxanthomonas*; Ps, Pseudomonadaceae; Ru, *Rubrivivax*; Si, Sinobacteraceae; Spb, Sphingobacteriaceae; Sph, *Sphingomonas*; Spm, Sphingomonadaceae; St, *Streptomyces*; Sx – *Sphingobium xenophagum*, TM1; TM7-1 (Saccharibacteria), TM3 – TM7-3 (Saccharibacteria); Xa, Xanthomonadaceae. Asterisks indicate OTUs not detected in the intercropped *L. serriola*.

The relative abundance of the OTUs did not significantly influence the number of co-occurrences (Spearman $R = 0.214$, $P = 0.338$, $n = 22$) which suggests that the most dominant organisms (such as Comamonadaceae) are not necessarily involved in intricate relationships. OTUs with highest numbers of connections in the correlation networks belonged to Chloracidobacteria, Anaerolineae and Sphingobacteriaceae (six correlations each), followed by *Flavobacterium succinicans* and Pseudomonadaceae (five correlations each) (Fig. 5). When grouped by phylum or class, Gammaproteobacteria showed the highest number of total correlations, followed by Bacteroidetes and Chloroflexi (16, 11 and 8 correlations, respectively, among 5, 2 and 3 OTUs, respectively, at 100% OTU cut-off level; Fig. 5 and Fig. S7D). Consequently, the highest connectivity was shown by Bacteroidetes (5.5 average correlations per OTUs), followed by Gammaproteobacteria (3.2 average correlations per OTUs) (Fig. 5 and Fig. S7D). Betaproteobacteria, however, were the less connected (only one correlation among four OTUs; Fig. 5 and Fig. S7D). The correlation network for the genotype-specific OTUs, which excludes any effects resulting from soil, revealed that 51 of these OTUs (out of 71) were involved in a total of 76 correlations (65 positive and 11 negative), organized in a core module with 'branches' and three small correlated modules (Fig. S8). Similar to the general correlation network, the Gammaproteobacteria and the Anaerolinae seem to play a key role for the establishment of the microbiota sociability. Moreover, the marginal involvement of Betaproteobacteria was also confirmed. As an important new feature compared with the general network, the Planctomycetes OTUs appear highly involved in co-occurrences, also in the smaller modules.

Colonization patterns analysed by FISH-CLSM

All major taxa (Gammaproteobacteria, Bacteroidetes, Chloroflexi, Betaproteobacteria, Actinobacteria, Acidobacteria, Alphaproteobacteria and Saccharibacteria) were detected by FISH-CLSM either on the root surface and/or in the endorhiza of lettuce. Alphaproteobacteria were found in 100% of confocal stacks where the alpha-specific probe was used, Chloroflexi in 95.2%, Betaproteobacteria in 88.9%, Gammaproteobacteria in 82%, Actinobacteria in 68.75%, Bacteroidetes in 62.9%, Acidobacteria in 42.9%.

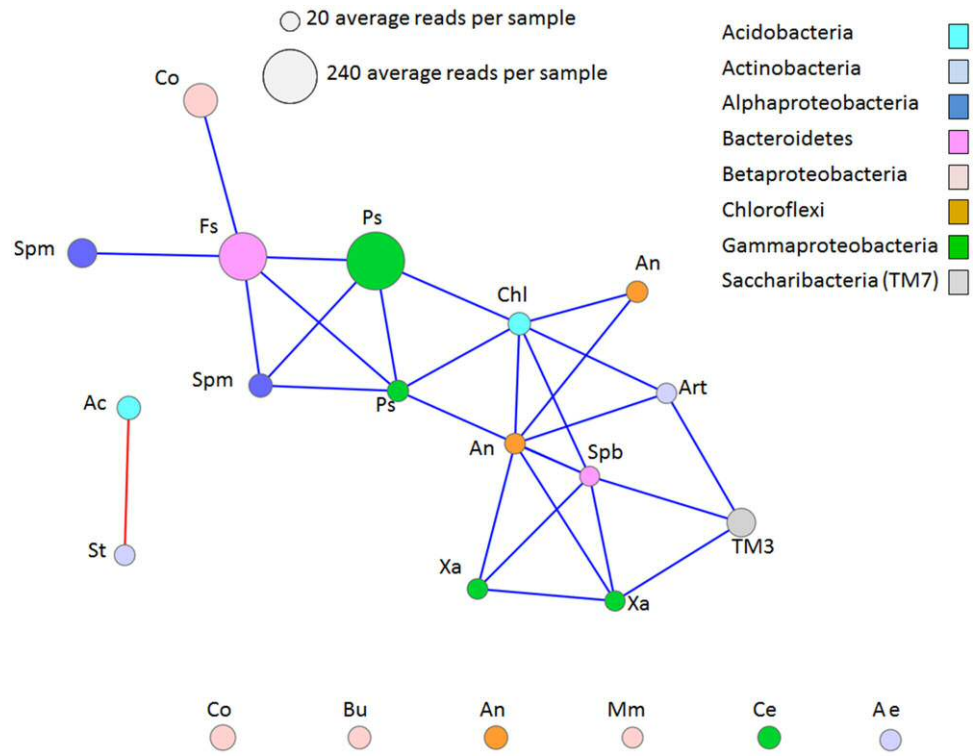

Fig. 5. Correlation of occurrence patterns between OTUs of the lettuce root microbiota, coloured by phylum/class. The network analysis shows correlations between 100% cut-off level OTUs (only abundant: > 0.5% of the total microbiota). Nodes represent OTUs and edges represent strong positive ($R > 0.6$, $P < 0.01$) or negative ($R < -0.6$, $P < 0.01$) Spearman correlations (blue and red lines respectively). OTU abundance (average number of reads per sample) and taxonomic affiliation (at phylum or class level) are indicated by node size and node colour respectively. Node labels indicate the taxonomic identification of the OTUs: Ac, Acidobacteria-6, Ae, *Aeromicrobium*, An, Anaerolineae, Art, *Arthrobacter*, Bu, *Burkholderia*, Ce, *Cellvibrio*, Ch, Chloracidobacteria, Co, Comamonadaceae, Fs, *Flavobacterium succinicans*, Me, *Metylotenera mobilis*, Ps, Pseudomonadaceae, St, *Streptomyces*, TM3, TM7-3 (Saccharibacteria), Spb, Sphingobacteriaceae, Spm, Sphingomonadaceae, Xa, Xanthomonadaceae.

Alphaproteobacteria and Gammaproteobacteria developed either as single cells or small clusters up to 50–60 cells. They colonized both the outer root surface as well as the endorhiza (Fig. S9). Alphaproteobacteria were detected in mixed colonies with Bacteroidetes (Fig. 6A), especially near the root tips where Bacteroidetes were more abundant. This colocalization indicates that the correlation *Flavobacterium succinicans*–Sphingomonadaceae (Fs-Spm, Fig. 5) agrees with potential interaction among these OTUs (cell size and morphology were also consistent with the genus *Flavobacterium* forming relatively long and flexible rods, Bernadet *et al*., 1996; Fig. S10). Bacteroidetes were heterogeneously distributed across the root system, but building dense colonies preferentially on the root tips. Otherwise, they occurred as single cells or small clusters (Fig. S11). The vicinity of Chloroflexi (filamentous Anaerolinae) within areas poorly colonized by Bacteroidetes is indicative of local enrichment (Fig. 6B, circles), and suggests commensalism for the correlation Anaerolinae-Sphingobacteriaceae (An-Spb, Fig. 5). The Chloroflexi was the most unexpected phylum among those dominating the lettuce. The OTU assignment to the class of the Anaerolineae was confirmed by microscopy (Fig. 6B and Fig. S12). They were ubiquitous at the outer root surface and often displayed cell-to-cell adhesion as well as involvement in mixed colonies (Fig. 6B, square, and Fig. S12, circles). Betaproteobacteria formed large colonies and often represented the great majority of total cell counts. Hence, the microscopically observed abundance exceeds the expectation of approximately 10% relative abundance according to pyrosequencing results (Fig. 6C,D). Both filamentous and non-filamentous Actinobacteria were detected, which is coherent with the pyrosequencing results (Fig. S4). Filamentous Actinobacteria seemed to preferentially colonize damaged root areas (Fig. S13, rectangle), which agrees with the wide catabolic activities typical of Actinobacteria. It might be of positive effect for the plant, when antibiotics-producing Streptomycetes could assist in protection against wound infections by plant pathogens. Non-filamentous Actinobacteria were ubiquitously distributed, and occurred also

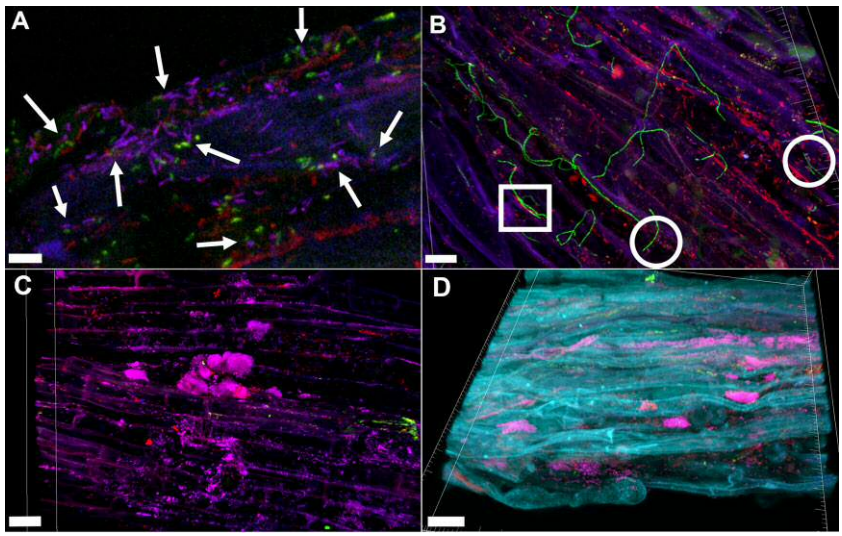

Fig. 6. FISH-CLSM analysis of root-associated bacteria in lettuce.
A. Colocalization and cell–cell interactions between Bacteroidetes (pink/purple) and Alphaproteobacteria (yellow). Red: other bacteria. Scale bar: 5 μm.
B. Filamentous Chloroflexi (green) colonize the root surface of lettuce ubiquitously. Interestingly, within areas low colonized by Bacteroidetes (pink/purple), vicinity of Chloroflexi seems to contribute to their local enrichment (circles). Chloroflexi also showed frequent cell–cell interactions with other bacteria (red) (square; see also Fig. S11). Scale bar: 20 μm.
C–D. Dominance of Betaproteobacteria in the lettuce root system. The relative abundance of Betaproteobacteria appeared much higher than the ~10% expected from the pyrosequencing results. Pink/purple: Betaproteobacteria; Green/yellow: Gammaproteobacteria; Red: other bacteria; Cyan (only in panel D): lettuce root stained with calcofluor. Scale bar: 20 μm.

endophytically inside root hairs (Fig. S14, arrows). When Gammaproteobacteria and Bacteroidetes were codetected in the same field of view, no colocalization could be observed (Fig. S15), suggesting that the correlation *Flavobacterium succinicans*–Pseudomonadaceae (Fs-Ps, Fig. 5) is more likely to be the effect of habitat sharing.

Discussion

In this work, we present new insights into the bacterial microbiota associated with the root system of lettuce (*Lactuca sativa* L.) as represented by eight cultivars and their wild relative prickly lettuce (*L. serriola* L.). Our approach was based on a step-by-step analysis from basic alpha- and beta-diversity to more sophisticated network analysis and integration of FISH-CLSM. The conditions of our sampling site provided an 'open laboratory' for testing the effect of the host genotype on the root microbiota. *Lactuca serriola* showed lower diversity than *L. sativa* cultivars, which indicates that the domestication led to a bacterial diversification within the root system. Also in case of lettuce, crop domestication can be compared with a human-driven evolutionary radiation with positive selection of heritable phenotypes displaying appealing qualities for consumers. We show that domestication of lettuce is supported by a diversified spectrum of associated microbiota and an overall increase of bacterial diversity.

Microbial diversity on plants is crucial to prevent pathogen invasion and a higher abundance of rare species also seems to represents a barrier against pathogens (Chapin *et al*., 2000; Gonzalez *et al*., 2011; Latz *et al*., 2012; Van Elsas *et al*., 2012). Interestingly, in the rare fraction of the microbiota (1 up to 10 reads) of our analysed root microbiota, we found a high number of potentially plant-associated taxa known for their beneficial effect, e.g. Rhizobiales (including Bradyrhizobiaceae), Actinobacteria, Bacillales (including *Paenibacillus*), Burkholderiales, *Pseudomonas*, *Stenotrophomonas*, methylotrophic bacteria and Planctomycetales. In contrast, no potential plant pathogens specific for lettuce were identified (e.g. *Pseudomonas cichorii*, *Rhizomonas suberifaciens* or *Xanthomonas campestris* pv. *vitians*), but Legionellales and Enterobacteriaceae, known to include some dreadful human pathogens (Brandl, 2006), were surprisingly frequent. Due to the presence of beneficials and the absence of plant pathogens in the lettuce root microbiota, we conclude that the high diversity along with a higher abundance of beneficial rare species could enhance the barrier effect against plant pathogens. FISH-CLSM also showed higher numbers of *Streptomyces* at damaged areas of the root, but we could not test whether these could also protect against invasive intruders by production of antimicrobials.

All lettuce cultivar groups displayed diverse root communities with 38 phyla detected (10 phyla > 1% relative

abundance) and an overall similarity in primary profiles and variations at lower taxonomic levels. Beta-diversity measures revealed that statistically significant differences between root microbiota are more pronounced at subspecies (and also cultivar) level than at convar level, which primarily denotes morphological types. Thus, host genetic variation shapes root associated bacterial communities in lettuce, similarly as in other crops such as potatoes (Weinert *et al*., 2011) and cottonwood (Schweitzer *et al*., 2008). Consequently, specific root microbiota not only could influence soil parameters, with effects on soil microbial community, ecosystem functioning and phytosociology, but also potentially modulate the metabolism of the host plant (and hence the nutrient values of different lettuce varieties). Such impact on the host has been demonstrated already for *Arabidopsis* (Badri *et al*., 2013). The influence of the cultivar groups exceeds that of the morphology (i.e. the convar level) which suggests that the host genotype rather than morphology is of influence on the root microbiota. We achieved insights into the structure of the core microbiota of lettuce roots, which comprises 68 OTUs, six of which showed significantly variable relative abundance across subspecies. Using *L. serriola* as a control for specificity, we found five core OTUs occurring exclusively in all lettuce samples, thereby demonstrating that at least a part of this core microbiota remains highly specific even after many years of co-cultivation. So far, only the root core microbiota of the model plant *Arabidopsis thaliana* has been unraveled (Bulgarelli *et al*., 2012; Lundberg *et al*., 2012). Because the main difference of our lettuce microbiota analysis is that we assessed shared OTUs between plants grown in the same soil since more than two decades. A direct comparison with the *Arabidopsis* core microbiota would therefore not be meaningful. Nonetheless, certain interesting similarities are noteworthy: (i) the observations of Comamonadaceae as one of the most abundant taxon, (ii) the ubiquitous occurrence of Sphingobacteriales and *Streptomyces*, (iii) the higher abundance of Pseudomonadeceae *versus* Xanthomonadaceae among the Gammaproteobacteria and (iv) the high abundance of Flavobacteriaceae. In comparison to *Arabidopsis*, the analysed lettuce roots harboured abundant Chloroflexi. They were discarded by Bulgarelli and colleagues (2012) due to both unreliable, extreme abundances in adjacent soil samples and failure to detect them through specific fluorescence in situ hybridization (FISH) staining. In our study, we did not analyse soil samples, but rather confirmed the presence of Chloroflexi in the root system through specific FISH staining and their morphology coincided with the typical filamentous Anaerolineae (Yamada *et al*., 2006). This class of environmental bacteria, typical in aquatic habitats and also found in agricultural soil but never reported as plant inhabitants (Shrestha *et al*., 2009; Yamada and Sekiguchi, 2009), was found here as one of the dominant lettuce-associated microorganisms. Previous works on several *L. sativa* cultivars using 16S rRNA gene clone libraries did not find any Chloroflexi member in the phyllosphere (Hunter *et al*., 2010). Recently, Podosokorskaya and colleagues (2013) described a new Anaerolineae bacterium able to degrade complex polysaccharides such as cellulose, thus suggesting a possible role as degraders of senescing root tissues. However, both the ubiquity and frequent cell–cell interactions observed here suggest more prominent and interactive functions within the root microbiota system.

Correlations between bacterial strains are likely of functional relevance (Van Elsas *et al*., 2012), and were here identified in the lettuce root microbiota. Such correlations are also thought to influence the sequential community assemblages, since communities rather aggregate in deterministic manner than at random (Horner-Devine *et al*., 2007; Barberán *et al*., 2012). However, the predominant organisms are not necessarily involved in modules comprising highly correlated strains in our study. This observation somehow differs from an analysis of plant flower microbiota successional patterns (Shade *et al*., 2013), where the most-prominent strains persisted and co-occurred with minor strains. On the other hand, it might explain why allochthonous species, including human pathogens, can successfully invade the native lettuce microbiota, replacing its dominating, harmless bacteria. The correlation network reconstructed with the genotype-specific OTUs showed that minor, specific OTUs are also involved in frequent co-occurrence. What extent these OTUs directly influence the host plant remains unclear so far. With their low relative abundance, it is tempting to speculate that they may not be important in shaping plant features. Nevertheless, highly correlated OTUs may belong to the same taxonomical group, such as Anaerolinae and Pilellulaceae, respectively (Fig. S8), and the potential role of their joint occurrences merits further study.

Microbiota-mediated colonization resistance against intestinal pathogens was first shown for the human microbiota by Buffie and Pamer (2013). The phenomenon that human pathogens can colonize lettuce plants as well as cause serious outbreaks was often described (Brandl, 2006; Teplitski *et al*., 2011; Ongeng *et al*., 2013) but it can be explained by linking this potential with the bacterial co-occurrence network structure. In addition, there are several reports showing exceptional biocontrol of soil-borne pathogens on lettuce (Scherwinski *et al*., 2008; Adesina *et al*., 2009), which can be also linked with the loose bacterial network, which was identified. According to our results, co-occurrence characterized the relationships among members of the root microbiota as opposed to co-exclusion. Presumably, the distance between

colonies of different organisms reduces competition throughout the whole root system. We were also able to largely reconcile the abundances assessed by 16S rRNA amplicon sequencing with FISH-CLSM results. Such a combined approach is useful, since variation in copy numbers at ribosomal RNA gene loci may bias sequence-based interpretations of abundances (Kembel *et al*., 2012). According to pyrosequencing results, Gammaproteobacteria (dominated by the genus *Pseudomonas*) was the prominent group, whereas Betaproteobacteria (mainly Comamonadaceae) were less frequent, whereas the FISH-CLSM approach suggested the alternative picture. A possible reason can thus be the higher average ribosomal operon copy number found in *Pseudomonas* spp. (5.04) with respect to Comamonadaceae (2.8; data obtained from the Ribosomal RNA Operon Copy Number Database, Klappenbach *et al*., 2001).

In this study, we developed an integrative approach based on co-occurrence analysis and FISH-CLSM, which results in a reliable reconstruction and interpretation of interaction networks in complex microbial communities. This approach thus serves to open new opportunities for future targeted studies on pathogen suppression but also on biocontrol on plants.

Experimental procedures

Sampling site and strategy

Root systems of 24 fully matured lettuce plants (*Lactuca sativa* L.) in the final growing stage (leaf development and partly flowering) and 3 *Lactuca serriola* L. (the wild ancestor) were collected in Schiltern, Austria (+48°31′ +15°37′) at the same time. Sampling was from the field of the Arche Noah: a non-profit association devoted to the preservation of plant cultivars in Europe including those which are no longer used in horticulture (http://www.arche-noah.at). The sampling site has been a field parcel since 1990, where numerous lettuce cultivars, representing all lettuce subspecies, along with the wild ancestor, are conserved and planted intermingled (distance between individuals: 30–50 cm) (Fig. S1). This special location represented a field laboratory for investigating the long-term effect of the plant genotype on the root-associated microbiota, under field conditions and under completely levelled environmental/pedological factors, and has the characteristic of an organic managed 'common garden'. At the sampling time, all plants were at the same growth stage (Fig. S1). Three plants per lettuce cultivar and three *L. serriola* plants were available for sampling and were collected. Two cultivars per subspecies were selected (thus, each subspecies included six samples). Since two subspecies belongs to the convar incocta and the other two ones to the convar sativa (Fig. 1), this sampling strategy allowed us to compare lettuce microbiota at convar, subspecies and cultivar level. Moreover, we used *L. serriola* to understand the effect of lettuce domestication on bacterial diversity and as a control for the specificity of the *L. sativa* core microbiota.

DNA extraction

The microbial fraction associated with lettuce roots was extracted according to Bragina and colleagues (2012). Briefly, after gentle removal of the adhering soil and washing into 0.85% NaCl to remove rhizosphere soil and loosely adhering microorganisms, ~ 5 g of roots were physically disrupted with a sterile pestle and mortar and resuspended in 10 ml of 0.85% NaCl. Two millilitre of suspension were then centrifuged (16 500 *g*, 20 min, 4°C) and the obtained pellet was used for isolation of the total community DNA with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). For mechanical lysis, the cells were homogenized twice in a FastPrep® FP120 Instrument (Qbiogene, BIO101, Carlsbad, CA, USA) for 30 s at a speed of 5.0 m s^{-1} and treated according to the manufacturer's protocol. Crude DNA extracts were purified with Geneclean turbokit (MP Biomedicals, Illkirch, France) to improve quality for downstream PCR reactions.

454-amplicon sequencing and data analysis

The V4 region of the 16S rRNA genes were amplified with multiplex identifier (MID) tagged universal bacterial primers F515 and R806 (Caporaso *et al*., 2011). The PCR reaction mixture (25 µl final volume) contained 1 × Taq&Go, 0.25 µM of each primer, 2 mM MgCl2 and 1 µl of template corresponding to 40–70 ng of DNA (96°C, 4 min; 32 cycles of 96°C, 1 min; 64°C, 1 min; 74°C, 1 min; and final elongation at 74°C, 10 min). The products of three independent PCR reactions per sample were pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Purified PCR products were pooled (200 ng each) and sequenced using the Roche GS FLX+ 454 Titanium platform by Macrogen Korea (Seoul, South Korea). The nucleotide sequences obtained in this work were submitted to the European Nucleotide Archive (www.ebi.ac.uk/ena) and are available under the accession number PRJEB5101.

Sequences ('reads') were analysed with the QIIME software version 6.0 (Caporaso *et al*., 2010). MID, primer and adapter sequences were removed prior to length and quality filtering (including option '-w') of sequences with final denoising. Four different OTU tables were created with similarity levels ('cut-off levels') of 90%, 95%, 97% and 100%. We decided to investigate the effect of different OTU similarity cut-off levels on the taxonomical structure and correlations because of discordant opinions about sequence similarity thresholds for species and genus delimitation (e.g. Stackebrandt and Ebers, 2006, Yarza *et al*., 2008).

For each OTU table, sequences of plastidal or mitochondrial origin, as well as chimeras (detected with chimera slayer; as part of QIIME) were removed. The dataset was normalized to 2200, 2150 or 2023) reads per sample (whole microbiota, no singletons and > 10 reads only respectively).

Statistical comparison of alpha diversity between samples was performed with the software SPSS 20 (IBM Corporation, Armonk, NY, USA), using *t*-test, ANOVA or non-parametric tests depending on both the number of groups (2 versus > 2) and the distribution of the variable (normal versus non-normal). Sample-specific OTUs (showing significantly different relative abundances between samples) were assessed

by ANOVA after FDR correction of the *P*-value (q-value), while the core microbiota comprises OTUs occurring in all *L. sativa* root microbiota.

