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The Seed & Fruit Microbiome: Health Implications for Crops and Consumers

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AFFIDAVIT

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ABSTRACT

Microorganisms are omnipresent, central to life on earth and closely linked to health and disease. Plants, animals and humans are no longer considered as standalone organisms, but, together with their associated microorganisms, referred to as assemblages of species or holobionts. However, we are still at the beginning to understand the complex interactions between microbial communities and their hosts. The present doctoral thesis covered manifold aspects of the plant microbiome, making use of amplicon sequencing, shotgun metagenomics, advanced microscopy and cultivation-dependent methods. While the most intimate interaction between the host and its microbiota was revealed by analyzing seed microbiomes from pristine environments, the apple fruit served as a model to understand the impact of modern crop production systems on the associated microbiome.

While long-time assumed to be sterile, plant seeds are adjusted with a diverse microbiome that is often dormant by the time point environmental conditions get convenient for germination. At this stage, the seed-associated microbiome is suggested to play a crucial role for plant development and resilience towards biotic and abiotic stresses. These stresses can be numerous, and several of them are directly associated with recent trends in human life style. Especially crop production systems are facing dramatic challenges that come along with large-scale industrialization, globalization and the changing climate. Considering the seed microbiome as primary inoculum of the mother plant to support the next generation, especially the conditions in pristine environments are of significant interest. Within the present thesis, seeds of native plant species were uncovered to harbor an exceptionally specific and interactive network of bacteria, fungi and archaea, which were newly discovered for seeds. While the microbiota was significantly driven by the plant genotype, no effect of fruit morphologies and the environment was observed. Reflecting on ancient plant domestication periods and the native seed microbiota, perspectives were provided for novel nature-based seed treatments. In addition, a comprehensive method collection, specifically optimized for seed microbiome analyses, was developed.

Apple fruits are among the most popular food commodities, well-known for beneficial impacts for human health. However, as a raw-eaten plant, also the associated microbiome potentially affects the consumers. The apple fruit microbiome was investigated to evaluate the impact of organic and conventional management practices, which was found significant for the microbiota composition but not abundance. Each tissue of the apple fruit was colonized by

distinct bacterial communities, while seeds represented bacterial hotspot. Bacterial and fungal dynamics during apple storage were assessed on an industrial scale and a method of microbiome-assisted postharvest treatment increasing the storability of apples was established. Finally, the antibiotic resistance potential of the apple microbiome was assessed via metagenomics shotgun sequencing. While in general, the apple resistome did not appear to be a cause for health concerns, the comparison of South African apples fresh from the tree to apples transported to Austrian supermarkets revealed an impact of the today´s global food transport on the produce resistomes, visible on a shift towards increasing antibiotic resistance gene diversity.

The ultimate goal in plant microbiome research is to protect environmental biodiversity, and simultaneously, establish methods for sustainable and high-yield crop production. The present study sheds light on the microbial composition of indigenous as well as highly domesticated plants and suggests environmental microbiomes to offer valuable opportunities for novel and sustainable crop cultivation systems.

ZUSAMMENFASSUNG

Mikrobiome sind allgegenwärtig; sie sind ein zentraler Bestandteil des Lebens und eng mit Gesundheit und Krankheit verbunden. Pflanzen, Tiere und Menschen gelten nicht länger als eigenständige Organismen, sondern, gemeinsam mit ihren assoziierten Mikroorganismen, als Holobionten. Die komplexen Wechselwirkungen innerhalb dieser Lebensgemeinschaft sind jedoch noch größtenteils unerforscht. In der vorliegenden Arbeit wurde deshalb das Pflanzenmikrobiom aus unterschiedlichen Perspektiven beleuchtet. Hierzu wurde eine Methodenkombination aus Amplikonsequenzierung, Shotgun-Metagenomik, Mikroskopie und Kultivierungs-Methoden genutzt. Eine intime Verbindung innerhalb des Holobionten wurde für das Samenmikrobiomen aus natürlichen Habitaten entschlüsselt; kontrastierend diente die Apfelfrucht als Modell für domestizierte Kulturpflanzen.

Pflanzensamen sind mit einem vielfältigen Mikrobiom ausgestattet, welches sich jedoch in einem ruhenden Zustand befindet, bis geeignete Keimungsbedingungen vorliegen. Während der Keimung spielt das Samenmikrobiom eine entscheidende Rolle und wirkt sich positiv auf die Pflanzenentwicklung und Widerstandskraft gegenüber biotischen und abiotischen Stressfaktoren aus. Stressfaktoren können von unterschiedlicher Art sein; einige stehen jedoch in direktem Zusammenhang zu anthropogenen Aktivitäten. Der Einfluss von Industrialisierung, Globalisierung und Klimawandel auf die Produktivität von Nutzpflanzen stellt die Agrarwirtschaft vor immense Herausforderungen. Das Samenmikrobiom, welches als primäres Inokulum der Mutterpflanze für die nächste Generation gilt, ist hier von Interesse. Die Analyse von indigenen Samenmikrobiomen ergab ein spezifisches und interaktives Netzwerk aus Bakterien, Pilzen und Archaeen; Letztere wurden erstmalig in Samen detektiert. Das Samenmikrobiom wurde hauptsächlich vom Genotyp der Mutterpflanze, jedoch nicht von der Morphologie und Entwicklung der Früchte beeinflusst. Weiters ermöglichte die retrospektive Betrachtung des Einflusses uranfänglicher Domestizierung auf Samenmikrobiome neue Perspektiven für naturbasierte Saatgutbehandlungen. Darüber hinaus wurde eine umfassende Methodensammlung, speziell zur Untersuchung von Samenmikrobiomen, erfasst.

Äpfel gehören zu den beliebtesten Früchten und ihre positiven Auswirkungen auf die Gesundheit sind weithin bekannt. Roh verzehrt könnte auch das Apfelmikrobiom Auswirkungen auf den Konsumenten haben. Der Einfluss biologischer und konventioneller Landwirtschaft auf das Apfelmikrobiom wurde untersucht und ergab einen signifikanten Unterschied in der Zusammensetzung, jedoch nicht in der Anzahl der Mikroorganismen. Jeder

Teil der Apfelfrucht wies unterschiedliche Bakteriengemeinschaften auf: die Schale war gering besiedelt, während Samen einen bakteriellen Hotspot darstellten. Die mikrobielle Dynamik während der Langzeitlagerung wurde im industriellen Maßstab untersucht und es wurden eine Mikrobiom-unterstützte Methode entwickelt, um die Apfel-Lagerfähigkeit zu verbessern. Weiters wurde das Antibiotikaresistenz-Potential (Resistom) des Apfels analysiert. Im Allgemeinen erscheint das Apfelresistom wenig besorgniserregend. Jedoch ergab der Vergleich von frisch geernteten südafrikanischen Äpfeln, zu jenen die kommerziell in österreichische Supermärkte transportiert wurden, dass der globale Lebensmitteltransport einen Einfluss auf das Resistom haben könnte: ein signifikanter Anstieg der Resistenzgen-Diversität wurde nach dem Transport festgestellt.

Das Ziel der Pflanzenmikrobiom-Forschung ist der Schutz der biologischen Vielfalt um gleichzeitig Methoden für nachhaltige, ertragreiche Agrarwirtschaft zu entwickeln. In der vorliegenden Studie wurden die Mikrobiome von natürlichen sowie domestizierten Pflanzen untersucht. Die Erkenntnisse erlauben den Schluss das speziell natürliche Pflanzen-Mikrobiome vielfältige Möglichkeiten für neuartige Anbausysteme und -technologien bieten können.

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INTRODUCTION

The importance of the microbiome for the holobiont

Continuous developments in high-throughput sequencing methods contributed to a paradigm shift in life sciences in which eukaryotic organisms are no longer considered as standalone entities, but as assemblage of species, also termed meta-organisms or holobionts (Zilber-Rosenberg and Rosenberg 2008; Theis et al. 2016; Vandenkoornhuyse et al. 2015). These terms pay respect to the intense relationship of humans, animals and plants to their associated microbiomes. By definition, the *microbiota* simply comprise the wealth of microscopically small organisms such as bacteria, fungi, archaea, algae and protists, occupying a reasonable well-defined habitat; and the *microbiome* encompasses the *microbiota* as well as the collection of all microbial genes, alias microbiota´s 'their theatre of activity' (Whipps et al. 1988; Berg et al. 2020).

The microbiome can play essential roles for the host´s growth, performance and survival (Vandenkoornhuyse et al. 2015; Cho and Blaser 2012) and its significance for planetary health is now reflected also in the *one health* concept (Flandroy et al. 2018). Even habitats that have been long-term assumed to be sterile, e.g. placenta and stomach of humans or plant seeds, have been confirmed to be colonized by diverse microbial communities. Despite being specific and deeply embedded within their host, these microbial communities certainly represent open and interconnected ecosystems, that coevolve, communicate and cross-feed (Berg 2015; Layeghifard et al. 2017). Accordingly, microbiota and their genes are continuously exchanged between the environment, humans, animals and plants. Such mutual exchanges have been vividly documented for the rhizosphere (Berg and Smalla 2009), the gut microbiome (Huttenhower et al. 2012) and the indoor microbiome (Mahnert et al. 2015). The specific characteristics of microbiome interactions are, however, still largely unknown. In particular, the plant microbiome plays a specific role for ecosystem functioning and the plasticity of the environment. Deep understanding of the underlying mechanisms by which plants acquire and exchange microbes and microbial exudates with their environment is of supreme importance for improved sustainability in agricultural settings, and in case of raw eaten plants, also for humans as consumers (Pennisi 2019).

The plant microbiome in general

The arbuscular mycorrhizal mutualism certainly represents the best-described model for plant-microorganism symbioses. Mycorrhiza have been studied already for several decades and are strongly believed as driving force in plant evolution, diversification and the functioning of the terrestrial ecosystem (Selosse and Le Tacon 1998; Heckman 2001); comparable key roles are nowadays suggested also for the entire plant microbiome (Vandenkoornhuyse et al. 2015). The plant microbiome represents a reservoir for an immense diversity of microbial species and genes which are involved in several functions for the holobiont, such as nutrition, resilience, support during germination and growth as well as resistance towards biotic and abiotic stresses (Berg et al. 2017; Mendes and Raaijmakers 2015). Microbiota can colonize plant surfaces as well as inner tissues and their composition is generally driven by the host species and genotype, soil type and quality, environmental factors and the host´s health status (Berg and Smalla 2009; Philippot et al. 2013). However, even within an individual plant, microbial signatures can be habitat-specific. Among these habitats, the rhizosphere is clearly the best-studied, although highly complex, as it represents the below-ground interface of plant roots and soil microbiota (Berg et al. 2005; Philippot et al. 2013; Mendes et al. 2013). Soil provides a huge reservoir of microorganisms and plant´s favorites may even be attracted by root exudates, becoming invited endophytes (Berg and Smalla 2009). The environment is an additional source for microorganisms to enter plant´s above-ground tissues, which are summarized as phyllosphere and subdivided into caulosphere (stems), phylloplane (leaves), anthosphere (flowers) and carposphere (fruits) (Vorholt 2012; Hardoim et al. 2015). While most of these habitats are frequently investigated, the seed microbiome arose scientific interest only recently; however, since then, strongly suggested to play important roles during seed germination and plant development (Hardoim et al. 2015).

Recent trends in human lifestyle and agricultural practices lead to severe reduction of world-wide biodiversity, resulting in a homogenization of plant genotypes (Purugganan and Fuller 2009); a loss of microbial diversity associated with plants and soil is the predictable consequence (Pérez-Jaramillo et al. 2016; Berg and Raaijmakers 2018). Reduced microbial diversity affects the capability of plants to combat biotic and abiotic hazards (Truyens et al. 2015) and can also supports the accumulation of both plant and human pathogens (Mendes et al. 2013; Pérez-Jaramillo et al. 2016; Berg et al. 2005). Ever-increasing management efforts and pesticide usage in agricultural settings is the unavoidable consequence (Gruber 2017; Oerke 2006). Thus, preservation and successive restoration of plant and soil microbial diversity is a

key objective to protect ecosystem health and to ensure food supply for a growing world population (Wall et al. 2015; Pérez-Jaramillo et al. 2016). The application of microorganisms in form of biopesticides or biofertilizers is furthermore a highly promising tool for sustainable agricultural management (Berg et al. 2014; Mitter et al. 2016); here also the seed microbiome represents a new research topic for biological crop production systems.

The functional capacity of the plant microbiome is, however, not bounded to host itself; in case of edible plants, it can also affect human health. The present doctoral thesis examines the plant microbiome from different perspectives, considering both plant and human health issues. The first part sheds light on the seed microbiome, encompassing i) novel perspectives for improved sustainability in agricultural settings, ii) fundamental research on plants from pristine environments, and iii) a comprehensive compilation of methods specific for seed microbiome screening. The second part focuses on the microbiome of the apple fruit as model to study i) the impact of different agricultural management and ii) postharvest practices as well as iii) the intercontinental fruit transport on the microbiota and their antibiotic resistance profile; potential assets and drawbacks for humans as consumers are discussed.

The seed microbiome and its potential for crop production

Seeds are small embryonic plants, protectively covered by maternal tissues, which serve to initiate a new life cycle of a plant. For a long time it was assumed that healthy seeds are sterile and that emerging seedlings are colonized mainly by soil microorganisms (Truyens et al. 2015). Thus, previous studies on seed microbiomes focused mainly on plant pathogens (Nelson 2018). However, recently, a plant specific core microbiota was observed to be vertically transmitted from the mother plant to the next generation by seeds (Adam et al. 2018; Bergna et al. 2018; Berg and Raaijmakers 2018; Rybakova et al. 2017), being even conserved across boundaries of evolution (Johnston-Monje and Raizada 2011).

Until now, several factors were described to contribute to the seed microbiome composition, including soil type (Hardoim et al. 2012), geographical sites (Johnston-Monje and Raizada 2013; Klaedtke et al. 2016) and beneficial and pathogenic microbial inoculants (Rezki et al. 2016). The plant genotype, however, is suggested as the main driver of the seed microbiota (Barret et al. 2015; Adam et al. 2018; Rybakova et al. 2017; Johnston-Monje and Raizada

2011), with the mother plant being responsible for the recruitment of environmental microorganisms (Nelson 2018). While endophytic, vertically transmitted microorganisms reside in internal seed tissues, the epiphytic microbiota colonize the seed surface and may be either vertically or horizontally transmitted. Both groups contribute to the spermosphere, representing the seed-surrounding zone where interactions between the germinating seed, soil and the present microbiota take place (Schiltz et al. 2015). Undoubtedly, the emerging seedlings represents the most vulnerable stage in a plant´s live cycle, being subjected to a variety of biotic and abiotic hazards. Seed endophytes are now considered as primary inoculum to equip the next generation of plants with specific microbial communities, potentially supporting the seedling in resilience towards this plethora of stressors (Hardoim et al. 2015; Truyens et al. 2015; Vujanovic and Germida 2017). However, despite the emphasis on plant microbiome analyses is rapidly increasing, seeds are poorly mentioned and our current understanding of microbial origins, colonization routes and especially their functions for germination and plant health is still only scratching the surface (Nelson 2018). Especially the indigenous seed microbiomes of native plants are almost unknown (Berg and Raaijmakers 2018). Seeds of natural environments are not simply responsible for germination, they are furthermore highly adapted for successful dispersal and the ability to persist and germinate in diverse environments under challenging biotic and abiotic conditions (Fenner and Thompson 2005). Consequentially, nature created a rich morphological diversity of seeds and fruit systems. In agricultural systems, however, most of these adaptive traits have been altered during millennials of breeding and domestication (Berg and Raaijmakers 2018); secretly, also their inherent microbiomes were affected. Today, crop seeds are almost entirely produced commercially and current seed treatments include disinfection, heavy handling and uniform planting across large-scale areas. These measures significantly increase the selective pressure on the inherent microbiota with yet unpredictable consequences for plant health and ecosystem diversity.

Objectives addressed on the native seed microbiome

Recently, Berg and Raijmakers (2018) proposed the *Saving Seed Microbiomes* concept, which suggests the re-definition of plant seeds as functional microbial entity and to restore beneficial microorganisms from seeds of wild ancestors of modern crop plants to use them as inoculants for modern crop seeds (Berg and Raaijmakers 2018). With that idea in mind, the following objectives were addressed on the seed microbiome:

i) **how was the seed microbiome shaped by domestication and modern agriculture and can we use this knowledge for sustainable crop production systems?**

In order to deeply understand, and successfully combat current agricultural issues, it can be helpful to take a glance at the past. **Chapter I: Publication I: 'Understanding the Indigenous Seed Microbiota to Design Bacterial Seed Treatments'** reflects upon how early plant domestication, starting 13,000 years ago, and current agricultural practices altered seed morphology, functionality and especially seed microbiomes; thus, widening the perspective to find new ideas for ecosystem-friendly agriculture.

ii) **how is the native and healthy seed microbiota composed and what can we learn from pristine environments?**

In **Chapter II: Publication II: 'Seeds of native alpine plants host unique microbial communities embedded in cross-kingdom networks'**, the indigenous seed microbiota of native plant populations from the European Eastern Alps were investigated. The selected habitat represents a glacial retreat for a high diversity of plant species, persisting over centuries under low anthropogenic influence. In-depth analysis of eight different plant genotypes, that differ in fruit morphology and life cycles, revealed novel insights into the bacterial, archaeal and fungal diversity, abundance, interconnectivity and specificity for plant seeds from pristine environments. The presented results can assist to understand a 'healthy seed microbiome' and to further translate this knowledge to crop plants.

iii) **how can we optimize biotechnological methods for specific and comprehensive analyses of seed microbiomes?**

In fact, the seed microbiome analysis was the last wasteland of plant microbiome research, mainly due to the complex circumstances, such as dormancy of seed microbiota, and limited methods for detection. Much of what we currently know about seed microbiome is based on culture-based studies, while recent advantages in sequencing and microscopic techniques offer deeper insights into the diversity and functionality of seed microbiota. In order to get a complete picture, a multidisciplinary analysis is essential. Methods, specifically established and optimized for a comprehensive analysis of seed microbiomes are summarized in the methodology review presented in **Chapter III: Manuscript III: 'Studying Seed Microbiomes'**.

The apple microbiome and the impact of modern agriculture

Apples constitute a major part of globally consumed fruits and their production currently comprises a significant proportion of world´s agriculture, increasing constantly (FAO 2019). Apples represent the most important dietary source for various flavonoids in European and North American diets (Shoji and Miura 2014) and health benefits of apple consumption are well-accepted. However, associated to a raw-eaten plant, the apple microbiome represents an important human-environment interface; thus, in-depth knowledge on the microbiome and its potential for human health is of crucial importance.

Alike the plant holobiont, humans are closely associated with their microbiota which were found to differ remarkably between individuals and body habitats, being driven by various factors such as diet, age, host genetics, mode of infant delivery and inheritance (Cho and Blaser 2012; Huttenhower et al. 2012). Especially the composition of the human gut microbiome is of significant value for the host´s health (Blaser 2017). Despite several pathologies, such as inflammatory bowel disease, colorectal cancer, diarrhea, diabetes and obesity, are associated with a dysbiosis in the gut microbiota (Pop et al. 2014; Gülden et al. 2015; Yu and Fang 2015; Perry et al. 2016; Layeghifard et al. 2017), especially, high compositional and functional microbial diversity can limit the invasion and proliferation of human pathogens (van Elsas et al. 2012; Pham and Lawley 2014). Raw-eaten vegetables and fruits represent an important source of a diverse microbiota and the so-called plant-gut microbiome axis could be of significant value for human health (Leff and Fierer 2013; Berg et al. 2014; Wassermann et al. 2017). Bacteria, fungi and viruses associated with the diet have recently been shown to colonize the gut system at least transiently (David et al. 2014); for bacteria associated with fermented foods it was even shown that 50% of ingested cells survived the gastric passage (Oozeer et al. 2006). The majority of plant-associated microbiota are non-pathogenic to humans (Bulgarelli et al. 2013; Berg et al. 2014). However, food-borne outbreaks, associated with the consumption of raw vegetables and fruits, continued to rise over the past two decades, despite intensive efforts for rapid detection and diverse decontaminating strategies (WHO 2015; Yoon and Lee 2018). Especially large scale crop production areas are assumed to be directly correlated with pathogen abundance (Olaimat and Holley 2012). Intense agricultural management and the tight interaction between humans, farm animals and crop plants, can provoke a shift in the microbiome towards high abundances of opportunistic bacteria and can make specific plants secondary hosts for human pathogens (Berg et al. 2005; Klerks et al. 2007; Berg et al. 2015).

The impact of different farming practices on the microbiome and the potential consequences for humans as consumers are, thus, a valuable object of investigation.

Regarding food safety, also the resistome (encompassing all antibiotic resistant genes of the present microbiota) of vegetables and fruits requires deep understanding and monitoring. In fact, the extensive use of antibiotics in clinical and agricultural environments over the past four decades has served as a driving force to disseminate antibiotic resistances world-wide (Hernando-Amado et al. 2019; Tripathi and Cytryn 2017). Apart from clinical settings, knowledge on resistances dissemination and evolution currently increases rapidly with particular focus on wastewater, agricultural soils, intensive animal-feed operations and the built environment (Chen et al. 2019; Mahnert et al. 2019; Rizzo et al. 2013; Baquero et al. 2008). In that regards, crop plants are still underexplored, despite serving as potential gateway for antibiotic resistant bacteria and antibiotic resistant genes to the human gut (Berger et al. 2010). The conditions influencing produce resistomes during the postharvest period are almost unknown.

The postharvest period is furthermore crucial in terms of food preservation. In total, 45% of all produced vegetables and fruits is lost on the way from the field to the consumer (FAO 2011); especially fungi causing food decay and spoilage represent a tremendous problem worldwide (FAO 2015b; Snowdon 1990). Currently, heavy countermeasures, mainly based on chemical fungicides, are taken to maintain the quality during the storage period. However, there is a growing public demand for sustainability over the whole supply chain and increasing pressure on industry to withdraw hazardous chemicals based on human health considerations (Droby et al. 2009; Kusstatscher et al. 2020). Biological control products for postharvest applications are therefore believed to become significantly more important in the future (Glare et al. 2012). Here, the antagonistic effect of specific microorganisms to combat fungal pathogens on vegetables and fruits is strongly believed to supplement current chemical treatments, however requiring intense research on the underlying mechanisms.

Objectives addressed on the apple microbiome

Within the doctoral thesis at hand, the apple holobiont served as model system to study the impact of current crop production and transport systems on the microbiome and resistome and to evaluate the microbial potential to reduce postharvest fruit decay. The three following questions were addressed on the apple microbiome:

i) how does organic and conventional management practices affect the apple fruit microbiome and is there a potential impact for human health?

In **Chapter 4: Publication IV: 'An apple a day: which bacteria do we eat with organic and conventional apples?'** novel insights into the apple fruit microbiome are presented and the impact of different management practice on the apple microbiome is discussed. The comparison of organically produced apples fresh from the tree to conventionally managed apples bought at the supermarket, allowed to decipher to which microbiota the consumer is usually exposed. Apple fruits were furthermore divided into six different tissues and the bacterial abundance in each tissue was evaluated. The data obtained can be applied to portray the contribution of the microbiota for quality and safety of apples.

ii) is there an effect of the global supply chain on the apple-associated microbiota, traceable on the antibiotic resistance gene composition?

Along the processing chain, vegetables and fruits are subjected to various treatments that can alter the metabolic composition of the plant; secretly, also the inherent microbiota respond on a structural and functional level (Droby and Wisniewski 2018). The resistome reflects the continuous co-evolution of microbial genomes and bioactive molecules in the environment (Wright 2007); thus, also the factors associated with postharvest processing and the global transport of vegetables and fruits may impact the inherent resistome composition. In order to get a deeper understanding of microbial resistances in fresh produce and the impact of the global supply chain on that, the resistome of apples was studied via metagenome sequencing **(Chapter 5: 'Manuscript V: From the tree to the consumer: deciphering the apple fruit resistome along global supply chain')**. Apples cultivated in South Africa were investigated fresh from the tree and after commercial transport to an Austrian supermarket in order to achieve novel insights into produce resistomes and the potential of intercontinental food transport to contribute to the emergence of environmental antibiotic resistance.

iii) how does the postharvest apple microbiome respond to currently in-use hot water treatment and can we increase storability by means of the indigenous microbiome?

A variety of plant-associated microorganisms possess antagonistic activity towards plant pathogens, building a protective shield for their host (Berg et al. 2016). This effect can be prolonged even after harvest (Droby and Wisniewski 2018) and thus, developed on industrial scale to increase storability of vegetables and fruits. **Chapter 6: 'Publication 6: Microbiome response to hot water treatment and potential synergy with biological control on stored apples'** discusses the apple microbiome response to postharvest disease and hot water treatment (HWT), which is a sustainable method to reduce pathogen-induced postharvest fruit decay. This study is the first providing deeper insights into the microbiome changes induced by currently in-use HWT on industrial scale and suggests improved protectivity by applying a combined process with biological control consortia.

CHAPTER 1

Publication I: Understanding the Indigenous Seed Microbiota to Design Bacterial Seed Treatments

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Abstract

Within millennia of domestication, crops and their seeds underwent traceably different adaptive trends, allowing rapid speciation and divergence that lead to phenotypic and genotypic distinction to their wild ancestors. Promoted by these dynamic processes, also the microbiotas have secretly co-evolved with the host plants. Recent studies revealed an unexpected microbial diversity and abundance within seeds with bacterial endophytes as symbiotic components. Soil type, climate, geography and plant genotype were identified as main drivers of the seed microbiota. In addition, domestication and intensive agricultural management changed the seed microbiota. This resulted in a loss of diversity, which has consequences for *one health*-related issues. In order to restore microbial diversity, bacterial seed treatments can be designed. They can be reconstructed based on the rich diversity of seeds of wild ancestors or other native plants. The resulting seed biologicals can be harnessed for sustainable agricultural approaches by improving stress tolerance and resilience of modern crops.

The Impact of Domestication on Plants and Seeds: Diversification and Diversity Loss

The domestication of plants and animals was a precondition for the expansion of civilization and the transformation of world-wide demography (Diamond 2002). It gave rise to the recent onset of rapid evolution and accompanied immense diversification and a global spread of domesticated plants. These dynamics inspired Charles Darwin in the process of formulating the *thesis on the origin of species through natural selection* (Darwin 1968). The cultivation of crop plants started 13,000 years ago and today´s divergence of domesticated plants to their wild ancestors emerged as a consequence of selecting wild plants that were gathered and cultivated by hunter-gatherers in early domestication periods (Darwin 1968). In contrast to the early periods of domestication, which resulted in a diversification of plant genotypes, today´s agriculture and human lifestyle push domestication processes to a distinct outcome: a global landscape highly dominated by modern crops, accompanied by the homogenization of plant genotypes (Purugganan and Fuller 2009). In addition, nutritional demand of the growing world population is constantly increasing and due to constraints in time and space, agriculture is focusing on extensive breeding and cultivation of specific crop cultivars with desired genotypic and phenotypic characteristics (Gruber 2017). Today, 90% of world´s energy demand is accomplished by only 15 crops and two-thirds of the world´s calorie intake depends on rice, maize and wheat (FAO 2017). Specialized breeding and crop selection engender the loss of heirloom breeds; a sheer amount of 70% of wild relatives of modern crops are in risk of getting lost (Castañeda-Álvarez et al. 2016). Due to recent developments, the genetic diversity of plants is in urgent need of protection as an untold numbers of plant genotypes are going extinct.

Altogether, domestication and especially intense agriculture causes long-lasting anthropogenic environmental impacts as it replaces natural vegetation, and thereby decreases diversity, and alters biogeochemical cycles. Therefore, a new human-dominated geological epoch, the Anthropocene was defined (Lewis and Maslin 2015). There are many examples for significant anthropogenic signatures, which are related to agriculture. The conversion of atmospheric nitrogen to ammonia by the Haber–Bosch process for fertilizer production, has altered the global nitrogen cycle so fundamentally, that the nearest suggested geological comparison refers to events about 2.5 billion years ago (Canfield et al. 2010). A likewise global effect was induced by the land use conversion for agriculture. Large-scale conversions resulted in species extinctions some 100 to 1,000 times higher than background rates and probably

constitute the beginning of the sixth mass extinction in Earth's history. Crops, domesticated animals and pathogens are efficiently exchanged around the world; this leads to a global homogenization of Earth's biota.

Seeds transmit the footprint of domestication (Berg and Raaijmakers 2018) and especially their altered morphology over time is therefore frequently studied by archeobotanic and genomic research. Cultivation pressure on plant seeds started about 8,000 years ago and was primarily applied on seeds of the plant family *Poaceae*. Targeted traits were the improvement of germination, with increased soil disturbance and sowing depth, as well as facilitated harvesting (Harlan 1973). Those traits were accompanied by the two major alterations on seed phenotypes, namely increased grain size and the selection of non-shattered cultivars (Baskin and Baskin 1998). The latter is considered as the most characteristic trait for plant domestication as it predicates successful seed-dispersal on human activity (Purugganan and Fuller 2009). Between then and now, extended multi-stage processes altered the genotype and phenotype of crop seeds. However, present seed treatments are considered to be among the most severe trends since the early stages of plant domestication.

The Plant and Seed Microbiota and their Main Drivers

Plants Harbor Distinct Habitat-specific and Species-specific Microbial Signatures

Plants and their associated microbes have been interacting with each other for a long time, forming assemblages of species that are referred to as holobionts (Vandenkoornhuyse et al. 2015). The plant-associated microbiota has the ability to contribute multiple aspects to the functioning of the plant holobiont, such as (i) seed germination and growth support, (ii) nutrient supply, (iii) resistance against biotic stress factors (pathogen defense), (iv) resistance against abiotic factors, and (v) production of bioactive metabolites (Berg et al. 2016). Plants harbor distinct habitat-specific microbial signatures, which are mainly shaped by abiotic factors. The phyllosphere comprises all above-ground organs, which are exposed to the air and permanently changing abiotic factors such as ultraviolet (UV) radiation, temperature and water, and a general low nutrient availability (Remus-Emsermann and Schlechter 2018). The phyllosphere can be further subdivided into the caulosphere (stems), phylloplane (leaves), anthosphere (flowers), and carposphere (fruits). Endophytic communities represent an intimate core of the plant microbiota and distinct connections of the different plant microhabitats and development stages

are of special importance for health issues (Hardoim et al. 2015). A reservoir for the plant's endophytes is the rhizosphere, which represents the below-ground interface with the highly diverse soil microbiota (Berg et al. 2005). Due to this importance for the holobiont, the factors that shape the plant microbiome have been studied for a long time. After a longer debate, it is accepted that the plant genotype and the soil quality are the crucial factors influencing the composition of the rhizosphere microbiota (Berg and Smalla 2009). Both have an impact, but the extent depends on many factors (plant's morphology and secondary metabolism and soil type) and is triggered by plant root exudates and signaling (Badri et al. 2013; Doornbos et al. 2012).

The spermosphere is the zone surrounding seeds where interactions between the soil, microbial communities and germinating seeds take place (Schiltz et al. 2015). This microenvironment links the above and below-ground microbiome of plants. Plant domestication processes have impacted the plant microbiota assembly and its functions via habitat expansion and via changes in crop management practices, root exudation, root architecture, and plant litter quality (Pérez-Jaramillo et al. 2016). The authors proposed a "back to the roots" framework that comprises the exploration of the microbiome of indigenous plants and their native habitats for the identification of plant and microbial traits, with the ultimate goal to reinstate beneficial associations that may have been undermined during plant domestication.

The Seed Microbiota and its Specific Microbial Signatures and Drivers

For a long time, it was assumed that the emerging seedling is colonized by microorganisms from its surrounding environment, with soil being the main source, controlled by the plant through different strategies, such as the specific profile of root exudates and its immune system (Truyens et al. 2015; Sánchez-Cañizares et al. 2017; Shade et al. 2017). Therefore, the study of the seed's microbiota was often neglected in the past or focused only on the occurrence of pathogens. Moreover, the relevant literature is largely based on culture-dependent investigations (Nelson 2018). The seed itself was the last "wasteland" in the landscape of plant microbiology. In the last decade, seeds have been discovered as source for the transmission of a plant-specific core microbiota; an overview of selected studies and their main findings is shown in Table 1.1.

Table 1.1 Overview of the current studies on seed microbiomes with their main findings.

Plant species/cultivar	**Main findings**	**Reference**
Maize (Zea mays)	*Domesticated maize and its wild ancestor share a significant core microbiota within seeds with potential plant growth promoting activities. The seed microbiota is conserved across boundaries of evolution, ethnography and ecology.*	(Johnston-Monje and Raizada 2011)
Rice (Oryza sativa)	Seeds of two consecutive rice generations were shown to share 45% of bacterial endophytes. Soil type is a major driver of the relative abundance of seed-borne strains.	(Hardoim et al. 2012)
Brassica and *Triticum* species	A conserved epiphytic core microbiota on seeds of geographically and ecologically distinct crops of the same species was identified. It included bacterial strains with antagonistic potential towards a fungal plant pathogen.	(Links et al. 2014)
Bean (Phaseolus vulgaris)	The seed microbiome is affected by the host´s terroir.	(Klaedtke et al. 2016)
Radish (Raphanus sativus)	The bacterial seed microbiome was not changed by application of phytopathogenic bacteria, while application of a fungal pathogen changed the seed mycobiome, without affecting bacterial assemblages.	(Rezki et al. 2016)
Pumpkin (*Cucurbita pepo)*	Seed microbiomes have stronger genotype specificity, but lower diversity compared to the rhizosphere.	(Adam et al. 2018)
Maize *(Z. mays)*	Plants grown in sterile and non-sterile soils shared the same dominant rhizosphere microbiota, suggesting seeds to be the primary inoculum.	(Johnston-Monje et al. 2016)
Tall fescue (Schedonorus arundinaceaus)	Infection by *Epichloë coenophyila* promoted fitness of the host plant by influencing the microbiome composition of seeds.	(Mormile 2016)
Sueda salsa	The dominant seed endophyte *Cladosporium cladosporioides* improved host germination rate.	(Qin et al. 2016)
Quinoa	Peculiarities of quinoa regarding stress resistance and germination ability is in part explainable by seed endophyte activity, particularly by seed-borne *Bacillus* species.	(Pitzschke 2018)
Cucurbitaceae species	Seeds of 21 cucurbit varieties shared a cultivable core microbiota consisting of *Bacillus* species, potentially promoting host plants.	(Khalaf and Raizada 2016)
Pepper (*Capsicum annuum*), soybean (*Glycine max*), *T. aestivum*	A potential biocontrol agent was introduced into seeds of various crop species. The accompanied modification of seed microbiota enhanced plant growth of treated seeds compared to control seeds in field trials.	(Mitter et al. 2017)
Malvaceae species	Natural cotton seeds harbored plant beneficial bacteria that promoted growth and alleviated salt stress when they were applied on cultivated plants under abiotic stress conditions.	(Irizarry and White 2017)
Oilseed rape (B. napus)	High genotype-specific bacterial diversity in seeds entailed colonization resistance towards potential pathogens and applied biologicals.	(Rybakova et al. 2017)
Soybean (Glycine max)	Seed microbial diversity was higher compared to sprout microbial diversity and taxonomy suggested sprouts to contain beneficial bacteria transmitted from seeds.	(Yang et al. 2018)
Rice (O. sativa)	Regardless of physiological salinity tolerance of the host, seeds of different plants shared a similar microbiota with stress tolerance alleviation and plant growth promoting activities.	(Walitang et al. 2017)

Radish (R. sativus)	The composition of seed microbiota was related to host community membership. Ecological drift and dispersal drives bacterial and fungal seed endophytes.	(Rezki et al. 2018)
Muskmelon (Cucumis melo)	Groups of seed endophytes are specialized to specific niches within seeds.	(Glassner et al. 2018)
Browntop millet (Brachiaria sp.)	Indigenous seed endophytes promoted seedling development and protected seedlings from fungal pathogens.	(Verma and White 2018)
Cucurbitaceae species	Cultivable seed endophytes possessed significant disease suppression potential against five major fungal and oomycete pathogens, by secretion of bioactive VOCs and extracelluar ribonucleases.	(Khalaf and Raizada 2018)
Bean (P. vulgaris)	Plant beneficial *Azospirillum brasilense* was vertically transmitted from the mother plant, forming significant intercellular population in seeds.	(Malinich and Bauer 2018)
Bean (P. vulgaris), radish (R. sativus)	Changing nutrient availability was followed by a selection of microbiota with functional traits linked to copiotrophy by different plant species.	(Torres-Cortés et al. 2018)
Barley (Hordeum vulgare)	Barley seed endophytes showed high rhizosphere competence and plant growth promoting effects. They induced resistance against a *Blumeria* pathogen in a greenhouse assay.	(Rahman et al. 2018)
Cucumber (Cucumis sativus)	Microbes, recruited by germinating seeds modified seed exudates to reduce encystment and germination of phytopathogenic *Pythium* species.	(Jack and Nelson 2018)
Crotalaria pumila	A bacterial microbiota was shared across three consecutive seed generations. It included a high abundance of bacteria that supported the host growing in metal mine residues.	(Sánchez-López et al. 2018)
Phragmites australis	Seed microbiota improved seed germination and plant growth of *P. australis* and protected the host from damping off disease, while mortality of competitor plants was increased.	(White et al. 2018)
Salvia miltiorrhiza	The seed core microbiome supports plant health and showed indications to supplement secondary metabolic capabilities of the host plant.	(Chen et al. 2018)
Ground-ivy *(Glechoma hederacea)*	A similar pool of bacteria and fungi were vertically transmitted from the mother plant to the offshoots in clonal plants. A significant effect of the distance between mother and daughter plants was found.	(Vannier et al. 2018)

Noteworthy, all of these studies revealed an unexpectedly high diversity and abundance of the seed-associated microbiota (Berg and Raaijmakers 2018). In some of these studies, up to 20,000 microbial species and up to two billion of bacterial cells were detected in one seed (Adam et al. 2018; Johnston-Monje et al. 2016; Shade et al. 2017). In general, the seed microbiota consists of bacteria, archaea and fungi. The presented studies also focus on the main drivers of the seed microbiota. Microbial compositions of seeds are described to vary between different geographical sites (Klaedtke et al. 2016), soil types and soil-associated microbiomes (Hardoim et al. 2012; Liu et al. 2013). In addition, microbial inoculants including pathogens and beneficials were shown to shape the seed´s microbiota (Mormile 2016; Rezki et al. 2016). However, plant genotype specificity of the seed microbiome has been described frequently

(Barret et al. 2015; Adam et al. 2018; Rybakova et al. 2017; Wassermann et al. 2019) and it was shown that seed endophytes can even be highly conserved across generations of a plant species (Johnston-Monje and Raizada 2011; Links et al. 2014). Besides the horizontal transfer of microbiota from diverse environmental sources, thus, vertical transfer of microbiota to the next generation via seeds plays a key role in adjusting the seed microbiome (Truyens et al. 2015). The mother plant is suggested to be responsible for the recruitment (Nelson 2018), and the plant genotype to be the main driver of a specialized seed microbiota. Hence, threats of plant extinction, driven by the implications of recent trends in human culture, affect the whole genomic entirety of the holobiont. Incidentally, the plant microbiota influences evolution of plants, as well as their phenotypic and epigenetic plasticity (van der Heijden et al. 2016); thereby biodiversity-loss forges ahead. A clear and drastic impact of domestication on seed microbiota was identified (Pérez-Jaramillo et al. 2017). Together with the centralized production and intensive treatment on seeds, plant genotype-specific seed microbiota are most probably homogenized and reduced, taking their functional and metabolic secrets with them (Figure 1.1).

