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Secret collaborations: How plant-associated archaea interact with their hosts

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AFFIDAVIT

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"Blessed are they who see beautiful things in humble places,

where other people see nothing."

(Camille Pissaro)

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Abstract

Plant holobionts are known to harbour a wide diversity of microorganisms, such as fungi and bacteria, which influence resistance to stress, nutrition and fitness of the plant, whereas archaea have been often overseen. Up to now, archaea have been found to be ubiquitous, colonizing multiple habitats with various conditions. Recent studies identified archaea even as members of plant microbiomes but their structure and function are less understood. The objective of this thesis was to study their structure in native and crop plants, and to identify their function, assembly and transmission in plant hosts.

To get a deeper insight into plant-associated archaea, 30 different agriculturally used plant-species from Austria and Eastern Africa were analysed and compared, such as arugula, tomato, and the leafy greens Okra, Nightshade, Black Jack and Spiderwisp, as well as the native vegetation of two alpine raised bogs from upper Styria (Austria). Therefore, a combined approach was conducted, including 16S rRNA gene fragment amplicon sequencing, real-time PCR (qPCR), metagenome shotgun sequencing and analysis, and fluorescence *in situ* hybridization and confocal laser scanning microscopy (FISH-CLSM).

Each plant harboured specific archaeal communities, while their abundance varied. Moreover, archaeal communities differed according to the plant compartments, whereat enrichment in the rhizosphere in comparison to soil was detected. In crops without supplementation of fertilizers *Thaumarchaeota* were clearly dominating the archaeal community structure, whereas in native bog vegetation *Euryarchaeota* were predominant. Although archaea were abundant in seeds of tomatoes, no indications of a plant-mediated vertical transmission of archaea could be found. The colonization of the plant rather origins from the soil than from the mother plant, indicating that archaea represent only bystander microorganisms in seeds. On plants, functional signatures were observed for putative adaptation mechanisms of archaea for their hosts, including those for nutrient cycling like CO_2 and N_2 fixation, stress response, higher chemotaxis, and possible plant growth promotion through auxin biosynthesis. Although archaea were found to have the potential to interact with their host, the life strategy of the host did not directly impact the archaeal community structure. All these findings combined reveal a so far unobserved role of archaea for plant holobionts, as substantial and functional constituents of plant microbiomes.

Zusammenfassung

Pflanzen-Holobionten beherbergen eine große Vielfalt von Mikroorganismen, wie z.B. Bakterien und Pilze, die die Nährstoffversorgung, Stressresistenz und Fitness beeinflussen. Archaeen an Pflanzen fanden in der Forschung allerdings weniger Beachtung. Bisher wurde festgestellt, dass Archaeen weit verbreitet sind und eine Vielzahl von Lebensräumen mit unterschiedlichen Bedingungen besiedeln. Neuere Studien identifizierten Archaeen sogar als Bestandteil von Pflanzenmikrobiomen, aber ihre Struktur und Funktion sind weniger bekannt. Das Ziel dieser Arbeit war es, die Struktur von Archaeen in einheimischen Pflanzen und Kulturpflanzen zu untersuchen, um ihre Funktion, Gemeinschaftsstruktur, Übertragung und Wechselwirkungen mit ihren Pflanzenwirten zu identifizieren.

Um einen tieferen Einblick in pflanzenassoziierte Archaeen zu erhalten, wurden 30 verschiedene landwirtschaftlich genutzte Pflanzenarten aus Österreich und Ostafrika analysiert und verglichen, darunter Rucola, Tomaten und die Blattgemüse-Arten (sog. „Leafy Greens“) Okra, Nightshade, Black Jack und Spiderwisp, sowie die heimische Vegetation alpiner Hochmoore aus der Obersteiermark (Österreich). Hierzu wurde ein kombinierter Ansatz aus quantitativer PCR (qPCR), 16S-rRNA-Genfragment-Amplikonsequenzierung, Genom-Shotgun-Sequenzierung und –Analyse, Fluoreszenz-*in-situ*-Hybridisierung und konfokaler Laser-Scanning-Mikroskopie (FISH-CLSM) verwendet.

Jede Pflanze beherbergte spezifische Gemeinschaften von Archaeen, wobei ihre Häufigkeit variierte. Darüber hinaus unterschieden sich die archaellen Gemeinschaften nach den Pflanzenkompartimenten, wobei eine Anreicherung in der Rhizosphäre im Vergleich zum Boden festgestellt wurde. Bei Kulturpflanzen ohne Zusatz von Düngemitteln dominierte das Phylum *Thaumarchaeota* deutlich die archaelle Gemeinschaftsstruktur, während bei der einheimischen Moorvegetation *Euryarchaeota* überwog. Obwohl in Tomatensamen Archaeen vorkamen, konnten keine Hinweise auf eine aktiv von der Pflanze ausgehende vertikale Übertragung von Archaeen gefunden werden. Die Besiedlung der Pflanze geht somit eher von dem Boden aus als von der Mutterpflanze, was darauf hindeutet, dass Archaeen in Samen nur eine nebensächliche Rolle spielen. In Pflanzen wurden funktionelle Gen-Signaturen für mögliche Anpassungsmechanismen von Archaeen an ihre Wirte beobachtet, einschließlich solcher für höhere Chemotaxis, Nährstoffkreisläufe wie CO_2- und N_2-Fixierung, Resistenz

gegen Stress und mögliche Förderung des Pflanzenwachstums durch Auxin. Obwohl festgestellt wurde, dass Archaeen das Potenzial haben mit ihrem Wirt zu interagieren, hatte die Anpassungsstrategie des Wirtes keinen direkten Einfluss auf die Struktur der Archaeen-Gemeinschaft. Diese Ergebnisse zeigen, dass Archaeen als funktionelle Bestandteile von Pflanzenmikrobiomen für Pflanzen-Holobionten eine bislang nicht beobachtete Rolle spielen.

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Introduction

Archaea - Characteristics and Phylogeny

Archaea were originally discovered in extreme environments, such as hot springs in the Yellowstone National Park (USA). Initially named as *Archaebacteria* assigned to the domain of Bacteria, Archaea were classified as a separate domain of life in 1977 (Woese and Fox, 1977). Although archaea are similar to bacteria, as they have no nucleus and a unicellular organization, their unique characteristics stand out, differentiating them from other domains. Archaea harbour a variation of unique cell-wall and cell-membrane components, such as a lack of peptidoglycan in their cell-wall (Howland, 2000) and cell-membrane structures, which are based on isoprene chains, L-glycerol, and ether linkages, as described in more detail in a review article **Chapter 1: 'Archaea are interactive components of complex microbiomes'**. Further, archaea possess unique cell appendages, such as the archaellum (Thomas, Bardy and Jarrell, 2001), and a special molecular processing machinery, which differentiates in enzymes for transcription, translation, and replication. Since their classification as a separate domain, archaeal classification has been continuously changing, as new archaea are frequently discovered. So far, the domain Archaea can be subdivided into several phyla and superphyla, such as *Euryarchaeota*, the DPANN superphylum (including *Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanoarchaeota*, and *Nanohaloarchaeota*), the TACK superphylum (including *Thaumarchaeota*, *Aigarachaeota*, *Crenarchaeota*, and *Korarchaeota*), and the newly discovered Asgard superphylum (including *Lokiarchaeota*, *Thorarchaeota*, *Odinarchaeota*, and *Heimdallarchaeota*), which are supposed to be the closest relatives to eukaryotic cells (Zaremba-Niedzwiedzka *et al.*, 2017). The phylum *Euryarchaeota* consists of methanogens, which play a crucial role in the carbon cycle by degrading organic matter, and halophiles, growing under high levels of salt. Many archaea of the phylum *Thaumarchaeota* are ammonia-oxidizing archaea (AOA), playing important roles in nitrogen and carbon cycles in the ocean and on land (Pester and Wagner, 2011). Furthermore, the phylum *Crenarchaeota* contains many extremophiles, such as hyperthermophiles, which have extreme optimal growth temperatures above 80°C.

Archaea - from extremophiles to ubiquitous colonizers

Due to new next-generation-sequencing methods, our view on archaea changed drastically during the last decades, as archaea have been found to be ubiquitous, being substantial parts of numerous microbiomes. In the ocean, archaea were found at high abundances of up to 40%, at which pelagic *Thaumarchaeota* (formerly classified as *Crenarchaeota*) are expected to be one of the most abundant cell types (Karner, DeLong and Karl, 2001). *Crenarchaeota* and *Thaumarchaeota* are thought to play primary roles in global biogeochemical cycles, such as carbon and nitrogen cycles (Könneke *et al.*, 2005; Offre, Spang and Schleper, 2013). Besides the world's oceans, archaea are common colonizers of soils and wetlands. In some soils AOA are actually found to be predominant over ammonia-oxidizing bacteria (Leininger *et al.*, 2006). Ammonia oxidation is part of the nitrification process, which is important for global nitrogen cycles. Further, archaea can be found in animals, such as termites or ruminants. Mainly *Euryarchaeota* colonize the gastrointestinal tract (GIT) of animals, causing high levels of methane emissions under certain circumstances (St-Pierre and Wright, 2013). But they can also be found in humans. Archaea, *Thaumarchaeota* in particular, are colonizing the human skin, whereas methanogens of the phylum *Euryarchaeota* are resident components of the gut microbiome (Koskinen *et al.*, 2017). Besides, archaea are also found to be substantial components of plant microbiomes, such as leafy greens, rice, maize and tomato, at which the rhizosphere is colonized at high abundances (Chelius and Triplett, 2001; Simon *et al.*, 2005).

Colonization and role of plant-associated archaea

Plant microbiomes are diverse, consisting of bacteria, fungi, and archaea. Structure and function of plant microbiomes are specific for each plant genotype and plant habitat, such as rhizosphere, root-endosphere, and phyllosphere (Berg *et al.*, 2009; Vorholt, 2012; Philippot *et al.*, 2013). Rhizospheres of plants can provide favourable conditions for methanogens or AOA, such as in the rhizosphere of maize or rice (Chelius and Triplett, 2001), as they can provide anoxic conditions. Methanogenic archaea were also found in the root-endosphere of rice plants, where they can contribute to up to 60% of rice field methane emissions (Edwards *et al.*, 2015; Pump, Pratscher and Conrad, 2015). Besides the rhizosphere, which provides rather stable conditions, archaea can also be found in the phyllosphere of plants, where they are exposed to rapid abiotic changes, although at lower abundances (Stapleton and Simmons,

2006). Though, archaea were preferentially colonizing the rather protected endosphere of leaves, such as in olive trees with a relative abundance of up to 36% (Müller *et al.*, 2015). However, archaea could also be found in seeds of native alpine plants (Wassermann *et al.*, 2019). Besides the habitat, the plant-genotype is influencing the archaeal community structure, such as in the vegetation of wetlands or perennial woody plants (Lee *et al.*, 2015; Müller *et al.*, 2015).

Host plants interact closely with their microbiome, together forming a "holobiont" (Vandenkoornhuyse *et al.*, 2015). These microbiomes are important factors for their hosts, as they can influence health, growth, and productivity of the plant (Berg *et al.*, 2016). Although bacteria and fungi are known to actively support their hosts, less is known about archaea. However, archaea play roles in nutrient cycles in environments associated to the plants, such as marshlands or soil, which is described in **Chapter 2: 'Archaea, tiny helpers on land plants?'**. Archaea, AOA in particular, are involved in reductive pathways of the nitrogen cycle and might provide the plants with nitrogen, as plants preferably absorb the reduced form (Cabello, Roldán and Moreno-Vivián, 2004). Besides the nitrogen-cycle, the carbon-cycle is of great environmental importance. Archaea are involved in CO_2 fixation as well as in methanogenesis, which is exclusively performed by anaerobic methanogens (Offre, Spang and Schleper, 2013). Furthermore, plants need phosphorous, which they take up in its solubilized form. Archaea have been shown to perform solubilisation of organic phosphorous and thereby making it available for the plant (Yadav *et al.*, 2015). Although archaea are an important constituent of plant microbiomes, biotic and abiotic factors driving the archaeal colonization of the plant and especially their role and interactions with the host plant remain mostly unclear.

Objectives and summary of the thesis

Although the plant microbiome has been intensively analysed, studies on plant-associated archaea are scarce. To get a broader and deeper understanding of the community structure, habitat specificities, and function of archaea on plants, the archaeal community of 30 different natural and domesticated plants was analysed with a comprehensive approach.

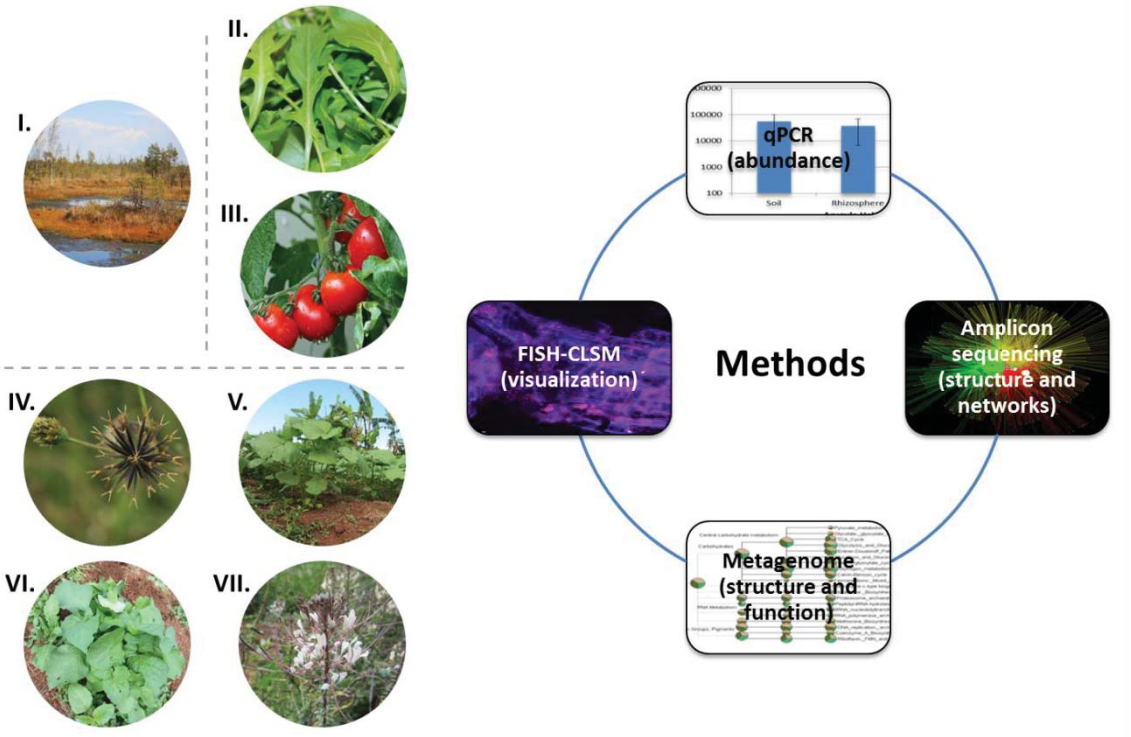

Figure 1: Illustrated overview of the methods used and studies carried out in this doctoral thesis. Studies grouped together regarding their environment: (I.) bog vegetation of two alpine raised bogs in upper Styria (Austria); (II.) arugula and (III.) tomato, from Graz (Austria); (IV.) Black Jack, (V.) Okra, (VI.) Nightshade and (VII.) Spiderwisp from a field in Kasangati (Uganda). The archaeal community of these plants was analysed with a combined approach of 16S rRNA gene fragment amplicon sequencing, whole genome shotgun sequencing, fluorescence *in situ* hybridization and confocal laser scanning microscopy (FISH-CLSM), and real-time PCR (qPCR).

First, to understand the colonization pattern of archaea on plants and to reveal plant-type- or habitat-specificities, 16S rRNA gene fragment amplicon sequencing, as well as fluorescence *in situ* hybridization and confocal laser scanning microscopy (FISH-CLSM) was conducted. Further, to get insights into functional capacities of archaea and plant host-archaeal interactions, an in-depth metagenomic sequencing approach was performed with samples of arugula (*Eruca sativa* Mill.) and bog vegetation (*Andromeda polifolia*, *Aulacomnium palustre*, *Bazzania trilobata*, *Calluna vulgaris*, *Carex nigra*, *Cladonia fimbriata*, *C. portentosa*, *Eriophorum*

vaginatum, *Molinia caerulea*, *Mylia anomala*, *Pinus mugo*, *Pleurozium schreberi*, *Polytrichum commune*, *P. strictum*, *Rhytidiadelphus triquetrus*, *Sphagnum angustifolium*, *S. capillifolium*, *S. cuspidatum*, *S. fuscum*, *S. magellanicum*, *Vaccinium myrtillus*, *V. vitis-idaea*, *V. uliginosum*, *V. oxycoccos*). By including real-time PCR (qPCR) into our analysis of archaea associated with tomatoes (*Solanum lycopersicum* L.) the archaeal colonization and transmission to the next generation could be investigated. Furthermore, analysing the archaeal community on rather natural leafy greens (*Abelmoschus esculentus; Solanum scabrum; Gynandropsis gynandra; Bidens pilosa)*, the impact of the host on its microbiome with special focus on the life strategy was studied. The combination and comparison of these studies lead to an identification of important factors influencing archaeal colonization on plants. This present doctoral thesis depicts colonization preferences of archaea associated to plants and provides first insights into the role of archaea as functional components of plant microbiomes.

Main scientific questions of the study:

1. Are archaea planttype-specific colonizers?
2. Are archaea habitat-specific colonizers?
3. Is there a functional specification of archaea on plants?
4. Are archaea actively transmitted to the next generation by the mother plant?
5. Which factors influence archaeal colonization on plants?

Archaea are planttype- and habitat-specific colonizers

Plants harbour a multitude of diverse microorganisms such as fungi, bacteria, and archaea, whereat the rhizosphere represents a peculiar habitat for the colonization of the plant (Buée *et al.*, 2009). So far habitat-preferences and plant colonization of bacteria and fungi are well studied, but less is known about archaea. To identify plantgenotype-specific colonization pattern of archaea, a total of 46 samples of 24 different vascular plants, bryophytes, and lichens were sampled in two alpine raised bogs in upper Styria (Austria) and analysed using 16S rRNA gene fragment amplicon sequencing. The study is described in detail in **Chapter 3: 'What is the role of archaea in plants? New insights from the vegetation of alpine bogs'**. The datasets revealed a high level of plant-specificity, whereat archaea showed high relative abundances in plants forming lignified parts, such as *Monocotyledons* and *Eudicotyledons*. In

bog vegetation this plant-specific colonization might be rather based on the archaeal preference to colonize habitats providing stable conditions. However, there was a strong archaeal core microbiome shared between all bog plants. Further studies could even show a cultivar-specific colonization of plants, which is described in **Chapter 5: 'Tomato-associated archaea show a cultivar-specific rhizosphere effect independently from soil quality'**. Therefore two tomato cultivars Moneymaker and Hildares F1 were analysed with a combination of 16S rRNA amplicon sequencing and qPCR. In tomatoes the diversity and abundance of the archaeal community was significantly higher in the rhizosphere of Moneymaker compared to Hildares F1. However, in natural leafy greens in Uganda, the planttype-specificity was not that distinct. In this study, which is described in **Chapter 6: 'Exploring the microbiome of novel leafy greens in Eastern Africa for plant health'**, the archaeal community of four leafy greens, Okra, Nightshade, Spiderwisp, and Black Jack, was analysed with 16S rRNA gene fragment amplicon sequencing. Archaeal diversity was only significantly different in Nightshade compared to the other three. However, the habitat-specificity was found to be significant, with the highest diversity of archaea in the rhizosphere. This habitat-specific colonization with a preference for the rhizosphere could also been shown in tomato plants and arugula. In arugula the archaeal community was studied using 16S rRNA gene fragment amplicon sequencing and habitat-specific colonization was further highlighted using FISH-CLSM, which is described in detail in **Chapter 4: 'Novel insights into plant-associated archaea and their functioning in arugula (*Eruca sativa* Mill.)'**. FISH-CLSM confirmed a habitat-specific colonization of archaea in plants, especially in hotspots with high nutrient levels, such as rotten roots.

Archaea are functional components of plant microbiomes

Bacteria are interacting with their plant hosts and thereby contributing to their functioning and health (Bulgarelli *et al.*, 2013). Archaea could be shown to be substantial components of plant microbiomes as well, but their role and interactions with their host remained unclear. In order to reveal the functional potential of plant-associated archaea 12 metagenomes of bog vegetation and three metagenomes of arugula of its habitats phyllosphere, rhizosphere and bulk soil were analysed and screened for functional signatures. The metagenomic study on the vegetation of two alpine raised bogs resulted in 285,058 functional hits of archaea (**Chapter 3**).

Assignment to functional subsystems of the SEED database revealed their genetic capacity to interact with fungi and functional signatures for N_2 and CO_2 fixation, indicating roles in nitrogen and carbon cycling. Further, in this study a so far undiscovered potential of archaea for their plant hosts could be demonstrated on three levels of interaction, via: i) plant growth promotion through biosynthesis of the phytohormone auxin, ii) supply and cycling of nutrients, and iii) response to abiotic stresses (especially oxidative and osmotic stress). A second metagenome study focussed on habitat-specific distribution of archaeal functions associated with arugula (**Chapter 4**). This dataset resulted in 5,804 archaeal reads, whereas the abundance of functional hits was decreasing from soil to rhizosphere, and was low in the phyllosphere. Functional signatures, found in the soil and the rhizosphere, were involved in the resistance to oxidative stress, nutrient cycling, such as CO_2 fixation, and glycogen degradation. In the phyllosphere functional signatures involved in the serine-glyoxylate cycle were relatively more abundant compared to the other habitats, allowing utilization of simple carbon sources, when complex ones are absent. These findings were in accordance with the preceding study on bog vegetation (**Chapter 3**) and strengthened the findings that archaea have the potential to interact with their plant host, as well as with their environment.

Archaea are not actively transmitted by plant seeds

In the studies described before (**Chapter 3 and 4**), the datasets showed that archaea have the potential to interact with their plant host and further to play a role in supporting plant growth. Bacteria are known to support plant growth and recently have been found to be actively transmitted from the mother to the next generation (Bergna *et al.*, 2018). As archaea have similar favourable features for the plant, plants might actively transmit beneficial archaea as well to support germination and health of the seedlings. In order to study archaeal transmission in plants, the archaeal community of two tomato cultivars Moneymaker and Hildares F1, which were nurtured in two different soil types, was analysed over two generations. Therefore a combination of qPCR and next-generation sequencing was conducted (**Chapter 5**). The archaeal abundance in the seeds decreased from the first to the second generation. The composition of the archaeal community showed random pattern and the diversity was low. The results of this study indicate that there is no vertical transmission of

archaea by the plant. In seeds, archaea might just represent bystander microorganisms, which could be based on syntrophic interactions with bacteria.

Plant host and the environment shape the archaeal community

Plants can be categorized based on their life strategy, which can be either oligotrophic (k-strategy) or copiotrophic (r-strategy) (Andrews and Harris, 1986). In previous studies, plants have been found to enrich bacteria with a similar life strategy in their associated soil (Gibbons et al. 2017). As archaea have been shown to be enriched in the rhizosphere and to have the potential to interact with their plant hosts (Simon *et al.*, 2005; Taffner *et al.*, 2018, 2019), a next-generation-sequencing based study was conducted focussing on the life strategies of archaea and their hosts. In the study, which is described in **Chapter 6**, the microbiome of four copiotrophic leafy greens (Okra, Nightshade, Black Jack, and Spiderwisp) from Uganda was analysed. In contrast to bacteria, at which the most abundant taxa were following the copiotrophic life strategy of the plant, the archaeal community almost exclusively consisted of the phylum *Thaumarchaeota*, which is known to be oligotrophic (Youssef *et al.*, 2015). This indicates that plant-associated archaea on leafy greens show no direct impact of the life strategy of the host.

Combining the results of all studies conducted within this thesis (**Chapter 3-6**), the planttype was found to influence the diversity and abundance of plant-associated archaea, whereat archaea do not necessarily follow the life strategy of their host. Comparison of the archaeal community of different habitats and studies revealed distinct biogeographical pattern. On phylum level the community associated to the bog vegetation was clearly dominated by methanogenic *Euryarchaeota*, whereas *Thaumarchaeota* mainly colonized plants from unfertilized fields in Austria and Uganda. However, it is the environment which rather defines the general community structure of archaea.

References

Andrews, J. H. and Harris, R. F. (1986) 'r- and K-Selection and Microbial Ecology', in. Springer, Boston, MA, pp. 99–147.

Berg, G. *et al.* (2009) 'Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere', *FEMS Microbiology Ecology*, 68(1), pp. 1–13.

Berg, G. *et al.* (2016) 'The plant microbiome explored: implications for experimental botany', *Journal of Experimental Botany*, 67(4), pp. 995–1002.

Bergna, A. *et al.* (2018) 'Tomato seeds preferably transmit plant beneficial endophytes', *Phytobiomes Journal*, p. PBIOMES-06-18-0029-R.

Buée, M. *et al.* (2009) 'The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors', *Plant and Soil*, 321(1–2), pp. 189–212.

Bulgarelli, D. *et al.* (2013) 'Structure and Functions of the Bacterial Microbiota of Plants', *Annual Review of Plant Biology*, 64(1), pp. 807–838.

Cabello, P., Roldán, M. D. and Moreno-Vivián, C. (2004) 'Nitrate reduction and the nitrogen cycle in archaea', *Microbiology*, 150(11), pp. 3527–3546.

Chelius, M. K. and Triplett, E. W. (2001) 'The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L.', *Microbial Ecology*, 41(3), pp. 252–263.

Edwards, J. *et al.* (2015) 'Structure, variation, and assembly of the root-associated microbiomes of rice.', *Proceedings of the National Academy of Sciences of the United States of America*, 112(8), pp. E911-20.

Howland, J. (2000) 'The surprising archaea: discovering another domain of life'.

Karner, M. B., DeLong, E. F. and Karl, D. M. (2001) 'Archaeal dominance in the mesopelagic zone of the Pacific Ocean', *Nature*, 409(6819), pp. 507–510.

Könneke, M. *et al.* (2005) 'Isolation of an autotrophic ammonia-oxidizing marine archaeon', *Nature*, 437(7058), pp. 543–546.

Koskinen, K. *et al.* (2017) 'First insights into the diverse human Archaeome: Specific detection of Archaea in the gastrointestinal tract, lung, and nose and on skin', *mBio*, 8(6).

Lee, S.-H. *et al.* (2015) 'Impact of elevated CO2 and N addition on bacteria, fungi, and archaea in a marsh ecosystem with various types of plants', *Applied Microbiology and Biotechnology*, 99(12), pp. 5295–5305.

Leininger, S. *et al.* (2006) 'Archaea predominate among ammonia-oxidizing prokaryotes in soils', *Nature*, 442(7104), pp. 806–809.

Müller, H. *et al.* (2015) 'Plant genotype-specific archaeal and bacterial endophytes but similar Bacillus antagonists colonize Mediterranean olive trees', *Frontiers in Microbiology*, 6.

Offre, P., Spang, A. and Schleper, C. (2013) 'Archaea in biogeochemical cycles.', *Annual review of microbiology*, 67(1), pp. 437–57.

Pester, M. and Wagner, M. (2011) 'The Thaumarchaeota: an emerging view of their phylogeny and ecophysiology', *Current Opinion in Microbiology*, 14(3), pp. 300–306.

Philippot, L. *et al.* (2013) 'Going back to the roots: the microbial ecology of the rhizosphere.', *Nature reviews. Microbiology*, 11(11), pp. 789–99.

Pump, J., Pratscher, J. and Conrad, R. (2015) 'Colonization of rice roots with methanogenic archaea controls photosynthesis-derived methane emission', *Environmental Microbiology*, 17(7), pp. 2254–2260.

Simon, H. M. *et al.* (2005) 'Cultivation of mesophilic soil crenarchaeotes in enrichment cultures from plant roots.', *Applied and environmental microbiology*, 71(8), pp. 4751–60.

St-Pierre, B. and Wright, A.-D. G. (2013) 'Diversity of gut methanogens in herbivorous animals', *animal*, 7(s1), pp. 49–56.

Stapleton, A. and Simmons, S. (2006) 'Plant control of phyllosphere diversity: genotype interactions with ultraviolet-B radiation', *Microbial ecology of the aerial plant*.

Taffner, J. *et al.* (2018) 'What Is the Role of Archaea in Plants? New Insights from the Vegetation of Alpine Bogs.', *mSphere*, 3(3), pp. e00122-18.

Taffner, J. *et al.* (2019) 'Novel insights into plant-associated archaea and their functioning in arugula (Eruca sativa Mill.)', *Journal of Advanced Research*.

Thomas, N. A., Bardy, S. L. and Jarrell, K. F. (2001) 'The archaeal flagellum: a different kind of prokaryotic motility structure', *FEMS Microbiology Reviews*, 25(2), pp. 147–174.

Vandenkoornhuyse, P. *et al.* (2015) 'The importance of the microbiome of the plant holobiont', *New Phytologist*, 206(4), pp. 1196–1206.

Vorholt, J. A. (2012) 'Microbial life in the phyllosphere', *Nature Reviews Microbiology*, 10(12), pp. 828–840.

Wassermann, B. *et al.* (2019) 'Seeds of native alpine plants host unique microbial communities embedded in cross-kingdom networks', *Microbiome*, 7(1), p. 108.

Woese, C. R. and Fox, G. E. (1977) 'Phylogenetic structure of the prokaryotic domain: The primary kingdoms (archaebacteria/eubacteria/urkaryote/16S ribosomal RNA/molecular phylogeny)', 74(11), pp. 5088–5090.

Yadav, A. N. *et al.* (2015) 'Haloarchaea Endowed with Phosphorus Solubilization Attribute Implicated in Phosphorus Cycle', *Scientific Reports*, 5(1), p. 12293.

Youssef, N. H. *et al.* (2015) 'Insights into the metabolism, lifestyle and putative evolutionary history of the novel archaeal phylum "Diapherotrites"', *The ISME Journal*, 9(2), pp. 447–460.

Zaremba-Niedzwiedzka, K. *et al.* (2017) 'Asgard archaea illuminate the origin of eukaryotic cellular complexity.', *Nature*, 541(7637), pp. 353–358.

1 Archaea are interactive components of complex microbiomes

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Abstract

Recent findings have shaken our picture of the biology of the archaea and revealed novel traits beyond archaeal extremophily and supposed 'primitiveness'. The archaea constitute a considerable fraction of the Earth's ecosystems, and their potential to shape their surroundings by a profound interaction with their biotic and abiotic environment has been recognized. Moreover, archaea have been identified as a substantial component, or even as keystone species, in complex microbiomes – in the environment or accompanying a holobiont. Species of the *Euryarchaeota* (methanogens, halophiles) and *Thaumarchaeota*, in particular, have the capacity to coexist in plant, animal, and human microbiomes, where syntrophy allows them to thrive under energy-deficiency stress. Due to methodological limitations, the archaeome remains mysterious, and many questions with respect to potential pathogenicity, function, and structural interactions with their host and other microorganisms remain.

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Review

Archaea Are Interactive Components of Complex Microbiomes

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Recent findings have shaken our picture of the biology of the archaea and revealed novel traits beyond archaeal extremophily and supposed 'primitiveness'. The archaea constitute a considerable fraction of the Earth's ecosystems, and their potential to shape their surroundings by a profound interaction with their biotic and abiotic environment has been recognized. Moreover, archaea have been identified as a substantial component, or even as keystone species, in complex microbiomes – in the environment or accompanying a holobiont. Species of the Euryarchaeota (methanogens, halophiles) and Thaumarchaeota, in particular, have the capacity to coexist in plant, animal, and human microbiomes, where syntrophy allows them to thrive under energy-deficiency stress. Due to methodological limitations, the archaeome remains mysterious, and many questions with respect to potential pathogenicity, function, and structural interactions with their host and other microorganisms remain.

Archaea – Unique, but Ubiquitous

Archaea is a separate domain of life, distinct from Bacteria and Eukarya. The archaea possess a unique cell wall and membranes as well as distinctive metabolic pathways and enzymes [1] (Box 1). As many archaea have the capacity to survive and thrive under extreme conditions, we have been amazed by the number of superlatives with respect to the chemical and physical borders of archaeal life [2]. By researching members of the Archaea, numerous novel insights into the evolution of life on our planet have been achieved [3], and speculations about possible (archaeal) life beyond Earth have been fuelled [4].

In the last few decades, important findings have been published from the archaeal research community, including the discovery of anaerobic methane oxidation [5], thaumarchaeal ammonia oxidation [6], the seventh order of methanogens [7–11], and the discovery of the Bathyarchaeota, a noneuryarchaeal lineage with methanogenic properties [12], and the evolutionarily important Lokiarchaeota [3], just to name a few. All of these findings have shaken our picture of the ecology and importance of the archaea, and have revealed novel traits beyond archaeal extremophily and supposed 'primitiveness'.

Meanwhile it is well accepted that the archaea constitute a considerable fraction of the microbial biomass in the Earth's moderate ecosystems: they have been recognized as widely distributed microorganisms that have the potential to shape their surroundings by a profound interaction with their biotic and abiotic environment. As the overwhelming majority of archaea resist cultivation in the laboratory, the availability of molecular methods, such as 16S rRNA gene

Trends

Archaea are substantial components of complex microbiomes in the environment and in holobionts.

Archaea interact closely with viruses, microorganisms, and holobionts such as plants, animals, and humans.

In holobionts, the archaeome reveals biogeographic patterns, indicating various functions.

Methanogens, in particular, are considered to be prominent partners in various settings, supporting bacterial fermentation processes based on syntrophy and driven by energy depletion.

No archaeal pathogen has been identified thus far.

Methodological problems hinder the proper analyses of the archaeome, including function and structural adaptations.

The archaeal double-membrane and anchored surface structures might support high-level interactions.

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cloning and high-throughput amplicon sequencing, has boosted insight into their astonishing diversity and omnipresence. Nevertheless, the basic principles, as well as the extent of archaeal interaction, remain largely obscure.

In this review, we emphasize the role of the archaea in complex **microbiomes** (see Glossary), their potential role as keystone organisms in various ecosystems, and their 'social' character. We discuss the archaea as important partners for members of the Archaea, Bacteria, and Eukarya – as parasites, symbionts, or syntrophic organisms [10] (Box 2).

Archaea Interacting with Other Microorganisms, in Laboratory Cultures or in Complex Ecological Ecosystems

In nature, most microorganisms grow in mixed consortia rather than in monocultures. Thus, microorganisms are assumed to be highly interactive either through defense while competing for nutrients or in cooperation while growing synergistically. The first evidence of (methanogenic) archaea that actively interact with other microorganisms was obtained from defined pure cultures, where syntrophy, mostly based on hydrogen transfer, was the major driving factor for increased benefit for both partners (overview given in [14]). However, meanwhile, archaea were found in stable microbial–microbial cocultures, consortia, or biofilms.

Archaeal Symbionts in Laboratory Cultures

Fascinating archaeal–archaeal cocultures have been described, comprising members of the phylogenetic clade *Nanoarchaeum*. To date, two symbiotic consortia with Nanoarchaea involvement have been studied in more detail, including the relationship of *Ca*. Nanopusillus acidilobi and its host *Acidilobus* sp. [15], and the well-known 'intimate association' of *Nanoarchaeum equitans* and *Ignicoccus hospitalis* [16]. *N. equitans* lacks a large number of the genes required for transcription, primary metabolism, and energy, and thus depends entirely on physical contact and exchange with its host *I. hospitalis* [16,17]. Notably, in coculture, the doubling times and final cell densities of the host remained unaffected by the presence of *N. equitans*, indicating a balanced interaction between the two partners [16]. Although the *I. hospitalis* and *N. equitans* system is considered the simplest interspecies community known so far, the 'intimate association' is based on a complex coregulation of metabolic pathways and transportation, during which no stress response or defense by *I. hospitalis* occurs [18].

Archaea in Naturally Occurring Microbial Consortia

A microbial consortium is defined as a (physical) association of two or more (few) types of microorganisms, in which syntrophy or other types of symbiosis occur [19]. Most described microbial consortia are characterized by a typical shape, physical interaction of the partners, and thus colocalization in a well-organized form. Quite well known examples including archaea

Glossary

Archaeome: entirety of archaeal cells, including their genetic material in a particular environment. Analogous to bacteriome, virome, mycobiome.
Microbiome: in the present context, microorganisms and their genetic material in a particular environment (i.e., plant, human body, environmental setting).
Syntrophy: obligately mutualistic metabolism (Box 2).

Box 1. Archaea – Characteristics and Phylogeny

In the three-domain concept, Archaea is considered to be a separate domain of life, distinct from Eukarya and Bacteria. At first glance, the archaea resemble bacteria due to their unicellular organization and lack of a nucleus. However, they possess unique features, such as a variety of cell-wall and cell-membrane components (no cell-wall peptidoglycan [but pseudomurein in some species], and cell-membrane structures based on isoprene chains, ether linkages [rather than ester linkages], and L-glycerol), cell appendages (e.g., archaellum), and different molecular processing machinery (in particular transcription-, translation-, and replication-related enzymes). The archaea cannot form spores, nor are they able to perform photosynthesis. The domain Archaea is divided into several phyla, including Euryarchaeota and two main 'superphyla', namely the TACK superphylum (Thaumarchaeota, Aigarchaeota, Crenarchaeota, and Korarchaeota) and the DPANN superphylum (Diapherotrites, Parvarchaeota, Aenigmarchaeota, Nanoarchaeota, Nanohaloarchaea; Figure I). Information on the DPANN superphylum is sparse, and it might include symbionts, thermophiles, acidophiles, and nonextremophiles with a broad environmental significance. Euryarchaeota represents the most extensively studied archaeal phylum as it includes the methanogens, which are widely distributed and play an important role in (syntrophic) anoxic degradation processes of organic matter, and the halophiles, which survive in extreme concentrations of salt. Thaumarchaeota, as a separate branch, was split from Crenarchaeota in 2008 [13], based on genomic and metabolic features. Many members of the Thaumarchaeota are meanwhile known as marine and terrestrial ammonia-oxidizers, contributing to global nitrogen and carbon cycles. The crenarchaeal branch contains numerous extremophiles, that is, hyperthermophiles (optimal growth temperature above 80°C) from terrestrial volcanic environments or submarine hydrothermal vent systems.

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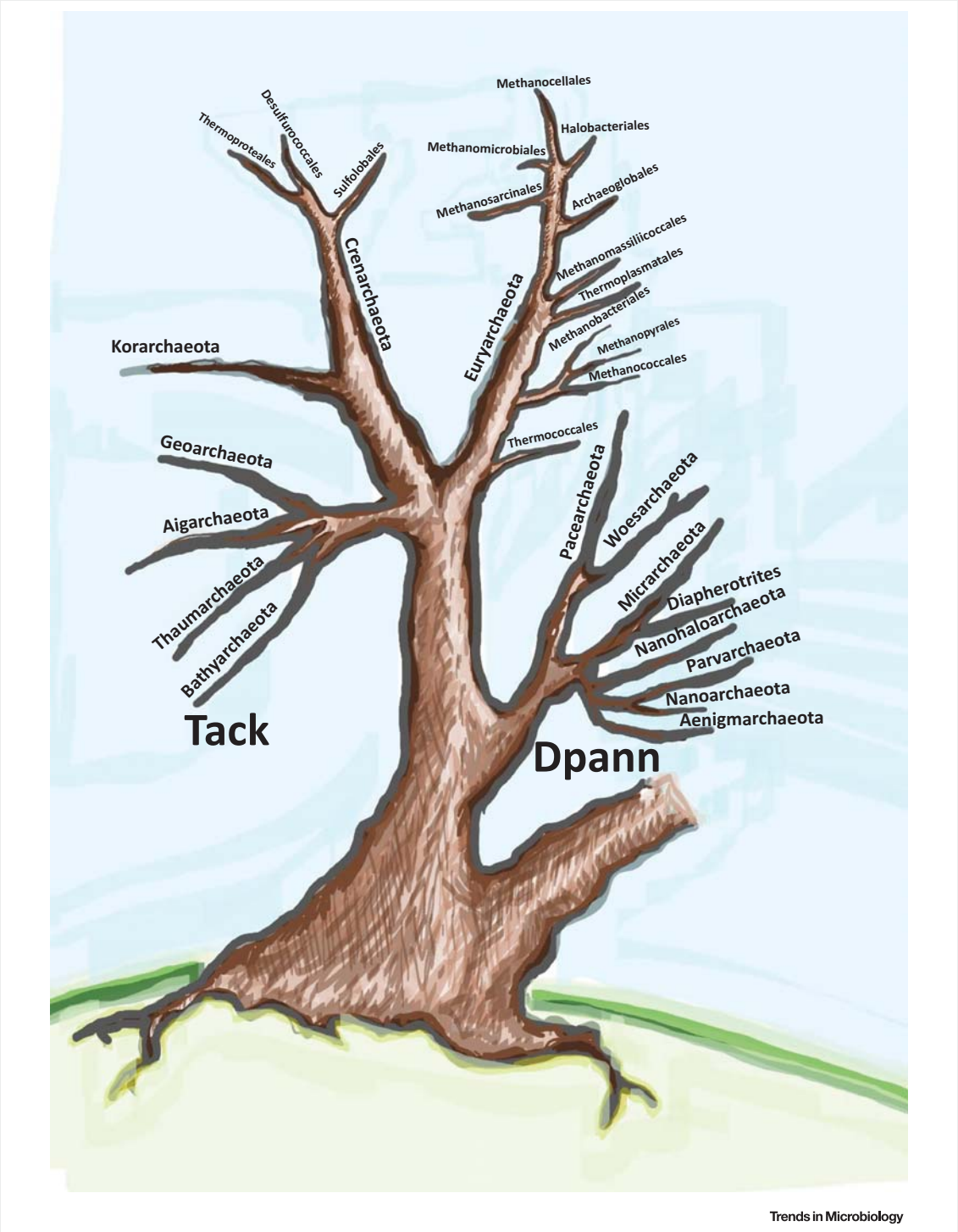

Figure I. Artist's View of the Archaeal Phylogenetic Tree, Showing the Three Major Groups, with the Phylum Euryarchaeota, and the TACK and DPANN Superphyla. The Euryarchaeota contains seven, polyphyletic orders of methanogens, namely, Methanosarcinales, Methanocellales, Methanomicrobiales, Methanomassiliicoccales, Methanobacteriales, Methanopyrales, and Methanococcales. Due to the former unclear taxonomic classification of Methanomassiliicoccales [11], signatures from this cluster were, in the first instance, affiliated to Thermoplasmatales, 'Methanoplasmatales' or so-called 'rumen-cluster C' (provided by Alexander Mahnert, Technical University Graz, Austria).
Drawing provided by Alexander Mahnert, Technical University Graz, Austria.

Box 2. Symbiosis, Syntrophy, and Parasitism

Symbiosis was originally defined as a long-term stable relationship between organisms, which can be either beneficial or nonbeneficial. Thus the definition of symbiosis includes mutualism (symbiosis, which is beneficial to all partners), commensalism (one partner benefits, the other is not harmed, nor derives benefit), and even parasitism (a nonmutual relationship, only one partner benefits, the other is harmed). In general, symbiotic relationships are not necessarily based on metabolism or nutrient requirements, but can provide protection against chemical or mechanical stress.

Syntrophy is defined as 'obligately mutualistic metabolism', emphasizing the overall metabolic process that benefits all microbial partners involved. Other words in this context are cross-feeding, metabolic cooperation, or resource–service mutualism. For a full review on microbial syntrophy please refer to [14].

are the 'string-of-pearls community' (a pearl-like association of *Thiothrix* sp. and *Ca*. Altiarchaeum hamiconexum) [20,21], the ARMAN (Archaeal Richmond Mine Acidophilic Nanoorganism)/*Thermoplasma* associations [22], and in particular the dual-species AOM (anaerobic oxidation of methane) consortia. Therein, anaerobic methanotrophic archaea (ANME) cooperate with sulfate-reducing bacteria, efficiently turning the greenhouse gas methane (emitted from the ocean floor) into the less harmful greenhouse gas CO_2–a globally relevant process [5,23]. Anaerobic methane oxidation is bioenergetically possible only when it is efficiently coupled with H_2 consumption through sulfate reduction. Due to the still extremely low energy yield, the two partners rely on an efficient electron exchange via reducing equivalents or nanowire-like cell–cell connections [23].

Archaea in Biofilms

Biofilms are surface-associated, microbial assemblages, characterized by the presence of (sticky) extracellular polymeric substances embedding the microbial cells; they represent the predominant lifestyle in nature. Biofilms are rewarding environments as they facilitate gene exchange, nutrient access, and protection [24]. Natural biofilms are mostly diverse assemblages, composed of numerous bacterial species, but mixed biofilms of bacteria and archaea are also known [25]. In particular, halophilic archaea seem to be predestined for biofilm formation. One well studied example is the Deep Lake (Antarctica) biofilm that contains 10% *Halorubrum lacusprofundi*. Interestingly, this biofilm is characterized by a high level of gene exchange across different haloarchaeal genera [26], which is facilitated by the well-structured biofilm architecture [24]. A quite unusual biofilm is formed by *Ca*. Altiarchaeum hamiconexum, a euryarchaeote thriving in deep groundwater aquifers [27]; this natural biofilm is formed by archaeal cells, which make up to 97% of all microbial cells and thus predominate [28]. The cells are enclosed in extrapolymeric spider-web and hook-bearing, proteinaceous filamentous surface appendages (the 'hami'; [29]) that allow the cells to connect and to interact, probably through electron transfer [21]. Recently, methanogenic archaea that are associated with the human gastrointestinal tract were also shown to form biofilms on nonbiological surfaces [30], thus indicating that they might also occur in mucosal biofilms. Microbial communities that occur in biofilms on the mucosal surface are expected to be crucially involved in immunomodulation of their host [31].

Archaea, Unicellular Eukarya, and Viruses

Many anaerobic protozoa rely on the interaction with methanogenic archaea. Instead of mitochondria, these protozoa contain so-called hydrogenosomes, which allow the production of ATP by fermentation with the end-products (H_2, CO_2, and acetate) serving as electron and carbon donors for intracellular, symbiotic methanogenic archaea [32]. Although the underlying molecular mechanisms remain largely unknown, it is obvious that this type of interaction requires sophisticated signalling, extensive adaptation, and protection of the endosymbiont [33].

Similar to other organisms, archaea can also be attacked by viruses; however, these viruses are unrelated to any other viruses, and they sometimes reveal unusual shapes, such as

bottle-shaped, spindle-shaped, droplet-shaped, or coil-shaped [34]. Besides their unique mechanism of interaction with the archaeal cell, archaeal viruses can have intrinsic egress mechanisms, as for instance described for the pyramidal egress structures of *Sulfolobus islandicus* rod-shaped virus 2 (SIRV2) [35]. Interestingly, recent discoveries of mixed virus infections of archaea indicate that interaction among genetic mobile elements might be crucial for the evolution of virus and hosts [36].

Archaea and Multicellular Eukarya: Plant-Associated Archaea

All plants harbour highly diverse microbiomes which are specific for each plant genotype [37,38] – but which are also specific for each plant organ, for example, roots [39], leaves [40], and spheres [41]. These microbiomes play an essential role for the plant as they can alter plant growth, productivity, adaptation, diversification, and health [38,42]. Research was focused on plant-associated bacteria and fungi, especially on pathogens and symbionts therein, but recent next-generation sequencing studies of the environmental microbiome revealed archaeal signatures in substantial amounts in diverse microbiomes associated with plants [43].

Plants Provide a Special Habitat for Archaea

As the roots and rhizospheres of plants can provide anoxic or oxygen-depleted micro-niches [44,45], they represent a special habitat for methanogens and ammonium-oxidizing archaea. Plants grown in oxygen-depleted wetland soils, such as rice plants (Box 3) or *Phragmites australis* (Cav.) *Trin. ex Steud.,* harbour a complex **archaeome** in their roots, including root tissue and rhizosphere [46,47]. Elevated atmospheric CO_2 was shown to force a shift in the archaeal community in the rhizosphere of different wetland plants [48].

The phyllosphere provides less stable but constantly oxygenated environmental conditions, and the archaeal community appears, in general, to be less represented therein [43,54]. Nevertheless, the endosphere of perennial plants was identified as a preferred habitat for archaea [55], and – as endophytes in leaves from Mediterranean olive trees (*Olea europaea L.*) – archaea were present in high abundance, representing up to 36% of the whole microbial community [56].

Interestingly, a plant-genotype specificity was also identified for archaea: the plant species was found to be an important key factor structuring the microbial community in wetland vegetation [48] and agricultural systems [56]. Perennial woody plants were identified as microbial hotspots of archaea [48,54–56]; however, this could potentially reflect a link between archaeal abundance and the host's age, as is seen for archaea on the human skin [57].

Function and Interaction of Plant-Associated Archaea

Without doubt, archaea form a substantial component of the plant microbiome, but less is known about their function and interaction with the host – or the overall biotic and abiotic factors that shape the composition of their community and the pattern of their colonization (Figure 1).

Box 3. The Archaeome of the Rice Plant Rhizosphere

Methane is a very effective greenhouse gas with a higher global warming potential (ca. 21 times) than carbon dioxide [49,50]. Rice fields account for about 10% of the global methane budget [51]. Up to 60% thereof is derived from rice plants' photosynthates by the activity of methanogenic archaea associated with the rice rhizosphere [52]. In particular, species of Methanocellales (Rice Cluster 1) and Methanosaetaceae are involved in this process.

Rice fields represent a special habitat for species of methane-producing Euryarchaeota as well as for ammonia-oxidizing archaea of the phylum Thaumarchaeota. Whereas methanogenic archaea were found to proliferate in the whole rice field microcosm, species of Thaumarchaeota were found to proliferate only on the rice roots, indicating a niche differentiation [53].

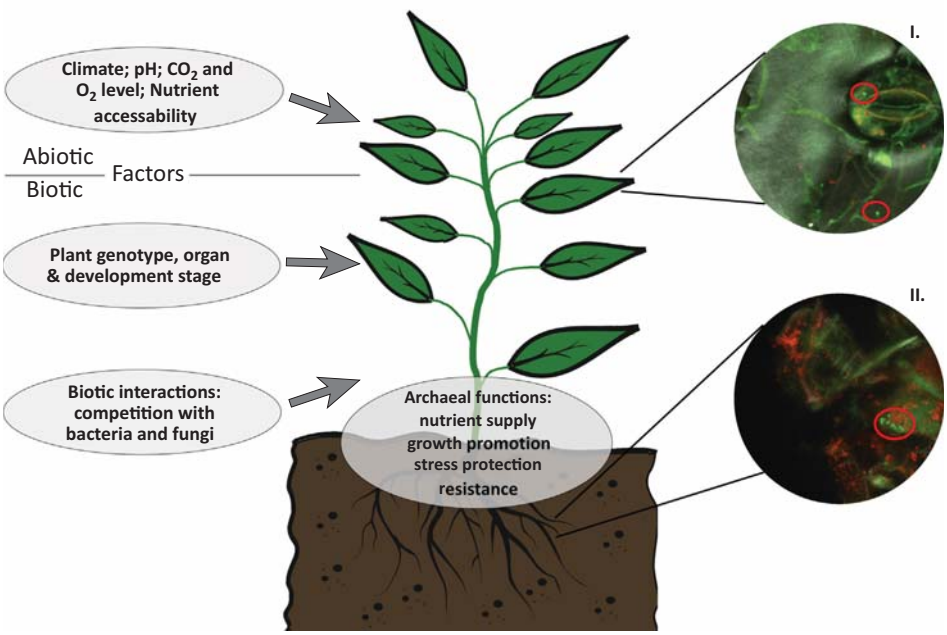

Figure 1. Plant–Archaea Interactions. Biotic and abiotic factors most probably influencing the colonization of archaea on plants, and potential functions for their host. Confocal laser scanning microscopy (CLSM) in addition to fluorescence *in situ* hybridization (FISH) was used in order to investigate archaeal colonization of the phyllosphere (I) and the rhizosphere (II) of cultivars of *Eruca sativa* (Arugula). Bacterial colonies are shown in red, and archaeal colonies are shown in green (red circles).