Correlation and network analysis

Spearman correlation between OTU occurrence patterns was calculated in order to identify potential interactions between OTUs, similar as in other studies (Barberán *et al*., 2012; Faust *et al*., 2012), but here we also used the 100% cut-off level OTUs. This rigorous approach avoided apocryphal occurrence patterns which may occur already at 99% cut-off level OTUs (Patin *et al*., 2013). Only strong correlations ($R > 0.6$ or < -0.6, and $P < 0.01$) were considered and visualized through network analysis using Cytoscape 2.8 (Smoot *et al*., 2011) by applying the spring embedded layout. Only abundant OTUs (> 0.5% of the total microbiota) were included due to the fact that our aim was to complement this analysis with FISH-CLSM whose detection limit ranges between 0.2% (Cardinale *et al*., 2012) and 1% (Mogge *et al*., 2000). Moreover, not including rare OTUs also eliminates PCR artefacts generated by the typical PCR error rates (Patin *et al*., 2013). In addition, we calculated the correlation of occurrence patterns between genotype-specific OTUs of the lettuce root microbiota (at 97% OUT cut-off level).

FISH-CLSM

Root subsamples were fixed with paraformaldehyde for 6 h, washed three times with cold PBS and then stored at –20°C in 1:1 (vol : vol) 96% ethanol: PBS until FISH-CLSM analysis. FISH was performed using the phylum- and class-specific probes listed in (Table S2) according to Cardinale and colleagues (2012). Briefly, after pre-treatment with Lysozyme, Cy3-, Cy5- and ATTO488-labelled FISH probes were applied to confirm the presence of all dominant phyla/classes detected by pyrosequencing. Additionally, we combined the FISH probes to attempt visualizing in situ the sequence-based correlated taxa. FISH-stained samples were mounted with SlowFade Gold Antifadent (Molecular Probes, Eugene, OR, USA) and stored at 4°C until observation with a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany). For each field of view, an appropriate number of optical slices were acquired with a Z-step of 0.15 – 0.5 μm ('confocal stacks'), and the software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used for visualization and post-processing. For each bacterial phylum/class, at least two independent FISH experiments were performed on two different lettuce subspecies, and a minimum of 20 confocal stacks were compared. Adobe Photoshop CS2 version 10.0.1 (Adobe Systems, USA) was used to assemble and label the figures.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Sampling site. Field plot of Arche Noah seed savers association (www.arche-noah.at, Schiltern, Austria; N48°31′, E15°37′) where numerous *Lactuca sativa* cultivars, representing all subspecies, along with the wild ancestor *Lactuca serriola*, are conserved and planted intermingled since 1990 (distance between individuals: 30–50 cm).

Fig. S2. Properties of four OTU tables created with different cut-off levels and corresponding rarefaction curves.

Fig. S3. Taxonomic structure of the lettuce root microbiota ($n = 24$, all lettuce samples including also *L. serriola*) as retrieved at different OTU cut-off levels.

Fig. S4. (A) Abundant OTUs (> 0.5% relative abundance to the whole microbiota) at different OTU cut-off levels. (B) Number of abundant OTUs at different OTU cut-off levels and corresponding number of sequences. Fraction (percentage) with respect to both the total OTUs and the total sequences are indicated into parenthesis.

Fig. S5. Beta-diversity plot created with weighted UniFrac distances. Nodes represent whole lettuce root microbiota at 97% OTU cut-off level. Different node colours represents different lettuce subspecies (also delimited by circles), whereas white edges connect samples belonging to the same cultivars. For this plot, Adonis values were $P = 0.0238$, $P = 0.0024$ and $P = 0.00002$ for convar, subspecies and cultivars respectively.

Fig. S6. OTUs showing a significant difference in their relative abundance between lettuce subspecies. Bars indicate the relative abundance to the whole microbiota (left Y-axis) and dots indicate significance values obtained by ANOVA after FDR correction (right Y-axis). OTUs labelled with 'C' are also occurring in the shared microbiota (Fig. 4).

Fig. S7. Network analysis showing the correlations between abundant OTUs (> 0.5% of the total microbiota) at different cut-off levels; coloured by phylum/class. Nodes represent phyla/classes and edges represent strong positive ($R > 0.6$, $P < 0.01$) or negative ($R < -0.6$, $P < 0.01$) Spearman correlations (blue and red lines respectively). Edge thickness represents the number of the interactions between OTUs belonging to the respective phylum/class. Node size indicates phylum/class abundance (percentage of the total microbiota) and node label indicates number of OTUs within the phylum/class. (A) 90% cut-off level OTUs; (B) 95%; (C) 97%; (D) 100%.

Fig. S8. Correlation of occurrence patterns between genotype-specific OTUs of the lettuce root microbiota, calculated at 97% cut-off level. Nodes represent OTUs and edges represent strong positive ($R > 0.6$, $P < 0.01$) or negative ($R < -0.6$, $P < 0.01$) Spearman correlations (blue and red lines respectively). OTU abundance (number of reads) and taxonomic affiliation (at phylum or class level) are indicated by node size and node colour respectively. Node labels indicate the taxonomic identification of the OTUs: Ac, Acidobacteria; Ac6, Acidobacteria-6; Acb, Actinobacteria; An, Anaerolineae; B, Bacteria; Bu, Burkholderia; Chi, Chitinophagaceae; Ch, Chloracidobacteria; Dy, Dyadobacter; El, Elusimicrobia; Fla, Flammeovirgaceae; Flb, Flavobacteria; Fle, Flexibacteraceae; Ga, Gammaproteobacteria; Ge, Gemmatimonadetes; Ge, Gemmata; Kr, Kribella; Meb, Methylobacillus; My, Myxococcales; No, Nocardioidaceae; Op, Opitutales; Ph, Phycispherae; Pi, Pirellulaceae; Pl, Planctomycetes; Pr, Pirellula; Ps, Pseudomonadaceae; Rh, Rhodobacteraceae; Ro, Roseiflexaceae; Si, Sinobacteraceae; Spb, Sphingobacteriales; Ste, Steroidobacter; Sx, *Sphingobium xenophagum*; TM1, TM7-1 (Saccharibacteria); Ve, Verrucomicrobiaceae; Xa, Xanthomonadaceae.

Fig. S9. FISH-CLSM analysis of root-associated bacteria in lettuce. Alphaproteobacteria (yellow) and Gammaproteobacteria (pink/purple) ubiquitously colonize the lettuce root both endophytically and on the surface. Red: other bacteria. A: volume rendering; B: 3D model; C: 3D model with transparent root signal (grey), to allow visualizing the endophytic bacteria. Scale bars: 20 μm.

Fig. S10. Pure culture of a *Flavobacterium* sp. stained by FISH using the CF319a/b FISH probe (Table S2). Scale bar: 5 μm.

Fig. S11. FISH-CLSM analysis of root-associated bacteria in lettuce. Bacteroidetes (pink/purple) were abundant on root tips (A) and less diffused on other parts of the root (B). Red: other bacteria. Scale bars: 20 μm.

Fig. S12. FISH-CLSM analysis of root-associated bacteria in lettuce. Anaerolinae (green) colonized the lettuce root ubiquitously and showed frequent cell–cell interactions with other bacteria (red). Scale bars: A, B and D, 5 μm; C, 10 μm.

Fig. S13. FISH-CLSM analysis of root-associated bacteria in lettuce. Filamentous Actinobacteria (likely *Streptomyces* spp.) colonize the lettuce root, forming more dense colonies on the damaged areas. This could determine a protection from plant pathogens due to the antimicrobial substances usually produced by Streptomycetes. A–D: individual confocal channels showing Actinobacteria (A), root tissues stained by calcofluor white (B), root autofluorescence (C) and all bacteria (D); E: overlap of the channels A–D. Here, the Actinobacteria appears yellow. Scale bar: 20 μm.

Fig. S14. FISH-CLSM analysis of root-associated bacteria in lettuce. A: XY-XZ-YZ projections of a confocal stack showing non-filamentous Actinobacteria (yellow) growing endophytically inside a root hair (arrows). B: volume rendering. C: three-dimensional model. Red: other bacteria. Grey: root tissues. Scale bars: A, 20 μm; B–C: 5 μm.

Fig. S15. FISH-CLSM analysis of root-associated bacteria in lettuce. Although abundant, Gammaproteobacteria and Bacteroidetes did not tend to be colocalized when detected in the same field of view. Yellow and purple circles delimitate the area of the root colonized by Gammaproteobacteria (yellow cells) and Bacteroidetes (pink/purple cells) respectively. Scale bars: A, 15 μm; B and C, 10 μm.

Table S1. Statistical significance of differences for Shannon, Equitability and Chao1 indices between samples (from species to cultivar level) at different cut-off levels, with and without singletons. Significant differences ($P < 0.05$) are in bold and marked with an asterisk.

Table S2. FISH probes used in this work.

Bacterial networks and co-occurrence relationships in the lettuce root microbiome

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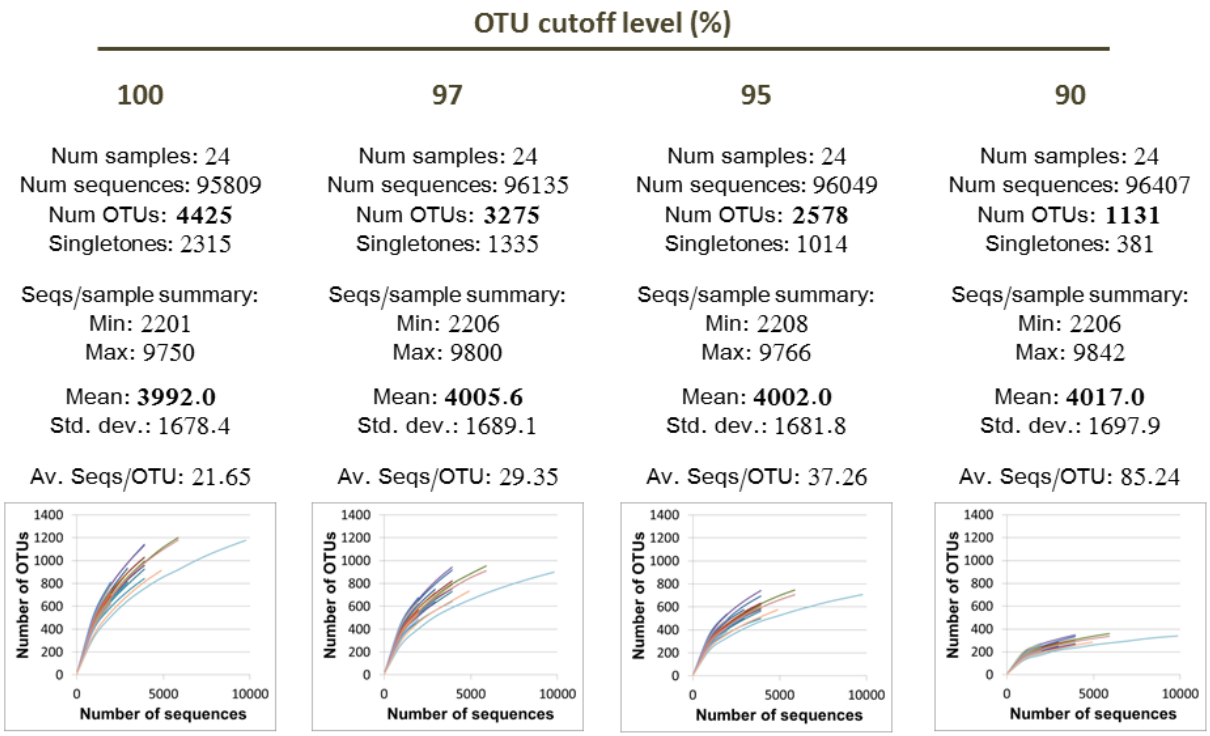
Supplementary figure material

Fig. S1



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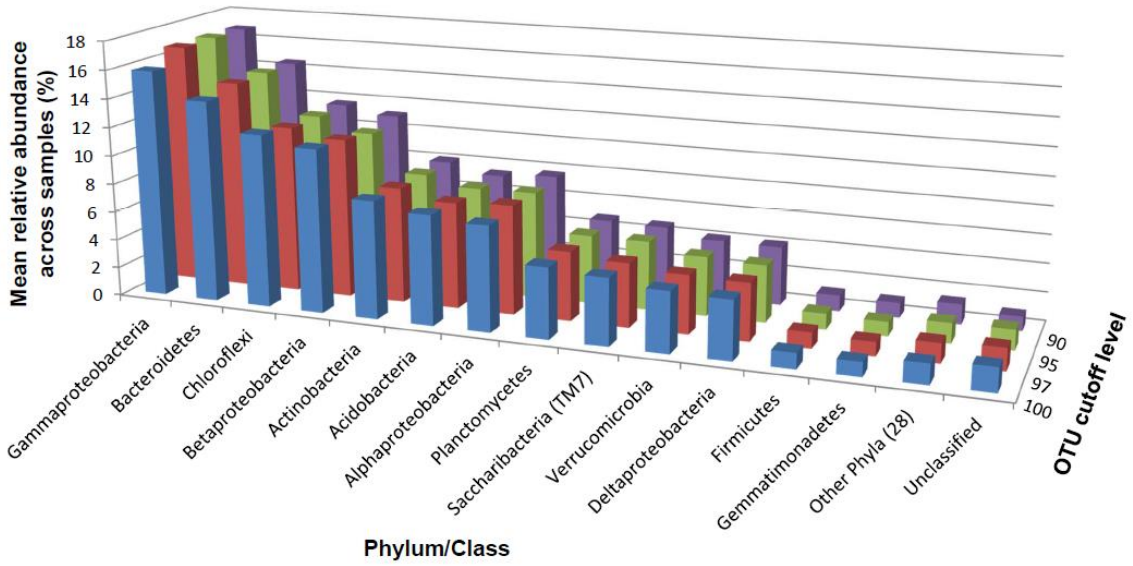
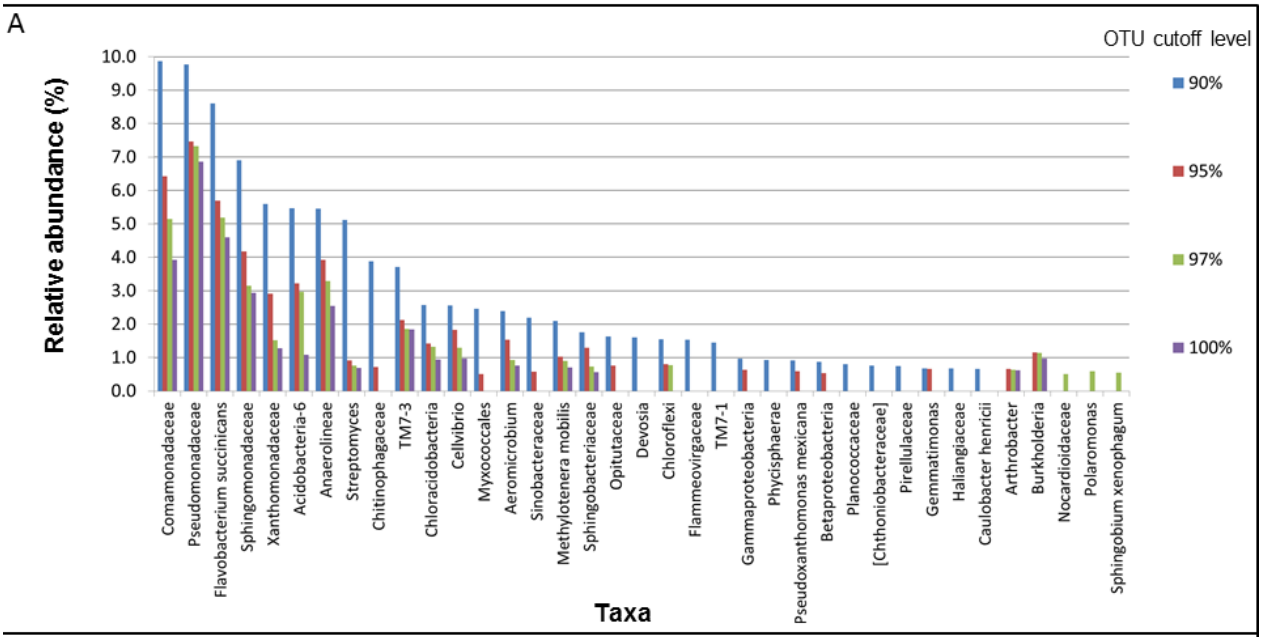


Fig. S3_SuppInfo

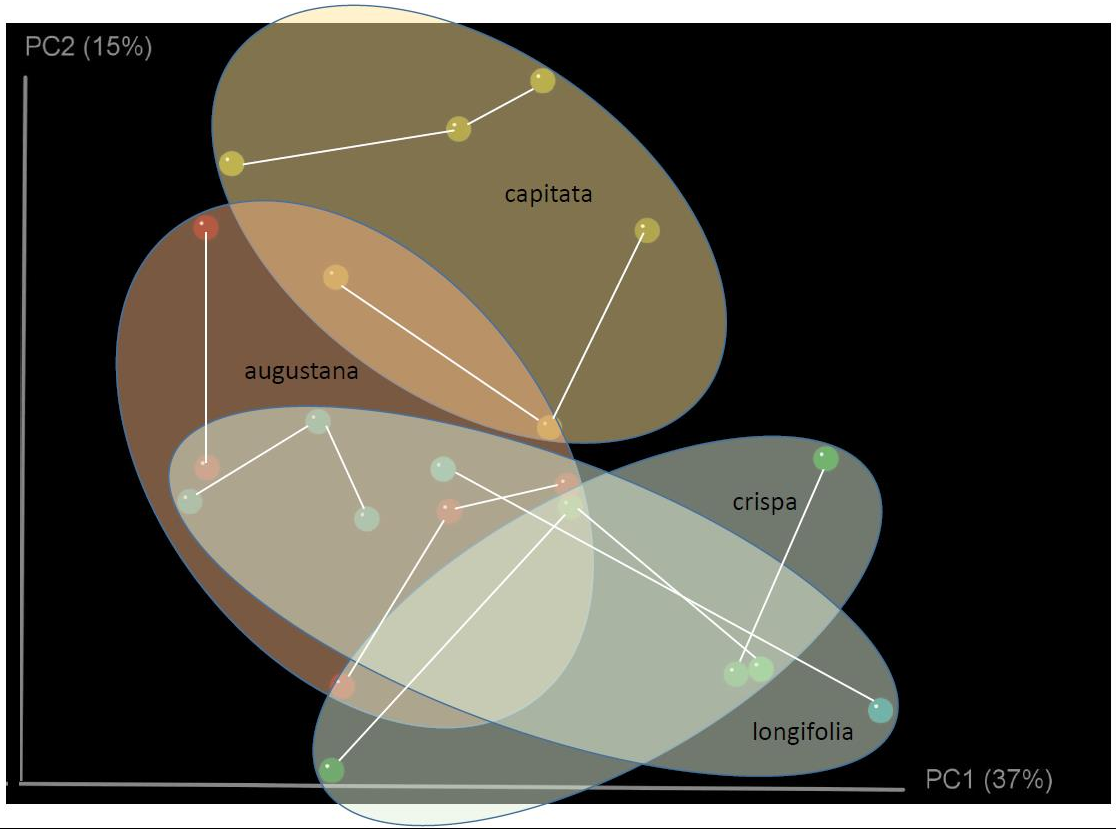
A



B

Abundant OTUs (>0.5% of the total microbiome)	OTU cutoff level			
	100%	97%	95%	90%
N. OTUs (% of total OTUs)	22 (0.5)	27 (0.82)	32 (1.24)	37 (3.27)
N. Seqs. (% of total seqs.)	29983 (31.3)	38980 (40.5)	49487 (51.5)	71923 (74.6)

Fig. S4_SuppInfo



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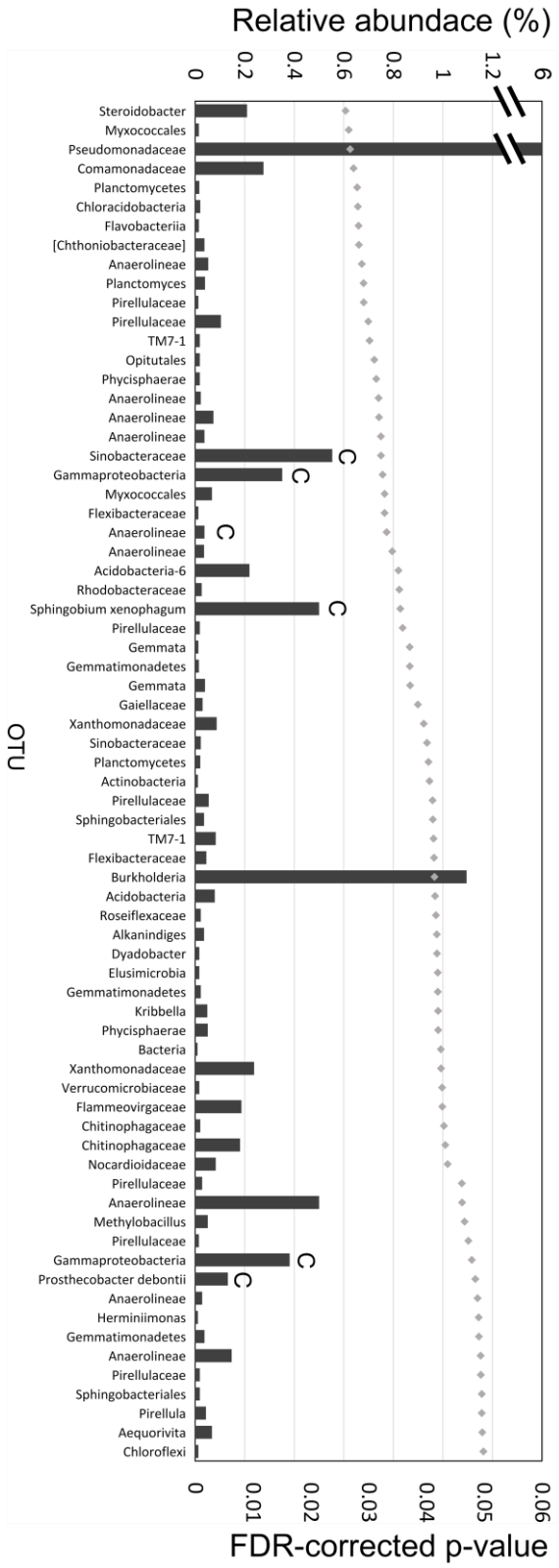

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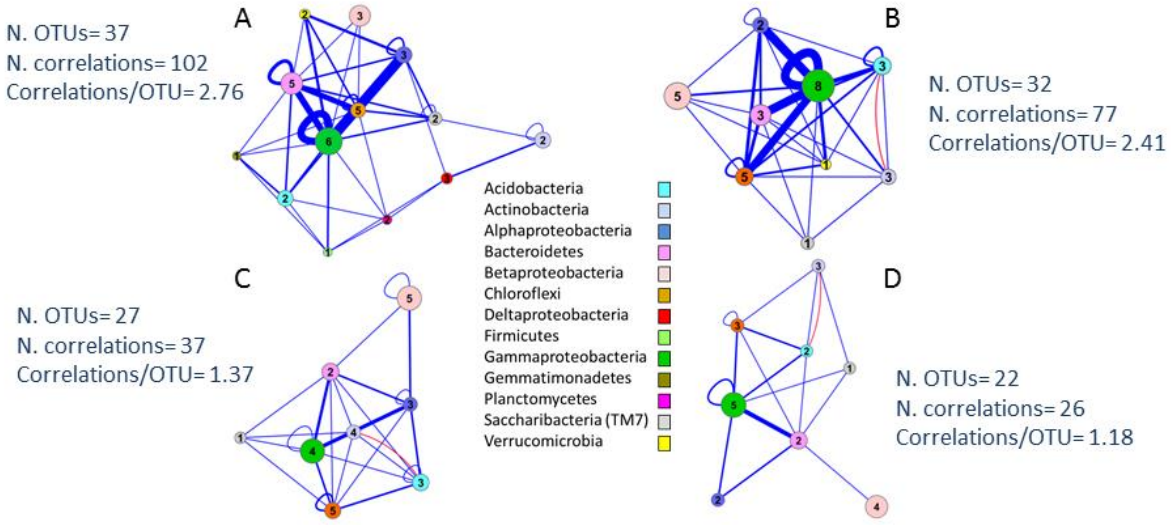