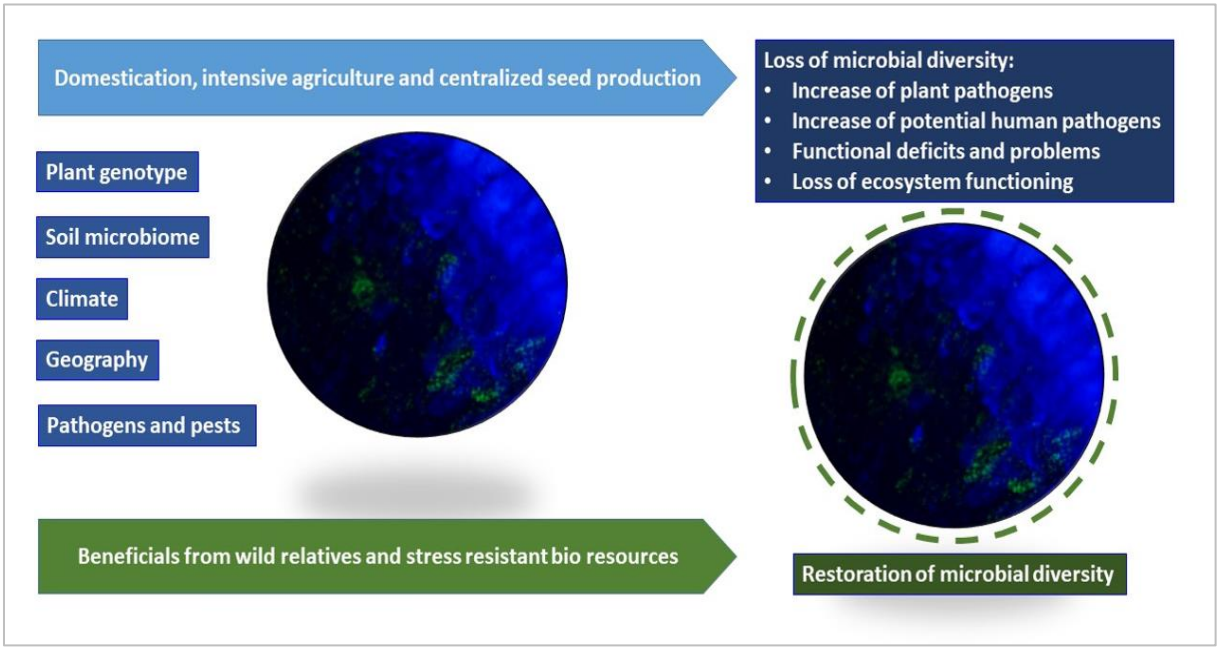

Figure 1.1 Factors influencing the composition of the seed microbiota. Negative effects of crop domestication are highlighted together with a potential countermeasure.

The indigenous seed microbiota is characterized by a high diversity and abundance of bacteria, archaea and fungi. The seed microbiome consists of up to 20,000 microbial species and up to two billions of bacterial cells in one seed. The composition of the microbiota is influenced by the soil type and its microbial population, by climate and geography, as well as by biotic factors such as pathogens and pests. However, the plant genotype is the main driver; therefore, crop domestication has a crucial impact on the seed microbiota.

When the impact of domestication on crop seed microbiomes is studied, the seed microbiomes of plants from natural ecosystems are especially of interest, as solely undisturbed environments are appropriate to explain indigenous plant-microbe-interactions. Seeds of plants from natural ecosystems have to feature high adaptations in dispersal, persistence and germinative ability under diverse environmental conditions (Fenner and Thompson 2005). Seeds and seedlings are exposed to a range of hazards like drought, resource limitation, herbivores and eukaryotic or prokaryotic pathogens (Bever et al. 2015). The seed microbiome, considered as the primary inoculum for plants (Barret et al. 2015), might have a major impact on the plant's possibilities to combat this plethora of biotic and abiotic stressors. Different visualization techniques can be applied to verify the colonization of seeds by distinct microorganisms. Scanning electron microscopy (SEM) and confocal laser scanning microscopy in combination with fluorescent *in situ* hybridization (FISH-CLSM) were used to visualize native micro niches of bacteria and fungi colonizing the seed surfaces (Figure 1.2) and internal seed tissues (Figure 1.3) of natural plants from the east alpine region of Austria. Studies that target seed microbiomes of plants from natural ecosystems are, however, still rare.

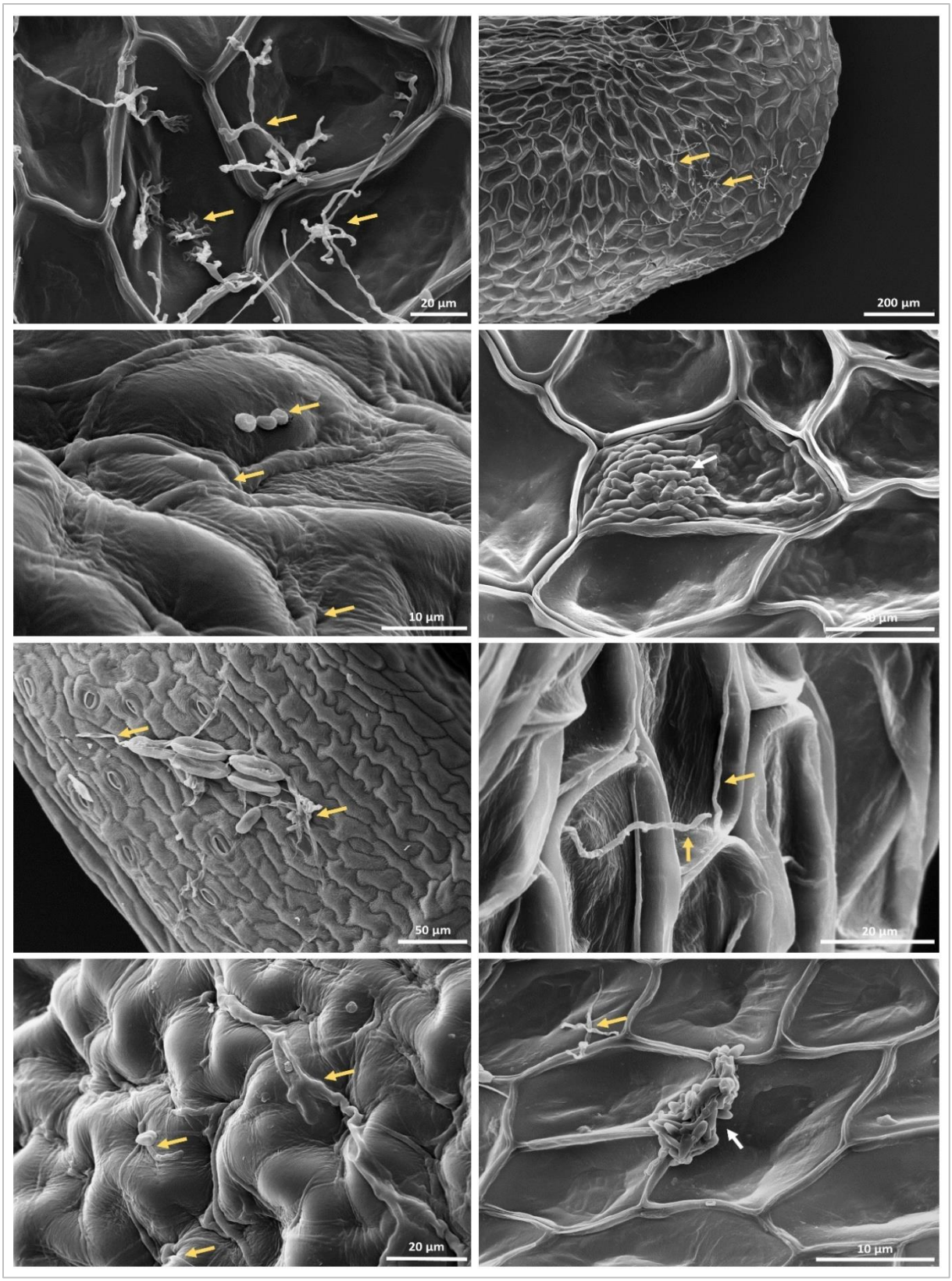

Figure 1.2 Scanning electron micrographs of the native, epiphytic colonization by bacteria (indicated by *white arrows*) and fungi (*yellow arrows*) on seeds of natural plants from the east alpine region of Austria (*Gentiana asclepiadea*, *Gentianella germanica* and *Parnassia palustris*).

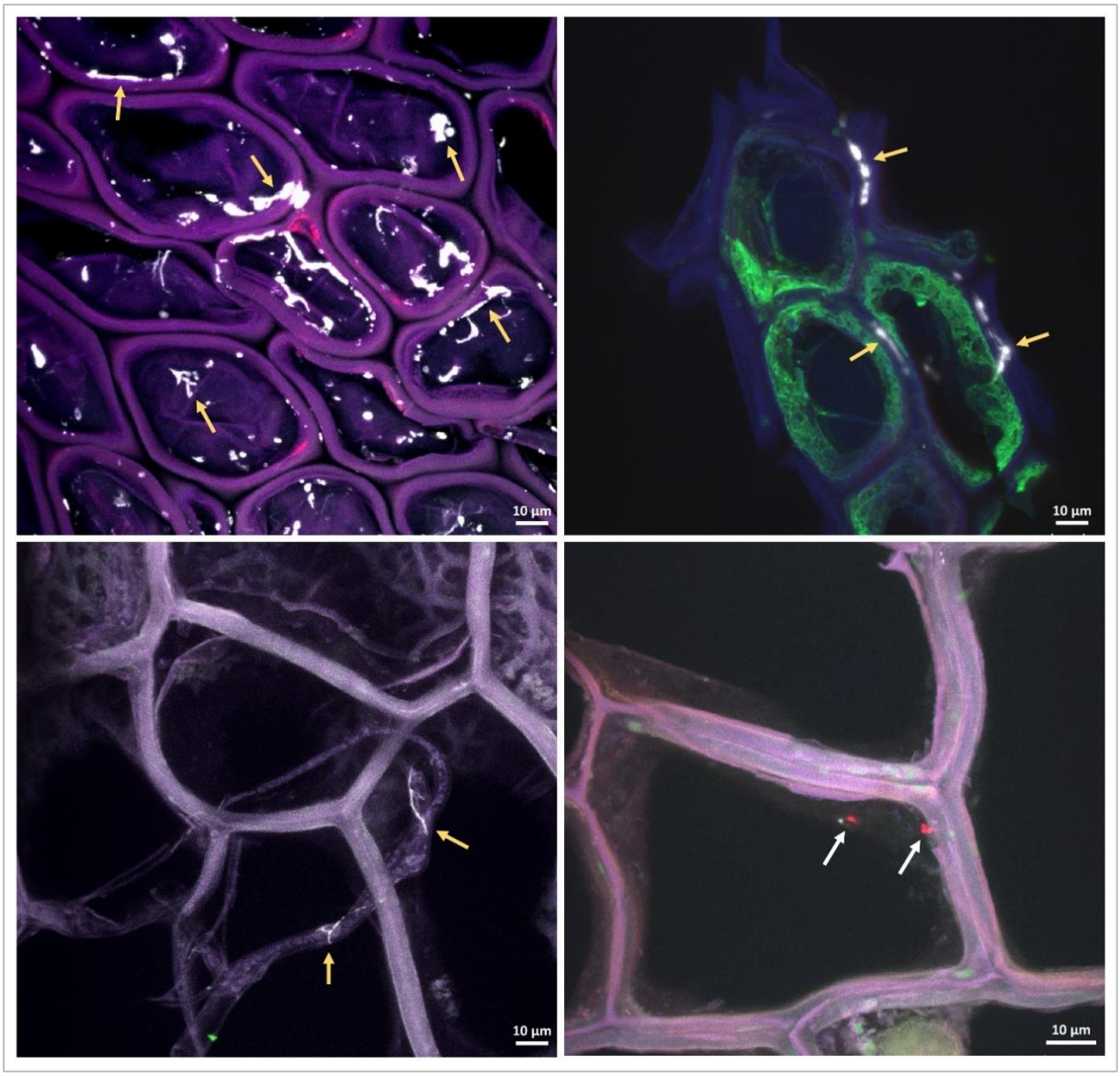

Figure 1.3 Confocal laser scanning micrographs of endophytic colonization patterns of *P. palustris* and *G. asclepiadea* seeds, by fungi (indicated by *yellow arrows*) and bacteria (*white arrows*) visualized by fluorescent *in situ* hybridization. Plants were gathered from the east alpine region of Austria.

Microbial Diversity and Health Issues

The Interconnected Microbiome Highlights the *One Health* Concept

The microbiota of soil and plants plays a crucial role in plant and ecosystem health (Berg et al. 2017; Laforest-Lapointe et al. 2017). Recently, the importance of the plant microbiota for human health was evidenced (David et al. 2014). The plant-associated microbial diversity can

be transferred to the gut microbiome, because fruits and vegetables are the major components of a healthy diet. However, loss of microbial diversity in the gut is associated with acute outbreaks as well as with chronic disease, e.g. allergies, obesity, and mental diseases (Turnbaugh et al. 2006). Increasing chronic diseases in children can be explained by the *missing microbe theory* published by Blaser (Blaser 2014). In 2017, this was further developed into the *theory of disappearing microbiota and the epidemics of chronic diseases*, which postulates that losses of particular bacterial species of our ancestral microbiota have altered the context in which immunological, metabolic and cognitive development occur in early life, resulting in increased disease susceptibility (Blaser 2017). Already in 2012, Hanski et al. showed that microbial biodiversity, human microbiota, and allergy are interrelated (Hanski et al. 2012). Structural and especially functional microbial diversity is already established as a key factor in preventing human diseases (Jakobsson et al. 2014), and is suggested as biomarker for plant health as well (Berg et al. 2017). Moreover, overlapping compositions, and interconnected microbiomes of human, animal and plant in connection with health should be considered, and used to expand the version of *one health*, that includes environmental health and its relation to human cultures and habits (Flandroy et al. 2018). Berg and Raaijmakers (Berg and Raaijmakers 2018) postulated the 'domestication syndrome' for plants and humans. It was found that changes in relative abundances of gut microbiota, more precisely between *Bacteroidetes* and *Firmicutes*, contribute remarkably on the pathophysiology of obesity in humans (Turnbaugh et al. 2006). For plants, the domestication footprint is expressed in a shift from *Bacteroidetes* to *Proteobacteria* (Germida and Siciliano 2001; Adam et al. 2018; Pérez-Jaramillo et al. 2017).

The Role of Soil and Seed Microbiomes to Maintain Microbial Diversity

Soil acts as a microbial seed bank. A protective and supportive impact of a diverse soil microbiome on plant health and resilience has been frequently reported (Raaijmakers and Mazzola 2016). Bender and Van der Heijden, for example, observed that soil microbial diversity is directly correlated with increased nutrient uptake efficiency of crop plants and subsequent increase of crop yields (Bender and van der Heijden 2015). A loss of microbial symbionts reduces the capability of plants and seeds to deal with pathogen attacks, adverse environmental conditions and impacts of a changing climate (Truyens et al. 2015; Nelson 2018). Moreover, reduced soil biodiversity is assumed to facilitate the proliferation of plant pathogens (Mendes et al. 2013; Raaijmakers and Mazzola 2016). Besides the direct impact on plant health and performance, a reduced dynamic reservoir of soil biodiversity is increasingly recognized to have profound impacts on human and ecosystem health (Wall et al. 2015). Low

microbial soil diversity is described to support the accumulation of soil-borne human pathogens (Berg et al. 2005) , that are hereinafter likely to contaminate staples, drinking water (Oliver and Gregory 2015) and even the air we breathe (Garrison et al. 2003). In fact, the reduction of soil microbial diversity might result in a decreased capacity of soil foodwebs to perform substantial functions for the whole ecosystem (Wall et al. 2015), with tremendous impact on health conditions of the human population. As a consequence, ever-stronger human interventions and pesticides are required (Oerke 2006; Gruber 2017). In between the microbiome connection, seeds also play a crucial role. Here, plants store their own beneficial inoculum to maintain plant health over generations.

Biotechnological Solutions for Sustainable Agriculture

Seed germination and seedling development are among the most vulnerable stages in a plant's life cycle (Leck et al. 2008), and the importance of the associated microbiome for seed and plant health is high. Seed-associated microbiomes contribute significantly to improve seed vigor and promote germination (Glick et al. 1998; Darrasse et al. 2010); Several plant families, e.g. *Orchidaceae* or *Sphagnaceae*, depend on beneficial microorganisms during germination. Today, various modern cultivars need chemical protection for their establishment in soil. However, many seed treatments are controversy discussed, e.g. copper seed treatments in organic agriculture. Neonicotinoids represent another one as they are the most widely used class of insecticides in the world for seed treatments. Due to their impact on bees, a ban by the European Union came into force in 2018. Modern cultivars, banned pesticides, and missing microbial diversity require novel solutions in plant biotechnology.

Several solutions are already suggested in literature and other ones are already commercialized. Berg and Raaijmakers proposed a so-called 'back to the future' approach: unraveling the seed microbiomes of wild relatives and ancient heirloom breeds of crop cultivars to save beneficial seed microbiomes for agriculture (Berg and Raaijmakers 2018). Harnessing seed microbiomes of wild relatives of crop plants from natural ecosystems, potentially enables a matching symbiosis between the plant and its specific seed microbiota. Conservational patterns of seed microbiota across boundaries of evolution were discovered by Johnston-Monje and Raizada, comparing the seed microbiomes of modern *Zea* cultivars and their wild ancestors (Johnston-Monje and Raizada 2011). Another solution was suggested by Zachow and colleagues (Zachow et al. 2013); the authors developed a direct selection strategy to obtain cultivable microorganisms from promising bio resources (alpine mosses, lichens and primrose)

using the bait plants and seeds. In a recent study, seeds of the Styrian oil pumpkin (*Cucurbita pepo* subsp. *pepo* var. Styriaca) were treated with fluorescent protein-tagged, beneficial *Serratia plymuthica* strains. These seeds naturally lack the lignification of the outer seed coat and thus provide less protection against microbial intrusion. It was found that the bacterial strains colonize outer and inner seed compartments after seed priming (Figure 1.4a and 1.4b). Due to the localization of beneficial bacteria in the inner seed compartments, this seed treatment leads to an early protection of the cotyledons and the rhizoplane of the emerging seedlings (Figure 1.4c). Moreover, the strains were highly abundant on roots (Figure 1.4d) and the first true leaves of young plants. This treatment is a promising alternative for conventional seed treatments with chemical fungicides containing, for example, Fludioxonil. In addition, Mitter and co-workers demonstrated the feasibility and promising utility of using seed microbiota for sustainable crop cultivation (Mitter et al. 2017). The authors succeeded to insert a potential biological control agent into seeds. During field trials, those treated seeds showed faster plant development compared to control seeds.

Current knowledge on the indigenous seed microbiota allows to draw some conclusions for several applied aspects and biotechnology.

1. **The structure of the seed microbiota can be used as novel biomarker in breeding strategies. Moreover, joint breeding strategies of the plant and the indigenous plant-associated microbiota are promising.**
2. **Breeding strategies can be successfully combined with biocontrol strategies. Biocontrol and stress protecting agents can be designed and applied as seed treatments.**
3. **Currently global seed production and management focus on uniform, pathogen-free and clean seeds. Learning from the seed studies would suggest a local production of indigenous cultivars. This could reduce the amount of required pesticides, because the plants are better adapted to the certain environment.**
4. **Seed cleanings and assessments can be evaluated using microbial diversity as criterion.**
5. **Conservation strategies for seeds to preserve genetic diversity, which already exists, should include conservation strategies for seed microbes as well.**

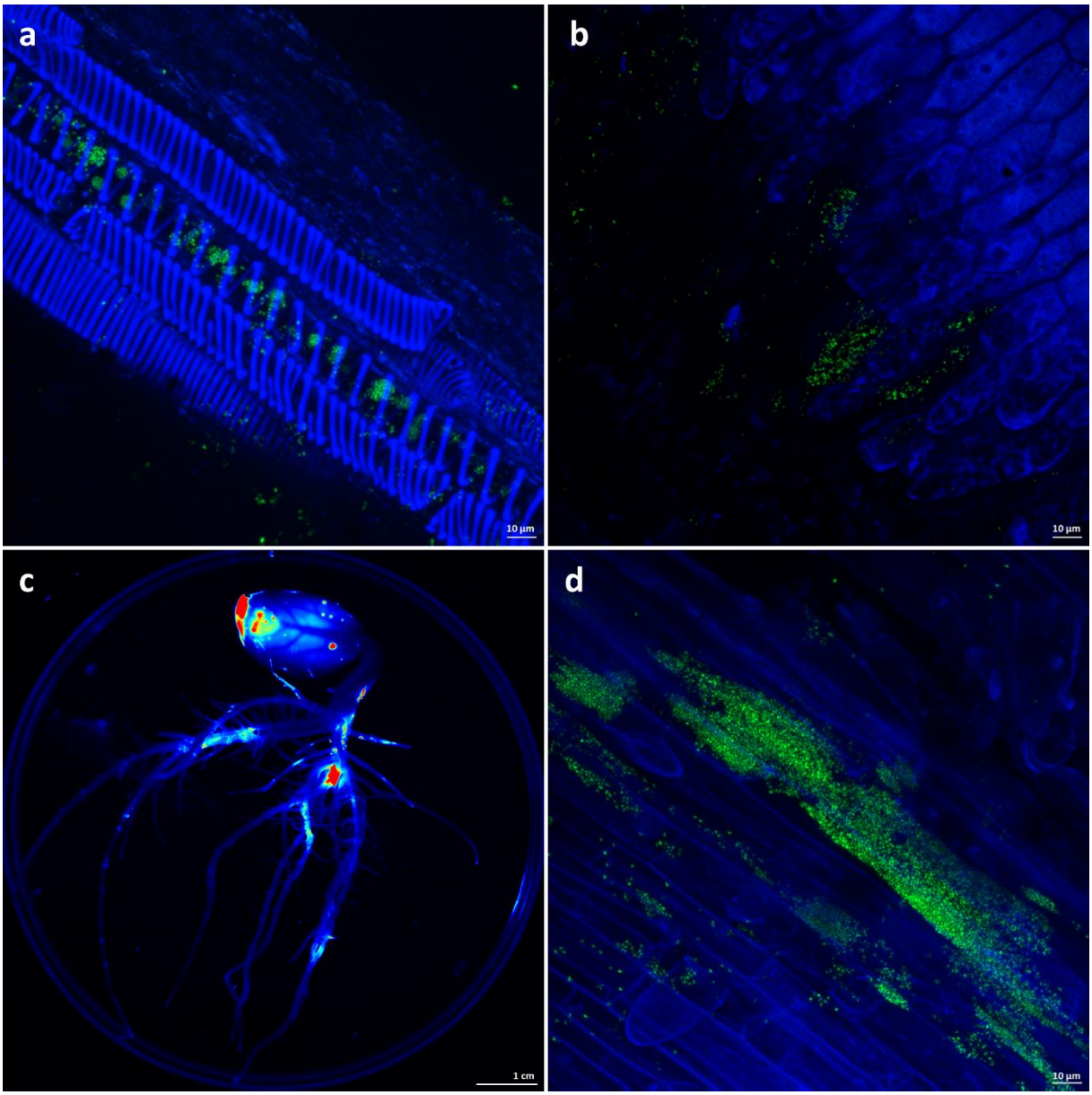

Figure 1.4 Confocal laser scanning micrographs (seeds and roots) and whole-plant visualization of the Styrian oil pumpkin (*blue signal*). Seeds were primed with fluorescent protein-tagged *Serratia plymuthica* strains (*green signal*) and the colonization was visualized two days after the treatment: (a) vascular bundle in the chlorenchyma layer of the outer seed coat and (b) root tip of the root-hypocotyl-embryo; (c) *S. plymuthica* colonization of a pumpkin seedling nine days after seed priming, visualized with a Bio-Rad ChemiDocTM XRS System; (d) CLSM of a densely colonized root of a seed primed pumpkin plant, 16 days after inoculation.

Conclusion

As a process of evolution and species diversification, domestication created a rich genetic diversity of early ancestors of modern crops. The co-evolution of plants and microorganisms resulted also in genotype-dependent seed microbiomes, which need to be better understood. Since multi-omics technologies allow us deeper insights into the functioning of the holobiont, we should intensively focus on the following issues: How do native seed microbiota perform under stressful conditions? How stable is the seed microbiome? How do horizontally transmitted seed microbiota overcome the plant´s defense strategies to become endophytes? Bacterial seed treatments can be designed, which allow a better functioning of the crop holobiont to cope with pathogen pressure and even climate change.

CHAPTER 2

Publication II: Seeds of Native Alpine Plants Host Unique Microbial Communities Embedded in Cross-Kingdom Networks

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Abstract

The plant microbiota is crucial for plant health and growth. Recently, vertical transmission of a beneficial core microbiota was identified for crop seeds but for native plants, complementary mechanisms are almost completely unknown. We studied the seeds of eight native plant species growing together for centuries under the same environmental conditions in Alpine meadows (Austria) by qPCR, FISH-CLSM and amplicon sequencing targeting bacteria, archaea and fungi. Bacteria and fungi were determined with approx. 10^{10} gene copy numbers g^{-1} seed as abundant inhabitants. Archaea, which were newly discovered as seed endophytes, are less and represent only 1.1% of the signatures. The seed microbiome was highly diversified and all seeds showed a species-specific, highly unique microbial signature, sharing an exceptionally small core microbiome. The plant genotype (species) was clearly identified as the main driver, while different life cycles (annual/perennial) had less impact on the microbiota composition, and fruit morphology (capsule/achene) had no significant impact. A network analysis revealed significant co-occurrence patterns for bacteria and archaea, contrasting with an independent fungal network that was dominated by mutual exclusions. These novel insights into the native seed microbiome contribute to a deeper understanding of seed microbial diversity and phytopathological processes for plant health, and beyond that for ecosystem plasticity and diversification within plant-specific microbiota.

Keywords: Seed microbiota, native plants, cross-kingdom networks, endophytes, plant resilience

Background

Plants and their associated microbes have been interacting with each other for a long time, forming an assemblage of species that is often referred to as a holobiont (Vandenkoornhuyse et al. 2015). The plant microbiome is essential for plant development, resilience and health (Gabriele Berg et al. 2016). Endophytic communities represent an intimate core of the plant microbiota and connect different plant microhabitats, with specific roles during development stages that are important for health issues (Hardoim et al. 2015). The rhizosphere is a reservoir for plant endophytes and represents the below-ground interface with the highly diverse soil microbiota (Gabriele Berg et al. 2013). For a long time, it was assumed that the emerging seedling is colonized by microbes from its surrounding environment, with soil being the main source and plant-controlled enrichment through different strategies, such as the specific profile of root exudates and its immune system (Truyens et al. 2015; Sánchez-Cañizares et al. 2017; Shade et al. 2017). Therefore, studies related to the seed microbiota have often been neglected or focused only on the presence of pathogens (Nelson 2018); there are less comprehensive studies including all components of the microbiome (Vujanovic and Germida 2017). In the past, only a few studies on seed-derived bacteria were published, because they are difficult to cultivate, while seed-borne archaea were not identified at all so far (Shahzad et al. 2018; Taffner et al. 2018). Our knowledge related to seed fungi is much broader as reviewed by Geisen et al. (Geisen et al. 2017), and Yang et al. (Yang et al. 2018). Especially in terms of information available on clavicipitaceous endophytes such as *Epichloë/Neotyphodium* species due to their beneficial and specific interaction with grasses, which is already commercially exploited (Schardl et al. 2004; Rodriguez et al. 2009). However, we still need a complete picture of the seed microbiota and its interactions and functions in the holobiont.

Recently, crop seeds were discovered as a source to transmit a plant-specific core microbiota (Adam et al. 2018; Johnston-Monje et al. 2016; Berg and Raaijmakers 2018). Studies focusing on the crop microbiome showed that domestication and intense agricultural management entailed alterations of the inherent microbiome of crop plants including a loss of plant-beneficial microbiota (Germida and Siciliano 2001; Pérez-Jaramillo et al. 2016; Pérez-Jaramillo et al. 2017). First results indicate similar effects on the seed microbiota (Adam et al. 2018; Rybakova et al. 2017). Understanding the key components of the indigenous seed microbiota of native plants can support the definition of a healthy microbiota and its translation to our crops. So far, little is known about the indigenous seed microbiota in natural ecosystems (Geisen et al. 2017). Our hypothesis was that seeds of native plants harbor a specific and diverse

microbiota, which allows plant populations to survive, persist and germinate under harsh natural conditions (Fenner and Thompson 2005; Bever et al. 2015).

To decipher the entire seed microbiota of native plants, we selected healthy plant populations persisting over centuries in the European Eastern Alps. The Hochschwab region (Northern Calcareous Alps, Austria) is a glacial retreat for a high diversity of plant species and is botanically and geologically well-studied (Dirnböck et al. 1999). To identify the composition and main drivers (plant genotype, life cycle, fruit morphology) of the native seed microbiota, we selected eight different alpine plant species; all of which were traditionally used as medicinal plants and produce a variety of antimicrobial compounds (Radulović et al. 2012; Teixeira and Silva 2013). Although nothing is known about bioactive compounds in the seeds of the selected species, we expect that those phytochemicals have profound impacts on the seed microbiota. In addition, we hypothesize a strong impact of fruit morphology; here we expected a higher microbial diversity for seeds of indehiscent fruits (achenes) than for seeds of dehiscent fruits (capsules). Achenes are monocarpellate structures, where the seeds are united with the pericarp forming a unit developed and distributed under the influence of the surrounding environment. A separation between seeds and fruit in achenes is not possible methodically, and we use the term seeds in the following text including achenes. Capsules are enclosed systems, where seeds develop inside, covered by the pericarp that splits apart to extrude the seeds at maturity. In these structures, the surrounding environment has a lower impact on the seed-microbiome. The selected plants are also characterized by a different life cycle (annuals/perennials). We hypothesize that perennials can accumulate a higher microbial diversity during their life cycle.

Materials and Methods

Experimental design and sampling procedure

For the microbiome analyses, eight different alpine plant species (in the following referred to as plant genotypes) were selected according to different life cycles and fruit morphologies. Our selection comprised the following species: Great masterwort *Astrantia major* L., Eyebright *Euphrasia rostkoviana* HAYNE, Willow gentian *Gentiana asclepiadea* L., Chiltern gentian *Gentianella germanica* (WILLD.) E.F.WARB., *Heliosperma quadrifida* WALDST. & KIT., Bog Star *Parnassia palustris* L., Yellow rattle *Rhinanthus glacialis* PERSONNAT and Pincushion flowers *Scabiosa lucida* VILL. These plant species differ in their fruit morphologies; *E. rostkoviana, R. glacialis, G. germanica, H. quadrifida, P. palustris* and *G. asclepiadea* produce capsules as dehiscent fruits and *S. lucida* and *A. major* seeds produce achenes as indehiscent fruits. Plants can furthermore be distinguished by their life cycle, which is either annual (*E. rostkoviana, R. glacialis and G. germanica*) or perennial (*H. quadrifida, P. palustris, G. asclepiadea, S. lucida* and *A. major*). All seeds were collected at time of dispersal in maturation state. The sampling was performed on September 4th 2016 at the Aflenzer Staritzen (Longitude: E15.183899, Latitude: N47.622001) in an area of approximately 100 000 square meters in the Hochschwab region (Northern calcareous Alps, Austria), which represent a botanically well-studied glacial retreat (Dirnböck et al. 1999). Each of the eight plant species was sampled from four different sites randomly selected across the total area. Each replicate consists of 15 to 20 plants that grew in close proximity (subpopulations). The distance between the replicates was 200 meter in minimum. Seeds of plants from one subpopulation were handled under sterile conditions and subsequently pooled. From each pool, 50 mg were weighted in, now referred to as one replicate, and total community DNA was extracted. We decided to use consistent seed weights for each replicate instead of seed counts due to strong variability in seed size and anatomy between the different plant genotypes (Figure 2.1, a).

Microbial DNA extraction and amplicon library construction

Seeds were physically disrupted under sterile conditions with liquid nitrogen and the total community DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, USA) and a FastPrep Instrument (MP Biomedicals, Illkirch, France) for 30 seconds at 5.0 ms^{-1}. Illumina amplicon sequencing was performed by using two different barcoded primer

combinations: 515f – 806r (Caporaso et al. 2010) to amplify 16S rRNA gene fragments and ITS1f – ITS2r (T. J. White et al. 1990) to amplify parts of the ITS region, with three technical replicates per sample. By adding peptide nucleic acid (PNA) clamps to the PCR mix, amplification of host plastid and mitochondrial 16S DNA was blocked (Lundberg et al. 2013). PCR for 16S rRNA gene amplification was performed in a total volume of 30 µl (5 x Taq&Go (MP Biomedicals, Illkirch, France), 1.5 µM PNA mix, 0.25 mM of each primer, PCR-grade water and 1 µl template DNA) under the following cycling conditions: 95°C for 5 min, 30 cycles of 96°C for 1 min, 78°C for 5 sec, 54°C for 1 min, 74°C for 60 sec and a final elongation at 74°C for 10 min. Amplification of the fungal ITS region was conducted in 20 µl (5 x Taq&Go, 25 mM $MgCl_2$, 10 µM of each primer, PCR-grade water and 1 µl template DNA) with the cycling conditions: 95°C for 5 min, 30 cycles of 94°C for 30 sec, 58°C for 35 sec, 72°C for 40 sec, final elongation at 72°C for 10 min. A nested PCR step was performed to add barcoded primers. Technical replicates were combined and purified by Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and DNA concentrations were measured with Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). Samples were combined in equimolar concentration and sequenced by Illumina MiSeq v2 (250 bp paired end) amplicon sequencing.

Illumina MiSeq data processing of 16S rRNA gene and ITS region amplicons and statistics

Raw sequence data preparation and data analysis was performed using QIIME 1.9.1 (Caporaso, et al. 2010). Paired reads were joined and quality filtered (phred q20) and chimeric sequences were identified using usearch7 (Edgar 2010) and removed. Operational taxonomic units (OTUs) were picked according to open references given by SILVA ver128_97_01.12.17 for 16S rRNA gene and UNITE ver7_99_01.12.17 for fungal ITS region. *De novo* clustering of OTUs was performed using usearch for bacterial and archaeal 16S rRNA and BLAST for fungal ITS region. Representative sequences were aligned, taxonomy was assigned and sequences assigned to host mitochondria and chloroplasts were discarded. OTU tables were rarefied to the lowest number of read counts (1 739 sequences for 16S rRNA gene and 5 807 sequences for ITS region). Bacterial and fungal core OTUs that were present in all alpine plant seeds investigated were further identified up to species level using NCBI BLAST alignment tool. Rarefied OTU tables served as input matrix for all upcoming alpha and beta diversity analyses. Statistics on microbial diversity and abundance were calculated in QIIME. Significant differences ($p < 0.05$) in Shannon diversity between groups for 16S rRNA gene fragments as

well as for the ITS region were calculated based on parametric two sample t-test at the greatest rarefaction depth using t-distribution to determine the p-value. Beta diversity, based on weighted UniFraq distance matrix for bacteria and bray curtis dissimilarities for fungi, was assessed by Principle Coordinates Analysis (PCoA) and the statistical significance between categorical variables was assessed by Analysis of Similarity (ANOSIM), including the pairwise option to compare differences between all plant genotypes. IBM SPSS program (version 25.0, IBM Corporation, Armonk, NY, USA) was used for calculating significant differences ($p < 0.05$) in microbial gene copy quantity, determined via quantitative PCR, based on ANOVA including Tukey-HSD test correction. Cytoscape version 3.4.0 and the add-on 'CoNet' were used to perform network analysis of significant ($q \geq 0.0004$) co-occurrence and mutual exclusion patterns of the microbiomes. Combined fungal and bacterial OTU table, collapsed on species level using absolute abundances, served as input matrix for the co-occurrence network. To ensemble inferences, Pearson and Spearman correlation coefficients for both positive and negative correlations, using the automatic threshold setting for the 1000 top and bottom edges for each method, the mutual information option, and Bray Curtis and Kullback-Leibler dissimilarity matrices were applied. For the final network, bootstrapping was selected as resampling method and Brown´s method was used to merge method- and edge-specific p-values, discarding unstable edges that showed scores outside the 0.95% range of their bootstrap distribution. The Benjamini-Hochberg method was selected for multiple test correction.

Quantitative Real-Time PCR (qPCR)

For quantifying gene copy numbers of bacteria, archaea and fungi within seeds, a qPCR was performed using the following primer pairs: 515f – 927r for bacteria (10 µM each; (Köberl et al. 2011)), 344aF – 517uR for archaea (5 µM each; (Probst et al. 2013)) and ITS1 – ITS2 for fungi (10 µM each; (White et al. 1990)). The reaction mix contained 5 µl KAPA SYBR Green, 0.5 µl of each primer, 3 µl PCR-grade water and 1 µl template DNA (diluted 1:10 in PCR grade water). Fluorescence intensities were detected in a Rotor-Gene 6000 real-time rotary analyzer (Corbett Research, Sydney, Australia) with the following cycling conditions. Bacteria: 95°C for 5 min, 40 cycles of 95°C for 20 sec, 54°C for 30 sec, 72°C for 30 sec and a final melt curve of 72 to 96°C. Archaea: 95°C for 5 min, 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec followed by melt curve of 72 to 96°C. Fungi: 95°C for 5 min, 40 cycles of 95°C for 30 sec, 58°C for 35 sec, 72°C for 40 sec with a melt curve of 72 to 96°C. Three individual

qPCR runs were conducted for each replicate. Intermittently occurring gene copy numbers that were found in negative controls were subtracted from the respective sample.

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

In-tube FISH technique, followed by visualization with CLSM was performed to observe the colonization patterns and penetration spots of seed-associated bacteria and fungi. Seeds were fixed with 4% paraformaldehyde/phosphate-buffered saline at 4°C over-night prior to FISH application according to the protocol of Cardinale et al. (Cardinale et al. 2008). To stain the overall bacterial community, Cy3-labeled EUB338MIX (Daims et al. 1999) was used and in order to contrast fungal structures from plant cell walls, FISH samples were treated with Calcoflour White.

Results

Visualization of microbial communities and their abundance in alpine plant seeds

Seed morphology and size of the eight alpine plants investigated was highly variable, specifically adapted to their mode of dispersal. Seed size ranged from 0.7 mm for *G. germanica* seeds up to 6.5 mm for *A. major* seeds (Figure 2.1, a). We used different observation methods to explore microbial colonization patterns on seeds. Scanning electron microscopy was applied to monitor seed surfaces for microbial colonization; here, only few epiphytes were detected. Among them, fungal structures were more frequent than bacterial ones (results not shown). In addition, CLSM in combination with specific FISH probes allows to localize endophytes in different sub-compartments of the seeds. Visualization was feasible for *P. palustris* and *G. asclepiadea* seeds (Figure 2.1, b). We found fungal structures more frequently than bacterial ones and especially the surface of *P. palustris* was covered with fungal hyphae. Comparably less Cy3-labeled bacteria were visualized colonizing seeds epi- as well as endophytically. Unfortunately, high autofluorescence of host tissues impeded imaging of microbiota in the seeds of the remaining plant genotypes.

In contrast, quantification via qPCR resulted in high microbial abundances in all seeds investigated, amounting for 2.8×10^{11}, 3.09×10^{9} and 4.2×10^{11} mean gene copy numbers per gram seeds for bacteria, archaea and fungi, respectively (Figure 2.2). Significant differences in microbial abundance were observed between the eight plant genotypes, whereas comparing fruit morphology (capsule or achene) or life cycle of the plant (annual or perennial) resulted in no statistical significance (Additional file 2.1, Table S2.1). This holds true for both the number of total microbial gene copies as well as for bacteria, archaea and fungi calculated separately. The total microbial gene copies per plant genotype, consisting of bacteria, archaea and fungi, ranged from 1.16×10^{11} gene copies in *G. germanica* seeds, up to 2.10×10^{12} gene copies in *R. glacialis* seeds. However, calculating the prokaryote to eukaryote ratio, indicated by percent values in Figure 2.2, resulted in high similarities between the different plant genotypes. Fungal ITS gene copies slightly prevailed over bacterial and archaeal 16S rRNA gene copies, except for *H. quadrifida* and *S. lucida* seeds. Archaeal gene copies were detected in all replicates, however, less than 1% out of total microbial genes per plant genotype were archaeal. This ratio was found to be consistent over the sample collection and no mutual exclusions between the three taxonomic groups were observed: seeds with high bacterial gene copies (*R. glacialis, P. palustris, G. asclepiadea* and *E. rostkoviana*) showed high copy numbers of archaeal and fungal

genes as well, while seeds with less bacterial copy numbers (*A. major*, *G. germanica* and *S. lucida*) exhibit also less archaeal and fungal gene copies.

With respect to both the microscopic and the quantitative evaluations, the majority of seed-associated microorganisms is most probably localized inside seeds.