Notably, ammonia-oxidizing archaea (AOA) were found to be dominant in the rhizosphere of rice plants and are influenced by root exudates, indicating a profound plant–archaea interaction [58]. This interaction could be based on syntrophic nitrogen cycling [59,60], similar to the intimate association of AOA with (cold) marine sponges, where AOA represent the major drivers of ammonia oxidation [61]. As part of the *sphagnum* microbiome in alpine raised bogs, archaea contribute to bog functioning [62]. Microbiome functions were found to be responsible for interaction via nutrient exchange – but also for coping with environmental stress, to which archaea, in general, are evolutionarily adapted [63,64].

Although there is initial evidence for specific interactions between archaea and plants, most of their ecological roles and interactions with their hosts still remain unclear. Further studies should conduct metagenomic and functional analysis to study the specific plant–archaea interaction in more detail.

Archaea and Multicellular Eukarya: Archaea In Animals

The study of archaea in animals was largely initiated based on the major concern regarding the high levels of methane emitted by livestock; this accounts for more than one quarter of all anthropogenic methane emissions [65]. Fermentation in the gastrointestinal tract (GIT) of animals is a widely known, syntrophic process. However, during fermentation, molecules such as CO_2 and methane are produced, and, as they cannot be absorbed, are released into the atmosphere [66] (Box 4).

Termites are another important natural source of atmospheric methane. The methanogenic community of one organism produces only one-half of a microgram of methane per day – which, however, due to the enormous world-wide population of termites, adds up to a global methane

Box 4. Methane Emission from Livestock

Methane is the most abundant organic trace gas in the atmosphere and has a significant impact on climate change, being considered the greenhouse gas with the highest global-warming potential. Half of the methane that is released annually into the atmosphere comes from anthropogenic sources, and, from these, around one quarter results from the agricultural sector, especially livestock [67,68] (Figure I). Biogenic methane is a by-product of microbial fermentation in the GIT of animals, especially in the rumen of ruminants; the methane thus produced is released through eructation, normal respiration, and flatus [68].

The methane emission from livestock has two negative aspects. Firstly, it reduces the animal productivity, and secondly it contributes to global warming, acting as a greenhouse gas [53]. As a reduction in methane emission has been identified as a priority, methods and strategies for reducing methane emissions from livestock need to be developed; this requires an understanding and exploration of archaeal communities in animals.

Much effort has been directed to reducing methane production. Several methods have been developed and can be grouped as: (a) nutritional changes, such as diet modification, defaunation, supplementation with propionate, ionophores, or tannins; (b) management strategies, such as improved nutrition, improved genetic selection, or reducing the number of animals; and (c) other strategies that include immunization, chemical inhibitors, rumen microbial intervention, and recombinant technology (reviewed in [68]).

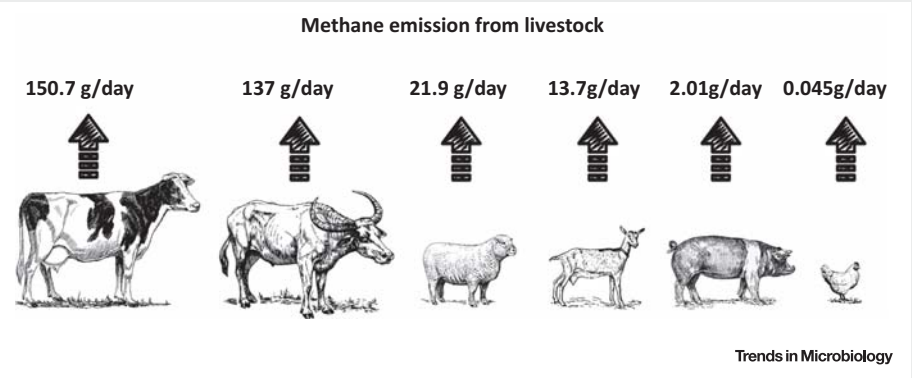

Figure I. Methane Emission Rate from Different Animals per Day [68,138,139].

emission of about 20 million tons each year (corresponding to about 3–5% of the total global methane budget) [69,70]. However, the final release of methane to the atmosphere is dependent on the balance of methane production and oxidation in direct vicinity of the termites [71].

Archaeal Communities in Animals

In animals, the archaeal communities are dominated by species belonging to the phylum Euryarchaeota, and just a few studies have reported archaeal signatures related to the phyla Crenarchaeota and Thaumarchaeota (Tables 1 and 2). Based on current data, the dominant archaea within animals' digestive tracts are the methanogens, accounting for up to 99% of all archaea. The rumen is one of the most studied organs with regard to the archaeal diversity (Table 1), with a diversity limited to four orders: Methanomicrobiales, Methanosarcinales, Methanobacteriales, and Methanomassiliicoccales (formerly referred to as Thermoplasmatales or Rumen Cluster C – RCC). Within the rumen, archaea account for only up to 4% of all microorganisms present [72].

Even though the rumen archaeal communities are better understood and explored, many studies have investigated archaeal communities in other animals (Table 2); it has been found that, in general, the archaea that predominate in the animal GIT are related to the genus *Methanobrevibacter*. Other archaeal signatures (*Methanosphaera, Methanosarcina, Methanomassiliicoccus, Methanimicrococcus*) have also been identified, but are less abundant [72].

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Table 1. The Gastrointestinal Archaeome of Ruminants

Ruminant	Detected archaeal diversity by isolation or 16S rRNA gene sequencing (16S)	Refs
Cattle	16S: *Methanobrevibacter* (*Mbb. ruminantium* accounting around 20–50% of all archaea), *Methanosphaera*, *Methanimicrococcus*, *Methanobacterium*, *Methanomicrobium*, Crenarchaeota Isolated: *Methanobacterium formicicum*, *Mb. bryantii*, *Mbb. ruminantium*, *Mbb. millerae*, *Mbb. olleyae*, *Methanomicrobium mobile*, and *Methanoculleus olentangyi*	[72,74,75]
Yak	16S: *Methanobrevibacter* (dominant species: *Mbb. ruminantium*)	[76]
Sheep	16S: *Methanobrevibacter* (*Mbb. gottschalkii, Mbb. millerae, Mbb. smithii, Mbb. thauerii, Mbb. ruminantium* and *Mbb. olleyae*), *Methanosphaera*, Thermoplasmatales[a], *Aciduliprofundum boonei*, *Picrophilus torridus*, *Methanosarcina barkeri* and *Methanoculleus palmolei*	[74,77]
Reindeer	16S: *Methanobrevibacter* (dominant species – *Mbb. millerae*) and Thermoplasmatales[a]	[78]
Goat	16S: *Methanobrevibacter*, Desulfurococcaceae, *Methanosphaera*, Archaeoglobus and Thermofilum	[79]
Water buffalo	16S: *Methanobrevibacter*, Methanomicrobiales (dominant species – *Methanomicrobium mobile*)	[80]
Deer	16S: *Methanobrevibacter* and *Methanosarcina*	[74]

[a]This taxon was most probably misclassified and is currently known as Methanomassiliicoccales; to avoid confusion, we are indicating the taxon affiliations as given in the original publication.

In nonhuman primates, the archaeal communities are similar to those of humans, being dominated by *Methanobrevibacter*, followed by *Methanosphaera*, *Methanomassiliicoccus*, and (less-abundant) species of Thaumarchaeota. Overall, the archaeal diversity was found to be higher when compared to the diversity in humans [73].

Table 2. Archaeal Diversity in Nonruminants, Detected by Cultivation-Independent Methods

Nonhuman primates		Methanobacteriales, Methanomassiliicoccales and Thaumarchaeota	[73]
Farmhouse animals	Horses and ponies	Methanobacteriales, Methanomicrobiales, Methanosarcinales, and 'Methanoplasmatales'[a] (second dominant clade in horse)	[81]
	Pigs	*Methanobrevibacter*, *Methanosphaera*, Thermoplasmatales[a] (<1%)	[82]
Macro-podidae	Wallaby	*Methanobrevibacter* (dominant species: *Mbb. gottschalkii*), and Thermoplasmatales[a]	[83]
	Kangaroos	*Methanobrevibacter, Methanosphaera stadtmanae*, *Thermoplasma acidophilum*	[84]
Captive white rhinos		*Methanobrevibacter, Methanobacterium, Methanosphaera, Methanomicrobium* and unidentified Euryarchaeota	[85]
Birds	Chicken	*Mbb. woesei* (accounting up to 97% of total archaea)	[86]
	Hoatzin	*Methanobrevibacter* (*Mbb. woesei*, and *Mbb. ruminantium*), *Methanosphaera*	[87]
Reptiles	Land iguanas	*Methanobrevibacter* (dominant), *Methanosarcina* and *Methanocorpusculum*	[88]
	Giant tortoises	*Methanocorpusculum*	[88]
Arthropods	Termites	*Methanobrevibacter*, *Methanimicrococcus*, 'Methanoplasmatales'[a], Methanomicrobiales, Methanosarcinales, Thaumarchaeota and uncultured archaea related to Crenarchaeota	[89]
	Shrimps	Euryarchaeota DHVE2, Thermococcales, Crenarchaeota Marine Group and Methanococcales	[90]
Sponges		Thaumarchaeota	[91]

[a]These taxa were most probably misclassified and are currently known as Methanomassiliicoccales; to avoid confusion, we are indicating the taxon affiliations as given in the original publication.

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Roles of Archaea in Animals

Methanogens are the dominant archaeal representatives within the GIT of animals. Methanogens produce methane based on **syntrophy** that relies on end-products from bacterial fermentation, such as CO_2, H_2, and other compounds (i.e., methyl groups such as methanol and methylamines). By consuming the hydrogen, methanogenic archaea represent keystone species, potentially influencing the overall community present in the GIT (reviewed in [92]). However, many aspects and details about the type and structure of interaction with host and bacteriome remain unclear. By studying the genome of specific methanogens, such as *Methanobrevibacter ruminantium (Mbb. ruminantium)*, *Methanobrevibacter smithii (Mbb. smithii)* and *Methanosphaera stadtmanae*, genes encoding adhesion-like proteins have been found that are speculated to play a role in cell–cell and cell–host interactions [93,94].

Archaea and Multicellular Eukarya: Archaea in Humans

Archaea have been known to be part of the endogenous human microbial community for more than 35 years [95,96]. However, until today, the human archaeal diversity was more or less limited to three identified (and cultivated) members of the family Methanobacteriaceae [*Mbb. smithii*, *Methanobrevibacter oralis (Mbb. oralis)* and *Ms. stadtmanae*]. Due to the establishment of high-throughput sequencing analyses, an increasing number of studies are identifying more and more archaeal species that are associated with the human microflora, and they report distinct archaeal communities across the human body landscape [97] (Box 5).

The Human Gut Archaeome

Within the human intestine, up to 10% of all anaerobes are methanogenic archaea, with *Mbb. smithii* being the predominant representative, found in almost every human subject [98–100]. The general occurrence of *Mbb. smithii* might be due mainly to its genetic adaptation to the human intestine as well as to its high flexibility in establishing syntrophic relationships with several gut bacteria [94,101]. Contrarily, *Ms. stadtmanae* has very restricted metabolic capabilities [102] and was found with variable abundance ranges from 30% [98] up to 90% in the human GIT [73]. Recently, the novel methanoarchaeal order Methanomassiliicoccales was identified based on the successful cultivation of several *Methanomassiliicoccus* strains from various anaerobic habitats [8,11,103–106]. Although earlier studies proposed a low abundance and prevalence of certain strains of the Methanomassiliicoccales [107–109], a recent publication clearly demonstrated the presence of *Methanomassiliicoccus* sp. in each human being sampled by using an archaeon-specific primer pair targeting the 16S rRNA gene [73]. To date, nine (draft) genomes of human-associated *Methanomassiliicoccus* species are available, giving insights into their metabolic properties and confirming their capacity to use methylamine substrates for methanogenesis. Notably, almost all human-associated strains of the Methanomassiliicoccales belong to the 'host-associated clade' (this clade also includes the signatures from animal digestive tracts), whereas the *Cand.* Methanomassiliicoccus intestinalis/luminyensis signatures are affiliated to the so-called 'free-living clade'. These findings indicate that at least two independent adaptation events towards the host's gut microbiome environment occurred [110].

Numerous studies identified various other members of eury- and crenarchaeal orders within the human gut microbiome by high-throughput sequencing approaches (particularly metagenomics) [111–115]; however, to date, only one nonmethanogenic strain could be successfully isolated and characterized from the human gut, namely *Haloferax massiliensis* [116].

Archaea in the Oral Cavity

The microbiome of the oral cavity has been shown to harbour a large bacterial diversity [117], and, for more than one decade, only two archaeal strains (*Mbb. oralis* and *Mbb. smithii*) have

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Box 5. The Human Archaeome

The human body is populated by trillions of microorganisms. Although bacterial species dominate the human gut microbiome, several members of the archaeal domain were also found to be resident components of this complex ecological community (Figure I). In detail, the oral cavity harbours various methanogenic strains of the genera *Methanobrevibacter* and *Methanomassiliicoccus*, which are often enriched in patients suffering from periodontal disease. The human skin is inhabited by thaumarchaeal species that might be responsible for ammonium turnover. The vast majority of human-associated archaea are found in the intestine, where they can comprise up to 10% of the anaerobic community. Most of the archaeal strains in the intestine are methanogens that are essentially indirectly involved in fermentation processes through H_2 removal. Although only few archaeal strains were successfully cultivated from human samples, modern high-throughput sequencing analyses indicated the existence of a larger number of additional archaeal signatures, including those of the orders Desulfurococcales, Crenarchaeales, Sulfolobales, Thermoproteales, Archaeoglobales, Halobacteriales, Methanosarcinales, Methanobacteriales, Methanococcales, Methanopyrales, or Thermococcales [99]. Up to now, no human-associated archaeal strain has been shown to be clearly pathogenic; however, it is currently hypothesized that methanoarchaea promote the growth of pathogenic microbes and thus might be indirectly involved in pathogenicity.

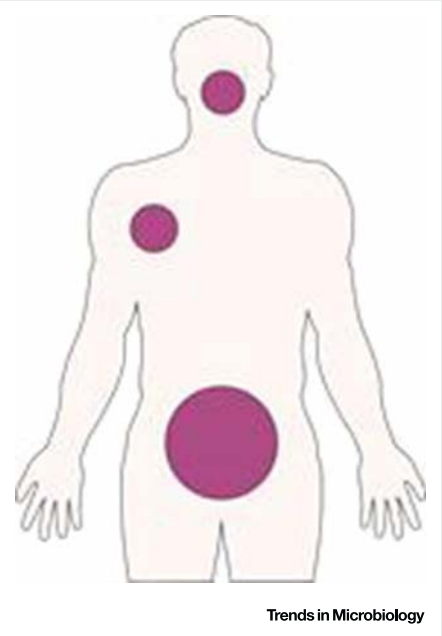

Figure I. Hotspots of the Human Archaeome.

been identified therein [118,119]. However, more recent studies, based on 16S rRNA gene amplicon sequencing, identified not only a greater diversity of methanogenic archaea within the oral microbiome (e.g., *Cand.* Methanobrevibacter massiliense and Methanomassiliicoccales), but also found relatives of halophilic and thermophilic Euryarchaeota as well as Thaumarchaeota [120,121].

Strains of Thaumarchaeota on Human Skin

Strains of Thaumarchaeota were initially found to be common members of the human skin microbiota [122]: the human skin appears to be populated by close relatives of ammonia-oxidizing *Nitrososphaera* that could potentially be associated with a reduction in smell and an improvement in the skin constitution [122]. However, a recent study has revealed a potential age- and skin-physiology-dependence of the human skin archaeome, as results revealed that the archaeal signatures were more abundant and diverse in human subjects either older than 60 years or younger than 12 years [57].

To date, data on archaea associated with other human body sites, such as the vagina, are extremely rare; this is also due to methodological problems [122].

Impact of Archaea on Human Health

Up to now, reports on the potential role of archaea in human health and disease are widely contradictory (reviewed in [99,100]).

With respect to positive effects on human health, strains of Methanomassiliicoccales are currently discussed as potential probiotics against metabolic disorders associated with trimethylamine produced by gut bacteria [110,123]. In addition, the unusual lipid moiety of several archaeal strains was proposed to be used as prospective adjuvants [124].

On the other hand, the question regarding the potential involvement of archaea during the development of microbial disorders, as well as diseases, remains largely unanswered. Several studies underline the hypothesis that methanogenic archaea are at least able to promote the growth of pathogenic bacteria and are thus most probably indirectly involved in the development of diseases – with particular focus on periodontal disease [119,125]. A recent article even indicates the copresence of *Methanobrevibacter oralis* in brain abscesses, and thus a possible involvement in the pathogenicity of this severe infection [126].

In addition, methane, the end-product of methanogenesis, has been shown to slow down the passage of material through the gut, and thus could support constipation, subsequent gastrointestinal disorders, or the development of obesity [127] – processes that could enhance the growth and activity of methanogens even more [128].

However, the high occurrence of *Mbb. smithii* and species of Methanomassiliicoccales in the gut, as well as their low immunogenic potential, argue that these strains are typical commensal microbes [100,129,130], whereas the lower abundance, as well as the high immunogenicity of *Ms. stadtmanae*, is currently being discussed in association with the development of inflammatory conditions involving the human gut [100,129,131]. In order to unravel these open questions, future studies on the general archaeal appearance and correlation with disease, as well as the archaeal molecular cross-talk with the human body, are urgently needed (see Outstanding Questions).

Basic Principles of Archaeal Interaction: Energy, Syntrophy, Structures, and Beyond

Basic Principles of Archaeal Interaction

Independently from the host and partners of the archaea, basic principles can be inferred from the above-discussed interactions and partnerships. Essentially, archaeal interactions are based on three driving factors: (i) (energetic) pressure deriving from the environment, (ii) the capability for exchange of metabolites and/or electrons, and (iii) genomic and structural adaptation capacity (by symbiont and host). Other aspects might be detoxification or facilitated horizontal gene transfer. In particular, strains of the Euryarchaeota, such as methanogens, seem to fulfil these requirements perfectly.

Archaea and Syntrophy

Although most interactions are not understood in full detail, syntrophy (Box 2) appears to be largely responsible for the known archaeal interactions. In general, syntrophy allows a consortium of microorganisms to gain energy by coupling processes that can, due to bioenergetic reasons, be accomplished only by microbial interlinkage [14]. The syntrophic associations involving archaea are mostly based on the transfer of reduced compounds, such as H_2 or

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formate (more details can be found in [14]). The utilization of H_2 by, for example, methanogens, keeps the partial pressure of hydrogen low during anaerobic degradation of organic matter and thus allows effective and complete degradation of organic matter to hydrogen and CO_2 by primary and secondary fermenters. Interestingly, such syntrophic interactions require overall changes or adjustments at the level of the transcriptome, metabolome, or even genome.

Archaeal Structures Involved in Interaction

In many cases, syntrophic associations require structural adaptations, including the formation of special cell-surface appendages, including nanowires (see above; [23]) or hami [29,132]. Besides, pili, archaella, and even S-layers have also been reported to mediate attachment to abiotic and biotic surfaces, allowing communication or electron transfer between cells [133,134]. Interestingly, the hami also seem to have evolved from S-layer proteins [132].

In general, the archaeal cell wall seems to play a major role in intercellular contact, as, on the one hand, it serves as an anchor for cell-surface appendages (pili, archaella, hami); on the other hand, it serves as a 'contact point' for interactions, attachment, and exchange.

The typical archaeal cell envelope is composed of a single cytoplasmic membrane (with archaea-typical lipids) and mostly with one to several layers of polymeric substance on top (such as the S-layers, heteropolysaccharides, or pseudomurein) [135]. However, in particular, interacting archaea were found to possess a double membrane, which is now thought to be involved in social processes.

An archaeal double membrane was originally discovered for *Ignicoccus* species, but it revealed a very special appearance: the 'outer cellular membrane' is possibly energized and might thus support interconnection with the respective symbiont, *Nanoarchaeum equitans* [136]. The presence of classical outer membranes, similar to those of Gram-negative bacteria, was discovered for *Methanomassiliicoccus luminyensis* [8], representatives of the ARMAN lineage [137], and *Ca*. Altiarchaeum hamiconexum [21]. It is speculated that many more archaeal species carry a double membrane; however, visualization of the second membrane is extremely difficult [135].

Concluding Remarks

Archaea, originally found in extreme environments, are now known to be ubiquitous. In most habitats they are assumed to interact with other microorganisms or eukaryotes. However, current knowledge on interacting archaea is largely limited to methanogens. Although the methanogens certainly represent keystone species, and exert a large impact on climate issues due to their efficient methane production (or consumption), archaeal diversity beyond the methanogens must not be overlooked. Archaea are crucial partners in numerous microbiomes, either in environmental settings or associated with holobionts.

Syntrophic relationships, in particular under energy-deficiency stress, appear to be a key strategy for the archaea; to date, however, it is unclear how archaea communicate or structurally interact with their environment. The double-membrane or attached cell-surface structure might be crucial in this regard. In addition, it remains completely unclear how archaea compete with bacteria and how they define their niche within a complex setting.

However, the most intriguing question remains. Do archaeal pathogens exist? So far, archaea appear to be truly salutogenic, as, *in vivo*, archaeal colonization alone has never been found to have caused pathogenic processes. Answering this question will certainly be a difficult task as we are currently facing a severe lack of appropriate methods to detect, quantify, and cultivate

Outstanding Questions

How diverse is the archaeome?

Do pathogenic archaea exist?

How abundant are archaea in the diverse microbiomes, and how diverse are they?

How do archaea physically interact with their environment?

How do archaea chemically communicate at the intra- and interspecies level, with their hosts or syntrophic partners?

How do archaea compete with bacteria?

What is the role of the archaeal double membrane?

How do archaea correlate with (human) disease; what is their impact on the host's health?

What are the functions of archaea in microbiomes?

How are (strictly anaerobic) archaea transferred from one microbiome to another?

Is archaeal gene transfer occurring in the holobiont's microbiomes?

Do archaea interact with the mycobiome?

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archaeal members from complex microbiomes. Increased effort is necessary to shed further light on the important archaeal component – its function, structure, and interaction.

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References

1. Woese, C.R. *et al.* (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl. Acad. Sci. U. S. A.* 87, 4576–4579
2. Stetter, K.O. (1999) Extremophiles and their adaptation to hot environments. *FEBS Lett.* 452, 22–25
3. Spang, A. *et al.* (2015) Complex archaea that bridge the gap between prokaryotes and eukaryotes. *Nature* 521, 173–179
4. Kendrick, M.G. and Kral, T.A. (2006) Survival of methanogens during desiccation: implications for life on Mars. *Astrobiology* 6, 546–551
5. Boetius, A. *et al.* (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. *Nature* 407, 623–626
6. Leininger, S. *et al.* (2006) Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* 442, 806–809
7. Paul, K. *et al.* (2012) '*Methanoplasmatales*', *Thermoplasmatales*-related archaea in termite guts and other environments, are the seventh order of methanogens. *Appl. Environ. Microbiol.* 78, 8245–8253
8. Dridi, B. *et al.* (2012) *Methanomassiliicoccus luminyensis* gen. nov., sp. nov., a methanogenic archaeon isolated from human faeces. *Int. J. Syst. Evol. Microbiol.* 62, 1902–1907
9. Mihajlovski, A. *et al.* (2008) A putative new order of methanogenic Archaea inhabiting the human gut, as revealed by molecular analyses of the mcrA gene. *Res. Microbiol.* 159, 516–521
10. Tajima, K. *et al.* (2001) Phylogenetic analysis of archaeal 16S rRNA libraries from the rumen suggests the existence of a novel group of archaea not associated with known methanogens. *FEMS Microbiol. Lett.* 200, 67–72
11. Iino, T. *et al.* (2013) *Candidatus* Methanogranum caenicola: a novel methanogen from the anaerobic digested sludge, and proposal of *Methanomassiliicoccaceae* fam. nov. and Methanomassiliicoccales ord. nov., for a methanogenic lineage of the class Thermoplasmata. *Microbes Environ.* 28, 244–250
12. Evans, P.N. *et al.* (2015) Methane metabolism in the archaeal phylum Bathyarchaeota revealed by genome-centric metagenomics. *Science* 350, 434–438
13. Brochier-Armanet, C. *et al.* (2008) Mesophilic Crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. *Nat. Rev. Microbiol.* 6, 245–252
14. Morris, B.E.L. *et al.* (2013) Microbial syntrophy: interaction for the common good. *FEMS Microbiol. Rev.* 37, 384–406
15. Wurch, L. *et al.* (2016) Genomics-informed isolation and characterization of a symbiotic Nanoarchaeota system from a terrestrial geothermal environment. *Nat. Commun.* 7, 12115
16. Jahn, U. *et al.* (2008) *Nanoarchaeum equitans* and *Ignicoccus hospitalis*: new insights into a unique, intimate association of two archaea. *J. Bacteriol.* 190, 1743–1750
17. Waters, E. *et al.* (2003) The genome of *Nanoarchaeum equitans*: insights into early archaeal evolution and derived parasitism. *Proc. Natl. Acad. Sci. U. S. A.* 100, 12984–12988
18. Giannone, R.J. *et al.* (2015) Life on the edge: functional genomic response of *Ignicoccus hospitalis* to the presence of *Nanoarchaeum equitans*. *ISME J.* 9, 101–114
19. Moissl-Eichinger, C. and Huber, H. (2011) Archaeal symbionts and parasites. *Curr. Opin. Microbiol.* 14, 364–370
20. Rudolph, C. *et al.* (2001) Natural communities of novel archaea and bacteria growing in cold sulfurous springs with a string-of-pearls-like morphology. *Appl. Environ. Microbiol.* 67, 2336–2344
21. Probst, A.J. *et al.* (2014) Biology of a widespread uncultivated archaeon that contributes to carbon fixation in the subsurface. *Nat. Commun.* 5, 5497
22. Comolli, L.R. and Banfield, J.F. (2015) Inter-species interconnections in acid mine drainage microbial communities. *Front. Microbiol.* 5, 367
23. Wegener, G. *et al.* (2015) Intercellular wiring enables electron transfer between methanotrophic archaea and bacteria. *Nature* 526, 587–590
24. Liao, Y. *et al.* (2016) Morphological and proteomic analysis of biofilms from the Antarctic archaeon, *Halorubrum lacusprofundi*. *Sci. Rep.* 6, 37454
25. Fröls, S. (2013) Archaeal biofilms: widespread and complex. *Biochem. Soc. Trans.* 41, 393–398
26. DeMaere, M.Z. *et al.* (2013) High level of intergenera gene exchange shapes the evolution of haloarchaea in an isolated Antarctic lake. *Proc. Natl. Acad. Sci. U. S. A.* 110, 16939–16944
27. Probst, A.J. and Moissl-Eichinger, C. (2015) 'Altiarchaeales': uncultivated archaea from the subsurface. *Life* 5, 1381–1395
28. Probst, A.J. *et al.* (2013) Tackling the minority: sulfate-reducing bacteria in an archaea-dominated subsurface biofilm. *ISME J.* 7, 635–651
29. Moissl, C. *et al.* (2005) The unique structure of archaeal 'hami', highly complex cell appendages with nano-grappling hooks. *Mol. Microbiol.* 56, 361–370
30. Bang, C. *et al.* (2014) Biofilm formation of mucosa-associated methanoarchaeal strains. *Front. Microbiol.* 5, 353
31. Macfarlane, S. *et al.* (2011) Mucosal biofilm communities in the human intestinal tract. *Adv. Appl. Microbiol.* 75, 111–143
32. Amils, R. *et al.* (2011) Hydrogenosomes. In *Encyclopedia of Astrobiology* (Gargaud, M., ed.), pp. 784–785, Springer
33. Wrede, C. *et al.* (2012) Archaea in symbioses. *Archaea* 2012, 596846
34. Prangishvili, D. *et al.* (2016) Viruses of the Archaea. *eLS* Published online August 16, 2016. http://dx.doi.org/10.1002/9780470015902.a0000774.pub3
35. Quax, T.E.F. *et al.* (2011) Simple and elegant design of a virion egress structure in Archaea. *Proc. Natl. Acad. Sci. U. S. A.* 108, 3354–3359
36. Erdmann, S. *et al.* (2014) Inter-viral conflicts that exploit host CRISPR immune systems of *Sulfolobus*. *Mol. Microbiol.* 91, 900–917
37. Berg, G. and Smalla, K. (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol. Ecol.* 68, 1–13
38. Bulgarelli, D. *et al.* (2012) Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* 488, 91–95
39. Philippot, L. *et al.* (2013) Going back to the roots: the microbial ecology of the rhizosphere. *Nat. Rev. Microbiol.* 11, 789–799
40. Vorholt, J.A. (2012) Microbial life in the phyllosphere. *Nat. Rev. Microbiol.* 10, 828–840

41. Hardoim, P.R. *et al.* (2015) The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol. Mol. Biol. Rev.* 79, 293–320
42. Berg, G. *et al.* (2015) The plant microbiome explored: implications for experimental botany. *J. Exp. Bot.* 67, 995–1002
43. Buée, M. *et al.* (2009) The rhizosphere zoo: an overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. *Plant Soil* 321, 189–212
44. Chelius, M.K. and Triplett, E.W. (2001) The diversity of archaea and bacteria in association with the roots of *Zea mays* L. *Microb. Ecol.* 41, 252–263
45. Herrmann, M. *et al.* (2008) Archaea dominate the ammonia-oxidizing community in the rhizosphere of the freshwater macrophyte *Littorella uniflora*. *Appl. Environ. Microbiol.* 74, 3279–3283
46. Liu, Y. *et al.* (2015) Archaeal communities associated with roots of the common reed (*Phragmites australis*) in Beijing Cuihu Wetland. *World J. Microbiol. Biotechnol.* 31, 823–832
47. He, Y. *et al.* (2017) Abundance and diversity of ammonia-oxidizing archaea and bacteria in the rhizosphere soil of three plants in the Ebinur Lake wetland. *Can. J. Microbiol.* 67, 573–582
48. Lee, S.-H. *et al.* (2015) Impact of elevated CO_2 and N addition on bacteria, fungi, and archaea in a marsh ecosystem with various types of plants. *Appl. Microbiol. Biotechnol.* 99, 5295–5305
49. Stocker, T. (2014) *Climate Change 2013: The Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, Cambridge University Press
50. Moss, A.R. *et al.* (2000) Methane production by ruminants: its contribution to global warming. *Annales de zootechnie* 49, 231–253
51. Conrad, R. (2009) The global methane cycle: recent advances in understanding the microbial processes involved. *Environ. Microbiol. Rep.* 1, 285–292
52. Pump, J. *et al.* (2015) Colonization of rice roots with methanogenic archaea controls photosynthesis-derived methane emission. *Environ. Microbiol.* 17, 2254–2260
53. Ke, X. *et al.* (2014) Different behaviour of methanogenic archaea and Thaumarchaeota in rice field microcosms. *FEMS Microbiol. Ecol.* 87, 18–29
54. Stapleton, A.E. and Simmons, S.J. *et al.* (2006) Plant control of phyllosphere diversity: genotype interactions with ultraviolet-B radiation. In *Microbial Ecology of the Aerial Plant Surface* (Bailey, M.J., ed.), pp. 223–238, CABI
55. Oliveira, M.N.V. *et al.* (2013) Endophytic microbial diversity in coffee cherries of *Coffea arabica* from southeastern Brazil. *Can. J. Microbiol.* 59, 221–230
56. Müller, H. *et al.* (2015) Plant genotype-specific archaeal and bacterial endophytes but similar *Bacillus* antagonists colonize Mediterranean olive trees. *Front. Microbiol.* 6, 138
57. Moissl-Eichinger, C. *et al.* (2017) Human age and skin physiology shape diversity and abundance of Archaea on skin. *Sci. Rep.* 7, 4039
58. Chen, X. *et al.* (2008) Ammonia-oxidizing archaea: important players in paddy rhizosphere soil? *Environ. Microbiol.* 10, 1978–1987
59. Treusch, A.H. *et al.* (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ. Microbiol.* 7, 1985–1995
60. Francis, C.A. *et al.* (2007) New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. *ISME J.* 1, 19–27
61. Radax, R. *et al.* (2012) Ammonia-oxidizing archaea as main drivers of nitrification in cold-water sponges. *Environ. Microbiol.* 14, 909–923
62. Bragina, A. *et al.* (2014) The *Sphagnum* microbiome supports bog ecosystem functioning under extreme conditions. *Mol. Ecol.* 23, 4498–4510
63. van de Vossenberg, J.L.C.M. *et al.* (1998) The essence of being extremophilic: the role of the unique archaeal membrane lipids. *Extremophiles* 2, 163–170
64. Valentine, D.L. (2007) Adaptations to energy stress dictate the ecology and evolution of the Archaea. *Nat. Rev. Microbiol.* 5, 316–323
65. Herrero, M. *et al.* (2013) Biomass use, production, feed efficiencies, and greenhouse gas emissions from global livestock systems. *Proc. Natl. Acad. Sci. U. S. A.* 110, 20888–20893
66. St-Pierre, B. and Wright, A.-D. (2013) Diversity of gut methanogens in herbivorous animals. *Animal* 7, 49–56
67. Pérez-Barbería, F.J. (2017) Scaling methane emissions in ruminants and global estimates in wild populations. *Sci. Total Environ.* 579, 1572–1580
68. Sejian, V. *et al.* (2011) Measurement and prediction of enteric methane emission. *Int. J. Biometeorol.* 55, 1–16
69. Fung, I. *et al.* (1991) Three-dimensional model synthesis of the global methane cycle. *J. Geophys. Res. Atmos.* 96, 13033–13065
70. Thorpe, A. (2009) Enteric fermentation and ruminant eructation: the role (and control?) of methane in the climate change debate. *Clim. Change* 93, 407–431
71. Ho, A. *et al.* (2013) Termites facilitate methane oxidation and shape the methanotrophic community. *Appl. Environ. Microbiol.* 79, 7234–7240
72. Janssen, P.H. and Kirs, M. (2008) Structure of the archaeal community of the rumen. *Appl. Environ. Microbiol.* 74, 3619–3625
73. Raymann, K. *et al.* (2017) Unexplored archaeal diversity in the great ape gut microbiome. *mSphere* 2, e00026 17
74. Kittelmann, S. *et al.* (2013) Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS One* 8, e47879
75. Wright, A.-D.G. *et al.* (2007) Molecular diversity of methanogens in feedlot cattle from Ontario and Prince Edward Island, Canada. *Appl. Environ. Microbiol.* 73, 4206–4210
76. An, D. *et al.* (2005) Prokaryote diversity in the rumen of yak (*Bos grunniens*) and Jinnan cattle (*Bos taurus*) estimated by 16S rDNA homology analyses. *Anaerobe* 11, 207–215
77. Snelling, T.J. *et al.* (2014) Diversity and community composition of methanogenic archaea in the rumen of Scottish upland sheep assessed by different methods. *PLoS One* 9, e106491
78. Sundset, M.A. *et al.* (2009) Rumen microbial diversity in Svalbard reindeer, with particular emphasis on methanogenic archaea. *FEMS Microbiol. Ecol.* 70, 553–562
79. Min, B.R. *et al.* (2014) Gastrointestinal bacterial and methanogenic archaea diversity dynamics associated with condensed tannin-containing pine bark diet in goats using 16S rDNA amplicon pyrosequencing. *Int. J. Microbiol.* 2014, 141909
80. Singh, K.M. *et al.* (2012) Methanogen diversity in the rumen of Indian Surti buffalo (*Bubalus bubalis*), assessed by 16S rDNA analysis. *Res. Vet. Sci.* 92, 451–455
81. Lwin, K.-O. and Matsui, H. (2014) Comparative analysis of the methanogen diversity in horse and pony by using *mcrA* gene and archaeal 16S rRNA gene clone libraries. *Archaea* 2014, 483574
82. Su, Y. *et al.* (2014) Early methanogenic colonisation in the faeces of Meishan and Yorkshire piglets as determined by pyrosequencing analysis. *Archaea* 2014, 547908
83. Evans, P.N. *et al.* (2009) Community composition and density of methanogens in the foregut of the Tammar wallaby (*Macropus eugenii*). *Appl. Environ. Microbiol.* 75, 2598–2602
84. Klieve, A.V. *et al.* (2012) Archaea in the foregut of macropod marsupials: PCR and amplicon sequence-based observations. *J. Appl. Microbiol.* 113, 1065–1075
85. Luo, Y. *et al.* (2013) Diversity of methanogens in the hindgut of captive white rhinoceroses, *Ceratotherium simum*. *BMC Microbiol.* 13, 207
86. Saengkerdsub, S. *et al.* (2007) Identification and quantification of methanogenic archaea in adult chicken ceca. *Appl. Environ. Microbiol.* 73, 353–356
87. Wright, A.-D.G. *et al.* (2009) Rumen-like methanogens identified from the crop of the folivorous South American bird, the hoatzin (*Opisthocomus hoazin*). *ISME J.* 3, 1120–1126

88. Hong, P.-Y. *et al.* (2011) Phylogenetic analysis of the fecal microbial community in herbivorous land and marine iguanas of the Galápagos Islands using 16S rRNA-based pyrosequencing. *ISME J.* 5, 1461–1470
89. Shi, Y. *et al.* (2015) Phylogenetic diversity of Archaea in the intestinal tract of termites from different lineages. *J. Basic Microbiol.* 55, 1021–1028
90. Durand, L. *et al.* (2010) Microbial diversity associated with the hydrothermal shrimp *Rimicaris exoculata* gut and occurrence of a resident microbial community. *FEMS Microbiol. Ecol.* 71, 291–303
91. Turque, A.S. *et al.* (2010) Environmental shaping of sponge associated archaeal communities. *PLoS One* 5, e15774
92. Tapio, I. *et al.* (2017) The ruminal microbiome associated with methane emissions from ruminant livestock. *J. Anim. Sci. Biotechnol.* 8, 7
93. Ng, F. *et al.* (2016) An adhesin from hydrogen-utilizing rumen methanogen *Methanobrevibacter ruminantium* M1 binds a broad range of hydrogen-producing microorganisms. *Environ. Microbiol.* 18, 3010–3021
94. Samuel, B.S. *et al.* (2007) Genomic and metabolic adaptations of *Methanobrevibacter smithii* to the human gut. *Proc. Natl. Acad. Sci. U. S. A.* 104, 10643–10648
95. Miller, T.L. *et al.* (1982) Isolation of *Methanobrevibacter smithii* from human feces. *Appl. Environ. Microbiol.* 43, 227–232
96. Miller, T.L. *et al.* (1984) Methanogens and anaerobes in a colon segment isolated from the normal fecal stream. *Appl. Environ. Microbiol.* 48, 449–450
97. Dridi, B. *et al.* (2011) Archaea as emerging organisms in complex human microbiomes. *Anaerobe* 17, 56–63
98. Dridi, B. *et al.* (2009) High prevalence of *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* detected in the human gut using an improved DNA detection protocol. *PLoS One* 4, e7063–e7063
99. Gaci, N. *et al.* (2014) Archaea and the human gut: new beginning of an old story. *World J. Gastroenterol. WJG* 20, 16062
100. Bang, C. and Schmitz, R.A. (2015) Archaea associated with human surfaces: not to be underestimated. *FEMS Microbiol. Rev.* 39, 631–648
101. Lurie-Weinberger, M.N. *et al.* (2012) Contribution of lateral gene transfer to the gene repertoire of a gut-adapted methanogen. *Genomics* 99, 52–58
102. Fricke, W.F. *et al.* (2006) The genome sequence of *Methanosphaera stadtmanae* reveals why this human intestinal archaeon is restricted to methanol and H_2 for methane formation and ATP synthesis. *J. Bacteriol.* 188, 642–658
103. Borrel, G. *et al.* (2012) Genome sequence of '*Candidatus* Methanomethylophilus alvus' Mx1201, a methanogenic archaeon from the human gut belonging to a seventh order of methanogens. *J. Bacteriol.* 194, 6944–6945
104. Borrel, G. *et al.* (2013) Genome sequence of '*Candidatus* Methanomassiliicoccus intestinalis' Issoire-Mx1, a third Thermoplasmatales-related methanogenic archaeon from human feces. *Genome Announc.* 1, e00453 13
105. Borrel, G. *et al.* (2013) Phylogenomic data support a seventh order of methylotrophic methanogens and provide insights into the evolution of methanogenesis. *Genome Biol. Evol.* 5, 1769–1780
106. Söllinger, A. *et al.* (2016) Phylogenetic and genomic analysis of *Methanomassiliicoccales* in wetlands and animal intestinal tracts reveals clade-specific habitat preferences. *FEMS Microbiol. Ecol.* 92, fiv149
107. Dridi, B. *et al.* (2012) Age-related prevalence of *Methanomassiliicoccus luminyensis* in the human gut microbiome. *APMIS* 120, 773–777
108. Mihajlovski, A. *et al.* (2010) Molecular evaluation of the human gut methanogenic archaeal microbiota reveals an age-associated increase of the diversity. *Environ. Microbiol. Rep.* 2, 272–280
109. Vanderhaeghen, S. *et al.* (2015) Methanogen communities in stools of humans of different age and health status and co-occurrence with bacteria. *FEMS Microbiol. Lett.* 362, fnv092
110. Borrel, G. *et al.* (2017) Genomics and metagenomics of trimethylamine-utilizing Archaea in the human gut microbiome. *ISME J.* Published online June 6, 2017. http://dx.doi.org/10.1038/ismej.2017.72
111. Gill, S.R. *et al.* (2006) Metagenomic analysis of the human distal gut microbiome. *Science* 312, 1355–1359
112. Rieu-Lesme, F. *et al.* (2005) Recovery of partial 16S rDNA sequences suggests the presence of Crenarchaeota in the human digestive ecosystem. *Curr. Microbiol.* 51, 317–321
113. Oxley, A. *et al.* (2010) Halophilic archaea in the human intestinal mucosa. *Environ. Microbiol.* 12, 2398–2410
114. Nam, Y.-D. *et al.* (2008) Bacterial, archaeal, and eukaryal diversity in the intestines of Korean people. *J. Microbiol.* 46, 491–501
115. Scanlan, P.D. *et al.* (2008) Human methanogen diversity and incidence in healthy and diseased colonic groups using *mcrA* gene analysis. *BMC Microbiol.* 8, 79
116. Khelaifia, S. and Raoult, D. (2016) *Haloferax massiliensis* sp. nov., the first human-associated halophilic archaea. *New Microbes New Infect.* 12, 96–98
117. Costello, E.K. *et al.* (2009) Bacterial community variation in human body habitats across space and time. *Science* 326, 1694–1697
118. Kulik, E.M. *et al.* (2001) Identification of archaeal rDNA from subgingival dental plaque by PCR amplification and sequence analysis. *FEMS Microbiol. Lett.* 196, 129–133
119. Vianna, M.E. *et al.* (2006) Identification and quantification of archaea involved in primary endodontic infections. *J. Clin. Microbiol.* 44, 1274–1282
120. Hong, H.A. *et al.* (2009) Defining the natural habitat of Bacillus spore-formers. *Res. Microbiol.* 160, 375–379
121. Horz, H.-P. *et al.* (2012) McrA and 16S rRNA gene analysis suggests a novel lineage of Archaea phylogenetically affiliated with Thermoplasmatales in human subgingival plaque. *Anaerobe* 18, 373–377
122. Probst, A.J. *et al.* (2013) Archaea on human skin. *PLoS One* 8, e65388
123. Brugère, J.-F. *et al.* (2014) Archaebiotics: proposed therapeutic use of archaea to prevent trimethylaminuria and cardiovascular disease. *Gut Microbes* 5, 5–10
124. Krishnan, L. and Sprott, G.D. (2008) Archaeosome adjuvants: immunological capabilities and mechanism(s) of action. *Vaccine* 26, 2043–2055
125. Bringuier, A. *et al.* (2013) Real-time PCR quantification of *Methanobrevibacter oralis* in periodontitis. *J. Clin. Microbiol.* 51, 993–994
126. Drancourt, M. *et al.* (2017) Evidence of archaeal methanogens in brain abscess. *Clin. Infect. Dis.* 65, 1–5
127. Triantafyllou, K. *et al.* (2013) Methanogens, methane and gastrointestinal motility. *J. Neurogastroenterol. Motil.* 20, 31–40
128. Tottey, W. *et al.* (2017) Colonic transit time is a driven force of the gut microbiota composition and metabolism: *in vitro* evidence. *J. Neurogastroenterol. Motil.* 23, 124
129. Bang, C. *et al.* (2014) The intestinal archaea *Methanosphaera stadtmanae* and *Methanobrevibacter smithii* activate human dendritic cells. *PLoS One* 9, e99411
130. Blais Lecours, P. *et al.* (2011) Immunogenic properties of archaeal species found in bioaerosols. *PLoS One* 6, e23326
131. Lecours, P.B. *et al.* (2014) Increased prevalence of *Methanosphaera stadtmanae* in inflammatory bowel diseases. *PLoS One* 9, 1–7
132. Perras, A.K. *et al.* (2015) S-layers at second glance? Altiarchaeal grappling hooks (hami) resemble archaeal S-layer proteins in structure and sequence. *Front. Microbiol.* 6, 543
133. Näther, D.J. *et al.* (2006) Flagella of *Pyrococcus furiosus*: multifunctional organelles, made for swimming, adhesion to various surfaces, and cell–cell contacts. *J. Bacteriol.* 188, 6915–6923
134. McGlynn, S.E. *et al.* (2015) Single cell activity reveals direct electron transfer in methanotrophic consortia. *Nature* 526, 531–535

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135. Klingl, A. (2014) S-layer and cytoplasmic membrane – exceptions from the typical archaeal cell wall with a focus on double membranes. *Front. Microbiol.* 5, 1–6

136. Küper, U. *et al.* (2010) Energized outer membrane and spatial separation of metabolic processes in the hyperthermophilic Archaeon *Ignicoccus hospitalis*. *Proc. Natl. Acad. Sci. U. S. A.* 107, 3152–3156

137. Comolli, L.R. *et al.* (2009) Three-dimensional analysis of the structure and ecology of a novel, ultra-small archaeon. *ISME J.* 3, 159–167

138. Brouček, J. and Čermák, B. (2015) Emission of harmful gases from poultry farms and possibilities of their reduction. *Ekologia* 34, 89–100

139. Daemmgen, U. *et al.* (2012) Enteric methane emissions from German pigs. *Agric. For. Res.* 3, 83–96

2 Archaea, tiny helpers on land plants?

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Abstract

Living organisms are divided into three kingdoms: eukaryotes, bacteria, and archaea. Archaea are found in extreme environments and diverse ecosystems including soils, oceans, and marshlands. Accumulating evidence shows that archaea are a constituent of plant-associated ecosystems in the aboveground and belowground phytobiome. However, few studies have investigated the role of archaea in plant health and its potential symbiosis in ecosystems. This Tansley Insight discusses recent progress in identifying how archaea contribute to plant traits such as growth, adaptation to abiotic stresses, and immune activation. We present the most recent functional and molecular data on archaea, including root colonization and the volatile emission to activate plant systemic immunity. These data represent a paradigm shift in our understanding of plant-microbiota interactions.

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Summary

Archaea are members of most microbiomes. While archaea are highly abundant in extreme environments, they are less abundant and diverse in association with eukaryotic hosts. Nevertheless, archaea are a substantial constituent of plant-associated ecosystems in the aboveground and belowground phytobiome. Only a few studies have investigated the role of archaea in plant health and its potential symbiosis in ecosystems. This Tansley Insight discusses recent progress in identifying how archaea contribute to plant traits such as growth, adaptation to abiotic stresses, and immune activation. We present the most recent functional and molecular data on archaea, including root colonization and the volatile emission to activate plant systemic immunity. These data represent a paradigm shift in our understanding of plant-microbiota interactions.

Introduction

Archaea were classified as the third kingdom of life along with prokaryotes and eukaryotes at the end of 1970 (Woese & Fox, 1977). Archaea are subdivided into four superphyla: *Diapherotrites*, *Parvarchaeota*, *Aenigmarchaeota*, *Nanoarchaeota,* and *Nanohaloarchaeota* (DPANN); *Thaumarchaeota*, *Aigarchaeota*, *Crenarchaeota,* and *Korarchaeota* (TACK); *Euryarchaeota*; and the newly discovered *Asgardarchaeota (*Zaremba-Niedzwiedzka *et al*., 2017). The most common phyla are *Euryarchaeota*, *Crenarchaeota*, and *Thaumarchaeota*. *Euryarchaeota* contains some halophilic archaeal species (haloarchaea) and methanogens (Cabello *et al*., 2004). *Crenarchaeota* primarily consists of hyperthermophilic archaea (Cabello *et al*., 2004). *Euryarchaeota, Crenarchaeota*, and *Thaumarchaeota* also contain ammonia-oxidizing archaea (Cabello *et al*., 2004; Chen *et al*., 2008; Muller *et al*., 2015). Archaea were initially found in extreme environments with extreme conditions such as high salinity and high temperature, and environments in which they evolved to metabolize organics such as methane and nitrogen (Cabello *et al*., 2004; Sakai *et al*., 2007; Chen *et al*., 2008; Singh *et al*., 2012). In the tree of life based on (meta)genomes, they occure ubiquitously but are are less prominent and less diverse in several ecosystems, e.g. , in the terrestrial subsurface and human-associated microbiomes than bacteria (Hug *et al.* 2016) .

Microbial communities associated with plant roots (microbiome) have been intensively analyzed (Philipot *et al*. 2013). However, most metagenome-based microbiome studies focused on dominant bacteria and fungi rather than archaea. However, the accumulating evidence indicates that archaea are important constituents of plant microbiomes, sparking scientific interest in plant-associated archaea (Song *et al*., 2019; Taffner *et al*., 2019). Previously, Archaea have been discovered in various land plant species, including rice, maize, and Scots pine, and in several aquatic plant species (Chelius & Triplett, 2001; Bomberg *et al.*, 2003; Dave *et al*., 2006; Ma *et al*., 2019) (Table 1). Archaea are primarily enriched in the plant rhizosphere (surrounding the root system), and have been identified at lower abundance in the endosphere (inside plant tissue) and phyllosphere (on the leaf) (Knief *et al*., 2012; Muller *et al*., 2015; Pump *et al*., 2015). The abundance and taxonomy of

archaea associated with plants differ depending on the plant species, environment, and developmental stage (Moissl-Eichinger *et al*. 2018). The combined results suggest that archaea might have developed specific plant-associated functions in plant ecosystems.

Two fundamental questions emerged about the interactions between archaea and plants. (1) What is the behavior of archaea during plant interactions? (2) What is the function of archaea within the plant environment? Some archaea have characteristics similar to bacteria, such as oxidizing ammonia and methane and environmental nutrient cycling and supply that supports plant health (Francis *et al.*, 2007; Verhamme *et al.*, 2011; Smith-Moore & Grunden, 2018). The main characteristic of archaea that differentiates them from other kingdoms is their viability in or adaptability to extreme environments (Valentine, 2007). This suggests that archaea might help plants adapt to abiotic stresses such as high metals, high salinity, and high temperature as well as other microbiome species including bacteria and fungi (Im *et al.*, 2005; Im *et al.*, 2009). There is evidence that archaea also are involved in enhancing plant immune responses, such as triggering induced resistance to pathogenic bacteria in Arabidopsis (Song *et al.*, 2019).

In this Tansley Insight, we discuss how archaea directly or indirectly affect plant health. We also consider how to maximize the potential of unique archaeal characteristics to optimize plant health.

How do archaea interact with plants?

Archaea survive in both aerobic and anaerobic conditions. A metagenomics analysis of plant rhizosphere identified both aerobic and anaerobic archaea colonizing plant roots (Knief *et al.*, 2012; Dubey *et al.*, 2016). The plant-soil interface generally contains both aerobic and anaerobic zones. Anaerobic zones are generated when oxygen consumption by soil biota exceeds oxygen diffusion into the soil, or when air flow is restricted due to high moisture or high groundwater level (Inglett *et al.*, 2005). Thus, the plant rhizosphere generates natural habitats for both aerobic and anaerobic archaea (Lecomte *et al.*, 2018).