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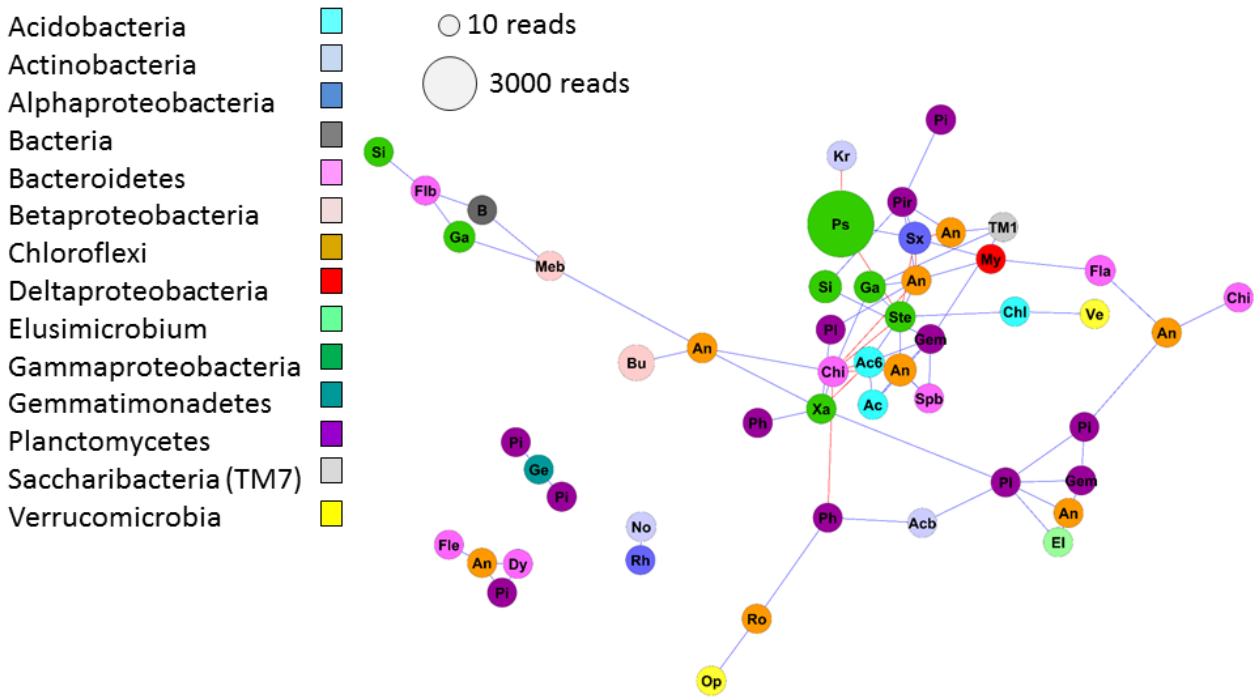

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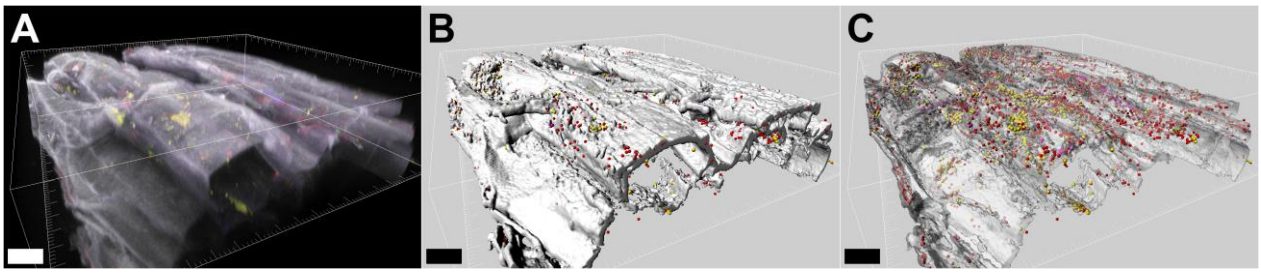

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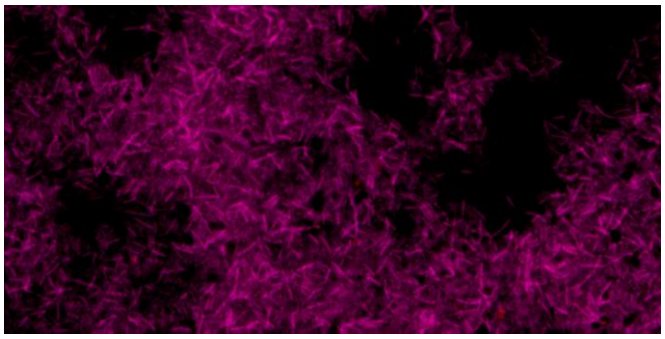

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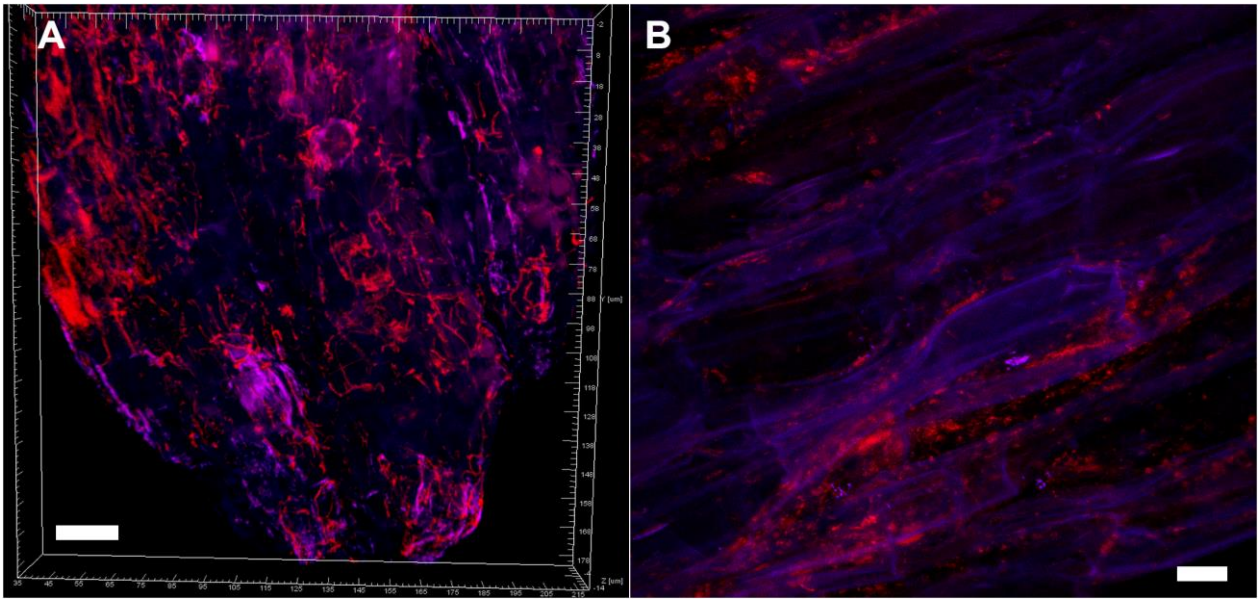

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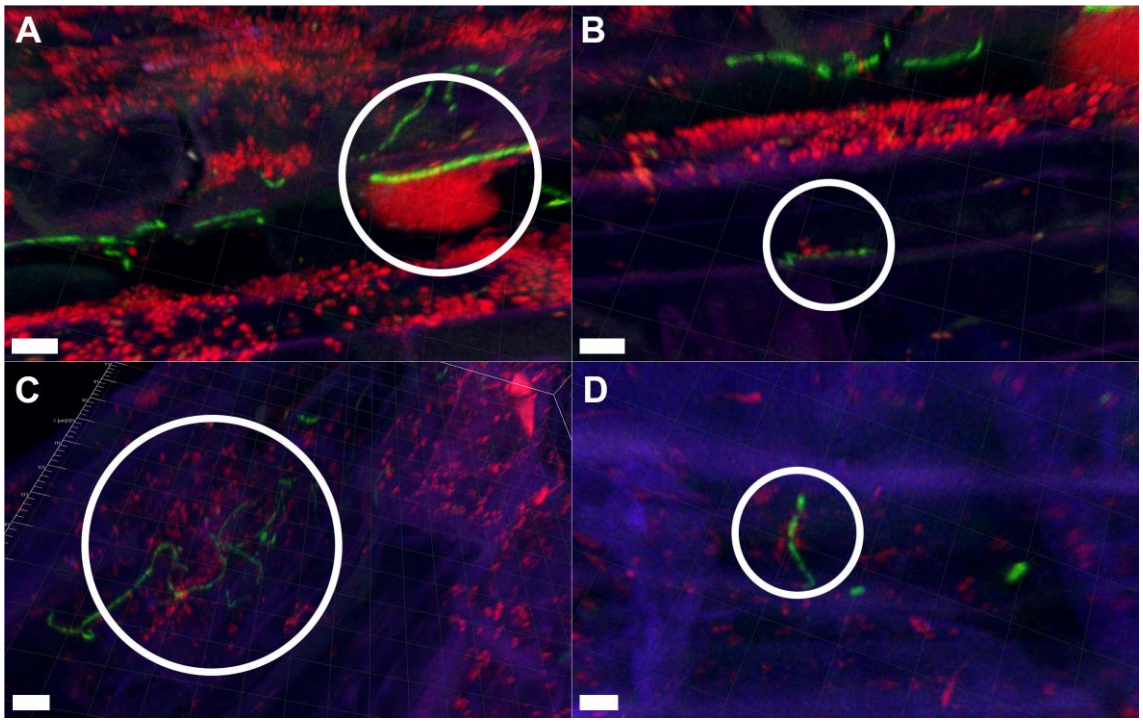

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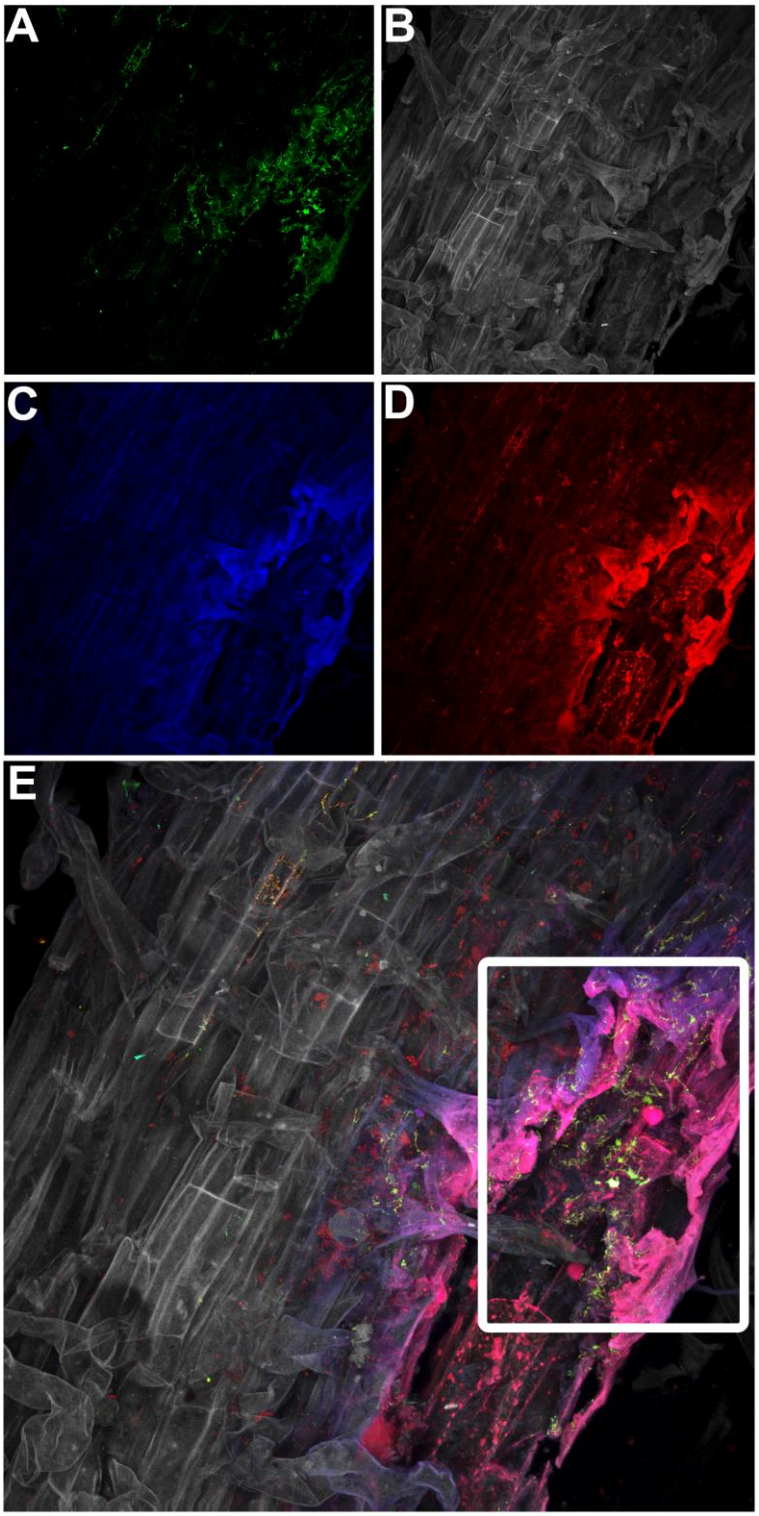

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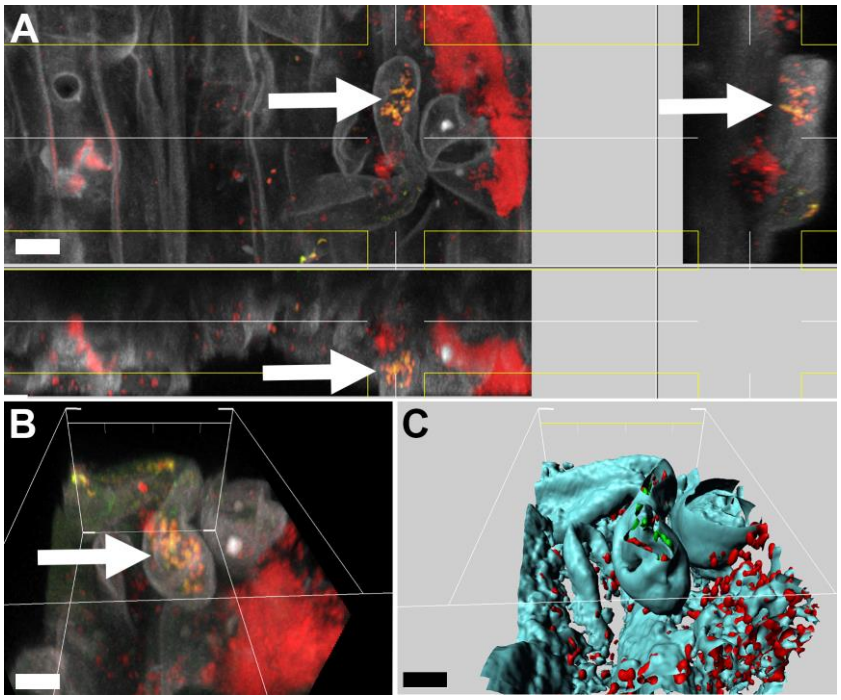

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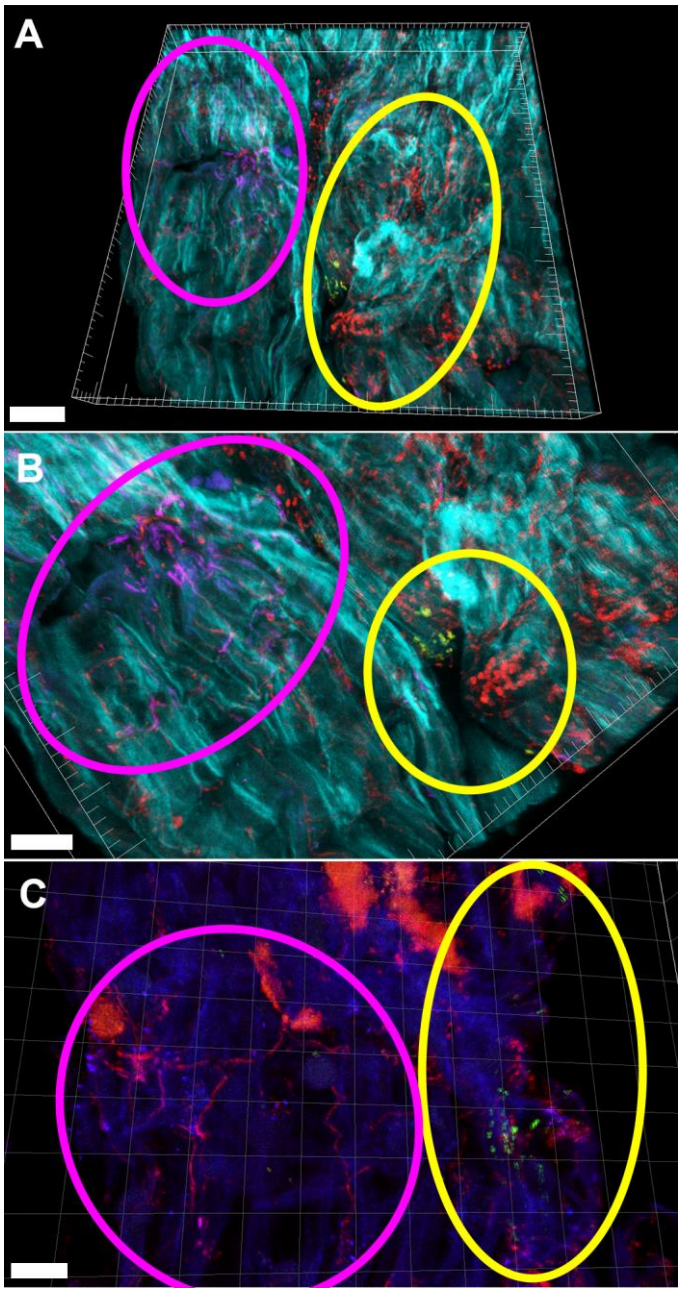

Fig. S15_SuppInfo

Supplementary material

Table S1 Statistical significance of differences for Shannon, Equitability, and Chao1 indices between samples (from species to cultivar level) at different cutoff levels, with and without singletons. Significant differences (p<0.05) are in bold and marked with an asterisk.

Plant taxonomic level	OTU cutoff level (%)	Shannon			Equitability			Chao1		
		Whole microbiome	No singletons	>10 reads	Whole microbiome	No singletons	>10 reads	Whole microbiome	No singletons	>10 reads
Species	100	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***	0.071	**0.006***	**0.006***
	97	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***	0.188	**0.034***	**0.001***
	95	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***	0.114	**0.016***	0.418
	90	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***	**<0.001***	0.164	**0.045***	0.914
Convar	100	0.219	0.202	0.296	0.190	0.153	0.251	0.325	0.506	0.968

	97	0.193	0.183	0.138	0.110	0.087	0.234	0.476	0.893	0.825
	95	0.200	0.132	0.163	0.140	0.087	0.088	0.191	0.213	0.563
	90	0.377	0.542	0.453	0.553	0.620	0.422	0.733	0.264	0.321
Subspecies	100	0.501	0.597	0.637	0.578	0.565	0.644	0.783	0.570	0.082
	97	0.522	0.595	0.663	0.469	0.415	0.521	0.376	0.556	0.673
	95	0.493	0.429	0.524	0.517	0.411	0.427	0.382	0.599	0.929
	90	0.224	0.236	0.250	0.350	0.332	0.198	0.367	**0.023***	0.441
Cultivar	100	0.071	0.091	**0.041***	**0.047***	0.088	**0.039***	0.369	**0.027***	0.092
	97	**0.040***	0.054	0.083	**0.021***	**0.033***	**0.046***	**0.042***	0.155	0.868
	95	**0.041***	0.065	0.077	**0.036***	0.054	**0.036***	0.262	0.495	0.450
	90	**0.041***	**0.034***	**0.023***	0.089	0.067	0.055	0.549	0.136	**0.029***

Supplementary material

Table S2 FISH probes used in this work

Probe name	Sequence (5'- -3')	Fluorescent dye	Target	% FA (43°C)	Reference
EUB338[1]	GCT GCC TCC CGT AGG AGT	Cy3	Most bacteria	10−20	Amann et al, 1990
EUB338II[1]	GCA GCC ACC CGT AGG TGT	Cy3	Planctomycetales	10−20	Daims et al, 1999
EUB338III[1]	GCT GCC ACC CGT AGG TGT	Cy3	Verrucomicrobiales	10−20	Daims et al, 1999
HGC236	AAC AAG CTG ATA GGC CGC	Cy5	Actinobacteria	10–20	Erhart, et al., 1997
ALF968	GGT AAG GTT CTG CGC GTT	Cy5	Alphaproteobacteria	40	Neef 1997
BET42a[2]	GCC TTC CCA CTT CGT TT	ATTO488	Betaproteobacteria	40	Manz et al, 1992
GAM42a[2]	GCC TTC CCA CAT CGT TT	Cy5	Gammaproteobacteria	40	Manz et al, 1992
SS_HOL1400	TTC GTG ATG TGA CGG GC	Alexa 488	Acidobacteria	20	Meisinger et al., 2007

CF319a/b	TGG TCC GTR TCT CAG TAC	ATTO488	Bacteroidetes	35	Manz et al, 1996
TM7905	CCG TCA ATT CCT TTA TGT TTT A	Cy5	Saccharibacteria (TM7)	20	Hugenholz et al., 200
CFX1223[3]	CCA TTG TAG CGT GTG TGT MG	Cy5	Chloroflexi	35	Björnsson et al, 2002
GNSB-941[3]	AAA CCA CAC GCT CCG CT	Cy5	Chloroflexi	35	Björnsson et al, 2002
NONEUB	ACT CCT ACG GGA GGC AGC	[4]	/	[5]	Wallner et al, 1993

1 and 3: used mixed in equimolar concentration; 2: used together in the same experiment (the probes are competitors each other); 4 and 5: the same fluorochrome and the same percentage of formamide than used for positive FISH probe in the same experiment.

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Publication IV

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Vegetable microbiomes: is there a connection among opportunistic infections, human health and our 'gut feeling'?

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Opinion

Vegetable microbiomes: is there a connection among opportunistic infections, human health and our 'gut feeling'?

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Summary

The highly diverse microbiomes of vegetables are reservoirs for opportunistic and emerging pathogens. In recent years, an increased consumption, larger scale production and more efficient distribution of vegetables together with an increased number of immunocompromised individuals resulted in an enhanced number of documented outbreaks of human infections associated with the consumption of vegetables. Here we discuss the occurrence of potential pathogens in vegetable microbiomes, the impact of farming and processing practices, and plant and human health issues. Based on these results, we discuss the question if vegetables can serve as a source of infection for immunocompromised individuals as well as possible solutions to avoid outbreaks. Moreover, the potentially positive aspects of the vegetables microbiome for the gut microbiota and human health are presented.

Pathogenicity, (opportunistic) pathogens and immunocompromised individuals

Pathogenicity to humans, animals and plants is the most acclaimed feature of microorganisms. Traditionally, pathogens are defined as causative agents of diseases, guided by Koch's postulates for more than a century and further improved by molecular criteria (Fredericks and Relman, 1996). Next generation sequencing-based technologies have revolutionized our knowledge not only on the microbiome, but also about pathogens drastically (Jansson *et al.*, 2012; Berg *et al.*, 2013; Bergholz *et al.*, 2014). The human microbiome is involved in many more human diseases than recently thought, and microbial imbalances can be responsible for severe diseases (Tremaroli and Bäckhed, 2012; Blaser *et al.*, 2013). Pathogen outbreaks are associated with shifts of the whole community including those supporting pathogens as well as opportunistic pathogens (Clemente *et al.*, 2012). On the other side, microbial diversity is an important factor determining the invasion of pathogens; reduced diversity supports opportunistic infections (van Elsas *et al.*, 2012; Pham and Lawley, 2014).