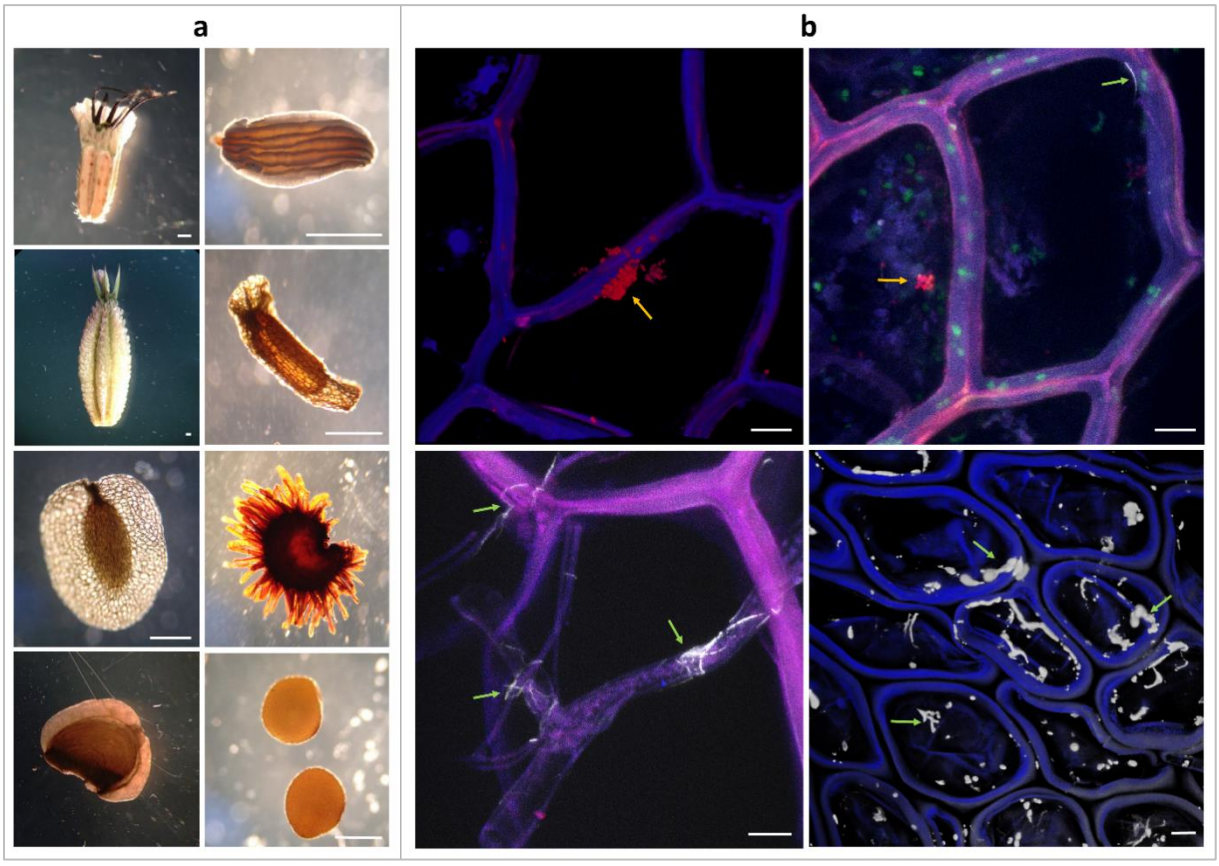
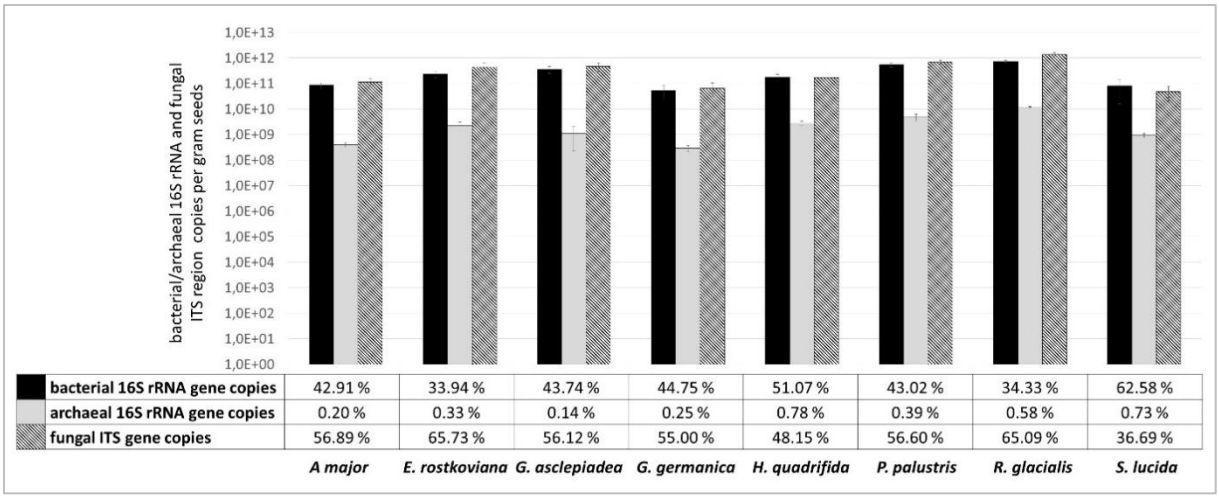


Figure 2.1: Micrographs of alpine plant seeds (including achenes). (a) Whole seed visualization shows the high morphological diversity of the alpine plant seeds investigated; from left to right and top to bottom: *S. lucida, E. rostkoviana, A. major, P. palustris, G. asclepiadea, H. quadrifida, R. glacialis and G. germanica.* Scale bars in (a) indicate 0.5 mm. (b) FISH-Confocal laser scanning micrographs visualize endophytic fungi (indicated by *green arrows*) and bacteria (*yellow arrows*) in *P. palustris* and *G. asclepiadea* seeds. Scale bars in (b) indicate 10 µm.



	A major	E. rostkoviana	G. asclepiadea	G. germanica	H. quadrifida	P. palustris	R. glacialis	S. lucida
bacterial 16S rRNA gene copies	42.91 %	33.94 %	43.74 %	44.75 %	51.07 %	43.02 %	34.33 %	62.58 %
archaeal 16S rRNA gene copies	0.20 %	0.33 %	0.14 %	0.25 %	0.78 %	0.39 %	0.58 %	0.73 %
fungal ITS gene copies	56.89 %	65.73 %	56.12 %	55.00 %	48.15 %	56.60 %	65.09 %	36.69 %

Figure 2.2: Microbial gene copy numbers in alpine plant seeds determined by qPCR. Values are given by primers targeting bacterial and archaeal 16S rRNA gene and fungal ITS region in seeds of the eight different alpine plants. Gene copy numbers are calculated per gram seeds used for the microbiome analysis. Table describes the prokaryote to eukaryote ratio within the total microbial gene copies detected in the seeds of the respective plant genotype. Total microbial gene copies can be looked up in Additional file 2.1, Table S2.1.

Unique microbial composition associated within alpine plant seeds

After quality-filtering and removing chimeric sequences, the amplicon dataset with the 16S rRNA gene fragments from alpine plant seeds contained 4,703,620 paired reads. Chloroplast and mitochondrial sequences were removed and 1,769,793 sequences remained in the dataset that were assigned to a total of 11,842 operational taxonomic units (OTUs). The quality-filtered and chimera-checked ITS dataset contained 10,443,899 paired reads that were assigned to 3,943 fungal OTUs. OTU tables were rarefied to 1,739 bacterial and archaeal sequences and 5,807 fungal sequences per sample, according to the samples with lowest amount of sequences.

The taxonomic assignment of 11,844 bacterial OTUs revealed 36 phyla, among them *Proteobacteria* were predominant in the dataset with 88.9%, followed by *Actinobacteria* (3.3%) and *Bacteroidetes* (2.2%). *γ-Proteobacteria* was the most abundant class received, amounting to 48.7% relative abundance and represented by 83 genera. *α-Proteobacteria* showed the highest diversity, being represented by 395 genera and amounting to 34.4% relative abundance. *Actinobacteria* and *Bacteroidetes* were represented by 200 and 68 genera, respectively. *Firmicutes* (141 genera), *Acidobacteria* (51 genera), *Chloroflexi* (45 genera), *Planctomycetes* (38 genera), *Verrucomicrobia* (26 genera) and *Cyanobacteria* (21 genera) showed each less

than 1% abundance in the whole dataset. Remaining, and less represented taxa are not described here.

Archaeal taxonomy was assigned to 32 OTUs and represented 0.05% of the 16S rRNA gene sequences. Archaea were mainly represented by *Thaumarchaeota* (98.3% rel. abundance of all archaeal sequences) with three genera of the Soil Crenarchaeotic Group and *Nitrosphaera*. *Euryarchaeota* were less represented, amounting to 1.7% of archaeal community but were more diverse, consisting of the genera *Haladaptatus*, *Methanobacterium*, *Methanobrevibacter*, *Natronorubrum*, *Methanosphaera* and one not further assigned genus of *Halobacteriaceae*.

The ITS amplicon library was assigned to 3,945 fungal OTUs. Most abundant OTUs included *Ascomycota* with 74.2% relative abundance and 274 genera and *Basidiomycota* with 25.8% abundance and 119 genera. Among *Ascomycota*, the majority of OTUs were assigned to *Dothideomycetes* (50.5% abundance and 93 genera), *Sordariomycetes* (1.3% and 72 genera), *Leotiomycetes* (5.9% and 48 genera) and *Eurothiomycetes* (2.1% and 16 genera). The most abundant class within *Basidiomycota* was *Tremellomycetes* (23.0%), although poorly diverse with only 19 genera. *Microbotryomycetes* (2.3% and 11 genera) and *Argaricomycetes* (0.3% and 77 genera) represented remaining *Basidiomycota*. *Chytridiomycota* (0.04% and three genera) and *Glomeromycota* (0.002%), *Rozellomycota* (0.001%) and *Zygomycota* (0.0005%), each represented by one genus, were much less abundant.

The composition of bacterial, archaeal and fungal genera, that were present in each replicate of a sample, is visualized in Additional file 2.1 (Figure S2.1). For bacteria and fungi, a threshold of 0.01% abundance was set. As most archaea were present with less than 0.01% in the 16S rRNA gene library, no threshold was set for archaeal composition to be included in Figure S2.1. Those highly diversified microbiomes included 70 bacterial, 10 archaeal and 58 fugal genera. Among them, some highly abundant genera were shared between the seed core microbiomes of all plant genotypes, but the relative abundance of those showed high variations. *Sphingomonas*, *Pseudomonas*, *Tatumella* or *Pantoea*, known for their ubiquitous association with plants, were present in all seed core microbiomes (Figure S2.1, A); the same was true for three different, but not further assigned archaeal taxa of Soil Crenarchaeotic Group (Figure S2.1, B). Fungal composition showed some consistencies among high abundant *Cryptococcus*, *Cladosporium* or *Davidiella* (Figure S2.1, C). The mean relative abundance of all bacteria and archaea and all fungi in the whole dataset with at least 0.01% abundance are listed in Additional file 2.1 (Table S2.2 and S2.3, respectively).

Identification of the main drivers of the native seed microbiome

The bacterial and fungal diversity within the alpine plant was assessed by Shannon diversity index and significant differences ($p < 0.05$) between plant genotypes were calculated using the parametric two sample t-test at the greatest rarefaction depth. The samples were either grouped by plant genotype, the life cycle or the fruit type, in order to identify dependencies of microbial diversity on either category (Additional file 2.2, Figure S2.2 and Table S2.4). When the samples were grouped by their plant genotype, values for bacterial diversity ranged from *E. rostkoviana* seeds (5.09) to *R. glacialis* seeds (2.4). Diversity of *E. rostkoviana* seed microbiome was found to be significantly higher than *R. glacialis* and *G. germanica* seed microbiomes. Significant differences in fungal diversity were observed between most diverse *G. asclepiadea* seeds (5.09) and *R.* glacialis, *P. palustris*, *G. germanica*, *E. rostkoviana* and *H. quadrifida* seeds. *H. quadrifida* seed mycobiome was furthermore significantly more diverse than the one of *G. germanica*. *A. major´*s seed mycobiome was significantly more diverse than *R. glacialis´*. The mycobiome diversity is therefore suggested to be more dependent on the plant genotype than the bacterial diversity. When the samples were grouped by their life cycle, no significant differences in diversity were observed for the bacterial microbiota, while the mycobiome of perennial plant seeds (4.53 ± 0.05) was significantly more diverse than the mycobiome (3.12 ± 0.05) of annuals. No significant difference in Shannon diversity for both bacteria and fungi were observed when the samples were grouped by their fruit type.

In order to evaluate the main driver of the seed microbiome composition, beta-diversity analysis was conducted using PCoA (Figure 2.3) in combination with ANOSIM (Additional file 2.2, Table S2.5). Among the selected categorical variables 'plant genotype', 'life cycle' and 'fruit type', the plant genotype was found be the main driver of the microbial composition of alpine plant seeds. This applies both for bacteria ($R=0.509$; $p=0.001$) and fungi ($R=0.612$; $p=0.001$). The bacterial composition seems to be further dependent on the plant´s life cycle ($R=0.198$; $p=0.004$), either annual or perennial, while the life cycle dependency was even higher for fungi ($R=0.395$; $p=0.001$). The fruit type (capsule or achene) had no impact on the microbial composition giving the following ANOSIM-values: $R=0.058$; $p=0.23$ for bacteria and $R=-0.029$; $p=0.584$ for fungi. The ANOSIM pairwise option was applied to compare the seed microbiomes of all plant genotypes; among the 28 combinations, 18 and 22 were significantly different for the bacterial and the fungal community, respectively (Additional file 2.2, Table S2.6). These results indicate that the fungal community has a higher plant genotype specificity than the bacterial community. However, it cannot be argued that two plant genotypes harbor

similar microbial communities, as for all combinations either the bacterial or the fungal microbiome was significantly different.

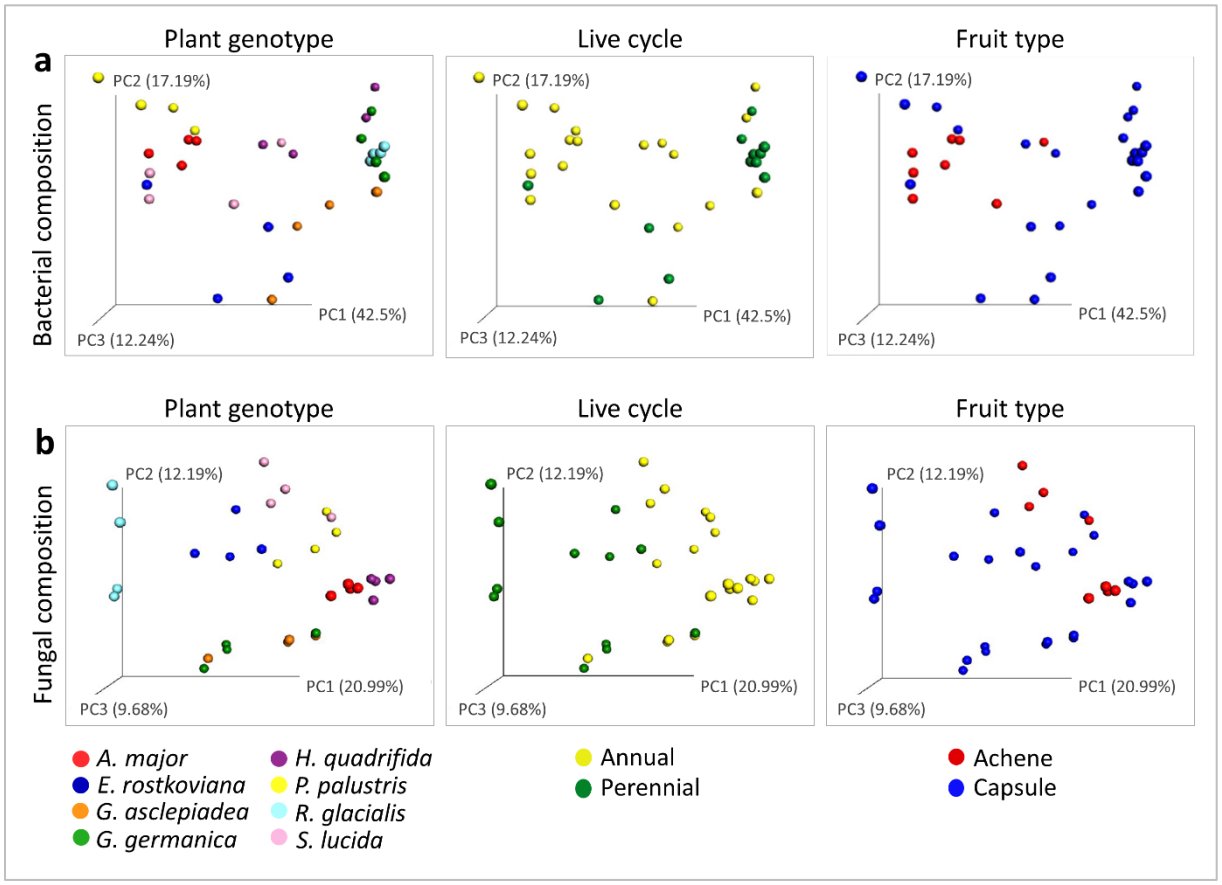


Figure 2.3: Beta-diversity analysis calculating microbiome composition dependencies on either categorical variable. PCoA plots are based on weighted UniFraq distance matrix for bacterial community (**a**) and on Bray-Curtis dissimilarities for the fungal community (**b**) of seed microbiomes. Bacterial and fungal composition of the samples are grouped by plant genotype, plant's life cycle, and the fruit type. Color codes are explained in the legends below

The limited seed core microbiome validates the unique signature of the plant genotype

In order to evaluate the dissimilarities between the seed microbiomes of the eight plant genotypes, the amount of shared OTUs was quantified. Only eleven out of 11,810 bacterial OTUs, and only five out of 3,945 fungal OTUs were present in all seeds (Table 2.1). This amounts to a percentage of 0.09% shared bacterial OTUs and 0.13% shared fungal OTUs, assigning bacteria a slight, but even higher plant genotype-dependent composition than fungi. Those bacterial and fungal OTUs represented the exceedingly undersized core microbiome

shared by all alpine plant seeds. Regarding archaea, not a single OTU out of 32 OTUs was shared. A genotype dependency of archaea is therefore highly assumed as well; however, a number of 32 OTUs is too low to give a clear assessment. The abundances of the shared bacterial and fungal OTUs varied considerably between the samples; hence, these shared OTUs are not coincidently high abundant in all seeds. The percentage amount of OTUs occurring exclusively in the core microbiomes of either plant genotype, while being absent in the core microbiomes of the other plants, was furthermore assessed (Table 2.1). Here, the core microbiome refers to OTUs that are present in all replicates of a plant species. The calculation revealed a highly specific seed microbiome for each plant genotype, reaching from 65.7% unique OTUs in *E. rostkoviana* seeds to 5.2% unique OTUs in *S. lucida* seeds. Unique fungal OTUs per plant genotype were even more frequent, reaching from 76.1% unique OTUs in *G. asclepiadea* seeds to 12.7% unique OTUs in *P. palustris* seeds. Those results further promote the clear plant genotype dependency of the seed microbiomes.

Contrasting interconnections of bacteria, archaea, and fungi within the microbial network

In order to illustrate general co-occurrence patterns of the seed microbiota across all plant genotypes, a network analysis was performed (Figure 2.4). The network, showing significant co-occurrence and mutual exclusion patterns of the seed-associated microbiota, consists of 223 nodes, a characteristic path length of 4.392 and a network density with 0.044. The vast majority of bacteria represent a very dense and highly interactive part of the network where exclusively positive interactions occur. The remaining bacteria, partially distantly located to this dense part, show only positive interactions as well. Archaea from the genus *Nitrososphaera* form a distinct and positive interacting cluster with some bacteria that are described for plant-beneficial properties. This distinct cluster is connected to the main network by a *Comamonadaceae* taxon. The entirety of negative interactions, i.e. mutual exclusions, were observed for fungi, located outside of the dense part of the bacterial network.

Table 2.1: Abundance of core OTUs in all seeds and percentage of OTUs exclusive for the core microbiomes of each plant genotype.

		A. major	*E. rostkoviana*	*G. asclepiadea*	*G. germanica*	*H. quadrifida*	*P. palustris*	*R. glacialis*	*S. lucida*
Bacterial core OTUs shared by all seeds*	*Tatumella sp.*	0.21	1.94	6.06	24.60	0.95	0.69	75.84	2.42
	Pseudomonas sp.	13.84	5.39	29.51	0.32	32.27	5.40	0.06	7.63
	Pseudomonas putida	0.17	0.26	1.73	27.40	9.48	0.14	0.24	0.65
	Pseudomonas fluorescens	0.41	0.47	2.02	0.49	12.43	0.34	0.62	2.64
	Serratia liquefaciens	0.75	1.15	0.77	0.37	1.11	0.72	1.35	4.17
	Burkholderia sp.	0.16	0.28	0.30	0.10	0.35	0.34	0.73	2.80
	Bacillus subtilis	0.20	0.19	0.23	0.08	0.34	0.28	0.70	0.13
	Pseudomonas protegens	0.13	0.26	0.16	0.17	0.62	0.08	0.12	0.21
	Curvibacter gracilis	0.05	0.07	0.09	0.03	0.14	0.10	0.17	0.61
	Pelomonas sp.	0.02	0.04	0.04	0.01	0.06	0.05	0.15	0.64
	Acinetobacter calcoaceticus	0.04	0.07	0.03	0.02	0.05	0.04	0.05	0.09
Percentage of bacterial OTUs unique for the plant´s core microbiome**		44.1	65.7	38.0	31.3	11.4	21.7	15.4	5.2
Fungal core OTUs shared by all seeds*	*Cladosporium cladosporioides*	7.31	27.37	14.55	46.33	2.41	3.59	25.77	6.53
	Cryptococcus victoriae	15.14	1.78	8.05	4.64	28.21	14.34	0.02	8.03
	Davidiella tassiana	6.40	7.14	14.79	15.52	12.34	14.54	1.18	5.07
	Boeremia exigua var. populi	0.46	10.95	3.11	0.14	1.98	29.00	0.03	19.77
	Epicoccum nigrum	4.94	8.76	1.45	2.20	6.53	1.16	1.57	11.40
Percentage of fungal OTUs unique for the plant´s core microbiome**		33.1	35.7	76.1	12.5	31.0	12.7	23.5	26.5

*Numbers denote for relative abundance (%) in seeds of each plant genotype. Taxonomy was assigned at species level to core OTUs using NCBI BLAST alignment tool.

** Percentage of OTUs occurring in all replicates of the respective plant species, while being absent in the core microbiomes of all other plant species.

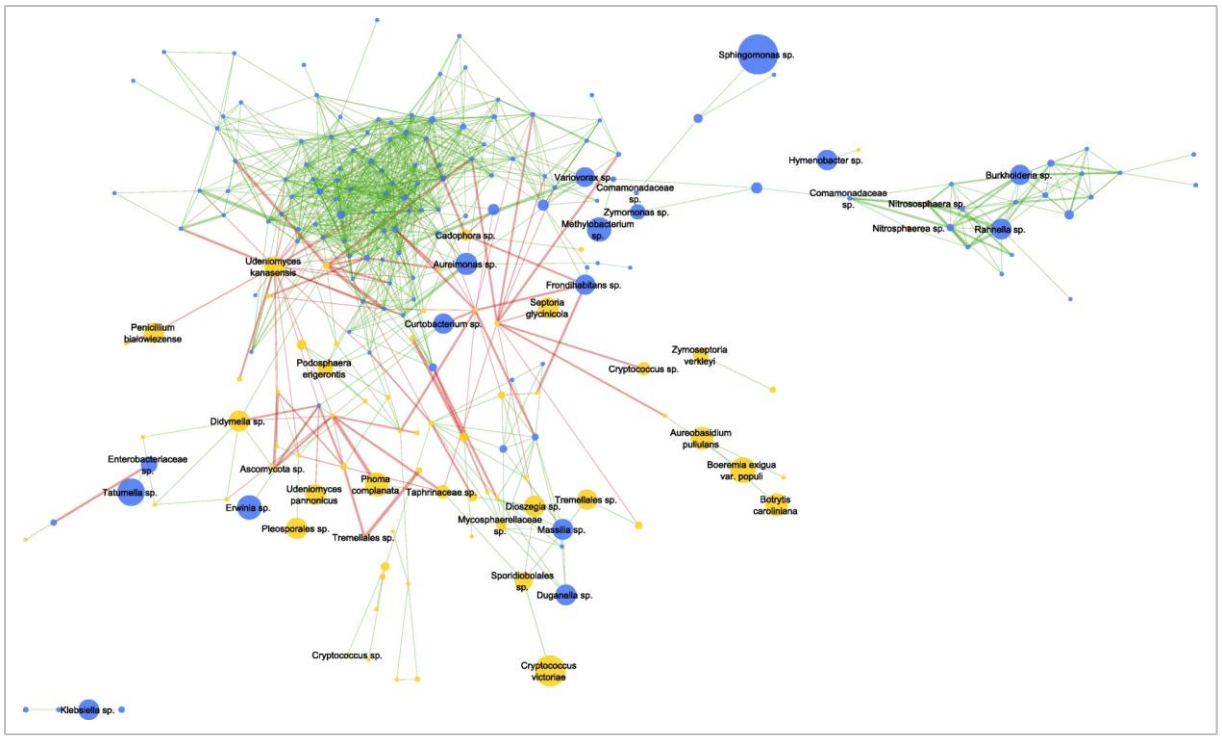


Figure 2.4: Co-occurrence and mutual exclusion relationships among seed-associated microbiota of alpine plants. Network was prepared by combining taxonomic assignment to OTUs on species level of 16S rRNA and ITS amplicon dataset. Only significant interactions are shown ($q \geq 0.0004$). Color of nodes represent the three taxonomic groups (*blue*: bacteria, *yellow*: fungi, *pink*: archaea), and the size of nodes is proportional to the abundance of the taxon. Color of edges indicates the type of the interaction (*green*: positive or co-occurrence, *red*: negative or mutual exclusion), and the edge width is proportional to the significance. Taxonomy for high-abundant taxa is included.

Discussion

The results of this study confirm our hypothesis that seeds of native plants harbor a more specific microbiota than already discovered for crop plants. The key findings of this study were i) the unexpected high microbial abundances mainly driven by the plant genotype, ii) the consistent eukaryote to prokaryote ratio across all seeds investigated iii) the high degree of plant specificity shown for the entire microbiome, iv) the first identification of plant-specific seed-borne archaea, v) an exceptionally small core microbiome although all plants grow together for centuries in the same soil and under the same environmental conditions, and vi) the network of bacteria and archaea, which was in contrast to the negatively interacting fungal network.

The alpine plants studied showed a unique degree of plant specificity compared to the present literature (Berg and Smalla 2009; Bulgarelli et al. 2015; 2013). Differences between seed microbiomes of the plant genotypes were found in terms of bacterial and fungal composition, abundance and diversity. Inter-kingdom symbiosis was genotype-specific as well, as seeds with high bacterial gene copy numbers also showed high copy numbers of archaea and fungi and *vice versa*. The seeds share an exceedingly undersized core microbiome where only eleven out of 11,810 bacterial OTUs, only five out of 3,945 fungal OTUs, and not a single OTU out of 32 archaeal OTUs, was shared. The five fungal OTUs, which form the core, are classified as black fungi (Diederich 1990) able to persist ecological harshness and even to convert radiation into metabolic activity (Dadachova et al. 2007). Rezki et al. (Rezki et al. 2018) recently described an even higher degree of individual-specific microbiota for radish seeds; only three bacterial and 19 fungal OTUs were shared. However, those OTUs covered 70% and 87% of all bacterial and fungal reads within the individual plants. Reduced diversity and low evenness might therefore be suggested for crop plants, in comparison to the alpine plants. Here, the core OTUs represented an average of 3% and 10% of all bacterial and fungal reads, respectively. The amount of OTUs specific for each plant genotype was furthermore substantial, reaching up to 65.7% for bacteria in *E. rostkoviana* seeds and 76.1% for fungi in *G. asclepiadea* seeds. However, direct comparison to (Rezki et al. 2018) is limited as *gyrB* instead of 16S rRNA was selected as bacterial marker gene. Seeds of *R. glacialis* were found most unique, probably caused by specific, antimicrobial secondary metabolites (Compean and Ynalvez 2014). Their microbial diversity was significantly lower, but abundance was significantly higher compared to the other samples. In addition to the plant genotype, plant´s life cycle was found to have an impact on the microbiome composition, where seed microbiota of perennial plants differed

significantly from seeds of annual plants. This can be explained by the possibility to accumulate microbial diversity protecting the plants also during winter time. By selecting plants with two different fruit types, capsules and achenes, we aimed to specify the influence of the surrounding environment on seed microbiomes, which was found surprisingly to be not significant. This indicates that the airborne contribution to the seed microbiome is negligible. Significant genotype and life cycle dependency of the seed-associated microbiota was obvious, although we decided not to distinguish between seed endo- and epiphytes and considered both as an entire community of the alpine plant seeds. However, our microscopic observations showed that seed surfaces were not or less colonized by microbes. Therefore, we promote the recent suggestion by Nelson (Nelson 2018) that the epiphytic seed community is less insignificant than previously expected.

According to present literature, a plant-pathogenic potential can be assigned to the majority of highly abundant seed-associated fungi (e.g. *Botrytis, Alternaria, Phoma, Didymella, Davidiella* (Dean et al. 2012; Tsuge et al. 2013; Keinath 2011; Thomma et al. 2005) while the abundance of fungal taxa with described parasitism towards other fungi was high as well (e.g. *Cryptococcus, Dioszegia* (Sterkenburg et al. 2015). However, all seeds were sampled from healthy plant populations, persisting under the given conditions for many growing seasons; germination ability is therefore highly assumed. Resilience towards emerging pathogens and adverse environmental conditions is probably supported by a highly abundant and competitive mycobiome. The inter-kingdom co-occurrence network illustrates the antagonistic features of the mycobiome; exclusively all mutual exclusions were observed for fungi. This stands in vast contrast to the positively interacting bacterial network, indicating synergism and stability. Competition for resources and space within the seed mycobiome has already been proposed by Rezki et al. (Rezki et al. 2016), where the invasion of a fungal plant pathogen altered the fungal, but not the bacterial seed community and Johnston-Monje and Raizada (Johnston-Monje and Raizada 2011) suggested stability of bacteria, based on conserved patterns of bacterial endophytes in *Zea* seeds across boundaries of evolution. Among archaea, two OTUs of *Nitrososphaera* were present in the significant inter-kingdom network, positively interacting with bacteria. Beyond that, archaea were detected in all seeds investigated. We therefore assume an important ecological function of this domain for plant health and development. The performance of co-occurrence networks can be interfered by the input matrix when metacommunities from different habitats are combined, which in such cases can result in co-occurrence due to the habitat sampled rather than direct biological interactions (habitat filtering effect) (Brisson et al. 2019). Simulation models and algorithms described in literature (Berry

and Widder 2014; Brisson et al. 2019) can remedy that issue, which however, would require a greater sample size than available in the present study. The sampling for the present study was conditioned by the number of plants per genotype grown on the alpine meadow. However, the sample size was still sufficient for the present network where statistical tests, reported to give highest specificity and sensitivity, were applied (Berry and Widder 2014). Interpretability of the present network inferring putative microbial interactions in alpine plant seeds is therefore feasible when keeping potential interferences in mind. Altogether, our results indicate specified functions within the whole microbial network: bacteria and archaea strengthen the beneficial interplay within the holobiont, while fungi are responsible for degradation of the organic matter, e.g. seed shell, and may be to condition and train the prokaryotic microbiome through their antagonistic pressure.

A comparison of the microbiota of native and crop seeds confirmed our hypothesis that seeds of native plants harbor a more specific and differentially composed microbiota in comparison to cultivated plants that were investigated so far. Links et al. (Links et al. 2014) compared seed microbiomes of *Brassica* and *Triticum* crops and found a hundredfold higher amount of shared OTUs (578 out of 5 477 OTUs) compared to alpine seeds. The contrast is increased by the fact that *Brassica* and *Triticum* seeds were originated from different locations, but showed still higher similarity than the seeds of alpine plants, sampled on less than 20 000 square meters. Truyens et al. (Truyens et al. 2015) reviewed that *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Micrococcus*, *Staphylococcus*, *Pantoea* and *Acinetobacter*, in ascending order, are the most common bacteria within seeds of very different crop species. This is only partially consistent with our results. Alpine plant seeds are dominated by *Pseudomonas*, *Sphingomonas*, *Tatumella*, *Methylobacterium* and *Pantoea*. The abundance of *Bacillus*, *Acinetobacter* and especially *Paenibacillus*, *Staphylococcus* and *Micrococcus* was very low. Differences to comparable studies on crop seeds (Johnston-Monje et al. 2016; Rybakova et al. 2017; Johnston-Monje and Raizada 2011; Links et al. 2014; Lopez-Velasco et al. 2013; Barret et al. 2015) were also observed on higher taxonomic levels: alpine plant seeds showed higher abundance of *α-Proteobacteria* but far lower abundance of *Actinobacteria* and *Firmicutes*.

Until now, mainly crop seeds were studied. What can we learn from the native seeds about a healthy seed microbiome? A healthy seed microbiome is: i) diverse, rich and evenly structured, ii) contains bacteria, archaea & fungi, iii) contains microorganisms known for beneficial as well as for pathogenic interaction and is iv) highly specific. We found substantial differences to the microbiomes of crop seeds; the same has already been reported for the

rhizosphere microbiome (Germida and Siciliano 2001). This is not surprising because cultivation pressure on seeds started around 8,000 years ago and included, among others, the two major alterations on seed morphology: increased grain size and non-shattered seeds (Fuller 2007), where the latter predicated successful seed-dispersal on human activity (Purugganan and Fuller 2009). Today, seed treatments focus on uniform, clean and pathogen-free seeds that are almost entirely produced commercially and traded globally (Gabriele Berg and Raaijmakers 2018). In contrast, nature created a rich diversity of seed and fruit systems, with their own genotype-specific microbiomes. Figure 1 illustrates the morphological diversity of seeds and associated microbiota of the alpine plants investigated. Recent agriculture lead to a global landscape highly dominated by only few crop plants with desired characteristics. An enormous amount of 70% of wild relatives of modern crop plants are threatened with extinction (Castañeda-Álvarez et al. 2016); consequentially, also their native microbiota with all their functional and metabolic skills are in risk of getting lost. For that reason, Berg and Raaijmakers (Berg and Raaijmakers 2018) recently proposed international seed banks like Svalbard Global Seed Vault and Millennium Seed Bank to include conservation strategies for seed-associated microbiota. Based on the seed microbiota of wild ancestors or natural plants, microbial communities could be reconstructed with the ultimate goal to improve resilience of modern crops and reduce the amount of required pesticides.

Conclusion

Undisturbed environments provide the best settings to explain indigenous plant-microbe-interactions. Under such conditions, in a protected Alpine meadow, we found highly diversified and abundant seed microbiomes consisting of bacteria, archaea, and fungi. Moreover, despite growing together in the same soil, we found a higher degree of plant specificity than already discovered for crop seeds. All results underline the importance of plant-specific seed microbiota to ensure best-matching microbial symbionts for the next generation. However, network analysis captured consistent patterns of co-occurrence between bacteria and archaea in contrast to exclusion within the fungal community across all plant genotypes. This outlines the importance of cross-kingdom microbial interactions. We suggest that diversity associated with seeds may contribute to maintain soil microbial diversity, with importance for plasticity of the whole ecosystem. This knowledge can be translated into a better understanding of disease outbreaks and could be used for the production of resilient, healthy and high quality crop seeds.

Supplementary Material

Additional file 2.1: Abundance and composition of seed microbiota.

Table S2.1: Mean microbial gene copy numbers in the seeds of different plant genotypes quantified by qPCR.

	Mean bacterial gene copy numbers/g seed	Mean archaeal gene copy numbers/g seed	Mean fungal gene copy numbers/g seed	Mean copy numbers of total microbiota/g seed
A. major	8.56E+10^{a}	3.97E+08^{a}	1.14E+11^{ab}	2.00E+11^{a}
E. rostkoviana	2.33E+11^{ab}	2.29E+09^{bc}	4.51E+11^{bc}	6.87E+11^{b}
G. asclepiadea	3.59E+11^{bc}	1.13E+09^{ab}	4.61E+11^{bc}	8.21E+11^{b}
G. germanica	5.20E+10^{a}	2.94E+08^{a}	6.39E+10^{a}	1.16E+11^{a}
H. quadrifida	1.78E+11^{ab}	2.71E+09^{c}	1.68E+11^{ab}	3.48E+11^{a}
P. palustris	5.30E+11^{c}	4.75E+09^{d}	6.97E+11^{c}	1.22E+12^{c}
R. glacialis	7.23E+11^{d}	1.22E+10^{e}	1.37E+12^{d}	2.10E+12^{d}
S. lucida	8.18E+10^{a}	9.53E+08^{a}	4.79E+10^{a}	1.31E+11^{a}

Upper case letters indicate significant differences within microbial abundances where samples with same letters are not significantly different ($p > 0.05$) according to ANOVA including Tukey-HSD test correction.