How do we know that archaea directly interact with plants and are not simply present in the rhizosphere? A recent study found archaeal cells exclusively colonizing and multiplying in plant roots without any soil components (Song *et al.*, 2019).In the absence of plants, no growth of these cells was detected. These results provide crucial evidence that archaea directly interact with plants in addition to colonizing the root surface. Subsequent sections of this Tansley Insight will explore why archaea populate the rhizosphere and investigate their functional niche within plant ecosystems.

Archaea are involved in environmental nutrient cycling in plant ecosystems

Nutrient cycling within the complex soil environment is mediated by bacteria, fungi, archaea, and their interactions. The most pronounced nutrient cycle is the nitrogen (N) cycle. Plant roots absorb N from the soil in the form of ammonium (NH^{4+}) and nitrate (NO^{3-}). However, the predominant form of N absorbed by plant roots is NO^{3-}, which indicates that NH^{4+} and atmospheric dinitrogen (N_2) should be reduced (Cabello *et al.*, 2004) (Fig. 1a). These N-cycling processes are mediated by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (Chen *et al.*, 2013; Trivedi *et al.*, 2019). In rice soils, AOA were more abundant in the rhizosphere than in bulk soil, indicating that AOA-mediated N-cycling is primarily associated with the plant root (Chen *et al.*, 2008) (Table 1). The *ammonia monooxygenase* (*amo1*) genes of *Crenarchaeota* were strongly enriched in the rhizosphere of the submersed macrophyte *Littorella uniflora* compared to the levels in surrounding sediments (Herrmann *et al.*, 2008; Buée *et al.*, 2009) (Table 1). These observations suggest that AOA are enriched in the plant rhizosphere and are involved in N-cycling to support plant growth and health.

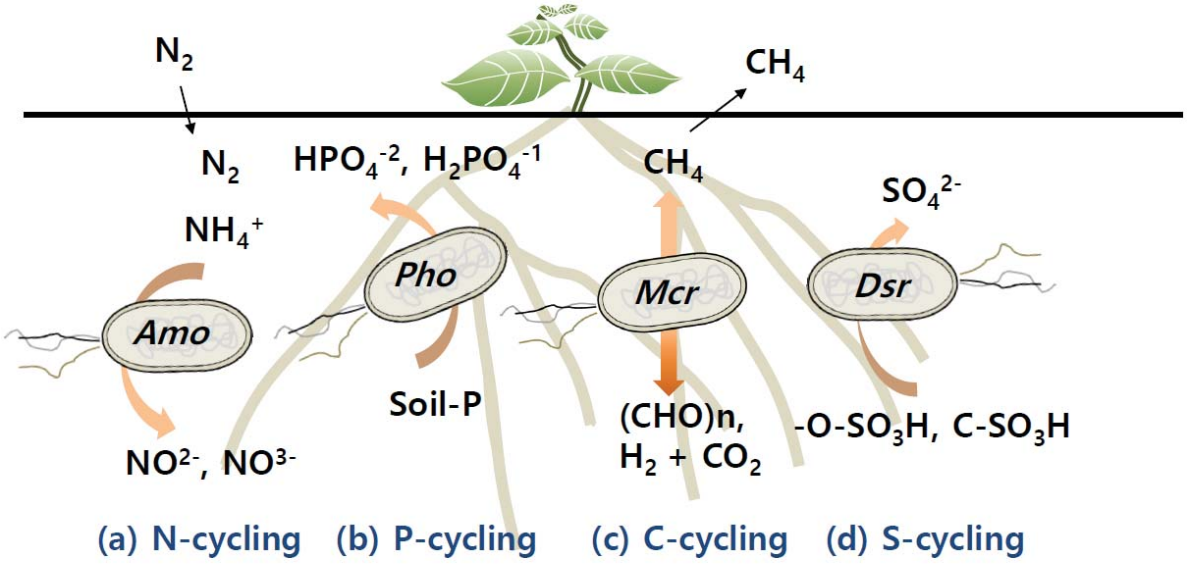

Fig. 1 Archaea are involved in environmental nutrient cycling in plant ecosystems. **(a)** Archaeal *ammonia monooxygenase* (*amo1*) gene catalyzes the oxidation of ammonium (NH^{4+}) to nitrite (NO^{2-}), which is subsequently oxidized to NO^{3-}, the bioavailable form of N that plants utilize. **(b)** The *alkaline phosphatase* (*Pho*) genes of archaea hydrolyze soil organic-P to HPO_4^{-2} and $H_2PO_4^{-1}$, which can be absorbed by plant roots. **(c)** *Methyl-coenzyme M reductase* (*Mcr*) catalyzes the last step in methanogenesis (CH_4 synthesis) and the first step in methanotrophy (CH_4 oxidation). Methanogenic archaea with high abundance of *mcr* are involved in C cycling. **(d)** S in the soil primarily exists in the form of sulfate-esters ($-O-SO_3H$) and sulfonates ($C-SO_3H$), which are reduced to bioavailable sulfides (SO^{4-}) for plant utilization by archaeal *dissimilatory sulfite reductases* (*dsr*).

Phosphorous (P) is another important element in plants. Plants and microbes obtain soluble organic-P from soil by hydrolyzing inorganic-P to orthophosphate when they are under P-deficiency conditions (Richardson & Simpson, 2011). Although both plant and microbial phosphatases effectively solubilize orthophosphate from soil organic-P, microbial phosphatases display higher efficiencies than those of plants (Richardson & Simpson, 2011; Teplitski *et al.*, 2011). Bacteria, fungi, and archaea contain alkaline phosphatases, phoD, and phoX, which hydrolyze soil organic-P (Ragot *et al.*, 2017) (Fig. 1b). Previous studies showed that *Euryarchaeota* isolated from arable, forest, and grassland soil expressed *phoD* and *phoX* genes (Ragot *et al.*, 2017) (Table 1). The *Euryarchaeota* species *halobactierum*, *halococcus*, and *halolamina* isolated from plant rhizosphere had measureable P solubilization activity (Al-Mailem *et al.*, 2010; Pires *et al.*, 2012; Yadav, Ajar Nath *et al.*, 2015; Gaba *et al.*, 2017) (Table 1). Further studies are needed to determine whether archaeal P solubilization contributes to and supports plant growth.

Carbon (C) cycling is crucial for plant ecosystems. Archaea are involved in C cycling by generating methane (CH_4) using H_2, CO_2, or methylated compounds (Fig. 1c) (Evans *et al.*, 2019). Previous studies showed that archaea were highly abundant in rice fields, which contribute 10–25% of global methane emissions (Sakai *et al.*, 2007) (Table 1). Methanogenic archaea of the *Euryarchaeota* phylum produce up to 60% of this methane emission (Pump *et al.*, 2015; Welte, 2018). *Methyl-coenzyme M reductase* (*Mcr*) is commonly expressed in methanogenic archaea; this enzyme catalyzes the last step in methanogenesis (CH_4 synthesis) and the first step in methanotrophy (CH_4 oxidation) (Evans *et al.*, 2019). Methanogenesis and anaerobic methane oxidation are important steps in the C cycle, and both are performed exclusively by anaerobic methanogens (Offre *et al.*, 2013).

Some bacteria and archaea are involved in sulfur (S) cycling, which is an important element in organisms (Finster *et al.*, 1997; Kertesz & Mirleau, 2004; Kertesz *et al.*, 2007; Liu *et al.*, 2012). Sulfur in the soil exists primarily in the form of sulfate-esters (-O-SO_3H) and sulfonates (C-SO_3H) (Kertesz & Mirleau, 2004), which need to be metabolized by soil microbes before the S becomes bioavailable for plants (Kertesz *et al.*, 2007). Archaea reduce these sulfates and sulfites to sulfides via enzymes encoded by *dissimilatory sulfite reductases* (*dsr*) (Anantharaman *et al.*, 2018) (Fig. 1d). *Euryarchaeota*, *Crenarchaeota*, and *Aigarchaeota* isolated from marine habitats or wetlands express the genetic capacity to reduce sulfite to sulfide via *dsr* (Anantharaman *et al.*, 2018) (Table 1). The combined studies suggest a potential role for archaea in rhizospheric S cycling, although further work is needed to verify direct archaeal involvement in S cycles.

The emerging evidence suggests that archaea have a beneficial role in enhancing land plant fitness through soil nutrient cycling. Future studies on archaeal metabolism and plant symbiosis will determine whether archaea are directly involved in plant growth and plant defense responses.

Plant growth-promoting archaea

Plant growth-promoting rhizobacteria (PGPR) promote plant growth by directly or indirectly interacting with plant roots (Kloepper *et al.*, 2004; Lugtenberg & Kamilova, 2009). As archaea are involved in nutrient cycling and making N, P, C, and S bioavailable to plants, they also should be considered as plant growth-promoting microorganisms (Yadav, A. N. *et al.*, 2015; Yadav *et al.*, 2017) (Fig. 2). For example, *Nitrosocosmicus oleophilus* MY3 cells oxidize N into plant-bioavailable forms and promote Arabidopsis growth (Song *et al.*, 2019) (Table 1). Archaea display functional metabolic signatures of CO_2 fixation; C is an essential nutrient for plants, and archaea might have a functional role in plant C supply (Taffner *et al.*, 2018; Evans *et al.*, 2019). Some halophilic archaea isolated from marine salterns around the Bhavnagar coast showed functional signatures of P siderophore production (Dave *et al.*, 2006) (Table 1). Some of these archaeal species were found in terrestrial plants, and it is likely that they might support plant growth by facilitating plant iron uptake (Al-Mailem *et al.*, 2010) (Fig. 2 and Table 1).

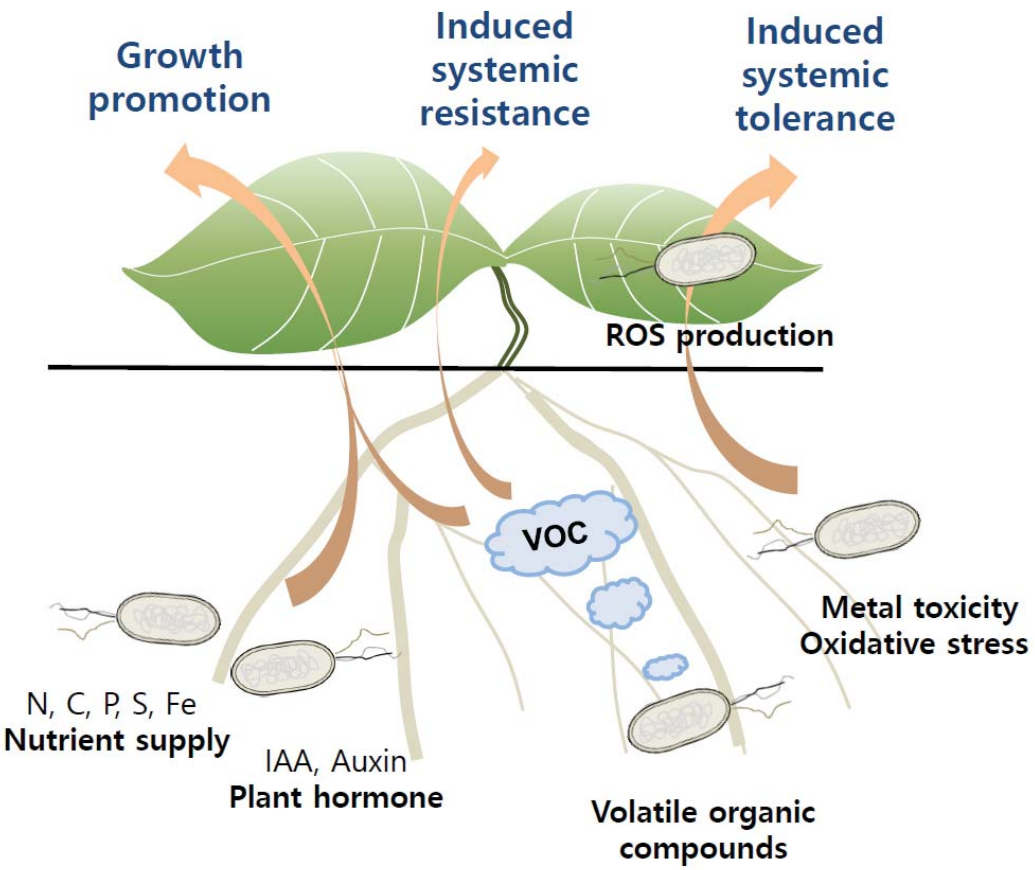

Fig. 2 Beneficial effects of archaea around plants. Archaea enhance plant health by promoting growth, inducing resistance, and elevating abiotic stress tolerance. Nutrient (N, C, P, and S) cycling and siderophore production by archaea could provide nutrients that support and promote plant growth. Indole acetic acid (IAA) produced by archaea and the genetic capacity for auxin biosynthesis in archaea also function to promote plant growth. The as-yet unidentified volatile organic compounds (VOC) of archaea trigger plant growth and induced resistance. Archaea can stabilize toxic metal via the dissimilatory sulfate reduction process, thereby supporting plant growth under environmental conditions with high metal levels. Functional signatures for resistance to oxidative stress and production of reactive oxygen species (ROS) suggest that archaea could protect plants under abiotic stress conditions.

PGPR also regulate plant growth by modulating hormone production, and archaea have a similar potential. Early studies reported that archaea promote the secretion of plant hormones. Thermophilic *Sulfolobus acidocaldarius* of the *Crenarchaeota* phylum produces the plant growth-promoting hormone indole acetic acid (IAA) at levels a thousand times higher than that observed in typical plant extracts (White, 1987) (Fig. 2 and Table 1). This was one of the first reports linking archaea to plant growth promotion (White, 1987). A recent metagenomic analysis of archaea associated with bog vegetation detected genetic evidence for auxin biosynthesis, which further supports the plant growth-promoting activity of archaea (Taffner *et al.*, 2018) (Fig. 2 and Table 1).

A recent study showed that *N. oleophilus* MY3 cells promote the growth of Arabidopsis plants grown on soil and in hypotonic medium. Treatment of plants with volatile compounds derived from *N. oleophilus* MY3 cells could also promote growth in the absence of any direct physical contact between archaea and plants. These results indicate that archaeal volatile compounds have a key role in plant growth promotion, similar to that of PGPR. Archaeal volatiles did not contain 2,3-butanediol, which is a well-known bacterial volatile that promotes plant growth (Ryu *et al.*, 2003; Song *et al.*, 2019). These observations lead to another emerging area of research, which investigates the effects of archaea or archaeal volatiles in plant growth promotion and host-microbe interactions.

Archaea are involved in enhancing abiotic and biotic stress resistance

Archaea can live in environments with extreme conditions, such as very high or low temperatures, high salt, and acidic or alkaline pH (Rampelotto, 2013). The hyperthermophilic archaea *Methanopyrus kandleri* live at 121℃, whereas the acidophilic archaea *Picrophilus* survive at pH 0.06 (Rampelotto, 2013). Plants can also grow in environments with high levels of abiotic stress, colonized by archaea, especially in their rhizosphere. Metagenomic analysis of the rhizosphere of *Jatropha curcas*, which adapted to grow under salt stress and high temperature conditions, showed high abundances of *Crenarchaeota* and *Euryarchaeota* (Dubey *et al.*, 2016) (Table 1). These archaea may help the plant adapt to environmental stresses.

Euryarchaeota and *Methanosarcina* species can methylate mercury (Hg) in rice fields, suggesting that these plant-associated archaea might have important roles in supporting plant growth under high Hg conditions (Ma *et al.*, 2019) (Table 1). Sulfate-reducing organisms stabilize metals such as Pb, Zn, and Cd in soils (Karna *et al.*, 2018). Bacterial and archaeal *dsrA*/*B* genes are key factors in metal sulfide formation via the dissimilatory sulfate reduction process (Anantharaman *et al.*, 2018; Karna *et al.*, 2018). Therefore, archaea could support plant growth under adverse environmental conditions with high metal levels (Fig. 2). Further, archaea from alpine bogs showed functional signatures in protecting plants from oxidative and osmotic stresses (Taffner *et al.*, 2018) (Table 1). Archaea found in the

rhizosphere and phyllosphere of arugula also displayed functional signatures for resistance to oxidative stress (Table 1) (Taffner et al. 2019). These combined results suggest that archaea could help plants survive and adapt to abiotic stress conditions (Fig. 2).

Archaea display functional traits that might enhance plant responses to biotic stresses. Genome analyses of 203 archaea including *Crenarchaeota* and *Euryarchaota* showed that genes involved in terpene and bacteriocin production were widely distributed in *Crenarchaota* genomes (Wang *et al.*, 2019). Terpene and bacteriocin deter herbivore feeding and microbial colonization, respectively; therefore, these archaea have potential functions for plant defense responses against herbivores and pathogenic bacteria (Singh & Sharma, 2015; Kumar & Tiwari, 2017).

Arabidopsis plants exposed to *N. oleophilus* MY3 cells displayed enhanced disease resistance when subsequently challenged with *Pectobacterium carotovorum* and *Pseudomonas syringae* (Song *et al.*, 2019). This induced resistance response depends on jasmonic acid rather than salicylic acid, indicating that archaea triggers induced systemic resistance (ISR) in Arabidopsis. NO_2^- promotes ISR in plants. However, the ISR response still occurs when archaea are completely sequestered from the plants, suggesting that archaeal volatile compounds elicit ISR responses against pathogens (Mayer *et al.*, 2018; Song *et al.*, 2019). These combined results suggest that archaea could produce novel plant protection compounds and could be used in innovative biotechnological applications.

Colonization and role of plant-associated archaea in the seeds

Archaea have a variety of properties that benefit the host plant and may support the plant progeny. In tomato (*Solanum lycopersicum* L.), beneficial bacteria are actively transmitted by the plant to the next generation via the seeds (Bergna *et al.*, 2018). Although plants do not actively select and transmit archaea to the offspring, recent work detected archaeal abundances of up to $3.09*10^9$ copies g^{-1} in seeds of native alpine plants and in tomatoes (Wassermann *et al.*, 2019; Taffner et al., 2019). Interestingly, in alpine seeds the composition of Archaea was highly specific for each plant species, which indicate a co-evolution in native

environments (Wassermann et al., 2019). Studies on transmission via clonal colonies in *Glechoma hederacea* also did not detect archaeal transmission from the mother to the daughter plant (Vannier *et al.*, 2018). In seeds, archaea appear to have evolved into bystander organisms based on synthrophic interactions with bacteria (Morris *et al.*, 2013). Instead, root exudates serve to attract and enrich archaea from the surrounding soil to the plant rhizosphere (Simon *et al.*, 2005). This archaeal colonization occurs during the latter phase of plant development (Edwards *et al.*, 2018). However, more studies are necessary to understand the co-evolution between plants and Archaea and their transmission routes.

Conclusions and perspectives

This Tansley Insight discussed the beneficial functions of archaea for plant health. Archaea have been detected in plant tissues and on plant surfaces, where they function to promote plant growth and enhance resistance to biotic and abiotic stresses. Therefore, we suggest that archaea could be used as possible biocontrol agents. Archaea have very slow growth rates, which makes archaeal genetic engineering a more suitable biotechnological strategy than direct field applications of archaea as biocontrol agents. *Superoxide reductase* (*SOR*) isolated from the thermophilic archaea *Pyrococcus furiousus* has been successfully expressed in *Arabidopsis thaliana* and tobacco cells; these transgenic plants displayed higher tolerance to heat, light, and methyl viologen than non-expressing plants (Im *et al.*, 2005; Im *et al.*, 2009). *SOR* expression in the chloroplast could further enhance plant stress tolerance, as a significant proportion of reactive oxygen species are generated in the chloroplast (Im *et al.*, 2009). Achaea are expected to have many beneficial properties for plants that have not yet been identified due to experimental and technical challenges. Further work is needed to cultivate and analyze plant-associated archaea and to determine their full potential in supporting plant health and growth. Today we have no single isolate of a plant-originated Archaeon! We predict that this work will greatly expand their beneficial applications for agriculture.

Table 1 Potential functions of archaea in the plant phytobiome.

Archaea	Discovery location	Potential function	Reference
Crenarchaeota			
	Mycorrhizospheres scots pine		(Bomberg *et al.*, 2003)
	Rhizosphere of *macrophyte Littorella uniflora*	Ammonia oxidation	(Herrmann *et al.*, 2008)
	Marine or wetland	Sulfur reduction (*dissimilatory sulfite reductases*)	(Anantharaman *et al.*, 2018)
Sulfolobus acidocaldarius		Indole acetic acid production	(White, 1987)
	Rhizosphere of *Jatropha curcas*	Nitrification	(Dubey *et al.*, 2016)
Thermoproteaceae, Sulfolobaceae Desulfurococcaceae		Bacteriosin or terpene in genome	(Wang *et al.*, 2019)
Euryarchaeota			
Methanogens, Rice cluster I	Rice fields	Methane-oxidizing archaea	(Cabello *et al.*, 2004; Sakai *et al.*, 2007; Welte, 2018)
	Marine or wetland	Sulfur reduction (*dissimilatory sulfite reductases*)	(Anantharaman *et al.*, 2018)
	Forest and grassland soil	Phosphatase enzymes *phoD* and *phoX*	(Ragot *et al.*, 2017)
Halobacteria	*Rhizophora mangle*	Phosphorous solubilization	(Pires *et al.*, 2012; Yadav, Ajar Nath *et al.*, 2015)
Halobactierum Halococcus	*Halonemum strobilaceum* (Chenopodiaceae)	Phosphorous Solubilization	(Al-Mailem *et al.*, 2010; Yadav, Ajar Nath *et al.*, 2015)
Halolamina	Rhizosphere of grasses in hypersaline soil	Phosphorous solubilization	(Yadav, Ajar Nath *et al.*, 2015; Gaba *et al.*, 2017)
Methanomicrobia, Halobacteriaceae	Rhizosphere of Bog vagetation	CO2 fixation, oxidative stress	(Taffner *et al.*, 2018)
Halococcus	Marine salterns around the	Siderophores production	(Dave *et al.*, 2006)

saccharolyticus, Halorubrum saccharovorum, Haloterrigena turkmenica, Halogeometricum sp., Natrialba sp	coast		
	Rhizosphere of Bog vegetation	Auxin biosynthesis	(Taffner *et al.*, 2018)
	Rhizosphere of *Jatropha curcas*		(Dubey *et al.*, 2016)
Methanosarcina	Rice paddy field	Hg-methylating	(Ma *et al.*, 2019)
Pyrococcus furiousus	*Cornus canadensis* L. f. (ornamental dogwood)	ROS generation and detoxification	(Im *et al.*, 2005; Im *et al.*, 2009; Geng *et al.*, 2016).
Halobacteriaceae		Terpene in genome	(Wang *et al.*, 2019)
Thaumarchaeota			
Nitrosocosmicus oleophilus MY3	Coal tar-contaminated sediment	Ammonia oxidation, growth promotion, disease resistance	(Jung *et al.*, 2016; Song *et al.*, 2019)
Non-classified			
	Roots of *Zea mays* L.		(Chelius & Triplett, 2001)
	Rhizosphere of rice	Ammonia oxidation	(Chen *et al.*, 2008)
	Soil from Bog vagetation	Oxidative stress	(Taffner *et al.*, 2018)
	Rhizosphere and phyllosphere of arugula	Oxidative stress	(Taffner *et al.*, 2019)

References

Al-Mailem DM, Sorkhoh NA, Marafie M, Al-Awadhi H, Eliyas M, Radwan SS. 2010. Oil phytoremediation potential of hypersaline coasts of the Arabian Gulf using rhizosphere technology. *Bioresour Technol* 101(15): 5786-5792.

Anantharaman K, Hausmann B, Jungbluth SP, Kantor RS, Lavy A, Warren LA, Rappé MS, Pester M, Loy A, Thomas BC, et al. 2018. Expanded diversity of microbial groups that shape the dissimilatory sulfur cycle. *Isme j* 12(7): 1715-1728.

Bergna A, Cernava T, Rändler M, Grosch R, Zachow C, Berg G. 2018. Tomato Seeds Preferably Transmit Plant Beneficial Endophytes. 2(4): 183-193.

Bomberg M, Jurgens G, Saano A, Sen R, Timonen S. 2003. Nested PCR detection of Archaea in defined compartments of pine mycorrhizospheres developed in boreal forest humus microcosms. *FEMS Microbiol Ecol* 43(2): 163-171.

Buée M, Boer Wd, Martin F, Overbeek LSv, Jurkevitch E. 2009. The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and some of their structuring factors. *Plant and Soil* 321(1-2): 189-212.

Cabello P, Roldan MD, Moreno-Vivian C. 2004. Nitrate reduction and the nitrogen cycle in archaea. *Microbiology* 150(Pt 11): 3527-3546.

Chelius M, Triplett EJMe. 2001. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. 41(3).

Chen XP, Zhu YG, Xia Y, Shen JP, He JZ. 2008. Ammonia-oxidizing archaea: important players in paddy rhizosphere soil? *Environ Microbiol* 10(8): 1978-1987.

Chen Y-L, Chen B-D, Hu Y-J, Li T, Zhang X, Hao Z-P, Wang Y-S. 2013. Direct and indirect influence of arbuscular mycorrhizal fungi on abundance and community structure of ammonia oxidizing bacteria and archaea in soil microcosms. *Pedobiologia* 56(4): 205-212.

Dave BP, Anshuman K, Hajela P. 2006. Siderophores of halophilic archaea and their chemical characterization. *Indian J Exp Biol* 44(4): 340-344.

Dubey G, Kollah B, Gour VK, Shukla AK, Mohanty SR. 2016. Diversity of bacteria and archaea in the rhizosphere of bioenergy crop Jatropha curcas. *3 Biotech* 6(2): 257.

Edwards JA, Santos-Medellin CM, Liechty ZS, Nguyen B, Lurie E, Eason S, Phillips G, Sundaresan V. 2018. Compositional shifts in root-associated bacterial and archaeal microbiota track the plant life cycle in field-grown rice. *PLoS Biol* 16(2): e2003862.

Evans PN, Boyd JA, Leu AO, Woodcroft BJ, Parks DH, Hugenholtz P, Tyson GW. 2019. An evolving view of methane metabolism in the Archaea. *Nat Rev Microbiol* 17(4): 219-232.

Finster K, Liesack W, Tindall BJ. 1997. Sulfurospirillum arcachonense sp. nov., a New Microaerophilic Sulfur-Reducing Bacterium. 47(4): 1212-1217.

Francis CA, Beman JM, Kuypers MM. 2007. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. *Isme j* 1(1): 19-27.

Gaba S, Singh RN, Abrol S, Yadav AN, Saxena AK, Kaushik R. 2017. Draft Genome Sequence of Halolamina pelagica CDK2 Isolated from Natural Salterns from Rann of Kutch, Gujarat, India. *Genome announcements* 5(6): e01593-01516.

Geng XM, Liu X, Ji M, Hoffmann WA, Grunden A, Xiang QY. 2016. Enhancing Heat Tolerance of the Little Dogwood Cornus canadensis L. f. with Introduction of a Superoxide Reductase Gene from the Hyperthermophilic Archaeon Pyrococcus furiosus. *Front Plant Sci* 7: 26.

Herrmann M, Saunders AM, Schramm A. 2008. Archaea dominate the ammonia-oxidizing community in the rhizosphere of the freshwater macrophyte Littorella uniflora. *Appl Environ Microbiol* 74(10): 3279-3283.

Im YJ, Ji M, Lee A, Killens R, Grunden AM, Boss WF. 2009. Expression of Pyrococcus furiosus superoxide reductase in Arabidopsis enhances heat tolerance. *Plant Physiol* 151(2): 893-904.

Im YJ, Ji M, Lee AM, Boss WF, Grunden AM. 2005. Production of a thermostable archaeal superoxide reductase in plant cells. *FEBS Lett* 579(25): 5521-5526.

Inglett P, Reddy K, Corstanje R 2005. Encyclopedia of soils in the environment: Academic Press Amsterdam.

Jung MY, Kim JG, Sinninghe Damste JS, Rijpstra WI, Madsen EL, Kim SJ, Hong H, Si OJ, Kerou M, Schleper C, et al. 2016. A hydrophobic ammonia-oxidizing archaeon of the Nitrosocosmicus clade isolated from coal tar-contaminated sediment. *Environ Microbiol Rep* 8(6): 983-992.

Karna RR, Hettiarachchi GM, Van Nostrand J, Yuan T, Rice CW, Assefa Y, Zhou J. 2018. Microbial Population Dynamics and the Role of Sulfate Reducing Bacteria Genes in Stabilizing Pb, Zn, and Cd in the Terrestrial Subsurface. 2(4): 60.

Kertesz MA, Fellows E, Schmalenberger A 2007. Rhizobacteria and Plant Sulfur Supply. *Advances in Applied Microbiology*: Academic Press, 235-268.

Kertesz MA, Mirleau P. 2004. The role of soil microbes in plant sulphur nutrition. *Journal of Experimental Botany* 55(404): 1939-1945.

Kloepper JW, Ryu CM, Zhang S. 2004. Induced Systemic Resistance and Promotion of Plant Growth by Bacillus spp. *Phytopathology* 94(11): 1259-1266.

Knief C, Delmotte N, Chaffron S, Stark M, Innerebner G, Wassmann R, von Mering C, Vorholt JA. 2012. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *Isme j* 6(7): 1378-1390.

Kumar V, Tiwari SK. 2017. Halocin HA1: An archaeocin produced by the haloarchaeon Haloferax larsenii HA1. *Process Biochemistry* 61: 202-208.

Lecomte SM, Achouak W, Abrouk D, Heulin T, Nesme X, Haichar FeZ. 2018. Diversifying Anaerobic Respiration Strategies to Compete in the Rhizosphere. 6(139).

Liu Y, Beer LL, Whitman WB. 2012. Sulfur metabolism in archaea reveals novel processes. *Environ Microbiol* 14(10): 2632-2644.

Lugtenberg B, Kamilova F. 2009. Plant-growth-promoting rhizobacteria. *Annu Rev Microbiol* 63: 541-556.

Ma M, Du H, Sun T, An S, Yang G, Wang D. 2019. Characteristics of archaea and bacteria in rice rhizosphere along a mercury gradient. *Sci Total Environ* 650(Pt 1): 1640-1651.

Mayer D, Mithöfer A, Glawischnig E, Georgii E, Ghirardo A, Kanawati B, Schmitt-Kopplin P, Schnitzler J-P, Durner J, Gaupels F. 2018. Short-Term Exposure to Nitrogen Dioxide Provides Basal Pathogen Resistance. 178(1): 468-487.

Morris BE, Henneberger R, Huber H, Moissl-Eichinger C. 2013. Microbial syntrophy: interaction for the common good. *FEMS Microbiol Rev* 37(3): 384-406.

Muller H, Berg C, Landa BB, Auerbach A, Moissl-Eichinger C, Berg G. 2015. Plant genotype-specific archaeal and bacterial endophytes but similar Bacillus antagonists colonize Mediterranean olive trees. *Front Microbiol* 6: 138.

Offre P, Spang A, Schleper C. 2013. Archaea in biogeochemical cycles. *Annu Rev Microbiol* 67: 437-457.

Pires AC, Cleary DF, Almeida A, Cunha A, Dealtry S, Mendonca-Hagler LC, Smalla K, Gomes NC. 2012. Denaturing gradient gel electrophoresis and barcoded pyrosequencing reveal unprecedented archaeal diversity in mangrove sediment and rhizosphere samples. *Appl Environ Microbiol* 78(16): 5520-5528.

Pump J, Pratscher J, Conrad R. 2015. Colonization of rice roots with methanogenic archaea controls photosynthesis-derived methane emission. *Environ Microbiol* 17(7): 2254-2260.

Ragot SA, Kertesz MA, Meszaros E, Frossard E, Bunemann EK. 2017. Soil phoD and phoX alkaline phosphatase gene diversity responds to multiple environmental factors. *FEMS Microbiol Ecol* 93(1).

Rampelotto PH. 2013. Extremophiles and extreme environments. *Life (Basel, Switzerland)* 3(3): 482-485.

Richardson AE, Simpson RJ. 2011. Soil Microorganisms Mediating Phosphorus Availability Update on Microbial Phosphorus. 156(3): 989-996.

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW. 2003. Bacterial volatiles promote growth in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* 100(8): 4927-4932.

Sakai S, Imachi H, Sekiguchi Y, Ohashi A, Harada H, Kamagata Y. 2007. Isolation of key methanogens for global methane emission from rice paddy fields: a novel isolate affiliated with the clone cluster rice cluster I. *Appl Environ Microbiol* 73(13): 4326-4331.

Simon HM, Jahn CE, Bergerud LT, Sliwinski MK, Weimer PJ, Willis DK, Goodman RM. 2005. Cultivation of Mesophilic Soil Crenarchaeotes in Enrichment Cultures from Plant Roots. 71(8): 4751-4760.

Singh A, Singh RS, Upadhyay SN, Joshi CG, Tripathi AK, Dubey SK. 2012. Community structure of methanogenic archaea and methane production associated with compost-treated tropical rice-field soil. *FEMS Microbiol Ecol* 82(1): 118-134.

Singh B, Sharma RA. 2015. Plant terpenes: defense responses, phylogenetic analysis, regulation and clinical applications. *3 Biotech* 5(2): 129-151.

Smith-Moore CM, Grunden AM. 2018. Bacteria and archaea as the sources of traits for enhanced plant phenotypes. *Biotechnol Adv* 36(7): 1900-1916.

Song GC, Im H, Jung J, Lee S, Jung MY, Rhee SK, Ryu CM. 2019. Plant growth-promoting archaea trigger induced systemic resistance in Arabidopsis thaliana against Pectobacterium carotovorum and Pseudomonas syringae. *Environ Microbiol* 21(3): 940-948.

Taffner J, Cernava T, Erlacher A, Berg GJJoAR. 2019. Novel insights into plant-associated archaea and their functioning in arugula (Eruca sativa Mill.).

Taffner J, Erlacher A, Bragina A, Berg C, Moissl-Eichinger C, Berg G. 2018. What Is the Role of Archaea in Plants? New Insights from the Vegetation of Alpine Bogs. *mSphere* 3(3).

Teplitski M, Mathesius U, Rumbaugh KP. 2011. Perception and degradation of N-acyl homoserine lactone quorum sensing signals by mammalian and plant cells. *Chem Rev* 111(1): 100-116.

Trivedi C, Reich PB, Maestre FT, Hu HW, Singh BK, Delgado-Baquerizo M. 2019. Plant-driven niche differentiation of ammonia-oxidizing bacteria and archaea in global drylands. *Isme j*.

Valentine DL. 2007. Adaptations to energy stress dictate the ecology and evolution of the Archaea. *Nat Rev Microbiol* 5(4): 316-323.

Vannier N, Mony C, Bittebiere AK, Michon-Coudouel S, Biget M, Vandenkoornhuyse P. 2018. A microorganisms' journey between plant generations. *Microbiome* 6(1): 79.

Verhamme DT, Prosser JI, Nicol GW. 2011. Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. *Isme j* 5(6): 1067-1071.

Wang S, Zheng Z, Zou H, Li N, Wu M. 2019. Characterization of the secondary metabolite biosynthetic gene clusters in archaea. *Computational Biology and Chemistry* 78: 165-169.

Wassermann B, Cernava T, Muller H, Berg C, Berg G. 2019. Seeds of native alpine plants host unique microbial communities embedded in cross-kingdom networks. *Microbiome* 7(1): 108.

Welte CU. 2018. Revival of Archaeal Methane Microbiology. *mSystems* 3(2).

White RH. 1987. Indole-3-acetic acid and 2-(indol-3-ylmethyl)indol-3-yl acetic acid in the thermophilic archaebacterium Sulfolobus acidocaldarius. *J Bacteriol* 169(12): 5859-5860.

Woese CR, Fox GE. 1977. Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America* 74(11): 5088-5090.

Yadav AN, Sharma D, Gulati S, Singh S, Dey R, Pal KK, Kaushik R, Saxena AK. 2015. Haloarchaea Endowed with Phosphorus Solubilization Attribute Implicated in Phosphorus Cycle. *Sci Rep* 5: 12293-12293.

Yadav AN, Sharma D, Gulati S, Singh S, Dey R, Pal KK, Kaushik R, Saxena AK. 2015. Haloarchaea Endowed with Phosphorus Solubilization Attribute Implicated in Phosphorus Cycle. *Sci Rep* 5: 12293.

Yadav AN, Verma P, Kaushik R, Dhaliwal H, Saxena AJEM. 2017. Archaea endowed with plant growth promoting attributes. 8(6): 294-298.

Zaremba-Niedzwiedzka K, Caceres EF, Saw JH, Bäckström D, Juzokaite L, Vancaester E, Seitz KW, Anantharaman K, Starnawski P, Kjeldsen KU, et al. 2017. Asgard archaea illuminate the origin of eukaryotic cellular complexity. *Nature* 541: 353.

3 What is the role of Archaea in plants? New insights from the vegetation of Alpine bogs

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Abstract

The *Archaea* represent a significant component of the plant microbiome, whereas their function is still unclear. Different plant species representing the natural vegetation of Alpine bogs harbour a substantial archaeal community originating from five phyla, 60 genera and 334 OTUs. We identified a core archaeome for all bog plants and ecosystem-specific, so far unclassified *Archaea*. In the metagenomic dataset archaea were found to have the potential to interact with plants by i) possible plant growth promotion through auxin biosynthesis, ii) nutrient supply, and iii) protection against abiotic (especially oxidative and osmotic) stress. The unexpectedly high degree of plant specificity supports plant-archaea interactions. Moreover, functional signatures of *Archaea* reveal genetic capacity for the interplay with fungi and an important role in the carbon and nitrogen cycle, e.g. CO_2 and N_2 fixation. These facts reveal an important, yet unobserved role of the *Archaea* for plants as well as for the bog ecosystem.

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RESEARCH ARTICLE
Host-Microbe Biology

What Is the Role of *Archaea* in Plants? New Insights from the Vegetation of Alpine Bogs

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ABSTRACT The *Archaea* represent a significant component of the plant microbiome, whereas their function is still unclear. Different plant species representing the natural vegetation of alpine bogs harbor a substantial archaeal community originating from five phyla, 60 genera, and 334 operational taxonomic units (OTUs). We identified a core archaeome for all bog plants and ecosystem-specific, so far unclassified *Archaea*. In the metagenomic data set, *Archaea* were found to have the potential to interact with plants by (i) possible plant growth promotion through auxin biosynthesis, (ii) nutrient supply, and (iii) protection against abiotic (especially oxidative and osmotic) stress. The unexpectedly high degree of plant specificity supports plant-archaeon interactions. Moreover, functional signatures of *Archaea* reveal genetic capacity for the interplay with fungi and an important role in the carbon and nitrogen cycle: e.g., CO_2 and N_2 fixation. These facts reveal an important, yet unobserved role of the *Archaea* for plants as well as for the bog ecosystem.

IMPORTANCE *Archaea* are still an underdetected and little-studied part of the plant microbiome. We provide first and novel insights into *Archaea* as a functional component of the plant microbiome obtained by metagenomic analyses. *Archaea* were found to have the potential to interact with plants by (i) plant growth promotion through auxin biosynthesis, (ii) nutrient supply, and (iii) protection against abiotic stress.

KEYWORDS *Archaea*, plant microbiome, plant-microbe interactions

During the last several decades, our picture of the diversity and metabolic potential of the *Archaea* in a wide variety of environments has been revolutionized (1, 2). For example, *Archaea* represent an important component of the human and plant microbiome, where their impact on their host is still unclear (3, 4). Within plants, *Archaea* are differently distributed (4). They have often been found in the rhizosphere and endosphere but rarely in the phyllosphere, which can be explained by the different abiotic conditions in these microenvironments (5–10). Besides abiotic factors and adaptation to chronic energy stress, archaeal colonization depends on biotic factors such as competition with bacteria, which might have led to microniche differentiation (11). Even though factors influencing archaeal functionality under specific anaerobic conditions in rice roots have been analyzed (9, 12), their ecological roles and interactions with plants remained largely unclear. The fact that most of *Archaea* are difficult to cultivate and that plant-associated archaeal pathogens are currently not known may be attributed to the lack of knowledge. However, due to their ubiquitous occurrence on healthy plants, we assume that *Archaea* interact positively with plants.

Plants harbor highly diverse and to a certain extent species-specific microbiomes (13–15). These microbiomes play an essential role for the plant as they can alter plant growth, productivity, adaptation, diversification, and health (16, 17). Especially in bog

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ecosystems, we have shown that plants and microbiota are closely interlinked (18–21). Bogs are one of the oldest terrestrial ecosystems on Earth (22); their functioning under extreme conditions is a result of a long period of coevolution between plants and microorganisms. Bog ecosystems fulfill important functions for the whole biosphere, as a reservoir for freshwater and for soil organic matter, acting as a carbon sink (23, 24). Since most of these ecosystems are extremely poor in accessible nutrients because they rely on rain water only (ombrotrophic lifestyle), plant-associated bacteria are known to play a crucial role in nutrient supply and cycling (25). Furthermore, raised bogs show a unique biodiversity, harboring a unique and highly specialized flora and fauna. The vegetation is often dominated by *Sphagnum* mosses, which play an important role in global carbon cycling and even in global climate (26). Especially the bacterial community associated with *Sphagnum* shows a supportive effect on plant health (18, 19), productivity (25), and peatland nutrient cycling (23, 27). The *Sphagnum* bacterial community is, to an extraordinary degree, host specific, is vertically transmitted, and contains different functional patterns that strongly support bog functioning under extreme environmental conditions: e.g., pH (highly acidic), nutrient availability (extremely low), and high water saturation (20, 28). In addition to *Sphagnum* mosses, there is diverse and well-adapted vegetation shaping this ecosystem: e.g., acidophytic bryophytes (*Polytrichum strictum* and *Aulacomnium palustre*), graminoids (*Eriophorum vaginatum* and *Carex nigra*), dwarf shrubs (*Andromeda polifolia* and *Vaccinium oxycoccus*), small trees (*Pinus mugo*), and lichens (e.g., *Cladonia fimbriata*). All components of the vegetation are embedded into *Sphagnum* mosses, forming the oxic acrotelm layer, which consists mostly of living plant material (see Fig. S1 in the supplemental material), in contrast to the anoxic catotelm, which consists of dead plant material (peat). The understanding of plant-microbe interactions in this specific bog environment still misses a relevant jigsaw puzzle piece: *Archaea* and their role in supporting functioning of this extreme ecosystem yet remain mostly unexplored. *Archaea* are expected to play an important role in nutrient supply (29) and stress protection (30).

The objective of our study was to find out if plants harbor specific archaeal communities and to identify potential modes of interaction of *Archaea* on plants in general. Another more specific objective was to integrate *Archaea* into the concept of the microbiome-driven functioning of the bog ecosystem. Therefore, we studied the archaeome of 46 plant samples originating from the green and oxic acrotelm layer, which represent the typical bog vegetation of alpine bogs. Samples were taken in Rotmoos and Pürgschachen Moor (Austria) and analyzed by a complementary approach of metagenomics and specific sequencing of the V4 region of the 16S rRNA gene fragment.

RESULTS

Composition of *Archaea* associated with bog vegetation. 16S rRNA amplicon analysis of 46 samples, including bryophytes, vascular plants, and lichens, resulted in amplicons for 41 samples (Table 1). Out of an overall data set of 305,430 sequences, 23,400 sequences (7.7%) were annotated to *Archaea* and clustered into 334 operational taxonomic units (OTUs). The data set was further normalized to 1,000 sequences per sample, resulting in the exclusion of 31 samples containing fewer. The estimated sequencing coverage for *Archaea* varied from 44.2% to 100%, with a mean value of 83.2%. The relative archaeal abundance differed from plant species to species and ranged from 0% to 33%. The highest relative abundances were detected in the samples of deep-rooted plants like blueberry (*Vaccinium myrthillus* [33%]) and cranberry (*V. oxycoccus* [31.7%]) and monocots like tussock cottongrass (*Eriophorum vaginatum* [29.1%]) and purple moor-grass (*Molinia caerulea* [20.2%]). However, also moss species harbored a substantial proportion of archaeal signatures: *Polytrichum commune* (25.4%), *Sphagnum capillifolium* (24.6%), *S. magellanicum* (18.2%), and *P. strictum* (16.7%). In a principal-coordinate analysis (PCoA) performed with representative species, archaeal communities in samples belonging to the class-level eudicots and monocots formed a distinct cluster, whereas samples belonging to the classes of *Sphagnopsida* and *Poly-*

TABLE 1 List of the complete set of 46 samples of overall representative vegetation of the bog, regarding their sampling location and plant coverage per plot[a]

Bog and plot[b]	Sample ID	Plant/lichen species	Clade	Growth form	Family	% of plant cover per plot	Relative archaeal abundance
Rotmoos							
Plot 1	MS1.1	*Pleurozium schreberi*	*Bryopsida*	Other mosses	Hylocomiaceae	3	0.1
	MS1.2	*Sphagnum angustifolium*	*Bryopsida*	Peat mosses	Sphagnaceae	37	0.33
	MS1.3	*Vaccinium myrtillus*	Eudicotyledons	Dwarf shrubs	Ericaceae	1	2.13
	MS1.4	*Calluna vulgaris*	Eudicotyledons	Dwarf shrubs	Ericaceae	10	2.27
	MS1.5	*Vaccinium oxycoccos*	Eudicotyledons	Dwarf shrubs	Ericaceae	3	31.72
	MS1.6	*Pinus mugo*	*Pinopsida*	Coniferous tree	Pinacaea	0.2	0.2
	MS1.7	*Sphagnum fuscum*	*Bryopsida*	Peat mosses	Sphagnaceae	10	3.18
	MS1.8	*Andromeda polifolia*	Eudicotyledons	Dwarf shrubs	Ericaceae	3	0.67
	MS1.9	*Sphagnum magellanicum*	*Bryopsida*	Peat mosses	Sphagnaceae	62	13.9
	MS1.10	*Polytrichum strictum*	*Bryopsida*	Other mosses	Polytrichaceae	5	16.71
	MS1.11	*Eriophorum vaginatum*	Monocotyledons	Graminoids	Cyperaceae	10	0.04
Plot 2	MS2.1	*Rhytidiadelphus triquetrus*	*Bryopsida*	Other mosses	Hylocomiaceae	3	0.24
	MS2.2	*Carex nigra*	Monocotyledons	Graminoids	Cyperaceae	5	3.36
	MS2.3	*Molinia caerulea*	Monocotyledons	Graminoids	Poaceae	10	1.04
	MS2.4	*Vaccinium vitis-idaea*	Eudicotyledons	Dwarf shrubs	Ericaceae	10	0
	MS2.5	*Vaccinium oxycoccus*	Eudicotyledons	Dwarf shrubs	Ericaceae	3	0.09
	MS2.6	*Polytrichum commune*	*Bryopsida*	Other mosses	Polytrichaceae	3	25.42
	MS2.7	*Vaccinium myrthillus*	Eudicotyledons	Dwarf shrubs	Ericaceae	3	0.32
	MS2.8	*Sphagnum magellanicum*	*Bryopsida*	Peat mosses	Sphagnaceae	20	18.2
	MS2.9	*Sphagnum angustifolium*	*Bryopsida*	Peat mosses	Sphagnaceae	87	0.04
	MS2.10	*Eriophorum vaginatum*	Monocotyledons	Graminoids	Cyperaceae	3	0.01
Pürgschachen Moor							
Plot 3	MS3.1	*Eriophorum vaginatum*	Monocotyledons	Graminoids	Cyperaceae	10	29.1
	MS3.2	*Sphagnum magellanicum*	*Bryopsida*	Peat mosses	Sphagnaceae	20	0
	MS3.3	*Vaccinium myrtillus*	Eudicotyledons	Dwarf shrubs	Ericaceae	3	33.02
	MS3.4	*Sphagnum capillifolium*	*Bryopsida*	Peat mosses	Sphagnaceae	3	24.61
	MS3.5	*Vaccinium oxycoccus*	Eudicotyledons	Dwarf shrubs	Ericaceae	3	0.24
	MS3.6	*Vaccinium vitis-idaea*	Eudicotyledons	Dwarf shrubs	Ericaceae	10	0.07
	MS3.7	*Vaccinium uliginosum*	Eudicotyledons	Dwarf shrubs	Ericaceae	10	0
	MS3.8	*Sphagnum angustifolium*	*Bryopsida*	Peat mosses	Sphagnaceae	62	0.05
	MS3.9	*Molinia caerulea*	Monocotyledons	Graminoids	Poaceae	3	20.24
	MS3.10	*Sphagnum cuspidatum*	*Bryopsida*	Peat mosses	Sphagnaceae	3	0
	MS3.11	*Aulacomnium palustre*	*Bryopsida*	Other mosses	Aulacomniaceae	3	0.42
Plot 4	MS4.1	*Calluna vulgaris*	Eudicotyledons	Graminoids	Ericaceae	20	0
	MS4.2	*Cladonia portentosa*	Ascomycota	Lichens	Cladoniaceae	3	0.04
	MS4.3	*Sphagnum magellanicum*	*Bryopsida*	Peat mosses	Sphagnaceae	37	0.1
	MS4.4	*Sphagnum fuscum*	*Bryopsida*	Peat mosses	Sphagnaceae	10	0.05
	MS4.5	*Mylia anomala*	*Bryopsida*	Liverworts	Myliaceae	3	0.03
	MS4.6	*Aulacomnium palustre*	*Bryopsida*	Other mosses	Aulacomniaceae	3	0.04
	MS4.7	*Sphagnum capillifolium*	*Bryopsida*	Peat mosses	Sphagnaceae	37	1.56
	MS4.8	*Pleurozium schreberi*	*Bryopsida*	Other mosses	Polytrichaceae	3	0
	MS4.9	*Andromeda polifolia*	Eudicotyledons	Dwarf shrubs	Ericaceae	1	0.18
	MS4.10	*Vaccinium oxycoccus*	Eudicotyledons	Dwarf shrubs	Ericaceae	3	0.5
	MS4.11	*Vaccinium vitis-idaea*	Eudicotyledons	Dwarf shrubs	Ericaceae	10	0.11
	MS4.12	*Bazzania trilobata*	*Bryopsida*	Liverworts	Lepidoziaceae	3	9.63
	MS4.13	*Eriophorum vaginatum*	Monocotyledons	Graminoids	Cyperaceae	10	0.03
	MS4.14	*Cladonia fimbriata*	Ascomycota	Lichens	Cladoniaceae	3	0.3

[a]All samples were analyzed with 16S rRNA sequencing, resulting in the displayed relative abundance of *Archaea* of the prokaryotic microbiota. Gray-shaded samples were additionally used for metagenomic studies, whereas the samples MS1.1 and -4.8, MS1.7 and -4.4, MS1.9 and -4.3, MS1.11 and -4.13, MS1.4 and -4.1, and MS1.8 and -4.9 were pooled prior to sequencing.

[b]Plot locations are as follows: plot 1, N47 41.029 E15 09.284, 695 m; plot 2, N47 41.059 E15 09.269, 695 m; plot 3, N47 34.835 E14 20.390, 632 m; and plot 4, N47 34.815 E14 20.482, 632 m.

trichopsida were more widespread (Fig. 1). Overall, the eudicot samples were more separate from the other groups, showing a lower diversity.