Opportunistic pathogens usually do not cause disease in a healthy, immunocompetent host; they take advantage of certain situations, for example, from compromised immune system of patients, which presents an 'opportunity' for the pathogen to infect. The number of immunocompromised individuals rises continuously worldwide and can be caused not only by recurrent infections, advanced human immunodeficiency virus (HIV) infection and genetic predisposition, but also by medical treatments, for example immunosuppressive agents for organ transplant recipients, chemotherapy for cancer or long-term antibiotic treatments (Klevens *et al.*, 2007; Fishman, 2013). A substantial number of opportunistic pathogens cause health-care-associated infections (HAIs) or nosocomial infections, because in health-care settings (e.g. wards, outpatient haemodialysis units, or same-day surgery), the number of immunocompromised individuals is high. In addition, the indoor environments of these settings contain a specific microbiome including diverse opportunistic pathogens (Oberauner *et al.*, 2013). HAIs are associated with significant morbidity, mortality and cost. According to the US National Nosocomial Infections Surveillance system, in 2002, the estimated number of HAIs in US hospitals was approximately 1.7 million (Klevens *et al.*, 2007). Opportunistic infections

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remain a major health problem worldwide and can limit immunosuppression therapies (Fishman, 2013). Interestingly, a worldwide study identified a significant association between the risk of death because of opportunistic infections in intensive care units and the global national income (Vincent *et al*., 2014). Although an excellent concordance between US and European definitions of HAIs was reported (Hansen *et al*., 2012), the taxonomic spectrum of opportunistic pathogens varies from hospital to hospital and is influenced by biogeographic aspects. Besides viruses, fungi and protozoa, a long list of bacterial pathogens causes opportunistic infections. The most reported species include the Gram-positive *Staphylococcus aureus* including methicillin-resistant *S. aureus*, *Enterococcus* species (*E. faecalis*, *E. faecium*), and Gram-negative bacteria like *Escherichia coli*, and *Pseudomonas aeruginosa* (Sydnor and Perl, 2011). Moreover, today, the antibiotic-resistant Gram-negative microorganisms, for example *Acinetobacter*, *Enterobacter*, *Klebsiella* (*K. pneumonia, K. oxytoca*), *Proteus*, *Pseudomonas*, *Serratia* and *Stenotrophomonas* are particularly troublesome, especially in the development of hospital-acquired infections (Sydnor and Perl, 2011). HAIs are associated with a broad range of diseases and symptoms: they can cause severe pneumonia, bloodstream infections, urinary tract infections, surgical site infections and other infections. In addition to the direct effects, opportunistic infections and the microbiome may adversely shape the host immune responses (Fishman, 2013).

Patients with cystic fibrosis are specifically prone to opportunistic infections. This hereditary disease affects the epithelial innate immune function in the lung, resulting in exaggerated and ineffective airway inflammation that fails to eradicate pulmonary pathogens. Pulmonary infection is therefore the most challenging problem in the management of cystic fibrosis and is the major determinant of life span and quality of life in affected individuals. Although the most important opportunistic pathogens are again *P. aeruginosa* and *S. aureus*, the number of causative species is higher and also includes the *Burkholderia cepacia* complex, *Burkholderia gladioli*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Ralstonia*, *Cupriavidus* and *Pandoraea* species (LiPuma, 2010).

Are there common characteristics of opportunistic pathogens? Although opportunistic pathogens have a broad phylogenetic background and include strains affiliated to *Firmicutes* (*Staphylococcus*, *Enterococcus*), *Betaproteobacteria* (*Burkholderia*) and *Gammaproteobacteria* (*Pseudomonas*, *Stenotrophomonas*, *Acinetobacter*, *Klebsiella*, *Escherichia*, *Enterobacter*, *Proteus*, *Serratia*), they share some properties. Opportunistic pathogens occur in natural environments and are often associated with other eukaryotic hosts such as plants. They are often characterized by several of the following properties: (i) r-strategists = copiotrophs, (ii) cultivable, (iii) antagonistic towards other microorganisms, (iv) highly competitive, (v) highly versatile in their nutrition, (vi) hypermutators, (vii) resistant against antibiotics and toxins and (viii) form biofilms. It is important to note that typically these traits were acquired via horizontal gene transfer and are strain specific (Rossi *et al*., 2014). It is predicted that in future decades, other lesser-known pathogens and new bacterial strains of bacteria will emerge as common causal agents of infections (Sydnor and Perl, 2011); therefore, it is important to understand the ecology of potentially emerging pathogens.

The vegetable microbiome

In a basic study, Leff and Fierer (2013) found that vegetables harboured diverse bacterial communities dominated by the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria*, but their composition was significantly different for each vegetable species. These differences were often attributable to distinctions in the relative abundances of *Enterobacteriaceae* taxa (Leff and Fierer, 2013). This large family of Gram-negative bacteria includes, along with many harmless symbionts, many of the more familiar so-called enteric pathogens that also play an important role as opportunistic pathogens (Brandl, 2006; Rastogi *et al*., 2012). However, according to these studies, they are an important component of the indigenous vegetable microbiome. In addition to raw vegetables, fermented fresh-like vegetables are a substantial part of our diet worldwide, and specific traditional products exist in different areas, for example 'Kimchi' in Korea or 'Sauerkraut' in Germany. Lactic acid fermentation using indigenous bacteria or starter cultures induce shifts to the bacterial community (Di Cagno *et al*., 2013).

Lettuce has a special position within the vegetable group; it is among the most popular raw-eaten vegetables with a global consumption of 24.6 Mio t (The Statistics Division of the Food and Agriculture Organization of the United Nations) and provides a habitat for specific microbes (Rastogi *et al*., 2012). The authors found high abundances 10^5–10^6 colony-forming unit (cfu) g^{-1} fw and diversities with a high proportion of *Enterobacteriaceae* in the phyllosphere of field-grown Romaine lettuce. *Enterobacteriaceae* taxa are present not only in the gammaproteobacterial microbiome of the lettuce phyllosphere und comprise potential beneficial bacteria, but also potential pathogens (Erlacher *et al*., 2014). In the German monitoring system of pathogens, verocytotoxin-producing *Escherichia coli* were found in 1.3% (0.4–3.4) and *E. coli* in 3.8% of the investigated lettuce samples (Käsbohrer *et al*., 2014). Washing steps and adding of detergents to sanitizer solutions failed in decontamination (Keskinen and Annous, 2011). This can be explained by an endophytic colonization of bacteria observed by Berg *et al*. (2014).

Omics approaches are starting to yield practical food safety solutions, but currently, only few studies are available (Bergholz *et al*., 2014). We used our metagenomic dataset of rucola (syn. arugula, *Eruca sativa* Mill.), which is widely popular as a salad vegetable, to detect frequently reported opportunistic pathogens (A. Erlacher and G. Berg, unpubl. data). Altogether, using the Greengenes database, the fraction of opportunistic pathogens comprised about 1.7% of the total bacterial community with the dominance of *Pantoea agglomerans* and *Stenotrophomonas maltophilia* – both are known for their ambivalent interactions with plants and humans (Fig. 1). In addition, a high proportion of genes involved in functions such as virulence, disease and defence were identified in the rucola phyllosphere, rhizosphere and the surrounding bulk soil (Fig. 2). This cluster contains functions for the subgroups responsible for adhesion, bacteriocin production and ribosomally synthesized antibacterial peptides, detection, invasion and intracellular resistance, resistance to antibiotics and toxic compounds, and toxins and superantigens. Interestingly, except the subgroup of toxins and superantigens, which is absent in the phyllosphere, comparable patterns for all three investigated habitats were found.

Farming and processing practices have an important influence on the composition of associated microbial communities (Leff and Fierer, 2013). Larger scale production and more efficient distribution of fresh vegetables over the past two decades have contributed to an increase in the number of illness outbreaks (Olaimat and Holley, 2012). Organic farming practices can differ from conventional farming practices, including the types of fertilizer and pesticides that are used, and these differences have the

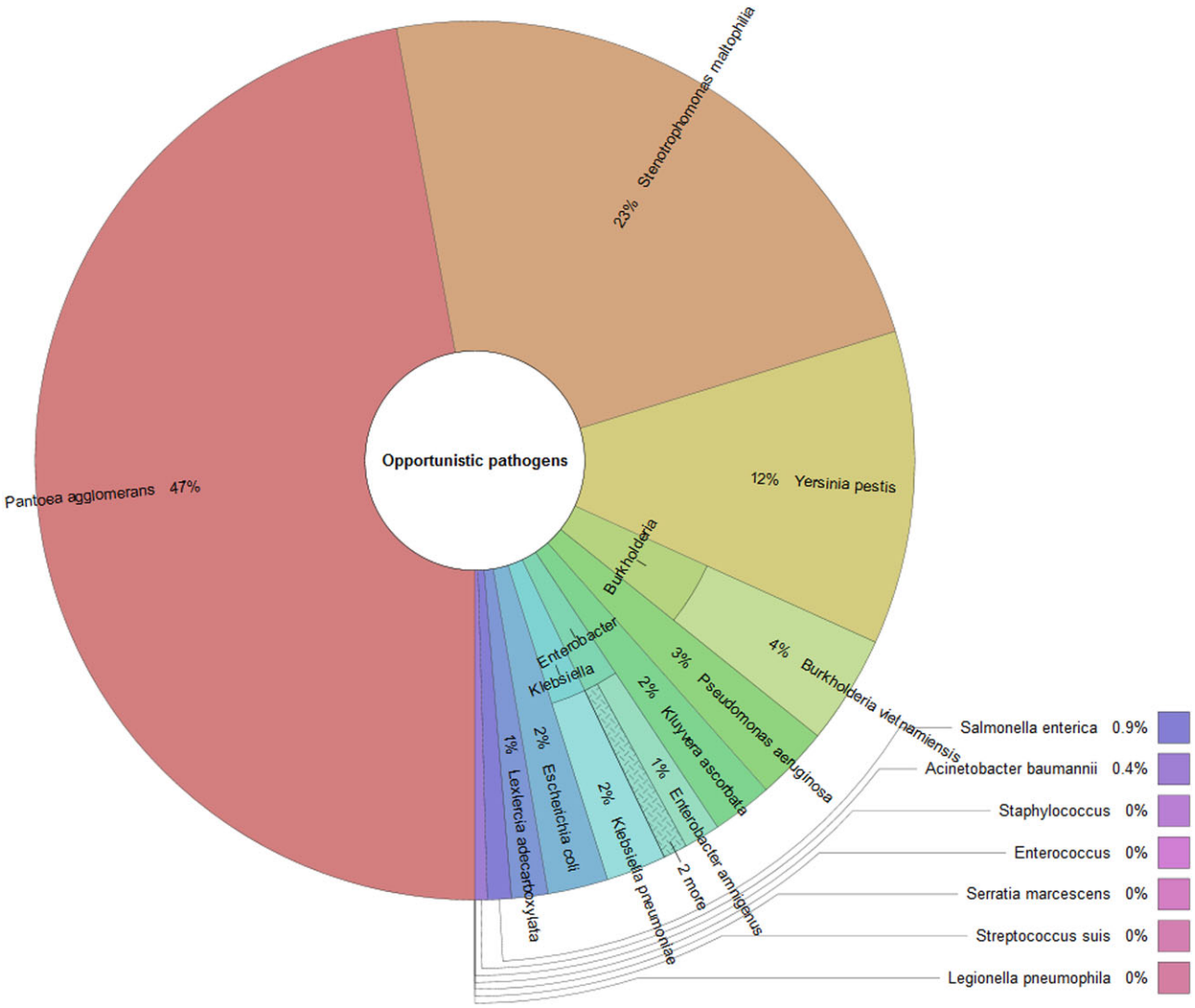

Fig. 1. Occurrence and taxonomic structure of opportunistic pathogens in the phyllosphere of *Eruca sativa* Mill. analyzed from a metagenomic data set. The relative abundance is based on the presented taxa and composed of 1.7% of the total bacterial fraction.

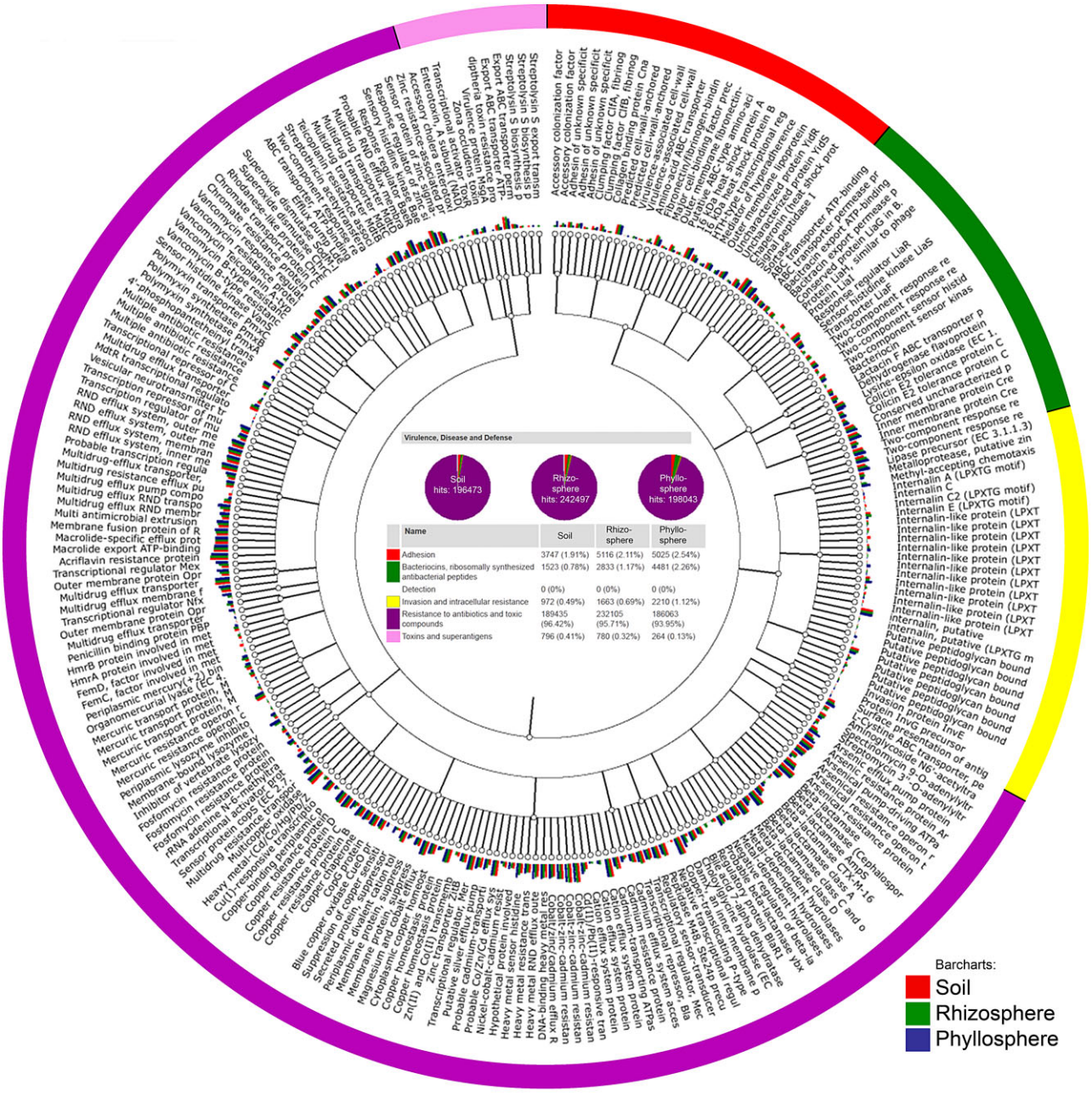

Fig. 2. Functional diversity tree of the virulence, disease and defence cluster of *Eruca sativa* Mill. The data were compared with SEED using a maximum e-value of 1e-5, a minimum identity of 60 % and a minimum alignment length of 15 measured in aa for protein and bp for RNA databases. Colour shading indicates classification membership and investigated habitat (bar charts).

potential to impact microbial community structure associated with vegetables; they are often characterized by a higher microbial diversity (Schmid *et al.*, 2011; Leff and Fierer, 2013). During the last decades, the usage of antibiotics in animal husbandry has promoted the development and abundance of antibiotic resistance in farm environments drastically (Woolhouse and Farrar, 2014). Especially, manure is a reservoir of resistant bacteria and antibiotic compounds, and its application to agricultural soils is assumed to significantly increase antibiotic resistance genes and selection of resistant bacterial populations in soil (Heuer *et al.*, 2011; Jechalke *et al.*, 2014). From the rhizosphere, these populations can invade into the endosphere of plants and here enter the food chain of humans. Pathogen contamination of fresh products may originate before or after harvest, but once contaminated, products are difficult to sanitize (Olaimat and Holley, 2012). However, food-processing practices also have an

important impact on the structure of the vegetable microbiome and food safety (Olaimat and Holley, 2012). For example, intermediate disturbances (e.g. by minor biotic or abiotic stresses) can enhance the relative abundance of *Enterobacteriaceae* (A. Erlacher and G. Berg, unpubl. data). Although outbreaks of enteric pathogens associated with fresh produce in the form of raw or minimally processed vegetables and fruits have recently increased, the ecology of enteric pathogens outside of their human and animal hosts is less understood (van Overbeek *et al*., 2014). The relatively infrequent outbreaks associated with pre-harvest contamination with *Shigella*, an organism with humans as its major reservoir, and the relative high frequency of those associated with *Salmonella* or *Shiga*-toxin-producing *Escherichia coli*, organisms with animals as their major reservoirs, underline the role of domestic and wild animals as dominant sources of pre-harvest contamination of vegetables like salads (Allerberger and Sessitsch, 2009).

Opportunistic pathogens in the vegetable microbiome

Plants, especially their endospheres and rhizospheres are important reservoirs for emerging opportunistic pathogens (Berg *et al*., 2005; Mendes *et al*., 2013). The number of documented outbreaks of human infections associated with the consumption of raw vegetables has increased in recent years (Buck *et al*., 2003). Diverse human pathogens are able to colonize vegetables including *E. coli* pathovars (Buck *et al*., 2003; van Overbeek *et al*., 2014). Figure 3 shows the invasion of *E. coli* cells into lettuce leaves via stomata after bacterial treatment. There are many plant-associated genera, including *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia*, *Staphylococcus* and *Stenotrophomonas* that enter bivalent interactions with plant and human hosts. Several members of these genera show plant growth promoting as well as excellent antagonistic properties against plant pathogens; therefore, they are utilized to control pathogens to promote plant growth (Berg *et al*., 2005). However, many strains also successfully colonize human organs and tissues and thus cause diseases. One reason is that similar or often identical factors allow recognition, adherence and invasion of plant and human hosts (Berg *et al*., 2005). Well-studied examples of this group are the Gram-negative, often multi-resistant species *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. Both were found as abundant members of plant microbiomes, and strains belonging to these species are characterized by a high versatility at genotypic and phenotypic level. Surprisingly, the pan-genome of *P. aeruginosa* has a larger genetic repertoire than the human genome, which explains the broad metabolic capabilities of *P. aeruginosa* and its ubiquitous distribution in habitats (Tümmler *et al*., 2014). Moreover, the popular plant model, *Arabidopsis thaliana*, has been used to successfully identify novel *P. aeruginosa* genes that are involved in virulence (Baldini *et al*., 2014). *Stenotrophomonas maltophilia* strains show a similar degree of diversity (Berg *et al*., 1999; Ryan *et al*., 2009; Alavi *et al*., 2014). Here, polymorphic mutation frequencies of clinical and environmental *S. maltophilia* populations explain the adaptation to new niches (Turrientes *et al*., 2010). Plant-associated populations have a broader diversity, and only those with a high mutation frequency (hypermutators) were able to adapt to clinical environments and human hosts. Although *S. maltophilia* strains cause a high number of nosocomial infections, only unspecific virulence factors, for example proteases and siderophores, were identified (Ryan *et al*., 2009). Strains belonging to this species persist and display multi-resistance; only a reduced indigenous microbiome gives an opportunity for the pathogen to infect humans. In natural habitats, *S. maltophilia* strains colonize dicotyledonous plants, which produce diverse secondary, antimicrobial metabolites, for example medicinal plants, eucalyptus and *Brassicaceae* (Ryan *et al*., 2009). To survive in such plant habitats, efflux pumps are used, which are also responsible for their multi-resistance against clinically used antibiotics (García-León *et al*., 2014). However, studies show

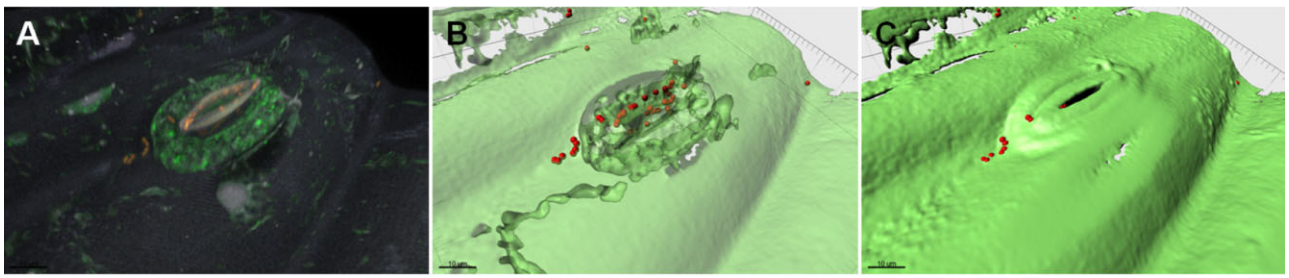

Fig. 3. *Escherichia coli* cells on lettuce leaves and colonization of stomata visualized by Fluorescence *in situ* hybridization coupled with confocal laser scanning microscopy.
A. Rendering of a confocal Z-stack volume.
B and C. Isosurface models of A showing bacteria inside the stoma.

a high plasticity as well as specificity of genomes and epigenomes at strain level, which can contribute to the development of virulent strains (Alavi *et al*., 2014).

The role of potential pathogens for plants and humans

The plant microbiome plays an important role for plant growth and health and depends on factors such as the plant species, the cultivar and the soil type (Berg and Smalla, 2009; Berg *et al*., 2013; Schreiter *et al*., 2014). Microorganisms can support the nutrient uptake and produce a broad range of phytohormons or influence the latter. Another important function is the involvement of plant-associated bacteria in pathogen defence (Mendes *et al*., 2013). Many pathogens attack plants, especially fungi, oomycetes and nematodes; it is estimated that they cause more than one third of yield losses worldwide. Whereas resistance against leaf pathogens is often encoded in the plant genome, it is difficult to find resistance genes against soil-borne pathogens. Cook and colleagues suggested already in 1995 that antagonistic rhizobacteria fulfil this function – this group acts also as human opportunistic pathogens. Besides direct antagonism, plant-associated bacteria can induce a systemic response in the plant, resulting in the activation of plant defence mechanisms (Pieterse *et al*., 2003).