Table S2.2: Composition of the bacterial/archaeal microbiome with at least 0.1% abundance

Family	Genus	Relative abundance in all samples (%)	Family	Genus	Relative abundance in all samples (%)
Pseudomonadaceae	*Pseudomonas*	18.51	*Phycisphaerae*	unidentified	0.02
Sphingomonadaceae	*Sphingomonas*	18.47	*Obscuribacterales*	unidentified	0.02
Enterobacteriaceae	*Tatumella*	12.87	*Opitutaceae*	*Opitutus*	0.02
Methylobacteriaceae	*Methylobacterium*	5.84	*Gaiellaceae*	*Gaiella*	0.02
Enterobacteriaceae	*Pantoea*	4.71	*Deinococcaceae*	*Deinococcus*	0.02
Enterobacteriaceae	*Erwinia*	4.04	*Acidobacteria*	unidentified	0.02
unidentified	*unidentified*	3.98	*Anaerolineaceae*	unidentified	0.02
Anaplasmataceae	*Wolbachia*	3.82	*Gemmatimonadaceae*	*Gemmatimonas*	0.02
Enterobacteriaceae	*Buchnera*	2.41	*Micrococcaceae*	*Micrococcus*	0.02
Rhizobiaceae	*Rhizobium*	2.40	*Xanthomonadaceae*	*Luteibacter*	0.02
Aurantimonadaceae	*Aureimonas*	1.96	*Roseiflexaceae*	*Roseiflexus*	0.02
Enterobacteriaceae	*Rahnella*	1.50	*Holophagae*	unidentified	0.02
Oxalobacteraceae	*Massilia*	1.40	*Soil Crenarchaeotic Group(SCG)*	unidentified	0.02
Oxalobacteraceae	*Duganella*	1.31	*Beijerinckiaceae*	unidentified	0.02
Enterobacteriaceae	*Klebsiella*	1.30	*Microbacteriaceae*	*Phycicola*	0.02
Cytophagaceae	*Hymenobacter*	1.29	*Acidobacteria*	unidentified	0.01
Enterobacteriaceae	unidentified	1.24	*Streptococcaceae*	*Streptococcus*	0.01
Xanthomonadaceae	unidentified	1.16	*Streptomycetaceae*	*Streptomyces*	0.01

Microbacteriaceae	*Curtobacterium*	0.82
Burkholderiaceae	*Burkholderia*	0.65
Microbacteriaceae	*Frondihabitans*	0.62
Oxalobacteraceae	unidentified	0.48
Sphingomonadaceae	*Zymomonas*	0.46
Bacillaceae	*Bacillus*	0.41
Comamonadaceae	*Variovorax*	0.37
Kineosporiaceae	*Kineococcus*	0.25
Rhizobiales	*unidentified*	0.22
Myxococcales	unidentified	0.22
Comamonadaceae	*Polaromonas*	0.22
Enterobacteriaceae	*Enterobacter*	0.21
Moraxellaceae	*Acinetobacter*	0.21
Microbacteriaceae	*Subtercola*	0.21
Other	unidentified	0.20
Flavobacteriaceae	*Flavobacterium*	0.20
Sphingobacteriaceae	*Pedobacter*	0.17
Comamonadaceae	*Curvibacter*	0.16
Comamonadaceae	*Aquabacterium*	0.15
Cytophagaceae	*Spirosoma*	0.15
Comamonadaceae	*Ramlibacter*	0.14
Acetobacteraceae	*Acidiphilium*	0.13
Moraxellaceae	*Moraxella*	0.12
unidentified	unidentified	0.11
Enterobacteriaceae	*Candidatus Hamiltonella*	0.11
Cyanobacteria	*Cyanobacteria*	0.11
Paenibacillaceae	*Paenibacillus*	0.10
Nakamurellaceae	*Nakamurella*	0.09
Comamonadaceae	*Pelomonas*	0.09
Nocardioidaceae	*Nocardioides*	0.09
Sanguibacteraceae	*Sanguibacter*	0.09
Mycobacteriaceae	*Mycobacterium*	0.08
Hyphomicrobiaceae	*Devosia*	0.08
Caulobacteraceae	*Brevundimonas*	0.08
Burkholderiaceae	*Ralstonia*	0.08
Xanthomonadaceae	*Stenotrophomonas*	0.08
Kineosporiaceae	*Kineosporia*	0.08
Comamonadaceae	unidentified	0.07
Geodermatophilaceae	*Modestobacter*	0.07
Sphingomonadaceae	*Novosphingobium*	0.07
Parcubacteria	unidentified	0.07
Bacillales	unidentified	0.07
Gaiellales	unidentified	0.07
Cytophagaceae	*Dyadobacter*	0.06
Micrococcaceae	*Arthrobacter*	0.06
Chthoniobacterales	unidentified	0.05
Simkaniaceae	*Candidatus Rhabdochlamydia*	0.01
Armatimonadetes	unidentified	0.01
Enterobacteriaceae	*Serratia*	0.01
unidentified	unidentified	0.01
Corynebacteriaceae	*Corynebacterium*	0.01
Acidobacteria	unidentified	0.01
Cytophagaceae	*Fibrella*	0.01
unidentified	unidentified	0.01
Microbacteriaceae	*Amnibacterium*	0.01
unidentified	unidentified	0.01
unidentified	unidentified	0.01
Comamonadaceae	*Caenimonas*	0.01
Xiphinematobacteraceae	*Xiphinematobacter*	0.01
Acidimicrobiaceae	uncultured	0.01
Acetobacteraceae	*Gluconobacter*	0.01
Cellulomonadaceae	*Cellulomonas*	0.01
Chitinophagaceae	*Chitinophaga*	0.01
Nocardiaceae	*Williamsia*	0.01
Coxiellaceae	*Aquicella*	0.01
Nitrosomonadaceae	uncultured	0.01
Micromonosporaceae	*Micromonospora*	0.01
Comamonadaceae	*Tepidimonas*	0.01
Planctomycetaceae	*Isosphaera*	0.01
Caulobacteraceae	*Caulobacter*	0.01
Micromonosporaceae	*Luedemannella*	0.01
Methylobacteriaceae	*Microvirga*	0.01
Rhodobacteraceae	*Paracocccus*	0.01
Rhodospirillales Incertae Sedis	*Candidatus Alysiosphaera*	0.01
Nocardioidaceae	*Marmoricola*	0.01
Acetobacteraceae	unidentified	0.01
Acidothermaceae	*Acidothermus*	0.01
Chthoniobacteraceae	*Chthoniobacter*	0.01
Bradyrhizobiaceae	*Bosea*	0.01
Planctomycetaceae	*Gemmata*	0.01
Beijerinckiaceae	*Methylorosula*	0.01
Flavobacteriaceae	*Cloacibacterium*	0.01
Cellvibrionaceae	*Cellvibrio*	0.01
Geodermatophilaceae	*Blastococcus*	0.01
Neisseriaceae	unidentified	0.01
Acidobacteria	*Bryobacter*	0.01
Chitinophagaceae	*Ferruginibacter*	0.01
Chloroflexi	*unidentified*	0.01
Cyanobacteria	unidentified	0.01
Chitinophagaceae	*Hydrotalea*	0.01
Chitinophagaceae	unidentified	0.01
Intrasporangiaceae	*Janibacter*	0.01

Sphingobacteriaceae	*Mucilaginibacter*	0.05
Patulibacteraceae	*Patulibacter*	0.05
Acetobacteraceae	*Roseomonas*	0.05
Comamonadaceae	unidentified	0.05
Microbacteriaceae	*Rathayibacter*	0.05
Xanthomonadaceae	*Dyella*	0.05
Enterobacteriaceae	*Candidatus Regiella*	0.05
Solirubrobacterales	unidentified	0.05
Flavobacteriaceae	*Chryseobacterium*	0.05
Acetobacteraceae	unidentified	0.05
Micromonosporaceae	*Actinoplanes*	0.04
Nocardiaceae	*Rhodococcus*	0.04
Solirubrobacteraceae	*Solirubrobacter*	0.04
Enterobacteriaceae	*Yersinia*	0.04
Aurantimonadaceae	unidentified	0.04
Bradyrhizobiaceae	*Bradyrhizobium*	0.04
Enterobacteriaceae	*Citrobacter*	0.04
Blattabacteriaceae	*Candidatus Brownia*	0.03
Comamonadaceae	*Delftia*	0.03
Bdellovibrionaceae	*Bdellovibrio*	0.03
Sphingomonadaceae	*Polymorphobacter*	0.03
Chloroflexi	unidentified	0.03
Rhizobiaceae	*Shinella*	0.03
Planctomycetaceae	uncultured	0.03
Planctomycetaceae	*Singulisphaera*	0.03
Pseudonocardiaceae	*Actinomycetospora*	0.03
Soil Crenarchaeotic Group(SCG)	unidentified	0.03
Flavobacteriaceae	*Empedobacter*	0.03
Nocardioidaceae	*Aeromicrobium*	0.03
Rhizobiaceae	*Ensifer*	0.03
Thermomicrobia	unidentified	0.03
Flavobacteriaceae	*Epilithonimonas*	0.03
Staphylococcaceae	*Staphylococcus*	0.02
Acidobacteria	unidentified	0.02
Pseudonocardiaceae	*Pseudonocardia*	0.02
Unknown Family	*Blastocatella*	0.02
Planococcaceae	unidentified	0.02
Comamonadaceae	*Comamonas*	0.02
Sphingomonadaceae	*Sphingobium*	0.02
Gemmatimonadaceae	uncultured	0.02
Bradyrhizobiaceae	*Tardiphaga*	0.02
Frankiaceae	*Jatrophihabitans*	0.02
Acidimicrobiales	unidentified	0.02
Oligoflexales	unidentified	0.02
Carnobacteriaceae	*Atopostipes*	0.01
Comamonadaceae	*Schlegelella*	0.01
Comamonadaceae	*Acidovorax*	0.01
Sphingobacteriaceae	*Sphingobacterium*	0.01
Microbacteriaceae	unidentified	0.01
Lactobacillaceae	*Lactobacillus*	0.01
Alcaligenaceae	*Achromobacter*	0.01
Clostridiaceae 1	*Clostridium sensu stricto 13*	0.01
Betaproteobacteria	unidentified	0.01
Thiotrichaceae	unidentified	0.01
Kineosporiaceae	*Quadrisphaera*	0.01
Xanthobacteraceae	*Variibacter*	0.01
Actinobacteria	unidentified	0.01
Latescibacteria	unidentified	0.01
Phyllobacteriaceae	*Mesorhizobium*	0.01
Solirubrobacterales	unidentified	0.01
Hyphomicrobiaceae	unidentified	0.01
Peptostreptococcaceae	*Intestinibacter*	0.01
Sphingomonadaceae	*Sphingopyxis*	0.01
Armatimonadales	unidentified	0.01
Rhodospirillaceae	*Defluviicoccus*	0.01
Legionellaceae	*Legionella*	0.01
Cyanobacteria	*Microcoleus*	0.01
Microbacteriaceae	*Clavibacter*	0.01
Actinobacteria	unidentified	0.01
Nocardioidaceae	unidentified	0.01
Chitinophagaceae	*Terrimonas*	0.01
Rhizobiales Incertae Sedis	*Rhizomicrobium*	0.01
Planctomycetes	unidentified	0.01
Acidimicrobiaceae	unidentified	0.01
Rhizobiaceae	*Neorhizobium*	0.01
Acidobacteria	*Candidatus Solibacter*	0.01
Rhodobacteraceae	*Amaricoccus*	0.01
Burkholderiales	unidentified	0.01
Rickettsiales	unidentified	0.01
Spiroplasmataceae	*Spiroplasma*	0.01
Rhodobiaceae	unidentified	0.01
uncultured bacterium	unidentified	0.01
Polyangiaceae	*Sorangium*	0.01
Planctomycetaceae	*unidentified*	0.01
Xanthomonadaceae	*Lysobacter*	0.01
Iamiaceae	*Iamia*	0.01
Enterobacteriaceae	*Escherichia-Shigella*	0.01
Myxococcales	unidentified	0.01

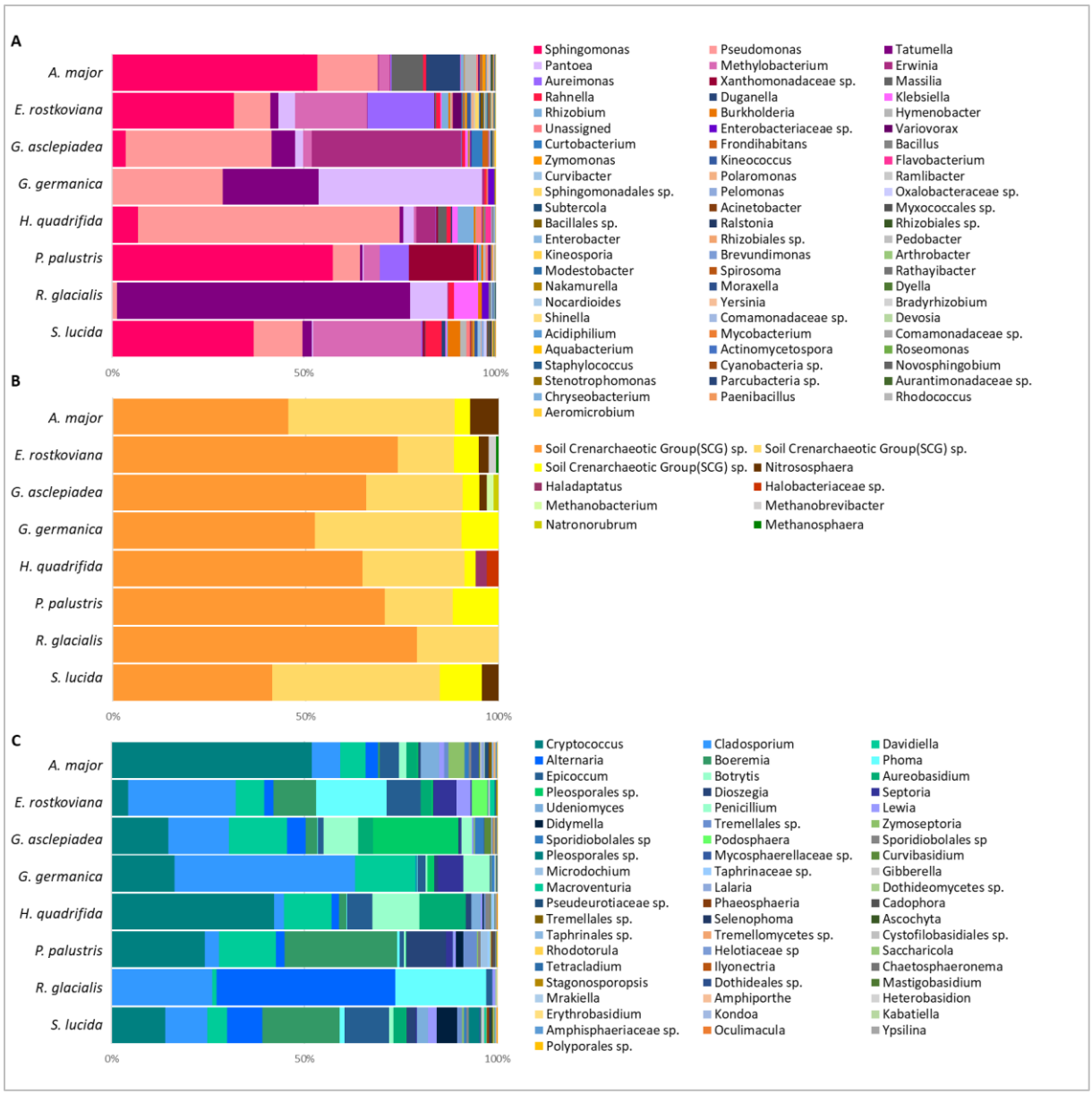


Figure S2.1: Microbial composition of the seed core microbiomes of each plant genotype. (A) shows bacterial, (B) archaeal and (C) fungal composition. Only bacteria and fungi occurring with at least 0.01% relative abundance in the whole dataset are shown. For low abundant archaea, no threshold was set to be included in the figure. Taxa not assigned to genus level are described by ‚sp.' after lower taxonomic description.

Table S2.3: Composition of the fungal microbiome with at least 0.1% abundance

Family	Genus	Relative abundance in all samples (%)	Family	Genus	Relative abundance in all samples (%)
Tremellales	*Cryptococcus*	17.03	*Phaeosphaeriaceae*	*Stagonospora*	0.07
Ascomycota	unidentified	12.79	*Helotiales*	*Oculimacula*	0.06
Davidiellaceae	*Davidiella*	7.92	*Dothideales*	unidentified	0.05
Pleosporales	*unidentified*	7.75	*Amphisphaeriaceae*	unidentified	0.05
Pleosporaceae	*Alternaria*	6.84	*Hypocreales*	*Myrothecium*	0.04
Pleosporales	*Phoma*	5.53	*Sporidiobolales*	*Sporobolomyces*	0.04
Pleosporales	*Boeremia*	5.20	*Erythrobasidiales*	*Erythrobasidium*	0.04
Pleosporaceae	*Epicoccum*	3.02	*Cystofilobasidiales*	*Mrakiella*	0.04
Dothioraceae	*Aureobasidium*	2.72	*Hypocreales*	*Ilyonectria*	0.04
Sclerotiniaceae	*Botrytis*	2.49	*Kondoaceae*	*Kondoa*	0.04
Tremellales	*Dioszegia*	2.27	*Spizellomycetaceae*	*Powellomyces*	0.03
Cystofilobasidiaceae	*Udeniomyces*	2.14	*Saccharomycetales*	*Debaryomyces*	0.03
Trichocomaceae	*Penicillium*	2.00	*Clavulinaceae*	*Clavulina*	0.03
Erysiphaceae	*Podosphaera*	1.73	*Lasiosphaeriaceae*	*Bagadiella*	0.03
Pleosporales	*Macroventuria*	1.71	*Lasiosphaeriaceae*	*Podospora*	0.03
Pleosporaceae	*Lewia*	1.49	*Ascomycota*	*Ypsilina*	0.03
Pleosporales	*Didymella*	1.46	*Parmeliaceae*	*Pseudevernia*	0.03
Pleosporales	*Mycocentrospora*	1.14	*Tremellales*	*Bullera*	0.03
Mycosphaerellaceae	*Septoria*	1.01	*Cystofilobasidiaceae*	*Cystofilobasidium*	0.03
Helotiales	*Cadophora*	0.85	*Davidiellaceae*	*Cladosporium*	0.03
Sporidiobolales	*unidentified*	0.79	*Ascomycota*	*Knufia*	0.03
Sporidiobolales	unidentified	0.79	*Nectriaceae*	*Flagellospora*	0.03
Dothideomycetes	*Zymoseptoria*	0.76	*Dothioraceae*	*Kabatiella*	0.02
Tremellales	*unidentified*	0.75	*Metschnikowiaceae*	*Metschnikowia*	0.02
Phaeosphaeriaceae	*Phaeosphaeria*	0.71	*Chaetothyriales*	unidentified	0.02
Dothideomycetes	unidentified	0.68	*Capnodiales*	unidentified	0.02
Pleosporales	unidentified	0.54	*Leucosporidiaceae*	*Leucosporidium*	0.02
Mycosphaerellaceae	unidentified	0.43	*unidentified*	unidentified	0.02
Tremellales	unidentified	0.42	*Helotiaceae*	*Hymenoscyphus*	0.02
Nectriaceae	*Gibberella*	0.39	*Cystofilobasidiaceae*	*Itersonilia*	0.02
Pseudeurotiaceae	unidentified	0.38	*Sordariomycetes*	unidentified	0.01
Taphrinaceae	unidentified	0.38	*Polyporales*	unidentified	0.01
Microbotryomycetes	*Curvibasidium*	0.34	*Leucosporidiaceae*	*Leucosporidiella*	0.01
Pleosporales	*Ascochyta*	0.28	*Phaeosphaeriaceae*	unidentified	0.01
Taphrinaceae	*Lalaria*	0.26	*Hypocreales*	*Acremonium*	0.01
Helotiales	*Chlorociboria*	0.26	*Tremellales*	*Tremella*	0.01
Pleosporaceae	*Pleospora*	0.25	*Ceratocystidaceae*	*Thielaviopsis*	0.01
Dothioraceae	*Selenophoma*	0.25	*Sporormiaceae*	*unidentified*	0.01
Helotiaceae	unidentified	0.24	*Sclerotiniaceae*	*Zoellneria*	0.01
Pleosporales	*Stagonosporopsis*	0.22	*Helotiales*	*Pilidium*	0.01
Bondarzewiaceae	*Heterobasidion*	0.19	*Clavicipitaceae*	*Claviceps*	0.01

Taphrinales	unidentified	0.18	*Mycosphaerellaceae*	*Cercospora*	0.01
Xylariales	*Microdochium*	0.18	*Saccharomycetales*	*Candida*	0.01
Massarinaceae	*Saccharicola*	0.18	*Botryobasidiaceae*	*Botryobasidium*	0.01
Tremellomycetes	unidentified	0.18	*Nectriaceae*	unidentified	0.01
Glomerellaceae	*Colletotrichum*	0.15	*Dothioraceae*	unidentified	0.01
Leucosporidiaceae	*Mastigobasidium*	0.15	*Hypocreales*	*Sarocladium*	0.01
Cystofilobasidiales	unidentified	0.12	*Helotiales*	*Helgardia*	0.01
Helotiales	*Tetracladium*	0.12	*Leptosphaeriaceae*	*Plenodomus*	0.01
Sporidiobolales	*Rhodotorula*	0.12	*Basidiomycota*	unidentified	0.01
Ascomycota	*Tumularia*	0.11	*Herpotrichiellaceae*	*Exophiala*	0.01
Gnomoniaceae	*Amphiporthe*	0.10	*Phaeosphaeriaceae*	*Ampelomyces*	0.01
Ascomycota	*Chaetosphaeronema*	0.10	*Diatrypaceae*	*Libertella*	0.01
Taphrinaceae	*Taphrina*	0.09	*Teratosphaeriaceae*	unidentified	0.01
Helotiales	unidentified	0.09	*Pleosporales*	*Periconia*	0.01
Diaporthaceae	*Diaporthe*	0.09	*Herpotrichiellaceae*	*Rhinocladiella*	0.01
Agaricostilbaceae	*Bensingtonia*	0.09	*Mytilinidiaceae*	*Lophium*	0.01
Leptosphaeriaceae	*Leptosphaeria*	0.08	*Lulworthiaceae*	*Zalerion*	0.01

Additional file 2.2: Comparison of microbial diversity and composition between alpine seeds investigated.

Table S2.4: Shannon diversity indices of seed samples grouped by plant genotype, life cycle and fruit type.

		Bacteria	Fungi
Plant genotype	*A. major*	3.59 ± 0.09	4.82 ± 0.06
	E. rostkoviana	5.09 ± 0.19	3.88 ± 0.05
	G. asclepiadea	3.94 ± 0.13	5.09 ± 0.08
	G. germanica	2.57 ± 0.08	3.21 ± 0.04
	H. quadrifida	3.28 ± 0.10	4.09 ± 0.03
	P. palustris	2.93 ± 0.08	4.01 ± 0.04
	R. glacialis	2.40 ± 0.08	2.27 ± 0.01
	S. lucida	4.51 ± 0.14	4.62 ± 0.05
Plant´s life cycle	Annual	3.36 ± 0.11	3.12 ± 0.03
	Perennial	3.65 ± 0.11	4.53 ± 0.05
Diaspore type	Capsule	4.05 ± 0.12	4.72 ± 0.05
	Achene	3.37 ± 0.11	3.76 ± 0.04

Table S2.5: ANOSIM results of community composition dependency for bacteria and fungi on the three categorical variables.

		R-value	p-value*
Bacteria	Genotype	0.509	0.001
	Life cycle	0.198	0.004
	Diaspore type	0.058	0.23
Fungi	Genotype	0.612	0.001
	Life cycle	0.395	0.001
	Diaspore type	-0.029	0.584

* Dependency of the community composition was highly significant on genotype and life cycle for bacteria and fungi.

Table S2.6: Pairwise ANOSIM results comparing differences in bacterial and fungal community composition between the seeds of the eight plant genotypes.

		Bacteria*		**Fungi***	
Group 1	**Group 2**	**R**	**p-value**	**R**	**p-value**
A.major	*E.rostkoviana*	**0.74**	**0.03**	**0.61**	**0.03**
A.major	*G.asclepiadea*	0.28	0.14	**0.94**	**0.03**
A.major	*G.germanica*	**0.64**	**0.03**	**0.66**	**0.03**
A.major	*H.quadrifida*	0.45	0.08	**0.45**	**0.03**
A.major	*P.palustris*	**0.67**	**0.02**	**0.81**	**0.03**
A.major	*R.glacialis*	**0.75**	**0.04**	**0.89**	**0.03**
A.major	*S.lucida*	0.30	0.14	**0.74**	**0.02**
E.rostkoviana	*G.asclepiadea*	0.41	0.11	**0.67**	**0.03**
E.rostkoviana	*G.germanica*	**0.79**	**0.03**	0.33	0.09
E.rostkoviana	*H.quadrifida*	**0.78**	**0.03**	**0.53**	**0.03**
E.rostkoviana	*P.palustris*	**0.96**	**0.03**	**0.35**	**0.05**
E.rostkoviana	*R.glacialis*	**0.85**	**0.03**	0.31	0.18
E.rostkoviana	*S.lucida*	**0.58**	**0.03**	0.32	0.06
G.asclepiadea	*G.germanica*	0.26	0.14	**0.51**	**0.03**
G.asclepiadea	*H.quadrifida*	0.11	0.32	**0.86**	**0.04**
G.asclepiadea	*P.palustris*	**0.51**	**0.03**	**0.74**	**0.03**
G.asclepiadea	*R.glacialis*	**0.29**	**0.05**	**0.84**	**0.03**
G.asclepiadea	*S.lucida*	0.13	0.24	**0.97**	**0.02**
G.germanica	*H.quadrifida*	**0.46**	**0.05**	0.53	0.06
G.germanica	*P.palustris*	**0.75**	**0.04**	0.48	0.07
G.germanica	*R.glacialis*	**0.46**	**0.05**	**0.65**	**0.03**
G.germanica	*S.lucida*	**0.58**	**0.03**	**0.77**	**0.03**
H.quadrifida	*P.palustris*	**0.57**	**0.03**	**0.57**	**0.05**
H.quadrifida	*R.glacialis*	0.02	0.41	**0.94**	**0.03**
H.quadrifida	*S.lucida*	0.13	0.25	**0.58**	**0.03**
P.palustris	*R.glacialis*	**0.64**	**0.04**	**0.83**	**0.03**
P.palustris	*S.lucida*	**0.64**	**0.04**	0.47	0.08
R.glacialis	*S.lucida*	0.20	0.10	**0.72**	**0.03**

* Significant differences are highlighted in bold.

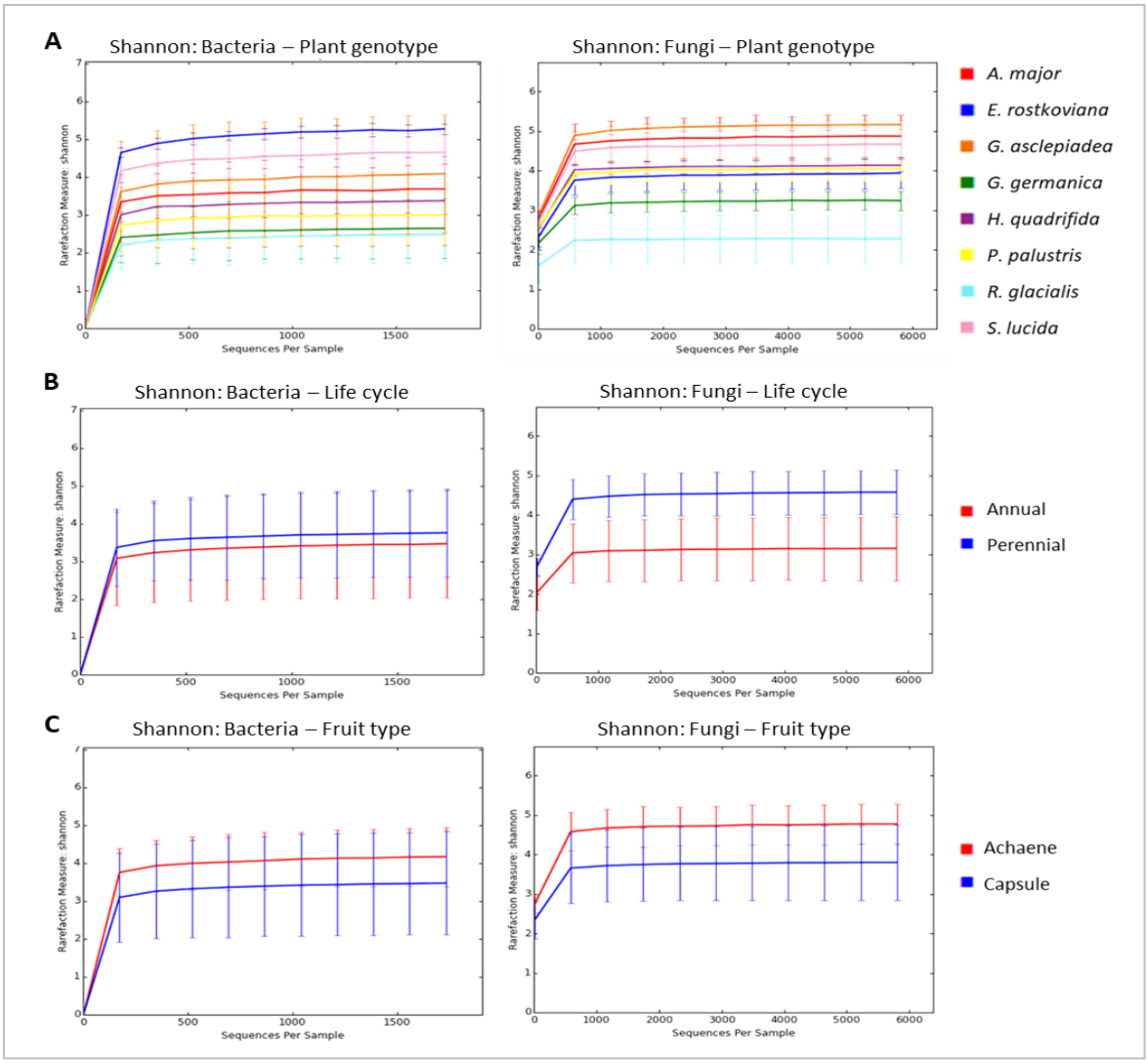


Figure S2.2: Comparison of bacterial and fungal diversity within alpine plant seeds. Shannon diversity indices were compared by grouping the samples according to their plant genotype (A), the life cycle of the plant (B), which is either annual or perennial and the fruit type (C), either achene or capsule. Colors of the grouping variables are shown on the either right. Calculated values and standard deviations can be looked up in Table S4.

CHAPTER 3

Manuscript III: Studying Seed Microbiomes

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Abstract

Recent studies on crop plants provided first insights into the structure of seed microbiomes, and indicated significant impact on germination and plant performance. However, the interplay between seed microbiota and plant health is still poorly understood. In order to get a complete picture of the system, a comprehensive analysis is required, comprising culture-dependent as well as independent techniques. In the following chapter we provide a combination of methods established and optimized for the analysis of the seed microbiome: i) activation and cultivation of dormant seed microbiota, ii) analysis of microbiota in germinated seeds (with and without substrate), iii) quantification of microbial DNA via real time PCR, iv) depletion of host DNA for amplicon and metagenome analysis, and v) slicing of the seeds using the microtome followed by fluorescent *in situ* hybridization (FISH)-on-slide for the visualization of seed endophytes via confocal laser scanning microscopy (CLSM). A deep understanding of the seed microbiome and its functions can help in developing new seed treatments and breeding strategies for sustainable agriculture.

Keywords: Seed microbiome, microbiota, germination, real time PCR, peptide nucleic acid, FISH-CLSM

Introduction

Seeds are small embryonic plants that regulate reproduction of gymnosperms and angiosperms and represent a remarkable phase in a plants' live cycle (Nelson 2018). Seeds have to feature resilience towards a diversity of biotic and abiotic stressors; likewise, their native microbiota is highly specialized. Recent multi-omics based analyses revealed that seeds host a highly diverse and abundant microbiota, which is plant genotype-specific and is affected by the environment and the management practice (Berg and Raaijmakers 2018). Up to two billion bacterial cells belonging to over 9,000 microbial species have been described to comprise the seed microbiota and a beneficial impact on plant health and performance is explicitly suggested (Adam et al. 2018; Johnston-Monje et al. 2016; Rybakova et al. 2017; Shade et al. 2017; Nelson 2018; Berg and Raaijmakers 2018; Bergna et al. 2018). Besides bacteria and fungi, recently, archaea were discovered as native members of the seed (Wassermann et al. 2019). Microbiota can enter seeds via horizontal transmission from the seed surrounding environment or via vertical transmission from the mother plant (Shade et al. 2017). The latter is considered as prenatal care for the successive plant generation by enhancing seed vigor, germination and resilience via the induction of plant defense mechanisms and antagonism towards plant pathogens (Vujanovic and Germida 2017).

The challenges in cultivating seed microbiota led to the long-time assumption that seeds are sterile and that the emerging seedling is colonized mainly by microbes from its surrounding environment, with soil being the main source (Truyens et al. 2015; Sánchez-Cañizares et al. 2017; Shade et al. 2017). Thus, our knowledge of the seed microbiome is still at initial stage and its potential for the promotion of host health and performance is largely unexplored. Understanding the specificity and efficiency of microbiota transmission from one plant generation to the next, as well as the transmission from seeds to roots and other plant organs might provide new opportunities for the development of healthy, resilient and high quality seeds for agriculture (Berg and Raaijmakers 2018).

Plants have evolved manifold strategies to produce seeds, and seed morphology, size and structure is accordingly highly diverse. Moreover, seeds contain plant species-specific secondary metabolites. Therefore, it is essential to adapt the methods to study seed microbiomes for each plant seed accordingly. Additionally, seed microbiome researchers should consider the following facts: i) seeds are composed of endophytes, which represent the microbial community within seeds, and of epiphytes, colonizing the seed surfaces; ii) inside the seeds, endophytes

can colonize different niches: e.g. seed coat, endosperm envelope, cotyledons and the root hypocotyl embryo (Wassermann, Adam, et al. 2019); iii) seed microbiota is very often in a dormant state until the seeds are exposed to a certain stimulus, mostly water; and last but not least, iv) the spermosphere, representing the zone surrounding germinating seeds, where seed microbiota interacts with soil microbial communities (Schiltz et al. 2015; Truyens et al. 2015). Herein, we describe different methods for studying relationships between seeds and its associated microbiota *in situ*, *in vitro* and *in silico*, and provide some examples of how these methods can be combined for an exhaustive study of the seed microbiome. Description of bioinformatics analyses of the seed microbiota would go beyond the framework of this chapter due to the ongoing change of standards. Figure 3.1 shows the guideline through the chapter 'Studying Seed Microbiomes'.

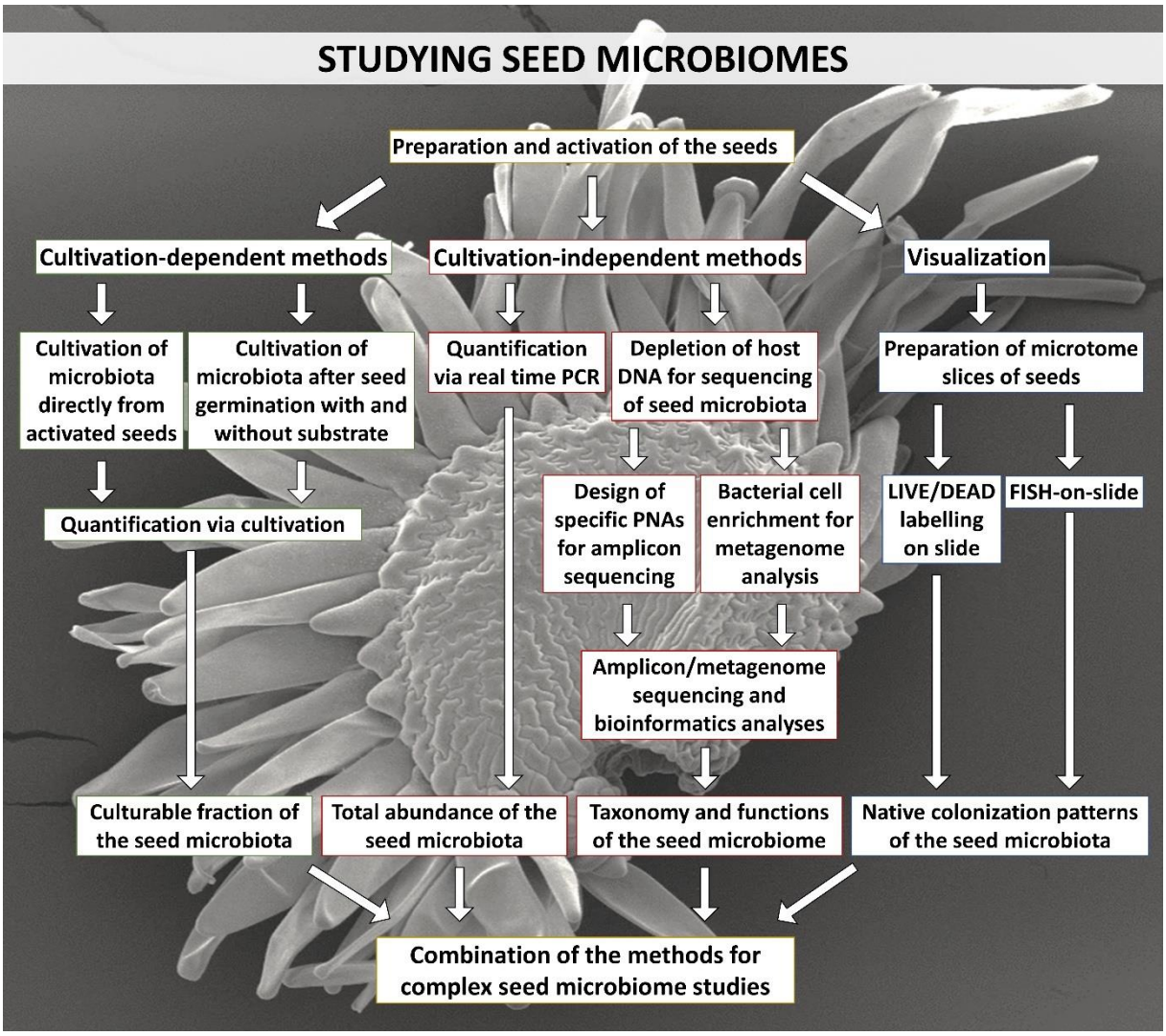

Figure 3.1: Overview of the methods described in the chapter 'Studying Seed Microbiomes'.

Materials

Preparation and activation of seeds

1. Laminar flow hood
2. Sterile distilled water
3. Laboratory platform shaker
4. (optional) Sodium hypochloride (e.g. for oilseed rape seeds: 2% in sterile distilled water) for surface sterilization of seeds

Cultivation of native seed microbiota and seed germination

1. Sodium chloride (0.85% in sterile distilled water)
2. Mortar and pistil (sterile)
3. Diverse solid media to determine microbial abundance and to isolate microorganisms
4. Centrifuge
5. Eppendorf tubes
6. FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, USA) (can be replaces by any other microbial DNA extraction kit appropriate for isolation of DNA from host-associated microbiota)
7. Germination pouches (Mega International, Newport, MN, USA) for gnotobiotic substrate-free seed germination
8. 1 L of soil-vermiculite mixture (1:4) and a plastic container with a volume of 5.6 liter per replicate (substrate composition and amount and size of plastic containers can be exchanged by adequate alternatives)
9. Greenhouse with controlled conditions
10. Stomacher laboratory blender (BagMixer, Interscience, St. Nom, France) or sterile whirl-packs® (Sigma-Aldrich, Vienna, Austria)

Design of specific PNAs for amplicon sequencing of seeds

1. Eppendorf tubes
2. PCR tubes
3. Taq&Go™ DNA Polymerase (Mastermix 5xC, MP Biomedicals, Solon, USA; can be exchanged by adequate alternatives)
4. Primer pair, specifically amplifying target gene sequence, including barcodes for amplicon sequencing

5. $MgCl_2$ (25 mM)
6. Nuclease-free water
7. PCR thermal cycler
8. Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA; can be exchanged by adequate alternatives)
9. NanoDrop™ 2000c Spectrophotometer (Fisher Scientific), or any appropriate UV-VIS spectrophotometric technology to determine nucleic acid concentration in samples
10. Customized peptide nucleic acid clamps (PNAs), if necessary including a) Gamma functional groups: lysine, miniPEG or alanine and glutamic acid, and/or b) O linker, E linker, X linker, or two lysines to enhance solubility of PNA probes (PNA Bio, California, USA)
11. (optional) Materials for single strand conformation polymorphism (SSCP) to check PNA functionality

Bacterial cell enrichment for metagenome analysis

1. Sterile water
2. Sterile 50 ml tubes
3. Eppendorf tubes
4. BCE buffer (bacterial cell extraction buffer): 50 mM Tris HCl pH 7.5, 1% Triton X-100 and 2 mM 2-mercaptoethanol, added prior to usage
5. 50 mM Tris HCl pH 7.5
6. Sterile mortar and pistil
7. Sterile Mesoft® filters
8. Refrigerated centrifuge appropriate for 50 ml Tubes
9. Histodenz™ (Merck, Vienna, Austria) solution: 8 g Histodenz dissolved in 10 ml of 50 mM Tris HCl pH 7.5
10. FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, USA) (can be replaces by any other DNA extraction kit, appropriate to extract microbial community DNA from eukaryotic hosts)

Microbiota quantification via real time PCR (qPCR)

1. Real-time PCR instrument; we use a Rotor-Gene 6000 real-time rotary analyzer (Corbett Research, Sydney, Australia)
2. RT PCR tubes

3. Primers for specific quantification of bacterial, archaeal, and/or fungal genes. We routinely use:
    a. Bacteria: primer pair 515f – 926r (Parada, Needham, and Fuhrman 2016), 10 µM each.
    b. Archaea: Primer pair 344aF – 517uR (Probst, Auerbach, and Moissl-Eichinger 2013), 5 µM each.
    c. Fungi: Primer pair ITS1 – ITS2 (T. J. White et al. 1990), 10 µM each.
4. Peptide nucleic acid clamps (PNAs) (Lundberg et al. 2013): pPNA and mPNA (PNA Bio, California, USA)
5. QuantiTect SYBR® Green PCR kit (QIAGEN GmbH, Hilden, Germany)
6. Nuclease-free water

Visualization of native seed microbiota *in situ*

1. Leica CM 3000 cryostat (GMI, USA) supplied with a stainless-steel rotary microtome
2. Confocal laser scanning microscope (CLSM), e.g. from Leica Microsystems (Wetzlar, Germany)
3. Water bath
4. Embedding media: a glycol-based tissue freezing medium (supplied by the manufacturer) which solidifies at low temperatures and binds the tissues to the tissue holder; e.g. EM-400 Embedding Medium (Leica Microsystems, Wetzlar, Germany) can be used.
5. Eppendorf tubes
6. Ice
7. Tweezers
8. 50, 70, 80 and 96% ethanol
9. 4% Paraformaldehyde (PFA)
10. PBS and ice-cold PBS. For 1 l PBS buffer add 8 g of NaCl to 800 ml of distilled water, then add 200 mg of KCl, 1.44 g of Na2HPO4, 240 mg of KH2PO4. Finally adjust solution to pH of 7.4 and add distilled water until volume is 1 l.
11. Ice-cold double distilled water
12. Microscopy slides
13. Lysozyme (Sigma-Aldrich); 1 mg/ml solution
14. (optional) Calcofluor white (CFW) staining, 0.15 %
15. ProLong Gold antifadent (Molecular Probes, Eugene, USA)

16. Translucent nail polish (any common supplier)
17. (optional) LIVE/DEAD® BacLight™ Bacterial Viability kit, Life technologies, California, USA)
18. Formamide (FA)
19. fluorescent *in situ* hybridization (FISH) probes (Cardinale et al. 2008). Probes used in this example are listed in the Table 1.
20. Hybridization buffer Hb1 for FISH-CLSM: For 200 µl of Hb1 buffer containing 35% FA, mix 36 µl of 5 M NaCl, 4 µl of 1 M Tris/HCl, 1 µl of 2% Sodium dodecyl sulfate (SDS), 70 µl FA and 88 µl ddH2O. Add 1 µl of each chosen probe shortly before you will use the solution. In this example the probes GAM42a, BET42a-competitor and ALF968 were used for the buffer Hb1.

    Please note that FA concentration in both hybridization and washing buffers is dependent on the probes you use (Cardinale et al. 2008).
21. Hybridization buffer Hb2 for FISH-CLSM: For 200 µl of Hb2 buffer containing 15% FA, mix 36 µl of 5 M NaCl, 4 µl of 1 M Tris/HCl, 1 µl of 2% SDS, 30 µl FA and add 128 µl ddH2O. Finally supplement with 1 µl of each chosen probe shortly before you will use the solution. In this example the probe EUB-mix was used.
22. Washing buffer Wb1 for FISH-CLSM: For 100 µl of Wb1 buffer (FA concentration 35%) mix 14 µl of 5 M NaCl, 20 µl of 1 M Tris/HCl, 50 µl of 6.5 M EDTA and 956 µl ddH2O.
23. Washing buffer Wb2 for FISH-CLSM: For 100 µl of Wb2 buffer (FA concentration 15%) mix 64 µl of 5 M NaCl, 20 µl of 1 M Tris/HCl and 956 µl ddH2O.