In total, based on the 16S rRNA gene data set, the archaeome associated with the bog vegetation showed low taxonomic diversity. A maximum likelihood phylogenetic tree was constructed based on the archaeal 16S rRNA amplicon sequences aligned to the complete archaeal 16S rRNA gene RefSeq database (Fig. 2). Based on the DNA

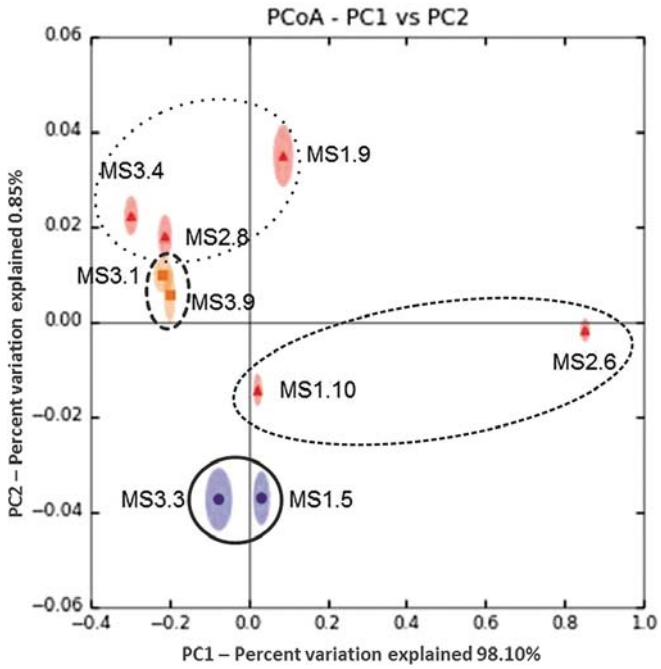

FIG 1 Comparison of archaeal communities associated with bog vegetation by principal-coordinate analysis (PCoA). The PCoA plot is based on a Bray-Curtis distance matrix of the 16S rRNA gene amplicon libraries and supported by 100 jackknife data resamplings using 1,000 sequences per library. The ellipses with symbols in the center show single samples with their IDs. Sample descriptions are as follows: *Vaccinium oxycoccus* (MS1.5), *S. magellanicum* (MS1.9, MS2.8), *Polytrichum strictum* (MS1.10), *P. commune* (MS2.6), *Eriophorum vaginatum* (MS3.1), *V. myrtilus* (MS3.3), *S. capillifolium* (MS3.4), and *Molinia caerulea* (MS3.9). Samples belonging to the clade Eudicotyledons are shown as solid circles circled by a solid line, samples of the clade Monocotyledons are shown as squares circled by a dashed line, and the samples of the clade *Bryopsida* (separated into the classes *Sphagnopsida* and *Polytrichopsida*) are shown as triangles circled by dotted and smaller dotted lines, respectively, in the center of the ellipses. Variation explained by each principal coordinate (PC) is defined on the plot.

distance maximum likelihood algorithm using 1,000 bootstraps, three main phylogeny clusters (A to C) were formed. Phylogenetic neighbor comparison allowed taxonomical identification, showing the highest abundant OTUs belonging to the phylum of *Euryarchaeota* could be assigned to the genera *Haloferax* (OTUs 8, 10, 12, 13, 15, and 17) and *Halogranum* (OTU 5), forming cluster A. The most abundant representative species were phylogenetically related to *Haloferax sulfurifontis*, *Haloferax prahovense*, and *Halogranum gelatinilyticum*. The second cluster B was formed within the phylum *Thaumarchaeota*, whereas OTUs were closely related to *Nitrososphaera viennensis* (OTUs 14 and 18) and *Nitrosopumilus maritimus* (OTU 1). Within cluster B, some OTUs (2, 4, 6, 7, 9, 11, and 16) formed a distinct branch, which was phylogenetically more distantly related to the classified *Thaumarchaeota* species of the RefSeq database. Furthermore, a third cluster, C, could be assigned to the *Euryarchaeota* species *Methanoregula boonei* (OTU 3) and *Methanosphaerula palustris* (OTU 19). A direct comparison of all OTUs revealed that the most abundant OTUs (4, 5, 10, 12, and 17) were present in all samples, forming an archaeal core microbiome (Fig. 3). Samples of *P. commune* (MS2.6) and *V. myrtillus* (MS3.3) showed the highest diversity of OTUs, whereas *S. magellanicum* (MS2.8) and *M. caerulea* (MS3.9) showed the lowest diversity.

The analysis of the archaeal sequences based on the 12 metagenomes revealed as expected a more detailed phylogenetic structure than the 16S rRNA amplicon data set (Fig. 4). Overall, the archaeal community made up 0.2% to 0.7% (842,752 hits) of all prokaryotic abundance (189,394,645 hits). The archaeal phylum *Euryarchaeota* was the dominant group accounting for 85.4% of the whole archaeal community, followed by *Crenarchaeota* (12.3%) and *Thaumarchaeota* (1.6%). *Archaea* belonging to the phylum *Korarchaeota* (0.8%) and *Nanoarchaeota* (0.1%) were less represented. At

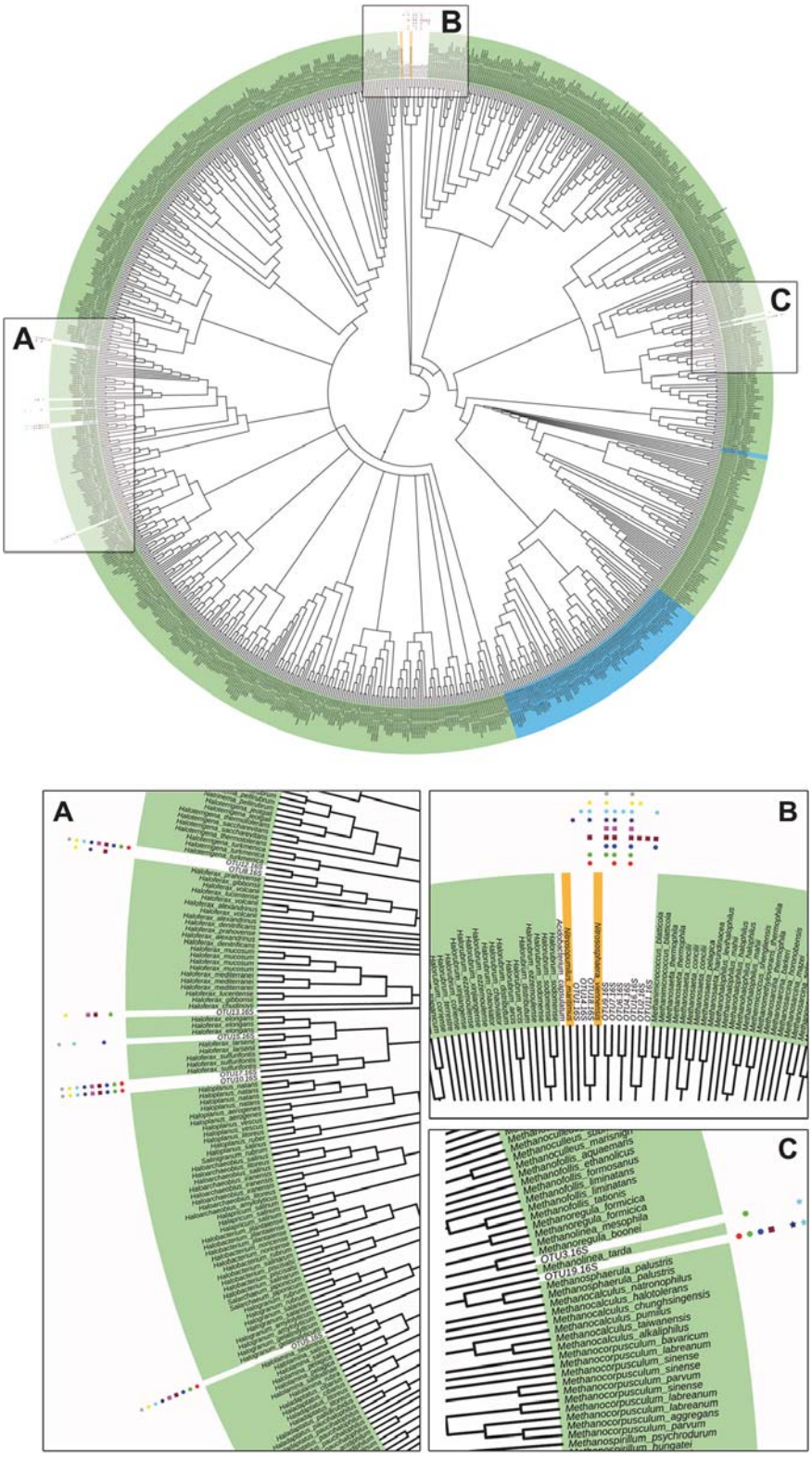

FIG 2 16S rRNA gene sequence-based DNA distance maximum likelihood tree of archaeal OTUs, of *Acidobacterium capsulatum* strain ATCC 51196 (NR_074106.1, outgroup), and the complete archaeal RefSeq database. Based on the neighboring clades, OTUs were taxonomically identified. Three main clusters (A to C) were formed, where the highest occurrence was found in the underrepresented phylum in the RefSeq database of *Euryarchaeota*. Symbols represent occurrence of the OTU, as indicated in the legend to Fig. 3. Green, *Euryarchaeota*; blue, *Crenarchaeota*; orange, *Thaumarchaeota*.

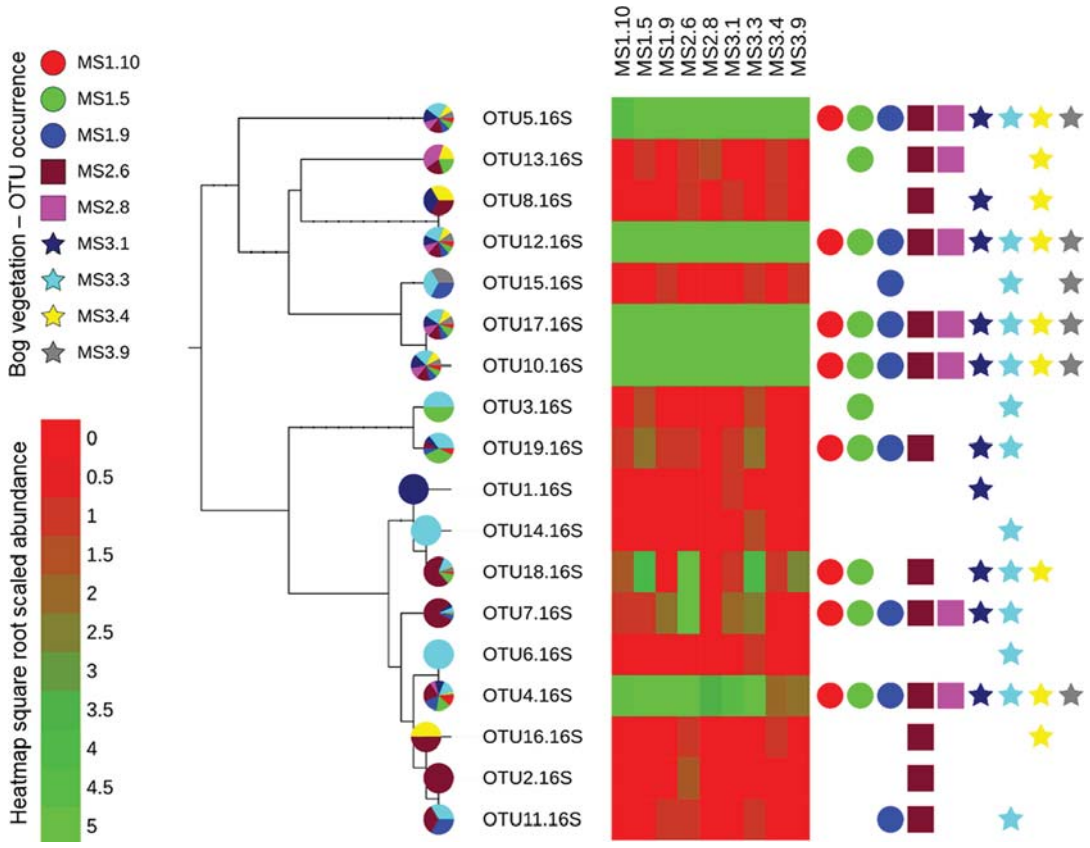

FIG 3 Pruned DNA distance maximum likelihood phylogenetic tree of archaeal 16S rRNA OTUs associated with bog vegetation: *Vaccinium oxycoccus* (MS1.5), *Sphagnum magellanicum* (MS1.9 and MS2.8), *Polytrichum strictum* (MS1.10), *P. commune* (MS2.6), *Eriophorum vaginatum* (MS3.1), *V. myrtilus* (MS3.3), *S. capillifolium* (MS3.4), and *Molinia caerulea* (MS3.9). Phylogenetic relationships are shown for 16S rRNA sequences representing the structure of archaeal OTUs. Pie charts show the OTU proportional distribution between the samples. Symbol charts represent the occurrence. Heat map abundance is based on square root scaled abundance: in order to discriminate between the lower-abundance groups, the upper heat map cap was set to 5.

the class level, the main annotated groups belonged to *Methanomicrobia* (40.6%), *Halobacteria* (16.2%), and *Thermoprotei* (12.3%). In total, 60 different archaeal genera could be determined. The most abundant genera could be identified as *Methanosarcina* (16.6%), *Methanoregula* (5.9%), *Sulfolobus* (3.8%), *Pyrococcus* (3. 5%), and *Thermococcus* (3.4%). In contrast to the 16S rRNA gene data set, *Archaea* of the genus *Haloferax* were less represented (1.2%).

Metagenome-inferred function of *Archaea* associated with bog vegetation. Functional analysis of 12 normalized metagenomes of the bog vegetation resulted in 285,058 archaeal hits, which could be assigned to certain functional subsystems of SEED database. Out of these annotations, a significant number of hits represented primary metabolic functions of *Archaea* (carbohydrates, 21. 6%; central carbohydrate metabolism, 7.6%; amino acids and derivatives, 20.1%; fatty acids, lipids, and isoprenoids, 2.3%; and cofactors, vitamins, prosthetic groups, and pigments, 6.4%). Besides functions of the central carbohydrate metabolism, 1.6% and 2.2% of archaeal functional hits were assigned to the fermentation and one-carbon metabolism subsystem, respectively. Functional signatures of *Archaea* involved in nutrient cycling were found as well, like signatures for CO_2 fixation, which were highly abundant (0.7%). In contrast, the functions assigned to subsystems of nitrogen fixation were detected with less than 0.1%. On the top SEED level, 1% were detected as contributing to nitrogen metabolism. Thereby the most abundant subsystem was found to contribute to ammonia assimilation (0.7%). Interestingly, the number of genetic attributes encoding a stress response was high (2%). Especially the abundance of subsystems involved in oxidative stress

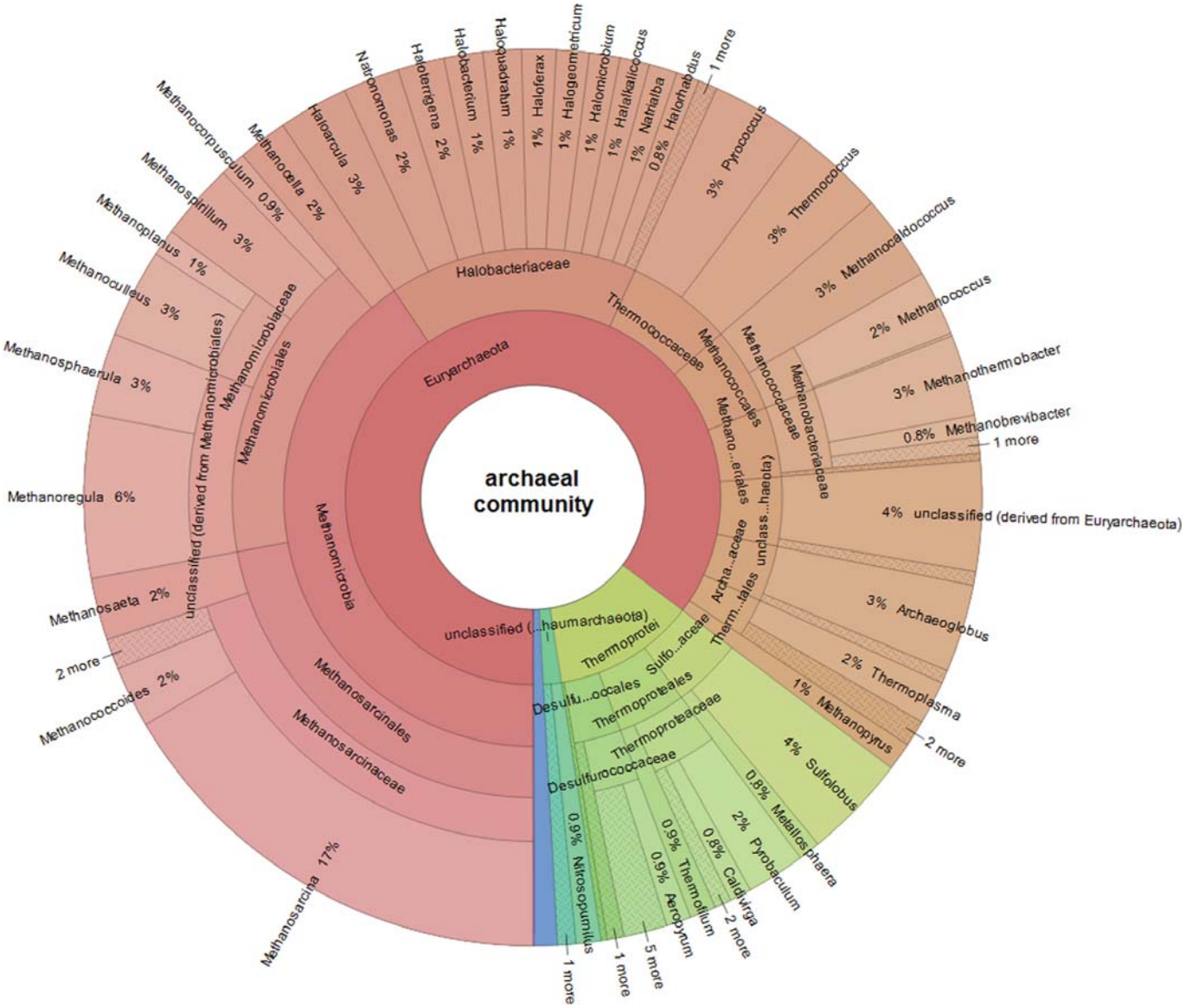

FIG 4 Krona chart representing taxonomic composition of the whole archaeal community associated with bog vegetation, revealed by metagenome sequencing. Abundances of archaeal genera are displayed relative to all sequences assigned to *Archaea* of the whole data set of 12 metagenomes (736,325 sequences). Metagenomes were obtained from 12 different plant species: *Pleurozium schreberi*, *Sphagnum angustifolium*, *Vaccinium myrtilus*, *Calluna vulgaris*, *V. oxycoccus*, *Pinus mugo*, *S. fuscum*, *Andromeda polifolia*, *S. magellanicum*, *Polytrichum strictum*, *Eriophorum vaginatum*, and *Molinia anomala*.

response was at 0.9%. Further subsystems contributing to the archaeome's stability, like attributes involved in DNA repair (2.3%) and osmotic stress (0.3%), were also abundant. In addition, archaeal subsystems involved in motility and chemotaxis (1.4%), like functions assigned to flagellar motility (0.2%), functional signatures for glycogen degradation, which is mainly found in fungi (0.3%), and interestingly subsystems involved in the plant-hormone biosynthesis of auxin (0.7%) were also found (see Tables S2 and S3 in the supplemental material). Nucleotide sequences for genes involved in auxin biosynthesis (EC 2.4.2.18, EC 5.3.1.24, EC 4.2.1.20, and EC 1.4.3.4) were further analyzed by using blastx. The taxonomic distribution of these genes among *Archaea* revealed a domain-wide distribution for EC 2.4.2.18, EC 5.3.1.24, and EC 4.2.1.20 (830, 213, and 877 archaeal hits, respectively), whereas the genes for monoamine oxidase (EC 1.4.3.4) were less represented and mainly found in *Euryarchaeota* (56 archaeal hits).

To further study the plant specificity of *Archaea* and archaeal functions on bog vegetation, the functional distribution among the clades of monocotyledons and

eudicotyledons and the class of *Bryopsida* was analyzed. In total, at the top SEED level, monocotyledons (*Eriophorum vaginatum*) and eudicotyledons (*Vaccinium myrtillus*, *Calluna vulgaris*, *V. oxycoccus*, and *Andromeda polifolia*) showed a similar distribution of abundance of archaeal functions (32% and 29%, respectively). Whereas 39% of all assigned archaeal functions belonged to the *Bryopsida* samples (*Polytrichum strictum*, *Pleurozium schreberi*, *Sphagnum angustifolium*, *S. fuscum*, *S. magellanicum*, and *Mylia anomala*). For the *Bryopsida*, a distinct predominance of attributes involved in subsystems of the regulation and cell signaling, the cell division and cell cycle, phosphorus metabolism, DNA metabolism, and the nucleosides and nucleotides were detected. On SEED level 2, *Bryopsida* showed an increased abundance of archaeal functional groups responsible for the response to osmotic stress and purine metabolism, compared to the two other groups. However, functions of the oxidative stress response, nitrogen metabolism, and especially nitrate and nitrite ammonification were mostly found in monocotyledons. Although eudicotyledons constantly showed a reduced relative abundance of archaeal functions, subsystems assigned to allantoin utilization in nitrogen metabolism were exclusively detected in eudicots. In more detail, on the plant species level, archaeal functions associated with auxin biosynthesis, response to oxidative stress, CO_2 fixation, and DNA repair were most abundant in *Sphagnum fuscum*, *S. magellanicum*, *Pleurozium schreberi*, *Polytrichum strictum*, *Mylia anomala*, and *Eriophorum vaginatum*. In general, in samples of *Vaccinium myrtillus* and *Pinus mugo*, a low abundance of these archaeal signatures was found. Functional signatures involved in glycogen degradation were especially represented in *Sphagnum angustifolium*, *Polytrichum strictum*, and *Sphagnum fuscum*.

The functional composition of *Archaea* in the bog ecosystem was further compared with the composition of bacterial functions (Fig. 5). The distributions of functions within the domains *Archaea* and *Bacteria* were similar, with the most dominant subsystems representing carbohydrate and amino acid metabolism, as the most important biochemical processes, although there were some functions that were represented relatively higher in *Archaea* than in *Bacteria*—like subsystems corresponding to DNA metabolism (DNA repair) and cell wall and capsule. Functional groups belonging to the less dominant subsystems cell division and cell cycle, motility and chemotaxis, and secondary metabolism (auxin biosynthesis) were also relatively more represented by *Archaea*.

Summarizing the results, we developed a model showing the contributions of bacteria and archaea to ecosystem functioning (Fig. 6). Both prokaryotic groups have the potential to interact with plants and are potentially able to protect their host against biotic and abiotic stresses. Moreover, they contribute to the stability of the ecosystem to a certain extent. *Archaea* are found to have the potential to be involved in (i) plant-microbe interaction, (ii) fungus-microbe interaction, (iii) nutrient supply and exchange, (iv) protection against abiotic (especially oxidative and osmotic) stress, and (v) plant secondary metabolite production.

DISCUSSION

Results of this study suggest that it is necessary to integrate *Archaea* into the synergistic concept of host-microbe interaction and bog functioning, which was established for bacteria (25). We could show that *Archaea* are a substantial component of the plant microbiomes and are able to fulfill functions for the host as well as for the ecosystem. *Archaea* are known as "food and survival artists" and for their ability to adapt to chronic energy stress (2, 30, 31). Both facts were confirmed for plant-associated *Archaea*, which were identified as being involved in nutrient supply and exchange. Our novel findings also suggest that they are involved in protection against abiotic stress as well as growth promotion and interact with plants as well as fungi.

In general, plant roots and rhizosphere provide microniches for specific microbial colonization. The anoxic and oxygen-limited conditions allow colonization of *Archaea* in high abundances like methanogenic and ammonium-oxidizing *Archaea* (5, 6). As for bog vegetation, we found these high abundances of *Archaea* filling specific niches in

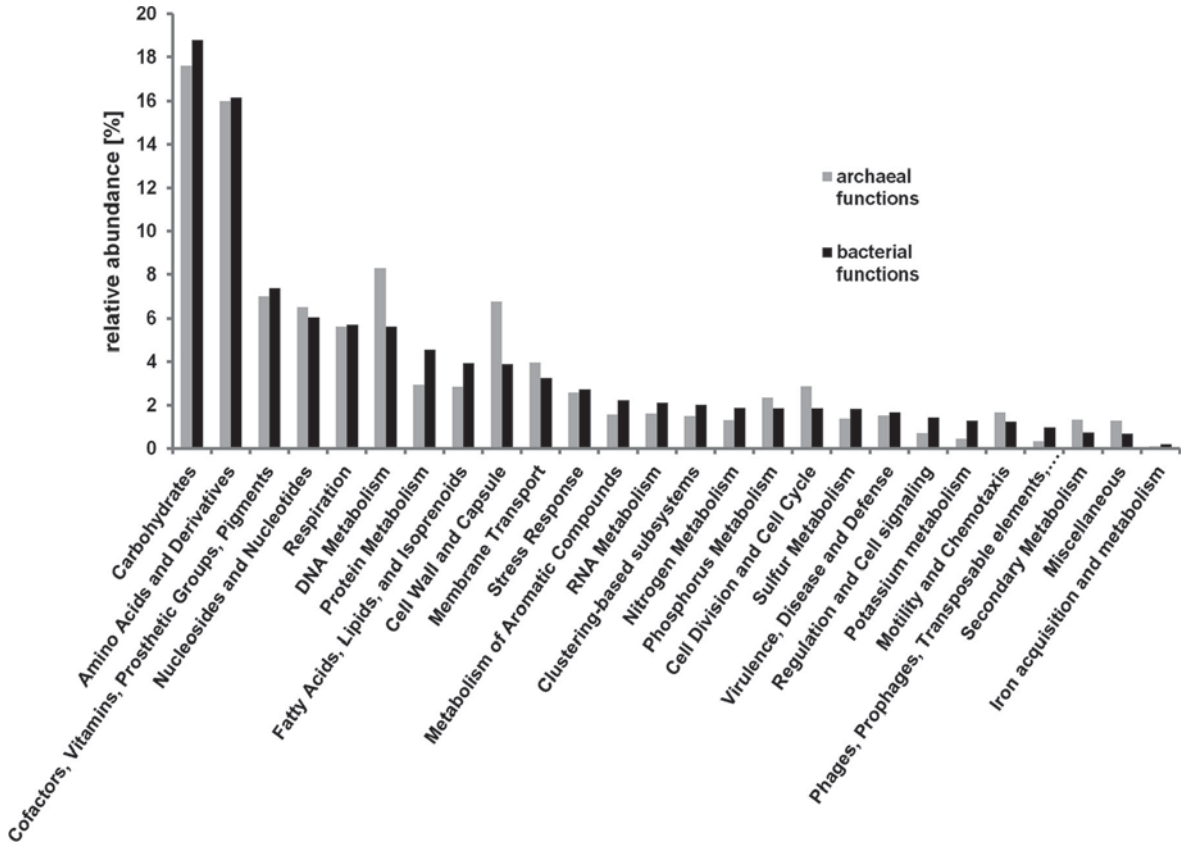

FIG 5 Comparison of functional compositions of archaeal (gray) and bacterial (black) communities associated with bog vegetation: *Pleurozium schreberi*, *Sphagnum angustifolium*, *Vaccinium myrtilus*, *Calluna vulgaris*, *V. oxycoccus*, *Pinus mugo*, *S. fuscum*, *Andromeda polifolia*, *S. magellanicum*, *Polytrichum strictum*, *Eriophorum vaginatum*, and *Molinia anomala*. Functional signatures were obtained from metagenomes, annotated using functional subsystems of SEED database, and processed with MG-RAST. Bar charts represent relative abundance of archaeal and bacterial functions of all functions annotated to *Archaea* (285,058 hits) and *Bacteria* (14,157,480 hits), respectively.

alpine raised bogs. Especially on bog vegetation forming lignified parts like plants of the classes Monocotyledons and Eudicotyledons, such as *Eriophorum vaginatum* and *Vaccinium oxycoccus*, respectively, high archaeal abundances were detected. Furthermore, PCoA clustering of 16S rRNA gene amplicons supported our observations of plant-specific colonization of *Archaea*. Moreover, the results are in accordance with previous findings showing the influence of changes in vegetation structure on the structure of methanogenic archaeal community in peatlands (32). Further, these bog plants were forming deep roots entering a special zone of the bog, the catotelm, which is characterized by anoxic and stable environmental conditions. As *Archaea* are more affected by abiotic than by biotic factors (7, 30), these stable and O_2-free conditions in connection with the plant type might have the greatest influence on archaeal colonization. Similar to rice roots, where mainly *Euryarchaeota* were colonizing the roots (33, 34), the archaeal community of bog vegetation consisted particularly of *Euryarchaeota*, more precisely *Methanosarcina* and *Methanoregula* species revealed by the metagenomic data set. Signatures belonging to *Crenarchaeota*, *Thaumarchaeota*, *Korarchaeota*, and *Nanoarchaeota* were rare. Overall, the diversity in the 16S rRNA gene data set was low compared to the whole metagenomics shotgun-sequencing approach, similar to observations in other motive-based studies of bog ecosystems such as 16S rRNA gene studies of methanogens in boreal peats (35). This issue of greater identification of phyla and genera among the metagenomic sequences compared to the 16S rRNA sequences is known to be due to the databases used and biases in PCR amplification and amplicon sequencing (36). In general, the resolution of current databases for *Archaea* is limited on the genus level, for which reason additional

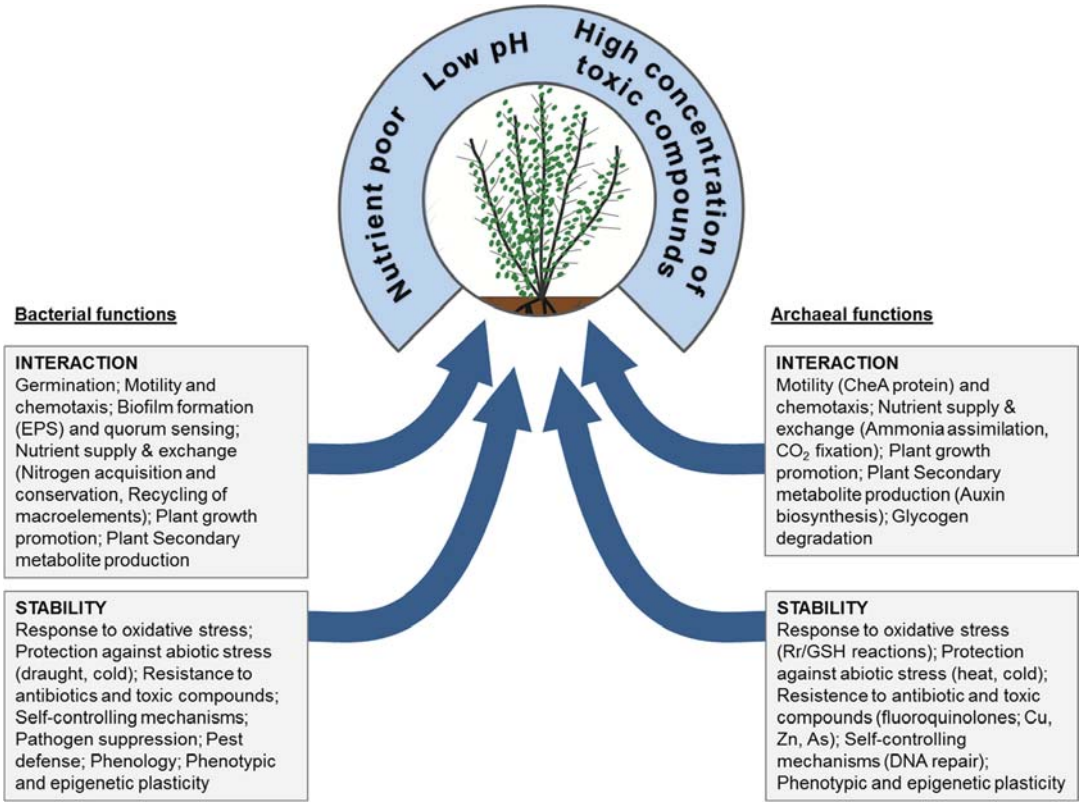

FIG 6 Model of *Archaea* and *Bacteria* contributing to bog functioning. Functions grouped as interaction and stability of the archaeal and bacterial microbiomes were deduced from metagenomic sequences that were annotated using functional subsystems of the SEED database. The examples in parentheses are the most distinctive and differentially abundant genetic signatures. EPS, extracellular polysaccharides; GSH, glutathione; Rr, rubrerythrin.

phylogenetic analyses possess the potential to improve the taxonomical classification by assignment of OTUs against a DNA-based distance matrix. In our study, we found a distinct branch of OTUs belonging to *Thaumarchaeota*, which could not be assigned to classified *Thaumarchaeota* species, based on the RefSeq database. These OTUs might represent bog plant-specific, so far unclassified *Archaea*. Comparatively, the taxonomic structure of the metagenomic and 16S rRNA amplicon data sets showed high homogeneity on the phylum level, whereas the abundances of most dominant taxa on the genus level differed.

Bog ecosystems provide stable but extreme environmental conditions for all (micro)organisms. The interaction of bacteria and the bog vegetation supports the survival of both partners (25). In our study, we found functional signatures of *Archaea* indicating a so far unknown interaction with plants and their importance for the bog ecosystem itself. First, plants in general and especially plants inhabiting open bog ecosystems are highly affected by oxidative stress (37). We found functional signatures involved in the response to oxidative stress for plant-associated *Archaea*, which are known to be evolutionarily adapted to energy stress (30). This adaptation might enable the plant colonization under such extreme conditions and thereby indirectly supporting plant growth by the archaeal capability of nutrient fixation, similar to what was previously shown for bacteria colonizing sphagna (25). Interestingly, the level of genetic attributes encoding general and oxidative stress responses was high, which can be explained by the extreme conditions in the ecosystem. In contrast, osmotic stress response indicates drought stress associated with climate change. Second, we found archaeal functions for direct interaction with the plant. In the metagenomes of the bog vegetation, we detected functional signatures of *Archaea* involved in auxin biosynthesis (Table S2). A more detailed analysis with blastx revealed that the auxin biosynthesis genes coding

for anthranilate phosphoribosyl-transferase, phosphoribosylanthranilate isomerase, and tryptophan synthase alpha and beta chains (EC 2.4.2.18, EC 5.3.1.24, and EC 4.2.1.20, respectively) are widely distributed among archaeal strains as they are also involved in the biosynthesis of amino acids (38). Whereas the gene encoding monoamine oxidase (EC 1.4.3.4), which is directly involved in auxin biosynthesis (39), was exclusively found in some archaeal species of the phylum *Euryarchaeota*. In this study, *Euryarchaeota* dominated the plant-associated archaeome on bog vegetation. Including the previous finding of the occurrence of the auxin biosynthesis genes, this might indicate that especially *Archaea* of the phylum *Euryarchaeota* adapted to the plant hosts. So far, auxin is known as a phytohormone regulating growth processes of the plant, and furthermore, it has been shown to be produced by bacteria (40); this is the first indication of direct interaction of archaeal strains with plants via auxin.

In addition to new aspects of the interaction with plants, we found functional signatures allowing interactions with other organisms (e.g., fungi), as well as relevance for the functioning of the entire bog ecosystem to a certain extent. The detected abundance of archaeal functions for glycogen degradation (Table S2) might give an explanation for previous observations of high abundances of *Archaea* in the mycorrhizosphere (41). Glycogen is a main storage unit of fungi and a part of fungal exudates, which support archaeal colonization (11). With respect to the entire ecosystem, *Archaea* associated with bog vegetation showed a high abundance in metagenome-derived functions associated with CO_2 fixation and ammonium assimilation and thereby present in nutrient cycling of the bog. This is of great importance for the plants in a nutrient-poor environment, such as an ombrotrophic bog. Furthermore, the high number of attributes involved in CO_2 fixation with regard to the main detected archaeal taxa *Methanomicrobia* and *Halobacteriaceae* is supported by previous work on the CO_2 fixation capacity of *Euryarchaeota* (23).

Conclusions. *Archaea* are still an underdetected and little-studied part of the plant microbiome, and their contributions to health or disease remain mostly unknown. Our data provide a first evidence of the importance of *Archaea* as a functional component of the plant microbiome. Under the harsh environmental conditions of the bog ecosystem, *Archaea* contribute to the functioning of the ecosystem and vegetation by performing functions involved in nutrient cycling, stress response, and phytohormone biosynthesis and by interacting with both bacteria and their hosts. These archaeal properties should be further taken into account for microbiome-based treatment of plants in agriculture, especially in sites with extreme conditions, like rice fields and permanent agriculture. More efforts are needed to cultivate plant-associated archaea and to learn more about plant-associated archaeal diversity. Thus, before *Archaea* become part of the "disappearing microbiota" (42), we should at least know if we are going to miss them when they are gone.

MATERIALS AND METHODS

Experimental design and sampling procedure. A microbiome-based analysis of the indigenous alpine peat bog vegetation in northern Styria, Austria, in November 2012 within two geographically distinct peat bogs, Rotmoos and Pürgschachen Moor, was conducted as described earlier (21). The plots for Rotmoos are located as follows: plot 1, N47 41.029 E15 09.284, 695 m; and plot 2, N47 41.059 E15 09.269, 695 m. The plots for Pürgschachen Moor are located as follows: plot 3, N47 34.835 E14 20.390, 632 m; and plot 4, N47 34.815 E14 20.482, 632 m. Both bogs have the typical structure of these ecosystems with an ombrotrophic, strongly acidic, large central part indicated by the dominance of different species of peat mosses (e.g., *Sphagnum magellanicum* and *S. fuscum*). In order to cover these typical ecological conditions, we selected randomly four plots (1 m^2) dominated by *S. magellanicum* Brid. (section *Sphagnum*) in both locations. Frequent accompanied species were *Sphagnum fuscum*, *S. angustifolium*, *S. capillifolium*, *Eriophorum vaginatum*, *Vaccinium myrtillus*, and *V. oxycoccus*. In total, 46 samples of higher plants, bryophytes, and lichens with a minimum required fresh biomass of 10 g per sample were collected from the four selected plots, representing exemplary species naturally growing there. Samples were taken from the oxic catotelm layer and comprise for mosses and lichens the whole organisms and for vascular plants leaves and mainly roots. Our plant-ecology-based strategy focuses on the green and aerobic *Sphagnum*-layer only because the anaerobic part is less important for plant growth. Samples were stored separately in sterile plastic bags and transported on ice to the laboratory. All samples ($n = 46$) were subjected to 16S rRNA gene sequencing; in addition, 12 representative samples

of the most frequently occurring species of bryophytes, vascular plants, and lichens were metagenome shotgun sequenced (Table 1).

Isolation of total community DNA. For DNA isolation, 5 g of sample material was physically disrupted with a sterile pestle and mortar and resuspended in 10 ml of 0.85% NaCl. Total community DNA was extracted from 2-ml aliquots after centrifugation (16,750 × *g* for 20 min at 4°C) using the FastDNA Spin kit for soil (MP Biomedical, Solon, OH). In a deviation from the manufacturer's protocol, pellets were homogenized twice in a FastPrep FP120 instrument (Qbiogene, Inc., Bio 101, Carlsbad, CA) for 30 s at speed 5.0 $m \cdot s^{-1}$.

Illumina sequencing and bioinformatics processing of 16S rRNA gene amplicons. Microbial diversity was investigated targeting the V4 region with the primer pair 515F/806R (43) of the 16S rRNA gene using an Illumina MiSeq v2 platform (LGC genomics, Berlin, Germany). The PCR was conducted in triplicates, purified with the Wizard SV Gel and PCR cleanup system (Promega, Madison, WI), and pooled in equimolar concentrations prior to sequencing. The generated 16S rRNA gene Illumina libraries were subjected to standardized initial quality processing by the sequencing company (LGC genomics, Berlin, Germany). The open source software package Quantitative Insights into Microbial Ecology (QIIME) version 1.8 (44) was used to analyze the reads. At first, the data set was length and quality filtered to remove low-quality sequences, sequences that contained ambiguous characters and homopolymers, and chimeric sequences. The sequences were then clustered into operational taxonomic units (OTUs) with a 97% similarity cutoff (45) with the pick_open_reference_otus.py script using the USEARCH algorithm v6.1544 against the SILVA reference data set version 128 (46). Representative sequences for each OTU were taxonomically assigned using the UCLUST-based consensus classifier with default settings (47). OTUs that were classified to the *Archaea* phylum were filtered from the OTU table and normalized to 1,000 sequences per sample. α and β diversity indices, including rarefaction analysis, observed OTUs, Shannon diversity, Chao1 diversity estimation, and coverage were calculated. Two-dimensional (2D) PCoA plots based on jackknifed β diversity were calculated using weighted UniFrac indices and multiple resampling (1,000 sequences ×100 times) (48). Statistical analyses were done using an Adonis test with 999 permutations. A DNA distance maximum likelihood phylogenetic tree was constructed using the software package Phylip (49). Representative sequences of the archaeal OTUs from the 16S rRNA gene data set were aligned with the complete 16S rRNA gene reference sequence database (RefSeq, NCBI, release 82) prefiltered for *Archaea* using ClustalX version 2.1 (50) and MEGA version 7.0 (51). The maximum likelihood tree was visualized and modified using the interactive Tree of Life platform (iTOL, version 3) (52).

Illumina metagenome sequencing and bioinformatics analysis. Selected total-community DNA samples of the bog vegetation (Table S1) were sent for sequencing to Eurofins MWG Operon (Ebersberg, Germany; http://www.eurofinsgenomics.eu/). The sequencing was performed with an Illumina HiSeq 2500 system. Prior to sequencing, samples of the same species (bryophytes, vascular plants, and lichens) but from different locations were pooled (Table 1; Table S1). Samples MS1.9 and MS4.3, MS1.7 and MS4.4, MS1.1 and MS4.8, MS1.11 and MS4.13, MS1.4 and MS4.1, and MS1.8 and MS4.9 were pooled in equimolar ratios, respectively. The single samples MS1.10, MS1.2, MS1.3, MS1.5, MS1.6, and MS4.5 were pooled in equimolar ratios with the combined samples and sent for sequencing. The functional composition of the microbiome was analyzed using the metagenomic RAST (MG-RAST) server (53). For this purpose, the complete metagenomes were uploaded to the server and initially processed with default parameters filtered for artificial replicate sequences (54), low-quality (61) and short sequences, and sequences containing ambiguous bases. The annotation was done using hierarchical classification with the following default parameters: SEED subsystems as an annotation source, a maximum E value of 10^{-5}, a minimum identity of 60%, and a minimum alignment length of 15 measured in amino acids for protein and base pairs for RNA databases. Within the annotated metagenomes, each single subsystem represented a group of sequences that encode a specific biological process or structural complex as defined by Overbeck et al. (55). The metagenomes were screened for functions annotated to *Archaea* within MG-RAST, and functional hits were subsequently exported for further analysis. The functional hits of each metagenome were normalized to the lowest number of sequences containing predicted proteins with known function (6,785,276). The structure and abundance of the functional subsystems were visualized using metagenome ANalyzer5 (MEGAN) (56) and in the latter compared with the relative distribution of bacterial functions. For further analysis of the distribution of the archaeal genes among the domain *Archaea*, blastx analysis was conducted (57). The taxonomic structure of the archaeal community was aligned and annotated with the RefSeq database as a reference (58). The taxonomic structure was then exported via the MG-RAST API server (59) and further visualized using krona tool version 2.7 (60).

Data availability. The 16S rRNA Illumina libraries obtained from the sequencing company were deposited in the European Nucleotide Archive (ENA) under project no. PRJEB8670 accession no. ERS667879 to ERS667924 and ERS668032 to ERS668033. The complete OTU table was deposited in the Dryad Digital Repository under the accession identifier doi:10.5061/dryad.8n2d5. The complete metagenomes of *Polytrichum strictum* (4550991.3), *Pleurozium schreberi* (4550992.3), *Sphagnum angustifolium* (4550993.3), *Vaccinium myrtillus* (4550994.3), *S. fuscum* (4550995.3), *S. magellanicum* (4550996.3), *Eriophorum vaginatum* (4551107.3), *Calluna vulgaris* (4551108.3), *V. oxycoccos* (4551109.3), *Pinus mugo* (4551110.3), *Andromeda polifolia* (4551111.3), and *Mylia anomala* (4551112.3) are publicly available at the MG-Rast server (https://www.mg-rast.org/linkin.cgi?project=mgp7657) under the corresponding accession numbers.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00122-18.

FIG S1, TIF file, 2.9 MB.

TABLE S1, DOCX file, 0.1 MB.

TABLE S2, DOCX file, 0.1 MB.

TABLE S3, DOCX file, 0.01 MB.

REFERENCES

1. Leininger S, Urich T, Schloter M, Schwark L, Qi J, Nicol GW, Prosser JI, Schuster SC, Schleper C. 2006. *Archaea* predominate among ammonia-oxidizing prokaryotes in soils. Nature 442:806–809. https://doi.org/10.1038/nature04983.
2. Adam PS, Borrel G, Brochier-Armanet C, Gribaldo S. 2017. The growing tree of *Archaea*: new perspectives on their diversity, evolution and ecology. ISME J 11:2407–2425. https://doi.org/10.1038/ismej.2017.122.
3. Probst AJ, Auerbach AK, Moissl-Eichinger C. 2013. *Archaea* on human skin. PLoS One 8:e65388. https://doi.org/10.1371/journal.pone.0065388.
4. Moissl-Eichinger C, Pausan M, Taffner J, Berg G, Bang C, Schmitz RA. 2018. *Archaea* are interactive components of complex microbiomes. Trends Microbiol 26:70–85. https://doi.org/10.1016/j.tim.2017.07.004.
5. Chelius MK, Triplett EW. 2001. The diversity of archaea and bacteria in association with the roots of *Zea mays* L. Microb Ecol 41:252–263. https://doi.org/10.1007/s002480000087.
6. Herrmann M, Saunders AM, Schramm A. 2008. *Archaea* dominate the ammonia-oxidizing community in the rhizosphere of the freshwater macrophyte *Littorella uniflora*. Appl Environ Microbiol 74:3279–3283. https://doi.org/10.1128/AEM.02802-07.
7. Buée M, De Boer W, Martin F, van Overbeek L, Jurkevitch E. 2009. The rhizosphere zoo: an overview of plant-associated communities of microorganisms, including phages, bacteria, archaea and of some of their structuring factors. Plant Soil 321:189–212. https://doi.org/10.1007/s11104-009-9991-3.
8. Berendsen RL, Pieterse CMJ, Bakker PAHM. 2012. The rhizosphere microbiome and plant health. Trends Plant Sci 17:478–486. https://doi.org/10.1016/j.tplants.2012.04.001.
9. Knief C, Delmotte N, Chaffron S, Stark M, Innerebner G, Wassmann R, von Mering C, Vorholt JA. 2012. Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. ISME J 6:1378–1390. https://doi.org/10.1038/ismej.2011.192.
10. Müller H, Berg C, Landa BB, Auerbach A, Moissl-Eichinger C, Berg G. 2015. Plant genotype-specific archaeal and bacterial endophytes but similar *Bacillus* antagonists colonize Mediterranean olive trees. Front Microbiol 6:138. https://doi.org/10.3389/fmicb.2015.00138.
11. Karlsson AE, Johansson T, Bengtson P. 2012. Archaeal abundance in relation to root and fungal exudation rates. FEMS Microbiol Ecol 80:305–311. https://doi.org/10.1111/j.1574-6941.2012.01298.x.
12. Pump J, Pratscher J, Conrad R. 2015. Colonization of rice roots with methanogenic archaea controls photosynthesis-derived methane emission. Environ Microbiol 17:2254–2260. https://doi.org/10.1111/1462-2920.12675.
13. Berg G, Smalla K. 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol Ecol 68:1–13. https://doi.org/10.1111/j.1574-6941.2009.00654.x.
14. Wagner MR, Lundberg DS, Del Rio TG, Tringe SG, Dangl JL, Mitchell-Olds T. 2016. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Nat Commun 7:12151. https://doi.org/10.1038/ncomms12151.
15. Yeoh YK, Dennis PG, Paungfoo-Lonhienne C, Weber L, Brackin R, Ragan MA, Schmidt S, Hugenholtz P. 2017. Evolutionary conservation of a core root microbiome across plant phyla along a tropical soil chronosequence. Nat Commun 8:215. https://doi.org/10.1038/s41467-017-00262-8.
16. Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat EVL, Schulze-Lefert P. 2013. Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol 64:807–838. https://doi.org/10.1146/annurev-arplant-050312-120106.
17. Berg G, Rybakova D, Grube M, Köberl M. 2016. The plant microbiome explored: implications for experimental botany. J Exp Bot 67:995–1002. https://doi.org/10.1093/jxb/erv466.
18. Opelt K, Berg C, Schönmann S, Eberl L, Berg G. 2007. High specificity but contrasting biodiversity of *Sphagnum*-associated bacterial and plant communities in bog ecosystems independent of the geographical region. ISME J 1:502–516. https://doi.org/10.1038/ismej.2007.58.
19. Opelt K, Chobot V, Hadacek F, Schönmann S, Eberl L, Berg G. 2007. Investigations of the structure and function of bacterial communities associated with *Sphagnum* mosses. Environ Microbiol 9:2795–2809. https://doi.org/10.1111/j.1462-2920.2007.01391.x.
20. Bragina A, Berg C, Cardinale M, Shcherbakov A, Chebotar V, Berg G. 2012. *Sphagnum* mosses harbour highly specific bacterial diversity during their whole lifecycle. ISME J 6:802–813. https://doi.org/10.1038/ismej.2011.151.
21. Bragina A, Berg C, Berg G. 2015. The core microbiome bonds the Alpine bog vegetation to a transkingdom metacommunity. Mol Ecol 24:4795–4807. https://doi.org/10.1111/mec.13342.
22. Succow M, Joosten H. 2001. Landschaftsökologische Moorkunde. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart, Germany.
23. Raghoebarsing AA, Smolders AJP, Schmid MC, Rijpstra WIC, Wolters-Arts M, Derksen J, Jetten MSM, Schouten S, Sinninghe Damsté JS, Lamers LPM, Roelofs JGM, Op den Camp HJM, Strous M. 2005. Methanotrophic symbionts provide carbon for photosynthesis in peat bogs. Nature 436:1153–1156. https://doi.org/10.1038/nature03802.
24. Dise NB. 2009. Environmental science. Peatland response to global change. Science 326:810–811. https://doi.org/10.1126/science.1174268.
25. Bragina A, Oberauner-Wappis L, Zachow C, Halwachs B, Thallinger GG, Müller H, Berg G. 2014. The *Sphagnum* microbiome supports bog ecosystem functioning under extreme conditions. Mol Ecol 23:4498–4510. https://doi.org/10.1111/mec.12885.
26. Clymo RS, Turunen J, Tolonen K. 1998. Carbon accumulation in peatland. Oikos 81:368. https://doi.org/10.2307/3547057.
27. Larmola T, Leppänen SM, Tuittila ES, Aarva M, Merilä P, Fritze H, Tiirola M. 2014. Methanotrophy induces nitrogen fixation during peatland development. Proc Natl Acad Sci U S A 111:734–739. https://doi.org/10.1073/pnas.1314284111.
28. Bragina A, Cardinale M, Berg C, Berg G. 2013. Vertical transmission explains the specific *Burkholderia* pattern in *Sphagnum* mosses at multi-geographic scale. Front Microbiol 4:394. https://doi.org/10.3389/fmicb.2013.00394.
29. Francis CA, Beman JM, Kuypers MMM. 2007. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. ISME J 1:19–27. https://doi.org/10.1038/ismej.2007.8.
30. Valentine DL. 2007. Adaptations to energy stress dictate the ecology and evolution of the *Archaea*. Nat Rev Microbiol 5:316–323. https://doi.org/10.1038/nrmicro1619.
31. Cavicchioli R. 2011. *Archaea*—timeline of the third domain. Nat Rev Microbiol 9:51–61. https://doi.org/10.1038/nrmicro2482.
32. Rooney-Varga JN, Giewat MW, Duddleston KN, Chanton JP, Hines ME. 2007. Links between archaeal community structure, vegetation type and methanogenic pathway in Alaskan peatlands. FEMS Microbiol Ecol 60:240–251. https://doi.org/10.1111/j.1574-6941.2007.00278.x.
33. Conrad R, Klose M, Noll M, Kemnitz D, Bodelier PLE. 2008. Soil type links microbial colonization of rice roots to methane emission. Glob Chang Biol 14:657–669. https://doi.org/10.1111/j.1365-2486.2007.01516.x.
34. Edwards J, Johnson C, Santos-Medellín C, Lurie E, Podishetty NK, Bhatnagar S, Eisen JA, Sundaresan V. 2015. Structure, variation, and assembly of the root-associated microbiomes of rice. Proc Natl Acad Sci U S A 112:E911–E920. https://doi.org/10.1073/pnas.1414592112.