Another hypothesis is that the plant microbiome has also a positive function for human health by stimulating our immune system and enhancing microbial diversity in the gut microbiome. Recently, Hanski and colleagues (2012) showed a correlation between bacterial diversity and atopy as shown through significant interactions with *Enterobacteriaceae*. Furthermore, they showed a positive association between the abundance of *Acinetobacter* and interleukin-10 expression in peripheral blood mononuclear cells in healthy human individuals. Interleukin-10 is an anti-inflammatory cytokine and plays a central role in maintaining immunologic tolerance to harmless substances (Lloyd and Hawrylowicz, 2009). Endotoxin derived from Gram-negative bacteria, such as *Enterobacteriaceae*, is known to have allergy-protective and immuno-modulatory potential (Doreswamy and Peden, 2011). If plants are a natural reservoir of *Enterobacteriaceae*, then these bacteria must have been a 'natural' part of our diet for a long time. Taking into account how many vegetables and fruits are eaten by people worldwide, these outbreaks seem to be more of an accident than the norm, particularly considering that traditionally, food was not processed and sterilized before eating. Therefore, the function of the plant-associated microbiome as an immune-stimulant or 'natural vaccination' was suggested by Berg and colleagues (2014). Interestingly, there is an overlap between the plant and human gut microbiome with respect to species composition and function (Ramírez-Puebla *et al*., 2013). Recent studies showed that the stomach does not pose a strict barrier for microbial passage as was previously thought; it is colonized by a broad diversity of species (von Rosenvinge *et al*., 2013). David and colleagues (2014) also recently provided additional evidence for the survival of food-borne microbes (both animal- and plant-based diet) after transit through the digestive system, and that food-borne strains may have been metabolically active in the gut. Microbial diversity in our gut ecosystem has an enormous impact on the host and *vice versa* connected by gut–brain crosstalk, which was revealed as complex, bidirectional communication system (Mayer, 2011). Interesting relationships were detected recently, for example between the gut microbiome and the development of obesity, between cardiovascular disease and metabolic syndromes (Tremaroli and Bäckhed, 2012) and also between motivation and higher-cognitive functions, including intuitive decision-making (Mayer, 2011). This important relationship is confirmed by the enormous success of faecal transplantations (De Vrieze, 2013). The impact of the vegetable microbiome on our health seems to be important and needs more attention in the future.

Solutions and conclusions

The gathered data indicate that the interplay of different microbiomes is very important. The microbiomes of vegetables, humans as well as in built environment such as hospitals seems to be well connected (Ramírez-Puebla *et al*., 2013; Berg *et al*., 2014). Microbial diversity is an important issue to avoid pathogen outbreaks, which can be often explained by microbial imbalances and poorness (van Elsas *et al*., 2012; Pham and Lawley, 2014). Therefore, to maintain and support microbial diversity is of interest to stabilize ecosystems. Here also, biotechnological solutions are already shown successfully for agriculture (Berg *et al*., 2013) or human health (Petrof and Khoruts, 2014). Probiotics, prebiotics, and synbiotics for plants as well as humans can provide support of the indigenous microbiome (De Vrese and Schrezenmeir, 2008). However, human activities contribute to fast changes of farming and processing practices of vegetables and also influence the structure and function of vegetable-associated bacteria. By horizontal gene transfer multiresistant super-bugs can develop – a scenario that should be avoided by a careful assessment of new techniques and processes. The new methods and omics technologies in microbial ecology allow these evaluations in great depth and can hopefully contribute to new environmentally friendly solutions. Moreover, to integrate epigenetics in multi-omics techniques opens existing opportunities for new discoveries (Chen *et al*., 2014).

The following points can be concluded:

i. Vegetable microbiomes are highly diverse; the composition of species varies for different vegetable species and is strongly influenced by biogeographic aspects and farming and food processing practices. *Enterobacteriaceae* belong to the indigenous microbiota and are key stone species.
ii. The vegetable microbiome is a reservoir for a long list of opportunistic and emerging pathogens. It is predicted that in future decades, other lesser-known pathogens and new strains of bacteria will emerge as common causes of infections.
iii. Opportunistic pathogens have a broad phylogenetic background (e.g. *Firmicutes*, *Beta-* and *Gammaproteobacteria*) and occur in natural environments or associated with eukaryotic hosts.
iv. Many potentially opportunistic pathogens have an endophytic lifestyle. This shows not only their intimate interactions with their host, but also results in difficulties of decontamination.
v. In immunocompetent hosts, these bacteria can stimulate the immunosystem and enhance microbial diversity to maintain our health. Moreover, they can contribute to the diversity of our gut microbiome. This diversity is important not only to avoid the development of diseases such as obesity, cardiovascular disease and metabolic syndromes, but also for our motivation and higher-cognitive functions, including intuitive decision-making.
vi. In immunocompromised individuals, opportunistic pathogens can cause severe infections. These infections include HAIs like pneumonia, bloodstream infections, urinary tract infections, surgical site infections and also diarrhoea.
vii. To understand the structure and function of microbiomes and their interplay is important to manipulate, reduce or maintain microbial diversity for human and ecosystem health. While multi-omics integration offers technical solutions, probiotics, prebiotics, and synbiotics can provide biotechnological solutions.

Conflict of Interest

None declared.

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Book Chapter I

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The Edible Plant Microbiome: Importance and Health Issues

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Chapter 44
The Edible Plant Microbiome: Importance and Health Issues

Gabriele Berg, Armin Erlacher and Martin Grube

Abstract Plants live together with microbial communities to form tight interactions that are essential for the performance and survival of the host. In recent decades, many studies have discovered a vast plant-associated microbial diversity. However, even though plants are a substantial part of a balanced diet including raw-eaten vegetables, fruits and herbs, the plant-associated microbial diversity has been largely ignored in this context. We hypothesize that the edible plant microbiome and its diversity can be important for humans as (i) an additional contributor to the diversity of our gut microbiome, and (ii) as a stimulus for the human immune system. Two specific examples for plant microbiomes, of lettuce and banana, are discussed in comparison with other relevant studies to explore these hypotheses. Moreover, the biotechnological potential of the edible plant microbiome is evaluated.

44.1 Plant-Associated Microbial Diversity

All Food Plants are Associated with a High Diversity of Microorganisms This diversity is still currently only partly characterized and is, to a certain degree, specific for the host species or even cultivars of food plants (Berg and Smalla 2009). This diversity is also specific for each microhabitat of plants which are usually distinguished as: the rhizosphere (roots), the phyllosphere (leaves), the caulosphere (stem), the anthosphere (flowers), the carposphere (fruits), and the endosphere

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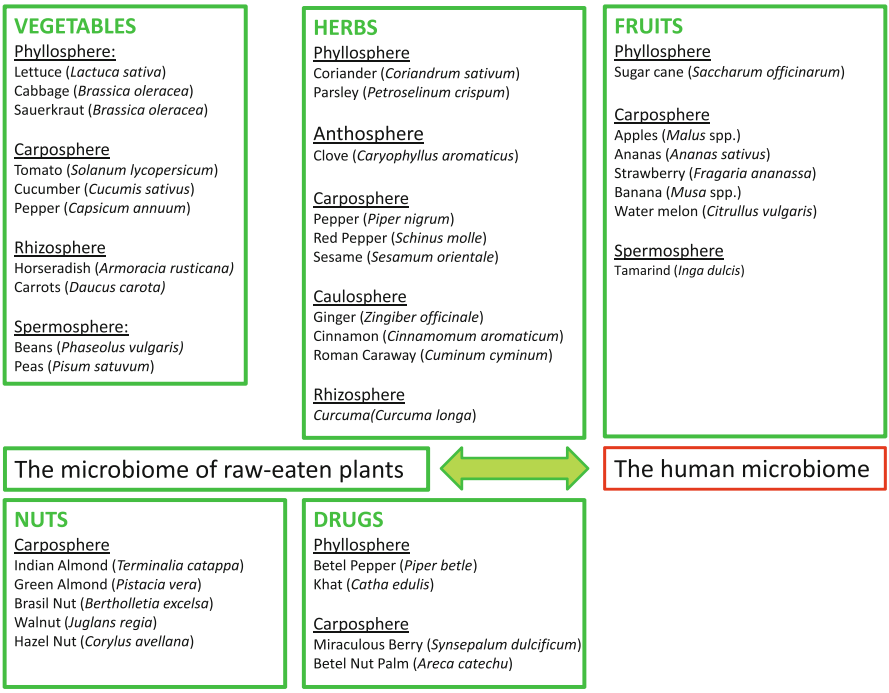

Fig. 44.1 Interactive microbiomes. Examples of the edible plant microbiome of fruits, herbs, nuts and drugs. We eat all parts of the plants including the phyllosphere (*lettuce, cabbage*), the rhizosphere (*carrots, turnip*), the carposphere (*tomato, banana*) as well as seeds (*beans, peas*) including all endospheres

(all inner parts). Although the discovery of specific microbiomes is primarily associated with the rhizosphere, there are currently only a few other compartments where species-specific diversity was detected, e.g. the carposphere (Leff and Fierer 2013). Given the estimated number of 370 000 species of higher plants, a great deal of work is still required before the details of global plant microbiome diversity will be fully understood.

Plants are a basic and substantial part of our daily diet. Vegetables, fruits, herbs, nuts, and medicinal herbs belong to the raw-eaten plants; several examples of each group are shown in Fig. 44.1. Our food thus comprises all parts of plants that include their microbial habitats and microhabitats that can be colonized by up to 10^4–10^{10} microorganisms per gram of plant. These microbial habitats include the phyllosphere (lettuce, cabbage), the rhizosphere (carrots, turnip), the carposphere (tomato, banana), as well as the seeds (beans, peas) and corresponding endospheres. An initial study published by Leff and Fierer (2013) demonstrated that fruits and vegetables harbored distinct and diverse bacterial communities, and interestingly showed that vegetables or fruits grown primarily close to the soil surface (i.e., sprouts, spinach,

lettuce, tomatoes, peppers, and strawberries) appear to share communities characterized by high relative abundance of *Enterobacteriaceae*. The authors concluded that humans are exposed to substantially different bacteria depending on the types of fresh produce they consume.

What could the effect of this exposure be on humans? Plant-associated microorganisms could have both a direct and indirect influence on human health. Indirect positive effects are linked to organisms that enhance the quality factors (including the content of active principles). Only a few examples are known for such effects, and most are related to medicinal plants and their bioactive substances (Köberl et al. 2013). For example, microorganisms are involved in the production of antimicrobial substances, e.g. taxol in endophytic fungi of *Taxus baccata* (Garyali et al. 2013), apigenin in *Chamomille matricaria* (Schmidt et al. 2014), or maytansine in *Putterlickia verrucosa* (Wings et al. 2013). Moreover, fruit-associated bacteria seem to influence the aroma expression in strawberries, where *Methylobacterium* treatment has been shown to enhance the production of aromatic furaneol substances (Verginer et al. 2010a). Evidence was also provided by Verginer et al. (2010b) for an influence of grape-associated microorganisms on the aroma of wine, indicating that the "terroir" effect can to some extent be attributed to bacteria. The indirect negative effects caused by plant-associated microorganisms are well-studied. The outbreak of plant pathogens is often associated with a microbiome shift and accompanied with minor pathogens. They do not only contribute to bad odor and taste, but also to the expression of mycotoxins which are among the world's most toxic and carcinogenic compounds (Wu et al. 2014). They have been responsible for numerous foodborne diseases and epidemics throughout history including *Claviceps purpurea*, the causative agent for the infamous Saint Anthony's Fire in Medieval times that occurred after eating contaminated bread (Belser-Ehrlich et al. 2013). Although such problems could be primarily solved by food hygiene, *Fusarium* mycotoxins still play an important role for our health (Wu et al. 2014). There is still very little knowledge concerning the long term effects of bioactive compounds at low concentration, and only recently has evidence been introduced for endophytes that produce novel and still poorly understood compounds. New technologies will contribute to increase the detection rate of specific beneficial plant-microbe interactions that are also relevant for human health.

What do we know about the direct effects of plant microbiomes that we consume along with our food? Most of our existing knowledge concerns fermented food, such as yoghurt as the foremost example for sources of probiotic strains. However, a substantial part of our plant diet is consumed fresh and may possibly include trillions of microorganisms during each meal. Even after washing or rinsing food surfaces, a substantial number of bacteria is expected to enter the body with our food. Our primary hypothesis is that the edible plant microbiome and its microbial diversity is important for humans as: (i) a contributor to the diversity within our gut microbiome, and (ii) as a stimulus for our immune response. We will present two examples for crop-associated microbiomes which are eaten raw by humans: of lettuce and banana. Furthermore, we will discuss our hypotheses as well as the impact of microbial diversity in general.

The Specific Structure of the Lettuce Microbiome Lettuce species such as *Lactuca sativa* L., *Eruca sativa* Mill., and their varieties belong to the most important raw-eaten vegetables world-wide and are a substantial part of a balanced, healthy diet. Several beneficial effects on health and lifestyle are attributed to the consumption of lettuce as it contains several vitamins, and is also a source of manganese and high amounts of dietary fibers. The relatively low amount of carbohydrates and fats correlates with its low calorie value. Lettuce provides habitats for a diverse range of microbes (Rastogi et al. 2012; Rastogi et al. 2013). Lettuce-associated microorganisms have currently only made it into the headlines in the context of scattered pathogen outbreaks. There are two crucial features that may be responsible for lettuce's vulnerability to pathogens: the variability and specificity of the associations within the microbial communities. Overall, a proportion of 12.5 % cultivar-specific bacteria were identified for the rhizosphere of eight different *Lactuca sativa* cultivars as well as the wild relative *L. serriola*. In addition, a large core microbiome was identified that includes 68 operational taxonomic units from nine major phyla (*Proteobacteria* the most abundant), and represents 48.8 % of the microbiome. A correlation analysis showed that within the lettuce microbiome co-occurrence prevailed over co-exclusion. Although predominant taxa (e.g. *Pseudomonas, Flavobacterium*, and *Sphingomonadaceae*) showed positive interactions, they were not necessarily involved in highly correlated modules of species. This loose bacterial network observed for lettuce allowed allochthonous organisms to colonize lettuce to interactive niching in microbial communities.

Little is known about the impact of biotic factors on the lettuce microbiota. Our hypothesis was that any disturbance of the native microbiomes (i) can induce drastic shifts in the community and that each pathogen outbreak (ii) could be accompanied by "minor", less virulent pathogens. In mesocosm and field experiments by using a combined approach including network analyses of 16S rRNA gene amplicon libraries and FISH microscopy (see Chap. 31), we found substantial impacts detectable as microbiome shifts by a plant pathogenic fungus, herbivorous gastropoda, or visiting pets. Although the genera *Enterobacter, Stenotrophomonas, Pseudomonas,* and *Acinetobacter* form a core microbiome, all three disturbing factors induced significant shifts in the community and increased species richness. In *Lactuca*, this was strongly correlated with an increase of *Enterobacter* and in *Eruca* with *Escherichia/Shigella* and *Pantoea*—all genera contain potential pathogens. A bacterial diversity associated with leaves is detectable by cultivation and bacterial DNA analysis, but very few bacteria are detected on the surface as only a few colonies occupy cavities along the external surface and in the vicinity of stomata (Fig. 44.2a). Through colonization experiments, we revealed unexpected colonization patterns of enteric species in lettuce leaves and found that bacterial populations do not colonize the surface, but rather intrude into the endosphere (Fig. 44.2b).

The Specific Structure of the Banana Microbiome Bananas and plantains are among the most important crops in the tropics and sub-tropical regions world-wide. Microhabitat-specific microbial communities for the rhizosphere, phyllosphere, and endosphere of bananas grown in three different traditional farms in Uganda were

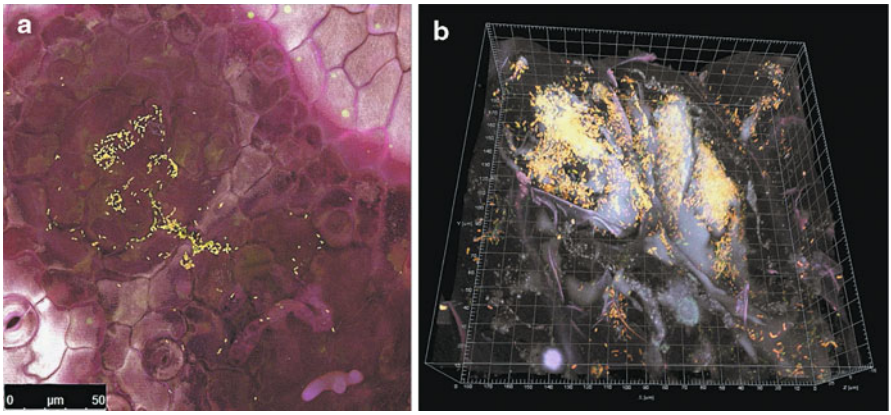

Fig. 44.2 Visualization of the lettuce microbiome. **a** Naturally occurring Gammaproteobacterial micro-colonies on the lettuce surface and in the vicinity of stomata visualized by FISH and CLSM. **b** Colonization patterns on lettuce leaves treated with *E. coli* cells. Both experiments are explained in detail in Erlacher et al. (2014)

detected (Rossmann et al. 2010). Interestingly, the banana stem endosphere showed the highest bacterial counts (up to 10^9 gene copy numbers g^{-1}), and *Enterobacteriacaea* provided 1/3 of the total bacteria. They comprise 14 genera including potential human pathogens, (*Escherichia, Klebsiella, Salmonella, Yersinia*) plant pathogens (*Pectobacterium*), but also disease-suppressive bacteria (*Serratia*). This dominant role of enterics can be explained by their permanent nature and the vegetative propagation of banana plants, as well as the addition of human and animal manure in traditional cultivations.

44.2 The Edible Plant Microbiome: Diversity and Human Health

Concerning our first hypothesis of a link between the plant and human gut microbiome, there is an interesting overlap between the plant and human gut microbiome with respect to species composition and function (Ramírez-Puebla et al. 2013). Recent studies showed that the stomach is colonized by a higher diversity of microbial species than has long been expected, and explained by the hostile conditions of low pH values. The stomach milieu thus does not pose a strict barrier for microbial passage as was previously thought (von Rosenvinge et al. 2013). Even though the effects of probiotics are often controversially discussed, it has now been shown that strains, including probiotics, survived the stomach passage to establish successfully in the gut (Iqbal et al. 2014). David et al. (2014) also recently provided additional evidence for the survival of foodborne microbes (both animal- and plant-based diet)

after transit through the digestive system, and that foodborne strains may have been metabolically active in the gut.

Our second hypothesis is that bacteria, associated with our diet, such as *Enterobacteriaceae*, act as stimuli for our immune system. Recently, Hanski et al. (2012) showed a correlation between bacterial diversity and atopy as shown through significant interactions with *Enterobacteriaceae*. Furthermore, they showed a positive association between the abundance of *Acinetobacter* and Interleukin-10 expression in peripheral blood mononuclear cells in healthy human individuals. Interleukin-10 is an anti-inflammatory cytokine and plays a central role in maintaining immunologic tolerance to harmless substances (Lloyd and Hawrylowicz 2009). Endotoxin derived from Gram-negative bacteria, such as *Enterobacteriaceae*, is known to have allergy-protective and immunomodulatory potential (Doreswamy and Peden 2011).

Microhabitats of plants are a reservoir for *Enterobacteriaceae* (Leff and Fierer 2013, Rastogi et al. 2012), which also include potentially human pathogenic bacteria such as human enteric pathogens (Brandl 2006). Particularly after intermediate disturbances, these human enteric pathogens are enhanced (Erlacher et al. 2014). Although outbreaks of enteric pathogens associated with fresh produce in the form of raw or minimally processed vegetables and fruits have recently increased (Holden 2010), the ecology of enteric pathogens outside of their human and animal hosts is less understood (Teplitski et al. 2011). If plants are a natural reservoir of *Enterobacteriaceae*, then these bacteria must have been a "natural" part of our diet for a long time. Taking into account how many vegetables and fruits are eaten by people worldwide, these outbreaks seem to be more of an accident than the norm, particularly considering that traditionally, food was not processed and sterilized before eating. A function of the plant- associated microbiome as an immunostimulant or "natural vaccination" is more likely than their pathogenic role.

44.3 Conclusions

Members of the prokaryotic and eukaryotic domains of life are often tied together by intricate interactions. While past research has paid much more attention to the pathogenic interactions, the results obtained over the last decade have taught us much more about a beneficial balance between microorganisms and their hosts (Blaser et al. 2013). It seems that in developing these interactions, diversity plays an incredibly important role. Diversity is intrinsically correlated with a low incidence of pathogen outbreaks in both plants and humans. Where does microbial diversity come from? The plants themselves as well as their secondary metabolites and microbiomes co-evolved together; microbes contribute to the diversification of plants and *vice versa* and continue to add to the high plant-associated microbial diversity. Interesting examples are medical as well as endemic plants which harbor a unique microbiome (Zachow et al. 2009; Köberl et al. 2013). Conversely, crops cultivated in intensive agriculture are often characterized by a reduced diversity in comparison with organic agriculture or natural ecosystems. In the past, breeding strategies induced a specific microbiome

as cultivar-specificity was very often reported (Berg and Smalla 2009). Our lettuce example revealed a higher diversity in comparison to its wild ancestor as well as a loose bacterial co-occurrence network in the modern cultivars. This could explain its susceptibility for pathogens as well as for biocontrol agents. Efficient biocontrol approaches were already shown for lettuce (Scherwinski et al. 2008; Erlacher et al. 2014). The enhancement of plant-associated microbial diversity is important for the sustainability of future agriculture. In addition, for human food and health, microbial diversity is an important issue, and we should take care of plant-associated diversity and produce our food in a way that is optimal for this purpose. Biotechnological strategies can be developed to contribute to this purpose. For example, "microbiome therapies" are a promising method to maintain or enhance plant-associated microbial diversity in combination with quality control (Gopal et al. 2013). Another interesting example is the biocontrol agent *Bacillus amyloliquefaciens* FZB42, which was able to enhance the overall plant-associated diversity (Erlacher et al. 2014). Next generation microbial inoculants should take both the diversity as well as human health issues into consideration (Berg et al. 2013), and someday in the future should have the potential to control plant diseases, generally enhance microbial diversity, and stimulate our immune system.

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Additional Publication I

Frontiers in Microbiology, (2014), **5**.

Cover page for the Frontiers research topic
"Berg, G., Grube, M., Schloter, M., & Smalla, K. (2014). The plant microbiome and its importance for plant and human health."

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THE PLANT MICROBIOME AND ITS IMPORTANCE FOR PLANT AND HUMAN HEALTH

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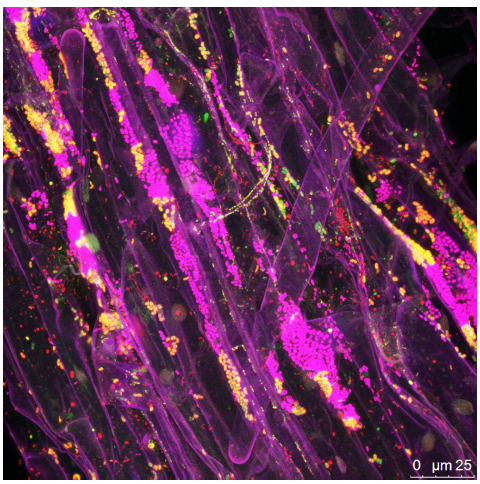

Plant-associated bacteria on lettuce roots visualized by Fluorescence In Situ Hybridization coupled with Confocal Laser Scanning Microscopy (FISH-CLSM). Gammaproteobacteria (yellow) and Betaproteobacteria (pink) form large colonies on the lateral roots of young lettuce plantlets. Micrograph provided by Armin Erlacher and Gabriele Berg (TU Graz).

The study of plant-microbe associations by new techniques has significantly improved our understanding of the structure and specificity of the plant microbiome. Yet, microbiome function and the importance of the plant's microbiome in the context of human and plant health are largely unexplored. Comparable with our human microbiome, millions of microbes inhabit plants, forming complex ecological communities that influence plant growth and health through its collective metabolic activities and host interactions. Viewing the microbiota from an ecological perspective can provide insight into how to promote plant health and stress tolerance of their hosts or how to adapt to a changing climate by targeting this microbial community. Moreover, the plant microbiome has a substantial impact on human health by influencing our gut microbiome by eating raw plants such as lettuce and herbs but also by influencing the microbiome of our environment through airflow. This research topic comprising reviews, original and opinion articles highlights the current knowledge regarding plant microbiomes, their specificity, diversity and function as well as all aspects studying the management of plant microbiomes to enhance plant growth, health quality and stress tolerance.