Methods

Preparation and activation of seeds

All procedures should be carried out under sterile conditions at room temperature, unless otherwise stated. In order to analyze the naturally composed seed microbiota, we recommend using at least four replicates per plant genotype or treatment.

1. To investigate the entire microbial community of the seeds, wash 20 seeds per replicate (or more, dependent on the seed size) three times with sterile distilled water and activate them under agitation (100 rpm) for four hours in an adequate amount of sterile water (e.g. for the oilseed rape seeds with app. 0.6 g/100 seeds, use 2 ml water for 20 seeds. For the pumpkin seeds with app. 22 g/100 seeds, use 13 ml sterile water for 20 seeds). (*see* **Note 1**).
2. (optional) Alternatively, to investigate the endophytic seed microbiota, you can either physically remove seed peel under sterile conditions or surface-sterilize seeds by incubating them with sodium hypochloride solution for five minutes under agitation (we use 2% solution to surface-sterilize oilseed rape seeds). Then, wash the seeds six times with sterile water (*see* **Note 2**).
3. (optional) In order to ensure whether the cultivable microorganisms have been inactivated by the surface-sterilization, the seed surface can be printed on solid media, or the final (sixth) wash solution can be plated out.

Now, you can either ground the activated seeds directly (**3.2)**, or allow the seeds to germinate under sterile conditions and extract microbiota from roots and green parts of the seedlings (**3.2)** (*see* **Note 3**).

Cultivation of microbiota directly from activated seeds and microbial DNA extraction

1. Mortar seeds with 2 to 10 ml sterile 0.85% sodium chloride (dependent on the size of the seeds) under sterile conditions.
2. Dilute the suspension serially for plating on diverse solid media to isolate microorganisms and determine microbial abundance of the cultivable fraction. The

cultivated isolates derived from seeds can be tested *in vivo* for desired qualities (e.g. activity against some plant pathogens).

3. For extraction of total microbial DNA, centrifuge mortared seed material at 16,500 × g for 20 min at 4°C and store pellets at -70°C or proceed directly to DNA extraction.
4. For DNA extraction we routinely use the FastDNA™ SPIN Kit for Soil according to the manufacturer protocol.

Cultivation of microbiota after seed germination with and without substrate and microbial DNA extraction

1. For cultivation without substrate, we recommend using germination pouches according to the manufacturer protocol with two or more pouches per replicate (Figure 3.2, A).
2. For cultivation with sterile substrate, we routinely use 1 liter of soil mixed with vermiculite (4:1) in plastic containers with a volume of 5.6 liter. The potting mixture must be autoclaved twice with a 48 h interval in order to inactivate microbial spores present in soil. We recommend using two or more plastic containers per replicate.
3. Pouches or pots with (sterile) substrate can be incubated under sterile conditions in a greenhouse for two to four weeks, dependent on the growth rate of the seedlings.
4. (optional) To visually check the colonization along the plant root, ‘root printing’ on solid media can be performed (Figure 3.2, B), prior to determination of microbial abundances or DNA extractions.
5. Separate roots from green parts (optional) and homogenize the plant material under sterile conditions in an adequate amount of sterile 0.85% sodium chloride (NaCl) solution, dependent on the plant size (e.g. use 2 ml of 0.85% NaCl for the roots of 14 oilseed rape seedlings grown for 14 days, or 50 ml of 0.85% NaCl for 7 g of pumpkin seedlings root material grown for 30 days).
6. Homogenization of plant material can be performed using sterile mortar and pistil or in a Stomacher laboratory blender using sterile whirl-packs® for three minutes.
7. For determining microbial abundances and extracting DNA, process with the homogenized plant material at the same way as described above for the homogenized seeds (3.2, 3-4).

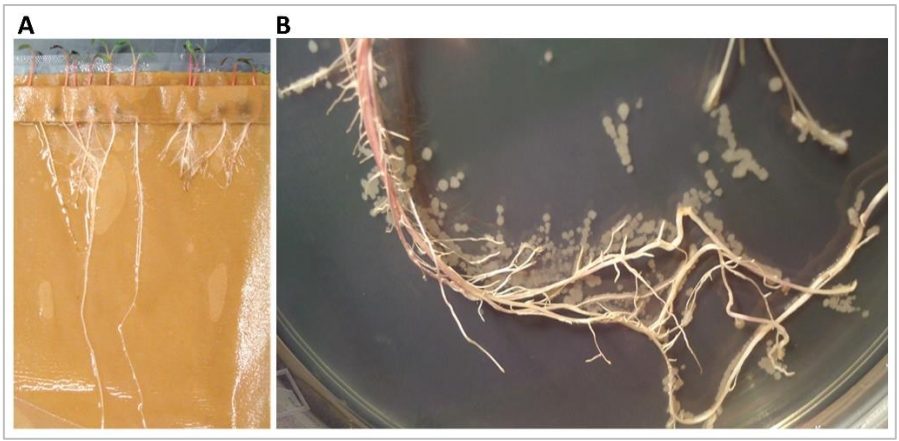

Figure 3.2: (A) sugar beet seedlings in a germination pouch. (B) root print of a sugar beet seedling on nutrient agar. ©Christin Zachow and Adrian Wolfgang (ACIB GmbH, Graz, Austria)

Host DNA depletion I: Design of specific PNAs for amplicon sequencing of seed microbiota

The peptide nucleic acid (PNA) is a single strand DNA oligomer, which specifically binds to target DNA causing a selective blocking of PCR synthesis (Lundberg et al. 2013). Ready-to-use pPNA and mPNA can be ordered as catalog items and used with recommended standard protocols to block the mitochondrial and plastid 16S rRNA derived from the host. Nevertheless, host sequences other than mitochondrial and plastid 16S rRNA may be amplified in a PCR reaction in addition to desired bacterial-derived 16S rRNA sequences and therefore impede microbiome studies. For such cases, specific PNA oligomers can be designed for each study. PCR reaction of the unwanted sequences can be additionally blocked by the means of elongation arrest of polymerase or by competitive binding between the forward or reverse primers and the PNA probe (von Wintzingerode et al. 2000) (*see* **Note 4**).

If your samples show a high proportion of host sequences, find a potential sequence for the PNA design as following:

1. Check a multiple sequence alignment of host sequences for a site with total base identity of all representative sequences.
2. Prevent that the sequence to be blocked is identical or similar to potential microbial sequences: blast the sequence against the NCBI nucleotide database for highly similar sequences (megablast) with default settings (Morgulis et al. 2008; Z. Zhang et al. 2000).

As PNA probes may also bind even if there is one mismatch in the formed heteroduplex, also different one-mismatch variants should be checked. Sequence hits outside of the DNA regions used for amplicon sequencing (rRNA or ITS regions) can be neglected.

3. Check whether the selected sequence can be found in the database which will be used in your study (e.g. UNITE reference database for ITS amplicons used by QIIME). Depending on the database and bioinformatics tools used, consider checking sense as well as reverse complement of the sequence.

Make sure that the sequence is suitable as a PNA blocking sequence:

4. The optimal length of a PNA oligomer for elongation arrest is between 13 bp and 17 bp (Lundberg et al. 2013), nevertheless a range between 12 and 21 bp is also possible ("PNA Bio Inc. (2018)" n.d.).
5. Avoid more than one mismatch between template DNA and PNA probe as it will affect PCR blocking (Terahara et al. 2011).
6. PNA melting temperature (T_m) has to be higher than that of primers (Lundberg et al. 2013). You can, for example, calculate T_m of your primers with the T_m calculator of New England Biolab available at https://www.nebiolabs.com.au/tools-and-resources/interactive-tools. Do not forget to include the barcodes in the calculation. PNA annealing temperature has to be higher than the annealing temperature of the primers used in the PCR reaction to be blocked.
7. PNA T_m should be above the temperature of the extension cycle (Terahara et al. 2011).
8. Further guidelines regarding orientation, self-complementarity, purine and guanine content and distribution can be found in the instructions of the PNA manufacturers. A PNA tool available at https://www.pnabio.com/support/PNA_Tool.htm is can be used for assistance in PNA design.
9. In case when problems fulfilling the rules arise, certain modifications of PNA are possible:
    a. Addition of Gamma functional groups (lysine, miniPEG or alanine and glutamic acid) results in a stereogenic centre at the γ-carbon atom that can convey advantages such as an increased T_m (5 - 8°C/substitution), thus providing higher affinity, improved solubility, less self-aggregation and more stable PNA-DNA duplexes.
    b. Addition of solubility enhancers such as O linker, E linker, X linker, or two lysines can enhance solubility of PNA probes.

10. Example of a 30 µl PCR batch for ITS amplicons including PNA (*see* **Note 5**):

    6 µl Taq&Go™ DNA Polymerase (Mastermix 5xC)

    1.2 µl of each primer including barcodes

    1 µl template DNA (diluted appropriately)

    0.9 µl $MgCl_2$ (25 mM)

    0.15 - 0.30 µl PNA (100 µM)

    19.40 – 19.55 µl nuclease-free water.

11. Extend the PCR cycler protocol with the PNA annealing step, considering the rule of the PNA annealing temperature. Be aware that low temperatures in the primer annealing step could cause unspecific binding of the PNA. Consult well-established protocols (e.g. for using mPNA and pPNA) in order to set the optimal annealing temperature in your specific case.

    Example of a PCR cycler protocol with a PNA annealing step:

    > 95°C for 5 min, 30 cycles of 95°C for 30 sec, 78°C for 5 sec (PNA annealing; set temperature 0 to 2°C below the predicted T_m of PNA including modifications), 58°C for 40 sec (primer annealing; set temperature 0 to 5°C below the T_m of your primer including the barcode with the lowest T_m) and final elongation at 72°C for 10 min.

12. After PCR amplification, the PCR products are purified using the Wizard® SV Gel and PCR Clean-Up System protocol for centrifugation.
13. DNA extracts can be sent to sequencing after determining purity and the nucleic acid concentration in the samples by UV-VIS spectrophotometric technology, e.g. NanoDrop™ Spectrophotometer.
14. (optional) Before re-sequencing your test samples for validation, you can check PNA functionality by a single strand conformation polymorphisms (SSCP) gel (Rochelle 2001). Use the purified PCR products resulting from various PNA concentrations as well those without PNA (standard PCR) for the SSCP gel. Deviating bands in the lanes of the standard PCR variants without PNA can be excised from the SSCP gel, purified accordingly and sequenced in order to verify that the blocked sequences refer to the targeted host sequences.
15. For problem handling, *see* **Note 6.**

Host DNA depletion II: Bacterial cell enrichment for metagenome analysis

The main problem when analyzing plant metagenomes are the host sequences that comprise up to 99% of the extracted DNA. In order to enrich the bacterial cell fraction, we suggest applying a series of centrifugation steps followed by density gradient centrifugation as described in protocols of Ikeda and colleagues and Tsurumaru and colleagues (Ikeda et al. 2009; Tsurumaru et al. 2015). We slightly updated the host DNA depletion protocol for the seeds:

1. To receive appropriate amounts of microbial DNA, homogenize up to 10 g of activated seeds in 50 ml BCE buffer using sterile pistil and mortar.
2. Filter the homogenate through a layer of sterile Mesoft® filter and transfer resulting suspension to a clean tube.
3. Centrifuge at 500 x *g* for 5 min at 10°C and transfer supernatant to a clean tube.
4. Centrifuge at 5,500 x *g* for 20 min at 10°C, discard supernatant and resuspend pellet in 5 ml BCE buffer.
5. To remove insoluble particles, filter suspension through a layer of sterile Mesoft filter, centrifuge filtrate at 10,000 x *g* for 10 min at 10°C discard supernatant and resolve pellet in 5 ml BCE buffer.
6. Repeat filtration and centrifugation steps two times.
7. Suspend the final filtrate in 6 ml of 50 mM Tris HCl (pH 7.5).
8. Prepare an Eppendorf tube with 4 ml Histodenz™ solution (8 g Histodenz dissolved in 10 ml of 50 mM Tris HCl) and add filtrate via pipetting on the tube bottom below Histodenz solution.
9. Centrifuge at 10,000 x *g* for 40 min at 10°C.
10. The bacterial cell fraction is now visible as whitish band, located at the interface of upper and lower phase.
11. Collect the whitish band, mix with equal volume of sterile water and centrifuge at 10,000 rpm for 1 min at 10°C.
12. In order to obtain appropriate amounts of microbial DNA, you can combine extracts from multiple samples. Store the resulting pellet at -20°C or proceed directly to the microbial DNA extraction.

Amplicon and metagenome sequencing and bioinformatics analyses

For appropriate primers and PCR protocols for 16S, ITS and 18S Illumina amplicon sequencing of seed-derived microbiomes, please refer to the suggestions described in the Earth Microbiome Project (Thompson et al. 2017). Specific adjustments for each study are necessary as the associations of microbial communities with various hosts are diverse. The most prominent bioinformatics tool to analyse host-associated amplicon sequences is the QIIME2 pipeline (Bolyen et al. 2018), providing a vast array of commands to depict microbiome composition as well as alpha-(within sample) and beta-(between sample) diversity of microbiota. Recommended databases for taxonomic binning are UNITE (Nilsson et al. 2019) for fungi, and SILVA (Quast et al. 2012) or GREENGENES (DeSantis et al. 2006) for bacteria and archaea. For amplicon sequencing, as well as for all other described analyses, an appropriate number of replicates is mandatory. From those replicates, so-called core microbiomes can be constructed, consisting of taxa which are specific for a certain sample.–Several online tools for data illustration are available; among others, Krona charts (Ondov, Bergman, and Phillippy 2011) (visualizing multi-level community compositions), Venn-diagrams (picturing shared and unique taxa within the sample pool), METAGENassist (Arndt et al. 2012) (to identify positively and negatively correlated taxa), MEGA X (Kumar et al. 2018) (for revealing phylogenetic relationships) and Circos (Krzywinski et al. 2009) (exploring relationships between samples and associated microbes). Cytoscape program (Shannon et al. 2003), used to visualize microbial interaction networks, and its add-on 'CoNet' (Faust and Raes 2016) assisting in inferring putative microbial interactions within their hosts, are valuable tools as well.

The analysis of the Shotgun Metagenomics data is highly dependent on the scientific questioning and the tools for its analyses are numerous. Mentionable are, for example, the open source online application MG-RAST (Meyer et al. 2008) for the automatic phylogenetic and functional analyses of the metagenome, MetaVelvet (Afiahayati, Sato, and Sakakibara 2015) and metaSPAdes (Nurk et al. 2017) for the assembly of multiple genomes from mixed sequence reads. In order to bin contigs into whole genomes, CONCOCT (Alneberg et al. 2014) can be used, resulting in highly representative metagenome-assembled genomes.

Microbiota quantification via real time PCR (qPCR)

For quantifying gene copy numbers of bacteria, archaea and fungi within seeds, a qPCR can be performed on extracted microbial DNA with kingdom-specific primer pairs or alternatively for specific taxa (*see* **Note 7**).

1. Prepare the following reaction mixture for one sample:
    a. For bacteria: 5 µl QuantiTect SYBR® Green PCR kit, 0.5 µl of each primer, 0.15 µl PNA mix (Lundberg et al. 2013), 2.85 µl nuclease-free water, and 1 µl template DNA.
    b. For archaea and fungi, prepare the following mixture: 5 µl QuantiTect SYBR® Green PCR kit, 0.5 µl of each primer, 3 µl nuclease-free water and 1 µl template DNA.
2. We use a Rotor-Gene 6000 real-time rotary analyzer, applying the following cycling conditions:
    a. Bacteria: 95°C for 5 min, 40 cycles of 95°C for 20 sec, 54°C for 30 sec, 72°C for 30 sec and a final melt curve of 72 to 96°C
    b. Archaea: 95°C for 5 min, 40 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec followed by melt curve of 72 to 96°C
    c. Fungi: 95°C for 5 min, 40 cycles of 95°C for 30 sec, 58°C for 35 sec, 72°C for 40 sec with a melt curve of 72 to 96°C.
3. Conduct three individual qPCR runs for each replicate and subtract intermittently occurring gene copy numbers that are found in negative controls from the respective sample.

Preparation seeds for microbial visualization via CLSM

As the seeds are often very hard and small, it is suggested to use the microtome to achieve a very thin slicing of the seeds for *in situ* visualization of microbiota using CLSM. The seed microbiota on the seed-slices can then be stained and visualized by either using FISH-on-slide method (**3.9**) to differentiate between microbial taxa or by using LIVE/DEAD® BacLight™ Bacterial Viability kit (**3.10**) to visualize the viability of the cells.

Figure 3.4 illustrates the method for preparing and visualizing seed microbiota *in situ*.

1. Activate the seeds (at least 4 seeds per condition) for 4 hours in water, followed by air-drying them for at least 12 hours.

2. Adjust the specimen temperature control of the cryostat cabinet down to the freezing temperature suggested by the manufacturer -30°C (unless other freezing temperature is suggested by manufacturer) and let it cool down to the desired temperature.
3. (optional) In order to visualize the endophytic microbial communities only, sterilize the seeds surface using 80% sterile ethanol three times. If the total seed microbiome (endophytes and epiphytes) is studied, this step can be omitted.
4. Pour the embedding media on dry surface of the tissue holders.
5. Place the seeds (one seed at a time) on embedding media.
6. Place the chuck (tissue holder) with seed and embedding media in the cryostat cabinet adjusted to a temperature of -30 °C (unless other freezing temperature is suggested by manufacturer).
7. Keep the chuck with the seed and embedding media in the cryostat cabinet for 15 minutes until the solidification of the embedding media and the seed occurs.
8. Adjust the microtome to obtain 100 µm sections as suggested by the manufacturer.
9. Cut the frozen seeds using the microtome into 100 µm sections.
10. Place the unfolded seed sections on glass slides by separating them from the media using pre-cooled tweezers.
11. (optional) Place one slice each in a pre-cooled Eppendorf tube and keep on ice until the fixation procedure which should occur within maximal 1-2 hours after the slicing.

Staining for CSLM I: FISH-on-slide

The advantages of the on-slide FISH method in comparison to the usual FISH in the tube is that the very thin and delicate sections of plant tissues can be kept directly on the slide and won't be washed away or damaged during repetitive washing and incubation steps. Additionally, much less volume of the buffers is required as compared to the conventional FISH in tube method (Rybakova et al. 2017).

1. Wash the sections with 600 µl PBS in the Eppendorf tube.
2. Add one volume PBS and three volumes 4% PFA to the tube to fix the samples.
3. Incubate the tubes over night at 4°C.
4. Remove the PBS/PFA solution using a pipette tip.
5. Wash the samples three times with PBS in the tube.

6. Place the seed sections on separate glass slides using tweezers and rinse three times with PBS.
7. Incubate the sections with 100 µl lysozyme (1 mg/ml) for 10 min at room temperature to increase the permeability of the bacterial cell wall.
8. Prepare buffer Hb1 containing FISH probes of your choice and pre-warm it at 43°C. (*see* **Note 8**).
9. Rinse the sections twice with ice-cold PBS on the slide.
10. Add a drop of 50% ethanol directly to the section on a slide so that it covers the seed section and incubate for 3 min.
11. Exchange the ethanol solution with 70% ethanol using the pipette and incubate for 3 min.
12. Exchange again the ethanol solution with 96% ethanol using the pipette and incubate for another 3 min.
13. Rinse the samples with ice-cold PBS on the slide once and incubate with PBS for 3 min at room temperature.
14. Apply 200 µl of pre-warmed (43°C) Hb1 directly to the section so that the tissue is completely covered with the liquid and incubate for 90 min at 43°C in the dark.
15. During the incubation time prepare Hb2 containing FISH probe EUB-mix (Table 1) or other probe of your choice and the washing buffer Wb1 and pre-warm at 43°C and 44°C, respectively.
16. Eliminate Hb1 and rinse the samples with 500 µl of pre-warmed Wb1. Add another 0.5 ml of Wb1 and incubate for 10 to 15 min at 44 °C.
17. Eliminate the washing buffer and add 200 µl of pre-warmed (44°C) Hb2 to the sample, followed by an incubation step at 43°C for 90 min.
18. Remove Hb2 and rinse the samples with pre-warmed (44°C) Wb2. Incubate the samples with additional 0.5 ml of Wb2 at 44°C for 10 to 15 min.
19. Remove the washing buffer and rinse the samples with ice-cold double distilled water in order to eliminate any salt residuals.
20. (optional) For the display of the plant structures, stain the samples with CFW, which binds to β-1,3 and β-1,4 polysaccharides as described by the manufacturer:
    a. Incubate the samples with 350 µl of 0.15 % CFW staining solution for 20 to 30 min in the dark and rinse afterwards with ice-cold double distilled water.
    b. Add 0.5 ml double distilled water to the samples and incubate for five to ten min in the dark.

21. Dry the samples very carefully with soft compressed air on the slide and apply up to 10 µl of ProLong Gold antifadent to the samples. Subsequently seal the coverslip with nail polish.
22. Leave the samples in the dark at room temperature for 24 h.
23. (Optional) Store the samples at 4°C and darkness until CLSM investigation for maximum of three days.

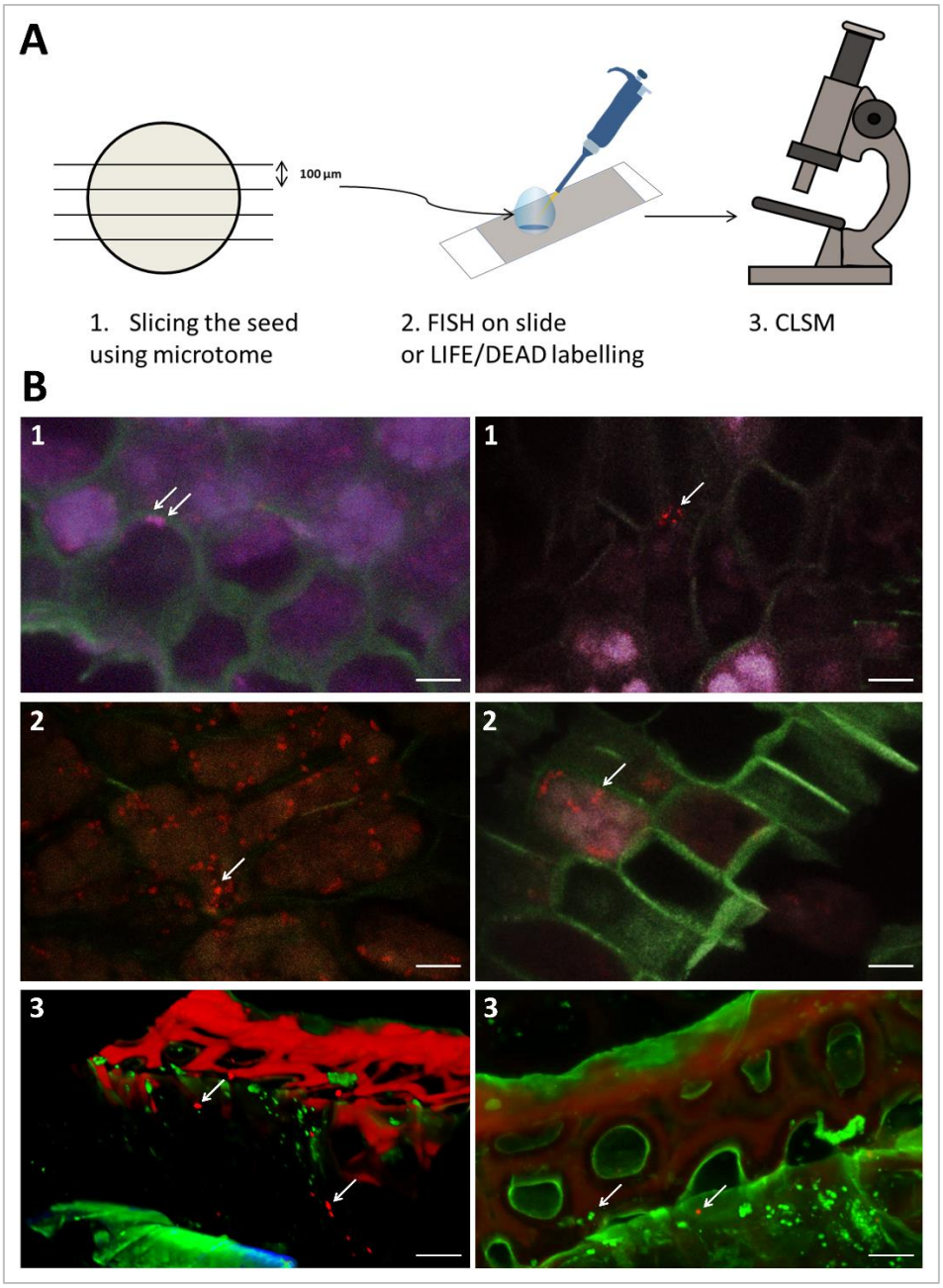

Figure 3.4: (A) A schematic of the seed microbiota visualization process. First, the seed is cut in approximately 100 µm thin sections using a microtome which are then labelled with the FISH probes using FISH-on-slide method or using a LIVE/DEAD staining method. (B) CLSM

visualization of bacterial colonization patterns in the untreated **(1)**, bio-primed with *Pseudomonas brassicacearum* CKB26 **(2)** and *Serratia plymuthica* HRO-C48 **(3)** oilseed rape seeds. The strains in **(1)** and **(2)** were visualized using FISH-CLSM with *Alphaproteobacteria*-specific ALF968 probe (Alexa488-labeled) and an equimolar ratio of eubacteria probe EUB338, EUB338II, and EUB338III (Cy5-labeled). In **(3)**, BacLight LIVE/DEAD stain was used to visualize alive (green) and dead (red) *S. plymuthica* HRO-C48 cells in 3D projection. ©Daria Rybakova and colleagues, Graz University of Technology (5).

Staining for CLSM I: LIVE/DEAD

1. Incubate the sections with 200 µl of 2% LIVE/DEAD® BacLight™ Bacterial Viability kit staining solution for 15 minutes at room temperature, as suggested by the manufacturer.
2. Remove the solution, cover the sample with the coverslip and seal with nail polish.
3. Proceed to CLSM immediately.

Combinations of the methods for complex seed microbiome studies

For a thorough representation of the seed microbiome, it is recommended to combine the data gained from the methods described above (Figure 3.1). The possibilities are numerous and highly dependent on the scientific questioning.

1. Follow a systemic approach by considering the absolute microbial abundance numbers resulting from the qPCR for the correct interpretation of relative microbial abundances resulting from amplicon sequencing. This will allow making a valid comparison between different states of one microbiome or between various microbiomes separated in space.
2. Sequence PCR-amplified 16S rRNA from the purified DNA of seed isolates, that showed desired properties during cultivation-dependent studies. Then, you can track those sequences in the amplicon pool in order to see the distribution of the isolates of interest in the microbial community.
3. Based on the results of the qPCR, amplicon studies or specific properties of isolates in cultivation-dependent studies, select specific FISH probes for verifying co-occurrences and co-localizations patterns *in situ* via CLSM.

4. By using a high number of replicates for cost-effective amplicon sequencing you can define a seed-specific core microbiome that can be used to determine core functions in the metagenome.
5. Activity of genes of interest in the metagenome can be quantified by cDNA amplification via qPCR by using designed primers specifically targeting genes of interest.

Notes

Note 1: Washing steps remove dust and other abiotic particles from the seeds surface. The majority of microorganisms native to seeds are strongly attached to the seed surface and will not be lost during the washing steps. Soaking dry or washed seeds in sterile water has the aim to activate the inherent but dormant seed microbiota which are considered to be viable but nonculturable microorganisms.

Note 2: The efficiency of surface sterilization is largely dependent on seed morphology, demanding specific adjustment in sodium hypochloride concentration and exposure time. Apart from that, we advise against surface sterilization of seeds, as the epiphytic community is a valuable part of the seed microbiome (Nelson 2018). Alternatively, you can remove of seed peel using sterilized dissecting instruments.

Note 3: During seed germination, various plant metabolites are synthesized and secreted that most likely boost the activation of (still dormant) seed microbiota. Seed germination under sterile conditions additionally provides information what kinds of microorganisms and to which extent seed microbiota can colonize roots and phyllosphere.

Note 4: If the host genome sequence is available, *in silico* analyses give first insights into the potential interference of the microbiota-targeted PCR with the host-sequences. We recommend to sequence a smaller part of the seed samples a prior to preparing a comprehensive pool. Pay attention to use several genetically distinct host genotypes for your test run in order to determine whether the PNA functionality is sufficient for all genotypes investigated in your study.

Note 5: Test several concentrations of your PNA in a PCR protocol that usually works well. For example, use a final concentration of 0.5 µM, 0.77 µM or 1 µM PNA and one without PNA for later validation in your test PNA PCR protocol.

Note 6: If blocking of desired sequences was not sufficient, the protocol can be improved by lowering the annealing temperature of PNA and by increasing the PNA concentration in the PCR batch (Orum 2000). If yielded PCR product is low, the PNA concentration could be decreased. In the case of very few microbial sequences in the template, higher template amounts might be necessary. In general, there is no upper limit for the amount of the template DNA. A nested PCR (Niepceron and Licois 2010) can be used in the case of too few microbial sequences in the sample. In a nested PCR the PNA may be used in both PCRs in order to prevent amplification of host sequences. In this case a lower PNA concentration may be sufficient.

Note 7: The PCR using bacteria-specific primer pair amplifies plant mitochondrial and plastid 16S DNA as well. Blocking amplification of the host sequences can be achieved by adding peptide nucleic acid (PNA) clamps (Lundberg et al. 2013) to the qPCR mixture. The disadvantage of this method is that PNAs were originally constructed to block 16S DNA sequences of *Arabidopsis* plants. Depending on the plant species, other host-plant specific sequences might be similar to primer target sequences, interfering the performance of the qPCR. Please refer to 3.4, describing the method to design host-specific PNAs.

Note 8: Here, we describe labelling of the seed microbiota with the probes ALF968 that target *Alphaproteobacteria*, GAM42a targeting *Gammaproteobacteria* and BET42a-comp which is a competitor probe that enhances the sensibility of the method (Cardinale et al. 2008). If other microbial taxa need to be visualized, the buffers and probes need to be adjusted as described by Cardinale and coworkers (Cardinale et al. 2008).

CHAPTER 4

Publication IV: An Apple a Day: Which Bacteria Do We Eat with Organic and Conventional Apples?

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Abstract

Apples are among the most consumed fruits world-wide. They represent a source of direct human exposure to bacterial communities, which is less studied. We analyzed the apple microbiome to detect differences between tissues and the impact of organic and conventional management by a combined approach of 16S rRNA gene amplicon analysis and qPCR, and visualization using fluorescence *in situ* hybridization and confocal laser scanning microscopy (FISH-CLSM). Each apple fruit harbors different tissues (stem, peel, fruit pulp, seeds and calyx), which were colonized by distinct bacterial communities. Interestingly, fruit pulp and seeds were bacterial hot spots, while the peel was less colonized. In all, approx. 10^8 16S rRNA bacterial gene copy numbers were determined in each g apple. Abundances were not influenced by the management practice but we found a strong reduction in bacterial diversity and evenness in conventionally managed apples. In addition, despite the similar structure in general dominated by *Proteobacteria* (80%), *Bacteroidetes* (9%), *Actinobacteria* (5%) and *Firmicutes* (3%), significant shifts of almost 40% of bacterial genera and orders were monitored. Among them, especially bacterial signatures known for health-affecting potential were found to be enhanced in conventionally managed apples. Our results suggest that we consume about 100 million bacterial cells with one apple. Although this amount was the same, the bacterial composition was significantly different in conventionally and organically produced apples.

Keywords: *Malus domestica*, management practice, plant protection, microbiota, carposphere, edible microbiome, one health concept

Introduction

The host-associated microbiota is involved in health issues of the host; this was shown for humans and plants as well (Derrien and van Hylckama Vlieg 2015; Berg et al. 2017). Despite being specifically composed and partly deeply embedded within the host, microbial communities are essentially open and interconnected ecosystems (Berg 2015). However, this connection and the exchange between microbiomes are less understood, despite their importance to health reflected now also in the one health concept (Flandroy et al. 2018). The plant-gut microbiome axis could be of special importance for human health, and raw-eaten plants seem an important source for microbes (Leff and Fierer 2013; Berg et al. 2014; Wassermann et al. 2017). Recently it was shown that plant-associated microbiota including bacteria, fungi and viruses transiently colonized the gut (David et al. 2014); thus, forming our transient microbiome (Derrien and van Hylckama Vlieg 2015). However, the microbial diversity associated with vegetables, fruits and herbs is less studied, especially in this context. In contrast, research and rules in this area focus on food-borne pathogens and food safety; food-borne diseases are recognized as a global burden (WHO 2015). First microbiome studies suggest that improved understanding of how certain ecologies provide supportive resources for human pathogens on plants, and how components of certain agro-ecologies may play a role in the introduction of human pathogens to plants (Ottesen et al. 2019). However, more knowledge on fresh produce-associated microbiota and a holistic view on the system is crucial for food safety inquiries (Blau et al. 2018).

The plant microbiota play an essential role in plant development and health and exert influence on resilience towards biotic as well as abiotic factors (Berg et al. 2016). In general, the plant microbiota is driven by the plant genotype, differs strongly between below and above ground parts and is affected by soil quality and biotic and abiotic conditions (Berg and Smalla 2009; Vorholt 2012; Phillipot et al. 2013). While a core plant microbiome is vertically transmitted by seeds, the surrounding environment is another source of the plant microbiota (Berg and Raaijmakers 2018). Many driving and assembly factors of the plant microbiome are already identified; in agricultural ecosystems management practices have a crucial influence on microbiota composition, diversity and functionality, subsequently affecting health and performance of the host plant (Philippot et al. 2013). Our understanding of the plant microbiome was improved by studies on the model plant *Arabidopsis thaliana* and important crops such as rice and maize (Bulgarelli et al. 2012; Lundberg et al. 2012; Peiffer et al. 2013) but the specific fruit and vegetable microbiome is understudied (Leff and Fierer 2013). Tomato is a model

vegetable for microbiome studies (Kwak et al. 2018; Bergna et al. 2018; Ottesen et al. 2019); in parallel, apples are models for fruit microbiomes.

Apples are among the most consumed fruits world-wide; their production is increasing constantly, and comprise about 83 million t (FAO 2019). Apples represent the most important dietary source for various flavonoids in our diets, and a beneficial impact on human health due to apple procyanidins and pectin has been frequently described (Shoji and Miura 2014; Okeke and Edelman 2001; Shtriker et al. 2018). Studies suggest that apple supplementation can induce substantial changes in microbiota composition and metabolic activity *in vitro*, which could be associated with potential benefits to human health (Koutsos et al. 2017; Garcia-Mazcorro et al. 2019). However, less is known about the apple microbiome; previous work has focused largely on plant pathogens and here, mainly the phyllosphere was studied (Pusey et al. 2009; Burr et al. 1996; Yashiro et al. 2011; Stockwell et al. 2010; He et al. 2012; Liu et al. 2018). Interestingly, apple flowers are colonized by thousands of bacterial taxa, and followed successional groups with coherent dynamics whose abundances peaked at different times before and after bud opening (Shade et al. 2013). The fungal community associated with the apple endosphere is pedigree-specific (J. Liu et al. 2018), and significantly dependent on different tissues (stem end, calyx end, peel and wounded flesh) within the apple carposphere (Abdelfattah et al. 2016). However, basic insights into the bacterial communities of apple fruits are still missing.

The objective of this study are basic insights into the apple fruit microbiome. In detail, we aim to identify i) differences between tissues of apple fruits and ii) the impact of organic and conventional management practices – which represent diverse defined abiotic treatments pre- and post-harvest - on abundance and composition of apple fruit-associated bacteria. We hypothesize i) that each apple provides different niches for bacterial communities and ii) that the management practice has substantial impact on the apple microbiome, which is crucial for plant (post-harvest) and human health issues. With our experimental design we targeted to decipher to which microbiota the consumer is usually directly exposed, and used an integrated design of methods combining 16S rRNA amplicon libraries and qPCR and FISH-CLSM.

Materials and Methods

Sampling and experimental design

In order to investigate and compare the microbiome of organically and conventionally managed apples (*Malus pumila* Mill.) the cultivar ‘Arlet’ was selected. Both the organically and the conventionally produced apples were cultivated in Styria (Austria) under AMAG.A.P. Certification (AMA-Gütesiegel-Produktion), which represent the Austrian law for the international guidelines for agricultural management program GLOBALG.A.P. Matured, fully developed apples were sampled at harvest time in September 2017 in Styria (Austria). Organically managed apples originated from an organic orchard, which follows the international “demeter” guidelines for organic farming (https://www.demeter.at/richtlinien/), using sterile gloves and instruments. Conventional apples originated from a conventional orchard in Styria. In contrast to the organically produced apples, they underwent the following post-harvest treatments: directly after harvest, apples were short-term stored under controlled atmosphere (1-2°C, 1.5%-2% CO_2), washed and wrapped in polythene sheets for sale. Both apple management groups (‘organic’ and ‘conventional’) were transported to laboratory immediately and processed under sterile conditions. All apples were visually examined for consistency in shape, size, color, flawlessness, firmness and freshness prior to processing. Four apples, weighing 190g ± 5g, were selected from each of the two management groups and each apple was divided into six tissues with the following weights: stem: 0.2 g, stem end: 2 g, peel: 9 g, fruit pulp: 12 g, seeds:_0.2 g and calyx end: 3 g. Thus, each tissue was represented by four replicates, where each replicate consists of the respective tissue of one apple. Here it has to be mentioned that seeds of conventionally managed apples contained on average only half as many seeds as organically managed ones.

Microbial DNA extraction and amplicon library construction

In order to extract microorganism, stem end, peel, fruit pulp and calyx end samples were homogenized in a Stomacher laboratory blender (BagMixer, Interscience, St. Nom, France) with 4 ml sterile NaCl (0.85%) solution for three minutes. Seeds and stems were physically disrupted in a sterilized mortar. For the upcoming cultivation-independent analyses, 2 ml of apple suspensions were centrifuged for 20 min at 16,000 g and pellets were used to extract bacterial genomic DNA using FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, USA) and

a FastPrep Instrument (MP Biomedicals, Illkirch, France) for 30 seconds at 5.0 m/s. For culture-independent Illumina MiSeq v2 (250 bp paired end) amplicon sequencing, the primers 515f – 806r (Caporaso, Kuczynski, Stombaugh, Bittinger, Bushman, Costello, Fierer, Pẽa, et al. 2010) were used to amplify the 16S rRNA gene using three technical replicates per sample. Peptide nucleic acid (PNA) clamps were added to PCR mix to block amplification of host plastid and mitochondrial 16S DNA (Lundberg et al. 2013). PCR for 16S rRNA gene amplification was performed in a total volume of 30 µl (5 x Taq&Go (MP Biomedicals, Illkirch, France), 1.5 µM PNA mix, 0.25 mM of each primer, PCR-grade water and 1 µl template DNA) under the following cycling conditions: 95°C for 5 min, 30 cycles of 96°C for 1 min, 78°C for 5 sec, 54°C for 1 min, 74°C for 60 sec and a final elongation at 74°C for 10 min. Technical replicates were pooled and purified by Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). For amplicon sequencing, DNA concentrations were measured with Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and samples were combined in equimolar concentration.

Illumina MiSeq data analysis and statistics

Raw sequence data preparation and data analysis was performed using QIIME 1.9.1 (Caporaso et al. 2010). After paired reads were joined and quality filtered (phred q20), chimeric sequences were identified using usearch7 (Edgar 2010) and removed. Representative sequences were aligned, open reference database SILVA (ver128_97_01.12.17) was used to pick operational taxonomic units (OTUs) and de novo clustering of OTUs was performed using usearch. After taxonomy assignment, sequences assigned to host mitochondria and chloroplasts were discarded. OTU tables were rarefied to 1,525 sequences per sample, according to the sample with lowest amount of sequences. Rarefied OTU tables served as input matrix for upcoming alpha and beta diversity analyses and according statistics were calculated in QIIME. Beta diversity, based on unweighted UniFraq distance matrix, was visualized by Principle Coordinates Analysis (PCoA) and statistical significance was calculated by Analysis of Similarity (ANOSIM). Box-and-Whiskers-Plots, based on Shannon diversity indices, were constructed to visualize microbiota diversity of apple samples using IBM SPSS program (version 25.0, IBM Corporation, Armonk, NY, USA) and statistics were calculated using non-parametric Kruskal-Wallis test and False Discovery Rate (FDR) multiple test correction. For taxonomy charts and in order to trace differentially abundant taxa between organically and conventionally managed apples, OTUs with less than 0.01% abundance were excluded from

the dataset. Significant differences (alpha < 0.05) in taxa abundance on genus and order level were calculated in QIIME, using non-parametric Kruskal-Wallis/FDR test. Taxonomy charts were constructed by merging the core microbiota (taxa occurring in 75% of all replicates) of each tissue of the corresponding management group and the taxonomic network was constructed using Cytoscape version 3.5.1 (Shannon et al. 2003).