35. Galand PE, Fritze H, Conrad R, Yrjälä K. 2005. Pathways for methanogenesis and diversity of methanogenic archaea in three boreal peatland ecosystems. Appl Environ Microbiol 71:2195–2198. https://doi.org/10.1128/AEM.71.4.2195-2198.2005.
36. Poretsky R, Rodriguez-R LM, Luo C, Tsementzi D, Konstantinidis KT. 2014. Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. PLoS One 9:e93827. https://doi.org/10.1371/journal.pone.0093827.
37. Daniels R, Eddy A. 1985. Handbook of European sphagna. Institute of Terrestrial Ecology, Natural Environmental Research Council, Cambrian News, Aberystwyth, United Kingdom.
38. Gory Vert G, Walcher C, Chory J, Nemhauser J. 2008. Integration of auxin and brassinosteroid pathways by auxin response factor 2. Proc Natl Acad Sci U S A 28:9829–9834.
39. Gaweska HM, Taylor AB, Hart PJ, Fitzpatrick PF. 2013. The structure of the flavoprotein tryptophan-2-monooxygenase, a key enzyme in the formation of galls in plants. Biochemistry 52:2620–2626. https://doi.org/10.1021/bi4001563.
40. Spaepen S, Vanderleyden J. 2011. Auxin and plant-microbe interactions. Cold Spring Harb Perspect 3:a001438. https://doi.org/10.1101/cshperspect.a001438.
41. Bomberg M, Jurgens G, Saano A, Sen R, Timonen S. 2003. Nested PCR detection of *Archaea* in defined compartments of pine mycorrhizospheres developed in boreal forest humus microcosms. FEMS Microbiol Ecol 43:163–171. https://doi.org/10.1111/j.1574-6941.2003.tb01055.x.
42. Blaser MJ. 2017. The theory of disappearing microbiota and the epidemics of chronic diseases. Nat Rev Immunol 17:461–463. https://doi.org/10.1038/nri.2017.77.
43. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci U S A 108(Suppl 1):4516–4522. https://doi.org/10.1073/pnas.1000080107.
44. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336. https://doi.org/10.1038/nmeth.f.303.
45. Schloss PD, Handelsman J. 2006. Toward a census of bacteria in soil. PLoS Comput Biol 2:e92. https://doi.org/10.1371/journal.pcbi.0020092.
46. Quast CT, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590–D596. https://doi.org/10.1093/nar/gks1219.
47. Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461. https://doi.org/10.1093/bioinformatics/btq461.
48. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. 2011. UniFrac: an effective distance metric for microbial community comparison. ISME J 5:169–172. https://doi.org/10.1038/ismej.2010.133.
49. Felsenstein J. 1989. Mathematics vs. evolution: mathematical evolutionary theory. Science 246:941–942. https://doi.org/10.1126/science.2464932.941.
50. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948. https://doi.org/10.1093/bioinformatics/btm404.
51. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054.
52. Letunic I, Bork P. 2016. Interactive Tree of Life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res 44:W242–WW245. https://doi.org/10.1093/nar/gkw290.
53. Meyer F, Paarmann D, D'Souza M, Olson R, Glass EM, Kubal M, Paczian T, Rodriguez A, Stevens R, Wilke A, Wilkening J, Edwards RA. 2008. The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 9:386. https://doi.org/10.1186/1471-2105-9-386.
54. Gomez-Alvarez V, Teal TK, Schmidt TM. 2009. Systematic artifacts in metagenomes from complex microbial communities. ISME J 3:1314–1317. https://doi.org/10.1038/ismej.2009.72.
55. Overbeek R, Begley T, Butler RM, Choudhuri JV, Chuang HY, Cohoon M, de Crécy-Lagard V, Diaz N, Disz T, Edwards R, Fonstein M, Frank ED, Gerdes S, Glass EM, Goesmann A, Hanson A, Iwata-Reuyl D, Jensen R, Jamshidi N, Krause L, Kubal M, Larsen N, Linke B, McHardy AC, Meyer F, Neuweger H, Olsen G, Olson R, Osterman A, Portnoy V, Pusch GD, Rodionov DA, Rückert C, Steiner J, Stevens R, Thiele I, Vassieva O, Ye Y, Zagnitko O, Vonstein V. 2005. The subsystems approach to genome annotation and its use in the project to annotate 1,000 genomes. Nucleic Acids Res 33:5691–5702. https://doi.org/10.1093/nar/gki866.
56. Huson DH, Auch AF, Qi J, Schuster SC. 2007. MEGAN analysis of metagenomic data. Genome Res 17:377–386. https://doi.org/10.1101/gr.5969107.
57. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol 215:403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.
58. Pruitt KD, Tatusova T, Maglott DR. 2007. NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. Nucleic Acids Res 35:D61–D65. https://doi.org/10.1093/nar/gkl842.
59. Wilke A, Bischof J, Harrison T, Brettin T, D'Souza M, Gerlach W, Matthews H, Paczian T, Wilkening J, Glass EM, Desai N, Meyer F. 2015. A RESTful API for accessing microbial community data for MG-RAST. PLoS Comp Biol 11:e1004008. https://doi.org/10.1371/journal.pcbi.1004008.
60. Ondov BD, Bergman NH, Phillippy AM. 2011. Interactive metagenomic visualization in a Web browser. BMC Bioinformatics 12:385. https://doi.org/10.1186/1471-2105-12-385.
61. Cox MP, Peterson DA, Biggs PJ. 2010. SolexaQA: at-a-glance quality assessment of Illumina second-generation sequencing data. BMC Bioinformatics 11:485. https://doi.org/10.1186/1471-2105-11-485.

4 Novel insights into plant-associated archaea and their functioning in arugula (*Eruca sativa* Mill.)

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Abstract

A plant's microbiota has various implications for the plant's health and performance; however, the roles of many microbial lineages, particularly *Archaea*, have not been explored in detail. In the present study, analysis of archaea-specific 16S rRNA gene fragments and shotgun-sequenced metagenomes was combined with visualization techniques to obtain the first insights into the archaeome of a common salad plant, arugula (*Eruca sativa* Mill.). The archaeal communities associated with the soil, rhizosphere and phyllosphere were distinct, but a high proportion of community members were shared among all analysed habitats. Soil habitats exhibited the highest diversity of *Archaea*, followed by the rhizosphere and the phyllosphere. The archaeal community was dominated by *Thaumarchaeota* and *Euryarchaeota*, with the most abundant taxa assigned to *Candidatus Nitrosocosmicus*, species of the 'Soil Crenarchaeotic Group' and, interestingly, *Methanosarcina*. Moreover, a large number of archaea-assigned sequences remained unassigned. Overall, analysis of shotgun-sequenced total-community DNA revealed a more diverse archaeome. Differences were evident at the class level and at higher taxonomic resolutions when compared to results from the 16S rRNA gene fragment amplicon library. Functional assessments primarily revealed archaeal genes related to response to stress (especially oxidative stress), CO_2 fixation, and glycogen degradation. Microscopic visualizations of fluorescently labelled archaea in the phyllosphere revealed small scattered colonies, while archaea in the rhizosphere were found to be embedded within large bacterial biofilms. Altogether, *Archaea* were identified as a rather small but niche-specific component of the microbiomes of the widespread leafy green plant arugula.

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Novel insights into plant-associated archaea and their functioning in arugula (*Eruca sativa* Mill.)

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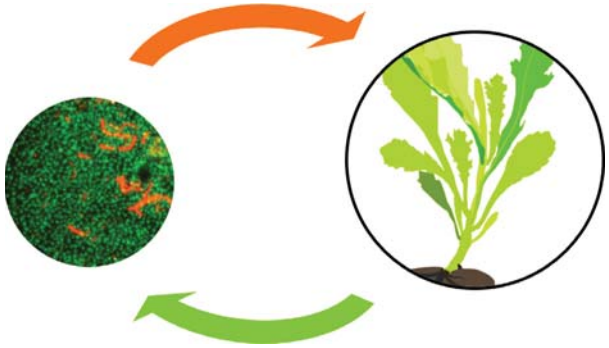
HIGHLIGHTS

- The first insights into archaea associated with a traditional plant are provided.
- Archaea showed habitat-specific colonization of arugula.
- The functional capacities of plant-associated archaea were revealed.
- Indications of archaea-host interactions were found.
- A basis is provided for developments that will benefit plant and human health.

GRAPHICAL ABSTRACT



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Holobiont

ABSTRACT

A plant's microbiota has various implications for the plant's health and performance; however, the roles of many microbial lineages, particularly *Archaea*, have not been explored in detail. In the present study, analysis of archaea-specific 16S rRNA gene fragments and shotgun-sequenced metagenomes was combined with visualization techniques to obtain the first insights into the archaeome of a common salad plant, arugula (*Eruca sativa* Mill.). The archaeal communities associated with the soil, rhizosphere and phyllosphere were distinct, but a high proportion of community members were shared among all analysed habitats. Soil habitats exhibited the highest diversity of *Archaea*, followed by the rhizosphere and the phyllosphere. The archaeal community was dominated by *Thaumarchaeota* and *Euryarchaeota*, with the most abundant taxa assigned to *Candidatus Nitrososcosmicus*, species of the 'Soil Crenarchaeotic Group' and, interestingly, *Methanosarcina*. Moreover, a large number of archaea-assigned sequences remained unassigned at lower taxonomic levels. Overall, analysis of shotgun-sequenced total-community DNA revealed a more diverse archaeome. Differences were evident at the class level and at higher taxonomic resolutions when compared to results from the 16S rRNA gene fragment amplicon library. Functional assessments primarily revealed archaeal genes related to response to stress (especially oxidative stress), CO_2 fixation, and glycogen degradation. Microscopic visualizations of fluorescently labelled archaea in the phyllosphere revealed small scattered colonies, while archaea in the rhizosphere were found to be embedded within large bacterial biofilms. Altogether, *Archaea* were identified as a rather small but niche-specific component of the microbiomes of the widespread leafy green plant arugula.

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Introduction

Leafy green plants have become a central element of a healthy diet, mainly due to their high fibre content but also due to the

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various micronutrients they contain. In modern diets, arugula (*Eruca sativa* Mill.) stands out due to its peppery, pungent taste that stems from various glucosinolates and other sulphur-containing compounds in the edible parts [1]. In addition to their flavour, isothiocyanates that are formed during the degradation of glucosinolates are thought to be involved in cancer prevention [2]. Arugula belongs to the *Brassicaceae* family and is commonly known as rucola (or garden rocket); it originated in the Mediterranean and has been cultivated at least since Roman and ancient Egyptian times [3]. In traditional medicine, arugula is used as a medicinal plant to treat disorders of the digestive system and has several other medical indications as well as aphrodisiac properties [4]. Moreover, various cultivars are broadly accepted in Western cuisine, where they are used in their raw form in various types of salads. Arugula has also been associated with *Salmonella* Thompson outbreaks causing severe illnesses in humans [5]. Therefore, it is important to understand the entire arugula microbiome because its structure, network and function as well as its colonization stability are crucial factors affecting outbreaks and the functioning of the holobiont [6]. To date, various important plant species-specific microbial key players have been identified; however, the focus of most studies is on bacterial and fungal communities. For example, the bacteriome of various *Brassicaceae* plants, including *E. sativa*, was previously identified [7,8]. It was shown that the phyllosphere of arugula harbours higher proportions of antibiotic-resistant bacteria than its rhizosphere and the surrounding soil [8]. However, details related to archaeal communities associated with *Brassicaceae* plants and their functioning still remain largely unknown.

Archaea have been identified as interactive components of complex microbiomes, such as those in the environment or associated with the gastrointestinal tract of animals, the gut and skin of humans and even the rhizosphere and endosphere of plants [9–12]. However, the function of archaea and their structural interactions with their host and other microorganisms remain mostly unclear, mainly due to methodological limitations. On plant hosts, *Archaea* have been found to colonize the rhizosphere and the endosphere at high abundances, whereas the phyllosphere is less colonized [13,14]. These different colonization patterns are influenced by different abiotic conditions but also by biotic factors, such as competition and interactions with bacteria and fungi [11,15]. Recent studies on the natural vegetation of alpine bogs revealed that the plant genotype also influences colonization by *Archaea*. On bog vegetation *Archaea* were further found to have the potential to interact with plants. These potential interactions based on functions such as plant growth promotion through auxin biosynthesis, nutrient supply, and protection against abiotic stress were identified by metagenomic mining [12]. Although factors influencing archaeal functionality in rice roots have been analysed [13], the interactions of archaea with cultivated plants remain mostly unclear. However, due to the ubiquitous occurrence of archaea and their important functions in healthy natural vegetation, *Archaea* presumably play a role in cultivated plants.

The objective of our study was to analyse the colonization patterns of *Archaea* with respect to micro-niche specificity on a widespread leafy green plant and to further increase our understanding of their role and functionality in plants in general. In addition, we aimed to fill gaps in our understanding of the microbiome-host interactions between *Archaea* and plants. Therefore, we analysed the specific archaeal communities of each habitat (soil, rhizosphere, and phyllosphere) of *E. sativa* grown under non-intensive horticultural conditions. Samples were obtained from home gardens (Graz, Austria) and analysed with a complementary approach combining metagenomics and targeted sequencing of the V4 region of the 16S rRNA gene fragment.

Material and methods

Sampling of arugula plants and isolation of total-community DNA

Arugula plants were grown in garden soil (hereafter referred to as bulk soil) in a suburban region of Graz (Austria; approx. 47°4′13″N, 15°28′19″E). Plants were watered by above-ground irrigation with a water hose. Plants were harvested in their final stage of leaf development in July. The plant leaves and their short stalks (edible plant parts) are called the phyllosphere throughout this paper when referring to the microbial habitat. In addition to the phyllosphere samples, rhizosphere samples were collected separately from the same plants, and bulk soil was included as a reference material. For each of the sample types, five equal specimens were obtained. All samples were stored on ice and immediately processed after arrival at a nearby laboratory. To homogenize the samples, 5 g of plant material or bulk soil per replicate was physically disrupted with a sterile mortar and pestle, re-suspended in 10 mL of 0.85% NaCl, transferred into two 2 mL Eppendorf tubes and subsequently centrifuged (16500*g*, 20 min, 4 °C). The obtained pellet was used to isolate the total-community DNA with a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, USA) according to the manufacturer's instructions. All DNA extracts were stored at −80 °C until further processing.

Preparation of the 16S rRNA gene fragment amplicon library for high-throughput sequencing

Community DNA extracted from the soil, rhizosphere and phyllosphere habitats of arugula plants was subjected to PCR-based barcoding. The approach entailed a nested PCR, with the archaea-specific primers 344f and 915r [16] in the first PCR and the modified primer pair S-D-Arch-0349-a-S-17/S-D-Arch-0519-a-A-16 (hereafter 349f/519r) [17] containing an additional 10 bp primer pad (TATGGTAATT/AGTCAGCCAG) and linker (GT/GG) in the subsequent PCR, as previously described in protocols of the Earth Microbiome Project [18]. In a third PCR, the Golay barcodes were annealed. The first PCRs (20 μL) comprised 4 μL of GC buffer (7.5 mM), 2 μL of bovine serum albumin (BSA) (10 mg/mL), 2 μL of dNTP mix (2 mM), 0.25 μL of Phusion polymerase (New England Biolabs, Frankfurt, Germany; 2 U/μL), 9.55 μL of PCR-grade water, and 0.6 μL each of forward and reverse primers (10 μM). Amplifications were conducted with the following settings: 95 °C for 2 min, followed by 10 cycles of 96 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min, 15 cycles of 94 °C for 25 s, 60 °C for 30 s, and 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. The nested PCRs with primers 349f and 519r were executed in the same way as the previous PCR but with different settings: 95 °C for 5 min, followed by 25 cycles of 95 °C for 40 s, 66 °C for 2 min, and 72 °C for 1 min and a final elongation step at 72 °C for 10 min. A final barcode-annealing PCR was conducted to attach sample-specific Golay barcodes to the primer pads on each forward and reverse primer, with the following settings: 95 °C for 2 min, followed by 10 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s and a final elongation step at 72 °C for 10 min. Amplified PCR products were checked by gel electrophoresis after each PCR, and 1 μL of PCR product from the previous PCR was used as a template for the subsequent PCR. All PCRs were conducted in triplicate, purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA), and pooled in equimolar concentrations prior to sequencing. The sequencing was then conducted using an Illumina MiSeq Personal Sequencer (GATC Biotech AG, Konstanz, Germany).

Bioinformatic analyses of archaeal 16S rRNA gene fragments

The generated 16S rRNA gene Illumina library reads were analysed and processed by using the open source software package

Quantitative Insights Into Microbial Ecology (QIIME) release 1.9.1 for pre-processing and pre-filtering, and QIIME2 release 2018.2 [19] was used for further analysis following tutorials provided on the QIIME2 homepage (https://docs.qiime2.org/2018.2/). First, the read quality was checked with FastQC, and barcodes were extracted in QIIME 1.9.1. Then, reads and metadata were imported into QIIME2, in which demultiplexing, denoising of truncated reads, and generation of ribosomal sequence variants (RSVs) were conducted using the DADA2 algorithm [20]. The RSVs were then summarized in a feature table and rarefied to a depth of 1000 RSVs. Feature tables subjected to additional filtering were used to calculate metrics of alpha and beta diversity, including the Shannon diversity index, Faith's phylogenetic diversity, evenness, the Jaccard index and the Bray-Curtis distance, with the QIIME2 core diversity metrics. For phylogenetic analysis, the MAFFT script was used to align representative sequences, and FastTree was used to generate a phylogenetic tree. For taxonomic composition analysis, the taxonomy was assigned to representative sequences by using a customized naïve Bayes classifier trained on 16S rRNA gene OTUs clustered at 97% similarities within the Silva 128 database [21]. In addition, 2D principal coordinate analysis (PCoA) plots were constructed using Emperor weighted and unweighted UniFrac distances. The distribution of taxa among the habitats was visualized with Cytoscape 3.3.0 [22] based on habitat-specific core archaeomes, which were identified by using a frequency threshold of 0.8 (present in 4 out of 5 samples). The most abundant sequences, which were showing a low taxonomic resolution, were further assigned by using nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Archaea-targeting functional metagenomics

Shotgun-sequenced datasets were available from a previous study [8] that utilized arugula plants from the same garden (repository IDs were provided in the respective section). The datasets (phyllosphere, rhizosphere, and bulk soil) were used to explore the plant's bacteriome and the functioning of the enterobacterial subpopulation therein. In the present study, functional and taxonomic analyses were performed on the Metagenomic RAST server (MG-RAST; http://www.mg-rast.org). Quality-filtered reads from HiSeq Illumina runs were uploaded to the server and initially processed with default parameters. The reads were filtered for artificial replicate sequences, low-quality sequences, short sequences, and sequences containing ambiguous bases. The filtered sequences were then annotated using hierarchical classification with default parameters: SEED subsystems as an annotation source, a maximum e-value of 10^{-5}, a minimum identity of 60% and a minimum alignment length of 15 measured in aa for protein and bp for RNA databases. Each subsystem within the metagenomes represents a group of sequences encoding for a specific biological process or a structural complex. Furthermore, the metagenomes were screened for functional signatures annotated to *Archaea* within MG-RAST. The functional hits were subsequently exported and normalized to the lowest number of sequences containing predicted proteins with known function from the soil habitat (8,400,892 sequences). Then, the structure and abundance of the functional signatures were visualized using MEtaGenome ANalyzer (MEGAN) 5 [23]. For taxonomic analysis, the structure of the archaeal community was aligned and annotated with the M5nr database as a reference and exported via the MG-RAST application programming interface (API) server (http://www.mg-rast.org).

In situ visualization of Archaea with confocal laser scanning microscopy

Archaeal colonization of the rhizosphere and phyllosphere samples of *E. sativa* was analysed by fluorescent *in situ* hybridization in combination with confocal laser scanning microscopy (FISH/CLSM). The microscope used for the imaging was a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) equipped with Leica ACS APO 40.0 × oil CS and Leica ACS APO 63 × oil CS oil immersion objective lenses. Plant tissues were fixed with 4% paraformaldehyde and 1x phosphate-buffered saline (3:1) for 6 h at 4 °C. For each habitat, three fixed replicates were analysed. The samples were then stained by in-tube FISH according to Cardinale and colleagues [24]. The FISH probes ARCH344-Cy5, ARCH1060-Cy5 [25], and ARCH915-Cy5 [26] and an equimolar mixture of Cy3-labelled EUB338, EUB338-II, and EUB338-III [27,28] were used to visualize colonization patterns of *Archaea* and bacteria, respectively (max. extinction/emission in nm, Cy3: 548/562 and Cy5: 650/670). As a positive control for visualization of *Archaea*, a culture of *Candidatus Altiarchaeum hamiconexum* was used. To visualize the structure of the plant, Calcofluor white staining was conducted (Sigma Aldrich, St. Louis, USA) using a stationary laser at a 405 nm wavelength. Further maximum projections of optical z-stack slices were used to generate micrographs of archaeal and bacterial colonization.

Repository deposition of next-generation sequencing data

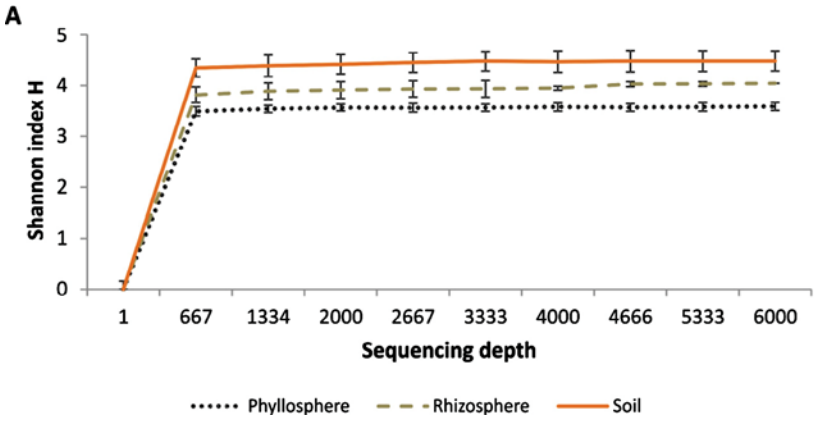
The metagenomes are publicly accessible on the MG-RAST server (http://www.mg-rast.org) under the accession numbers mgm4551355.3 (phyllosphere), mgm4551391.3 (rhizosphere), and mgm4551574.3 (bulk soil). All amplicon sequencing data sets were submitted to the European Nucleotide Archive (ENA) (http://www.ebi.ac.uk/ena) and are accessible under the project accession number PRJEB28404.

Results

Archaeal diversity in arugula plants

Quality-filtering of the 16S rRNA gene fragment dataset of *E. sativa* resulted in 1668 features (RSVs) with a total abundance of 1,040,565. The 16S rRNA amplicon analysis included three habitats: soil (413,055 reads), the rhizosphere (146,847 reads) and the phyllosphere (470,663 reads). Alpha diversity was analysed by phylogenetic and non-phylogenetic based methods; outliers were excluded (n = 4). The highest diversity of *Archaea* was found in the soil habitat (Shannon index (H): 4.44; Faith: 83.06), followed by the rhizosphere (H: 3.95; Faith: 71.63) and the phyllosphere (H: 3.56; Faith: 52.47) (Fig. 1).

Weighted and unweighted PCoA plots with all 15 samples revealed a distinct clustering of archaeal communities belonging to each of the habitats (Fig. 2A and B). In the phyllosphere (77.8%), rhizosphere (73.4%) and soil (74.2%), the archaeal community was dominated by *Thaumarchaeota*. The second most abundant archaeal phylum throughout all habitats was *Euryarchaeota*, totalling 15.1% in the phyllosphere, 20.9% in the rhizosphere, and 15.3% in the soil. *Archaea* assigned to the phyla *Woesearchaeota* (phyllosphere: 0.4%; rhizosphere: 3.0%; soil: 7.1%) and *Bathyarchaeota* (phyllosphere: 0.1%; rhizosphere: 0.2%; soil: 0.1%) were less represented. However, 4.0% of all sequences remained unassigned. Overall, at the genus level, the most abundant feature was assigned to *Candidatus Nitrosocosmicus* (27.8%), as further revealed by BLAST analysis. Other abundant genera were assigned to an uncultured *Thaumarchaeota* strain (14.2%) and to *Methanosarcina thermophila* (8.8%). The most abundant taxa were shared among all three habitats, representing the core archaeome of arugula (Fig. 3). Taxa shared exclusively between two habitats were found only between the soil and the rhizosphere. Unique taxa, i.e., those found exclusively in one habitat, were detected in the



B

Habitat	Shannon index H
Phyllosphere	3.56 ± 0.03
Rhizosphere	3.95 ± 0.07
Soil	4.44 ± 0.05

Fig. 1. Visualization of Shannon index H, as function of sequencing depth of habitats of *E. sativa* (A). The applied method is alpha rarefaction with 10 repeats at 10 different sequencing depths. Displayed habitats are soil (solid line), rhizosphere (dashed line) and phyllosphere (dotted line). Shannon index H values are displayed with their corresponding standard-deviation (B).

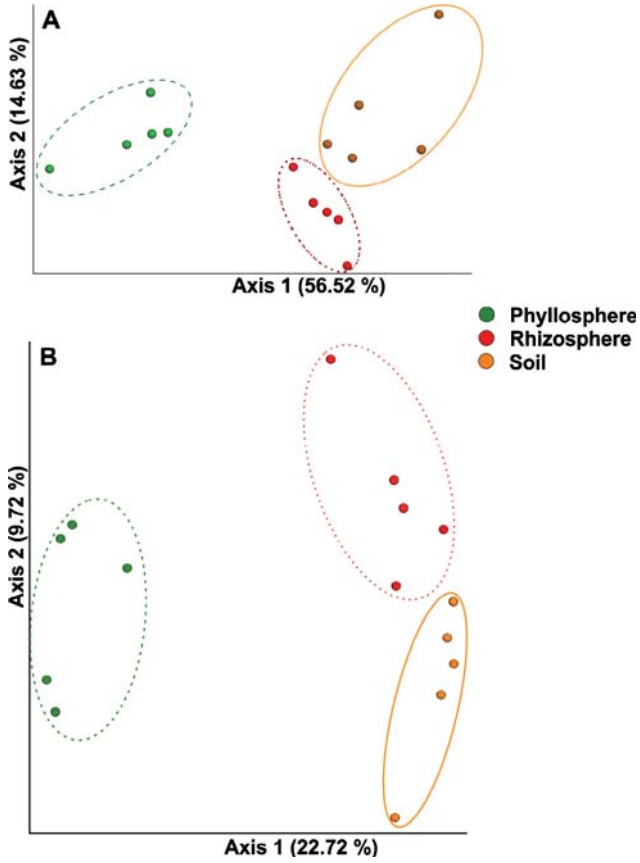


Fig. 2. Comparison of archaeal communities from the soil, rhizosphere and phyllosphere of *Eruca sativa* by principal coordinate analysis (PCoA). Plots were calculated using Emperor weighted UniFrac distances (A) and unweighted UniFrac distances (B). Each dot represents a distinct sample of a habitat: the phyllosphere in green (dashed circle), the rhizosphere in red (dotted circle) and the soil in orange (solid circle). The variation explained by each principal coordinate (PC) is defined on the plot.

soil and (to a lesser extent) in the rhizosphere. All taxa found in the phyllosphere were also present in the other habitats.

The three metagenomes revealed a different taxonomic structure than the 16S rRNA amplicon dataset (Fig. 4). The relative archaeal abundance was highest in the soil, at 0.7% (48,603 sequences) of all prokaryotic sequences (7,396,616 sequences). The relative abundance of *Archaea* was slightly lower in the rhizosphere, at 0.5% (45,140 sequences) of all prokaryotic sequences (9,822,615 sequences). The lowest archaeal abundance was found in the phyllosphere, accounting for 0.1% (5949 sequences) of the total prokaryotic community (8,531,239 sequences). At the phylum level, the distribution of the archaeal community was similar in all habitats, whereas the dominant archaeal group was *Euryarchaeota*, accounting for 67.1–74.5% of all archaea. In contrast to the results from the 16S rRNA gene fragment dataset, *Thaumarchaeota* accounted for only 10.5–15.6% of archaea, followed by the phylum *Crenarchaeota*, which accounted for 13.0–14.8%. At the class level, *Methanomicrobia* and *Halobacteria* were the most abundant taxa, representing 27.0–31.8% and 15.6–18.5% of archaea, respectively, followed by *Thermoprotei* (11.6–12.6%) and unclassified *Thaumarchaeota* (10.5–15.6%). *Thermococci*, *Methanococci*, *Methanobacteria*, *Archaeoglobi* and *Thermoplasmata* were less represented. Similar to the results for the 16S rRNA gene fragment dataset, the relative abundance of unclassified reads was high, ranging from 7.4–8.8% of archaea.

Visualization of archaeal communities of arugula

Archaeal colonization patterns in the phyllosphere and the rhizosphere of *E. sativa* plants was visualized using a FISH/CLSM approach (Fig. 5). In the analysed phyllosphere samples, small archaeal colonies were spatially distant from each other, mainly forming colonies in close proximity to plant stomata (Fig. 5A). The colonies were clearly separated from each other and mostly consisted of fewer than 100 individual cells. In contrast, larger colonies were found in the rhizosphere (Fig. 5B). These colonies were also found to be embedded within large bacterial biofilms. Archaeal colonies were mainly found on lignified plant parts and especially on rotten roots. Bacteria that were labelled with a different fluorescent dye were visualized with the same approach. These bacteria often co-localized with archaeal colonies in the rhizosphere but not in the phyllosphere and were predominant in the plant samples.

Metagenome-based functional analysis of Archaea associated with arugula

From the three normalized metagenomes, functional analysis resulted in 5804 archaeal sequences. These sequences were assigned to certain functional subsystems of the SEED database (Table 1). Most of the sequences were assigned to primary metabolic functions of *Archaea*, such as carbohydrates (4161 hits; 71.7%), including central carbohydrate metabolism (1706 hits;

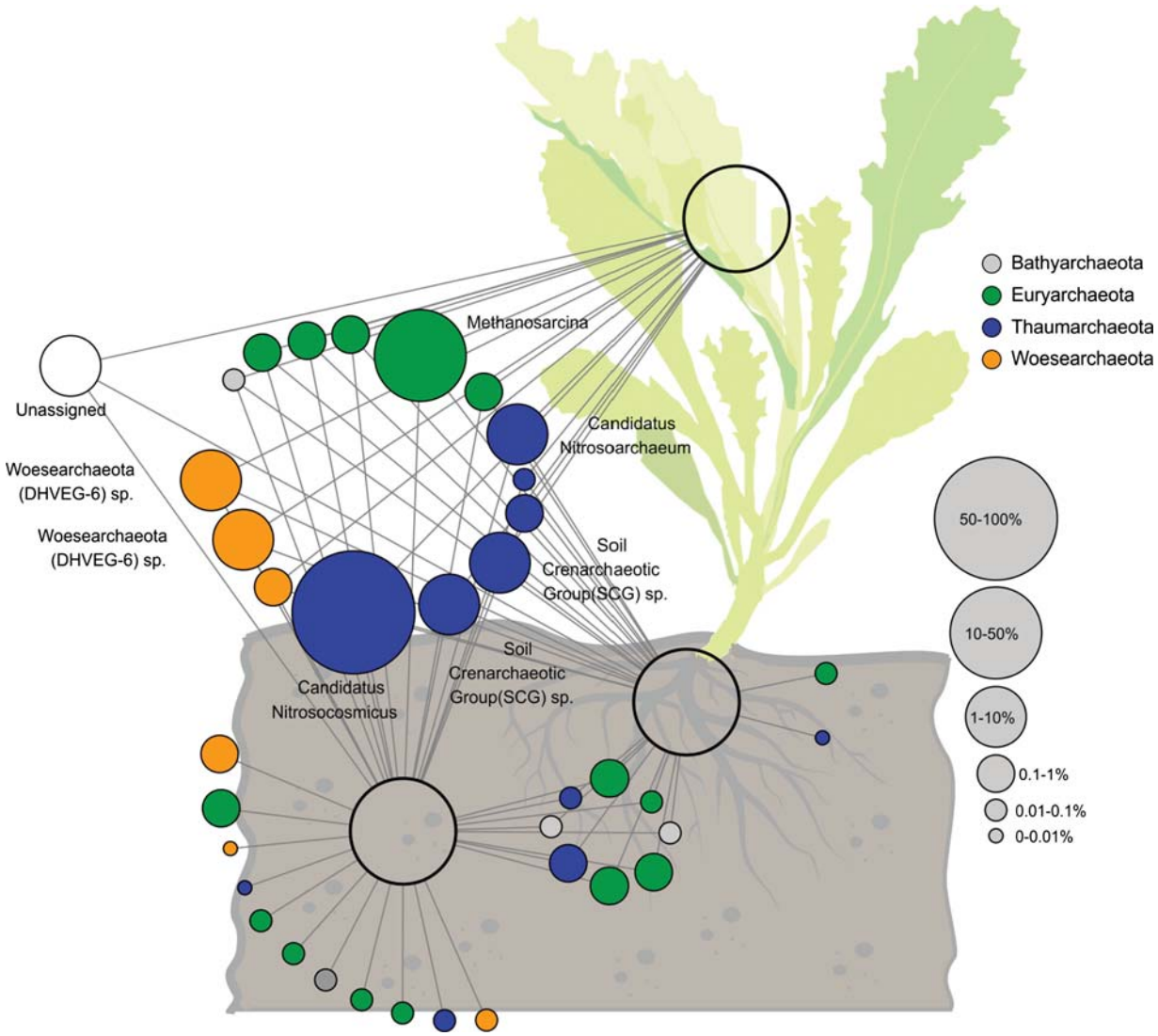


Fig. 3. Feature network of the plant's archaeal communities at the genus level, based on 16S rRNA gene fragment datasets. The datasets were obtained from the soil, rhizosphere and phyllosphere habitats of *Eruca sativa*. For each habitat, a core archaeome was identified with a frequency threshold of 0.8 (4 out of 5 samples). Archaeal phyla are indicated by coloured bubbles: *Bathyarchaeota* in grey; *Euryarchaeota* in green; *Thaumarchaeota* in blue; and *Woesearchaeota* in orange. The size of the bubble represents the relative abundance of the archaeal taxa throughout all habitats.

29.4%) and polysaccharides (2115 hits; 36.4%), and cofactors, vitamins, prosthetic groups and pigments (621 hits; 10.7%). Functions involved in one-carbon metabolism (657 hits; 11.3%) and fermentation (21 hits; 0.4%) were also found. Furthermore, archaeal functions were assigned to subsystems involved in nutrient cycling, such as functional signatures for CO_2 fixation (400 hits; 6.9%), whereas signatures for nitrogen fixation were not detected. Functions assigned to cofactors, vitamins, prosthetic groups and pigments were mainly involved in the pyrimidine deaminase pathway (535 hits; 9.2%). A high proportion of archaeal functions were also assigned to subsystems involved in glycogen degradation (1022 hits; 17.6%) and DNA metabolism, especially DNA replication (472 hits; 8.1%). In contrast, functions involved in RNA metabolism were less represented (86 hits; 1.5%). In addition, functional signatures involved in response to stress, especially oxidative stress (17 hits; 0.3%), and signatures involved in protein degradation (215 hits; 3.7%) were also found.

Furthermore, the habitat specificity of archaeal functions in *E. sativa* was analysed. To do so, the functional distributions of the normalized metagenomes were compared among the habitats. In general, most assigned functions belonged to the soil habitat (48.8%), followed by the rhizosphere (36.2%) and the phyllosphere (15.0%). Functional signatures involved in glycogen degradation, and amino acids and derivatives were found at a higher relative abundance in the soil than in the other habitats, with representations of 40.6%, and 4.0%, respectively (Fig. 6). Additional functions involved in CO_2 fixation and DNA replication were similarly distributed in the soil and the rhizosphere with a representation of 7.4% and 8.8% in the soil and 7.7% and 9.0% in the rhizosphere, respectively, whereas the relative abundance of these functions in the phyllosphere was below 3.7%. Functions involved in fermentation were not found in the phyllosphere. Further, functions involved in stress response and oxidative stress were represented in low relative abundance in the phyllosphere (0.2%), compared to the soil (0.4%) and the rhizosphere (0.2%). The only exceptions included signatures assigned to one-carbon metabolism, more precisely the serine-glyoxylate cycle, the TCA cycle, and the biosynthesis of riboflavin, flavin mononucleotide (FMN) and flavin

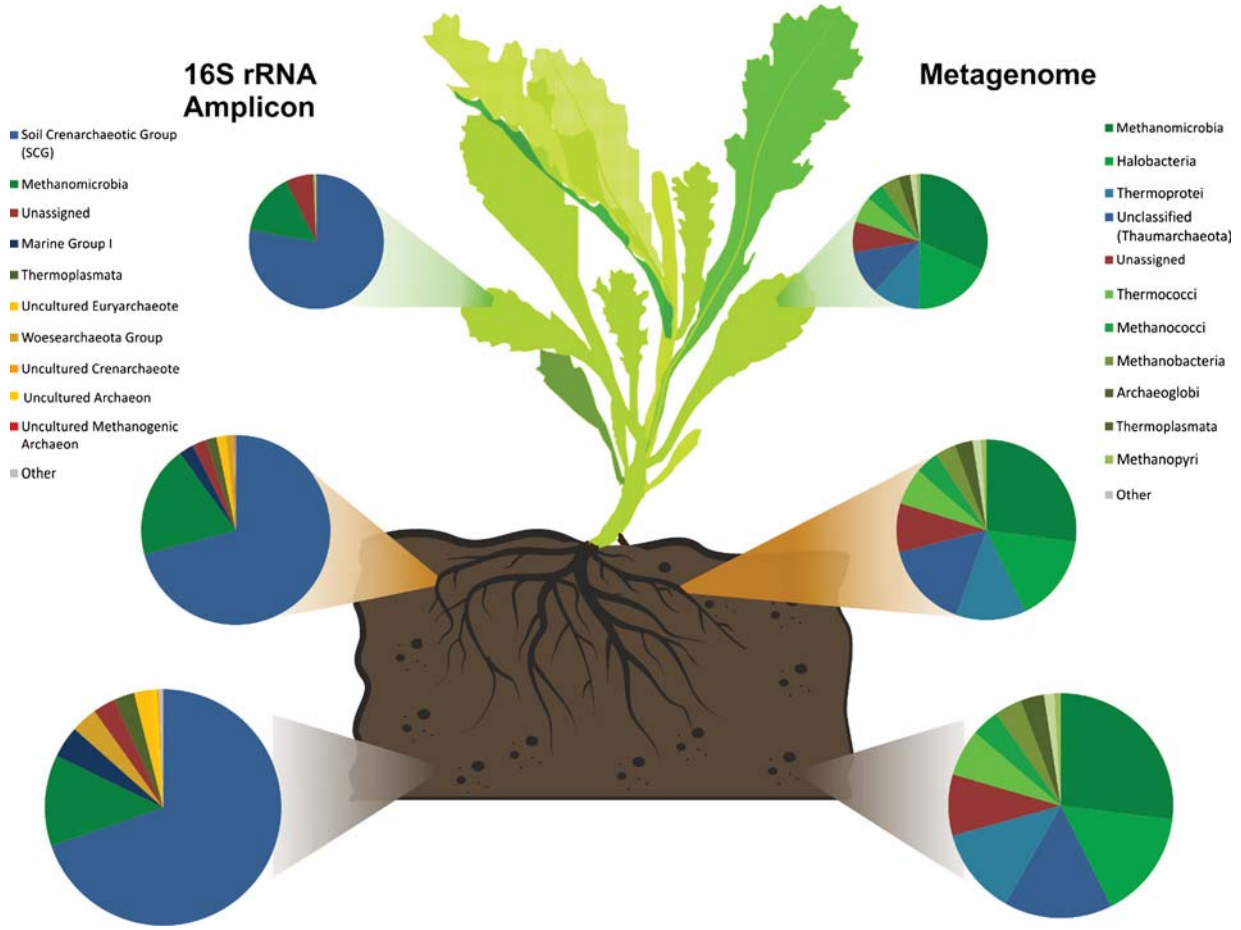


Fig. 4. Taxonomic composition of archaeal communities of *Eruca sativa* revealed by 16S rRNA amplicon and shotgun sequencing-based metagenomics analysis. The archaeal community is described at the class level for each habitat: soil, rhizosphere and phyllosphere. The abundances of archaeal genera are displayed relative to all sequences assigned to *Archaea* in the metagenomics dataset (soil: 48,603 sequences; rhizosphere: 45,140 sequences; phyllosphere: 5949 sequences) as well as relative to all sequences assigned to the 16S rRNA gene fragment dataset (soil: 82,611 sequences; rhizosphere: 31,369 sequences; phyllosphere: 94,133 sequences).

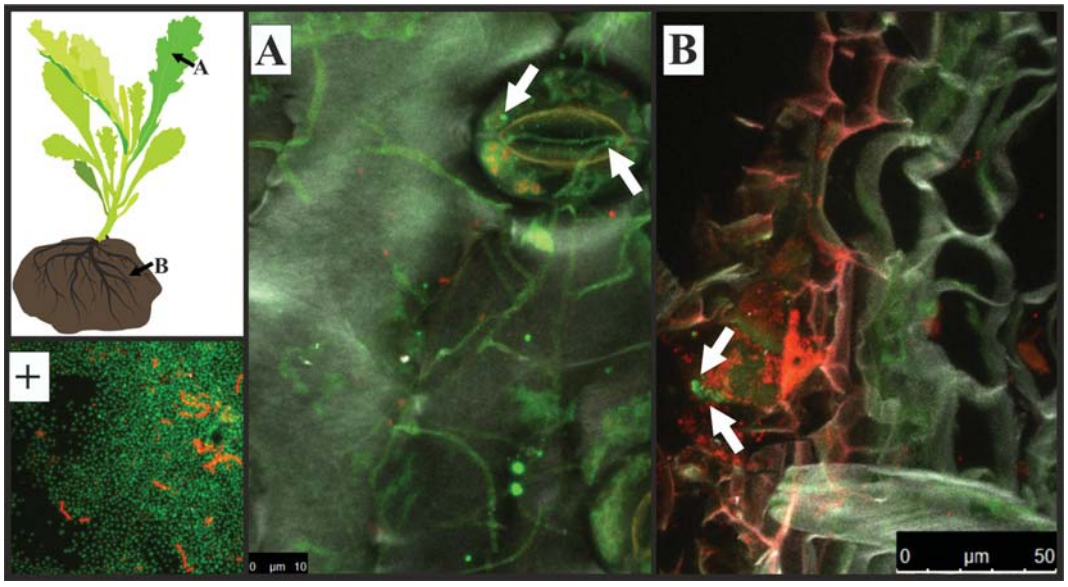


Fig. 5. FISH/CLSM visualization of archaeal colonization patterns in the phyllosphere (A) and rhizosphere (B) of *Eruca sativa*. *Archaea* were stained with the fluorochrome Cy5 and are shown in green and highlighted with white arrows. For better contrast, bacteria were stained with the fluorochrome Cy3 and are shown in red. To visualize the structure of the plant, Calcofluor white staining was conducted. As a positive control for visualization of *Archaea*, a culture of *Candidatus Altiarchaeon hamiconexum* was used (+).

Table 1
List of functional signatures of *Archaea* associated with *E. sativa*. Functional signatures were obtained from three metagenomes of the habitats soil, rhizosphere and phyllosphere of *E. sativa*, annotated using functional subsystems of SEED database, processed with MG-Rast. Total abundances of each signature are separately shown for each habitat.

SEED Level				Habitat		
SEED L1	SEED L2	SEED L3	SEED L4	Soil	Rhizosphere	Phyllosphere
Total archaeal functional hits				**2831**	**2102**	**871**
Carbohydrates	Central carbohydrate metabolism			**833**	**626**	**247**
		Pyruvate metabolism I: anaplerotic reactions, PEP	Phosphoenolpyruvate carboxylase, archaeal (EC 4.1.1.31)	14	7	0
		Glycolate, glyoxylate interconversions	Phosphoglycolate phosphatase, archaeal type (EC 3.1.3.18)	19	9	1
		TCA Cycle	Archaeal succinyl-CoA ligase [ADP-forming] alpha chain (EC 6.2.1.5)	10	10	2
		TCA Cycle	Archaeal succinyl-CoA ligase [ADP-forming] beta chain (EC 6.2.1.5)	27	17	1
		TCA Cycle	Putative malate dehydrogenase (EC 1.1.1.37), similar to archaeal MJ1425	112	116	89
		Glycolysis and Gluconeogenesis,	2,3-bisphosphoglycerate-independent phosphoglycerate mutase, archaeal type (EC 5.4.2.1)	410	276	120
		Glycolysis and Gluconeogenesis,	Fructose-1,6-bisphosphatase, type V, archaeal (EC 3.1.3.11)	98	87	16
		Glycolysis and Gluconeogenesis,	Fructose-bisphosphate aldolase, archaeal class I (EC 4.1.2.13)	20	9	2
		Glycolysis and Gluconeogenesis,	Glucose-6-phosphate isomerase, archaeal (EC 5.3.1.9)	11	20	4
		Glycolysis and Gluconeogenesis,	NAD(P)-dependent glyceraldehyde 3-phosphate dehydrogenase archaeal (EC 1.2.1.59)	112	75	12
		Entner-Doudoroff Pathway	2,3-bisphosphoglycerate-independent phosphoglycerate mutase, archaeal type (EC 5.4.2.1)	410	276	120
		Glycolysis and Gluconeogenesis	Fructose-bisphosphate aldolase, archaeal class I (EC 4.1.2.13)	20	9	2
	One-carbon Metabolism			**169**	**243**	**245**
		Serine-glyoxylate cycle	Putative malate dehydrogenase (EC 1.1.1.37), similar to archaeal MJ1425	112	116	89
		Serine-glyoxylate cycle	Serine-pyruvate aminotransferase/archaeal aspartate aminotransferase	57	127	156
	Polysaccharides			**1134**	**752**	**229**
		Glycogen metabolism	Glycogen branching enzyme, GH-57-type, archaeal (EC 2.4.1.18)	541	400	152
		Glycogen metabolism	Putative glycogen debranching enzyme, archaeal type, TIGR01561	593	352	77
	CO2 fixation			**210**	**162**	**28**
		Calvin-Benson cycle	Fructose-1,6-bisphosphatase, type V, archaeal (EC 3.1.3.11)	98	87	16
		Calvin-Benson cycle	NAD(P)-dependent glyceraldehyde 3-phosphate dehydrogenase archaeal (EC 1.2.1.59)	112	75	12
	Fermentation			**14**	**7**	**0**
		Fermentations: Mixed acid	Phosphoenolpyruvate carboxylase, archaeal (EC 4.1.1.31)	14	7	0
Stress Response	Oxidative stress			**10**	**5**	**2**
		Glutathione: Biosynthesis and gamma-glutamyl cycle	Glutamate–cysteine ligase archaeal (EC 6.3.2.2)	10	5	2
Protein Metabolism	Protein degradation			**110**	**87**	**18**
		Proteasome archaeal	Proteasome subunit alpha (EC 3.4.25.1), archaeal	30	27	6
		Proteasome archaeal	Proteasome subunit beta (EC 3.4.25.1), archaeal	63	50	6
		Proteasome archaeal	Proteasome-activating AAA-ATPase (PAN), archaeal	17	10	6
RNA Metabolism				**46**	**36**	**4**
	RNA processing and modification	tRNA nucleotidyltransferase	tRNA nucleotidyltransferase, archaeal type (EC 2.7.7.21) (EC 2.7.7.25)	24	23	2
	Transcription	RNA polymerase archaeal initiation factors	Archaeal transcription factor E	22	13	2
DNA Metabolism				**250**	**190**	**32**
	DNA replication	DNA replication, archaeal	Archaeal DNA polymerase I (EC 2.7.7.7)	106	72	11
		DNA replication, archaeal	Archaeal DNA polymerase II large subunit (EC 2.7.7.7)	113	94	16
		DNA replication, archaeal	Archaeal DNA polymerase II small subunit (EC 2.7.7.7)	31	24	5

(*continued on next page*)

Table 1 (*continued*)

SEED Level				Habitat		
SEED L1	SEED L2	SEED L3	SEED L4	Soil	Rhizosphere	Phyllosphere
				258	**200**	**163**
Cofactors, Vitamins, Prosthetic Groups, Pigments	Coenzyme A	Coenzyme A Biosynthesis	Dephospho-CoA kinase archaeal, predicted (EC 2.7.1.24)	10	4	2
		Coenzyme A Biosynthesis	Pantoate kinase, archaeal (EC 2.7.1.-)	6	2	1
		Coenzyme A Biosynthesis	Phosphopantothenate synthetase, archaeal	34	21	4
	Riboflavin, FMN, FAD	Riboflavin, FMN and FAD metabolism	CTP-dependent archaeal riboflavin kinase	1	1	0
		Riboflavin, FMN and FAD metabolism	Pyrimidine deaminase archaeal predicted (EC 3.5.4.26)	207	172	156
Miscellaneous	Miscellaneous	Peptidyl-tRNA hydrolase, archaeal type (EC 3.1.1.29)	Peptidyl-tRNA hydrolase, archaeal type (EC 3.1.1.29)	20	14	12
Amino Acids and Derivatives	Methionine	Methionine Biosynthesis	Archaeal S-adenosylmethionine synthetase (EC 2.5.1.6)	113	65	8

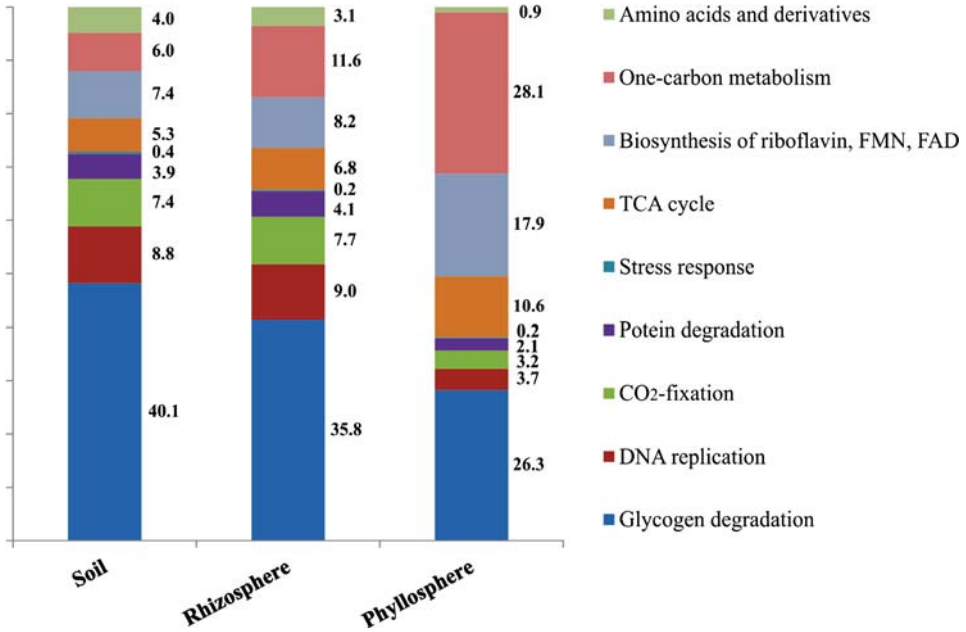


Fig. 6. Comparison of the relative distributions of specific archaeal functions in the soil, rhizosphere and phyllosphere of *Eruca sativa* based on metagenomics datasets. Abundances of the functional signatures are shown as proportion of all functions assigned to *Archaea* in the metagenomics dataset of the corresponding habitat (soil: 2831 total hits; rhizosphere: 2102 total hits; phyllosphere: total 871 hits). The values next to distinct segments indicate their respective percentages in the archaeal fraction.

adenine dinucleotide (FAD), which were more represented in the phyllosphere (28.1%, 10.6%, and 17.9%) than in the soil (6.0%, 5.3%, and 7.4%) and rhizosphere (11.6%, 6.8%, and 8.2%).