Additional Publication II

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Rhizobiales as functional and endosymbiontic members in the lichen symbiosis of *Lobaria pulmonaria* L.

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Rhizobiales as functional and endosymbiontic members in the lichen symbiosis of *Lobaria pulmonaria* L.

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Rhizobiales (*Alphaproteobacteria)* are well-known beneficial partners in plant-microbe interactions. Less is known about the occurrence and function of *Rhizobiales* in the lichen symbiosis, although it has previously been shown that *Alphaproteobacteria* are the dominating group in growing lichen thalli. We have analyzed the taxonomic structure and assigned functions to *Rhizobiales* within a metagenomic dataset of the lung lichen *Lobaria pulmonaria* L. One third (32.2%) of the overall bacteria belong to the *Rhizobiales*, in particular to the families *Methylobacteriaceae*, *Bradyrhizobiaceae,* and *Rhizobiaceae*. About 20% of our metagenomic assignments could not be placed in any of the *Rhizobiales* lineages, which indicates a yet undescribed bacterial diversity. SEED-based functional analysis focused on *Rhizobiales* and revealed functions supporting the symbiosis, including auxin and vitamin production, nitrogen fixation and stress protection. We also have used a specifically developed probe to localize *Rhizobiales* by confocal laser scanning microscopy after fluorescence *in situ* hybridization (FISH-CLSM). Bacteria preferentially colonized fungal surfaces, but there is clear evidence that members of the *Rhizobiales* are able to intrude at varying depths into the interhyphal gelatinous matrix of the upper lichen cortical layer and that at least occasionally some bacteria also are capable to colonize the interior of the fungal hyphae. Interestingly, the gradual development of an endosymbiotic bacterial life was found for lichen- as well as for fungal- and plant-associated bacteria. The new tools to study *Rhizobiales*, FISH microscopy and comparative metagenomics, suggest a similar beneficial role for lichens than for plants and will help to better understand the *Rhizobiales*-host interaction and their biotechnological potential.

Keywords: *Rhizobiales*, lichen symbiosis, *Lobaria pulmonaria*, metagenomics, *Rhizobiales*-specific FISH probe, endosymbiont

INTRODUCTION

Lichen symbioses are estimated to cover up to 8% of the global land surface. Many habitats colonized by lichens are characterized by unfavorable environmental conditions, such as low nutrient availability and/or high temperature fluctuations (Ahmadjian, 1995). Although lichens are able to resist extreme environmental conditions via a dormant stage, they are highly specialized for their habitats and vulnerable to slight changes in the microclimate (or air pollution), which can easily disrupt the integrity of the fine-tuned symbiotic interplay. Lichen symbioses appear as composite organisms with a shape-forming fungus (the mycobiont) and a photosynthetic partner (the photobiont), which is often sheltered by complex fungal structures, into which a complex, stable and thallus-specific microbiome is incorporated. Recently, a bacterial microbiome was identified as a third component of this symbiosis (Grube et al., 2009). Lichens are densely colonized by diverse and host-specific communities of bacteria that occur in specific ecological niches of their hosts (Cardinale et al., 2008, 2012a,b; Grube et al., 2009). Specific above-ground niches in higher plants comprize for example the phyllo- and rhizosphere, or the endosphere (Ryan et al., 2008; Berg et al., 2014). However, lichens do not produce the same organs as found in plants, which develop their organs from meristems. Lichens instead produce a thallus of densely conglutinated fungal hyphae which can form foliose, filamentous, crustose, leprose, squamulose, gelatinous, or fruticose shapes (Grube and Hawksworth, 2007), each hosting specific sets of ecologically specific niches, which can be occupied by bacteria.

Rhizobiales are well-studied associates of plants; they commonly exert beneficial functions for their hosts by providing various nutrients, phytohormons as well as precursors for essential plant metabolites (Ivanova et al., 2000; Delmotte et al., 2009; Verginer et al., 2010). The order contains many genera of nitrogen-fixing, methanotrophic, legume-nodulating, microsymbiotic bacteria (Jourand et al., 2004; Garrity et al., 2005). Recently, nitrogen-fixation was shown not to be limited

to *Rhizobiales* in leguminous plants, but also to be expressed within various endophytic compartments of non-leguminous plants (Fischer et al., 2012). Besides their almost ubiquitous presence with higher plants, *Rhizobiales* are also found associated with mosses and lichens (Lundberg et al., 2012; Vorholt, 2012; Erlacher et al., in press). Pink-pigmented-facultative-methylotrophs (PPFMs) are a specific group of *Rhizobiales*, which can affect the host metabolism including production of vitamins and phytohormones, such as auxines and cytokinines (Ivanova et al., 2000; Delmotte et al., 2009). *Methylobacterium* spp. can utilize methanol emitted by the plants, methylamine and further C2, C3, and C4 compounds as solely carbon and energy source (Green and Bousfield, 1983; Lidstrom and Chistoserdova, 2002). Schauer and Kutschera (2013) suggest that ferns, liverworts and moss protonemata have an intimate association with methylobacteria, and they argue that the haploid phases of cryptogames are preferred host organisms of these pink-pigmented microbial phytosymbionts. However, less is known for lichen-associated *Rhizobiales,* including methylobacteria. We postulate that they also play a beneficial role in the lichen symbiosis.

According to recent publications, *Rhizobiales* are a particularly common order on lichens (Bates et al., 2012; Cardinale et al., 2012b). Although there are several reports about endofungal bacteria in ascomycetous fungi (Bertaux et al., 2005; Sharma et al., 2008), until know there is no evidence for them in lichens (Grube and Berg, 2009). While *Alphaproteobacteria* have been detected by fluorescence *in situ* hybridization and confocal laser scanning microscopy in lichens (Cardinale et al., 2008; Grube et al., 2009), there are no suitable FISH probes available to specifically stain the order *Rhizobiales,* except RHIZ1244 (according to Probebase) (http://www.microbial-ecology.net/probebase; accession nr. pB-02665; Thayanukul et al., 2010). *In silico* analysis using probematch (https://rdp.cme.msu.edu/probematch/) revealed that the RHIZ1244 probe detection spectrum is incomplete and fails to recognize important families such as *Methylobacteriaceae*, *Bradyrhizobiaceae,* or *Beijerinckiaceae*.

The lung lichen, *Lobaria pulmonaria* L., is a tripartite lichen, with one ascomycete fungus hosting both a dominant green algal partner (*Dictyochloropsis reticulata*) and a minor cyanobacterial partner (internal herds of *Nostoc*). This lichen is known as a sensitive biological indicator of air pollution that experienced a massive decline in Europe during the twentieth century (Scheidegger and Goward, 2002). Nonetheless, it may develop prolific populations in suitable cool and humid habitats, both by the efficient spread with symbiotic propagules and by its growth rate, which is one of the highest among all lichens (**Figure 1**). In this work, we have investigated *L. pulmonaria* collected in the high montane forest zone in Alps. We pursued a metagenomic approach to assess functional diversity of *Rhizobiales* associated with this lichen species and used *in situ* visualization to localize and reveal colonization strategies of this bacterial order. For this purpose, we designed a novel FISH probe to efficiently and specifically target members of *Rhizobiales*.

FIGURE 1 | *Lobaria pulmonaria* from a mountain maple in an Abieti-Fagetum forest in Johnsbach (Austria) at an altitude of 1200 m above sea level. (Styria, Austria, 47°32′29.7″N 14°37′36.6″E).

MATERIALS AND METHODS

SAMPLING

L. pulmonaria samples were collected in two mountain forests from mountain maple (*Acer pseudoplatanus* L.) in Austria (Styria, Gstatterboden, 47°34′20″N, 14°35′4.4″E and Styria, Johnsbach, 47°32′29.7″N 14°37′36.6″E). Ten individual lichen thalli were collected and stored in sterile plastic bags on ice.

LOBARIA PULMONARIA METAGENOME AND ANALYZES

All metagenome-based analyzes were carried out on the assembled dataset described in a previous study by Grube et al. (2015). The number of actual contigs used for the *in silico* FISH-probe evaluation was 368,424, while 28,526 contigs assigned to the taxon *Rhizobiales* were used for the functional analysis of *Rhizobiales*. CLUSTER CONTROL (Stocker et al., 2004) was used to search with the blastn algorithm for FISH-probe matches within the dataset (*e*-value cutoff = 1.6). The assembled metagenomic dataset is publicly available on MG-RAST (http://metagenomics.anl.gov; project ID: 4529136.3). To obtain taxonomic assignments, the Tera-BLASTN program (www.timelogic.com/documents/TeraBLAST2009.pdf) was run on the 368,424 contigs, using TimeLogic (Active Motif, Carlsbad, CA, USA) DeCypher boards against the "nt" (nucleotide sequence) database from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db). The blastn results were imported into MEGAN (Metagenome Analyzer, v4.70.4) (Huson et al., 2011) to produce several taxonomy profiles. For functional analysis, we used a similar approach as above but used Tera-BLASTX (www.timelogic.com/documents/TeraBLAST2009.pdf), which was run against the "nr" (non-redundant protein sequence) database from NCBI (ftp://ftp.ncbi.nlm.nih.gov/blast/db). The blastx results were imported into MEGAN (v4.70.4) as well for functional analysis. The 28,526 contigs that were previously assigned to *Rhizobiales* were used for assignment of SEED functions (Overbeek et al., 2005) within MEGAN. SEED-based analysis allows hierarchical organization of complete and partial gene sequences allocated within the utilized contig collection and thus quantification of specific functions on different levels.

IN SILICO ANALYSIS OF rRNA-TARGETED OLIGONUCLEOTIDE PROBES

ProbeBase (http://www.microbial-ecology.net/probebase; Loy et al., 2007) was used to screen for available FISH probes targeting the order *Rhizobiales*. RDP Probe Match (http://rdp.cme.msu.edu/probematch; Cole et al., 2005) and the Silva RNA database using TestProbe 3.0 (http://www.arb-silva.de/search/testprobe; Quast et al., 2013) with taxonomy browser were utilized to

evaluate the amplitude and coverage of available and designed FISH probes. The Probe sequences (5′–3′) were aligned (reversed and complement search allowed) to RDP and Silva SSU r119 databases with the REFNR sequence collection.

FLUORESCENCE *IN SITU* HYBRIDIZATION COMBINED WITH CONFOCAL LASER SCANNING MICROSCOPY (FISH-CLSM)

FISH-CLSM was applied on *L. pulmonaria* samples to investigate colonization patterns of *Rhizobiales* and all bacteria. Within 3 h after collection, samples were fixed with 4% paraformaldehyde and 1x phosphate-buffered saline (PBS) (3:1 ratio, respectively) for 6 h at 4°C. Fixed *Lobaria* thallus samples were cut with a cryotome.

We designed FISH probe RHIZ3r (**Table 1**) specific to our metagenomic data. The oligonucleotide sequence based on the Primer 3r (Nishio et al., 1997) was synthetized and labeled with a Cy5 fluorochrome (Biomers, Wiener Neudorf, Austria). FISH was applied according to Cardinale et al. (2008) to visualize and decipher the nature of the correlations detected by metagenomics. Briefly, the cryosections were transferred into 1.5 ml Eppendorf tubes and rinsed with 1x PBS. Lysozyme (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) treatment was applied and incubated at RT for 10 min. After an ethanolic series (50–70–96% EtOH solutions; 3 min each) samples were rinsed and further washed for 3 min with ice-cold 1x PBS. All hybridizations were performed at 43°C for 2 h in a buffer containing 0.9 M NaCl, 0.02 M Tris-HCl, 0.01% sodium dodecyl sulfate, 10–50% (**Table 1**) ultrapure formamide (FA; Invitrogen), and 5.0 ng of each FISH probe μl^{-l} (pH 8). An equimolar mixture of Cy3-labeled EUB338, EUB338-II and EUB338-III probes (Amann et al., 1990; Daims et al., 1999) was used for staining all Bacteria. RHIZ3r and RHIZ1244 (Thayanukul et al., 2010) was used to stain taxa within the bacterial order *Rhizobiales*. NONEUB probes (Wallner et al., 1993) labeled to fluorochromes analogous to the positive probes were used as negative controls. The hybridization buffer was replaced by a prewarmed (44°C) washing buffer [20 mM Tris-HCl, 450/46/18 mM NaCl (10/40/50% FA), and 5 mM EDTA (for 40% and 50% FA)] and incubated for 15 min in a water bath (44°C). The hybridization and washing step were repeated sequentially for the utilized FISH-probes in dependency of the specific FA requirements. After eliminating the washing buffer the sections were again rinsed with ice-cold double-distilled H_2O in order to remove residual salt crystals. FISH stained samples were transferred on optical slides, dried and mounted with SlowFade Gold antifadent (Molecular Probes, Eugene, USA). For visualization a Leica TCS SPE confocal laser-scanning microscope (Leica Microsystems, Mannheim, Germany) was used. Autofluorescence and additional calcofluor white (Sigma-Aldrich) staining of the lichen tissues was used for imaging the host structures. The fluorescent dyes Cy3, and Cy5 labeling the FISH probes were sequentially excited with 532 and 635 nm laser beams. Autofluorescence and calcofluor staining was excited with a 405 nm laser beam. The confocal stacks were acquired with a LeicaACS APO 40x oil CS objective lens (NA, 1.15) and a Leica ACS APO 63x oil CS objective lens (NA, 1.30) and for each field of view, an appropriate number of optical slices were acquired within a Z-step ranging from 0.15 to 0.5 µm. The software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used for imaging and post-processing of the confocal stacks and maximum projections. Adobe Photoshop (Adobe Systems Inc., USA) was used to label the final figures.

RESULTS

ANALYSIS OF *LOBARIA*-ASSOCIATED *RHIZOBIALES*

An assembled *Lobaria*-associated metagenome consisting of 362,424 contigs was utilized for taxonomic and functional studies of assigned *Rhizobiales*. In total, 88,602 contigs were assigned to bacteria. *Alphaproteobacteria* was the most frequently identified bacterial phylum in the metagenome and comprized 53,688 contigs (46.8% of all identified cellular organisms and 60.6% of identified bacteria). Of these, 28,526 assigned contigs belong to the predominant *Rhizobiales* (32.2% of identified bacteria), with families *Methylobacteriaceae* (11,421 contigs or 12.9% of identified bacteria), *Bradyrhizobiaceae* (5230 contigs or 5.9% of identified bacteria), and *Rhizobiaceae* (2403 contigs or 2.7% of identified bacteria). *Methylobacterium* was the only identified genus of *Methylobacteriaceae* and *Methylobacterium radiotolerans* (8% of all *Rhizobiales*) the most frequent species. Less frequent species were identified as *M. nodulans*, *M. populi* and members of the *M. extorquens* group. A total of 21% of present *Rhizobiales* was assigned to the cluster *Methylobacterium* sp. 4–46 or remained unclassified. Identified genera within the family of *Bradyrhizobiaceae* were more diverse and represented by four distinctive genera: *Bradyrhizobium* (7% of all *Rhizobiales*), *Rhodopseudomonas* (6% of all *Rhizobiales*), *Nitrobacter* (1% of all *Rhizobiales*) and *Oligotropha* (0.5% of all *Rhizobiales*).

Table 1 | Oligonucleotide probes utilized for FISH in this study.

Name	Sequence (5′-3′)	Fluorochrome	Target	Formamide (% at 43°C)	References
EUB338*	GCTGCCTCCCGTAGGAGT	Cy3	Most bacteria	10	Amann et al., 1990
EUB338II*	GCAGCCACCCGTAGGTGT	Cy3	*Planctomycetales*	10	Daims et al., 1999
EUB338III*	GCTGCCACCCGTAGGTGT	Cy3	*Verrucomicrobiales*	10	Daims et al., 1999
NONEUB**	ACTCCTACGGGAGGCAGC	Cy5 or Cy3	/	**	Wallner et al., 1993
RHIZ3r	GGCTTATCACCGGCAGTCTCC	Cy5	*Rhizobiales*	40	Nishio et al., 1997
RHIZ1244	TCGCTGCCCACTGTCACC	Cy5	*Rhizobiales*	50	Thayanukul et al., 2010

*Probes were used in equimolar concentration.

**NONEUB was applied as negative control; formamide concentrations were analog to the positive FISH probes.

Three percent of all *Rhizobiales* remained unclassified genera of the *Bradyrhizobiaceae* family. The most abundant species within *Bradyrhizobiaceae* was identified as *Rhodopseudomonas palustris* (6% of all *Rhizobiales*). *Rhizobiaceae* included the *Rhizobium/Agrobacterium* group (6% of all *Rhizobiales*) and the *Sinorhizobium/Ensifer* group (2% of all *Rhizobiales*). Less abundant *Rhizobiales* families were assigned to the genera of *Beijerinckiaceae* (5% of all *Rhizobiales*), *Xanthobacteraceae* (4% of all *Rhizobiales*), *Phyllobacteriaceae* (4% of all *Rhizobiales*) and *Brucellaceae* (0.4% of all *Rhizobiales*). A detailed taxonomic composition of *Rhizobiales* up to species level was visualized with Krona ((Ondov et al., 2011); **Figure 2**).

SEED-based functional analysis (**Figure 3**) focused on *Rhizobiales* and its three most abundant families. Included clustering and hierarchical organization of identified functions was utilized to retrieve quantitative information for highly abundant taxa. The abundance of function-related genes assigned to specified taxonomic ranks was compared to their overall occurrence in the entire metagenome. Thereby we obtained a comprehensive overview of functions with high relevance to the symbiotic system (Table S1). *Lobaria*-associated *Rhizobiales* were shown to be involved in all candidate functions (except biosynthesis of plant alkaloids). They were found to be involved in the biosynthesis of auxins and plant octadecanoids. Notably *Bradyrhizobiaceae* accounted for 24 contigs assigned to biosynthesis of plant octadecanoids, while *Methylobacteriaceae* only accounted for one contig containing this function. Nitrogen fixation was represented by 5 contigs assigned to *Rhizobiales*, and one assigned to either *Bradyrhizobiaceae* or *Methylobacteriaceae*. Type III secretion systems were found in 35 contigs, with 16 assigned to *Methylobacteriaceae*, 7 assigned to *Bradyrhizobiaceae*, one assigned to *Rhizobiaceae* and 11 without assignment to a specific family. One-carbon metabolism and carbon dioxide fixation were represented within *Rhizobiales* by 224 and 286 contigs, respectively. Biosynthesis of cofactors, vitamins, prosthetic groups and pigments was particularly frequent and represented by 848 contigs within *Rhizobiales*. Notably *Bradyrhizobiaceae* were found to contribute to chlorophyll biosynthesis (59 contigs), synthesis of folate and pterines (46 contigs) and coenzyme B12 biosynthesis (99 contigs). Conversely, *Rhizobiaceae* were rather underrepresented with 5 contigs assigned to synthesis of folate and pterines and 7 contigs assigned to coenzyme B12 biosynthesis. Overall stress response was found within 632 contigs associated with *Rhizobiales*. *Methylobacteriaceae* accounted

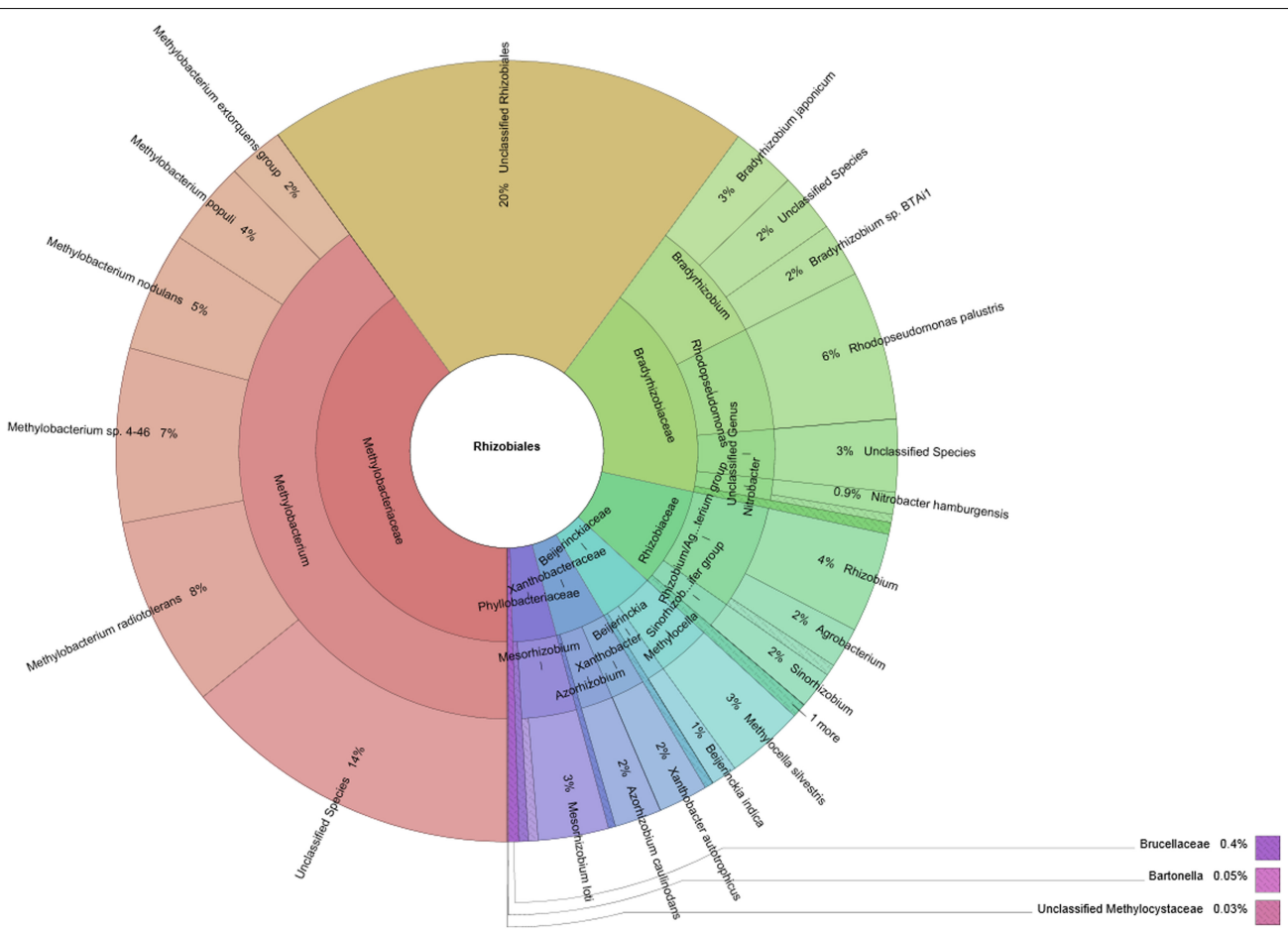

FIGURE 2 | *Rhizobiales* taxa within the *Lobaria*-associated metagenome are presented in a multi-level chart. Taxonomic information was retrieved through blastn assignments of assembled contigs, followed by processing with MEGAN. Circles represent taxonomic classifications in ascending order up to the species level (outermost circle). Less abundant taxa are listed outside the charts together with their relative abundance.