Quantitative PCR (qPCR)

For determining bacterial abundance, qPCRs were conducted with the bacterial directed primer pair 515f – 927r (10 µm each; (Köberl et al. 2011)). The qPCR reaction mix contained 5 µl KAPA SYBR Green, 0.15 µl PNA mix, 0.5 µl of each primer, 2.85 µl PCR-grade water and 1 µl template DNA (fruit pulp and seed samples were diluted 1:10 in PCR grade water). Quantification of fluorescence was detected in a Rotor-Gene 6000 real-time rotary analyzer (Corbett Research, Sydney, Australia) with the following cycling conditions: 95°C for 5 min, 40 cycles of 95°C for 20 sec, 54°C for 30 sec, 72°C for 30 sec and a final melt curve of 72 to 96°C. Three individual qPCR runs with R^2 values of standard curves of 0.12 were conducted separately and each replicate was measured in triplicate. Intermittently occurring gene copy numbers that were detected in negative control reactions were subtracted from the respective sample. Significant differences ($p < 0.05$) of bacterial gene copy numbers per gram of tissue between management groups and apple tissues were calculated using IBM SPSS program by applying non-parametric Kruskal-Wallis test including FDR multiple test correction.

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

Native colonization patterns of bacteria associated with the apple tissues were visualized by FISH-CLSM, using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany) with oil immersion objective lenses Leica ACS APO 40.0 x oil CS and Leica ACS APO 63 x oil CS. Apple samples were fixed with 4% paraformaldehyde/phosphate-buffered saline over-night at 4°C prior to FISH application, according to the protocol of Cardinale et al. (Cardinale et al. 2008). Cy3-labelled EUB338MIX (Amann et al. 2001; Daims et al. 1999) was used to stain overall bacterial colonization and for specific visualization of *Firmicutes* and *Gammaproteobacteria*, Cy5-labelled LGC-mix (Meier et al. 1999) and ALEXA-labelled GAM42a (Manz et al. 1992), respectively, were applied. For

contrasting host cell walls, FISH samples were treated with Calcoflour White. By maximum projections of optical z-stack slices, micrographs of the bacterial colonization were generated.

Results

Quantitative records of bacterial 16S rRNA gene abundance in apple tissues

Gene copy numbers of bacterial 16S rRNA per gram tissue of organic and conventional apples were measured by qPCR inquiry (Figure 4.1). Bacterial abundances were observed to be mostly consistent between the management analogs of each tissue; no significant differences ($p < 0.05$) were observed according to non-parametric Kruskal-Wallis/FDR. In contrast, bacterial abundance was strongly tissue-specific. Overall, stem (mean value 1.54×10^8 16S rRNA gene copy numbers per gram) and seeds (mean value 1.26×10^8) showed highest bacterial abundance, followed by calyx end, stem end and fruit pulp; peel microbiota (mean value 4.49×10^4) were lowest abundant. Table 1, therefore, shows only the significant difference in 16S rRNA gene abundance per gram between the tissues within the two management groups. Combining all tissue samples of the corresponding management group resulted in the mean values 4.85×10^7 and 4.67×10^7 per gram organic and conventional apple, respectively. The difference was not significant. In order to give a notion on the amount of bacteria ingested during the consumption of a whole apple, we excluded stem samples and multiplied the values of 16S rRNA gene copy numbers per g tissue with the mean weight of the respective tissue within one ‘Arlet’ apple: stem end: 6 g, peel: 35 g, fruit pulp: 145 g, seeds: 0.3 g, calyx end: 5 g. Calculated values were then added up; accordingly, consumption of one organic and one conventional ‘Arlet’ apple includes ingestion of 1.39×10^8 and 4.19×10^7 16S rRNA gene copy numbers, respectively. If you eat only peel and fruit pulp, 3.87×10^7 and 3.39×10^6 16S rRNA gene copies are ingested with one organic and one conventional apple, respectively. The differences were not statistically significant. ‘Arlet’ apples represent a relatively small apple variety; considering the standard size of an apple with 240 g, consuming the whole apple includes a mean uptake of 1.14×10^8 16S rRNA gene copy numbers.

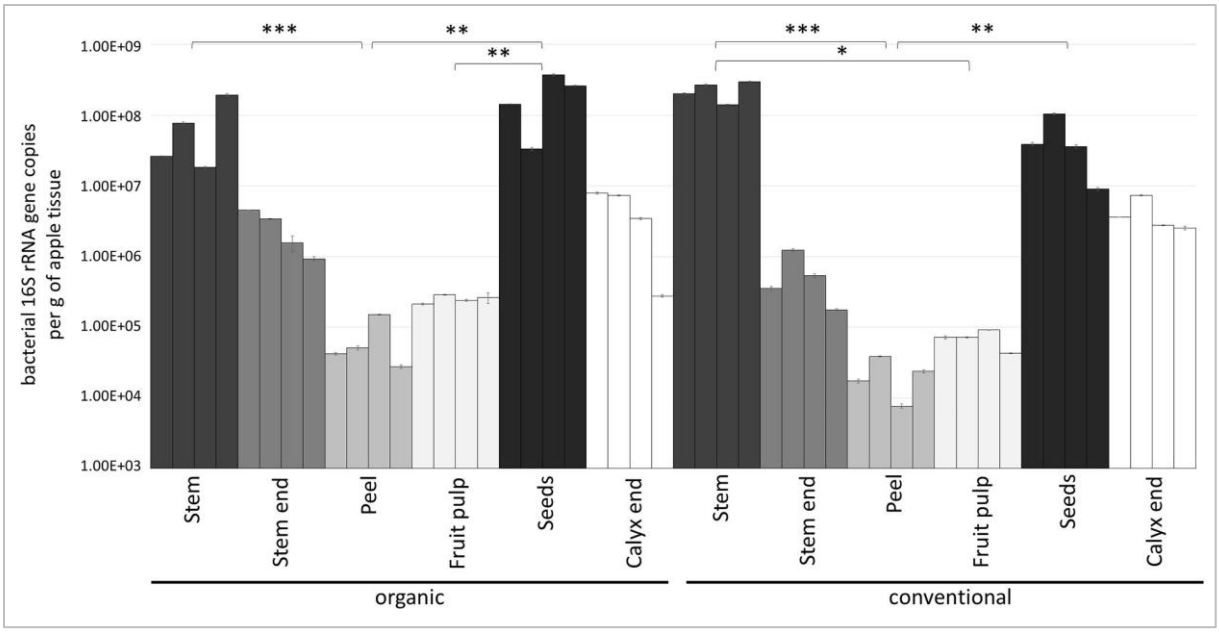

Figure 4.1: Bacterial abundance in carposphere tissues of organically and conventionally managed apples. Microbial community abundance within each tissue was measured in four replicates by qPCR using PNAs to block mitochondrial and plastid 16S DNA. Asterisks indicate significant differences in 16S rRNA gene abundance (calculated per g of apple tissue) between the tissues within a management group.

Table 4.1: Significant differences in 16S rRNA gene abundance per gram of tissue between organically and conventionally managed apple tissues.

	Group1*	**Group2***	**Group1 mean**	**Group2 mean**	**p-value**
Organic tissues	Stem O	Peel O	7.91E+07 ± 6.99E+07	6.81E+04 ± 4.89E+04	0.001
	Peel O	Seeds O	6.81E+04 ± 4.89E+04	2,04E+08 ± 1.28E+08	0.002
	Fruit pulp O	Seeds O	2.51E+05 ± 2.80E+04	6.81E+04 ± 1.28E+08	0.004
Conventional tissues	Seeds C	Peel C	4.71E+07 ± 3.50E+07	2.18E+04 ± 1.12E+04	0.002
	Stem C	Peel C	2.28E+08 ± 6.16E+07	2.18E+04 ± 1.12E+04	0.001
	Stem C	Fruit pulp C	2.28E+08 ± 6.16E+07	6.96E+04 ± 1.76E+04	0.02

* Abbreviations O and C denote for organically and conventionally managed apples, respectively. Only significant differences in microbial abundance between apple tissues are listed.

Quantitative records of diversity estimates of apple microbiota

Shannon diversity estimates revealed organically managed apples to harbor a significantly more diverse microbiota than conventionally managed ones (Figure 4.2 and Table 4.2). The difference was even more significant when the two management analogs of each tissue were compared; Shannon diversity index was significantly higher for the microbiota of all organic tissues, compared to conventional ones, with the sole exception of calyx end microbiota. Table 2 shows furthermore the comparison of the tissues within one management group. For organic apples, fruit pulp showed highest microbial diversity, followed by peel and stem, stem end, seed and calyx end, in ascending order. Diversity of the fruit pulp microbiota was significantly higher than stem, seeds and calyx end microbiota. Regarding conventional tissues, Shannon diversity index was highest for peel microbiota, followed by stem, stem end, fruit pulp, calyx end and seed microbiota. Here, peel microbiota was significantly more diverse than seed, calyx end and fruit pulp microbiota.

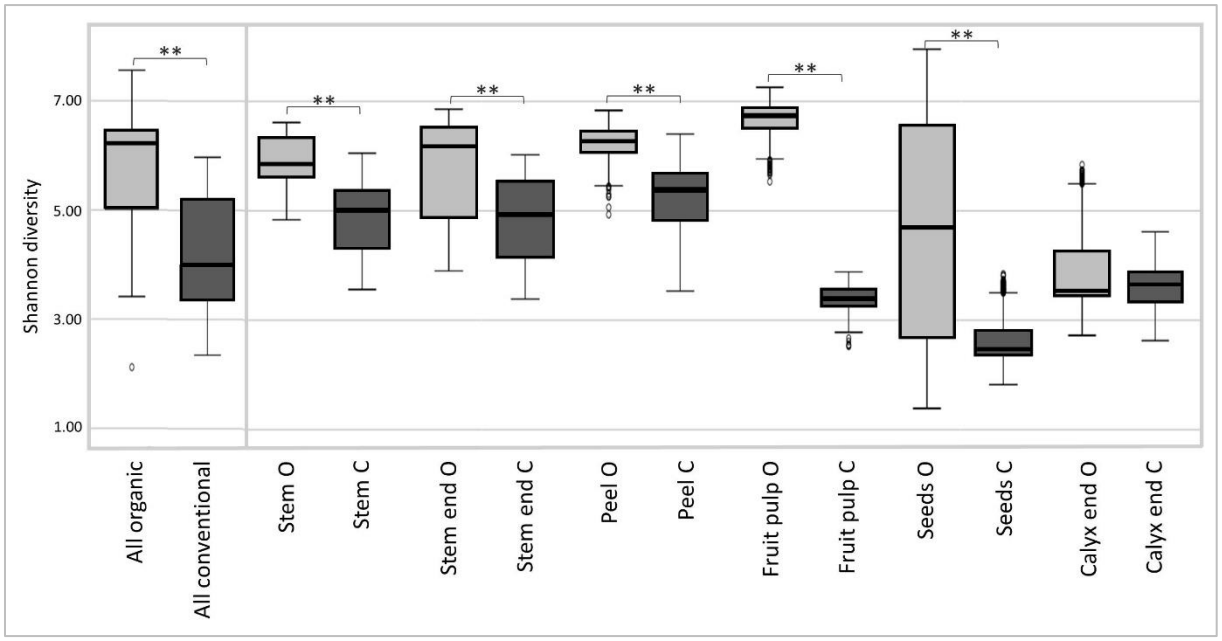

Figure 4.2: Microbial diversity estimates of organically and conventionally managed apples and apple tissues. Suffixes O and C of carposphere tissue in the bottom legend, denote for organic and conventional management, respectively. Significant differences in Shannon diversity estimates of the apple management analogs are indicated by brackets and asterisks.

Table 4.2: Alpha diversity measures of differentially managed apples and apple tissues based on Shannon diversity estimates.

	Group1*	Group2*	Group1 mean		Group2 mean		p-value**
Whole apple	All Organic	All Conventional	5.60	± 1.36	4.17	± 1.11	0.003
Organic versus conventional tissues	Stem end O	Stem end C	5.87	± 0.81	4.94	± 0.71	0.001
	Stem O	Stem C	5.92	± 0.41	4.98	± 0.64	0.001
	Peel O	Peel C	6.22	± 0.32	5.32	± 0.57	0.001
	Fruit pulp O	Fruit pulp C	6.67	± 0.35	3.39	± 0.25	0.001
	Seeds O	Seeds C	4.97	± 2.13	2.68	± 0.50	0.001
	Calyx end O	Calyx end C	3.96	± 0.87	3.70	± 0.47	0.782
Organic tissues	Peel O	Stem end O	6.22	± 0.32	5.87	± 0.81	1
	Peel O	Stem O	6.22	± 0.32	5.92	± 0.41	1
	Peel O	Seeds O	6.22	± 0.32	4.97	± 2.13	0.157
	Peel O	Calyx end O	6.22	± 0.32	3.96	± 0.87	0.002
	Peel O	Fruit pulp O	6.22	± 0.32	6.67	± 0.35	0.157
	Stem end O	Stem O	5.87	± 0.81	5.92	± 0.41	0.157
	Stem end O	Seeds O	5.87	± 0.81	4.97	± 2.13	0.002
	Stem end O	Calyx end O	5.87	± 0.81	3.96	± 0.87	0.001
	Stem end O	Fruit pulp O	5.87	± 0.81	6.67	± 0.35	1
	Stem O	Seeds O	5.92	± 0.41	4.97	± 2.13	1
	Stem O	Calyx end O	5.92	± 0.41	3.96	± 0.87	0.175
	Stem O	Fruit pulp O	5.92	± 0.41	6.67	± 0.35	0.002
	Seeds O	Calyx end O	4.97	± 2.13	3.96	± 0.87	1
	Seeds O	Fruit pulp O	4.97	± 2.13	6.67	± 0.35	0.001
	Calyx end O	Fruit pulp O	3.96	± 0.87	6.67	± 0.35	0.001
Conventional tissues	Peel C	Stem end C	5.32	± 0.57	4.94	± 0.71	1
	Peel C	Stem C	5.32	± 0.57	4.98	± 0.64	0.157
	Peel C	Seeds C	5.32	± 0.57	2.68	± 0.50	0.001
	Peel C	Calyx end C	5.32	± 0.57	3.70	± 0.47	0.001
	Peel C	Fruit pulp C	5.32	± 0.57	3.39	± 0.25	0.001
	Stem end C	Stem C	4.94	± 0.71	4.98	± 0.64	1
	Stem end C	Seeds C	4.94	± 0.71	2.68	± 0.50	0.001
	Stem end C	Calyx end C	4.94	± 0.71	3.70	± 0.47	0.003
	Stem end C	Fruit pulp C	4.94	± 0.71	3.39	± 0.25	0.116
	Stem C	Seeds C	4.98	± 0.64	2.68	± 0.50	0.002
	Stem C	Calyx end C	4.98	± 0.64	3.70	± 0.47	0.209
	Stem C	Fruit pulp C	4.98	± 0.64	3.39	± 0.25	1
	Seeds C	Calyx end C	2.68	± 0.50	3.70	± 0.47	1
	Seeds C	Fruit pulp C	2.68	± 0.50	3.39	± 0.25	0.209
	Calyx end C	Fruit pulp C	3.70	± 0.47	3.39	± 0.25	1

* Abbreviations O and C denote for organically and conventionally managed apples, respectively.

** Statistics were calculated based on Kruskal-Wallis/FDR test.

Highest beta diversity measures were observed when the replicates were grouped by the tissue of the respective management group (ANOSIM values: R=0.8, p=0.001; Figure 4.3, A). Grouping samples by organic and conventional management revealed the ANOSIM values R=0.26, p=0.001 (Figure 4.3, B). Hence, we had a closer look on the management effect on each tissue separately, resulting in the ANOSIM values R>0.8, p<0.05 for all tissues, except seeds (ANOSIM values for seeds: R=0.4, p=0.05). The management practice therefore seems to have a profound impact on the microbiota composition of all tissues while the management effect on seed microbiota was lower. This observation was confirmed when seed samples were excluded from the dataset; ANOSIM values increased to R=0.45 and p=0.001 (Figure 4.3, C).

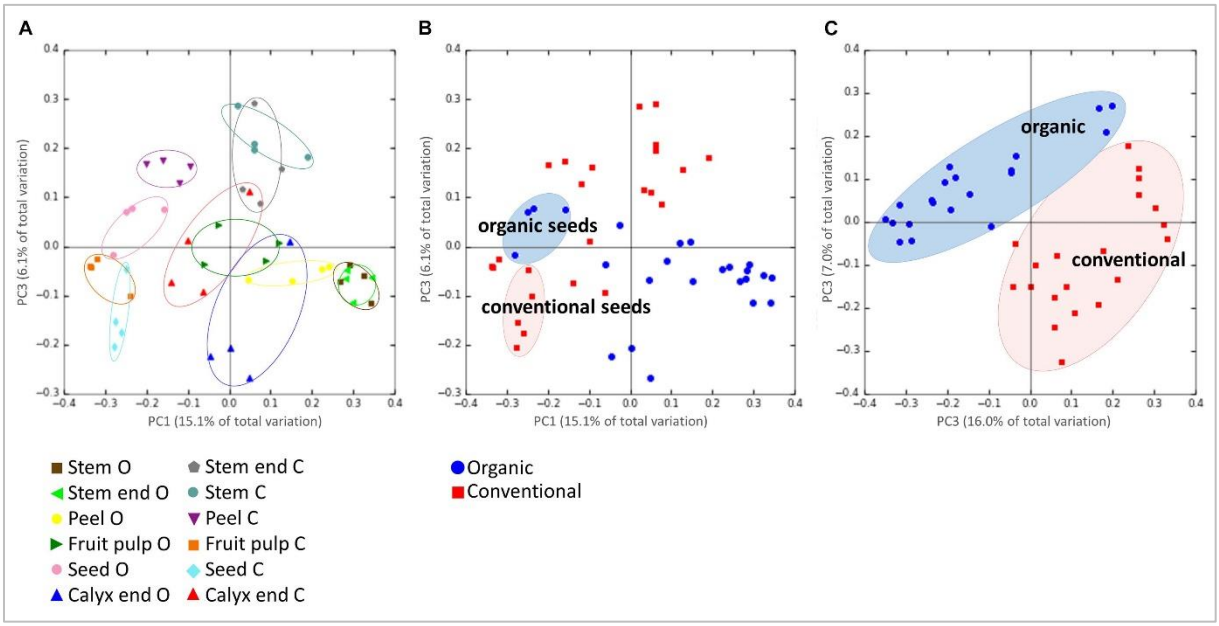

Figure 4.3: Beta-diversity analysis on microbiota composition dependencies. Panel (A) shows the microbiota composition grouped by the tissue of the respective management group, where O and C in the bottom legend denote for organically and conventionally managed apples, respectively. Panel (B) visualizes composition of all tissue replicates, colored by organic (blue circles) and conventional (red squares); seeds of organically and conventionally managed apples are highlighted. In Panel (C), same dataset is shown but seed samples of both management groups were excluded. PCoA plots are based on unweighted UniFraq distance matrix.

The general structure of the bacterial apple microbiota

After removing chimeric, mitochondrial and chloroplast sequences, the overall bacterial community of all apple samples, assessed by 16S rRNA gene amplicon sequencing, contained 6,711,159 sequences that were assigned to 92,365 operational taxonomic units (OTUs). The taxonomic assignment of OTUs revealed 44 different phyla, 325 orders and 1,755 genera. Among bacterial phyla, *Proteobacteria* highly dominated with 80%, followed by *Bacteroidetes* (9%), *Actinobacteria* (5%) and *Firmicutes* (3%). *Burkholderiales* were highly abundant concerning bacterial orders (31% abundance), followed by *Sphingomonadales* (14%), *Rhizobiales* (12%), *Pseudomonadales* (11%), *Enterobacteriales* (7%) and *Cytophagales* (5%); *Micrococcales*, *Sphingobacteriales*, *Bacillales*, *Rhodospirillales* and *Flavobacteriales*, in ascending order, represented between 5% and 1% of total OTUs. OTUs assigned to the genus *Ralstonia* were most frequent with 13%, while *Sphingomonas* (12%), *Pseudomonas* (11%), *Massilia* (7%), *Methylobacterium* (7%), *Burkholderia* (5%), *Pantoea* (5%) and *Hymenobacter* (5%) were furthermore high abundant.

The specific structure of the microbiota in tissues of organic and conventional apples

A clustering network based on the core taxa of the tissues of each apple management group was constructed to visualize the taxa present in all apples as well as the taxa that are specific for each management group (Figure 4.4). Only taxa occurring with at least 0.01% abundance in the whole dataset were included in the network analysis. All apples were found to share a high abundant core microbiota; 73 out of 141 genera were shared. Among them, highly abundant *Proteobacteria* were most dominant and abundant with 45 genera. In total 16 genera were found only in organically managed apples, and 50 genera, predominated by *Proteobacteria* (33 genera) were specific for conventional apples. Overall, the specific microbiota for each management group were less abundant than the shared microbiota.

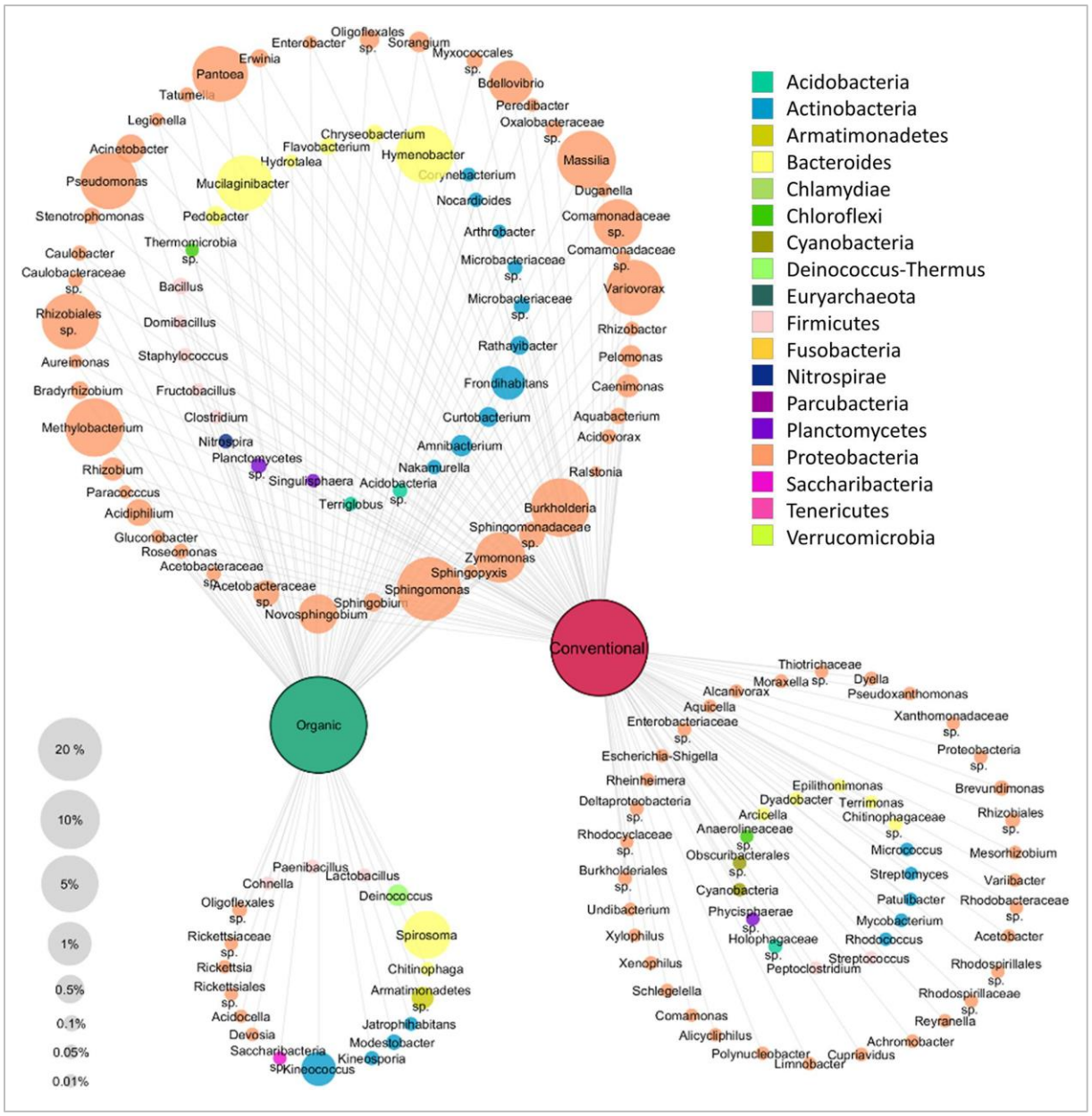

Figure 4.4: Core and specific microbiota for organic and conventional apples. Core microbiota (taxa occurring in 75% of all replicates) of each management group (conventional and organic) were combined for network analysis. To be included, taxa had to exhibit at least 0.01% abundance in the whole dataset. Node size correspond to relative abundance in the dataset as denoted in the legend on the bottom left, node labels display taxonomic identification of OTUs on genus level wherever possible and node color indicates appropriate phylum, as described in the legend on the top right.

In order to visualize the differences between the community compositions of the management analog of each tissue on a taxonomic level, Figure 4.5 was prepared. Pie charts include only taxa that are abundant with at least 0.1% in the whole dataset. Here, differences between organically and conventionally managed apples are obvious for all tissues. Contradictory to beta diversity analysis (described above and Figure 4.3), seeds appear to feature very different microbiota, especially due to the dominance of *Ralstonia* in conventional seeds. The inconsistency of the results can be explained by the fact that beta diversity measures were calculated on the entire OTU table and Figure 4.5 was constructed on the high abundant (> 0.1%) core taxa of each tissue.

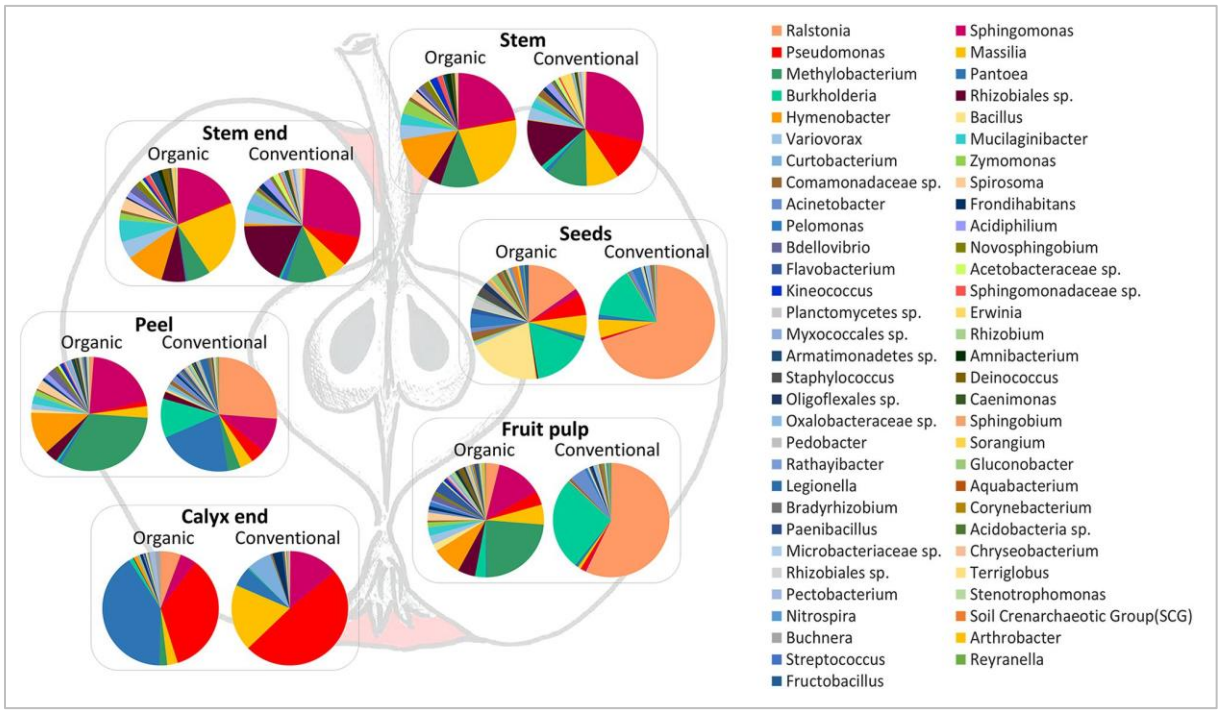

Figure 4.5: Taxonomic composition of organic and conventional apple tissue microbiota. Pie charts visualize taxa occurring in the core microbiomes of each tissue, with at least 0.1% abundance in the whole dataset, and visualize differences between conventional and organic apples.

Indicator species for organically and conventionally managed apples

Differences in abundance of specific bacterial taxa associated with either organically or conventionally managed apples, were assessed by applying non-parametric Kruskal-Wallis/FDR test. Priorly, OTU table was filtered by excluding OTUs with less than 0.01%

abundance, resulting in a total of 172 taxa on genus level. Calculations assigned 67 taxa a significantly higher abundance in either organically or conventionally managed apples (Supplementary Table 4.1); accordingly, 39% of the taxa were significantly different abundant. Noteworthy among them are *Methylobacterium*, *Hymenobacter*, *Spirosoma* and *Zymomonas* which were high abundant in organically managed apples, and *Burkholderia*, *Pantoea*, *Erwinia* and *Acinetobacter,* especially high abundant in conventional apples. Significantly different abundance between microbiota of organically and conventionally managed apples was furthermore calculated on higher taxonomic level. The 172 genera were condensed to 66 different bacterial orders; among them, 25 orders were significantly different abundant, accounting to 37.8% (Supplementary Table 4.1). Among those, *Cytophagales* were high abundant in organic apples while the orders *Burkholderiales, Pseudomonadales, Enterobacteriales* and *Flavobacteriales* prevailed in conventional apples.

Indicator species for health with focus on *Enterobacteriales*

The microbiota of conventional and organic apples were screened for their potential to feature health-relevant properties for humans. For that purpose, we constructed an OTU table containing only *Enterobacteriales*, as especially this order is described to contain taxa responsible for food-borne outbreaks. In our dataset the order *Enterobacteriales* was found to be significantly more abundant in conventionally managed apples (described above and Supplementary Table 4.1). Figure 4.6 shows the relative abundance of taxa to total *Enterobacteriales* in the tissues of organically and conventionally managed apples. *Pantoea* was most abundant among all samples, representing between 60% and 99% of *Enterobacteriales* microbiota; however, *Pantoea* was significantly more abundant in conventionally managed apples (Supplementary Table 4.1). *Pectobacterium*, *Tatumella* and *Enterobacter* were furthermore abundant in almost all tissues, independent of their management practice. Abundance of a not further assigned *Enterobacteriaceae* taxon (*Enterobacteriaceae* sp. in Figure 4.6), *Erwinia* and *Escherichia-Shigella* were significantly more abundant in conventional apples.

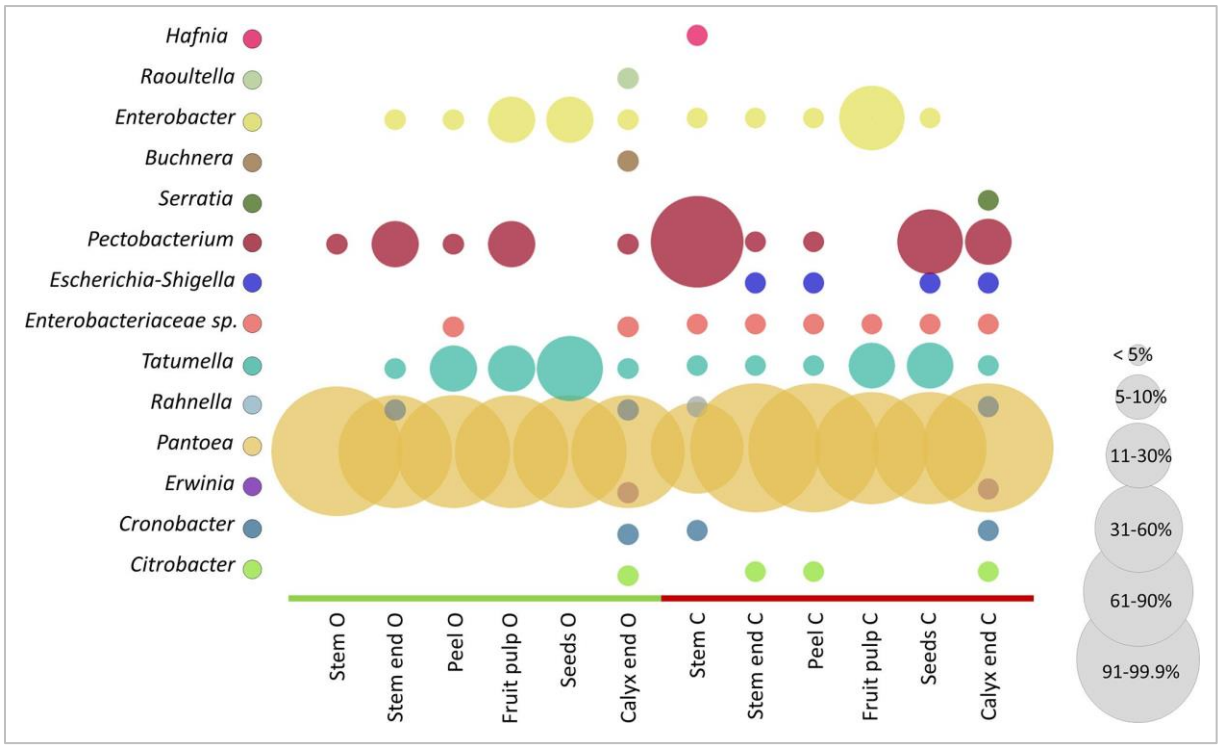

Figure 4.6: Comparison of conventional and organic apple tissues regarding *Enterobacteriales* abundance. Color code for bubbles is depicted in the legend on the left and bubble size indicates relative abundance of taxa within total *Enterobacteriales* microbiota, as explained in the legend on the right. The abbreviations O and C denote for organically and conventionally managed apple tissues, respectively.

Native colonization patterns of microbiota in apple tissues

By using CLSM in combination with FISH we were able to visualize bacteria native to all carposphere tissues *in situ* (Figure 4.7). Visualization of stem, stem end, peel and calyx end microbiota turned out to be successful; *Gammaproteobacteria* (fluorescing pink) and *Firmicutes* (yellow) were distinguishable from remaining bacteria (red). In fruit pulp and seed samples, few bacteria were detected as well, however, due to high autofluorescence of host tissues, imaging was more challenging compared to remaining tissues. During microscopic observations, no differences were observed between organic and conventional apples, therefore Figure 4.7 illustrates only tissues of organic apples.

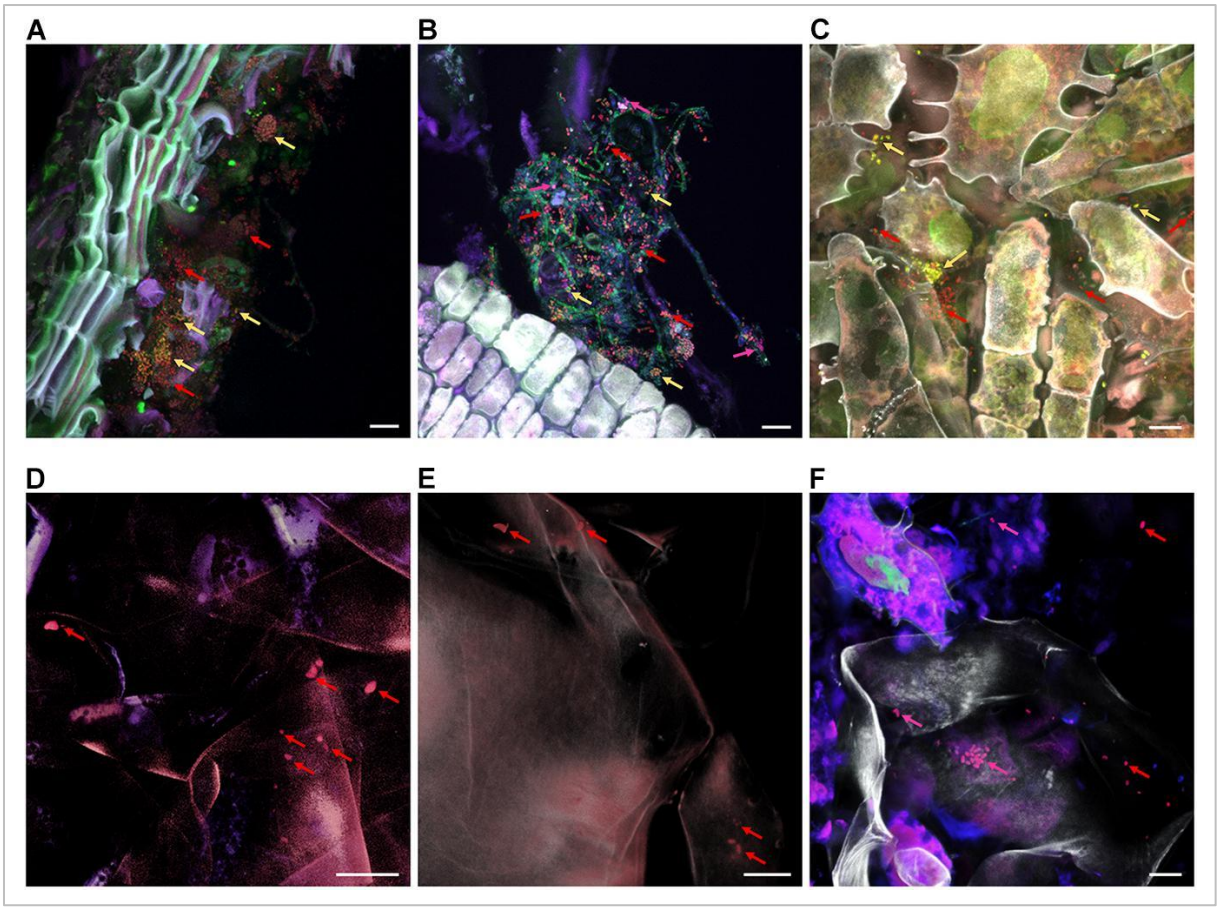

Figure 4.7: FISH-CLSM micrographs showing bacterial colonization of organic apple tissues. Panels (A), (B), (C), (D), (E) and (F) visualize stem, stem end, peel, fruit pulp, seeds and calyx end samples, respectively. Bacteria were stained with FISH probes specific for *Gammaproteobacteria* (fluorescing pink and indicated by pink arrows), *Firmicutes* (yellow) and remaining bacteria of other classes (red); host structures are fluorescing white. Bar on the bottom right of each panel denotes for 10µm.

Discussion

In the present study we identified tissue-specific and management-specific microbial communities for apple fruits. This specificity was apparent for all tissues regarding both microbiota composition and diversity, but not abundance. The observed differences between organic and conventional apples could certainly be attributable to a variety of factors within farming and storage conditions.

Deciphering the bacterial microbiota of Austrian 'Arlet' apples resulted in a drastic diversification between the six tissues within the apple carposphere for bacterial abundance, diversity and composition. Interestingly, alpha diversity estimates and calculations of bacterial abundance (according to qPCR) were pretty much inconsistent. Whereas fruit pulp and peel featured highest values for diversity, microbiota abundance was lowest in those tissues. Seeds, on the other hand, were less divers than other tissues, but showed highest abundance. Those results were partially confirmed by FISH-CLSM; high microbial abundance was visualized on stem, stem end and calyx end samples, whereas peel and fruit pulp turned out to be less colonized. However, for seeds it was not possible to visualize the high bacterial abundances indicated by qPCR which was due to exceptionally high autofluorescence in seed tissues. Differences between the tissue-associated microbiota were expected beforehand, as varying metabolic and nutrient conditions in the specific parts are certain. The sole responsibility of all the different parts of a fruit is to protect the seeds and enable their dispersal for a successful reproduction of the plant. Apple seed microbiota showed interesting features: among all tissues, seeds, together with stem, were found to significantly prevail in bacterial quantity, hosting an average of 126 billion bacterial gene copy numbers per gram seeds. Seed microbiota composition was most similar to fruit pulp microbiota which underline the vertical microbiome transmission in plants (Hardoim et al. 2012).