Discussion

In the present study, we found various indicators for *Archaea* to be important components of the microbiomes of plants domesticated by humans. In *E. sativa*, *Archaea* exhibited a habitat-specific structure, colonization patterns and functions. Similar to the ways in which biotic and abiotic factors shape bacterial and fungal communities, the archaeome is likely also affected by its environment. Each plant habitat is affected by different environmental conditions, such as low nutrient availability and exposure to environmental changes in the phyllosphere; the availability of root exudates in the plant rhizosphere; and the more stable conditions in the soil. These conditions might be among the factors influencing the habitat-specific diversity of *Archaea*. In the present study, the highest archaeal diversity was found in soil samples, and the lowest diversity was found in the phyllosphere. Furthermore, the composition of the archaeal community at the phylum and class levels was similar among the habitats, which was also observed in the metagenomics analysis. However, the predominant lineages in the metagenomics dataset, namely, *Euryarchaeota* and *Thaumarchaeota*, were inverted in the 16S rRNA gene fragment dataset. Overall, the metagenomics shotgun-sequencing approach revealed a more diverse taxonomy than the 16S rRNA gene fragment amplicons. This bias was described previously and can occur due to differences in database entries and errors during PCR amplification and amplicon sequencing [29]. In general metagenomic sequencing reveals a higher richness than the 16S rRNA approach, whereat the 16S rRNA approach additionaly misses 10% of yet uncharacterized *Archaea*, showing the limitations of the accurate identification of microbes within a microbiome [30,31].

An in-depth analysis of the 16S rRNA gene fragment dataset with a feature network highlighted the habitat-specific colonization of plants by *Archaea*. Soil samples exhibited the greatest number of habitat-specific features. However, the rhizosphere also harboured unique features, whereas the phyllosphere had no unique features. Overall, a large core archaeome was shared among

the habitats, with the most abundant taxa assigned to *Candidatus Nitrosocosmicus*, a member of the ammonium-oxidizing *Archaea* (AOA) that plays an important role in nitrification processes and is expected to possess key genes associated with protection from abiotic stress [32]. *Archaea* assigned to *Methanosarcina*, *Candidatus Nitrosoarchaeum* and *Woesearchaeota* were also abundant in the core archaeome. These lineages were previously found in animal digestive tracts, sediments, and the human gut, respectively [33–35]. Methanogens are strict anaerobes; therefore, the detection of *Methanosarcina* in the phyllosphere might be explained by anaerobic niches in the phyllosphere or, more likely, by contact between the plant and animals, as arugula was grown under field conditions [36]. The visualization of archaeal colonization of *E. sativa* revealed a habitat-specific distribution of the overall population. In the phyllosphere, we found small scattered colonies, whereas in the rhizosphere, *Archaea* formed larger colonies and colonies in close proximity to or even within bacterial biofilms without any obvious zone of inhibition. No negative interactions between archaea and bacteria have been observed to date, suggesting mostly synergistic relationships between the two groups [37]. Moreover, *Archaea* were found to accumulate in nutrient-rich hotspots such as rotten roots, indicating that they play a direct or indirect role in decomposition processes. In Finnish forests, *Archaea* and *Thaumarchaeota* were previously found to be active components of the decaying wood microbiota [38].

The arugula archaeome harboured specific archaeal functions mainly assigned to central carbohydrate metabolism and polysaccharides. We also found functional signatures involved in nutrient cycling such as CO_2 fixation but no signatures involved in nitrogen fixation, although *E. sativa* was mainly colonized by AOA. These findings are in accordance with our previous study on *Archaea* associated with bog vegetation [12]. Arugula has low nutrient requirements, therefore we hypothesize that archaea are not selected by arugula in order to complement the nitrogen balance of the holobiont. In the current study, we also detected functions involved in glycogen degradation, even at higher relative abundances. Glycogen is used by fungi as a main storage unit and is also excreted by them as part of common exudates. This relationship indicates potential interactions with fungi, as fungal exudation rates and fungal colonization were previously shown to be correlated with archaeal abundance [39]. Functions involved in response to stress, especially oxidative stress, were less represented, which might be due to the specific micro-environments of arugula examined. The highest abundances of functional hits for *Archaea* were found in the soil and rhizosphere, whereas the phyllosphere was relatively low in sequences corresponding to archaeal functions. This discrepancy indicates that soil and the rhizosphere are the preferred habitats of *Archaea* and the habitats with the highest archaeal metabolic activity. Functions that were relatively more abundant in the phyllosphere were involved in the serine-glyoxylate cycle and assigned to "serine-pyruvate aminotransferase/archaeal aspartate aminotransferase" (EC 2.6.1.51) and "putative malate dehydrogenase" (EC 1.1.1.37). "Serine-pyruvate aminotransferase" is involved in the glyoxylate cycle, which enables the utilization of simple carbon sources when complex and energetically more valuable carbon sources (*e.g.*, glucose) are absent [40], as is the case in the phyllosphere.

Conclusions

Archaea might show less functional adaptation to agricultural plants such as *E. sativa* than to their wild ancestors due to differences in genotype and the environment. These differences include altered nutrient and energy levels in the soil caused by introducing fertilizers and the accompanying phenotypic changes of plants. Since *Archaea* are adapted to energy deficiency, stress and energy limitations, they might lose their advantage over bacteria in terms of environmental tolerance and subsequently be outcompeted by bacteria, which focus on exploiting energy-rich resources. In summary, *Archaea* are small but potentially important niche-specific components of plant microbiomes, and therefore, we must advance our understanding of plant-associated *Archaea* before they disappear [12] due to our agricultural practices.

Conflict of interest

The authors declare no conflicts of interest.

Compliance with Ethics Requirement

This article does not contain any studies with human or animal subjects.

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References

[1] Piślewska-Bednarek M, Nakano RT, Hiruma K, Pastorczyk M, Sanchez-Vallet A, Singkaravanit-Ogawa S, et al. Glutathione Transferase U13 functions in pathogen-triggered Glucosinolate metabolism. Plant Physiol 2018;176:538–51.
[2] Halkier BA, Gershenzon J. Biology and bioechmistry of glucosinolates. Annu Rev Plant Biol 2006;57:303–33.
[3] Zeven A, Wet J De. Dictionary of cultivated plants and their regions of diversity: excluding most ornamentals, forest trees and lower plants. Centre for Agricultural Publishing and Documentation; 1982.
[4] Garg G, Sharma V. *Eruca sativa* (L.): botanical description, crop improvement, and medicinal properties. J Herbs Spices Med Plants 2014;20:171–82.
[5] Nygård K, Lassen J, Vold L, Andersson Y, Fisher I, Löfdahl S, et al. Outbreak of *Salmonella* Thompson infections linked to imported rucola lettuce. Foodborne Pathog Dis 2008;5:165–73.
[6] Erlacher A, Cardinale M, Grosch R, Grube M, Berg G. The impact of the pathogen *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous lettuce microbiome. Front Microbiol 2014;5:175.
[7] Wassermann B, Rybakova D, Müller C, Berg G. Harnessing the microbiomes of *Brassica* vegetables for health issues. Sci Rep 2017;7:17649.
[8] Cernava T, Erlacher A, Soh J, Sensen CW, Grube M, Berg G. Enterobacteriaceae dominate the core microbiome and contribute to the resistome of arugula (*Eruca sativa* Mill.). Microbiome 2019;7:13.
[9] Fierer N, Breitbart M, Nulton J, Salamon P, Lozupone C, Jones R, et al. Metagenomic and small-subunit rRNA analyses reveal the genetic diversity of bacteria, archaea, fungi, and viruses in soil. Appl Environ Microbiol 2007;73:7059–66.
[10] Probst AJ, Auerbach AK, Moissl-Eichinger C. Archaea on human skin. PLoS ONE 2013;8:e65388.
[11] Moissl-Eichinger C, Pausan M, Taffner J, Berg G, Bang C, Schmitz RA. Archaea are interactive components of complex microbiomes. Trends Microbiol 2018;26:70–85.
[12] Taffner J, Erlacher A, Bragina A, Berg C, Moissl-Eichinger C, Berg G. What is the role of Archaea in plants? New insights from the vegetation of alpine bogs. MSphere 2018;3:e00122–e218.
[13] Pump J, Pratscher J, Conrad R. Colonization of rice roots with methanogenic archaea controls photosynthesis-derived methane emission. Environ Microbiol 2015;17:2254–60.
[14] Müller H, Berg C, Landa BB, Auerbach A, Moissl-Eichinger C, Berg G. Plant genotype-specific archaeal and bacterial endophytes but similar *Bacillus* antagonists colonize mediterranean olive trees. Front Microbiol 2015;6:1–9.
[15] Bengtson P, Sterngren AE, Rousk J. Archaeal abundance across a pH gradient in an arable soil and its relationship to bacterial and fungal growth rates. Appl Environ Microbiol 2012;78:5906–11.
[16] Casamayor EO, Massana R, Benlloch S, Ovreas L, Diez B, Goddard VJ, et al. Changes in archaeal, bacterial and eukaryal assemblages along a salinity gradient by comparison of genetic fingerprinting methods in a multipond solar saltern. Environ Microbiol 2002;4:338–48.
[17] Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, et al. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-

generation sequencing-based diversity studies. Nucleic Acids Res 2013;41. e1 e1.
[18] Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A, et al. Improved bacterial 16S rRNA Gene (V4 and V4–5) and fungal Internal Transcribed Spacer marker gene primers for microbial community surveys. MSystems 2016;1:e00009–e15.
[19] Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat Methods 2010;7:335–6.
[20] Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods 2016;13:581–3.
[21] Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;41:590–6.
[22] Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res 2003;13:2498–504.
[23] Huson DH, Auch AF, Qi J, Schuster SC. MEGAN analysis of metagenomic data. Genome Res 2007;17:377–86.
[24] Cardinale M, Grube M, Erlacher A, Quehenberger J, Berg G. Bacterial networks and co-occurrence relationships in the lettuce root microbiota. Environ Microbiol 2015;17:239–52.
[25] Moissl C, Rudolph C, Rachel R, Koch M, Huber R. In situ growth of the novel SM1 euryarchaeon from a string-of-pearls-like microbial community in its cold biotope, its physical separation and insights into its structure and physiology. Arch Microbiol 2003;180:211–7.
[26] Stahl AD. Development and application of nucleic acid probes. Nucleic Acid Tech Bact Syst 1991:205–48.
[27] Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl Environ Microbiol 1990;56:1919–25.
[28] Daims H, Brühl A, Amann R, Schleifer K-H, Wagner M. The Domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. Syst Appl Microbiol 1999;22:434–44.
[29] Poretsky R, Rodriguez-R LM, Luo C, Tsementzi D, Konstantinidis KT. Strengths and limitations of 16S rRNA Gene amplicon sequencing in revealing temporal microbial community dynamics. PLoS One 2014;9:e93827.
[30] Eloe-Fadrosh EA, Ivanova NN, Woyke T, Kyrpides NC. Metagenomics uncovers gaps in amplicon-based detection of microbial diversity. Nat Microbiol 2016;1:15032.
[31] Ranjan R, Rani A, Metwally A, McGee HS, Perkins DL. Analysis of the microbiome: advantages of whole genome shotgun versus 16S amplicon sequencing. Biochem Biophys Res Commun 2016;469:967–77.
[32] Sauder LA, Albertsen M, Engel K, Schwarz J, Nielsen PH, Wagner M, et al. Cultivation and characterization of *Candidatus Nitrosocosmicus exaquare*, an ammonia-oxidizing archaeon from a municipal wastewater treatment system. ISME J 2017;11:1142–57.
[33] Miller TL, Wolin MJ. Methanogens in human and animal intestinal Tracts. Syst Appl Microbiol 1986;7:223–9.
[34] Mosier AC, Allen EE, Kim M, Ferriera S, Francis CA. Genome sequence of "*Candidatus Nitrosoarchaeum limnia*" bg20, a low-salinity ammonia-oxidizing archaeon from the San Francisco ay estuary. J Bacteriol 2012;194:2119–20.
[35] Koskinen K, Pausan MR, Perras AK, Beck M, Bang C, Mora M, et al. First insights into the diverse human Archaeome: Specific detection of Archaea in the gastrointestinal tract, lung, and nose and on skin. MBio 2017. doi: https://doi.org/10.1128/mbio.00824-17.
[36] Probst AJ, Weinmaier T, Raymann K, Perras A, Emerson JB, Rattei T, et al. Biology of a widespread uncultivated archaeon that contributes to carbon fixation in the subsurface. NatureCom 2014;5:5497.
[37] Morris BEL, Henneberger R, Huber H, Moissl-Eichinger C. Microbial syntrophy: interaction for the common good. FEMS Microbiol Rev 2013;37:384–406.
[38] Rinta-Kanto JM, Sinkko H, Rajala T, Al-Soud WA, Sørensen SJ, Tamminen MV, et al. Natural decay process affects the abundance and community structure of Bacteria and Archaea in *Picea abies* logs. FEMS Microbiol Ecol 2016;92:fiw087.
[39] Karlsson AE, Johansson T, Bengtson P. Archaeal abundance in relation to root and fungal exudation rates. Microbiol Ecol 2012;80:305–11.
[40] Lorenz MC, Fink GR. Life and death in a macrophage: role of the glyoxylate cycle in virulence. Eukaryot Cell 2002;1:657–62.

5 Tomato-associated archaea show a cultivar-specific rhizosphere effect independently from soil quality

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Abstract

Archaea have recently been identified as substantial members of the plant microbiome. As for other microorganisms, the rhizosphere is a favorable habitat for archaea but less is known about their community assembly, composition, or origin. Therefore, we investigated archaeal communities in the rhizosphere of tomato plants (*Solanum lycopersicum* L., cv. Moneymaker and Hildares F1) nurtured in two different soil types over two generations of seeds. The abundance of the archaea was significantly different for each plant genotype and habitat showing highest abundances of 2×10^{12} 16S rRNA gene copies per g rhizosphere in Moneymaker. Differences in abundance, diversity and composition between cultivars were so distinct that they masked any effect determined by the different composition of soil. Overall, the archaeal community in tomato was dominated by *Thaumarchaeota* and *Euryarchaeota*. The core community in tomato consisted of species assigned to the *Soil Crenarchaeotic Group* (*Thaumarchaeota*; 60.7%), *Methanosarcina* (*Euryarchaeota*; 12.6%), *Methanoculleus* (*Euryarchaeota*; 3.4%), and unassigned archaeal species (7.2%). In seeds, archaeal abundance and diversity was low and composition showed random patterns; no indications of a plant-mediated vertical transmission were found. We assume that archaea represent only bystander microorganisms in seeds, while their cultivar-specific enrichment in the rhizosphere suggests a role in functioning of the plant holobiont.

MANUSCRIPT

Introduction

The plant microbiome was identified as a key for the next green revolution towards sustainable agriculture (Bender et al., 2016). The focus of plant microbiome research is mainly on bacteria and fungi, whereas archaea are often overseen. So far, archaea have been found as part of numerous microbiomes, adapted to a great variety of conditions, colonizing soil, plants and animals, humans, and especially ruminants and termites (Moissl-Eichinger et al., 2018). In soils both archaeal abundance and community structure can differ, as they are mainly shaped by the soil type and layer (Azziz et al., 2016; Chen et al., 2010). In plants, including domesticated plants such as arugula, olive trees, and maize, archaea represent a stable component of the microbiome (Hardoim et al., 2015). They have been found at high abundances in the rhizosphere and endosphere, mostly in nutrient-rich hotspots like rotten roots, and in lower abundances in the phyllosphere (Chelius & Triplett, 2001; Müller et al., 2015; Taffner et al., 2019). Several abiotic factors, such as climate, pH, and accessibility to nutrients, but also biotic factors, such as plant genotype, development stage, and competition with bacteria and fungi, have been found to influence the archaeal fraction of the plant microbiome, reshaping community structure and abundance (Bengtson et al, 2012; Edwards et al., 2018; Nicol et al., 2008). Metagenomic analyses revealed the potential of Archaea to interact with the plant holobiont by three different modes of action: i) competition and support (syntrophic interaction) with bacteria and fungi, ii) nutrient supply for plants, and iii) plant growth promotion through auxin biosynthesis (Stams & Plugge, 2009; Taffner et al., 2019; Taffner et al., 2018; Song et al., 2018). Some archaeal functions were especially found in specific plant microhabitats just as different archaeal communities, e.g. archaea were enriched in the rhizosphere, but less is known about their assembly and transmission (Taffner et al., 2018).

Domesticated plants in particular harbour specific conditions for microorganisms due to intensive long-standing breeding, which may affect the microbiome assembly and the interaction with the host (Pérez-Jaramillo et al., 2016). To unveil the composition and structure of plant archaeal communities, tomato (*Solanum lycopersicum* L.) was selected as a model crop. Tomato plants represent the most important vegetable with a total yield of up to 177 million tons per year (FAOSTAT, 2016). Together with other vegetables, tomatoes

represent a significant part of a healthy diet linked to a reduced risk of heart disease and stroke, lower blood pressure, cancer prevention and other numerous beneficial effects for human health (He et al., 2006). To date, the production and processing is commonly associated with conspicuous losses that reach up to 45% (FAO, 2018). Soil-borne pathogens, e.g. fungi from the genera *Fusarium*, *Rhizoctonia* and *Verticillium*, are among the major microbiological threats for this crop that significantly limit its yields (Oerke, 2006). For both its relevance and the problems connected with tomato production, the microbiome of the tomato plant has been widely characterized with several studies focusing on the below ground plant compartments in correlation with its resistance to biotic and abiotic stresses (Liu et al., 2017; Upreti & Thomas, 2015; Yan et al., 2003). In a recent study Bergna and colleagues (2018) identified tomato seeds as a key compartment for the vertical transmission of beneficial bacteria representing a significant portion of the plant microbiome in early developmental stages.

As of today, less is known about archaea in tomato plants apart from the strong impact of root exudates that enrich archaea in this habitat (Simon et al., 2005). Therefore, we studied the archaeal community in tomato plants to identify i) if factors such as plant genotype and soil quality shape the community structure of plant-associated archaea, and ii) if archaeal taxa are transmitted from one generation to the next. As it has been shown for bacterial communities (Bergna et al. 2018), we want to assess if and to what extent archaea are transmitted to the offspring, supporting germination and development. To achieve this, we combined qPCR and next-generation sequencing to quantify and describe the archaeal community of the tomato plant with a focus on the rhizosphere, two generations of seeds and the soil in which plants were generated.

Material and Methods

Experimental design

Surface-sterilized seeds (1st generation) of tomato plants (*Solanum lycopersicum* L.) of the cultivars Moneymaker (Austrosaat AG, Austria) and Hildares F1 (Hild Samen GmbH, Germany) were sown in 8 L pots (one seed per pot). Each pot contained a defined soil mixture obtained by adding sterile quartz sand and commercial loamy soil (Ökohum GmbH, Herbertingen, Switzerland) or diluvial sand (Rühlmann and Ruppel 2005) (proportion 10:1). A total of 100 planted pots were kept in a non-acclimated greenhouse (approximately 24/20°C day/night temperature) of the Graz Botanical Garden (Graz, Austria) together with unplanted pots containing only the soil mixture.

Sample collection

At the late flowering stage of the tomato plants (85 days post planting) soil and plant specimens were collected followed by a second sampling at the ripening of fruits of Moneymaker plants. Soil samples were collected from the central section of the pots containing the soil-sand mixture by only removing the top layer of soil (2-3 cm) with sterile tools. Rhizospheric soil has been obtained by shaking the root compartment and by collecting the material that was adhering to the roots in sterile bags. At ripening of tomato fruits, the seeds of the 2nd generation were collected from 10 Moneymaker plants and cleaned from leftover fruit tissues using sterile tools.

Samples were processed using a modified protocol of Bragina and colleagues (2012) following sample processing as described in the work of Bergna and colleagues (2018). Briefly, collected specimens of both soil and rhizosphere were suspended in 0.85% sodium chloride solution (NaCl) and shaken for 30 minutes. After this first step, the liquid phase was extracted with laboratory pipettes and the microorganisms-containing pellets were obtained by centrifugation (20 min at 16,750 g) and stored at -70°C. Seeds (I and II generation) of Moneymaker and Hildares F1 were washed in sterile water, divided in plastic vials (20 seeds per vial) with 4 ml of 0.85% NaCl and gently shaken for 4 hours. The seeds were then homogenized with mortar and pestle and suspended in 0.85% NaCl. After centrifugation (20 min at 16,750 g) microorganisms-containing pellets were stored at -70°C.

DNA extraction and preparation of the 16S rRNA gene fragment amplicons

The aforementioned pellets were used for the total community DNA isolations. DNA was isolated with the FastDNA® SPIN Kit for Soil and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. Extracted community DNA samples were used for PCR-based barcoding. In order to strictly amplify the archaeal 16S rRNA gene alone, we performed a nested PCR using the archaea-specific primers 344f and 915r in the first PCR and modified primer pair S-D-Arch-0349-a-S-17/S-D-Arch-0519-a-A-16 (here 349f/519r (Klindworth et al., 2013)) with an additional 10 bp primer-pad (TATGGTAATT/AGTCAGCCAG) and linker (GT/GG) in the subsequent PCR, as previously described by protocols of the Earth Microbiome Project (Walters et al., 2016). Afterwards the Golay barcodes were annealed in a third PCR. The PCR reactions were conducted as previously described (Taffner et al., 2019). All PCR reactions were conducted as triplicates, purified with the Wizard SV Gel and PCR cleanup system (Promega, Madison, WI), and pooled in equimolar concentrations prior to sequencing. The Sequencing was then conducted using an Illumina HiSeq Personal Sequencer (GATC Biotech AG, Konstanz, Germany).

Quantitative real-time PCR with archaea-specific primers

The quantification of archaea in soil, rhizosphere, and two generations of seeds was conducted with primer pairs 344aF and 517uR (Probst et al., 2013). For the qPCR one µl of extracted DNA was used in each 10 µl reaction mix. The reaction mix contained 5 µl KAPA SYBR Green 2X MM Mix (BIO-RAD, Hercules, CA, USA), 0.5µl forward and reverse primers (344aF and 517uR) at a concentration of 10 pmol/µl and 3 µl of PCR grade water. 16S rRNA gene standards from *Haloferax denitrificans* were used as a standard. PCR amplifications were conducted in triplicates using a Rotor-Gene 6000 series (Corbett Research) thermal cycler with the following program settings: 95°C / 5 min, 95°C / 15 sec, annealing 60°C / 30 sec, extension 72°C / 30 sec; amplification steps were repeated 39 times. Final elongation was done from 72°C - 96°C.

Data analysis of 16S rRNA gene amplicons for determination of archaeal community structure

16S rRNA gene paired-end sequences were joined (SeqPrep), reoriented and demultiplexed in the Qiime1 environment (Quantitative Insights into Microbial Ecology, version 1.9.0) (Caporaso et al., 2010). Sequences were then denoised, dereplicated and clustered using the DADA2 pipeline integrated within Qiime2 environment (2019.4 release). Chimeras were identified and filtered. The features' taxonomy assignment was conducted using a fitted classifier (Scikit-learn) (Pedregosa et al., 2011) and the Silva 16S (349af - 519ar 99 otusversion 128) Archaeal database. Unassigned and non-archaeal features contaminants were filtered from the resulting feature table.

A graphical rendering of the archaeal community structure at class level was produced using the open-source software Circos (Krzywinski, 2009) (Fig. 1). In order to display a more reliable differential abundance among samples, the number of reads of each sample has been normalised with the concentration value obtained with the qPCR. In this way, we coupled the superior quantification accuracy of qPCR with the capacity of amplicon sequencing to describe complex microbial communities. Alpha diversity distances were calculated and rendered at feature level in the Phyloseq package (McMurdie & Holmes, 2013) within R environment using Observed Species, Chao 1, Shannon and Inverse Simpson measures. Phyloseq package (McMurdie & Holmes, 2013) was also employed for generating the PCoA plot with Bray Curtis. Statistical analysis for sample difference significance was performed using the package vegan v. 2.5.5 (Oksanen et al., 2019) with the Adonis test (999 permutations). In order to visualize the archaeal distribution among the habitats of the tomato plant Cytoscape 3.3.0 software was used (Shannon et al., 2003).

Results

Quantification of archaeal population density in tomato plants

Using a quantitative polymerase chain reaction (qPCR) with specific archaeal primers, targeting the 16S ribosomal RNA gene, we quantified the archaeal population of two tomato cultivars (Moneymaker and Hildares F1) grown in two soil mixtures. Archaeal rRNA gene abundances spanned between $2.9*10^{9}$ and $2.3*10^{13}$ copies per g of sample (Fig. 1). The highest archaeal abundance was found in the rhizosphere of Moneymaker plants. Irrespective of the soil mixture in which these plants were grown, archaea were significantly ($p < 0.05$) more abundant in the rhizosphere of this cultivar than in the rhizosphere of Hildares F1, showing archaeal abundances of $1.9\text{-}2.0*10^{12}$ 16S rRNA gene copies g^{-1}. The composition and texture of the two soil mixtures did not result in significantly different archaeal abundance ($p > 0.05$) that has been quantified in $5.9*10^{11}$ and $2.9*10^{12}$ 16S rRNA copies g^{-1} in loamy and sandy soil mixtures, respectively. However, there was a distinct difference in the number of archaeal gene copies among the two analysed generations of Moneymaker seeds: we found the latter (second generation) characterised by a lower abundance of archaea with $2.9*10^{9}$ to $7.1*10^{9}$ 16S rRNA copies g^{-1}. In contrast, the archaeal gene copy numbers in the seeds of the first generation were higher with $2.1*10^{12}$ 16S rRNA gene copies g^{-1}, but with a high standard deviation. Further statistical analysis with Kruskal-Wallis test did not show a significant result for the difference of both seed generations ($p > 0.05$).

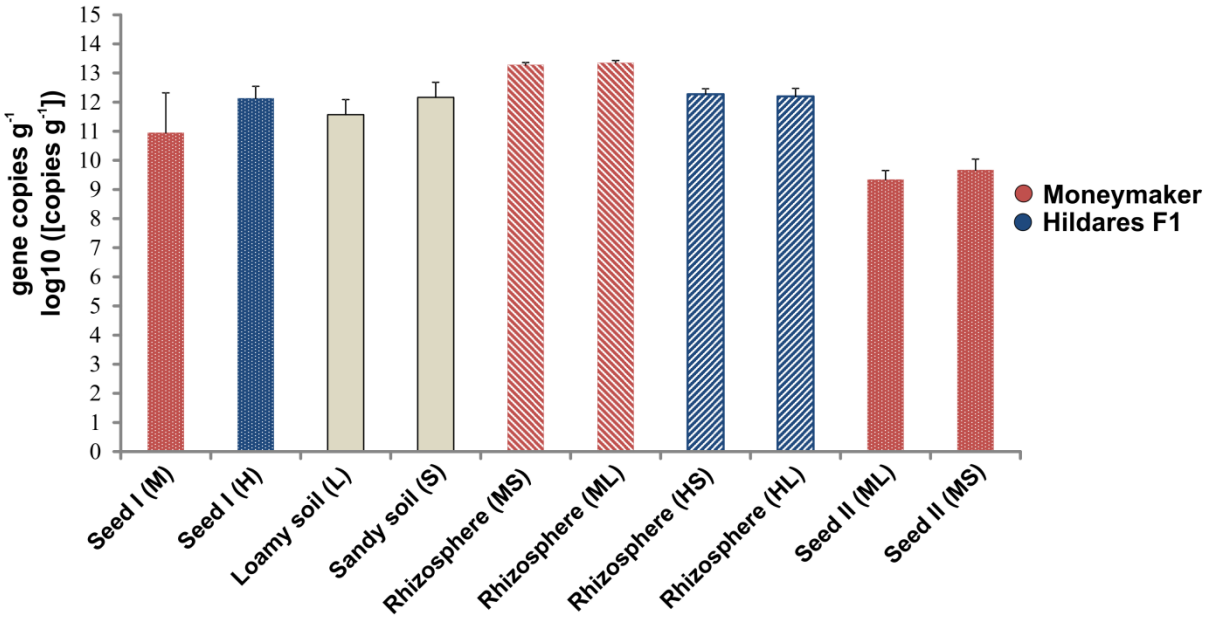

Figure 1: qPCR-based quantitative analysis of archaea in different samples. Archaeal abundances were determined for the habitats: seeds from the first (Seed I) and the second generation (Seed II), and the rhizosphere of the cultivars Moneymaker (M) and Hildares F1 (H). Abundances in the loamy (C) and sandy soil (G) were measured as well.

Structure of tomato-associated archaeal communities and diversity analyses

High throughput sequencing of 16S rRNA gene fragments of two soil mixtures, rhizosphere and seeds (1st and 2nd generation) of two tomato cultivars (Moneymaker and Hildares F1) yielded in a total of 748,221 high quality archaeal reads that have been clustered in a total of 1,133 distinct features.

The composition of archaeal communities in tomato was dominated by two main phyla: *Thaumarchaeota* and *Euryarchaeota* (Fig. 2). In all analysed microenvironments, members of these two phyla accounted for more than 80% of the whole community. More precisely, *Thaumarchaeota* consistently accounted for more than 60% of the archaeal community and lower abundances of this phylum have been found in the Hildares F1 seeds used for generating the plants (1st generation) as well as in the loamy soil. The low abundance of *Thaumarchaeota* (46%) in the Hildares F1 seeds was accompanied by a high abundance of *Euryarchaeota* (34%) and unassigned reads (20%). The archaeal community composition of the commercial loamy soil mixture represented a rather equal distribution with *Euryarchaeota* representing 49% of the community and *Thaumarchaeota* 48%. At the same time, among the two soil types a great difference in the abundance of these phyla was

observed. While *Thaumarchaeota* represented 79% of the archaeal community in sandy soil, in commercial loamy soil their abundance was only of 51%. The abundance of *Euryarchaeota*, on the other hand, varied between 11% in sandy soil to 48% in loamy soil.

At class level, both community-dominating phyla were mostly represented by a single class; the *Soil Crenarchaeotic Group* for *Thaumarchaeota* and *Methanomicrobia* for *Euryarchaeota*. Other *Euryarchaeota* classes, which were relevant for the archaeal community, were identified as *Thermoplasmata* and *Methanobacteria* that found to be specific for the sandy soil (respectively 2% and 1%) and representing up to 4% and 8% in the rhizosphere. A more complete description of the archaeal community is provided in Table S2 (supplementary material).

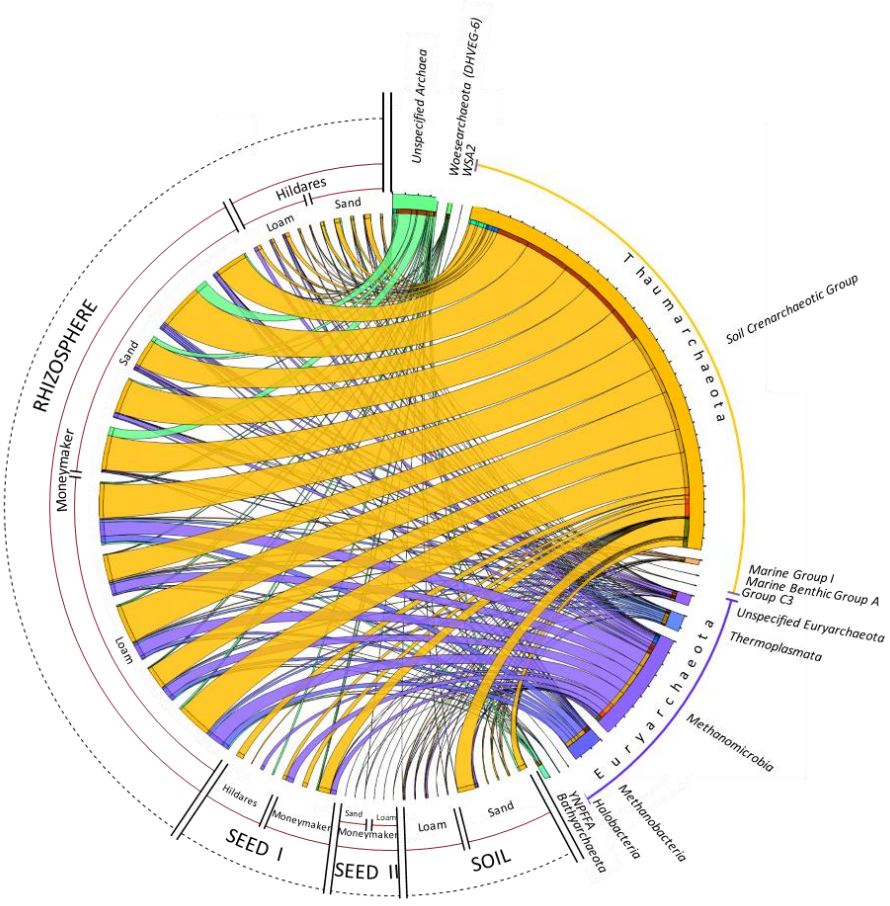

Figure 2: Archaeal communities of two soil-sand mixtures, seeds, and the rhizosphere of two tomato plant cultivars (Moneymaker and Hildares F1). Total abundances of the respective archaeal populations were adjusted with a qPCR-based quantification. The graph was obtained using the open-source software Circos (http://circos.ca).

Soil and cultivar driven variabilities

The diversity within archaeal communities (Table S2) was evaluated with metrics sensitive to dominant OTUs (Inverse Simpson's index), to rare OTUs (observed species and Chao1) and incorporating both richness and evenness (Shannon). While alpha diversity values were not inferable for seed samples due to their reduced number of reads (low abundance across samples confirmed by qPCR results), the analysis showed a consistently higher diversity in the rhizosphere of Moneymaker plants regardless the soil mixture employed (Fig. 3). The comparison of diversity levels of soil mixtures indicated that the sandy soil mixture has a higher archaeal diversity when compared to the commercial loam mixture. Interestingly, the rhizosphere of plants grown in these two substrates was not determined by the archaeal community characteristics of the soil. The rhizosphere of Moneymaker plants was found to harbour an increased diversity, which was 3-fold higher than soil and the Hildares F1 rhizosphere (Observed and Chao1 indices).

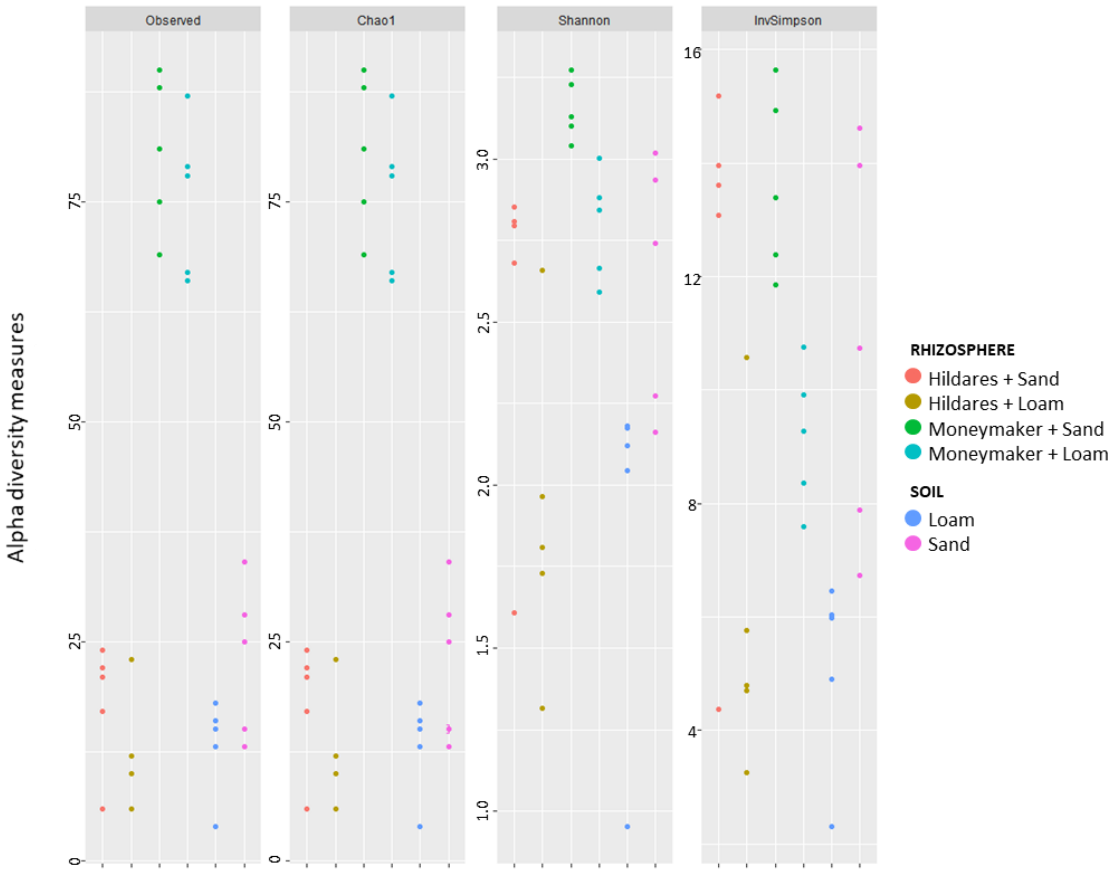

Figure 3: Assessment of alpha diversity across the rhizosphere and soil samples. Four different diversity measures were used: Observed Species, Chao1, Shannon and Inverse Simpson. A combination of measures sensitive to rare OTUs (Observed species and Chao1) and to dominant OTUs (Inverse Simpson's index) was performed in order to provide a comprehensive assessment of bacterial diversity in the plant system.

In order to analyse similarities and dissimilarities among the archaeal communities of different samples, the beta diversity analysis has been graphically rendered with a principal coordinate analysis (PCoA) using the Bray Curtis distance (Fig. 4). Complementary to the alpha-diversity analysis, the beta-diversity analysis highlighted the unique composition of the rhizospheric communities of Moneymaker plants in this system. Rhizosphere archaeal communities of Moneymaker plants showed slightly different archaeal communities (R^2 = 0.69006, Pr (>F) = 0.01) among soil qualities. Regardless of the soil quality, the archaeal community of this sample group significantly differed to all other samples (Hildares F1 rhizosphere, 1st and 2nd generation seeds, and both soil mixtures) (R^2 = 0.49868, Pr (>F) = 0.001)). In parallel, in the PCoA no inter-sample similarities linked to the soil mixture quality were observed.

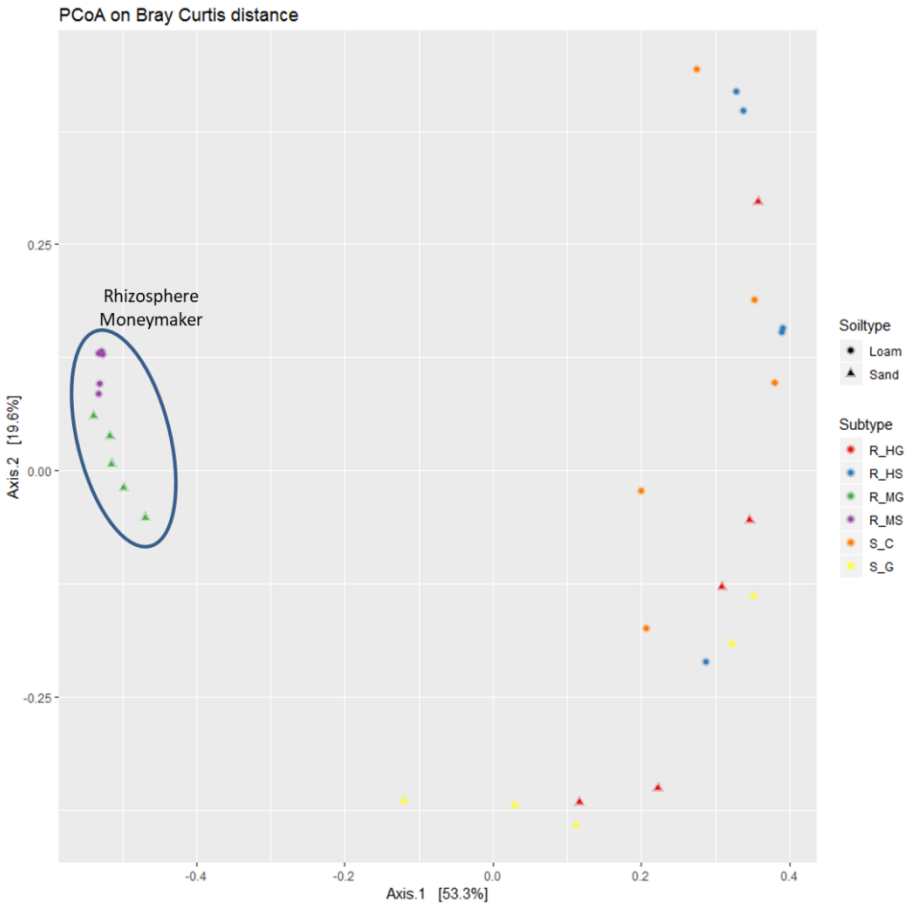

Figure4: PCoA plot calculated using Bray Curtis metrics plotting the similarities/dissimilarities among samples based on their archaeal community composition. Samples are coloured according to the different microhabitats and the shape refers to the soil mixture employed.

Composition of the archaeal community in tomato

In tomatoes the archaeal core community consisted of species assigned to the *Soil Crenarchaeotic Group* (*Thaumarchaeota*; 60.7%), *Methanosarcina* (*Euryarchaeota*; 12.6%), *Methanoculleus* (*Euryarchaeota*; 3.4%), and unassigned archaeal species (7.2%), which were shared among all habitats, including the seeds (Fig. 5). Further, the seeds of Moneymaker harboured archaea of the genus *Candidatus Nitrososphaera* and several *Euryarchaeota* genera. Overall, a higher archaeal diversity was found associated to the cultivar Moneymaker, than in Hildares F1. In the rhizosphere of Moneymaker, several archaeal taxa specific for this habitat were found. Furthermore, the minor phylum of *Bathyarchaeota* was exclusively found associated to Moneymaker and loamy soil.

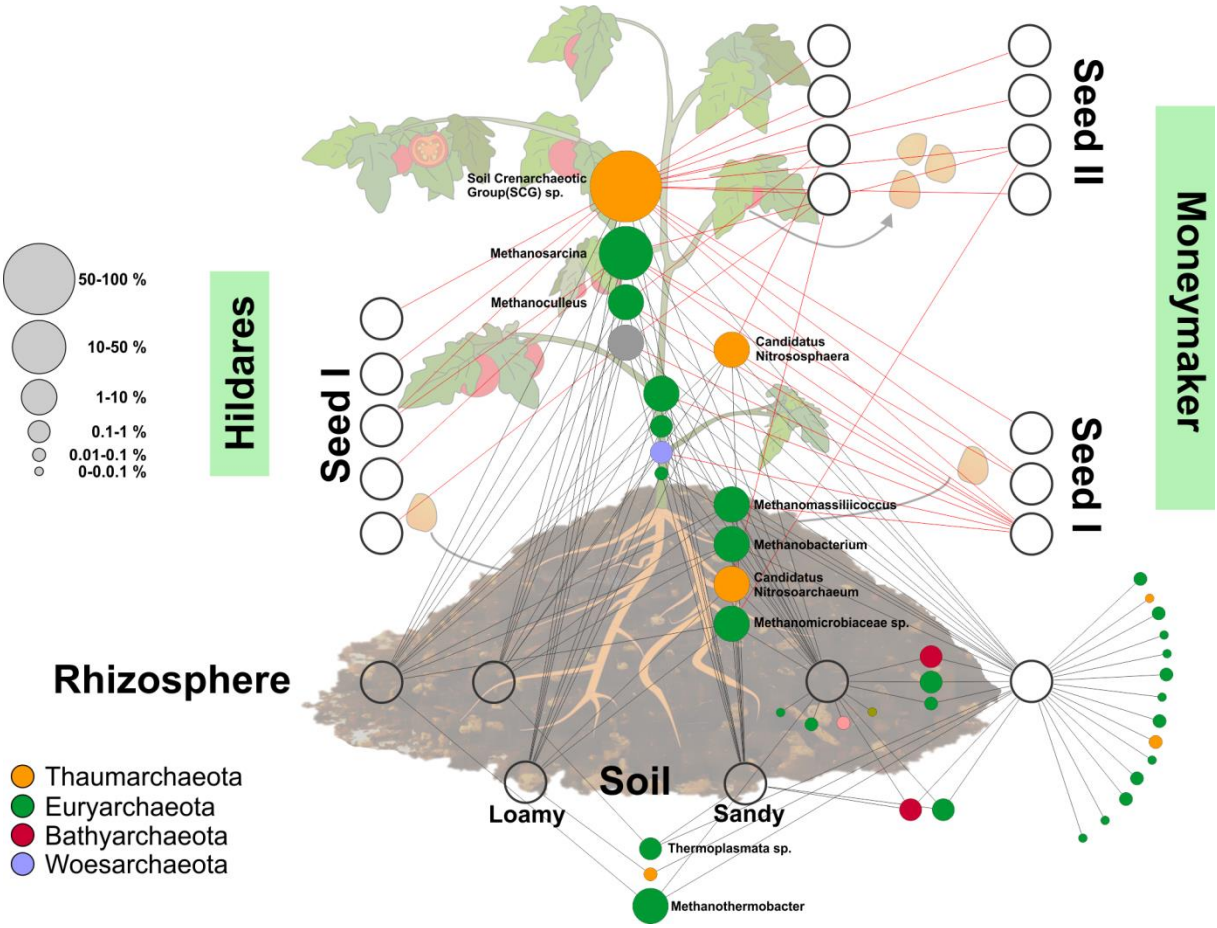

Figure 5: Feature network of the archaeal communities at the genus level, based on 16S rRNA gene fragment datasets. The datasets were obtained from the habitats soil, rhizosphere, and first and second generation of seeds of tomato plants of the cultivars Moneymaker and Hildares F1. Coloured bubbles indicate archaeal phyla: *Thaumarchaeota* in orange; *Euryarchaeota* in green; *Bathyarchaeota* in red; and *Woesarchaeota* in purple; unassigned taxa are shown in grey. Red lines connecting the associated sample highlight taxa found in the seeds. The size of the bubble represents the relative abundance of the archaeal taxa throughout all habitats.

Discussion

Habitat specificity and rhizosphere enrichment of archaeal communities in tomato plants

In the present study, the abundance of archaea across the tomato plant was found to be highly habitat-specific and showed a strong rhizosphere effect. This is in line with previous findings for soil *Crenarchaeota,* which were shown to be enriched in the rhizosphere by the presence of nutrient-rich root exudates (Simon et al., 2005). On the other hand, it is possible that the high archaeal abundance found in this habitat is connected to a specific bacterial community setup that favours archaeal colonisation, e.g. by using bacterial metabolites.

Soil quality shapes the archaeal community in soil

The archaeal community in the two soil qualities selected for this study (loamy and sandy) showed differences in their diversity and in the abundance of specific dominant archaeal taxa. Our findings confirm that soil quality is a main determinant for the soil archaeal community structure (Chen et al., 2010; Di et al., 2010), and that it can favour archaeal taxa with specific characteristics (Azziz et al., 2016). An example is the increased abundance of ammonia oxidizing archaea (AOA) in sandy soil compared to loamy soil. AOA are part of the phylum *Thaumarchaeota*, which composed most of the archaeal community structure associated to the tomato plants in this study. The second most abundant phylum was assigned to *Euryarchaeota*. This phylum consists for the most part of methanogens such as *Methanomicrobia*, which represented the most abundant taxa of *Euryarchaeota* found. These anaerobic methanogens are usually part of microbiomes of crops, such as maize or arugula, mainly located in anoxic niches in the rhizosphere of the plants (Chelius & Triplett, 2001; Taffner et al., 2019).

The impact of soil quality on the archaeal community in the rhizosphere

In contrast to the differences observed in bulk soils, we could not detect nor infer any significant soil quality-related effect on the abundance of the archaeal population in the rhizosphere. On the one hand, it is possible that the absence of the effect might be due to the experimental design of this study; the substantial dilution of both loamy and sandy soils

with sterile sand might have mitigated the effects of soil texture on the rhizosphere. On the other hand, a previous analysis with an identical experimental setup (Bergna et al., 2018) observed that the differences in the bacterial communities of these two specific soils were highly conserved for the bacterial community of the rhizosphere of tomato plants. This different sensitivity of archaea and bacteria to soil quality is not new, but still not fully understood. While it is known that these microorganisms respond differently to soil depth, where the ratio of archaea to bacteria increases (Leininger et al., 2006b), archaea inhabit a far more restricted ecological niche in soils compared to bacteria (Bates et al., 2011). In addition, a recent study defined the process of rhizosphere formation as a dynamic and almost bacterial-exclusive process, that does not include archaea until the last stages of plant development (Edwards et al., 2018). For these reasons, it is possible to hypothesize that, in contrast to what applies for bulk soil, archaeal rhizosphere communities are much less affected by soil quality compared to bacteria. Instead, archaea in the rhizosphere might be deeply influenced by the coexistence with bacterial communities and by the adjacent plant root system (Kang et al., 2019).

The plant genotype is a main driver for archaeal community in the rhizosphere

The archaeal diversity and abundance observed in the rhizosphere of tomato plants was consistently higher compared to both bulk soils employed. This rhizosphere effect is likely to be connected with the production of root exudates that represent a constant source of nutrients (Mendes et al.,2011) that attracts and allows the colonisation of archaea. Interestingly, the rhizospheric diversity shift was observed to be even clearer in Moneymaker plants that hosted a three times more diverse archaeal community than Hildares F1 plants (Fig. 6). This is the first time that a plant-genotype effect of this magnitude has been observed on archaeal communities in the rhizosphere of agricultural plants. So far, a similar plant-genotype driven effect has been reported only for archaeal methanogens in the rhizosphere of rice in an aquatic environment (Wu et al., 2009). This highly specific effect might not only be explained by the differences in the quality and quantity of root exudates, but also by different nutrient-uptake strategies of each plant cultivar (Grayston et al., 1997). It is in fact known that specific archaea, and notably AOA, highly accumulate in N-demanding plants (Thion et al., 2016). Further, another explanation of this effect can be found in the

inter-domain interactions that archaea can establish (Taffner et al., 2019). In this framework, the effects that different root exudates have on bacterial and fungal microorganisms were shown, such as modifying the presence of metabolites in the rhizosphere and the soil-plant interface. Similarly, archaeal abundance is known to correlate with mycorrhizal abundance (Grayston et al., 1997); for this reason it is valuable to also integrate fungal communities in the analysis of archaea-plant interactions.