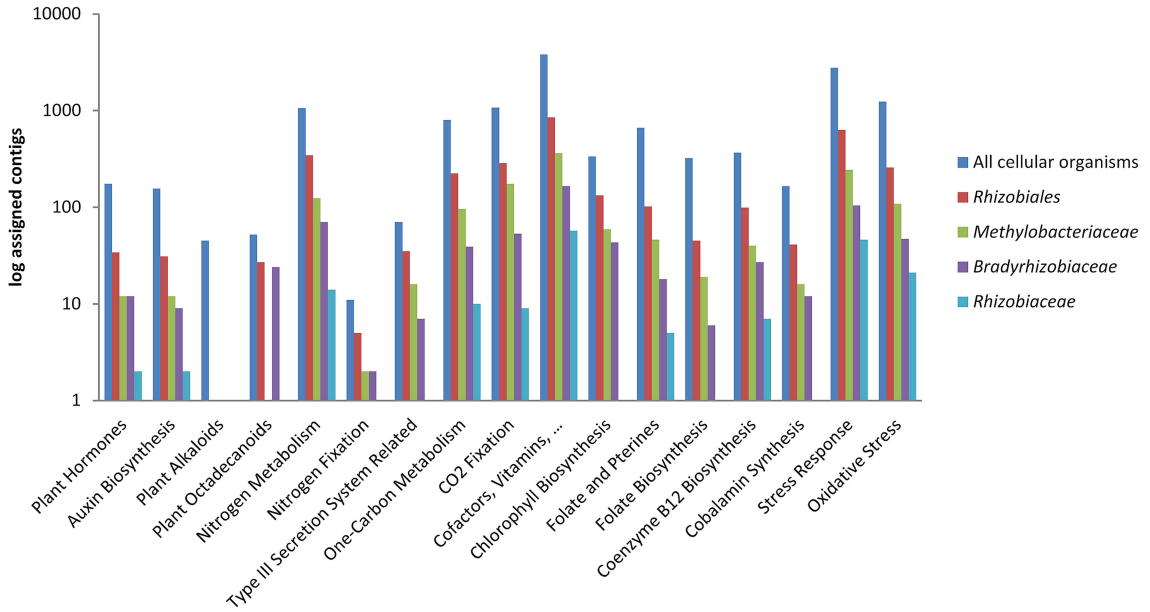

FIGURE 3 | Selected functions within the *Lobaria*-associated metagenome were assigned to taxa at order (*Rhizobiales*) and family (*Methylobacteriaceae, Bradyrhizobiaceae* and *Rhizobiaceae*) level together with hits without assignment to a specific taxon. Taxonomic information was retrieved through blastx assignments of assembled contigs, followed by processing with MEGAN.

for 243 contigs, while *Bradyrhizobiaceae* and *Rhizobiaceae* accounted for 104 and 46 contigs, respectively. Response to oxidative stress was present with 257 contigs (108 assigned to *Methylobacteriaceae*, 47 to *Bradyrhizobiaceae* and 21 to *Rhizobiaceae*).

EVALUATION OF THE RHIZ3r FISH PROBE FOR *RHIZOBIALES* STAINING

Alignments to sequences of the Silva (Quast et al., 2013) and Probematch databases (Cole et al., 2005) revealed that the only available FISH probe RHIZ1244 targeting *Rhizobiales* was not suitable to label taxa retrieved in the *Lobaria* microbiome (**Figure 4**; Table S2). The designed FISH probe RHIZ3r, based on the primer 3r (Nishio et al., 1997), was therefore evaluated and we could demonstrate a high coverage for specific taxa, including the most abundant families *Methylobacteriaceae* and *Bradyrhizobiaceae* (**Figure 4**). According to the Probematch analysis (Table S3), RHIZ3r (11166 hits) shows slightly reduced coverage in the order *Rhizobiales* compared to RHIZ1244 (14312 hits). However, the latter probe does not match well with the highly abundant families *Bradyrhizobiaceae* (7/11453) and *Methylobacteriaceae* (5/9098 hits), whereas RHIZ3r performs much better in this respect (*Methylobacteriaceae*: 3821/9098 hits; *Bradyrhizobiaceae:* 5586/11453 hits; Table S3). *In silico* alignment of the sequence to genus level shows that the FISH probe RHIZ3r targets bacteria belonging to the order *Rhizobiales*, families *Methylobacteriaceae* (genus *Methylobacterium*, coverage: 86%) and *Bradyrhizobiaceae* (genus *Bradyrhizobium*, coverage: 99%; genus *Afipia*, coverage: 100%; genus *Nitrobacter*, coverage: 100%; genus *Oligotropha,* coverage: 100%; genus *Rhodoblastus,* coverage: 100%; genus *Rhodopseudomonas*, coverage: 92%). The results of the *in silico* analysis were confirmed by identification of excised SSCP bands amplified with primer RHIZ3r (Erlacher et al., in press) and with the metagenomic data.

IN SILICO EVALUATION OF POTENTIAL FISH PROBES FOR *RHIZOBIALES* IN THE METAGENOME

We used Blastn to search for FISH probe binding sites within the entire assembled *Lobaria*-associated metagenome, and found different *Rhizobiales* taxa, including members of the families *Rhizobiaceae, Beijerinckiaceae, Bradyrhizobiaceae, Methylobacteriaceae, Methylocystaceae,* and *Phyllobacteriaceae*. Hits for non-targeted taxa included mostly unspecific chloroplast and plastid DNA as well as two hits for *Xanthomonadaceae* and one hit for *Pseudomonadaceae* (Table S2).

VIZUALISATION OF *LOBARIA*-ASSOCIATED *RHIZOBIALES*

Fluorescence *in situ* hybridization with both the RHIZ3r and the EUB338-MIX probes resulted in unambiguously strong signals. Image analysis and three-dimensional reconstructions of confocal stacks showed that most of the bacteria colonize *L. pulmonaria* at the outer surface of the lichen cortex (**Figures 5A–C**). Mixed colonies formed by putative *Rhizobiales* and other bacteria were frequently detected (**Figures 5A–C,E**), and morphological diversity of bacteria was apparent (**Figure 5E**). The autofluorescent fungi and algae in *Lobaria* allowed us to reconstruct the host structure (**Figures 5A,B,D**). Close co-existence between the bacteria and the hydrophilic fungal cortex were observed (**Figure 5D**), whereas intra-thalline hydrophobic spaces as well as photobionts were not colonized by bacteria (**Figures 5A,C**). Free

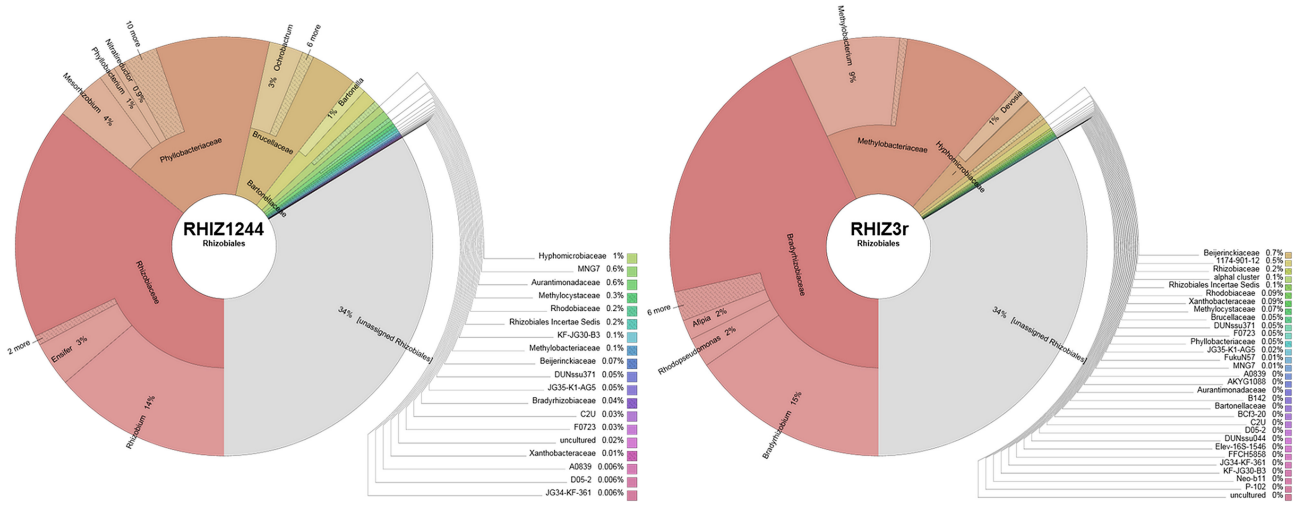

FIGURE 4 | TestProbe 3.0 (Silva ribosomal RNA database) analysis showing the targeted coverage of bacterial taxa in a comparison of RHIZ3r- and RHIZ1244- FISH Probes. The maximum amount of mismatches was set to 0 (see also Table S3 for RDP Probmatch comparison).

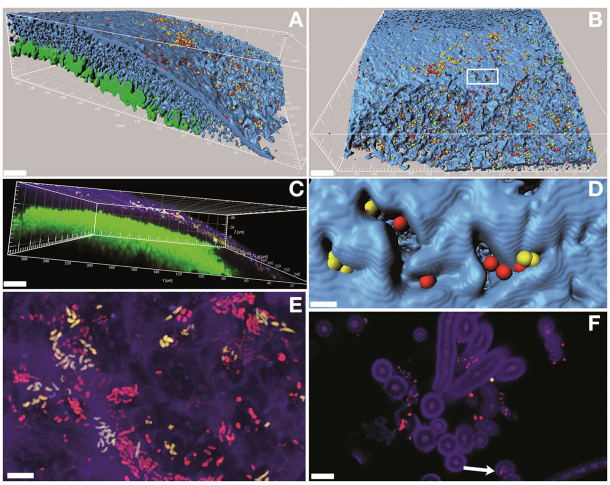

FIGURE 5 | Confocal laser scanning microscopy (CLSM) micrographs showing the bacterial colonization of the lichen *L. pulmonaria* stained by fluorescence *in situ* hybridization (FISH). Green, algae; blue/purple, fungi; yellow, *Rhizobiales*; red, other bacteria. (**A,B,D**), three-dimensional models made of isosurfaces and spheres; **(C)**, volume rendering; **(E)**, maximum projection; **(F)**, single optical slice. In **(B)**: white box indicates the region shown in **(D)**. Arrow in **(F)** indicates an endophytic bacterial cell (see also Figure S1). Scale bars: **(A-C)** (30 μm); **(D-F)** (5 μm).

hyphae protruding from the lower surfaces were often covered by bacteria. In rare cases, bacteria colonized the hyphae internally (**Figure 5F**; Figures S1, S2).

DISCUSSION

Our new study provides a first insight into the functional potential of *Rhizobiales,* which are the predominant order of bacteria associated with the lichen symbiosis. *Rhizobiales* are responsible for more than one third of all bacterial taxonomic assignments. About 20% of our metagenomic assignments could not be placed in any of the *Rhizobiales* lineages, which indicates that there might be numerous yet undescribed bacterial diversity colonizing lichens. One taxonomically undescribed phylogenetic lineage of *Rhizobiales*, not present in our dataset, was detected in diverse lichens from North America, and named LAR1 (Hodkinson and Lutzoni, 2009). Most of the classified bacteria in our dataset belong to the families *Methylobacteriaceae, Bradyrhizobiaceae* and *Rhizobiaceae*, which are therefore expected to play an important role within the lichen symbiosis.

Because *Rhizobiales* are common in growing lichen parts, we argue that they could play a role in development and growth of lichens. This hypothesis is well supported by the potential functions encoded in the metagenomic contigs of *Alphaproteobacteria* and *Rhizobiales* in our dataset. SEED-based functional analysis revealed functions supporting the symbiosis, including auxin and vitamin production, nitrogen fixation and stress protection. Taxonomical assignments showed high proportions of beneficial nitrogen fixing at species level. However, we think that nitrogen-fixation is not a required rhizobial function in the *L. pulmonaria* symbiosis, because fixed nitrogen is provided by the associated cyanobacterial partners (which is located in clustered colonies, in so-called internal cephalodia), and because excessive nitrogen (e.g., agricultural contamination) is rather a problem affecting the survival of *L. pulmonaria* in many localities. It is therefore interesting to observe a significant number of contigs that is assigned to nitrogen metabolism. Metabolism related to cofactors and vitamin production is also well represented in our dataset, suggesting that the corresponding products are valuable to support the growing lichen thallus. In addition, the high abundance of *Methylobacterium* species might be a promising source to find novel compounds or bioconversion as in higher plants (Verginer et al., 2010). In comparison with a study of *Methylobacterium* spp. on mosses by Erlacher et al. (in press), using fingerprinting methods, we

detected higher species diversity in the *L. pulmonaria* microbiome, which is also confirmed by the metagenomic data. Stress protection for the symbiosis by bacteria was detected, which seem to play a unique and important function of host-associated microbiomes. Stress protection was already detected for mosses (Bragina et al., 2014) but also for plant-associated bacteria (Alavi et al., 2013). The biotechnological potential of stress-protecting bacteria was already shown (Alavi et al., 2013; Berg et al., 2013), which shows new solutions for agriculture in a changing climate.

So far, colonization of lichens was mostly shown on surfaces of lichens (e.g., Cardinale et al., 2008). The present study clearly shows that *Rhizobiales* members are not restricted to the thallus surface. It is thus tempting to consider endobiotic life style of bacteria, similar to endophytism in plants. However, there are marked differences to an endophytic lifestyle of higher plants. Plants are typically characterized by a protective cuticula which forms a clear boundary between the plant and the external environment. By the cuticula internal tissues, plants are protected against uncontrolled water loss or contamination from external water, dirt, and from invasion of microorganisms. Such a layer is missing in thalline organisms such as mosses or lichen thalli (which are also known as "lower plants" or "cryptogams"). Both mosses and lichens belong to poikilohydric organisms, desiccating with atmospheric drought. Without a cuticula it is also more difficult to differentiate between endosphere and phyllosphere. The present data confirm that there is no clear external border of the lichen surface. We have already observed a depletion of bacterial abundance in other lichens but no qualitative differences when we analyzed bacterial associates of lichens after increasing duration of surface sterilization (unpublished data). By studying lichens we uncover interesting new insights about the endophytic strategies. In some lichens, the internal parts of lichens can be colonized. This is clearly shown in *Cladonia*, where hollow thalli are internally colonized by biofilm like bacterial communities (Cardinale et al., 2008). In cases of crust-forming lichens we observed that bacteria can partly enter the lateral parts of neighboring thallus segments (areoles; e.g., *Lecanora polytropa*, Grube et al., 2009). The case of *Lobaria* now shows that the external polysaccharide matrix between the hyphae of the lichens can, at varying depths, be penetrated by *Rhizobiales*. The loose aggregation of hyphae and the lack of a cuticula found in higher plants facilitate mutualistic bacterial colonization which gradually develops from ecto- to endo-symbiotic lifestyles. We have not observed bacteria so far in the algal layer or in the aerated medulla part beneath the algal layer. We suppose this is due to the fact that particularly the medulla layer of lichens has strongly hydrophobic surfaces (due to a hydrophobin cell layer, which enwrap the cells of the eukaryote partners). However, we also found first indications of endohyphal occurrences of bacteria in *L. pulmonaria* (**Figure 5F**; Figures S1, S2). While our findings of intracellular colonization of lichenized-fungal hyphae still require additional methodological prove to be validated, the endohyphal bacterial occurrence in non-lichenized fungi has repeatedly been found in very different lineages (e.g., Bertaux et al., 2005; Partida-Martinez and Hertweck, 2005; Sharma et al., 2008). Recent work sheds light on their diverse functions (Ghignone et al., 2012), and also revealed new details regarding how bacteria penetrate fungal hyphae (Moebius et al., 2014). We argue that occasional endohyphal bacteria in lichens might be particularly efficient strains to digest the rather thick fungal cell walls in lichens. This observation may also spur new interest in lichens as a bioresource for biotechnological applications.

Beneficial plant-microbe interactions were extensively studied in the past and reviewed by Berg (2009). Such interactions include diverse and important functions including the suppression of pathogens and the increase in plant growth and fitness. While the traits involved in bacterial adaption and exchange of particular metabolites to higher plants are partially deciphered (Vorholt, 2012), less is known about microbe-lichen interactions. Functional assignments from the metagenome suggest *Rhizobiales* as a vital component supporting the lichen symbiosis. Results indicate that they are able to supply auxiliary as well as essential metabolites to their host. This study is the first to relate the abundance of bacteria with potential functions of their representatives within the lichen structure. Our present study also provides first indications for lichen endosymbiosis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00053/abstract

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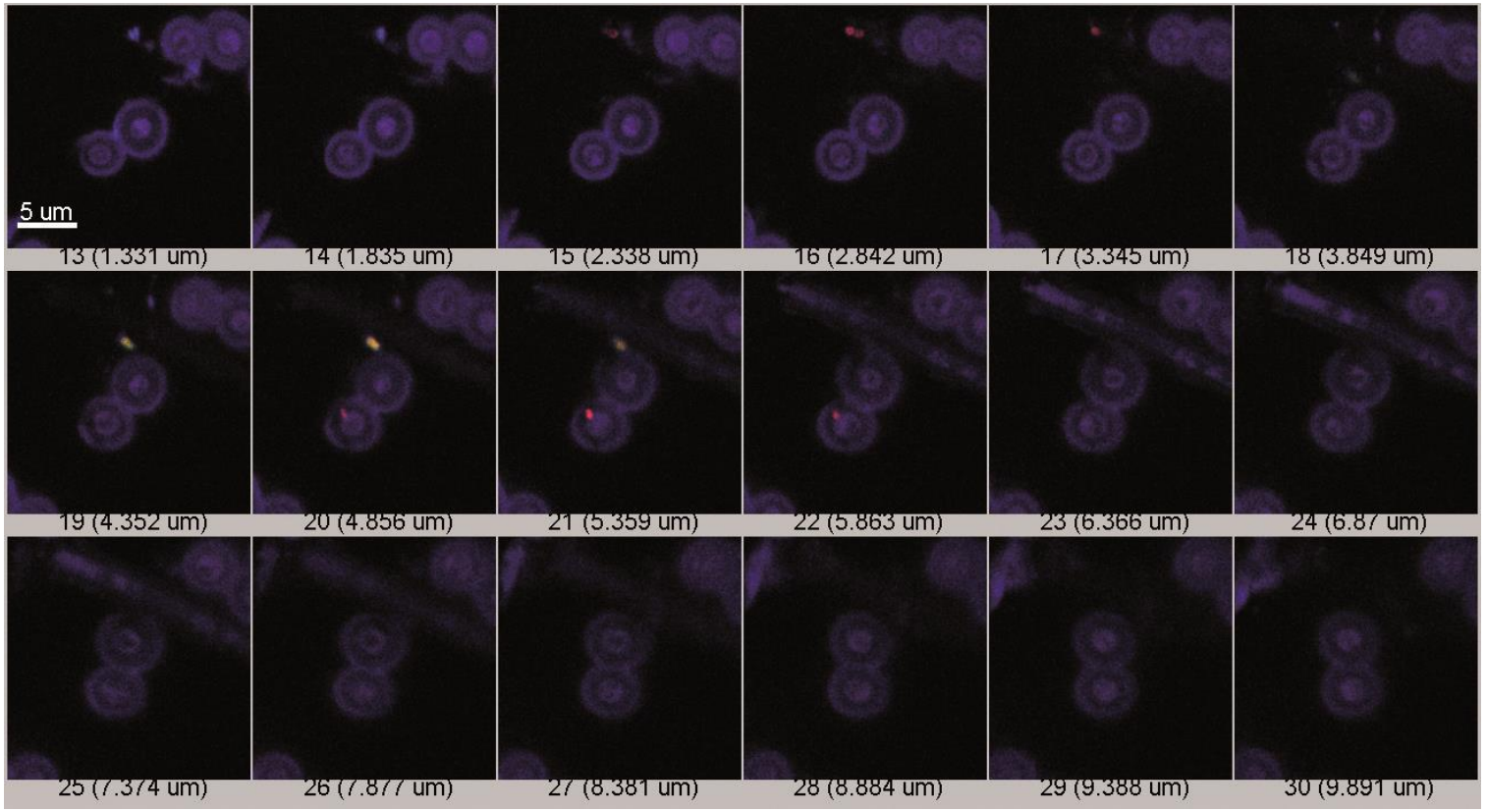

Figure S1 Endohyphal bacterial occurrence in *Lobaria pulomonaria*. Confocal microscopy image series showing a FISH-stained bacterial cell endophytically colonizing a fungal hypha. The distance between the confocal planes is 0.5 µm. Blue/purple = fungi (autofluorescence); yellow = RHIZ3r-FISH+EUB338-MIX stained bacteria; red = only EUB338-MIX stained bacteria.

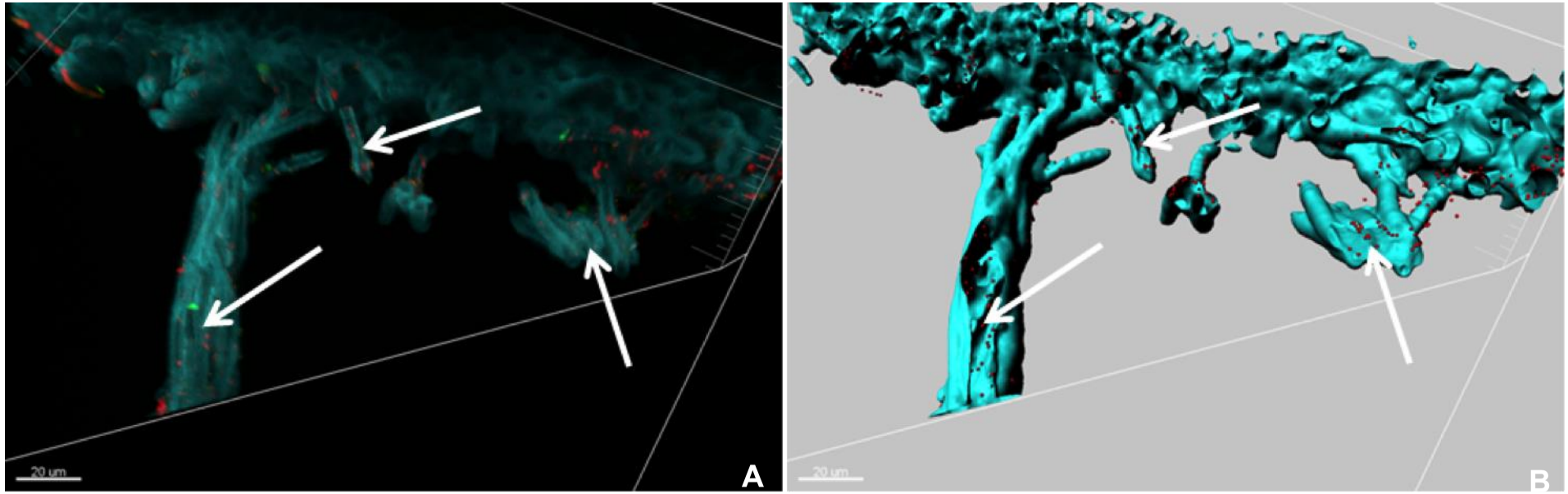

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Figure S2 Endohyphal bacterial occurrence in *Lobaria pulomonaria*. Confocal microscopy volume (A) and iso-surface model (B) showing FISH-stained bacterial cells (red, arrows) endophytically colonizing fungal hyphae. The distance between the confocal planes is 0.5 µm.

Table S1 Assignment of SEED functions to bacterial taxa within the *Lobaria* metagenome. Functions were assigned to overall *Rhizobiales* and the three most abundant families therein. Higher SEED level functions are highlighted in grey with more specific functions thereunder (not highlighted).

	Assignment of SEED functions to specific bacterial taxa				
	All cellular organisms	Rhizobiales	Methylobacteriaceae	Bradyrhizobiaceae	Rhizobiaceae
Contigs assigned to specified taxon	**114821**	**28526**	**11421**	**5230**	**2403**
SEED functions of interest					
Plant Hormones	175	34	12	12	2
Auxin Biosynthesis	156	31	12	9	2
Plant Alkaloids	45	0	0	0	0
Plant Octadecanoids	52	27	1	24	0
Nitrogen Metabolism	1060	344	124	70	14
Nitrogen Fixation	11	5	2	2	0
Type III Secretion System Related	70	35	16	7	1
One-Carbon Metabolism	802	224	96	39	10
CO2 Fixation	1072	286	175	53	9
Cofactors, Vitamins, Prosthetic Groups, Pigments	3799	848	361	165	57
Chlorophyll Biosynthesis	334	133	59	43	0
Folate and Pterines	662	102	46	18	5
Folate Biosynthesis	323	45	19	6	0
Coenzyme B12 Biosynthesis	365	99	40	27	7
Cobalamin Synthesis	165	41	16	12	0
Stress Response	2769	632	243	104	46
Oxidative Stress	1238	257	108	47	21

Table S2 *In silico* evaluation of the FISH probe with the whole *Lobaria*-associated metagenome and blastn analysis.