The management practice was found to significantly drive the microbiota of all tissues within the apple. Diversity was significantly higher in all organically grown tissues (except for calyx end) and the microbiota composition was distinct between organic and conventional tissue analogs. Compared to the other tissues, seed microbiota was lowest affected by the management practice, while the exclusion of low abundant taxa from the dataset resulted in dramatic dissimilarities between organic and conventional seeds. Organic seeds showed a much more even composition than conventional seeds which were highly dominated by *Ralstonia*. Altogether, organic apple microbiota was significantly more divers and differentially composed; the remarkable amount of 39% of genera and 38% of bacterial orders was

significantly different abundant. Referring to a previous work on the apple flower microbiome, *Deinococcus-Thermus* and *Saccharibacteria* (formally known as TM7) dominated the community (Shade et al. 2013). In the present study, both taxa were present in almost all replicates of organic apples (0.6% and 0.08%, respectively), in contrast to conventional ones (0.01% *Deinococcus-Thermus* and 0.007% *Saccharibacteria*). This promotes exceptional specificity and functionality of the microbiota for successive development stages from the flower to the mellow fruit and potentially suggests organic management to rather allow the formation of a stable and beneficial community. Conventional apple microbiota was furthermore found to be less even constructed and highly dominated by *Burkholderiales*, accounting to almost 43% abundance. The order *Enterobacteriales* was one of the signature taxa of conventional apples as well; among them, we would like to highlight the almost ubiquitous occurrence of OTUs assigned to *Escherichia-Shigella* in the tissues of conventional apples (although low abundant) and their absence in organically managed apples. Higher abundances of *Enterobacteriales* in conventional fresh produces compared to organic equivalents have already been reported by Leff and Fierer (Leff and Fierer 2013). Controversially, *Lactobacillus*, which is frequently used within probiotics (Derrien and van Hylckama Vlieg 2015), was one of the core taxa of organic apples. The highly diverse microbiome of organically managed apples might probably limit or hamper the abundance of human pathogens, simply by outcompeting them; negative correlations between human pathogen abundance and the natural microbiome of fresh produce has already been described (Cooley et al. 2006). The described microbial patterns in organic apples resemble the impact of apple polyphenols on human health, which have not only been shown to alleviate allergic symptoms (Zuercher et al. 2010), but also to promote growth of *Lactobacillus* and *Bifidobacterium* in the human gut and to reduce abundance of food-borne pathogens (Bialonska et al. 2010; Taguri et al. 2004). Considering that specific microbial signatures have potential to reduce food allergies (Kalliomäki et al. 2010), the native microbiome of organic and unprocessed apples could be an advantageous tool to manage and prevent allergic diseases. *Methylobacterium*, identified to enhance the biosynthesis of strawberry flavor compounds (Verginer et al. 2010), was significantly higher abundant in organic apples; here especially on peel and fruit pulp samples. In contrast, *Ralstonia* and *Erwinia*, frequently described for adverse impact on plant health (Pirhonen et al. 2018; Denny 2007), prevailed in conventional apples. Our results are in significant accordance to a recent study on the apple fruit-associated fungal community (Abdelfattah et al. 2016), where the authors observed specificity of the fungal microbiota to different tissues and management practices. Concordantly, the management

practice is suggested to be accountable for the different bacterial and fungal community composition. The lowest effect was observed on seed microbiota, which is mainly cultivar-driven (Berg and Raaijmakers 2018).

Calculations of 16S rRNA gene abundance resulted in significant differences between tissues but not for the management. This suggests bacteria to occupy the tissues of organically and conventionally produced apples in a similar quantity, while the management practice drives composition and diversity. For the quantitative analyses we used PNAs to block amplification of 16S rRNA of host origin; nevertheless, there is still a possibility that non-bacterial 16S rRNA genes are amplified. Furthermore, qPCR results do not exclusively represent the viable bacterial community. However, comparing gene abundances between tissues and management groups is possible and reliable in this regard.

Conclusion

Investigating the apple fruit microbiota resulted in profound differences between the tissues, applicable for microbiota diversity, composition and abundance. A significant management effect on the microbiota was furthermore apparent for all tissues, even for seeds. Organic and conventional apples are occupied by a similar quantity of microbiota; consuming the whole apple includes an approximate uptake of 100 million bacterial gene copy numbers. However, freshly harvested, organically managed apples harbor a significantly more diverse, more even and distinct microbiota, compared to conventional ones; the abundance of almost 40% of bacterial genera and orders differed significantly between organically and conventionally managed apples. Moreover, organic apples conceivably feature favorable health effects for the consumer, the host plant and the environment in contrast to conventional apples, which were found to harbor potential food-borne pathogens.

Supplementary Material

Supplementary Table 4.1: Bacterial taxa on genus and order level with significantly different abundance between organically and conventionally managed apples.

Organic	Conventional	Bacterial genera
6.76	3.25	*Methylobacterium*
5.97	0.21	*Hymenobacter*
1.04	0.01	*Spirosoma*
0.99	0.24	*Zymomonas*
0.77	0.06	*Bdellovibrio*
0.57	0.02	*Kineococcus*
0.48	0.00	*Deinococcus*
0.48	0.01	*Armatimonadetes sp.*
0.44	0.08	*Amnibacterium*
0.26	0.04	*Paenibacillus*
0.25	0.04	*Sorangium*
0.16	0.00	*Rickettsiaceae sp.*
0.11	0.00	*Oligoflexales sp.*
0.10	0.01	*Modestobacter*
0.10	0.01	*Cohnella*
0.10	0.00	*Chitinophaga*
0.06	0.00	*Rickettsiales sp.*
0.06	0.00	*Nakamurella*
0.05	0.00	*Kineosporia*
0.04	0.04	*Variibacter*
0.02	0.00	*Anaerococcus*
0.02	0.00	*Rhodanobacter*
1.55	8.26	*Burkholderia*
3.79	3.96	*Pantoea*
0.47	1.26	*Erwinia*
0.26	1.24	*Acinetobacter*
0.01	0.47	*Rhizobiales sp.*
0.00	0.18	*Brevundimonas*
0.10	0.16	*Chryseobacterium*
0.06	0.16	*Reyranella*
0.00	0.12	*Holophagaceae sp.*
0.06	0.11	*Obscuribacterales sp.*
0.04	0.11	*Caulobacter*
0.04	0.10	*Moraxella*
0.00	0.10	*Arcicella*
0.01	0.07	*Rhizobacter*
0.00	0.07	*Dyella*
0.04	0.06	*Micrococcus*
0.01	0.06	*Rhodospirillales sp.*
0.00	0.05	*Deltaproteobacteria sp.*
0.00	0.04	*Rhodobacteraceae sp.*
0.00	0.04	*Mesorhizobium*
0.03	0.04	*Schlegelella*
0.01	0.04	*Cupriavidus*
0.01	0.04	*Alcanivorax*
0.01	0.04	*Xenophilus*
0.00	0.04	*Pseudoxanthomonas*
0.00	0.04	*Cyanobacteria sp.*
0.00	0.04	*Rheinheimera*
0.00	0.04	*Dermacoccus*
0.01	0.03	*Achromobacter*
0.00	0.03	*Burkholderiales sp.*
0.01	0.03	*Rhodospirillaceae sp.*

Organic	Conventional	Bacterial orders
7.11	0.45	*Cytophagales*
0.83	0.08	*Bdellovibrionales*
0.62	0.02	*Kineosporiales*
0.51	0.00	*Deinococcales*
0.48	0.01	*Armatimonadetes p.*
0.31	0.03	*Frankiales*
0.25	0.02	*Nitrosomonadales*
0.12	0.10	*Clostridiales*
16.71	42.67	*Burkholderiales*
5.61	13.27	*Pseudomonadales*
5.19	5.41	*Enterobacteriales*
0.56	1.09	*Flavobacteriales*
0.19	0.19	*Rhodobacterales*
0.00	0.12	*Holophagales*
0.06	0.11	*Obscuribacterales*
0.04	0.08	*Rhodocyclales*
0.01	0.05	*Deltaproteobacteria p.*
0.02	0.04	*Oceanospirillales*
0.00	0.04	*Cyanobacteria p.*
0	0.04	*Chromatiales*
0.00	0.03	*Chlamydiales*
0.00	0.03	*Pseudonocardiales*
0.00	0.03	*Thiotrichales*
0.00	0.02	*Anaerolineales*

0.01	0.03	*Enterobacteriaceae sp.*
0.00	0.03	*Mycobacterium*
0.00	0.03	*Epilithonimonas*
0.00	0.03	*Sandaracinobacter*
0.02	0.02	*Rhodocyclaceae sp.*
0.00	0.02	*Thiotrichaceae sp.*
0.00	0.02	*Escherichia-Shigella*
0.00	0.02	*Polynucleobacter*
0.00	0.02	*Sphingomonadaceae sp.*
0.00	0.02	*Terrimonas*
0.00	0.02	*Anaerolineaceae sp.*
0.01	0.02	*Undibacterium*
0.00	0.02	*Rhodococcus*

Relative abundance (%) that was significantly higher in the respective management group, is highlighted in bold. Significances were calculated on taxa occurring with 0.01% abundance within the whole dataset by applying non-parametric Kruskal-Wallis/FDR-P (alpha=0.05).

CHAPTER 5

Manuscript V: The Apple Resistome: Deciphering the Impact of the Global Supply Chain

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In preparation.

Abstract

The expanding antibiotic resistance crisis calls for a more in-depth understanding of the importance of antimicrobial resistance genes (ARGs) in food. We, therefore, studied the apple resistome via metagenomic sequencing from two different apple cultivars (Royal Gala and Braeburn), selecting apples freshly harvested in South African orchards and apples stored and transported to Austrian supermarkets. A pool of 132 resistance determinants was identified, targeting 19 different antibiotic classes including natural as well as synthetic compounds. Multidrug resistances prevailed in all apples, deriving mainly from highly abundant efflux pumps (70-75%), while the overall set of ARGs was underlying all major resistance mechanisms. Among target-specific ARGs, resistances against polymyxin, quinolones and mupirocin appeared as predominant. The apple core resistome comprises 24 highly abundant ARGs with a target spectrum of eight antibiotic classes. Furthermore, we found ARG diversity to be significantly higher in stored/transported apples of both cultivars, which were represented by increased abundances of ARGs associated with quinolines, rifampicin, fosfomycin and aminoglycosides. Our results shed light onto the antibiotic resistance background of edible plants and the potential impact of the global food chain on that.

Introduction

The extensive use of antibiotics over the past four decades has served as a driving force to disseminate antibiotic resistances world-wide. WHO lists antibiotic resistance among the major health risks of our time and the ongoing trends may already predict a post-antibiotic era (WHO 2014; Kåhrström 2013). The development of microbial antibiotic resistance is based on either de-novo mutation or the acquisition of mobile genes from the versatile pool in the environment, which comprises both naturally evolved antibiotic resistance genes (ARGs) as well as ARGs introduced by anthropogenic practices (Wellington et al. 2013). Microbial communities are deeply embedded within their host; nevertheless, they represent open and interlinked ecosystems that coevolve, communicate and cross-feed (Berg 2015; Layeghifard et al. 2017). Accordingly, human, animal and environmental habitats are strongly interconnected and the effects of applied antibiotics to any of these habitats can extend beyond the site of use (Hernando-Amado et al. 2019). In this regard, especially large-scale agricultural resistomes are of particular interest due to extensive human intervention, posing high-risk habitats for antibiotic resistance selection and dissemination (Blau et al. 2018). Resistomes of fresh produce are, however, still largely overlooked even though their microbiomes represent an important environment-human interface and can serve as a gateway for antibiotic resistant bacteria to humans (Chen et al. 2019; Blau et al. 2018; Cernava et al. 2019). Detailed understanding of the spread of resistances within and between microbial communities across ecological boundaries is therefore crucially important (Tripathi and Cytryn 2017).

Antibiotic resistance, however, not only transcends ecological, but also geographical borders, calling for both *one health* and *global health* actions (Okeke and Edelman 2001; Hernando-Amado et al. 2019). Here, the global food trade might be a crucial era to control. Our contemporary food chain provides consumers globally with the availability of extensive choice and all-season fresh produce. Not just recently, concerns are rising about ecological and environmental burdens associated with that, including extensive energy demands for the large-scale industrialized production, packaging and preservation requirements as well as the transport-related fossil-fuel energy consumption and carbon dioxide emissions (Pretty 1999; Carlsson-Kanyama 1998; Jones 2002). Within a case study on dessert apples, Jones and colleagues (Jones 2002) assessed the environmental impact of transport components of different food supply chains and concluded that transportation is responsible for a significant percentage of the total energy consumption in the life cycle of an apple. However, the contribution of the

global supply chain to exacerbate the world-wide dissemination of antibiotic resistance via fresh produce-associated microorganisms was proposed only recently (Chen et al. 2019).

Apples are among the most consumed fruits world-wide and represent a valuable commodity with over 83 million tons being produced each year (FAO 2019). While recent studies shed light on the native apple microbiota and its response to management and postharvest practices (Leff and Fierer 2013; Abdelfattah et al. 2016; Wassermann et al. 2019; Wassermann et al. 2019; Angeli et al. 2019), the apple resistome has not been investigated so far. Moreover, the selection and emergence of ARGs and antibiotic resistant bacteria (ARBs) during the postharvest period is still rather unknown. In frame of an international program to investigate fruit and food microbiome for food safety and security, we studied the microbiome and resistome of apple fruits fresh from the tree and compared them to apples at the end of the global supply chain, *i.e.* a supermarket located about 9.000 km away (linear distance: Cape town, South Africa – Graz, Austria). Since the resistome reflects a response of the microbial community to changing environmental conditions as well as the host´s metabolism (Wright 2007), we assume an impact of the factors associated with the global supply chain on the resistome composition of apple fruits and aim to assess whether those changes may contribute to the emergence of a global resistance.

Materials and Methods

Sampling procedure and experimental design

In order to investigate and compare the microbial composition and ARG profiles of apples (*Malus pumila* Mill.) at their point of harvest (unprocessed and freshly harvested from trees) and their point of consumption (processed, stored and transported to Austrian Supermarkets), two apple cultivars, Braeburn and Royal Gala, were selected. For the purpose of clarity, samples are hereinafter referred to as 'Braeburn fresh' and 'Royal Gala fresh', for apples harvested fresh from the trees, and 'Braeburn stored' and 'Royal Gala stored' for apples sampled at the Austrian retailer. All apples investigated were cultivated in South African orchards under conventional and certified conditions. 'Braeburn fresh' and 'Royal Gala fresh' were sampled during harvest time in February 2019 in South African orchards (Braeburn: 33°11'16.1"S 19°15'45.0"E; Royal Gala: 33°11'23.5"S 19°15'12.1"E), using sterile gloves and instruments, and kept on ice until further processing. 'Braeburn stored' and 'Royal Gala stored' were purchased in an Austrian supermarket (N47° 4' 2.891" E15° 26' 33.432") 30 days after South African harvest time. This reflects the usual transport time starting at the day of harvest and includes processing, transport via ship to Northern European harbors and further truck transport to Austrian supermarkets; here, selected apples were presented open-layered in trays. Apples were transported to the laboratory immediately after purchase and kept on ice until upcoming DNA isolation. All apples were visually evaluated for consistency in size, shape and flawlessness prior to processing.

Microbial DNA extraction for shotgun metagenomics sequencing

For each sample category ('Braeburn fresh', 'Royal Gala fresh', 'Braeburn stored', 'Royal Gala stored') three whole apples were separately subjected to total microbial DNA extraction. DNA extracts of the three replicates were than pooled in order to reach sufficient amounts of DNA for subsequent metagenomics shotgun sequencing. In order to specifically enrich the microbial cell fraction, the density gradient centrifugation method, based on the methods developed by Ikeda et al. 2009 and Tsurumaru at al. 2015 (Ikeda et al. 2009; Tsurumaru et al. 2015), was applied. In short, one whole apple was cut in pieces; per 100 mg apple, 500 ml of BCE buffer was added and homogenized with a blender. The mixture was filtered through a layer of sterile Mesoft® filters and the filtrate was divided into ten 50 ml tubes. The filtrates were centrifuged (5 min, 10°C, 500 x *g*) and the resulting supernatants were transferred to clean tubes. After an additional centrifugation step (20 min, 10°C, 5,500 x *g*) supernatants were discarded and pellets

was resuspended in 50 ml BCE buffer. Suspensions were filtered again through layers of Mesoft® filters and centrifuged (10 min, 10°C, 10,000 x *g*); the resulting pellet was resuspended in 50 ml BCE buffer. Filtration and centrifugation steps were repeated twice. The final filtrates from ten tubes per apple were suspended in 0.5 ml 50 mM Tris HCl (pH 7.5) and pooled. The resulting suspension was pipetted below 4 ml Histodenz™ (Merck, Vienna, Austria) solution (8 g Histodenz dissolved in 10 ml of 50 mM Tris HCl pH 7.5; utilized as alternative to Nycodenz®), and centrifuged (40 min, 10°C, 10,000 x *g*). The bacterial cell fraction, visible as whitish band at the interface of upper and lower phase, was collected and DNA was extracted using FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, United States) and a FastPrep Instrument (MP Biomedicals, Illkirch, France) for 30 s at 5.0 m s^{-1}. The three replicates per sample category were combined into one tube, DNA concentrations were measured with Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Wilmington, DE, United States) and the whole DNA extract was sent for whole metagenomics shotgun sequencing at Vienna BioCenter (Vienna, Austria) using NovaSeq 6000 instrument.

Shotgun metagenomics bioinformatics

Sequencing adaptors and low-quality sequences were removed using Trimmomatic (Bolger, Lohse, and Usadel 2014). In order to reduce host-derived sequences in the dataset, forwards and reverse reads were aligned against the reference genome of *Malus domestica*, available at NCBI database (GCF_002114115.1_ASM211411v1_genomic), using Bowtie2 v2.4.1 (Langmead and Salzberg 2012) in very-fast-local alignment mode and SAMtools (Li et al. 2009), and aligned reads were discarded. Kaiju (Menzel, Ng, and Krogh 2016) was used for taxonomic classification of sequencing reads at the protein level, by translating reads into amino acids. All resistome analyses were conducted focusing on assembly-based data by using contigs and bins. Paired-end reads were subjected to *de novo* assembly into contigs using MEGAHIT (D. Li et al. 2016). Contigs with less than 500 nucleotides in length were discarded for further resistome analyses. Reads were mapped back to assemblies using Bowtie2 v2.4.1 prior to resistance gene annotation with DIAMOND BLASTX (v0.9.29.130) against deepARG (Arango-Argoty et al. 2018) database. A cutoff of 80% similarity to the reference genes and an e-value of 10^{-11} was set for antibiotic resistant genes to be retained in the dataset. To overcome false positive results due to sequencing depth, ARG counts were normalized to the lowest number of reads present in one sample after removal of host reads. PlasFlow (Krawczyk et al. 2018) was used to check whether resistance genes with at least 1,000 bp length are located on

either chromosomes or plasmids and RAWGraphs (Mauri et al. 2017) was used to visualize abundance and distribution of plasmid-encoded genes in apple resistomes. Networks of resistance genes were conducted in Cytoscape version 3.5 (Shannon et al., 2003) and a dendrogram, based on Euclidean distance and average clustering method, was produced in R version 4.0.2 to visualize hierarchical relationship between the resistomes of the four apple samples. CIRCOS Table Viewer v0.63-9 (Krzywinski et al. 2009) was used for circular representation of ARG relative abundance within the apple samples.

Contigs were further binned into draft genomes using MaxBin 2.0 (Wu et al. 2015) and binning quality was validated with CheckM (Parks et al. 2015). Draft genomes with more than 70% completeness and less than 25% contamination were considered for downstream analysis. Contigs of each genomes bin were re-annotated using AmphoraNet (Kerepesi, Bánky, and Grolmusz 2014) and resistance gene annotation was conducted using DeepARG database with the same parameters as described above for contigs-based analysis. Abundance of bins within the respective metagenome was calculated based on the proportion of bin copies in the host sequence-filtered reads and a phylogenetic tree based on average nucleotide identity (ANI) was generated using ANI online tool (www.ezbiocloud.net/tools/ani)

Results

Bacterial community profiles of fresh and stored apples

After the removal of host sequences, the four metagenomes sequenced ('Braeburn fresh', 'Braeburn stored', 'Royal Gala fresh', 'Royal Gala stored') produced between 15 and 21 million quality trimmed reads each. Taxonomic classification of host cell-depleted and host sequence-filtered metagenomes was performed using KAIJU. The domain Bacteria was predominant in all apple metagenomes sequenced; in total, 85-87% were affiliated to Bacteria, 11-14% were assigned to Eukaryota, while Archaea and Viruses each covered 0.2-0.3% of metagenomic reads. The bacterial community profile of all apple samples was highly dominated by *Proteobacteria*, covering 57-61% of all bacterial reads assigned, followed by *Actinobacteria* (9-15%), *Bacteroidetes* (10-13%) and *Firmicutes* (8-13%). *Gammaproteobacteria* was the dominating bacterial class in all apples (31-47%), followed by *Alphaproteobacteria* (10-22%), *Actinobacteria* (9-14%), *Bacteroidetes* (10-13%), *Bacilli* (7-11%) and *Betaproteobacteria* (1-2%). No impact of either the apple cultivar or the point of sampling (fresh from the tree or after storage and transport) was observed for bacterial phyla, nor for classes, except for *Gammaproteobacteria*. The latter were found to be reduced during transport and storage in both apple cultivars. Thus, the gammaproteobacterial community was investigated in more detail and is in visualized in Figure 5.1. For both apple cultivars a decrease of *Pseudomonadales* and *Xanthomonadales* and an increase of *Enterobacteriales* after storage and transport was clearly apparent. *Pseudomonas* represents the dominating genus in 'Braeburn fresh' and 'Royal Gala fresh', covering 15% and 32% of all bacterial reads, respectively. 'Braeburn stored' and 'Royal Gala stored' apples showed a clear decrease in relative *Pseudomonas* abundance (3% and 4%, respectively); the same was true for reads assigned to *Stenotrophomonas.* In stored and transported apples, the *Enterobacteriales* genera *Rahnella* (18% of all bacterial reads in 'Braeburn stored') and *Pantoea* (12% in 'Royal Gala stored') prevailed. Apart from the gammaproteobacterial community, an increase of *Methylobacterium* and a decrease of *Microbacterium* was observed for stored and transported apples from both cultivars. Bacterial core genera, present in all apple metagenomes, with a minimum of 1% abundance in at least one of the samples are listed in Table 5.1.

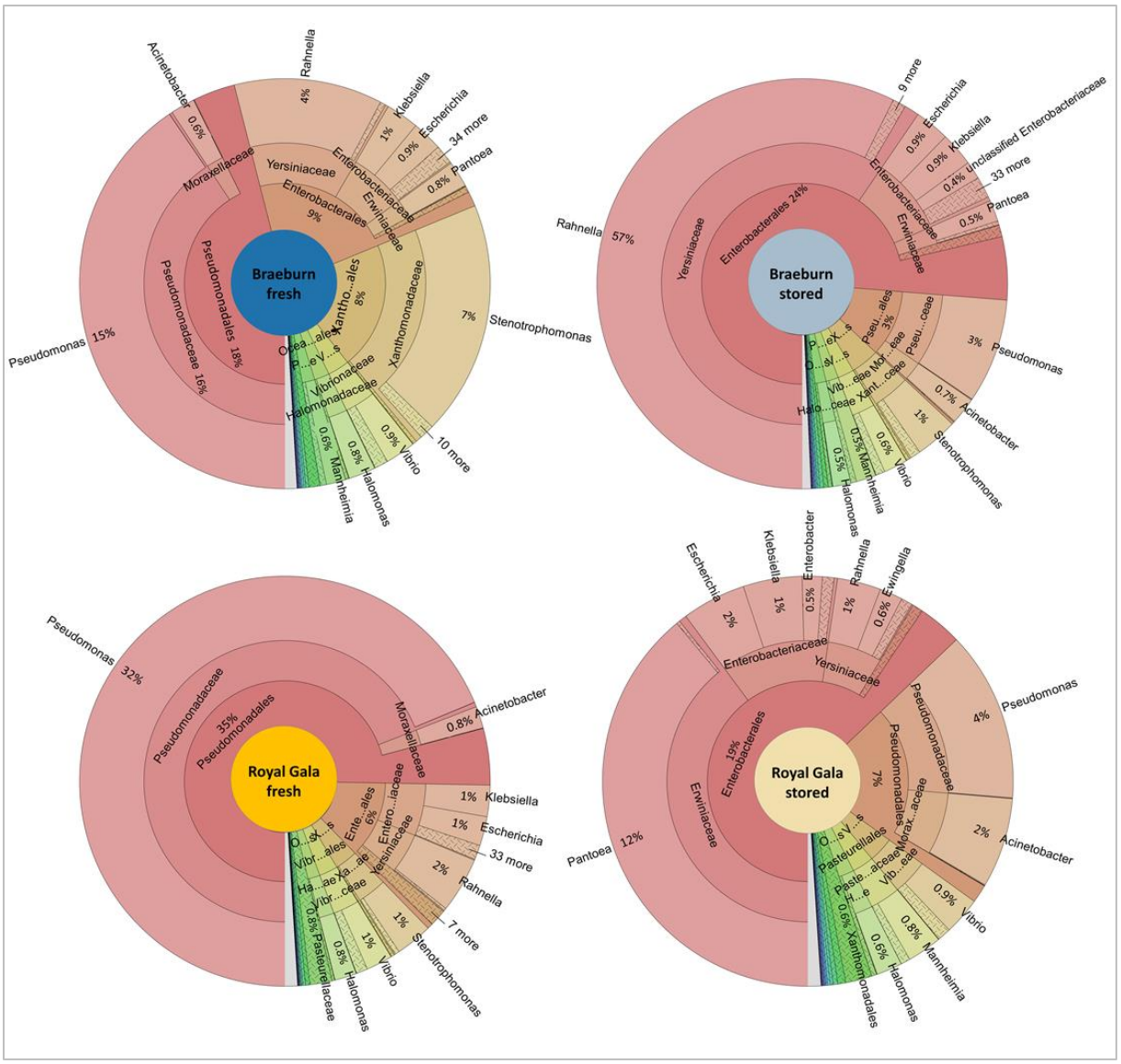

Figure 5.1: Gammaproteobacterial community profile of apple samples. Multi-level krona plots depict relative abundances of bacterial genera from the class *Gammaproteobacteria* in fresh and stored apples from the cultivars Braeburn and Royal Gala. Please note that percentage values indicate proportion of the respective genus to the whole bacterial community. Bacterial taxonomy was annotated using Kaiju.

Table 5.1: Relative abundance of the 18 most abundant bacterial core genera.

	Braeburn		Royal Gala	
	fresh (%)	stored (%)	fresh (%)	stored (%)
Pseudomonas	15	3	32	4
Rahnella	4	18	2	1
Acetobacter	4	3	3	6
Frankia	4	2	4	4
Solibacillus	3	3	3	5
Pantoea	0.8	0.5	0.4	12
Staphylococcus	3	2	3	3
Stenotrophomonas	7	1	1	0.6
Sphingomonas	3	4	0.5	0.5
Bradyrhizobium	3	0.9	0.8	2
Microbacterium	2	0.5	2	0.4
Paenanthrobacter	2	0.9	1	1
Escherichia	0.9	0.9	1	2
Methylobacterium	0.4	3	0.07	0.8
Acinetobacter	0.7	0.7	0.8	2
Ochrobactrum	1	0.08	0.9	2
Klebsiella	1	0.9	1	1
Bacillus	0.6	0.6	0.7	1

Resistome profiles of fresh and stored apples

The apple resistome was analyzed in terms of total ARGs detected, their relative distribution within samples, the drug classes to which they encode resistance, and the underlying resistance mechanisms; Figure 5.2 gives an overview of resistance gene distribution within tested apples. In total, 132 different ARGs were assigned according to the DeepARG database, which code for resistance against 19 different antibiotic classes. Efflux pumps highly prevailed among resistance mechanisms, while target alteration, target protection, antibiotic inactivation and target replacement, in ascending order, were detected in all samples as well. ARGs associated to efflux pumps and target protection were slightly higher in both stored apple samples compared to fresh ones (Figure 5.2, A). The apple core resistome, consisting of ARGs shared by all apples is displayed in Figure 5.2, B at pie charts, representing fractions detected within each apple metagenome. It comprises all highly abundant ARGs detected in the dataset; in total 25 ARGs contributed to the core resistome with a target spectrum of eight different antibiotic classes. Target-unspecific ARGs are summarized as multidrug, referring to the DeepARG assignment. For the majority (16 out of 25) of ARGs constituting the core resistome, abundance correlated with the state of apple freshness, regardless of the cultivar.

ARG diversity was significantly lower in both fresh apples compared to the stored samples (p=0.004; according to independent *t*-test). In total, 'Braeburn fresh' contained 42 different ARGs, four of which were unique, and 'Royal Gala fresh' contained 39 ARGs including one unique ARG. Increased numbers were found for apples stored and transported to Austrian supermarkets: 'Braeburn stored': 69 ARGs in total, including 39 unique; 'Royal Gala stored': 74 ARGs in total, including 24 unique. Interestingly, stored apples shared 17 ARGs, that were not present in fresh apples, hereinafter referred to as the 'storage-specific resistome' (framed red in Figure 5.2, B). The 'storage-specific resistome' consists of seven drug-specific ARGs and ten ARGs associated with multidrug resistance. In contrast, fresh apples shared only two low abundant ARGs (one multidrug, one beta-lactam resistant ARG), although both cultivars were sampled on the same day, from the same orchard, which was subjected to the same in-field management. Moreover, except for three low abundant ARGs for Breaburn and one for Royal Gala, no cultivar-specific ARGs were observed. Correspondingly, resistomes of the two stored apples as well as the two fresh apples were more similar to each other than samples within a cultivar, as shown in the dendrogram in Figure 5.2, C.

Table 5.2 lists the ARGs constituting the 'apple core resistome' and the 'storage-specific resistome' including resistance mechanism, target drug class, and the bacterial organisms the respective ARG has been assigned to by either Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al. 2013) or UniProt (Consortium 2019) database. ARGs are listed in descending order according to their total abundance in all samples. The ARGs *mfd*, and *oqxP*, conferring resistance towards quinolones were most abundant across all samples, representing 7.7% and 7.5% of all resistance hits in the contig dataset, respectively. Polymyxin resistance-conferring *arnA*, and mupirocin resistance-conferring *ileS1* were furthermore high abundant, representing 7.1% and 6.7% of resistance hits, respectively.

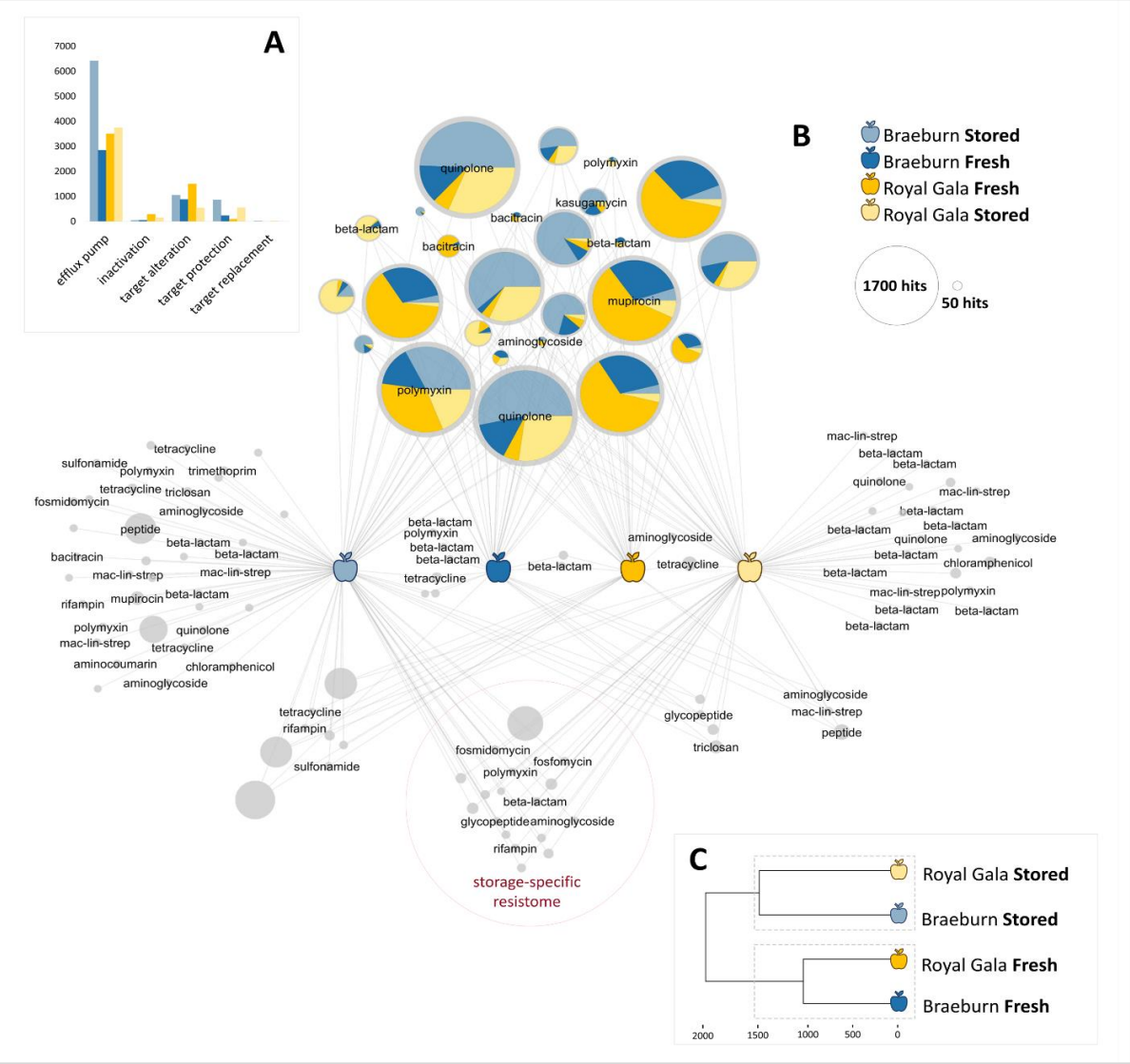

Figure 5.2: ARG profiles of fresh and stored apple samples. Results are based on DeepARG annotations of contigs for resistance genes, target drug classes and resistance mechanisms, including only ARGs with at least 80% similarity to reference ARGs and an e-value of 10^{-11}. All datasets were rarefied to the sample with the lowest read counts. Color code for apple metagenomes is depicted in the legend on the upper right: Braeburn: *blue;* Royal Gala: *yellow*, both fresh from the tree (*dark blue* and *dark yellow*, respectively) and stored/transported (*light blue* and *light yellow*, respectively). **A:** Distribution of resistance mechanisms based on absolute hits of annotated ARGs within the four apple samples. **B:** Network representation of core and specific ARGs in apple metagenomes. Nodes represent different ARGs detected and node labels point to the antibiotic target class, while unlabeled nodes indicate multidrug resistance of the respective ARG. Node size corresponds to absolute abundance of ARGs in the rarefied datasets as indicated in legend on the lower right. Pie charts of nodes shared by all samples, representing the 'apple core resistome', indicate fractions detected within each apple metagenome. ARGs

that were shared by stored apples but absent in fresh apples are highlighted as the 'storage-specific resistome'. **C**: Dendrogram visualizes connection between different apple samples based on their ARG composition. Calculations were executed in R using Euclidean distance with average clustering method.

Table 5.2: ARGs constituting the 'apple core resistome' and the 'storage-specific resistome'

	ARG	**resistance mechanism**	**target drug class**	**known bacterial organism**
apple core resistome	mfd	target protection	quinolone	*Escherichia coli*
	oqxB	efflux pump	quinolone	*Citrobacter freundii, E. coli*
	arnA	target alteration	polymyxin	*P. aeruginosa*
	ileS1	target alteration	mupirocin	*Pseudomonas fluorescens*
	mexK	efflux pump	multidrug	*P. aeruginosa, P. fluorescens*
	MexB	efflux pump	multidrug	*P. aeruginosa*
	MexF	efflux pump	multidrug	*P. aeruginosa*
	acrB	efflux pump	multidrug	*E. coli*
	msbA	efflux pump	multidrug	*E. coli*
	cpxA	efflux pump	multidrug	*E. coli*
	tolC	efflux pump	multidrug	*E. coli*
	CRP	efflux pump	multidrug	*E. coli*
	mdtb	efflux pump	multidrug	*E. coli*
	cpxr	efflux pump	multidrug	*E. coli*
	KsgA	target alteration	kasugamycin	*E. coli*
	emrB	efflux pump	multidrug	*E. coli*
	mrdA	efflux pump	beta lactam	*E. coli*
	uppP	inactivation	bacitracin	*E. coli*
	H-NS	efflux pump	multidrug	*E. coli*
	bepE	efflux pump	multidrug	*Brucella suis*
	ampC	inactivation	beta lactam	*P. aeruginosa*
	BacA	target alteration	bacitracin	*E. coli*
	rosB	efflux pump	polymyxin	*Yersinia sp.*
	ceoB	efflux pump	multidrug	*Burkholderia cenocepacia*
	acrD	efflux pump	aminoglycoside	*E. coli*
storage-specific resistome	mdfA	efflux pump	multidrug	*E. coli*
	mexW	efflux pump	multidrug	*P. aeruginosa*
	arpB	efflux pump	multidrug	*P. putida*
	MuxB	efflux pump	multidrug	*P. aeruginosa*
	mtrA	efflux pump	multidrug	*Mycobacterium tuberculosis*
	arpC	efflux pump	multidrug	*Dictyostelium discoideum*
	murA	target alteration	fosfomycin	*E. coli*
	RbpA	target alteration	rifampin	*Streptomyces coelicolor*
	mexi	efflux pump	multidrug	*P. aeruginosa*
	mepA	efflux pump	multidrug	*Staphylococcus aureus*
	RosA	efflux pump	fosmidomycin	*Yersinia sp.*
	ttgC	efflux pump	multidrug	*P. putida*
	APH(6)-Id	inactivation	aminoglycoside	*E. coli*
	arnC	target alteration	polymyxin	*E. coli*
	arnD	target alteration	glycopeptide	*E. coli*
	MexD	efflux pump	multidrug	*P. aeruginosa*
	pbp2	target replacement	beta lactam (penam)	*S. aureus*

Storage and transport correlate with ARG composition and diversity

Based on resistance gene annotations using DeepARG database, multidrug resistances of contigs within the rarefied dataset were found to be exclusively conferred by efflux pumps. For the purpose of a more in-depth description of target-specific ARGs, multidrug resistant ARGs were excluded from the analysis described in the following and visualized in Figure 5.3. Resistance against quinolones, polymyxin and mupirocin prevailed in all apples investigated. For both apple cultivars after storage and transport, increased counts for ARGs conferring resistance against quinolone, rifampicin, fosfomycin and aminoglycoside were observed; resistance towards the latter two antibiotics were unique for stored apples. Furthermore, ARGs conferring resistance against trimethoprim, tetracycline, fosmidomycin, chloramphenicol and the combined group of macrolide-lincosamide-streptogramin antibiotics were only detected in either 'Braeburn stored' or 'Royal Gala stored', being absent in fresh apples. However, abundances of resistance determinants acting on mupirocin and bacitracin were higher in both apple cultivars fresh from the tree. The observed differences were, however, not significant.