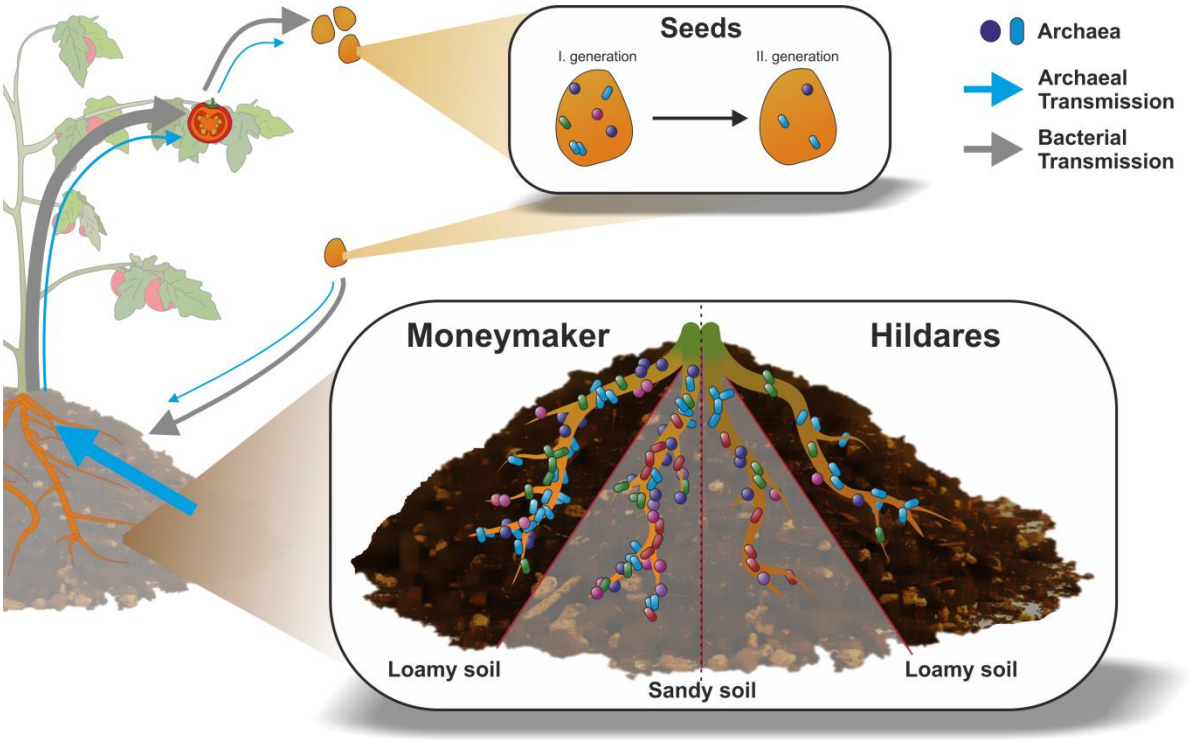

Figure 6: Graphical illustration of the colonization and transmission of archaea in tomato plants. The rhizosphere of both analysed tomato cultivars (Moneymaker and Hildares F1) in loamy and sandy soil, as well as the 1st and 2nd generation of the seeds of Moneymaker are included in the model. Blue arrows highlight archaeal transmission between the different habitats. For comparison to a previous study by Bergna and colleagues (2018), the bacterial transmission is indicated by grey arrows. The size of the arrow indicates the relative proportion of transmitted microorganism.

The potential ecological role of archaea in tomato seeds

Recently we described how the seed can represent an important vehicle for the vertical transmission of beneficial bacteria across generations (Bergna et al., 2018). Since archaea play relevant roles for plant nutrient cycling in the soil and the rhizosphere (Erkel et al., 2006; Leininger et al., 2006; Mendes et al., 2013), we initially hypothesized that archaea would have

been conveyed by the plant to the seed where, as for bacteria (Bergna et al., 2018), they might support the germination and development of the offspring plant. The archaeal abundance registered by qPCR in tomato seeds was in the range of 10^9 copies g^{-1} in seeds, which goes in line with recent observations of Wassermann and colleagues (2019) in seeds of native alpine plants. However, the analysis of the archaeal community of tomato seeds did not provide any evidence that could indicate a selection of archaea for the offspring plant. As previously discussed, the recent work of Edwards and colleagues (2018) describes how the rhizosphere can be highly dynamic during the vegetative phase of plant growth and how it compositionally stabilises only for the remainder of the life cycle. Only in this latter phase, archaea are found colonising the rhizosphere and are for this reason indicated as "late colonisers". Interestingly, a development-based colonization was observed for plants and humans as well; archaea are more abundant in older organisms (Probst et al. 2013; Moissl-Eichinger et al. 2018). Contrary to bacteria which are early colonizers and transmitted through the seed, that represents the primary vehicle of beneficial microorganisms for the early stages of plant development. Therefore, we suggest that archaea, which appear to be non-essential for the first stages of plant development, are not found in the seed. Moreover, it is more likely that archaea might have developed as bystander microorganisms in seeds, possibly based on syntrophic relationships with bacteria (Morris et al., 2013).

Unassigned archaeal features in tomato plants

The bioinformatic reconstruction of the archaeal community associated with the plant habitats was performed using an up-to-date established pipeline. This approach resulted in a well-defined archaeal community structure that was though not exempt from several taxonomically unassigned features. Features without taxonomical assignment represented 15% of total features found in these habitats, but can represent up to 20% at class level in seed samples. This is a well-known limitation for the characterisation of novel habitats, especially for archaea. In fact, the rather low frequency of archaeal community investigations resulted in the use of smaller and often incomplete taxonomy databases. The relatively low ratio of unassigned features of this analysis excludes the presence of significant PCR off-target effects or low read length. On the other hand, the high ratio of unassigned taxa in a low characterised habitat, such as the seed, indicates that this problem is seemingly due to

still poorly defined reference databases that can be increased only with further investigation of the archaeal domain.

Conclusion:

Archaea are substantial components of the tomato microbiota with specific compositions in the rhizo- and endosphere. The plant genotype (tomato cultivar) was identified as main factor influencing abundance and diversity, while soil quality did not notably affect archaeal communities. Our results show for the first time transmission of archaea from the parent to the offspring plant but we found no indication for a targeted selection as shown for bacteria. In comparison to the seed, the rhizosphere showed cultivar-specific increased abundance and diversity of archaea indicating a role for the developed plant. The plant microbiome is known to change during its life cycle; accordingly, different members of the plant microbiome seem to have different abundances and functions. This has to be considered in management strategies developed for healthy plant microbiomes in sustainable agriculture.

References

Azziz, G., Trasante, T., Monza, J., & Irisarri, P. (2016). The effect of soil type, rice cultivar and water management on ammonia-oxidizing archaea and bacteria populations. Appl. Soil Ecol. 100:8–17.

Bates, S. T., Berg-Lyons, D., Caporaso, J. G., Walters, W. A., Knight, R., & Fierer, N. (2011). Examining the global distribution of dominant archaeal populations in soil. ISME J. 5:908–917.

Bender, S. F., Wagg, C., & van der Heijden, M. G. A. (2016). An underground revolution: biodiversity and soil ecological engineering for agricultural sustainability. Trends Ecol. Evol. 31:440–452.

Bengtson, P., Sterngren, A. E., & Rousk, J. (2012). Archaeal abundance across a pH gradient in an arable soil and its relationship to bacterial and fungal growth rates. Appl. Environ. Microbiol. 78:5906–5911.

Bergna, A., Cernava, T., Grosch, R., Zachow, C., & Berg, G. (2018). Tomato seeds preferably transmit plant beneficial endophytes. Phytobiomes J. 2:183–193.

Bragina, A., Berg, C., Cardinale, M., Shcherbakov, A., Chebotar, V., & Berg, G. (2012). *Sphagnum* mosses harbour highly specific bacterial diversity during their whole lifecycle. ISME J. 6:802–813.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335–336.

Chelius, M. K., & Triplett, E. W. (2001). The diversity of Archaea and Bacteria in association with the roots of *Zea mays* L. Microb. Ecol. 41:252–263.

Chen, X., Zhang, L.-M., Shen, J.-P., Xu, Z., & He, J.-Z. (2010). Soil type determines the abundance and community structure of ammonia-oxidizing bacteria and archaea in flooded paddy soils. J. Soils Sediments 10:1510–1516.

Di, H. J., Cameron, K. C., Shen, J.-P., Winefield, C. S., O'Callaghan, M., Bowatte, S., & He, J.-Z. (2010). Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. FEMS Microbiol. Ecol. 72:386–394.

Edwards, J. A., Santos-Medellín, C. M., Liechty, Z. S., Nguyen, B., Lurie, E., Eason, S., … Sundaresan, V. (2018). Compositional shifts in root-associated bacterial and archaeal microbiota track the plant life cycle in field-grown rice. PLoS Biology 16(2): e2003862.

Erkel, C., Kuba, M., Heinhardt, R., & Liesack, W. (2006). Genome of rice cluster I archaea - The key methane producers in the rice rhizosphere. Science 313:370-2.

Grayston, S. J., Vaughan, D., & Jones, D. (1997). Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability. Appl. Soil Ecol. 5:29–56.

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., … Sessitsch, A. (2015). The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol. Mol. Biol. Rev. 79:293–320.

He, F. J., Nowson, C. A., & MacGregor, G. A. (2006). Fruit and vegetable consumption and stroke: meta-analysis of cohort studies. Lancet 367:320–326.

Janssen, P. H., & Kirs, M. (2008). Structure of the archaeal community of the rumen. Appl. Environ. Microbiol. 74:3619–3625.

Oksanen, J., Guillaume Blanchet, F. , Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., & Solymos, P., Henry, M., Stevens, H., Szoecs, E., and Wagner, H. (2019). vegan: Community Ecology Package. R package version 2.5-5.

Kang, M.-S., Hur, M., & Park, S.-J. (2019). Rhizocompartments and environmental factors affect microbial composition and variation in native plants. J. Microbiol. 1–12.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41:e1–e1.

Krzywinski, M. et al. (2009). Circos: an Information Aesthetic for Comparative Genomics. Genome Res 19:1639–1645.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G. W., … Schleper, C. (2006)a. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442:806–809.

Liu, K., Newman, M., McInroy, J. A., Hu, C.-H., & Kloepper, J. W. (2017). Selection and Assessment of Plant Growth-Promoting Rhizobacteria for Biological Control of Multiple Plant Diseases. Phytopathology 107:928-936.

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 8:e61217.

Mendes, R, Kruijt, M., Bruijn, I. De, … E. D.-, & 2011, undefined. (n.d.). Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science 332:1097-100.

Mendes, Rodrigo, Garbeva, P., & Raaijmakers, J. M. (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol. Rev. 37:634-63.

Moissl-Eichinger, C., Pausan, M., Taffner, J., Berg, G., Bang, C., & Schmitz, R. A. (2018). Archaea are interactive components of complex microbiomes. Trends Microbiol. 26:70–85.

Morris, B. E. L., Henneberger, R., Huber, H., & Moissl-Eichinger, C. (2013). Microbial syntrophy: interaction for the common good. FEMS Microbiol. Rev. 37:384–406.

Müller, H., Berg, C., Landa, B. B., Auerbach, A., Moissl-Eichinger, C., & Berg, G. (2015). Plant genotype-specific archaeal and bacterial endophytes but similar *Bacillus* antagonists colonize Mediterranean olive trees. Front. Microbiol. 6:138.

Nicol, G. W., Leininger, S., Schleper, C., & Prosser, J. I. (2008). The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environ. Microbiol. 10:2966–2978.

Oerke, E. C. (2006). Crop losses to pests. J. Agric. Sci. 144:31–43.

Pedregosa, F., Varoquaux, G., Gramfort, A., Michel, V., Thirion, B., Grisel, O., … Duchesnay, É. (2011). Scikit-learn: Machine learning in Python. J. Mach. Learn. Res. 12:2825–2830.

Pérez-Jaramillo, J. E., Mendes, R., & Raaijmakers, J. M. (2016). Impact of plant domestication on rhizosphere microbiome assembly and functions. Plant Mol. Biol. 90:635–644.

Probst, A. J., Auerbach, A. K., & Moissl-Eichinger, C. (2013). Archaea on human skin. PLoS One 8:e65388.

Samuel, B. S., Hansen, E. E., Manchester, J. K., Coutinho, P. M., Henrissat, B., Fulton, R., … Gordon, J. I. (2007). Genomic and metabolic adaptations of *Methanobrevibacter smithii* to the human gut. Proc. Natl. Acad. Sci. U. S. A. 104:10643–10648.

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., … Ideker, T. (2003). Cytoscape: A software environment for integrated models of biomolecular interaction networks. Genome Res. 13:2498–2504.

Shi, Y., Huang, Z., Han, S., Fan, S., & Yang, H. (2015). Phylogenetic diversity of Archaea in the intestinal tract of termites from different lineages. J. Basic Microbiol. 55:1021–1028.

Simon, H. M., Jahn, C. E., Bergerud, L. T., Sliwinski, M. K., Weimer, P. J., Willis, D. K., & Goodman, R. M. (2005). Cultivation of mesophilic soil crenarchaeotes in enrichment cultures from plant roots. Appl. Environ. Microbiol. 71:4751–4760.

Song, G. C., Im, H., Jung, J., Lee, S., Jung, M.-Y., Rhee, S.-K., & Ryu, C.-M. (2018). Plant growth-promoting archaea trigger induced systemic resistance in *Arabidopsis thaliana* against *Pectobacterium carotovorum* and *Pseudomonas syringae*. Environ. Microbiol. 21:940-948.

Stams, A. J. M., & Plugge, C. M. (2009). Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat. Rev. Microbiol. 7:568–577.

Taffner, J., Cernava, T., Erlacher, A., & Berg, G. (2019). Novel insights into plant-associated archaea and their functioning in arugula (*Eruca sativa* Mill.). J. Adv. Res. 19:39-48.

Taffner, Julian, Erlacher, A., Bragina, A., Berg, C., Moissl-Eichinger, C., & Berg, G. (2018). What s the role of Archaea in plants? New insights from the vegetation of alpine bogs. MSphere 3:e00122-18.

Thion, C. E., Poirel, J. D., Cornulier, T., De Vries, F. T., Bardgett, R. D., & Prosser, J. I. (2016). Plant nitrogen-use strategy as a driver of rhizosphere archaeal and bacterial ammonia

oxidiser abundance. FEMS Microbiol. Ecol. 92:fiw091.

Upreti, R., & Thomas, P. (2015). Root-associated bacterial endophytes from *Ralstonia solanacearum* resistant and susceptible tomato cultivars and their pathogen antagonistic effects. Front. Microbiol. 6: 255.

Walters, W., Hyde, E. R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., … Knight, R. (2016). Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. MSystems 1:e00009-15.

Wassermann, B., Cernava, T., Müller, H., Berg, C. & Berg, G. (2019). Seeds of native alpine plants host unique microbial communities embedded in cross-kingdom networks. Microbiome 7:108

Wu, L., Ma, K., Li, Q., Ke, X., & Lu, Y. (2009). Composition of archaeal community in a paddy field as affected by rice cultivar and N fertilizer. Microb. Ecol. 58:819–826.

Yan, Z., Reddy, M. S., & Kloepper, J. W. (2003). Survival and colonization of rhizobacteria in a tomato transplant system. Can. J. Microbiol. 49: 383-9.

Supplementary Material

Table S2: feature table reporting the relative abundance of archaeal taxa across the tomato plant.

	Rhizosphere				Seeds 1st gen.		Seeds 2nd gen.		Soil	
	Hildares		Moneymaker		Moneymaker		Moneymaker			
	Loam	Sand	Loam	Sand	Hildares		Loam	Sand	Loam	Sand
Phylum *Bathyarchaeota*	0.00%	0.00%	1.47%	0.24%	0.00%	0.00%	0.00%	0.00%	0.00%	0.24%
Crenarchaeota (class: *YNPFFA*)	0.00%	0.00%	0.18%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Euryarchaeota (class: *Halobacteria)*	0.00%	0.00%	0.00%	0.03%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Euryarchaeota (class: *Methanobacteria*)	3.90%	1.40%	8.04%	2.20%	0.00%	0.00%	0.00%	0.00%	0.00%	1.11%
Euryarchaeota (class: *Methanomicrobia*)	38.46%	6.96%	22.76%	5.45%	34.02%	18.58%	16.29%	11.28%	49.82%	7.92%
Euryarchaeota (class: *Thermoplasmata*)	0.95%	0.48%	3.76%	1.54%	0.00%	0.78%	0.00%	0.00%	0.00%	2.09%
Unspecified *Euryarchaeota*	0.56%	2.18%	1.34%	2.32%	0.00%	0.20%	0.00%	0.00%	0.90%	0.56%
Thaumarchaeota (class: *Group C3*)	0.00%	0.00%	0.00%	0.08%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thaumarchaeota (class: *Marine Benthic Group A*)	0.00%	0.00%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Thaumarchaeota (class: *Marine Group I*)	0.00%	0.00%	1.35%	0.00%	0.00%	0.00%	0.00%	2.54%	12.40%	0.32%
Thaumarchaeota (class: *Soil Crenarchaeotic Group*)	53.88%	71.83%	58.31%	76.18%	45.98%	77.17%	83.71%	70.62%	36.26%	82.28%
Phylum *WSA2*	0.00%	0.00%	0.02%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%
Woesearchaeota (*DHVEG-6*)	0.57%	0.03%	1.18%	0.10%	0.00%	1.14%	0.00%	0.00%	0.00%	0.00%
Unspecified *Archaea*	1.68%	17.12%	1.60%	11.84%	20.00%	2.13%	0.00%	15.56%	0.61%	5.48%

6 Exploring the microbiome of novel leafy greens in Eastern Africa for plant health

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Abstract

In Eastern Africa, indigenous leafy greens recently regained popularity for healthy diet, due to their robustness, high level of nutrients and, sometimes, special medicinal properties. Nothing is known about their associated microbiota, which could be involved in the specific resilience of greens. Therefore, we studied the archaeal and bacterial communities of four leafy greens Okra (*Abelmoschus esculentus*), Nightshade (*Solanum scabrum*), Spiderwisp (*Gynandropsis gynandra*) and Black Jack (*Bidens pilosa*) in a combined approach of amplicon libraries, qPCR and isolate characterization. The diversity in leafy greens was higher for bacteria (26,388 OTUs / H=6.91) than for archaea (2,995 OTUs / H=4.91). Members of *Enterobacteriacaea, Pseudomonacaea, Bacillacaea and Streptococcaceae* dominated bacteria, while *Thaumarchaeota* present the majority of archaea. The leafy green microbiota was characterized by an abundant and diverse rare and unidentified part. All four plants share a large core consist of *Bacillus*, *Sphingobium*, *Comamonadaceae*, *Pseudomonas*, and one archaeon from the Soil Crenarchaeotic Group. The habitat (phyllosphere, root endosphere, rhizosphere, soil) was crucial to microbiota composition, not the plant species. Leafy greens enrich bacteria with copiotrophic life strategy, whereas for archaea mostly oligotrophic taxa were found. In screening strategies for stress and health protecting traits, 24 promising candidates were found. They belong to *Bacillus* (*B. siamensis*, *B. velenzensis*, *B. amyloliquefaciens*, *B. methylotrophicus*, *B. vallismortis, B. subtilis)* and *Sphingomonas* (*S. echinoides* and *S. glacialis).* The strains will be used to develop a stress protecting consortium, in order to improve plant health and ecosystem health, and to ensure food security for smallholders in East Africa.

MANUSCRIPT

Introduction

Worldwide more than 60% of the population directly depend on agriculture, with a growing global demand for food and crop production of 100-110% from 2005 to 2050 (Food and Agriculture Organization of the United Nations., 2012; Tilman, Balzer, Hill, & Befort, 2011). Most of the land which is used to cover this demand is located in Latin America and sub-Saharan Africa, such as the Democratic Republic of the Congo or Uganda. In Uganda more than 80% of the population work in the agricultural sector, with a large share of smallholders, producing for their livelihoods. Although Uganda is largely harbouring very favourable soil conditions and climate, many households are suffering from food insecurity and malnutrition, mainly due to the high population growth (3.4%), weather variabilities, which are accumulating because of climate change, and plant pathogenic fungi in particular (Agriculture & Development, 2017; Venkateswarlu, Shanker, & Shanker, 2011). Phytopathogenic fungi like *Botrytis cinerea*, *Fusarium oxysporum*, *F. verticillioides*, *Sclerotium rolfsii*, and *Verticillium dahliae* are listed among the top fungal pathogens with economic importance in Eastern Africa (Dean et al., 2012). To counteract food insecurity, pests are mainly controlled by using chemical pesticides. Besides their desired effects, pesticides also have poisonous effects on the environment and on humans. In humans these pesticides cause severe disease pattern ranging from cancer over adverse reproductive outcome to immunological effects and neuropsychological dysfunctions (al-Saleh, 1994; Rosenstock et al., 1991). Furthermore, pesticides also heavily affect the environment, contaminating soil, ground and drinking water. Due to the growing demand for healthier and more productive crops going along with the problems arising from the excessive use of chemical pesticides, safer and more sustainable alternatives are needed to ensure future food supply.

One alternative to cut the dependency on pesticides and to counteract food-insecurity could be to switch to more robust and healthier plants. Commercial crops were bred for an increase in productivity, incidentally loosing resistance to stress factors (Venkateswarlu et al., 2011). Some indigenous plants are found to be much more robust and healthier, leading to a growing popularity in Eastern Africa (Cernansky, 2015). Although leafy greens were so far mostly ignored by African companies and science, researchers try now to experimentally improve their productivity. Especially the leafy greens Okra (*Abelmoschus esculentus*),

Nightshade (*Solanum scabrum*), Spiderwisp (*Gynandropsis gynandra*), and Black Jack (*Bidens pilosa*) show many beneficial traits such as higher levels of proteins, iron, vitamins and other valuable nutrients compared to popular non-native crops (e.g. kale and cabbage), medicinal properties, and the ability to stand biotic and abiotic stresses (Bartolome et al., 2013; Cernansky, 2015; S. Kumar et al., 2010; Onyango et al., 2013; Ronoh et al., 2019). Further, these plants can be harvested much earlier and are therefore less vulnerable to irregular rainfall due to climate change. Therefore they are increasingly used in rural agriculture, cuisine and medicine (Cernansky, 2015). But the question remains why these plants are so robust and if this robustness can be transferred to other crops.

Plant fitness is not solely depending on plant-genotype. Plants host highly diverse, and to a certain extent, species-specific microbial communities, which can influence plant growth, productivity, adaptation, and health (Berg et al., 2009; Bulgarelli et al., 2013; Wagner et al., 2016; Yeoh et al., 2017). Plant growth can be directly influenced by improving nutrient supply of micro- and macronutrients, e.g. by nitrogen fixation or phosphorous solubilisation, as shown for bacteria (Chanway, Hynes, & Nelson, 1989; Unno et al., 2005). Further, bacteria can also alter plant growth through hormonal stimulation, such as the production of ethylenes, cytokins, and indole-3-acetic acid (IAA) (Idris, Iglesias, Talon, & Borriss, 2007). So far, research on plant-microbe interactions has been mainly focussing on bacteria and fungi, whereas studies on plant-associated archaea are scarce. Recent studies have found archaea to be widespread as stable components of the microbiome of plants in pristine environments, such as in the vegetation of bogs, as well as in domesticated plants like arugula, rice, and maize (Erkel et al., 2006; Hardoim et al., 2015; Moissl-Eichinger et al., 2018; Pump, Pratscher, & Conrad, 2015). There they especially colonize the rhizosphere at high abundances (Chelius & Triplett, 2001b). Furthermore, metagenomics analysis have shown that even archaea have the potential to interact with the plant by supporting nutrient supply and plant growth promotion via auxin biosynthesis (Taffner et al., 2018; Taffner et al., 2019). Microorganisms can also indirectly support plant growth by antagonizing pathogens, through production of inhibitory substrates such as volatile organic compounds (VOC), toxins or diffusible antibiotics (Lugtenberg & Kamilova, 2009; Whipps, 2001). However, archaea are not known yet for their antagonistic potential, as most studied interactions with other microorganisms were found to be symbiotic (Moissl-Eichinger & Huber, 2011). Our hypothesis is that the microbiome

strongly contributes to the fitness and health of the host plant, and accordingly contributes to the robustness of the leafy greens.

The goal of this study is to characterize the colonization and the role of the bacterial and archaeal community in natural leafy greens, to identify key species in order to develop a biocontrol agent to increase robustness and health in crops, supporting smallholders sustainably in rural areas of Uganda. To achieve this, we combined next-generation sequencing and characterization of bacterial isolates as well as antagonistic testing against main pathogenic fungi (*B. cinerea*, *F. oxysporum*, *F. verticillioides*, *S. rolfsii* and *V. dahliae*).

Material and Methods

Experimental design

The leafy greens Black Jack (B. pilosa), Okra (A. caillei), Nightshade (S. scabrum), and Spiderwisp (G. gynandra) were sampled in Kasangati, Uganda (0° 26' 33''N, 32° 36' 19''E) in April 2017. Four samples of each leafy green consisting of one individual specimen were harvested. Except for Nightshade, where three specimens per sample were taken. Throughout the study plant leaves and stalks were termed as phyllosphere. Additionally, 4 bulk soil samples were collected as reference. Plant and soil samples were placed in air-tight plastic bags, kept cool and transferred to the laboratory. Soil parameters were analysed by "AGROLAB Agrar und Umwelt GmbH" (Sarstedt, Nümbrecht, Germany). The soil texture was sandy loam with pH= 5.9, 3,7% organic matter, and the nutrient contents [mg/1000g soil]: 413 K, 86 P, and 214 Mg. All further experiments described were performed at the Institute of Environmental Biotechnology, University of Technology, Graz in Austria.

Isolation of bacterial strains and total-community DNA

Samples were processed in order to isolate bacterial strains and total-community DNA. Three g of the phyllosphere and 5-10 g of the rhizosphere and the soil per replicate were physically disrupted in a Bag Mixer (Interscience) with 15 ml of 0.85% NaCl. Rhizosphere samples were further surface sterilised with a 4% sodium hypochloride solution (NaClO) for 3 min, washed 4 times with 0.85% sodium chloride (NaCl), resuspended in 15 ml NaCl, and physically disrupted with a sterile mortar and pestile, according to the protocol described by Bragina et al., 2012. Bacterial strains were isolated according to the protocol of Berg and colleagues (Berg et al., 2006). Briefly, 100 µl of the 15 ml 0.85% NaCl suspensions of each sample were plated onto nutrient broth II (NBII) agar plates, incubated for 5 days at 20°C and CFU were counted. In total 512 strains were randomly selected and stored in 20% Glycerol at -70°C for further characterization. Pellets of microorganisms were obtained by centrifugation (16,500 g, 20 min, 4°C) of 2 x 2 ml of the suspension in Eppendorf tubes and stored at -70°C for further processing. Community DNA was further extracted from sample pellets. using "FastDNA Spin Kit for soil" (MP Biomedical, Eschwege, Germany) and cleaned with GENECLEAN TurboTM Kit (MP Biomedicals, Eschwege, Germany) following the manufacturer's instructions for genomic DNA.

Preparation of the 16S rRNA gene fragment amplicon library for Illumina sequencing

Total-community DNA from the three habitats of the four leafy greens and soil was subjected to PCR based barcoding. The bacterial PCR approach was carried out with the universal bacterial primer set 515f-806r (Caporaso et al., 2011) and PNA Mix (Lundberg, Yourstone, Mieczkowski, Jones, & Dangl, 2013) to remove plastid DNA. In order to amplify the archaeal 16S rRNA gene, a nested PCR was performed using the archaea-specific primers 344f and 915r in the first PCR. In a second PCR approach the modified primer pair S-D-Arch-0349-a-S-17/S-D-Arch-0519-a-A-16 (here 349f/519r (Klindworth et al., 2013)) with an additional 10 bp primer-pad (TATGGTAATT/AGTCAGCCAG) and linker (GT/GG) was used, according to the protocols of the Earth Microbiome Project (Walters et al., 2016). In a third PCR the Golay barcodes were annealed. All PCR reactions were conducted as previously described (Julian Taffner et al., 2019). Bacterial and archaeal PCR reactions were conducted as triplicates, purified with the Wizard SV Gel and PCR cleanup system (Promega, Madison, WI), and pooled to equimolarity. Sequencing was carried out by Eurofins MWG Operon (Eurofins, Ebersberg, Germany) with an Illumina HiSeq 2500 system.

Bioinformatic processing of 16S rRNA gene fragments

The generated 16S rRNA gene libraries were pre-processed using the bioinformatics tool Quantitative Insights Into Microbial Ecology (QIIME) release 1.9.1 (Caporaso et al., 2010) and QIIME 2 (2018.2). First the read quality was checked with fastqc and barcodes were extracted. After the length- and quality-filtering, features' taxonomy assignment was conducted. As a classifier, SILVA reference data base version 128 and the Silva 16S (349af - 519ar 99 otusversion 128) Archaeal database were used with a 97% and 99% similarity cut-off, for bacteria and archaea respectively. OTUs containing mitochondria or chloroplasts were removed. Data analysis was performed according to the "moving pictures" tutorial from QIIME 2, provided at the QIIME2 homepage (https://docs.qiime2.org/2018.2/). For evaluating alpha diversity, Kruskal-Wallis all groups and pairwise, alpha rarefaction and Shannon diversity index were calculated. Beta diversity was analysed by Principal coordinate analysis (PCoA) plots and ANOSIM test. The PCoA plot was based on phylogenetic distance metrics of weighted UniFrac and visualised with EMPEROR. The non-parametric ANOSIM test was evaluated on the basis of 999 permutations. To visualise the bacterial distribution among the four leafy greens Cytoscape 3.3.0 software was used (Shannon et al., 2003). Abundant sequences with a low

taxonomical resolution were additionally assigned by using the nucleotide BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Screening for antagonistic activity of bacterial strains

The 512 randomly selected bacterial isolates were screened for antagonistic activity towards main phytopathogenic fungi Botrytis cinerea, Fusarium oxysporum, Fusarium verticillioides, Sclerotium rolfsii and Verticillium dahlia, provided by the Institute of Environmental Biotechnology (Berg et al., 2006). For the screening dual culture in vitro assays were conducted on Waksman agar (WA) plates with four bacterial isolates each, based on protocols described by Berg et al., 2002. All isolates were screened in independent triplicates and were evaluated regarding their antagonistic activity: 0 (No antagonistic effect; fungi overgrow bacteria); 1 (Bacteria are not overgrown but in touch with fungi); 2 (Fungi and bacteria do not touch; halo is visible but small (< 5 mm)); 3 (Clear halo between fungi and bacteria of at least 5 mm).

Sequencing of antagonistic bacteria

DNA of 20 identified bacterial isolates with strong antagonistic traits against all tested fungi was extracted by ribolysing in glass-bead filled tubes. BOX-PCR was performed according to the protocol of Rademaker & de Bruijn, 1997to resolve genetic diversity of bacterial isolates, using the BOXA1R primer 5′-CTA CGG CAA GGC GAC GCT GAC G-3′. After separation by gelelectrophoresis, resulting band pattern were compared with "Gel Compar II" V.5.1 (Applied Maths, Kortrijk, Belgium) and similar isolates were grouped accordingly (Berg et al., 2002). Different isolates were further sequenced based on the 16S rRNA gene fragment and taxonomically identified by manual BLAST search (https://blast.ncbi.nlm.nih.gov/).

Screening for antifungal VOCs production

The screening for antifungal VOC-producing strains was carried out using a two-clamp VOC assay (Cernava et al., 2015). Bacterial isolates and pathogenic fungi were streaked onto 6-well plates containing nutrient agar (for bacteria) or WA (for fungi). Bacteria were put up side down onto fungal growth plates, separated by a sterile, perforated silicone foil. The arrangement was fixed with clamps. After 7 d of incubation the diameter of fungal hyphae was measured and compared to a reference.

Abiotic stress assays and phosphate-solubilization tests

Potential antagonistic isolates were screened for resistance to abiotic stress such as drought, salinity and reactive oxygen, as well as their potential to solubilize phosphate, as previously described by Zachow and colleagues (Zachow et al., 2013). For reactive oxygen species tests, bacterial isolates were cultivated with different tellurite concentrations (1, 3, 5, 7, 9, 10, 13, 15, 18 and 20 μg/ml). In an additional test, bacterial isolates were cultivated with different hydrogen peroxide concentrations (100, 300, 500, 700, 900, 1000, 1300, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3500, 3800 and 4000 μmol). Growth has been measured after 24 h at 30°C under agitation in four replicates using the plate reader (Infinite 200, Tecan Trading AG, Switzerland) at a wavelength of 600 nm. For evaluation of the tolerated osmolarity level, bacterial isolates were cultivated in LB media with sodium chloride concentrations from 0%-15% in steps of 1%). Growth has been measured in four replicates after 24 h, 48 h, 72 h and 144 h using the plate reader. For the desiccation assay 20 μl of bacterial overnight culture were dried under sterile conditions in a 96-well plate and resuspended after 24 h, 48 h, 5d (120 h), 7d (168 h), 14 d (336 h), 30 d (720 h), 60 d (1440 h) and 88 d (2112 h) in 20 μl 0.9% NaCl. Further 10 μl of the resuspended cells were dropped onto LB-agar plates in a dilution series. Growth was evaluated by counting colonies and calculating the Colony Forming Units (CFU).

Screening for plant-growth promoting activities

Growth-promoting activities of bacterial isolates were tested on tomato (Lycopersicon esculentum cv. Moneymaker, Austrosaat AG, Austria) plants (Zachow et al., 2013). Tomato seeds were primed with bacterial cultures suspended in 20 ml sterile water and incubated for 4 h under agitation. CFU and OD600 were determined. Two pouches were prepared per strain with 8-9 seeds each. After 15 d the plants were harvested, leaves and roots weighted, and roots mortared for CFU determination.

Results

The general community structure of Procaryota associated to leafy greens

Sequencing of the 16S rRNA gene fragments originating from the phyllosphere, root-endosphere, rhizosphere, and soil of the leafy greens Okra, Nightshade, Spiderwisp, and Black Jack resulted in a total of 9,643,229 high quality bacterial and 2,663,458 archaeal reads. These reads were then clustered in a total of 26,388 and 2,995 distinct features, for bacteria and archaea respectively.

The bacterial core microbiome revealed similarities and differences between the phytobiome composition referring to the plant genotype and micro-habitat (Fig. 1).

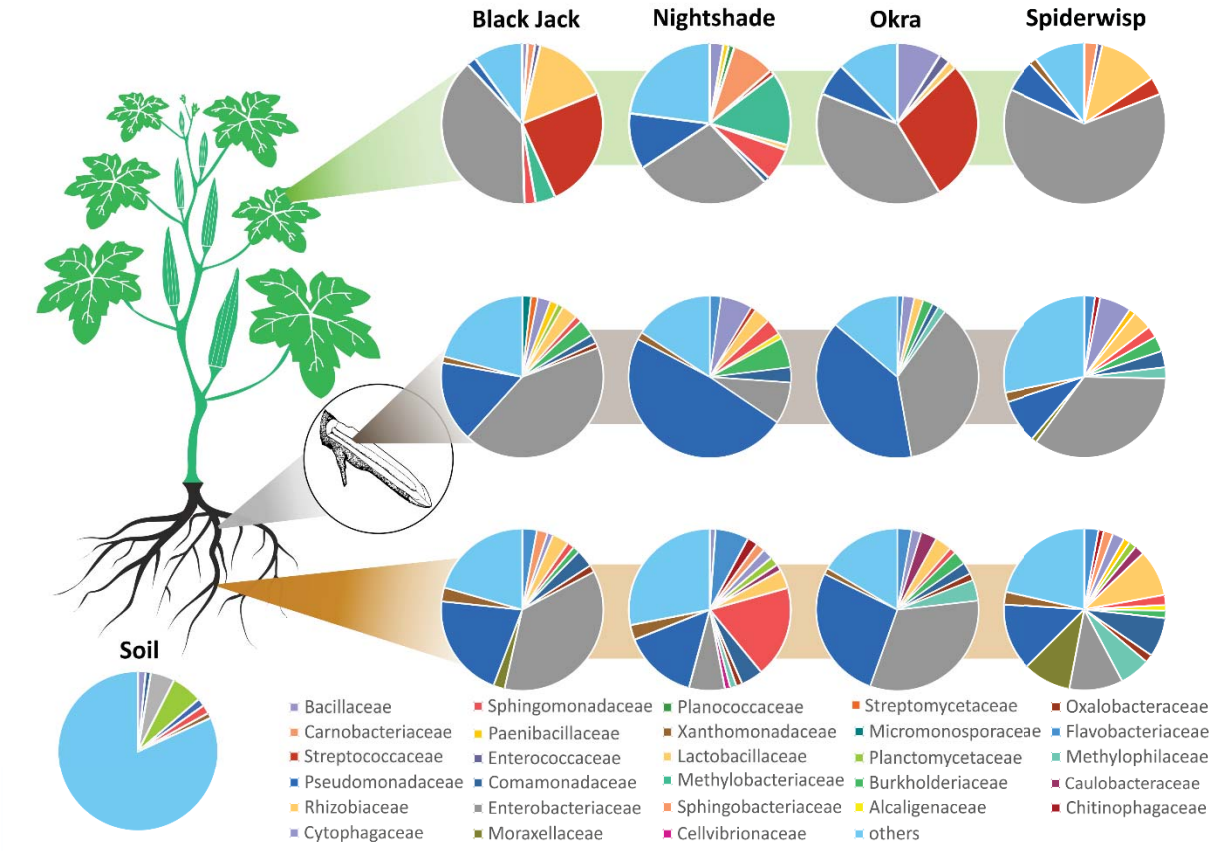

Figure 1: Bacterial core microbiome of leafy greens. The composition of the microbiome of Okra, Black Jack, Nightshade and Spiderwisp and their microhabitats are displayed at family level: phyllosphere (green stripe), root-endosphere (gray stripe) and rhizosphere (brown stripe). Families with abundances below 1% of total microbiome are captured within "others".

In the phyllosphere of leafy greens *Enterobacteriacaea* (42.2%) and *Streptococcaceae* (14.4%) were dominating the bacterial community, whereas in the root-endosphere and rhizosphere *Enterobacteriacaea* (30.7% and 21.6%, respectively) and *Pseudomonadacaea* (28.0%

and 19.0%, respectively) were predominant. In general, *Sphingomonadaceae* (4.2%), *Lactobacillaceae* (3.3%), *Bacillaceae* (2.9%), *Rhizobiaceae* (2.7%), *Comamonadaceae* (2.5%), *Flavobacteriaceae* (2.0%), and *Xanthomonadaceae* (1.5%) were ubiquitous on leafy greens but less abundant. In the phyllosphere of Okra and Black Jack *Streptococcaceae* were dominant with around one fourth of the core microbiome. Black Jack and Spiderwisp harboured both *Lactobacillaceae* with 12.0% - 15.3% in the phyllosphere. *Bacillaceae* and *Pseudomonadaceae* were part of the core microbiome of each plant in each habitat (1.09% - 6.33%), with the exception of the phyllosphere of Spiderwisp, where no *Bacillaceae* could be found. Throughout all habitats and plants, the fraction of families with lower abundance than 1% ("others") was relatively high (13.9% - 21.6%). These bacteria were mainly belonging to *Oxalobacteraceae* (0.9%), *Caulobacteraceae* (0.9%), unknown *Acidobacteria* family (0.9%), *Sphingobacteriaceae* (0.8%), *Paenibacillaceae* (0.8%), unknown *Rhizobiales* family (0.7%), *Chitinophagaceae* (0.7%), *Planctomycetaceae* (0.6%), *Enterococcaceae* (0.6%), and *Alcaligenaceae* (0.5%).

The archaeal community in leafy greens was clearly dominated by the phylum *Thaumarchaeota* (89.0%). In general, a high proportion of unassigned reads of up to 20.7% was detected, especially associated to the phyllosphere of Black Jack and Okra. In all leafy greens *Euryarchaeota* could be found as well but at low relative abundances (0.7% - 1.0%), except of Spiderwisp, in which no *Euryarchaeota* were detected. At class level archaea of the Soil Crenarchaeotic Group (SCG) were relatively most abundant (56.2%), followed by unassigned *Thaumarchaeota* (22.9%). Archaea of the SCG were especially abundant in Nightshade and Spiderwisp. Methanogenic archaea of the class *Methanomicrobia* were mostly found in the phyllosphere and the root-endosphere of all leafy greens, but not in Spiderwisp.

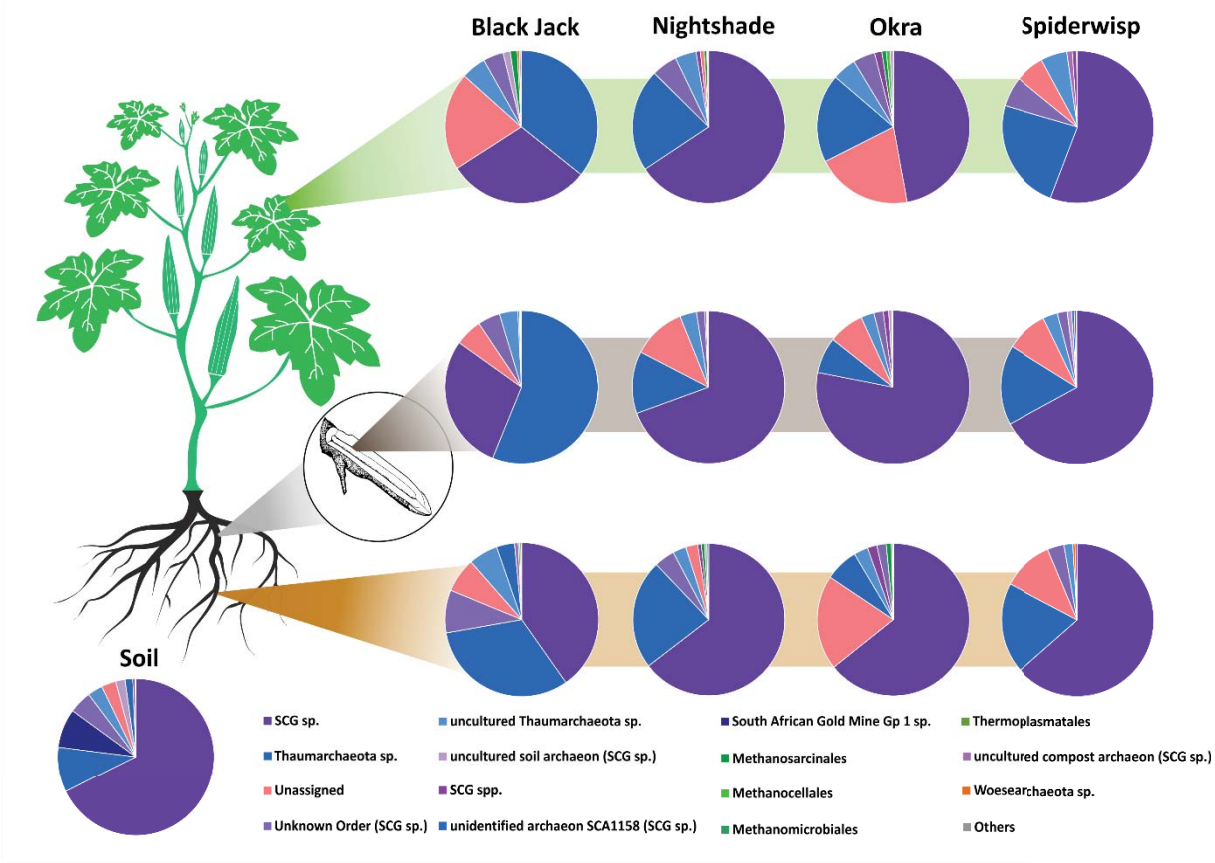

Figure 2: Archaeal community in leafy greens. The composition of the archaeal community of Okra, Black Jack, Nightshade and Spiderwisp and their microhabitats is displayed at order level: phyllosphere (green stripe), root-endosphere (gray stripe) and rhizosphere (brown stripe).

Bacterial diversity associated to leafy greens

Diversity metrics based on phylogeny were calculated with QIIME 2 to show assimilarities and dissimilarities of the bacterial community of leafy greens. To gain insights into the species diversity within the bacterial community, Shannon's diversity index H was calculated for the habitats as well as for the plants. The diversity in the plant-habitats increased from rhizosphere to phyllosphere, with an exception for Nightshade which showed the least diversity in root-endosphere. However, diversity within Nightshade's rhizosphere was the highest comparing all plants (H= 7.81 ± 0.21). Diversity within bulk soil samples was far higher (H= 9.41 ± 0.42) compared to the plants (H (average)= 6.91 ± 0.16, ranging from H= 5.31 to H= 6.24).

Further, the alpha- and beta-diversity analysis, which is visualised by principal coordinate analysis (weighted UniFrac) and faith's phylogenetic diversity, is shown in Figure 3.

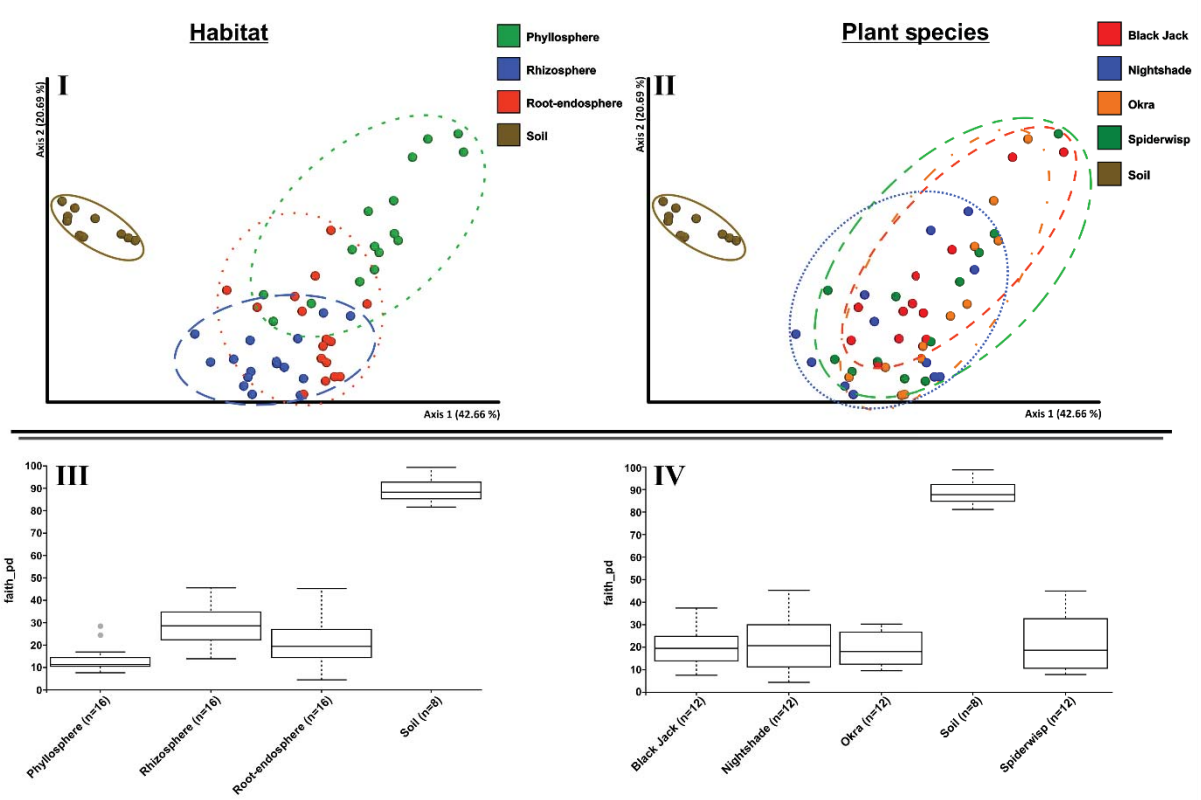

Figure 3: Bacterial alpha- and beta-diversity of leafy greens. PCoA plots of the 16S rRNA amplicon datasets of the four leafy greens (Okra, Nightshade, Spiderwisp and Black Jack) were constructed based on phylogenetic distance metrics (weighted UniFrac). The distance between the data points negatively correlates with the similarity of the communities. **I** clusters the communities based on habitat (phyllosphere, rhizosphere, root-endosphere, and soil), **II** based on organisms (Black Jack, Nightshade, Okra, Spiderwisp, and soil). Comparison of bacterial alpha-diversity based on faith's phylogenetic diversity of the habitats and plant-types is shown in (III) and (IV), respectively.

Regarding the differences between microhabitats a cluster formation (Fig.3**Figure 3**, I) as well as a trend from rhizosphere to phyllosphere could be detected, whereas the bacterial community of rhizosphere was overlapping to some extend with root-endosphere. However, phyllosphere samples were more distinct. The soil showed a clear cluster and was significantly different to the other habitats with quantitative measures (ANOSIM test: R=0.504 and p-value=0.001). When assigning the same communities to their respective plants (Fig. 3, II), no distinct clustering could be detected (ANOSIM: R= 0.048 and p= 0.064). Only Nightshade showed a slightly different clustering pattern. For statistical investigation of relationships between plants (within-sample), alpha-diversity indices were calculated by Kruskal-Wallis (all

groups and pairwise). Alpha-diversity was significantly different between the habitats (p= 0.001), whereas the differences in diversity between the four plants were not significant (p= 0.080). The overall group of habitats differed in diversity, but with respect to pairwise investigations, this was due to differences of rhizosphere as well as root-endosphere to phyllosphere. Further group statistics showed that bacterial diversity of these leafy greens was not plant-type-specific. However, principal coordinate analysis and Kruskal-Wallis test revealed that microbial diversity was habitat-specific.

Archaeal diversity associated to leafy greens

Diversity was further analysed for the archaeal community associated to the leafy greens. Diversity calculated by Shannon index H of all plants was similar (H= 4.51 - 4.95), with the highest archaeal diversity in Nightshade (H= 4.95 ± 0.21). Within the plants, the diversity of the habitats did not differ much, ranging from H= 4.42 ± 0.37 in the root-endosphere to H= 4.92 ± 0.23 in the rhizosphere. The Shannon index for archaeal communities in bulk soil was similar at H= 5.26 ± 0.27.

Further alpha- and beta-diversity of the archaeal community was conducted and visualised in Figure 4. In a PCoA-plot (Fig.4, I), soil and the rhizosphere samples were clustering, whereas root-endosphere and phyllosphere were more widespread. Again, there was a pattern from rhizosphere to phyllosphere, as the clusters were overlapping, with the soil being embedded within the rhizosphere samples. In general habitats showed significant differences in diversity (ANOSIM: R= 0.226; p= 0.001), with soil showing the highest diversity (Fig.4, III). Analysing the beta-diversity regarding plant-type-specific differences, a cluster formation of Nightshade and Spiderwisp could be seen (Fig.4, II). These plant-type-specific differences were confirmed by ANOSIM-test (R= 0.131; p= 0.002), and found to be due to Nightshade and Black Jack (q< 0.05), based on pairwise comparison, whereas Spiderwisp and Okra showed similarities (q> 0.377). Further alpha-diversity analysis with Kruskal-Wallis (all groups and pairwise) confirmed that archaeal diversity was significantly different depending on the habitat (p= 0.001), as well as on the plant-type (p= 0.01), which is due to the significant different diversity of Nightshade (Fig.4, III&IV). However, pairwise comparison did not find

any significant differences between any of the plants (q> 0.08). Soil was significantly different to all plant habitats (q< 0.004), as well as the phyllosphere to the root-endosphere (q= 0.038).