Contig ID	Score (bits)	E-value	Closest BLASTn hit
NODE_342079_length_71_cov_5.422535	42	4,00E-04	*Methylobacterium* sp.
NODE_215355_length_99_cov_22.181818	42	4,00E-04	Uncultured bacterium/Chloroplast DNA
NODE_13033_length_932_cov_5.468884	42	4,00E-04	*Afipia* sp.
NODE_1705_length_71_cov_25.323944	42	4,00E-04	Uncultured bacterium/Plastid DNA
NODE_48805_length_113_cov_49.150444	40	0,002	*Microvirga* sp.
NODE_500503_length_5127_cov_5.032768	38	0,006	Plastid/Chloroplast DNA
NODE_245160_length_460_cov_4.478261	38	0,006	*Methylocella* sp.
NODE_469663_length_91_cov_3.560440	36	0,025	*Mesorhizobium* sp.
NODE_419997_length_203_cov_4.650246	36	0,025	Uncultured bacterium
NODE_419761_length_91_cov_9.362638	36	0,025	*Rhodanobacter* sp./*Luteibacter* sp.
NODE_398947_length_86_cov_3.941860	36	0,025	Uncultured *Rhizobiales* bacterium
NODE_398053_length_107_cov_4.093458	36	0,025	*Rhodanobacter* sp.
NODE_239641_length_131_cov_18.465649	36	0,025	*Pseudomonas* sp.
NODE_79526_length_86_cov_16.546511	36	0,025	*Methylocystis* sp.
NODE_400044_length_11426_cov_5.588395	34	0,1	Uncultured *Rhizobiales* bacterium
NODE_81818_length_110_cov_3.336364	34	0,1	Chloroplast DNA
NODE_3958_length_3680_cov_154.217941	34	0,1	*Rhizobiales* sp.
NODE_367687_length_1456_cov_5.146978	30	1,6	*Beijerinckia* sp.
NODE_345763_length_80_cov_6.550000	30	1,6	*Rhizobum* sp.
NODE_305066_length_80_cov_10.550000	30	1,6	Chloroplast DNA

Table S3 RDP Probematch analysis showing the comparison of RHIZ3r- and RHIZ1244-FISH Probes. Bold letters indicate taxa occurring in the *Lobaria* Metagenome; Asterisks indicate the two most dominant groups *Methylobacteriaceae* and *Bradyrhizobiaceae*. The Maximum amount of mismatches was set to 0.

RHIZ3r	Root (13311/3122829) ; Bacteria (13311/2879170) ; "Proteobacteria" (13126/944978) ; Alphaproteobacteria (12664/215572)
	order Rhizobiales (11166/71864) (hits/total searched)
	family "Aurantimonadaceae" (1/1437)
	family Bartonellaceae (0/475)
	family Beijerinckiaceae (169/1791)
	* **family Bradyrhizobiaceae (5586/11453)**
	family Brucellaceae (0/2902)
	family Cohaesibacteraceae (0/112)
	family Hyphomicrobiaceae (236/6126)
	* **family Methylobacteriaceae (3821/9098)**
	family Methylocystaceae (7/1575)
	family Phyllobacteriaceae (2/5718)
	family Rhizobiaceae (6/12475)
	family Rhodobiaceae (6/1269)
	family Xanthobacteraceae (5/1813)
	family Rhizobiales_incertae_sedis (1/290)
	unclassified_Rhizobiales (1326/15330)
RHIZ1244	Root (16831/3122829) ; Bacteria (16831/2879170) ; "Proteobacteria" (16777/944978) ; Alphaproteobacteria (16398/215572)
	order Rhizobiales (14312/71864) (hits/total searched)
	family "Aurantimonadaceae" (33/1437)
	family Bartonellaceae (395/475)
	family Beijerinckiaceae (11/1791)
	* **family Bradyrhizobiaceae (7/11453)**
	family Brucellaceae (1670/2902)
	family Cohaesibacteraceae (0/112)
	family Hyphomicrobiaceae (143/6126)
	* **family Methylobacteriaceae (5/9098)**
	family Methylocystaceae (13/1575)
	family Phyllobacteriaceae (3475/5718)
	family Rhizobiaceae (7736/12475)
	family Rhodobiaceae (26/1269)
	family Xanthobacteraceae (4/1813)
	family Rhizobiales_incertae_sedis (20/290)
	unclassified_Rhizobiales (774/15330)

Additional Publication III

IOBC WPRS Bulletin, in press

Mosses and lichens provide specific micro-habitats for pink pigmented facultative methylotrophs (PPFMs)

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Mosses and lichens provide specific micro-habitats for pink pigmented facultative methylotrophs (PPFMs)

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Abstract: Methylotrophic bacteria, also known as pink pigmented facultative methylotrophs (PPFMs) colonize the surfaces of almost all known plants and often exert beneficial functions for the host. Little is known about their occurrence on mosses and lichens. Here we selected two *Sphagnum* spp. (*S. fallax* and *S. magellanicum*) and *Lobaria pulmonaria* as representatives to study the abundance and diversity of associated methylotrophic bacteria. By isolation, three distinctive *Methylobacterium* species were detected but only one of them occurred in *S. magellanicum* at a much lower extent. Very acidic pH of *S. magellanicum* could partially explain the difference in *Methylobacterium* abundance compared to the morphologically similar species *S. fallax*. Phylogenetic analysis showed cryptogam-specific clades, indicating potentially new *Methylobacterium* species. To investigate further uncultivated methylotrophs, we have amplified the 16S rRNA genes from the metagenomic DNA with a broad−spectrum primer set; single strand conformation polymorphism (SSCP) was used to recover bands belonging to methylotrophs. By SSCP band sequencing we were able to detect abundant genera shared by the hosts, whereas rare species were host-specific. These results enhance our knowledge of mehtylotrophic bacteria in cryptogams and show the co-occurrence of shared and host-specific taxa.

Key words: Pink Pigmented Facultative Methylotrophs (PPFMs), *Methylobacterium*

Introduction

Pink pigmented facultative methylotrophic bacteria (PPFMs) inhabit the above-ground parts of almost all known plants so far studied. PPFMs and, in particular, members of the genus *Methylobacterium* can affect the host metabolism in various ways, including production of vitamins and phytohormones, such as auxines and cytokinines (Ivanova *et al.*, 2000; Delmotte *et al.*, 2009). Some PPFM species are able to nodulate legumes and fix atmospheric nitrogen (Jourand *et al.*, 2004). *Methylobacterium* spp. can utilize methanol emitted by host-plants, methylamine and further C_2, C_3, and C_4 compounds as sole carbon and energy source (Green and Bousfield, 1983; Lidstrom and Christoserdova, 2002). Although the importance of methylotrophs for higher plants is well-established, rather little is known about their occurrence on certain cryptogams. In contrast to higher plants, metabolism and photosynthetic activity of cryptogams mainly depend on their actual hydration level. In addition, they do not propagate by seeds, but produce spores, making them evolutionary and microbiologically more stable. Among cryptogams, mosses and lichens can be interpreted as symbiotic mini-ecosystems containing a functionally active microbiome, which contributes to both survival and growth under even extreme abiotic conditions (Bragina *et al.* 2012). To investigate PPFMs in these evolutionary old organisms can help in understanding their importance within the indigenous microbiome as well as possible interactions with the host. The latter might suggest, in light of the PPFM functions in higher plants, active roles in supporting cryptogams' fitness.

In this work we investigated the PPFM communities associated with three cryptogams: two *Sphagnum* spp. (*Sphagnum magellanicum* Brid. and *Sphagnum fallax* (H. Klinggr.) H. Klinggr.) and *Lobaria pulmonaria* (L.) Hoffm., collected within a close geographical distance, in an alpine region characterized by relatively mild climatic conditions. We applied a polyphasic approach including cultivation−dependent and independent methods with the aim to assess diversity, to individuate potential novel lineages of methylotrophs and to study their colonization strategies.

Material and methods

Sampling and experimental design

Samples of the peat mosses *Sphagnum magellanicum* and *Sphagnum fallax* were collected in the Pürgschachen peat bog (Ardning, Styria, Austria, GPS 47°59´N, 14°36´E). *Lobaria pulmonaria* samples were collected in an Abieti-Fagetum forest from mountain maple in Nationalpark Gesäuse near Admont (Gstatterboden, Styria, Austria, 47°57´N, 14°61´E). Both samplings were carried out on 16. November 2011. For each species, four independent replicates consisting of approximately 30 gametophytes plantlets or five to ten lichen thalli, respectively, were collected and stored in sterile plastic bags on ice.

Isolation and identification of methylotrophs

Cultivation of *Methylobacterium* sp. was achieved by imprinting sample-tissues on methylothrophs-selective methanol-inorganic salt medium (MIS; Corpe 1985). Genomic DNA was extracted from pure cultures following the method described by Nishio *et al.* (1997) and ITS-PCR was carried out as described by Cardinale *et al.* (2004) using the primers ITS-F (5´-GTC GTA ACA AGG TAG CCG TA-3´) and ITS-R eub (5´-GCC AAG GCA TCC ACC-3´). ITS amplicons were separated on 1.5% agarose together with 1 kb DNA Ladder (Fermentas) as a reference and stained with ethidium bromide. GelComparII software (version 4.1; Applied Maths, Kortrijk, Belgium) was used for grouping the ITS profiles into OTUs based on their fingerprint similarity (dice coefficient). For the identification of the OTUs, the 16S rRNA gene of one or more representative isolates per OTU was amplified with the primers 27f/1492r (Lane *et al.*, 1991; Blackwood *et al.*, 2005), sequenced by the ZMF – Center for Medical Research, Graz, Austria, and aligned with reference sequences using BLASTn (Altschul *et al.* 1997). To infer phylogenetic relationships, 16S rRNA gene sequences, representative of all OTUs, and reference sequences of several *Methylobacterium* strains were aligned by Clustal X (Thompson *et al.* 1994) and used to build a neighbor joining tree with the PHYLIP package version 6.69. (Felsenstein, 2005).

Molecular fingerprints of methylotrophic bacteria

Samples were prepared according to Opelt *et al.* (2007) and SSCP was carried out according to Schwieger and Tebbe (1998).

Results

Isolation and identification of methylotrophs

Methylobacterium colonies, characterized by the typical pink pigmentation, were visible on plates after nine days of incubation at 30°C. In total, 131 pure cultures were obtained from *S. fallax* (48), *S. magellanicum* (5) and *L. pulmonaria* (78) using a comparable sampling

approach. Their ITS fingerprints were clustered into 37 operational taxonomic units (OTUs) on the base of the profile similarity. The most dominant OTUs were shared among all hosts but were present at different abundances. Species-specificity was found among minor OTUs, which were the most numerous: in fact, 30 OTUs out of 37 undergo 4% of relative abundance. Taxonomical identification of the OTUs by BLASTn alignment resulted in three different species: *Methylobacterium adhaesivum* (22), *M. mesophilicum* (11) and *M. rhodesianum* (4). *M. adhaesivum* was detected in all three hosts, whereas *M. mesophilicum* and *M. rhodesianum* could be identified from both *S. fallax* and *L. pulmonaria* but not in *S. magellanicum*. Phylogenetic analysis of representative isolates and reference sequences confirmed the taxonomic affiliation suggested by BLASTn alignment, and showed that various cryptogam-associated isolates cluster within the species *M. adhaesivum*, *M. mesophilicum* and *M. rhodesianum* (Fig. 1).

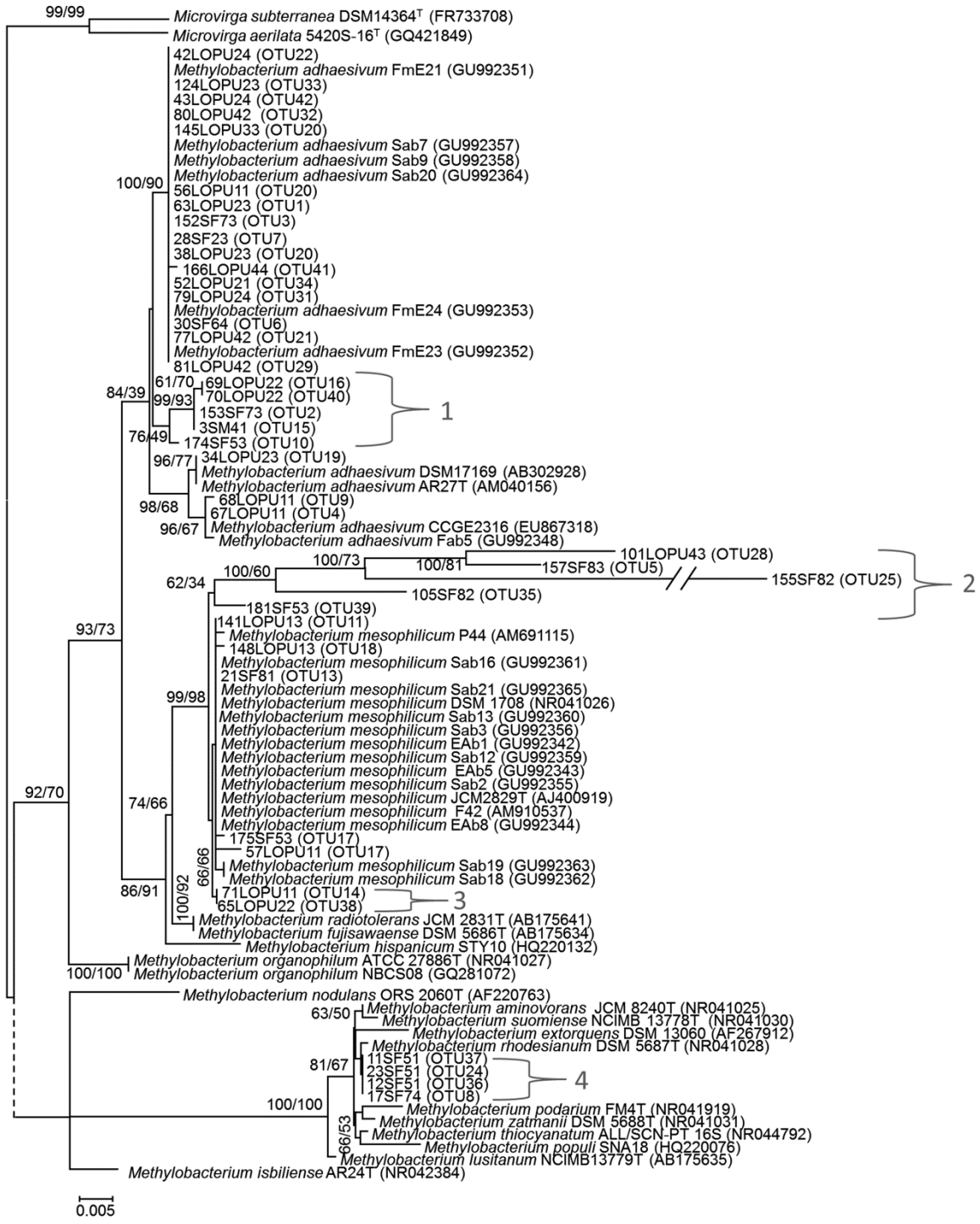

Figure 1. Neighbor joining tree showing the phylogenetic relationships between 16S rRNA gene sequences of PPFMs isolated in this study and reference sequences. 1, 2, 3 and 4: monophyletic clades exclusively including isolates from this study. The abbreviations LOPU, SF and SM represent the isolation source of the representative isolate sequenced (*Lobaria pulmonaria*, *Sphagnum fallax* and *Sphagnum magellanicum*, respectively).

Interestingly, several monophyletic clades formed exclusively by our isolates were found. One clade included OTUs from all three hosts (Fig. 1, clade 1), whereas clades 2 and 4 included OTUs from *S. fallax* and *L. pulmonaria,* and clade 3 from *L. pulmonaria* only.
A pH depending growth assay showed that none of the tested strains was able to grow in nutrient broth II media acidified to a pH of 4 or lower. At pH 5 only strain 21SF81 identified as *M. mesophilicum* grew, whereas other strains were already inhibited (data not shown). Additionally, alkalinization of liquid medium was observed after bacterial growth for all tested strains.

Molecular fingerprints of methylotrophic bacteria
SSCP fingerprints showed higher variability between host individuals than within them (Fig. 2). The variability within independent replicates was higher within *L. pulmonaria* (up to 80 % of the banding pattern), in comparison to the *Sphagnum* spp.

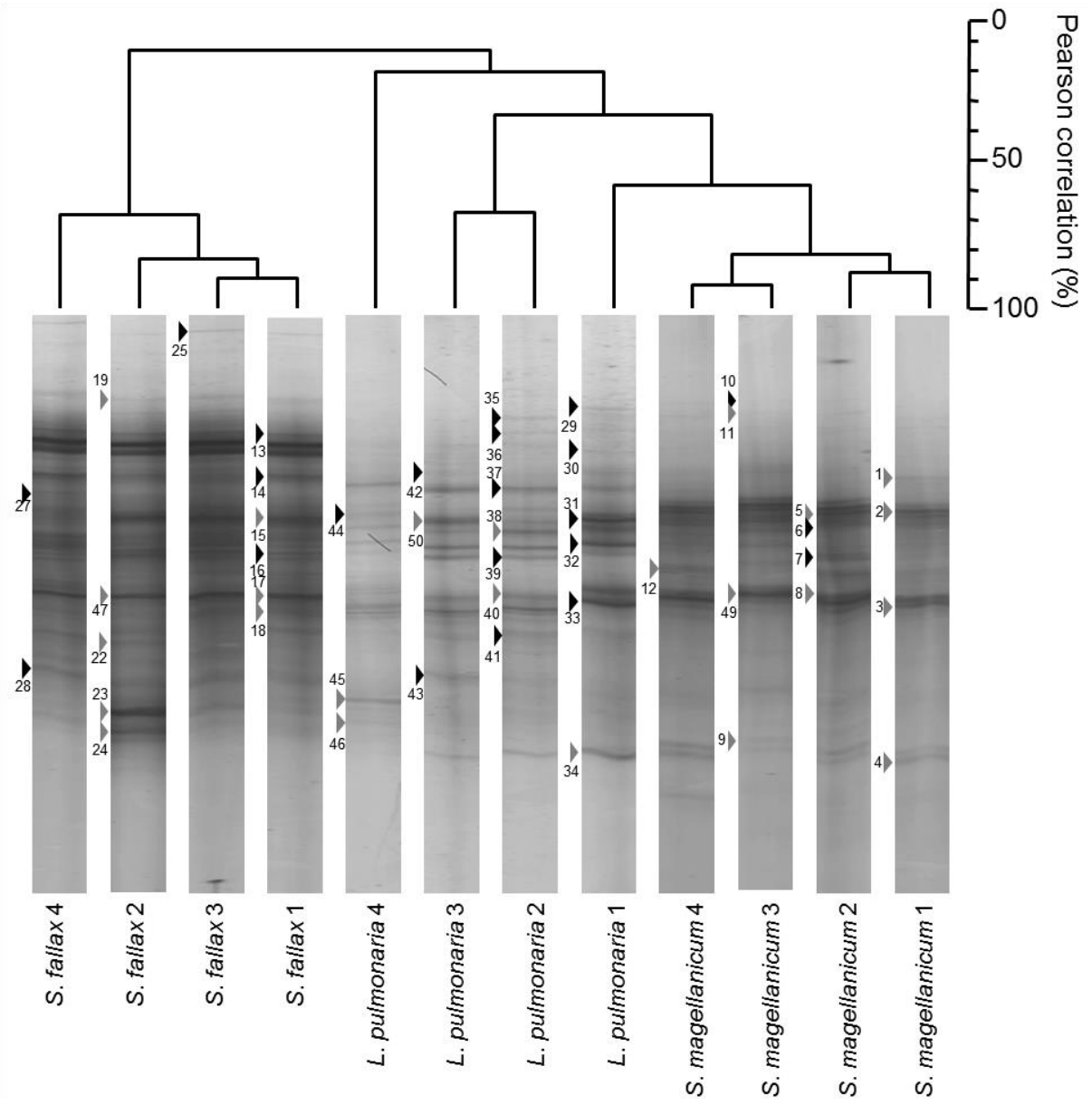

Figure 2. Microbial fingerprints of methylotrophic bacteria obtained from *S. fallax, S. magellanicum, L. pulmonaria*. Arrow heads indicate the excised bands identified as members of Rhizobiales. *Methylocystis* and *Methylocapsa* were found exclusively in *Lobaria* and in *S. fallax*, respectively. Further bands recovered from both *Sphagnum* ssp. were putatively affiliated with *Methylosinus*, although BLASTn alignment showed the same sequence similarity with some *Beijerinckia* spp. In addition to the methylotrophs, bands representing members of the families *Hyphomicrobiaceae, Bradyrhizobiaceae* and *Beijerinckiaceae* were retrieved from all hosts but with different relative abundance.

Discussion

We found remarkable differences between *S. fallax, S. magellanicum* and *L. pulmonaria* in terms of colonization, structure and diversity of total bacteria and PPFMs. These differences could reflect different roles as well as different stability of association with the host. The fact that almost no *Methylobacterium* could be isolated from *S. magellanicum* demonstrates that even very closely related hosts, co−occurring in the same site, can surprisingly exhibit an unexpected difference in terms of specific bacterial populations. A reason for such difference between the two *Sphagnum* spp. here investigated might be their environmental requirements. Bragina *et al.* (2012) showed that the habitat of *S. magellanicum* is acidic and we showed that our isolates belonging to the species *M. adhaesivum, M. mesophilicum* and *M. rhodesianum* have limited growth at a pH of 5. Moreover the habitat of *S. magellanicum* is also extremely poor in nutrients (Bragina *et al.*, 2012). These two abiotic parameters may represent a growth limit for possible colonization by *Methylobacterium* spp.

The phylogenetic clusters formed exclusively by our isolates suggest the occurrence of putatively new *Methylobacterium* species (clade 2) or new strains specific to the geographical region (clade 1) or to the host (clades 3 and 4). This could reflect a stable association with the host and may be the effect of a long lasting co-evolution. So far, there is not enough data to reveal ubiquitism of certain species, but in our study we could demonstrate that, among the cryptogams studied here, new lineages were retrieved from all hosts, whereas others were more specific. New methylotrophic species were isolated already from lower plants (Schauer and Kutschera, 2011; Schauer et al, 2011), and the exploitation of new bioresources can lead to the discovery of biotechnologically interesting new microbial strains. *Sphagnum* spp., among other mosses, and lichens are known for their healing and antiseptic properties from ethnic medicine (Painter, 2003; Shukla *et al.*, 2010).

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-Thank you-

Curriculum Vitae

PERSONAL DATA

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EDUCATION

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Sep. 1998 – Nov. 2001	Technical College Bulme Graz-Gösting
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WORKING EXPERIENCE

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Jul. 2010	Participated in BEST biotechnology course "I made it, I ate it", University of Tallinn, Tallinn, Estonia
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Statistical/Analytical: R, SPSS
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PUBLICATION LIST

WHITE PAPERS/REPORTS

Erlacher, A., Cardinale, M., Grosch, R., Grube, M., & Berg, G. (2014). The impact of the pathogen *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous lettuce microbiome. *Frontiers in microbiology*, 5: 175.

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Erlacher, A., Grube, M., & Berg, G. Metagenom (Manuscript I).

Erlacher, A., Spanberger, Grube, M., & Berg, G. Impact of Biocontrol on the lettuce microbiome.

ORAL PRESENTATIONS

Erlacher, A., Cardinale, M., Grosch, R., Grube, M., & Berg, G (2012). The lettuce and bananas associated microbiome and its impact on human health. *COST FA1103 - Endophytes in Biotechnology and Agriculture (WORKING GROUPS 1 - 4 MEETING)*. San Michele all'Adige (Trento), Italy.

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