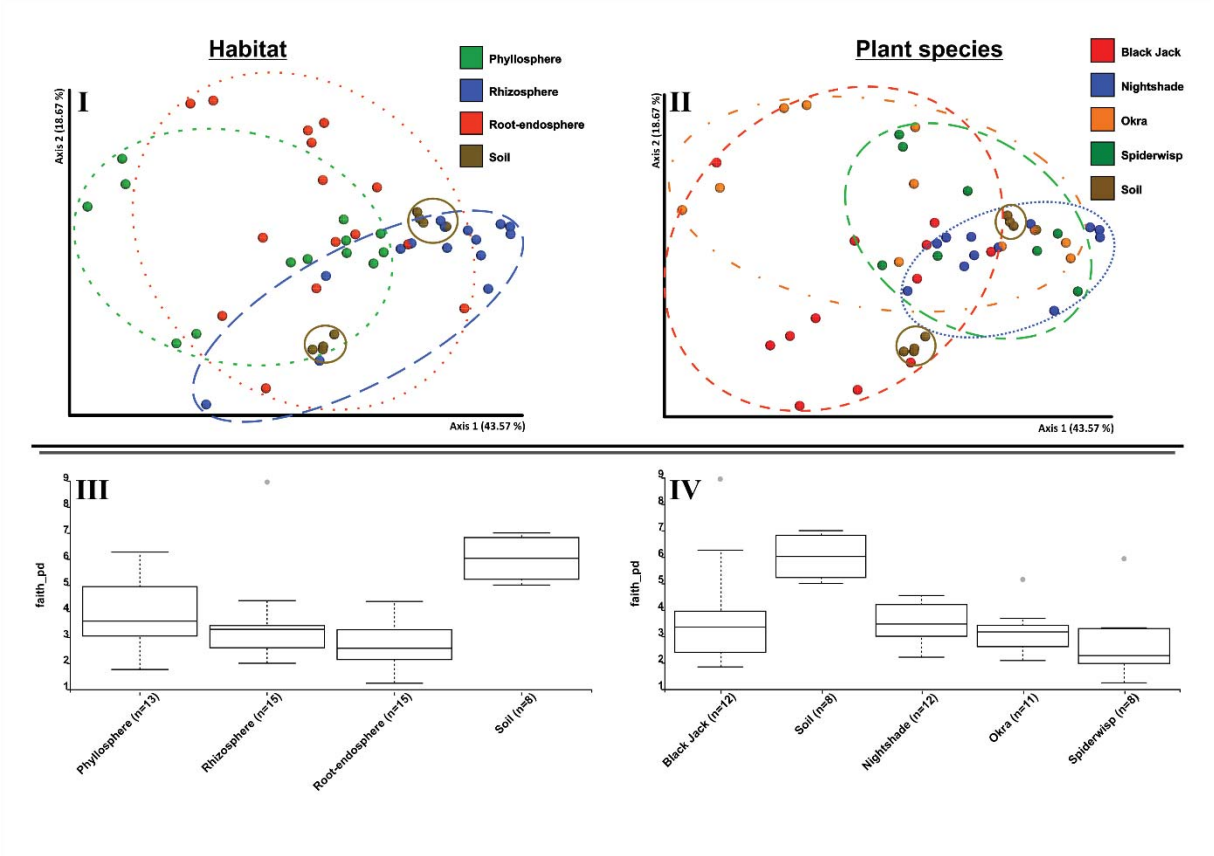

Figure 4: Alpha- and beta-diversity analysis of archaeal communities associated to leafy greens. PCoA plots based on the weighted Unifrac diversity metrics show the archaeal community of samples of the habitats phyllosphere, rhizosphere, root-endosphere, and soil (I) of the four leafy greens Black Jack, Nightshade, Okra, and Spiderwisp (II). Comparison of alpha-diversity based on faith's phylogenetic diversity of the habitats and plant-types is shown in (III) and (IV).

Analysis of the core microbiota of leafy greens

Microbial core communities across Nightshade, Okra, Spiderwisp and Black Jack were cross-linked based on taxonomic analysis at family level and visualised as a network using Cytoscape (Fig.5).

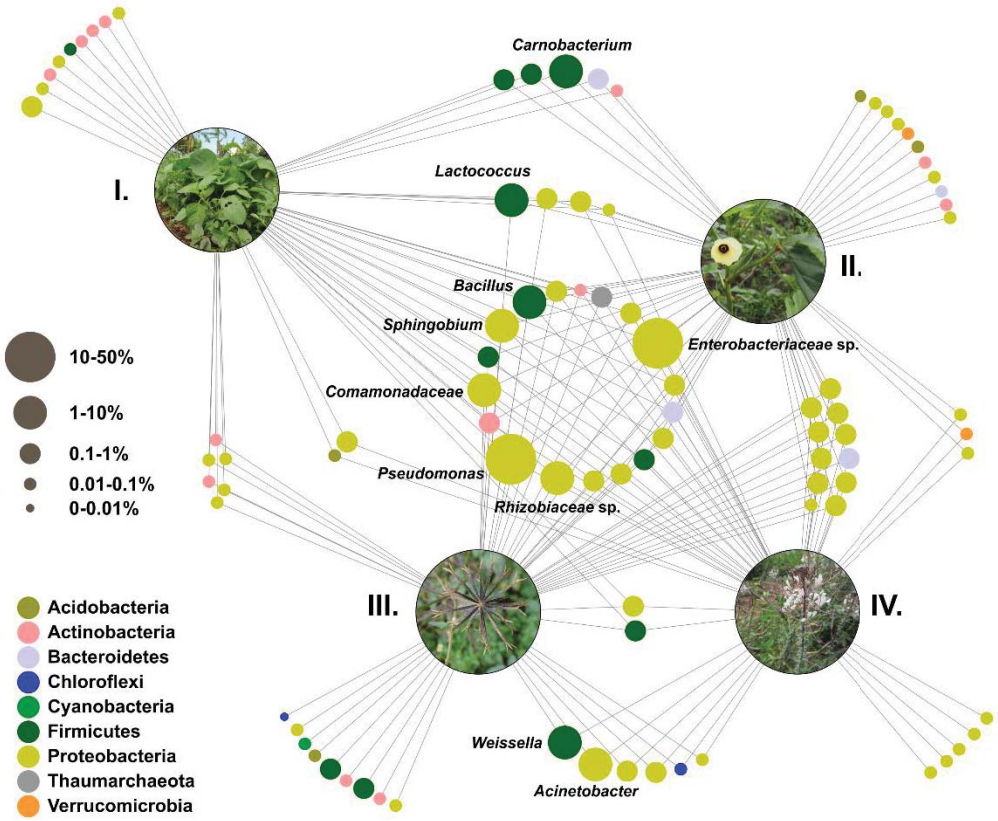

Figure 5: Feature-network based on taxonomic analysis at family level. Each node represents a family of the core microbiome and is coloured according to its phylum. Cross-linked nodes express families shared between the plants Nightshade, Okra, Black Jack and Spiderwisp. Legend: I: Nightshade, II: Okra, III: Black Jack and IV. Spiderwisp. Taxonomic 16s rRNA gene fragment data was obtained by using a universal bacterial primerset.

In total 91 features were identified, whereas only one belonged to *Archaea*. A big core microbiome of 18 families, such as *Bacillus*, *Sphingobium*, *Comamonadaceae*, *Pseudomonas*, and *Rhizobiaceae* (including the archaeal Soil Crenarchaeotic Group), mainly assigned to *Proteobacteria*, were shared between all four plants. Additional 11 families, also mostly *Proteobacteria*, were common in Okra, Spiderwisp, and Black Jack, thus communities associated to Nightshade were more specific and therefore further apart. Nightshade and Okra shared specific taxa of the family *Carnobacterium*, as well as Black Jack and Spiderwisp shared *Weissella* and *Acinetobacter*. Each plant showed some specific bacterial families that were unique in the core microbiome of the respective plant. The number of such distinctive communities ranged from five (Spiderwisp; IV) over nine (Black Jack and Nightshade; III and I) to 11 (Okra; II).

Screening and identification of bacterial antagonists against biotic and abiotic stress

Out of 512 randomly selected bacterial isolates from four leafy greens and bulk soil, 108 showed high antagonistic activity of category 3 (clear halo between fungi and bacteria ≥ 5 mm) against at least one pathogenic fungus (*Botrytis cinerea*, *Fusarium oxysporum*, *F. verticillioides*, *Sclerotium rolfsii* and *Verticillium dahliae*), 23 against even four fungi (Fig. 6). Screening tests results against *V. dahliae* needed different categories as cultivation of the fungi demanded a different procedure and was therefore not included into Venn diagram.

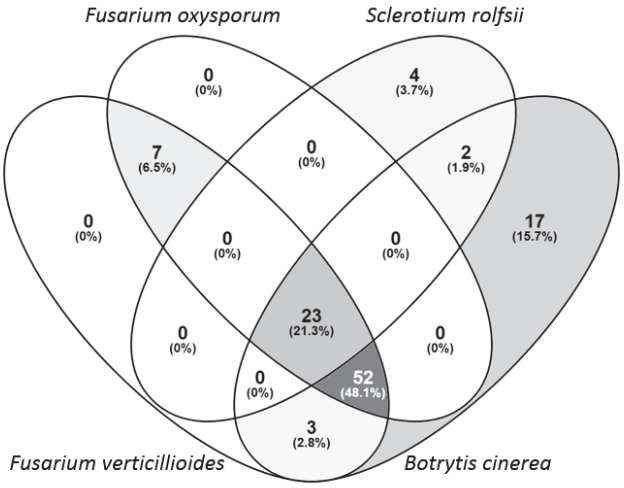

Figure 6: Quantity of bacterial isolates with antagonistic effects against fungal pathogens. Fungal pathogens considered are Fusarium oxysporum, Sclerotium rolfsii, F. verticillioides and Botrytis cinerea. Only bacterial antagonists showing high antagonistic activity (category 3) were assigned to their respective fungi.

44 isolates were highly active (category 3) against *V. dahliae*. Based on these results, a selection of 24 antagonists, mostly antagonising all tested pathogens, was chosen for further characterization. 12 of the isolates originated from soil and 12 antagonists were exclusively isolated from root-endosphere (nine isolates) and rhizosphere (three isolates). Antagonistic isolates were further identified using BOX-PCR and 16S sequencing. After excluding similar replicates based on BOX-PCR, 16 isolates were identified as *Bacillus* sp. with suggested species *B. siamensis*, *B. velenzensis*, *B. amyloliquefaciens*, *B. methylotrophicus*, *B. vallismortis* and *B. subtilis*. Further eight isolates were assigned to *Sphingomonas* sp. with hits for *S. echinoides* and *S. glacialis*. Combining the alignment results with similarity pattern of BOX PCR bands, isolates were clustered into five similarity groups.

The antagonistic bacterial strains were further characterized in order to evaluate their potential for application as future biocontrol agents (BCAs). Therefore, abiotic stress tests, comprising reactive oxygen species stress tests were conducted. None of the listed isolates showed ability to solubilize phosphate, neither could any of the isolates grow in presence of tellurite (TeO_2) concentrations between 1 and 20 µg/ml. Further results are summarized in table 1.

Table 1: Abiotic stress confrontation assays. Growth after desiccation was measured by CFU/ml: 0=CFU below 10^5 after drought for 2112h, 1= CFU above 10^5 after drought for 2112h. Reactive oxygen species test performed with hydrogen peroxide (H_2O_2): 0=growth lies below the threshold OD of 0.3. Other values show the highest concentration of H_2O_2, the culture could still tolerate. Osmolarity stress was tested with sodium chloride for various incubation times and concentrations: 0=growth lies below the threshold OD of 0.4. Other values show the highest concentration of NaCl, the culture could still tolerate. Stress tolerant results are highlighted in grey.

Origin	Microhabitat	Species	Drought	H_2O_2	NaCl 24 h	NaCl 48 h	NaCl 72h	NaCl 6d
Soil	Soil	*Bacillus sp.*	1	0	0	0	8%	6%
Soil	Soil	*Bacillus sp.*	1	2000 µmol	5%	7%	7%	7%
Soil	Soil	*Sphingomonas sp.*	1	0	0	0	8%	11%
Okra	Root-endosphere	*Sphingomonas sp.*	1	100 µmol	0	0	8%	10%
Okra	Root-endosphere	*Sphingomonas sp.*	1	0	0	0	8%	11%
Nightshade	Root-endosphere	*Bacillus sp.*	1	900 µmol	0	0	0%	0

The desiccation assay showed that all tested isolates were highly resistant to drought with CFU/ml of above 10^5 after almost three months (2112 h). The ability to resist reactive oxygen could not be shown against tellurite, but using hydrogen peroxide, three isolates were able to grow. Whereas the *Sphingomonas* sp. only tolerated 100 µmol, one *Bacillus* sp. still grew at a concentration of 2000 µmol H_2O_2. This species, showed additionally resistance to high levels of sodium chloride, already after 24 h. Other species needed longer to adapt to higher NaCl concentrations and showed tolerance only after an adaption phase of 72 h.

To further characterize and investigate the mechanism of antagonism of the isolates, two clamp VOC assays (TCVA) were performed. With this assay, we could not show antagonistic effects of bacteria against fungi, based on volatile organic compounds.

Plant growth promotion of bacterial antagonists

Isolated bacteria identified to antagonize fungal pathogens were additionally tested for their ability to promote plant growth. Therefore, tomato seeds (*Lycopersicon esculentum*) were primed with identified antagonistic isolates. The growth performance of seeds primed with *Bacillus* isolates resulted in an increased biomass of the seedlings of up to 70%, compared to the control. Priming with *Sphingomonas* species did not show any effect.

Embedding antagonists within the microbial network of leafy greens

Identified taxa with antagonistic activity were *Bacillaceae*, *Comamonadaceae*, *Pseudomonadaceae* and *Sphingomonadaceae*. The abundance of all four mentioned families within the microbiome of leafy greens is depicted in Figure 7.

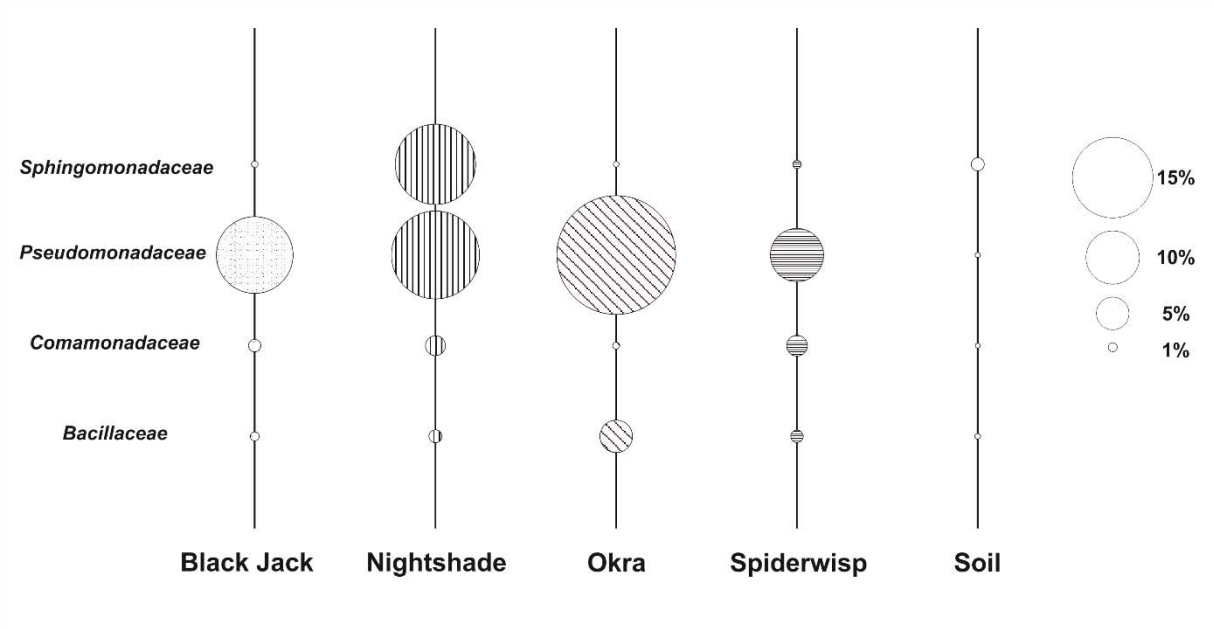

Figure 7: Relative abundance of antagonistic families in the microbiome associated to leafy greens. The diameter of the bubble represents the relative abundance of each family within the microbiome of leafy greens (Black Jack, Nightshade, Okra, and Spiderwisp) and Soil. Soil is used as a reference.

Pseudomonadaceae was the most abundant antagonistic family within the microbiome of each plant, with the highest relative portion in Okra. All antagonistic families were found in the soil but at lower levels, only *Sphingomonadaceae* was relatively more abundant in the soil than in the microbiome of all plants except Nightshade, which was showing the highest relative abundance of *Sphingomonadaceae*. Most antagonistic families were relatively enriched within the plant's microbiomes compared to their relative abundance within soil. The microbiome of Nightshade consisted of the highest share of antagonistic families (31.2%), followed by Okra (25.5%), Black Jack (16.3%) and Spiderwisp (14.8%). Within soil, antagonistic families comprised only 4.5% of all occurring microorganisms.

Discussion

Leafy greens enrich common bacteria with copiotrophic life style

In this study, the diversity and community structure of bacteria and archaea in uncultivated leafy greens was found to be habitat-specific, rather than plant genotype-specific. The impact of different factors (plant genotype, habitat, developmental stage and soil quality) is an old question in microbial ecology; where in many studies especially from natural vegetation the "plant genotype" was the winner (Berg & Smalla 2009;Berg et al., 2009). The less pronounced impact of the plant genotype can be explained by the life strategy of plants. to explain their behaviour and response to the environment. Naturally occurring leafy greens studied here are ubiquitous, mostly invasive, and produce a lot of offsprings, and are therefore categorized as copiothrophs (Andrews & Harris, 1986). The life strategy of these plants might also affect the life strategy of their associated microorganisms or enrich them. It has been shown that invasive plants, such as Cheatgrass, Knappweed, and Leafy spurge, enrich copiotrophic bacteria in their associated soil (Gibbons et al., 2017). In our study, the microbiome associated to leafy greens was neither specific nor depending on the plant-type. We found the most abundant bacterial phyla to follow the same life strategy as their host, such as *Proteobacteria*, *Firmicutes*, *Bacteriodetes*, and *Actinobacteria*, which can be categorized as copiotrophic (Ho, Lonardo, & Bodelier, 2017). In contrast, archaea, *Thaumarchaeota* in particular, are mostly considered to be oligotrophic (Uksa et al., 2015; Youssef et al., 2015). This indicates high substrate specificity, supports the assumption of a niche-colonization of archaea and a role as followers of bacteria.

Here, the habitat could be identified as a driving factor determining microbial diversity with the highest diversity found in the rhizosphere, which has been described before as the "rhizosphere effect" (Buée et al., 2009; Foster, Rovira, & Cock, 1983). The rhizosphere is of special interest, as in this habitat direct exchange of metabolites between the plant host and the microorganisms takes place. While the rhizosphere effect and assembly are well studied for bacteria(Berendsen, Pieterse, & Bakker, 2012; Philippot et al., 2013), we found this for archaea too, especially for Nightshade. The enrichment through root exudates has already been shown for archaea in tomato plants (Simon et al., 2005), and might explain the differences in archaeal diversity in Nightshade compared to the other leafy greens, as Nightshade is phylogenetically closer related to tomato.

Increased microbial diversity of leafy greens compared to cultivated crops

All leafy greens, Okra, Nightshade, Black Jack, and Spiderwisp, showed similarities in their microbial diversity, with Shannon index H-values ranging from 5.31 and 4.51 (Okra) to 6.24 and 4.95 (Nightshade), for bacteria and archaea respectively. Compared to the bacterial diversity of the phyllosphere of the four leafy greens, ranging from 4.40 (Okra) to 5.74 (Nightshade), the diversity of cultivated leafy greens, such as spinach (*Spinacia oleraceae*) was lower, with an H-value of 3.15 (Lopez-Velasco et al., 2013). Another main cultivated crop worldwide is maize (Development, 2013). Its Shannon diversity index for the rhizosphere was found to be 3.42, which is distinctly lower than the diversity of the rhizosphere of the leafy greens (H= 6.91) (García-Salamanca et al., 2013). Further, also the archaeal diversity in leafy greens was higher than in cultivated plants. Shannon index H of cultivated plants such as rice (*Oryza sativ*), Barbados nut (*Jatropha curcas*), tomato (*Solanum lycopersicum* L.) was at 4.08–4.43, 3.16, 3.4, respectively, whereas the diversity of leafy greens was between 4.51 to 4.95 (Dubey, Kollah, Gour, Shukla, & Mohanty, 2016; Lee, Jeong, Kim, Madsen, & Jeon, 2015). Indigenous leafy greens are not overbred, as they used to be collected in the wild and just recently found their way into agriculture. In previous studies organic farming or sustainable practices led to increased diversity loss of the microbiome compared to conventional intensive farming practices (Lupwayi, Rice, & Clayton, 1998). Comparing organic farming with conventional farming, significant differences in the microbiome of corn, melon, pepper and tomato (p-value=0.049), as well as on the soil, were found (Hartmann et al., 2015; Xia et al., 2015). Comparing diversity indices of uncultivated leafy greens from Uganda with crops grown in intensive agriculture, we conclude that intensive breeding as well as intensive agricultural practices are the main factors for the loss of diversity in the microbiome of crops (Pérez-Jaramillo, Mendes, & Raaijmakers, 2016). Natural grown vegetables, like leafy greens, have a high microbial diversity, which is directly correlated to healthier plants, less vulnerable to pathogenic outbreaks (Berg, Erlacher, & Grube, 2015).

Traits of the core microbiome in leafy greens

Multiple taxa found in the core microbiome of leafy greens were previously shown to have favourable traits. *Enterobacteriaceae* and *Pseudomonadaceae* were the most dominant bacterial families in leafy greens and are also found to be dominant in other crops, such as sugarcane (de Souza et al., 2016) or maize (Johnston-Monje & Raizada, 2011). *Enterobacteriaceae* are reported to have plant-growth promoting activities and can compete pathogens such as *Rhizoctonia solani* (Shoebitz et al., 2009). Though *Enterobacteriaceae* also include human enteric pathogens , they are rather seen as immune stimulant or "natural vaccination" than a pathogen, as *Enterobacteriaceae* have always been part of human diet as part of plant microbiomes (Berg et al., 2015; Brandl, 2006). Besides, Pseudomonads are also reported to have plant growth promoting activities (Hayat et al., 2010). Additionally, *Pseudomonadacea* can antagonize phytopathogenic fungi by competition and production of antimicrobial metabolites (Couillerot, Prigent-Combaret, Caballero-Mellado, & Moënne-Loccoz, 2009; Haas & Défago, 2005). *Actinobacteria* and *Proteobacteria* are also important for plant protection against fungal infections (Mendes et al., 2011), of which *Streptomycetaceae* (*Actinobacteria*) as well as *Sphingomonadaceae*, *Pseudomonadaceae* and *Enterobacteriaceae* (*Proteobacteria*) were broadly distributed throughout the core microbiome of leafy greens. Besides the dominant families mentioned, *Xanthomonadaceae* (*Proteobacteria*), *Bacillaceae* and *Paenibacillaceae* (*Firmicutes*) were less abundant but also ubiquitous in the leafy green's microbiomes. *Bacillaceae* are known to comprise growth-promoting species such as *Bacillus subtilis*, *B. amyloliquefaciens* and *B. cereus* (Hayat et al., 2010). Furthermore, the families *Xanthomonadaceae* and *Paenibacillaceae* were also reported to have plant growth-promoting properties, comprising species such as *Stenotrophomonas rhizophila*, *S. maltophilia* and *Paenibacillus amylolyticus* among others (Hariprasad, Venkateswaran, & Niranjana, 2014).

The archaeal community was clearly dominated by *Thaumarchaeota*, which are common colonizers of leafy greens, such as arugula (Julian Taffner et al., 2019). This phylum consists mostly of ammonia oxidizing archaea (AOA), which are important for nitrogen cycling (Francis, Beman, & Kuypers, 2007) and therefore for the nutrient support of the plant . Further, recent studies could show that they have the potential to directly support plant growth via auxin biosynthesis, a plant growth hormone (Taffner et al., 2018). Besides *Thaumarchaeota*,

methanogens of the phylum *Euryarchaeota* could be found. These are also common in plants, colonizing anoxic niches e.g. in the rhizosphere, such as in maize or arugula (Chelius & Triplett, 2001a; Taffner et al., 2019). However, there was a high relative abundance of taxonomically unassigned archaeal features, although an up-to-date established pipeline was used for the bioinformatic analysis. This limitation is well-known for archaea, especially in novel, so far less studied habitats like Uganda, and is mainly due to still poorly defined reference databases. We can conclude that the core microbiome of leafy greens contained several taxa with the potential to support plant growth and protection against pathogenic fungi, and thereby contributing to the robustness and health of its plant host.

Promising key species for future biocontrol agents

In the core microbiome of the leafy greens we could identify *Bacillus* sp. and *Sphingomonas* sp. playing a pivotal role in suppressing main pathogenic fungi *B. cinerea*, *F. oxysporum*, *F. verticillioides*, *S. rolfsii* and *V. dahliae*. Isolated antagonists were tested for their resistance to abiotic stresses and their plant-growth promotion capabilities. *Bacillus* strains were previously reported as solubilizers of inorganic phosphate (Hayat et al., 2010), though we could not identify phosphate solubilizers among our isolates. However, priming of tomato seeds with *Bacillus* isolates resulted in significant plant-growth promotion of up to 70% compared to the control, whereas *Sphingomonas* did not show any effect. Abiotic stresses, such as oxidative stress, are important factors for plants, though just one *Bacillus* sp. was able to tolerate a higher load of hydrogen peroxide. Further, salinity reduces plant's water-uptake efficiency and photosynthesis rate, but microorganisms capable of dealing with osmolarity stress may also confer resistance in plants to salt stress (Mayak, Tirosh, & Glick, 2004). All our isolates were able to grow under saline conditions up to 10% salinity. Furthermore, episodic drying and re-wetting of soil cause fluctuations in the soil's water potential and challenges microbes. We could show that all our candidates were highly resistant to desiccation. *Bacillus* sp. are reported to have plant growth-promoting properties and to produce antimicrobial substances, such as *B. subtilis* producing mycosubtilin and lipopeptides (Leclère et al., 2005), or *B. amyloliquefaciens* antagonising through bacillomycin D (Koumoutsi et al., 2004; A. Kumar & Johri, 2012). *Sphingomonas* are mainly known for their ability to degrade refractory contaminants, but have also been reported to antagonize *Verticillium dahlia*, *Pseudomonas syringae* (Innerebner, Knief, &

Vorholt, 2011; White, Sutton, & Ringelberg, 1996), and several *Fusarium* species (Wachowska, 2013). Furthermore, they can promote plant growth by producing gibberellins (GA) and indole acetic acid (IAA), improving agricultural and horticultural productivity, which has also been reported for *Bacillus* sp. (Khan et al., 2014). These antagonistic and plant-growth-promoting activities of *Bacillus* and *Sphingomonas* make them promising candidates for application against fungal infections and to increase robustness and plant health in Ugandan agriculture.

Conclusion:

In our study we found the microbiome of natural leafy greens from Uganda to be significantly more diverse than of cultivated crops. We could identify the habitat to be rather the driving force of microbial diversity than plant species. However, Okra, Nightshade, Black Jack, and Spiderwisp enriched a core microbiome with the same copiotrophic life strategy as its host, harbouring microbes with strong antagonistic activities against main pathogenic fungi, mechanisms to stand abiotic stresses and plant-growth promotion activities. Especially 6 isolates assigned to the families *Sphingomonadaceae* and *Bacillaceae* showed to be promising key-candidates for future biocontrol agents, supporting smallholders in rural areas of Uganda. The biocontrol approach is a chance to reduce or even replace excessive pesticide use for crops in Eastern Africa, supporting smallholders, and reducing risks for human and environmental health.

References

Agriculture, I. C. for T., & Development, U. S. A. for I. (2017). Climate-Smart Agriculture in Uganda.

al-Saleh, I. A. (1994). Pesticides: a review article. J. Environ. Pathol. Toxicol. Oncol. 13:151–161.

Andrews, J. H., & Harris, R. F. (1986). r- and K-Selection and Microbial Ecology (pp. 99–147).

Bartolome, A. P., Villaseñor, I. M., & Yang, W.-C. (2013). Bidens pilosa L. (Asteraceae): Botanical Properties, Traditional Uses, Phytochemistry, and Pharmacology. Evid. Based. Complement. Alternat. Med. 2013:340215.

Berendsen, R. L., Pieterse, C. M. J., & Bakker, P. A. H. M. (2012). The rhizosphere microbiome and plant health. Trends Plant Sci. 17:478–486.

Berg, G., Erlacher, A., & Grube, M. (2015). The Edible Plant Microbiome: Importance and Health Issues. In *Principles of Plant-Microbe Interactions* (pp. 419–426).

Berg, G., Opelt, K., Zachow, C., Lottmann, J., GÃ¶tz, M., Costa, R., & Smalla, K. (2006). The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus Verticillium differs depending on plant species and site. FEMS Microbiol. Ecol. 56:250–261.

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A., & Smalla, K. (2002). Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different Verticillium host plants. Appl. Environ. Microbiol. 68:3328–3338.

Berg, G., Smalla, K., G, B., R, M., M, K.-Z., IS, D., … S, C. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol. Ecol. 68:1–13.

Bragina, A., Berg, C., Cardinale, M., Shcherbakov, A., Chebotar, V., & Berg, G. (2012). Sphagnum mosses harbour highly specific bacterial diversity during their whole lifecycle. ISME J. 6:802–813.

Brandl, M. T. (2006). Fitness of Human Enteric Pathogens on Plants and Implications for Food Safety. Annu. Rev. Phytopathol. 44:367–392.

Buée, M., De Boer, W., Martin, F., van Overbeek, L., & Jurkevitch, E. (2009). The rhizosphere zoo: An overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. Plant Soil 321:189–212.

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E. V. L., & Schulze-Lefert, P. (2013). Structure and Functions of the Bacterial Microbiota of Plants. Annu. Rev. Plant Biol. 64:807–838.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7:335–336.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., … Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. U. S. A. 4516–4522.

Cernansky, R. (2015). The rise of Africa's super vegetables. Nature 522:146–148.

Cernava, T., Aschenbrenner, I. A., Grube, M., Liebminger, S., & Berg, G. (2015). A novel assay for the detection of bioactive volatiles evaluated by screening of lichen-associated bacteria. Front. Microbiol. 6:398.

Chanway, C. P., Hynes, R. K., & Nelson, L. M. (1989). Plant growth-promoting rhizobacteria: Effects on growth and nitrogen fixation of lentil (Lens esculenta moench) and pea (Pisum sativum L.). Soil Biol. Biochem. 21:511–517.

Chelius, M. K., & Triplett, E. W. (2001)a. The diversity of Archaea and Bacteria in association with the roots of Zea mays L. Microb. Ecol. 41:252–263.

Chelius, M. K., & Triplett, E. W. (2001)b. The Diversity of Archaea and Bacteria in Association with the Roots of Zea mays L. Microb. Ecol. 41:252–263.

Couillerot, O., Prigent-Combaret, C., Caballero-Mellado, J., & Moënne-Loccoz, Y. (2009). Pseudomonas fluorescens and closely-related fluorescent pseudomonads as biocontrol agents of soil-borne phytopathogens. Lett. Appl. Microbiol. 48:505–512.

de Souza, R. S. C., Okura, V. K., Armanhi, J. S. L., Jorrín, B., Lozano, N., da Silva, M. J., … Arruda, P. (2016). Unlocking the bacterial and fungal communities assemblages of sugarcane microbiome. Sci. Rep. 6:28774.

Dean, R., Van Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., … Foster, G. D. (2012). The Top 10 fungal pathogens in molecular plant pathology. Mol. Plant Pathol. 13:414–430.

Development, (NEPAD) New Partnership for African. (2013). African Agriculture, Transformation and Outlook.

Dubey, G., Kollah, B., Gour, V. K., Shukla, A. K., & Mohanty, S. R. (2016). Diversity of bacteria and archaea in the rhizosphere of bioenergy crop Jatropha curcas. 3 Biotech 6:257.

Erkel, C., Kuba, M., Heinhardt, R., & Liesack, W. (2006). Genome of rice cluster I archaea - The key methane producers in the rice rhizosphere. Science (80-.).

Food and Agriculture Organization of the United Nations. (2012). *FAO Statistical Yearbook 2013 - World Food and Agriculture.* FAO.

Foster, R. C., Rovira, A. D., & Cock, T. W. (1983). Ultrastructure of the root-soil interface. Ultrastruct. Root-Soil Interface.

Francis, C. A., Beman, J. M., & Kuypers, M. M. M. (2007). New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. ISME J. 1:19–27.

García-Salamanca, A., Molina-Henares, M. A., van Dillewijn, P., Solano, J., Pizarro-Tobías, P., Roca, A., … Ramos, J. L. (2013). Bacterial diversity in the rhizosphere of maize and the surrounding carbonate-rich bulk soil. Microb. Biotechnol. 6:36–44.

Gibbons, S. M., Lekberg, Y., Mummey, D. L., Sangwan, N., Ramsey, P. W., & Gilbert, J. A. (2017). Invasive Plants Rapidly Reshape Soil Properties in a Grassland Ecosystem. MSystems 2:.

Haas, D., & Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonads. Nat. Rev. Microbiol. 3:307–319.

Hardoim, P. R., van Overbeek, L. S., Berg, G., Pirttilä, A. M., Compant, S., Campisano, A., … Sessitsch, A. (2015). The Hidden World within Plants: Ecological and Evolutionary Considerations for Defining Functioning of Microbial Endophytes. Microbiol. Mol. Biol. Rev. 79:293–320.

Hariprasad, P., Venkateswaran, G., & Niranjana, S. R. (2014). Diversity of cultivable rhizobacteria across tomato growing regions of Karnataka. Biol. Control 72:9–16.

Hartmann, M., Frey, B., Mayer, J., Mäder, P., & Widmer, F. (2015). Distinct soil microbial diversity under long-term organic and conventional farming. ISME J. 9:1177–1194.

Hayat, R., Ali, S., Amara, U., Khalid, R., & Ahmed, I. (2010). Soil beneficial bacteria and their role in plant growth promotion: a review. Ann. Microbiol. 60:579–598.

Ho, A., Lonardo, D. P. Di, & Bodelier, P. L. E. (2017). Revisiting life strategy concepts in environmental microbial ecology. FEMS Microbiol. Ecol. 93:fix006.

Idris, E. E., Iglesias, D. J., Talon, M., & Borriss, R. (2007). Tryptophan-Dependent Production of Indole-3-Acetic Acid (IAA) Affects Level of Plant Growth Promotion by *Bacillus amyloliquefaciens* FZB42. Mol. Plant-Microbe Interact. 20:619–626.

Innerebner, G., Knief, C., & Vorholt, J. A. (2011). Protection of Arabidopsis thaliana against leaf-pathogenic Pseudomonas syringae by Sphingomonas strains in a controlled model system. Appl. Environ. Microbiol. 77:3202–3210.

Johnston-Monje, D., & Raizada, M. N. (2011). Conservation and Diversity of Seed Associated Endophytes in Zea across Boundaries of Evolution, Ethnography and Ecology. PLoS One 6:e20396.

Khan, A. L., Waqas, M., Kang, S.-M., Al-Harrasi, A., Hussain, J., Al-Rawahi, A., … Lee, I.-J. (2014). Bacterial endophyte Sphingomonas sp. LK11 produces gibberellins and IAA and promotes tomato plant growth. J. Microbiol. 52:689–695.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 41:e1–e1.

Koumoutsi, A., Chen, X.-H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., … Borriss, R. (2004). Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in Bacillus amyloliquefaciens strain FZB42. J. Bacteriol. 186:1084–1096.

Kumar, A., & Johri, B. N. (2012). Antimicrobial Lipopeptides of Bacillus: Natural Weapons for Biocontrol of Plant Pathogens. In *Microorganisms in Sustainable Agriculture and Biotechnology* (pp. 91–111).

Kumar, S., Dagnoko, S., Haougui, A., Ratnadass, A., Pasternak, N., & Kouamé, C. (2010). Okra (Abelmoschus spp.) in West and Central Africa: Potential and progress on its improvement. African J. Agric. Res.

Leclère, V., Béchet, M., Adam, A., Guez, J.-S., Wathelet, B., Ongena, M., … Jacques, P. (2005). Mycosubtilin overproduction by Bacillus subtilis BBG100 enhances the organism's antagonistic and biocontrol activities. Appl. Environ. Microbiol. 71:4577–4584.

Lee, H. J., Jeong, S. E., Kim, P. J., Madsen, E. L., & Jeon, C. O. (2015). High resolution depth distribution of Bacteria, Archaea, methanotrophs, and methanogens in the bulk and rhizosphere soils of a flooded rice paddy. Front. Microbiol. 6:639.

Lopez-Velasco, G., Carder, P. A., Welbaum, G. E., & Ponder, M. A. (2013). Diversity of the spinach (*Spinacia oleracea*) spermosphere and phyllosphere bacterial communities. FEMS Microbiol. Lett. 346:146–154.

Lugtenberg, B., & Kamilova, F. (2009). Plant-Growth-Promoting Rhizobacteria. Annu. Rev. Microbiol. 63:541–556.

Lundberg, D. S., Yourstone, S., Mieczkowski, P., Jones, C. D., & Dangl, J. L. (2013). Practical innovations for high-throughput amplicon sequencing. Nat. Methods 10:999–1002.

Lupwayi, N. Z., Rice, W. A., & Clayton, G. W. (1998). Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. Soil Biol. Biochem. 30:1733–1741.

Mayak, S., Tirosh, T., & Glick, B. R. (2004). Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiol. Biochem. 42:565–572.

Mendes, R., Kruijt, M., de Bruijn, I., Dekkers, E., van der Voort, M., Schneider, J. H. M., … Raaijmakers, J. M. (2011). Deciphering the Rhizosphere Microbiome for Disease-Suppressive Bacteria. Science (80-.). 332:1097–1100.

Moissl-Eichinger, C., & Huber, H. (2011). Archaeal symbionts and parasites. Curr. Opin. Microbiol. 14:364–370.

Moissl-Eichinger, C., Pausan, M., Taffner, J., Berg, G., Bang, C., & Schmitz, R. A. (2018). Archaea Are Interactive Components of Complex Microbiomes. Trends Microbiol. 26:70–85.

Onyango, C. M., Kunyanga, C. N., Ontita, E. G., Narla, R. D., & Kimenju, J. W. (2013). Current status on production and utilization of spider plant (Cleome gynandra L.) an underutilized leafy vegetable in Kenya. Genet. Resour. Crop Evol. 60:2183–2189.

Pérez-Jaramillo, J. E., Mendes, R., & Raaijmakers, J. M. (2016). Impact of plant domestication on rhizosphere microbiome assembly and functions. Plant Mol. Biol. 90:635–644.

Philippot, L., Raaijmakers, J. M., Lemanceau, P., & van der Putten, W. H. (2013). Going back to the roots: the microbial ecology of the rhizosphere. Nat. Rev. Microbiol. 11:789–799.

Pump, J., Pratscher, J., & Conrad, R. (2015). Colonization of rice roots with methanogenic archaea controls photosynthesis-derived methane emission. Environ. Microbiol. 17:2254–2260.

Rademaker, J. L. W., & de Bruijn, F. J. (1997). Characterization and classification of microbes by rep-PCR genomic fingerprinting and computer assisted pattern analysis. DNA

Markers Protoc. Appl. Overviews.

Ronoh, R., Linde, M., Winkelmann, T., Abukutsa-Onyango, M., Dinssa, F. F., & Debener, T. (2019). Morphological characterization, genetic diversity and population structure of African nightshades (section Solanum L.). Genet. Resour. Crop Evol. 66:105–120.

Rosenstock, L., Keifer, M., Daniell, W. E., McConnell, R., & Claypoole, K. (1991). Chronic central nervous system effects of acute organophosphate pesticide intoxication. Lancet 338:223–227.

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., … Ideker, T. (2003). Cytoscape: A Software Environment for Integrated Models of Biomolecular Interaction Networks. Genome Res. 13:2498–2504.

Shoebitz, M., Ribaudo, C. M., Pardo, M. A., Cantore, M. L., Ciampi, L., & Curá, J. A. (2009). Plant growth promoting properties of a strain of Enterobacter ludwigii isolated from Lolium perenne rhizosphere. Soil Biol. Biochem. 41:1768–1774.

Simon, H. M., Jahn, C. E., Bergerud, L. T., Sliwinski, M. K., Weimer, P. J., Willis, D. K., & Goodman, R. M. (2005). Cultivation of mesophilic soil crenarchaeotes in enrichment cultures from plant roots. Appl. Environ. Microbiol. 71:4751–4760.

Taffner, J., Erlacher, A., Bragina, A., Berg, C., Moissl-Eichinger, C., & Berg, G. (2018). What is the role of Archaea in plants? New insights from the vegetation of alpine bogs. MSphere 3:.

Taffner, Julian, Cernava, T., Erlacher, A., & Berg, G. (2019). Novel insights into plant-associated archaea and their functioning in arugula (Eruca sativa Mill.). J. Adv. Res.

Taffner, Julian, Erlacher, A., Bragina, A., Berg, C., Moissl-Eichinger, C., & Berg, G. (2018). What Is the Role of Archaea in Plants? New Insights from the Vegetation of Alpine Bogs. MSphere 3:e00122-18.

Tilman, D., Balzer, C., Hill, J., & Befort, B. L. (2011). Global food demand and the sustainable intensification of agriculture. Proc. Natl. Acad. Sci. U. S. A. 108:20260–20264.

Uksa, M., Schloter, M., Endesfelder, D., Kublik, S., Engel, M., Kautz, T., … Fischer, D. (2015). Prokaryotes in Subsoil-Evidence for a Strong Spatial Separation of Different Phyla by Analysing Co-occurrence Networks. Front. Microbiol. 6:1269.

Unno, Y., Okubo, K., Wasaki, J., Shinano, T., & Osaki, M. (2005). Plant growth promotion abilities and microscale bacterial dynamics in the rhizosphere of Lupin analysed by phytate utilization ability. Environ. Microbiol. 7:396–404.

Venkateswarlu, B., Shanker, A., & Shanker, C. (2011). Crop stress and its management: perspectives and strategies.

Wachowska, U., Katarzyna, K., Malgorzata, J., & Natalia, L. (2013). Microorganisms as Biological Control Agents against Fusarium Pathogens in Winter Wheat. Polish J. Environ. Stud. 22:591–597.

Wagner, M. R., Lundberg, D. S., del Rio, T. G., Tringe, S. G., Dangl, J. L., & Mitchell-Olds, T. (2016). Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Nat. Commun. 7:12151.

Walters, W., Hyde, E. R., Berg-Lyons, D., Ackermann, G., Humphrey, G., Parada, A., … Knight, R. (2016). Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. MSystems 1:.

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. J. Exp. Bot. 52:487–511.

White, D. C., Sutton, S. D., & Ringelberg, D. B. (1996). The genus Sphingomonas: physiology and ecology. Curr. Opin. Biotechnol. 7:301–306.

Xia, Y., DeBolt, S., Dreyer, J., Scott, D., & Williams, M. A. (2015). Characterization of culturable bacterial endophytes and their capacity to promote plant growth from plants grown using organic or conventional practices. Front. Plant Sci. 6:490.

Yeoh, Y. K., Dennis, P. G., Paungfoo-Lonhienne, C., Weber, L., Brackin, R., Ragan, M. A., … Hugenholtz, P. (2017). Evolutionary conservation of a core root microbiome across plant phyla along a tropical soil chronosequence. Nat. Commun. 8:215.

Youssef, N. H., Rinke, C., Stepanauskas, R., Farag, I., Woyke, T., & Elshahed, M. S. (2015). Insights into the metabolism, lifestyle and putative evolutionary history of the novel archaeal phylum 'Diapherotrites.' ISME J. 9:447–460.

Zachow, C., Müller, H., Tilcher, R., Donat, C., & Berg, G. (2013). Catch the Best: Novel Screening Strategy to Select Stress Protecting Agents for Crop Plants. Agronomy 3:794–815.

Additional Publications

7 Novel strategies for soil-borne diseases: exploiting the microbiome and volatile-based mechanisms toward controlling Meloidogyne-based disease complexes

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Abstract

Under more intensified cropping conditions agriculture will face increasing incidences of soil-borne plant pests and pathogens, leading to increasingly higher yield losses world-wide. Soil-borne disease complexes, in particular, are especially difficult to control. In order to better understand soil-borne Meloidogyne-based disease complexes, we studied the volatile-based control mechanism of associated bacteria as well as the rhizospheric microbiome on Ugandan tomato plants presenting different levels of rootgalling damage, using a multiphasic approach. The experimental design was based on representative samplings of healthy and infected tomato plants from two field locations in Uganda, to establish species collections and DNA libraries. Root galling symptoms on tomato resulted from a multispecies infection of root-knot nematodes (*Meloidogyne* spp.). Results revealed that 16.5% of the bacterial strain collection produced nematicidal volatile organic compounds (nVOC) active against *Meloidogyne*. Using SPME GCMS, diverse VOC were identified, including sulfuric compounds, alkenes and one pyrazine. Around 28% of the bacterial strains were also antagonistic toward at least one fungal pathogen of the disease complex. However, antagonistic interactions appear highly specific. Nematicidal antagonists included *Pseudomonas*, *Comamonas*, and *Variovorax* and fungicidal antagonists belonged to *Bacillus*, which interestingly, were primarily recovered from healthy roots, while nematode antagonists were prominent in the rhizosphere and roots of diseased roots. In summary, all antagonists comprised up to 6.4% of the tomato root microbiota. In

general, the microbiota of healthy and diseased root endospheres differed significantly in alpha and quantitative beta diversity indices. Bacteria-derived volatiles appear to provide a remarkable, yet wholly unexploited, potential to control *Meloidogyne*-based soil-borne disease complexes. The highly specific observed antagonism indicates that a combination of volatiles or VOC-producing bacteria are necessary to counter the range of pathogens involved in such complexes.

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8 Unravelling native plant resistomes - The *Sphagnum* microbiome harbours versatile and novel antimicrobial resistance genes

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Abstract

The expanding antibiotic resistance crisis calls for a more in depth understanding of the importance of antimicrobial resistance genes (ARGs) in pristine environments. We, therefore, studied the microbiota associated with *Sphagnum* forming the main vegetation in undomesticated, evolutionary old bog ecosystems. In our complementary analysis of a culture collection, metagenomic data and a fosmid library, we identified a low abundant but highly diverse pool of resistance determinants, which targets an unexpected broad range of antibiotics including natural and synthetic compounds. This derives both, from the extraordinarily high abundance of efflux pumps (80%), and the unexpectedly versatile set of ARGs underlying all major resistance mechanisms. The overall target spectrum of detected resistance determinants spans 21 antibiotic classes, whereby β-lactamases and vancomycin resistance appeared as the predominant resistances in all screenings. Multi-resistance was frequently observed among bacterial isolates, e.g. in *Serratia*, *Pandorea, Paraburkholderia* and *Rouxiella.* In a search for novel ARGs we identified the new class A β-lactamase Mm3. The native *Sphagnum* resistome comprising a highly diversified and partially novel set of ARGs contributes to the bog ecosystem´s plasticity. Our results shed light onto the antibiotic resistance background of non-agricultural plants and highlight the ecological link between natural and clinically relevant resistomes.

MANUSCRIPT

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Education

2015-present	Graz University of Technology, Graz, Austria Institute of Environmental Biotechnology PhD thesis: "Secret collaborations: How plant-associated archaea interact with their hosts"
2012-2015	Saarland University, Saarbrücken, Germany **Master of Science** in Biotechnology
2014-2015	University of Otago, Dunedin, New Zealand Department of Biochemistry Master thesis: "Investigating the roles of low-molecular-weight proteins belonging to the CP43 subcomplex in the assembly of Photosystem II"
2009-2012	Saarland University, Saarbrücken, Germany **Bachelor of Science** in Human and Molecular Biology
2012	Universidad Pompeu Fabra, Barcelona, Spain Infection Biology Group Bachelor thesis: "Effect of *in vitro* induced regulatory T cells on the LCMV-specific immune response"

Work Experience

2015-2020	**University Assistant** Graz University of Technology, Graz, Austria Institute of Environmental Biotechnology Supervision of bachelor and master students; Leading laboratory courses for students; Administration of institute duties; IT officer for web appearance
2013-2015	**Research Assistant** Saarland University, Saarbrücken, Germany Institute of Neurobiology and Physiology Molecular processes of learning and memory formation in honey bees; beekeeper training
2014	**Internship** Helmholtz Institute for Pharmaceutical Research, Saarbrücken, Germany Preparation of nanosized starch coacervatives

Teaching

2018-present	Chairing and leading the seminar "Environmental Microbiology", Institute of Environmental Biotechnology, TU Graz
2017-present	Chairing and leading the Institute's seminar, Institute of Environmental Biotechnology, TU Graz
2016-present	Chairing and leading the Journal Club "Molecular Microbiology", Institute of Environmental Biotechnology, TU Graz
2016-present	Leading the laboratory course "Biotechnology", Institute of Environmental Biotechnology, TU Graz
2016-present	Leading the laboratory course "Microbiology", Institute of Environmental Biotechnology, TU Graz
2018-2019	Co-Supervision of master thesis "Exploring the microbiome of novel leafy greens in Eastern Africa"
2017-present	Co-Supervision of the master thesis "Effect of land use type and rhizosphere microbiome on bio-control of tomato bacterial wilt disease in Uganda"
2017-2018	Co-Supervision of the master thesis "Controlling the Meloidogyne disease complex in Ugandan tomatoes"
2018	Co-Supervision of bachelor thesis "Isolation of antagonistic bacteria from Ugandan leafy greens in order to develop novel bio-control agents"

Professional Affiliations & Administrative Experience

2016-present	Member of Austrian Association of Molecular Biosciences and Biotechnology (ÖGMBT)
2016-present	Original member and representative for Young Life Scientists Austria (YLSA) branch south for network building and event management
2017-2018	Organising committee of the 19th DocDay of the life science doctoral schools, Graz University of Technology and Karl-Franzens University

Additional Activities

2018-present	PhD student representative of the Doctoral School of Molecular Biosciences and Biotechnology, TU Graz, Graz
2016-present	Active member of the PhD student council for doctoral studies (PhD Union), TU Graz, Graz
2016-present	Founder and head of the documentary screening society "Dokuabende Graz"
2016, 2018	1st at the styrian academic fencing championships in sabre, Graz

2010-2014	Active member of student council of Biology and Biotechnology, Saarland University, Saarbrücken
2014	5th in single and 2nd in team competition at the national fencing championships of New Zealand in sabre

Publications

1) "Nanosized Coacervates Of Positive And Negative Starch Derivatives For Pulmonary Delivery Of Proteins"; S. Barthold, S. Kletting, J. Taffner, et al., Journal of Materials Chemistry B, 2016
2) "Archaea Are Interactive Components Of Complex Microbiomes"; C. Moissl-Eichinger, M. Pausan, J. Taffner, et al., Trends in Microbiology, 2017
3) "What Is The Role Of Archaea In Plants? New Insights From The Vegetation Of Alpine Bogs"; J. Taffner, A. Erlacher, A. Bragina, et al., mSphere, 2018
4) "Novel insights into plant-associated archaea and their functioning in arugula (*Eruca sativa* Mill.)"; J. Taffner, T. Cernava, A. Erlacher, and Gabriele Berg, Journal of Advanced Research, 2019
5) "Novel strategies for soil-borne diseases: exploiting the microbiome and volatile-based mechanisms towards controlling Meloidogyne-based disease complexes"; A. Wolfgang, J. Taffner, R. A. Guimarães, et al., Frontiers in Microbiology, 2019

Scientific Outreach

2018	**EMBO**, Vienna, Austria Oral presentation: "Secret collaborations: How plant-associated Archaea interact with their hosts" **ESM**, Helsinki, Finland Oral presentation: "Secret collaborations: plant-associated Archaea and their interaction with their hosts"
2017	**18th Frankfurt meeting on Genome function and gene regulation in Archaea**, Schmitten, Germany Oral presentation: "Secret collaborations: first insight into the life of plant-associated Archaea" **International Conference on Holobionts**, Paris, France Oral presentation: "Understanding the role of Archaea for plant holobionts"
2016	**ISME16**, Montreal, Canada Oral presentation: "Metagenomic data provide new insights into plant-specific archaeal communities" **ÖGMBT Annual Meeting**, Graz, Austria Poster presentation: "Archaea are species- and habitat-specific plant colonizers unravelled by metagenomics"

References

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Personal Skills & Awards

German	Native language
English	Professional speaking and writing skills
French	Intermediate
Computer skills	Microsoft Office, CorelDRAW, Photoshop, Final Cut Pro X, Qiime1&2, TYPO3
2019	European Solidarity Corps Grant
2014	DAAD scholarship (PROMOS)

Interests

Research	Microbiology, Archaea, Sustainable Development, Environmental Biotechnology, Science Communication, Environmental Conservation
Others	Communicating environmental science to the public by writing short articles and giving oral presentations Event management and especially organisation of documentary screening events with additional discussion rounds with experts Fencing, Photography, Environmental Conservation, Travelling and Exploring