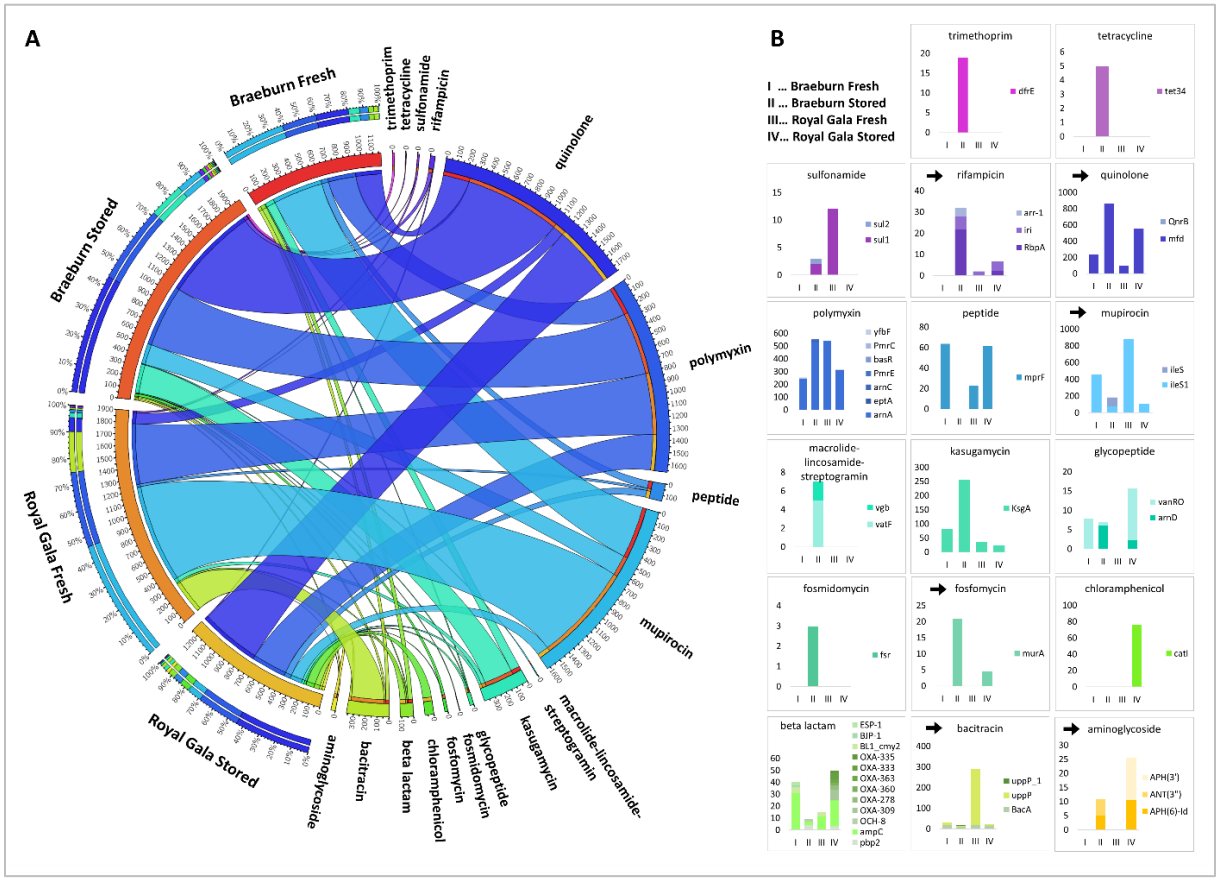

Figure 5.3: Distribution of drug-specific ARGs in fresh and stored apples. Only ARGs with at least 80% similarity and an e-value of 10^{-11} to reference ARGs are included and ARGs conferring via efflux pumps, were excluded. **A:** Circular representation of the proportional

abundance of drug classes conferred by target-specific ARGs (right part of the circle) detected in fresh and stored Braeburn and Royal Gala apples (left part of the circle). Thickness of ribbons refers to abundance of specific ARGs in the rarefied dataset. Visualization was generated using default settings of Circos software. **B:** More detailed classification of the data shown in **A**, where each drug class is visualized in a separate panel. Barcharts represent total abundance (y-axis, note the different scaling) of target-specific ARGs within the normalized dataset of each apple sample (I: Braeburn fresh, II: Braeburn stored, III: Royal Gala fresh, IV: Royal Gala stored). Stacked bars depict ARGs associated to the same antibiotic class within each sample; color-code for ARGs is shown on the right of each panel. *Black arrows* point to antibiotic classes to which resistance is either increased or decreased in both stored apples compared to their fresh equivalents.

The antibiotic resistome of fresh and stored apples revealed by reconstructed genomes and plasmids

Assembled contigs could be binned into 95 draft genomes ('Braeburn fresh': 18 bins, 'Braeburn stored': 34 bins, 'Royal Gala fresh': 15 bins, 'Royal Gala stored': 28 bins). From them, 19 draft genomes (representing 43.1% of all assembled contigs) were sufficient in quality and were further analyzed in terms of taxonomic annotation, abundance, nucleotide similarity and resistance gene profiles (Figure 5.4). All high-quality genomes were either assigned to *Gammaproteobacteria* (10 bins) or *Alphaproteobacteria* (9 bins). From the 'Braeburn fresh' und 'Royal Gala fresh' metagenomes, nine (*Rhizobiales, Rahnella, Pseudomonas, Xanthomonadaceae, Bradyrhizobiaceae, Microbacterium testaceum, Sphingomonadaceae, Pantoea vagans, Alphaproteobacteria*) and four (*Pseudomonas, Xanthomonadaceae, Brucellaceae, Rahnella*) genome bins were reconstructed, respectively. Three genomes were each reassembled from the two stored apple samples ('Braeburn stored': *Rahnella* sp. Y9602, *Myxococcales, Xanthomonadaceae*; 'Royal Gala stored': *Pantoea vagans, Ochrobactrum, Bradyrhizobiaceae*). Interestingly, except a *Microbacterium testacaeum* bin, to which no resistance profile could be assigned, only genomes of gram-negative bacteria were reconstructed from the metagenomes in a sufficient quality. Calculations of the percentage abundance of binned genomes within the respective metagenome revealed particularly high values for several of them; e.g. *Pseudomonas* with 17.9 % abundance in 'Royal Gala fresh', *Pseudomonas* with 8.6 % abundance in 'Braeburn fresh', *Pantoea vagans* in 'Royal Gala stored' (6.3 %), *Rahnella* sp. Y9602 in 'Braeburn stored' (15 %) and a closely related *Rahnella*

bin in ‘Braeburn fresh’ with 3.6 % abundance. All binned genomes identified are represented by cultivable bacteria and the here annotated ARGs have been previously identified for the respective bacterial genomes. ARGs which were found highest abundance in contigs (Table 5.2) were represented by binned genomes as well, with the exception of the efflux pump-related ARGs *tolC*, *mdfA*, *mdtK*, *YojI*, *MdtH* and *H-NS*.

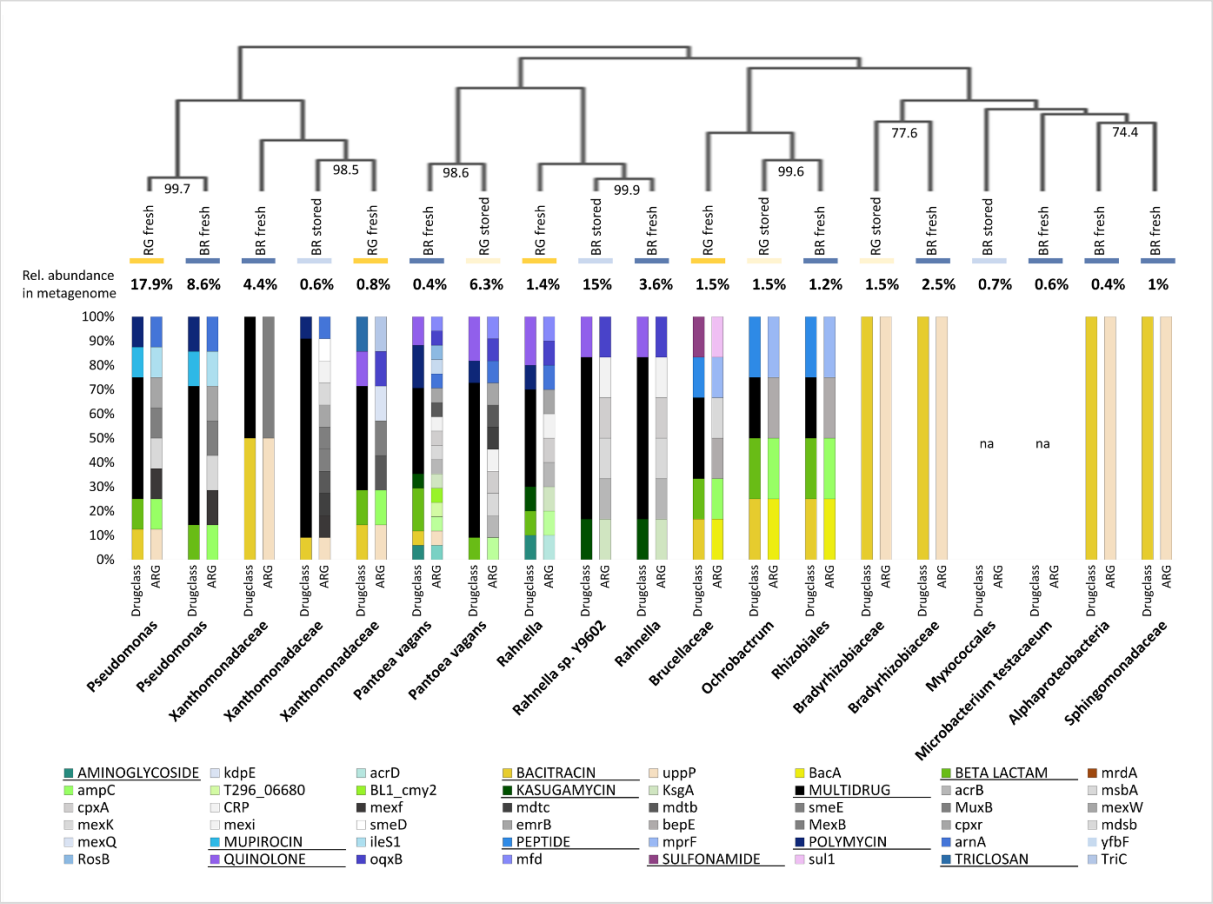

Figure 5.4: Resistome profiles of reconstructed genomes from fresh and stored apples samples. Binned genomes with sufficient quality are clustered by average nucleotide identity (ANI) and values for percentage identity are included for highly identical bins. Reconstructed genomes are resolved to highest taxonomic levels; their relative abundance within the respective metagenome was calculated and is indicated by percentage value on top of each bar. The left bar of each bin depicts the relative proportion of drug classes to which resistance is conferred by the respective bin and drug classes are capitalized and underlined in the legend. The right bar represents the ARGs conferring resistance towards the respective drug class. No antibiotic resistant profile was annotated to binned genomes assigned to *Myxococcales* (reconstructed from ‘Braeburn stored’ metagenome) and *M. testacaeum* (‘Braeburn fresh’), as indicated by ‘na’ (not assigned).

The fraction of horizontally transferable determinants within the apple resistome was evaluated using PlasFlow, predicting ARG location on either chromosomes or plasmids. In total 18 determinants were assigned to plasmids; three for 'Braeburn fresh' and five for each of the remaining apple samples (Figure 5.5). Plasmid-encoded ARGs confer resistance to nine different antibiotics while efflux-mediated multidrug resistance prevailed with six determinants. *TetC*, responsible for tetracyline resistance and the multidrug-resistant *bepE* were most abundant with 124 and 65 hits, respectively. Total abundance of plasmid-encoded genes in the rarefied contig dataset ranged from 25 hits for 'Braeburn stored' to 148 hits for the resistome of 'Royal Gala stored'. Thus, no consistency for either apple freshness or apple cultivar can be stated based on abundance or diversity of plasmid-encoded ARGs.

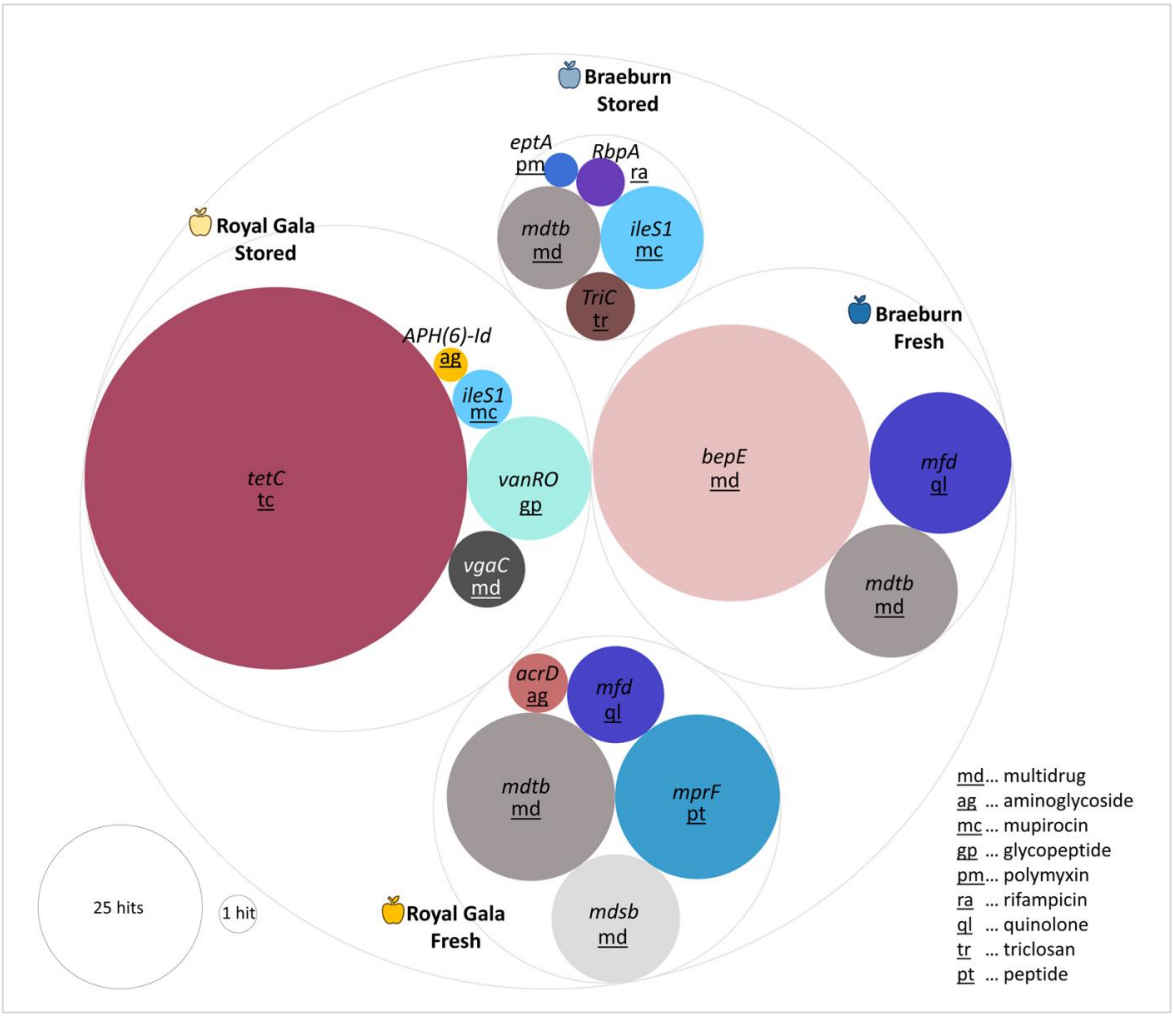

Figure 5.5: Apple-associated ARGs located on plasmids. Each bubble represents one determinant specifically assigned to be located on plasmids. Bubble size corresponds to total hits of the specific ARG in the rarefied datasets, as indicated in the legend on the lower left and ARGs are grouped by apple sample. Bubbles are labeled with gene names and target drug class (underlined; abbreviations are explained in the legend on the lower right).

Discussion

Within the present study we analyzed the resistome and microbiome composition of Braeburn and Royal Gala apples fresh from the trees and after intercontinental fruit trade via deep metagenomic shotgun sequencing. This way, we observed i) consistency for apple freshness but not for cultivars on taxonomic and resistome levels, ii) an apple core resistome, mainly composed of efflux-mediated multi drug resistance as well as resistance against quinolones, polymyxins and mupirocin, iii) a significant increase of ARG diversity, but not abundance, for both apple cultivars after storage/transport, iv) higher abundances of ARGs associated with quinolones, rifampicin, fosfomycin and aminoglycosides in stored/transported apples, opposed to v) fresh apple-dominating resistance to mupirocin and bacitracin.

We assessed abundance and composition of detected resistance genes, their location on mobile genetic elements, thus, their potential to be horizontally transferred to other bacteria, as well as their context in metagenome-assembled draft genomes. However, besides our effort for comprehensive data analysis, we must point out the limitations of the present study. These include a lack of replicates for the different apple metagenomes and the unavailability of metadata on origins and specific treatments of stored and transported apples, except for the information provided by the reseller. These factors limit the representativeness of our analysis and constrain our effort to compare the results. Thus, additional studies including detailed monitoring of potential contamination hot spots across the processing line are required to confirm the hypothesis formulated in the present study. Nevertheless, we provide novel insights into the potential of the world-wide food chain to affect the apple-associated resistome available to the consumer and attempt to address the influencing parameters.

These parameters can be numerous. Antibiotic treatment is prohibited in South African apple production, however, associated with the mobilome, resistance genes can be acquired, principally, from any source (Blau et al. 2018). Contamination can occur already in the field through irrigation water, organic fertilizers, wild animals, and soil; especially antibiotic usage in animal husbandry or waste water treatment plants is described to co-select for mobile genetic elements that carry multiple resistant genes (Berendonk et al. 2015). The harvest and postharvest period, however, represents a critical component as well, which is still less understood. The apple resistome might be shaped by postharvest conditions by three aspects. First of all, the aspect of handling. South African apple cultivation is largely based on manual labor, especially during harvest, and in Austrian supermarkets, apples were presented open-

layered, assuming further handling. Secondly, the aspect of storage time. During storage, host-associated bacteria may evolve towards antibiotic resistance as a natural response to changes of both the host´s physiology, *e.g.* ripening processes and altering metabolic conditions, as well as the metacommunity, including fungi. And thirdly, the aspect of postharvest treatments. Here, the main purpose is certainly the reduction of microbial loads and diversity (Kusstatscher et al. 2020); however, high diversity of the natural microbiome is strongly suggested to negatively correlate with pathogen and alien species abundance (Kennedy et al. 2002; Cooley et al. 2006; Blaser and Falkow 2009; van Elsas et al. 2012; Berg et al. 2014; Blaser 2016). In particular, this high degree of human intervention during harvest and postharvest periods may correlate with increased ARG diversity observed in stored apples; comparable results were recently documented for the built environment (Mahnert et al. 2019).

All apples were dominated by efflux pumps conferring multidrug resistance. Efflux pumps are understood to confer general resistance against a variety of toxic compounds in the environment and play a role in bacterial colonization and persistence ability within the host (Piddock 2006). As recently suggested, high proportion of efflux pumps might be a common characteristic for the diversified plant microbiota enabling successful co-existence within the host (Obermeier et al. 2019). High abundance of polymyxin resistance genes was furthermore in common for all apples investigated; six genome bins (assigned to *P. vagans*, *Rahnella*, *Xanthomonadaceae*, *Pseudomonas*) from fresh and stored apples carried respective ARGs and within stored Braeburn apples, a plasmid-borne polymyxin resistance gene was found. Polymyxin antibiotics are highly effective against many multidrug-resistant gram-negative bacteria and are currently used as last-resort treatment option (Olaitan et al. 2014). Resistances are increasingly documented for environmental, food, animal and human isolates (Baron et al. 2016), and here, even without previous drug exposure, calling for high alertness (Olaitan et al. 2016). However, the recently discovered, globally spreading, and thus highly concerning plasmid-mediated *mcr-1* polymyxin resistance gene (Liu et al. 2016) was not detected in apple resistomes. Although core resistance determinants and core bacteria were shared by all apples, their abundance was still consistent with the state of freshness, regardless of the cultivar. A clear shift within the gammaproteobacterial community, from *Pseudomonadales*-dominated fresh apples towards *Enterobacteriales*-dominated stored apples, was found. *Pseudomonas* was highest abundant in both fresh apples, while ‘Braeburn stored’ and ‘Royal Gala stored’ were dominated by *Rahnella* and *Pantoea*, respectively. All of these genera are widely distributed in natural habitats, including apples (Wassermann et al. 2019); several members show biological control potential (*e.g.* against *Erwinia amylovora*, the causative agent of apple´s ‘fire blight’

disease) (Stockwell et al. 2010) and are represented by emerging opportunistic pathogens, known to carry important resistance determinants as well (Gabriele Berg, Erlacher, et al. 2014; Pidot et al. 2014). Higher abundances of *Enterobacteriales* in stored apples might correlate with processing, handling and storage conditions, which has been previously observed (Wassermann et al. 2019; Abdelfattah et al. 2020). Bacteria associated with freshly harvested apples may feature functional capabilities to adapt to fluctuating conditions in the field, including UV radiation, temperature and humidity as well as nutrient availability; handling, processing and the constant conditions during storage may select for different genetic functionality of the microbial community, including potentially also a more diversified resistome.

Differences in abundance of the dominating taxa can be correlated with changes in the resistome composition, which was also revealed by genome bins reconstructed of the respective apples. Both stored apple cultivars are characterized by insignificantly higher abundances of resistance genes against quinolones, rifampicin, fosfomycin and aminoglycoside; ARGs against the latter two antibiotics were even found unique in apples after storage and transportation. In total, we found 17 different ARGs that were shared by both stored, but absent in both fresh apples, consituting the 'storage-specific resistome'. These ARGs are potenially aquired during the global transport. Additionally, genes conferring resistance to trimethoprim, tetracycline, fosmidomycin, chloramphenicol and macrolide-lincosamide-streptogramin antibiotics were not present in fresh apples, but still in one of the two stored cultivars. However, with a maximum of 76 hits per apple metagenome (chloramphenicol resistance genes in 'Royal Gala stored'), genes associated with these antibiotics were generally very low abundant compared to high abundant quinolone resistance genes (860 hits in 'Braeburn stored'). These were also detected in reconstructed genomes from fresh as well as stored apples (two *P. vagans*, three *Rahnella* and one *Xanthomondaceae* bin). Additionally, a plasmid-encoded quinolone resistance gene was assigned to fresh apples of both cultivars. Interestingly, most of the drug classes to which resistance increased after storage and transport represent (semi-)synthetics that are extensively used in clinical environments or life stock treatment including growth promotion (B. Li et al. 2015).

However, fresh from the tree apple cultivars, which were highly similar regarding their ARG composition, predominate in mupirocin and bacitracin resistance genes. Mupirocin is a natural antibiotic produced by *P. fluorescens* (Fuller et al. 1971), typically used to prevent colonization of methicillin-resistant *S. aureus* (MRSA), which are however, evolving increasing rates of resistance (Hosseini et al. 2017). While low-level mupirocin resistance

(chromosomally-encoded by *IleS*) has low clinical importance, high-level resistance of MRSA strains (plasmid-encoded mainly by *mupA*) has been reported critical (Gurney and Thomas 2011). *MupA* was not detected in the present study, while *IleS* was high abundant in fresh apples and revealed in assembled *Pseudomonas* bins. This suggests prevalence of mupirocin resistance to be associated with high abundant *Pseudomonas* strains carrying their native *IleS* gene. However, we detected a plasmid-encoded *IleS* gene in both of the stored apple metagenomes. This may raise concerns, since as plasmid-borne *IleS* is described to confer critical high-level resistance of MRSA strains (Hodgson et al. 1994; Gilbart et al. 1993). However, no taxonomic assignment was possible for the plasmid-encoded *IleS* detected in stored apples. High abundance of bacitracin resistance genes in fresh apples is most likely associated with assembled genomes assigned to *Rhizobiales.* Bacitracin is used as therapeutic agent against gram positives in human and veterinary medicine and as animal growth promoter (Manson et al. 2004). Consequentially, usage of bacitracin has impact on distinct environments (Matos et al. 2009). Across a large-scale metagenomic survey of environmental samples, bacitracin resistance was among the main mechanisms detected in river water and soil (B. Li et al. 2015). Irrigation systems using river water may represent a transmission route for bacitracin resistant bacteria to apple fruits, and *Rhizobiales* are general members of soil and rhizosphere communities; explanatory variables for higher abundance of bacitracin resistance in apples sampled directly from trees.

The composition of the apple carposphere resistome must, however, not necessarily raise concerns for several reasons. Antibiotics are natural products of bacterial secondary metabolism; equally, resistance to antibiotics is a natural and ancient microbial feature, and thus present even in pristine environments that pre-date the anthropogenic influence on resistance dissemination (Crofts et al. 2017; Walsh 2003). Two recent studies, on the *Sphagnum* moss resistome from an undomesticated bog ecosystem (Obermeier et al. 2019), and the resistome of *Eruca sativa* (Cernava et al. 2019), found more than 900 and more than 800 associated ARGs, respectively; sizeable, compared to 132 ARGs detected in the study at hand. Berendonk and colleagues suggested 16 different ARGs as indicators for the antibiotic resistance status of environments (Berendonk et al. 2015); among them, only two sulfonamide resistance genes (*sul1 and sul2,*) were detected with very low abundance in apples. And finally, the resistome genotype must be distinguished from the resistance phenotype, meaning that the presence of a specific ARG does not encode resistance, inevitably (Dantas and Sommer 2012).

Conclusion

In agreement with the recent suggestion of coordinated *global health* actions to combat world-wide transmission of ARBs and ARGs (Hernando-Amado et al. 2019), we promote the consideration of the global food chain as a potential vector for resistance dissemination. Certainly, political actions of developed nations are required; however, the single consumer may contribute by choosing fresh, seasonal and local produce, thereby reducing both ‘food miles’ and eventually also the burden of antibiotic resistance and its impact on human and planetary health.

CHAPTER 6

Publication 6: Microbiome Response to Hot Water Treatment and Potential Synergy with Biological Control on Stored Apples

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Abstract

Postharvest food decay is one major issue for today's food loss along the supply chain. Hot water treatment (HWT), a sustainable method to reduce pathogen-induced postharvest fruit decay, has been proven to be effective on a variety of crops. However, the microbiome response to HWT is still unknown, and the role of postharvest microbiota for fruit quality is largely unexplored. To study both, we applied a combined approach of metabarcoding analysis and real time qPCR for microbiome tracking. Overall, HWT was highly effective in reducing rot symptoms on apples at industrial scale. The efficiency was rather due to induced plant response than due to alterations of the microbiome; the fungal microbiota was only slightly, and the bacterial community insignificantly affected. Pathogen infection, however, significantly decreased the bacterial and fungal diversity, and especially rare taxa were almost eradicated in diseased apples. Here, almost 90% of the total fungal community was composed by co-occurring storage pathogens *Neofabraea alba* and *Penicillium expansum*. Additionally, the prokaryote to eukaryote ratio, almost balanced in apples before storage, was shifted to 0.6% bacteria and 99.4% fungi in diseased apples, albeit the total bacterial abundance was stable across all samples. Healthy stored apples shared 18 bacterial and four fungal taxa that were not found in diseased apples, therefore defining a health-related postharvest microbiome. In addition, applying a combined approach of HWT and a biological control consortium consisting of Pantoea vagans 14E4, Bacillus amyloliquefaciens 14C9 and Pseudomonas paralactis 6F3, were proven to be efficient in reducing both postharvest pathogens. Our results provide first insights into the microbiome response to HWT, and suggest a combined treatment with biological control agents.

Keywords*: Malus domestica*; apple; microbiota; amplicon; postharvest losses; biological control consortium; hot water treatment (HWT); *Neofabraea* sp.; bull´s eye rot; *Penicillium expansum*; blue mold

Introduction

Food loss is one of the major problems of modern society; about one third of all produced food is either lost or wasted globally (FAO 2015a). Especially the postharvest period plays a crucial role and has a lot of potential for improvements (Aulakh and Regmi 2013; Kader 2003). A high proportion of postharvest food loss is induced by microbial postharvest pathogens colonizing and damaging the fruits (Johnston et al. 2002; Morales et al. 2010). Until now, mainly chemical and physical treatments are used to suppress pathogens; microbiome research is expected to bring notable understanding and improvements into future biological applications and treatments (Droby and Wisniewski 2018; Janisiewicz and Korsten 2002).

Plants closely interact with their colonizing microorganisms which are crucial for plant health and growth (Berendsen et al. 2012; Berg 2009; Vandenkoornhuyse et al. 2015). Microorganisms not only protect the plant before harvest, even after harvest the shielding effect is prolonged (Droby et al. 2016). Studying plant-microbe interactions, beneficial bacteria and their functions were shown to be substantial for advanced biotechnological agriculture applications (Berg et al. 2017). However, the development of biocontrol application for postharvest use can be difficult due to the challenging in-use storage conditions (Castoria et al. 2001). Nevertheless, biocontrol products were intensively researched over the last decades as a biological alternative to classical synthetic pesticides not only for on-field, but also for postharvest applications. Additionally, health considerations and potential prohibition of currently used pesticides as well as trends towards a fully biological production increased the demand for highly efficient biological alternatives over the last years (Droby et al. 2009).

Apple, with worldwide over 83 million tons harvested each year and China, the US and Poland being the top producers is one of the major fruit crops worldwide (FAOSTAT 2017). Facing extensive storage times of several months, apple storage technologies are a major research topic around the globe. Qualitative and quantitative food loss along the supply chain and investigations of pathogens and mycotoxins just being a few examples (Johnston et al., 2002; Morales et al., 2002). *Penicillium expansum* Link, causing blue mold and the three *Neofabraea* species *N. alba* Jacks, *N. malicorticis* (Jacks) Nannfeld and *N. perennans* Kienholz, being the causal agents of bull´s eye rot, also referred to as gloeosporium rot (Snowdon 1990) or bitter rot (Corke 1956) are of particular interest. Apart from chemical treatments to control postharvest pathogens, hot water treatment (HWT) for 3 min at 50-53°C, a relatively simple method that is used since the 20th century, was shown to be rather effective in reducing

pathogen-induced postharvest losses (Fallik et al. 2001; Maxin et al. 2012); both bull´s eye rot and blue mold haven been proven to be successfully controlled by HWT (Maxin et al. 2005; Trierweiler et al. 2003). Rather than a direct killing of the fungus, the efficiency of HWT is based on a physiological plant response by inducing transcription and translation of heat shock proteins, where a subset of which comprise pathogenesis-related proteins (Fallik et al. 2001; Pavoncello et al. 2001; Maxin et al. 2014). Recently, combined approaches of HWT with bioactive molecules and biocontrol agents were proven to be efficient (Conway et al. 2004; Spadaro et al. 2004). Even though these developments show a bright future towards a pesticide-free postharvest storage, there are still a lot of missing links between postharvest diseases on apples, their colonizing microbiota and the impact of HWTs on the latter.

The present study provides the first investigation of the apple microbiome changes induced by the currently in-use HWT at an industrial scale. Stored apples that were not subjected to HWT remaining unaffected by fungal infestation were investigated, potentially contributing to postharvest pathogen resistance. Additionally, the indigenous apple microbiome was harnessed for biocontrol agents to combat postharvest pathogens *P. expansum* and *N. malicorticis*. Their additive protective effect as well as their applicability in the HWT process was evaluated, providing the first evaluation of a combined process with biological control consortia. This way, an integrative strategy combining the knowledge of the inherent apple microbiome and its postharvest changes with the development of a novel postharvest treatment was applied.

Material and Methods

Experimental design and sample processing

Organically produced apple fruits (*Malus domestica*) of the cultivar 'Topaz' were obtained from the organic storage company Rosenbaum Franz GmbH & Co KG (Pöllau, Austria). Apple samples were taken directly after harvest and after a six-months storage period. Freshly harvested apples were immediately taken to the laboratory and processed under sterile conditions (in the following named 'before storage'). For analyzing impact of HWT on the apple microbiota, 100 apples were stored untreated and 100 apples were subjected to HWT by immersing apples in a 53°C water bath for three minutes. Both groups were stored in the company´s storage chamber under controlled conditions for six months. Directly after opening storage chambers, fungal infection rate on apples was evaluated. HWT was found to be highly efficient as no disease patterns were observed. Among the 100 apples that were untreated and stored in a separate chamber 10% were infected, exhibiting disease patterns of 2.5 to 4 cm in diameter. A subset of each group, consisting of 10 randomly selected apples, was subjected to amplicon analyses; untreated apples were defined into 'untreated healthy' and 'untreated diseased'. The apples were transported to the laboratory and processed under sterile conditions. Apples of each category ('before storage', 'HWT', 'untreated healthy' and 'untreated diseased') were cut into smaller pieces and homogenized in a Stomacher laboratory blender (BagMixer, Interscience, St. Nom, France) with 40 ml sterile NaCl (0.85%) solution for three minutes. A total of four ml of the solution was centrifuged at 16.000 g for 20 min and the pellet stored at -70°C for further DNA extraction.

Microbial DNA extraction and metabarcoding library construction

The resulting pellets from the previous step were subjected to total microbial DNA extraction using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, USA) and a FastPrep Instrument (MP Biomedicals, Illkirch, France) for 30 seconds at 5.0 m/s. Amplicons were prepared in three technical replicates using the primer pair 515f - 926r, specific for bacteria and ITS1f - ITS2r specific for fungi. Sequences of primers are listed in Supplementary Table 6.1. Peptide nucleic acid (PNA) clamps were added to the PCR mix to block amplification of host plastid and mitochondrial 16S DNA (Lundberg et al. 2013). Amplification of the 16S rRNA gene was performed in a total volume of 20 µl (5 x Taq&Go (MP Biomedicals, Illkirch, France), 1.5 µM

PNA mix, 10 µM of each primer, PCR-grade water and 1 µl template DNA) under the following cycling conditions: 95°C for 5 min, 35 cycles of 78°C for 5 sec, 55°C for 45 sec, 72°C for 90 sec and a final elongation at 72°C for 5 min. PCR for amplifying the fungal ITS region was conducted in 20 µl (5 x Taq&Go, 10 µM of each primer, 25 µM $MgCl_2$, PCR-grade water and 2 µl template DNA) using the cycling conditions: 94°C for 5 min, 30 cycles of 94°C for 30 sec, 58°C for 35 sec, 72°C for 40 sec and a final elongation at 72°C for 10 min. A nested PCR step was performed to add barcoded primers (10 µM) in a total volume of 30 µl for both 16S rRNA gene and ITS region: 95°C for 5 min, 15 cycles of 95°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec and a final elongation at 72°C for 5 min. Three technical replicates, conducted for each sample, were combined and purified by Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). DNA concentrations were measured with Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA) and samples were combined in equimolar concentration. The amplicons were sequenced on a Illumina MiSeq v2 (2 × 250 bp) machine.

Illumina MiSeq data evaluation of 16S rRNA gene and ITS region and statistics

After joining forward and reversed paired end reads in QIIME 1.9.1, sequencing data was imported into QIIME 2 2019.1 and demultiplexed following the QIIME 2 tutorials. The DADA2 algorithm was applied for quality filtering, discarding chimeric sequences and to obtain a feature table (containing sequence variants (SVs)) and representative sequences. Feature classification was performed using a Naïve-Bayes feature classifier trained on the Silva132 release (16S) (Quast et al. 2013) or the UNITE v7.2 release (ITS) (Kõljalg et al. 2013). Sequences of features of interest were further identified on species level using NCBI blast alignment tool. Mitochondria and chloroplast reads were discarded from 16S data. Alpha and beta diversity was investigated running the core diversity script in QIIME 2 rarefying feature tables to the lowest value of reads present in one sample. Core microbiomes (features present in 50% of the samples) were defined for each sample group and core tables were rejoined to obtain barplots and evaluate taxonomic differences. A taxonomy network was constructed on core genera using Cytoscape version 3.5. (Shannon et al. 2003).

Statistical analysis of metabarcoding data was performed using scripts in QIIME 1.9 as well as QIIME2 2019.1. Alpha diversity was tested using the Kruskal-Wallis test and beta diversity using Analysis of Similarity (ANOSIM) test. Significant differences (alpha$\leq$ 0.05) in

taxa abundance on genus level were calculated using non-parametric Kruskal-Wallis test and False Discovery Rate (FDR) multiple test correction.

Quantitative Real-Time PCR (qPCR)

A qPCR was conducted to quantify overall bacterial 16S and fungal ITS gene copy numbers, as well as postharvest pathogens *P. expansum* and *Neofabraea* spp.. For specific quantification of bull´s eye rot-causing *Neofabraea* strains, a primer pair was selected that specifically targets the highly conserved β-tublin gene which was found to amplify the three major pathogens associated with bull´s eye rot (*N. alba, N. malicorticis, N. perennans*), but no other related fungi (Cao et al. 2013). Primer pairs were used each in 5 pmol/µl concentration and are listed in Supplementary Table 6.1. All reaction mixes contained 5 µl KAPA CYBR Green, 0.5 µl of each primer, 1 µl template DNA, adjusted with PCR-grade water to a final volume of 10 µl. Reaction mix for bacterial amplification was supplemented with 0.15 µl PNA mix to block amplification of host-derived 16S gene copies. Fluorescence intensities were detected using a Rotor-Gene 6000 real-time rotary analyzer (Corbett Research, Sydney, Australia) with the following cycling conditions: Bacteria: 95°C for 5 min, 45 cycles of 95°C for 20 sec, 54°C for 30 sec, 72°C for 30 sec and a final melt curve of 72 to 96°C. Fungi: 95°C for 5 min, 45 cycles of 95°C for 30 sec, 58°C for 35 sec, 72°C for 40 sec and a final melt curve of 72 to 96°C. *P. expansum*: 95°C for 5 min, 45 cycles of 95°C for 20 sec, 65°C for 15 sec, 72°C for 15 sec and a final melt curve of 96 to 72°C. *Neofabraea* sp.: 95°C for 5 min, 45 cycles of 95°C for 20 sec, 57°C for 15 sec, 72°C for 40 sec followed by melt curve of 96 to 96°C. Three individual qPCR runs were conducted for each replicate. Intermittently occurring gene copy numbers that were found in negative controls were subtracted from the respective sample. Significant differences ($p \leq 0.05$) of bacterial and fungal gene copy numbers per apple between the different apple groups were calculated using a pairwise Wilcox test (Bonferroni correction) and visualized using ggplot2 in R version 3.5.1.

Small-scale storage experiments

Small scale experiments were conducted to test the efficacy of potential biocontrol agents with and without combined HWT against infection of the fungal pathogens *P. exopansum* ATCC 7861 (Origin: CBS 325.48) and *N. malicorticis* (Jacks) Nannfeld (Origin: DSMZ 62715), selected as representative for bull´s eye rot-causing fungal pathogens. More than 800 bacterial

strains, isolates from apples, were tested for antagonistic properties towards the two pathogens by dual-culture in vitro assay on Waksman agar (Berg et al. 2002). Bacterial isolates showing highest antagonistic properties towards both fungi were identified by Sanger sequencing (LGC Genomics, Berlin, Germany) and NCBI BLAST alignment tool: *Pantoea vagans* 14E4, *Bacillus amyloliquefaciens* 14C9 and *Pseudomonas paralactis* 6F3. For *in vivo* tests, 30 apples from the cultivar 'Topaz' per treatment and pathogen were rinsed with water and four artificial wounds were cut with a sterile knife around the radius of the fruits. Each apple was artificially infected with *N. malicorticis* (submerged in a 1.6×10^5 conidia/mL solution) or *P. expansum* (10µL of a 5×10^4 spores/mL solution) and incubated for 24h at 20°C. Overnight cultures of bacterial biocontrol strains were centrifuged at 5,000 rpm for 15 min. The supernatant was discarded and bacterial pellets were resuspended in sterile sodium chloride solution (0.85%). A consortium of all three biocontrol strains was prepared. Suspensions were diluted to an OD_{600} of 0.2 (approximately 10^6 cells/mL). Apples infected by the fungal pathogens were treated either with *P. vagans* 14E4 or the consortium by submerging the apples in the prepared solution. HWT groups were previously submerged in 53°C hot water for 3 min and allowed to dry. Negative control samples were stored directly after wounding without pathogen infection and positive control samples were stored after infection with *N. malicorticis* and *P. expansum* without further treatment. Results were evaluated after three weeks (*P. expansum*) and five weeks (*N. malicorticis*) storage period under controlled conditions at 4°C. Supplementary Figure 6.1 exemplifies the temporally resolved disease progression of *P. expansum* infection, directly, one and three weeks after wounding. The diameter of infected areas as well as the length of the cuts was measured and statistical significance tested using a pairwise Wilcox test (Bonferroni correction) and visualized using ggplot2 in R version 3.5.1.

Results

The structure of the core postharvest microbiota in apples

After quality filtering and removing of chimeric sequences using the DADA2 algorithm and excluding mitochondrial and chloroplast sequences from the 16S rRNA gene fragments, the 16S and ITS datasets contained 1,071,751 and 880,909 paired reads, respectively. Sequences were assigned to 2,297 bacterial and 613 fungal features and the datasets were rarefied to 1,638 bacterial and 1,319 fungal sequences, according to the sample with the lowest amount of sequences. Core microbiota were defined for each sample group ('before storage', 'HWT', 'untreated healthy' and 'untreated diseased'), by keeping only the features present in 50% of the replicates of the respective group. In total, 205 core bacterial and 89 core fungal features remained that were condensed to 60 and 44 genera, respectively. From those taxa, an OTU network was constructed to visualize shared taxa and taxa being unique for a specific group (Figure 6.1). Among 104 bacterial and fungal genera, 23 were shared by all apples, while 22 genera were present in 'HWT' and 'untreated healthy' apples but absent in all other samples, probably indicating a health-related postharvest microbiome. Additionally, 'HWT', 'untreated healthy' and 'before storage' samples hosted 13, 16 and 10 unique taxa, respectively, while no unique taxa were found for 'untreated diseased' apples. *N. alba* was present in all apples, including 'before storage' samples, whereas *P. expansum* only occurred in stored apples.

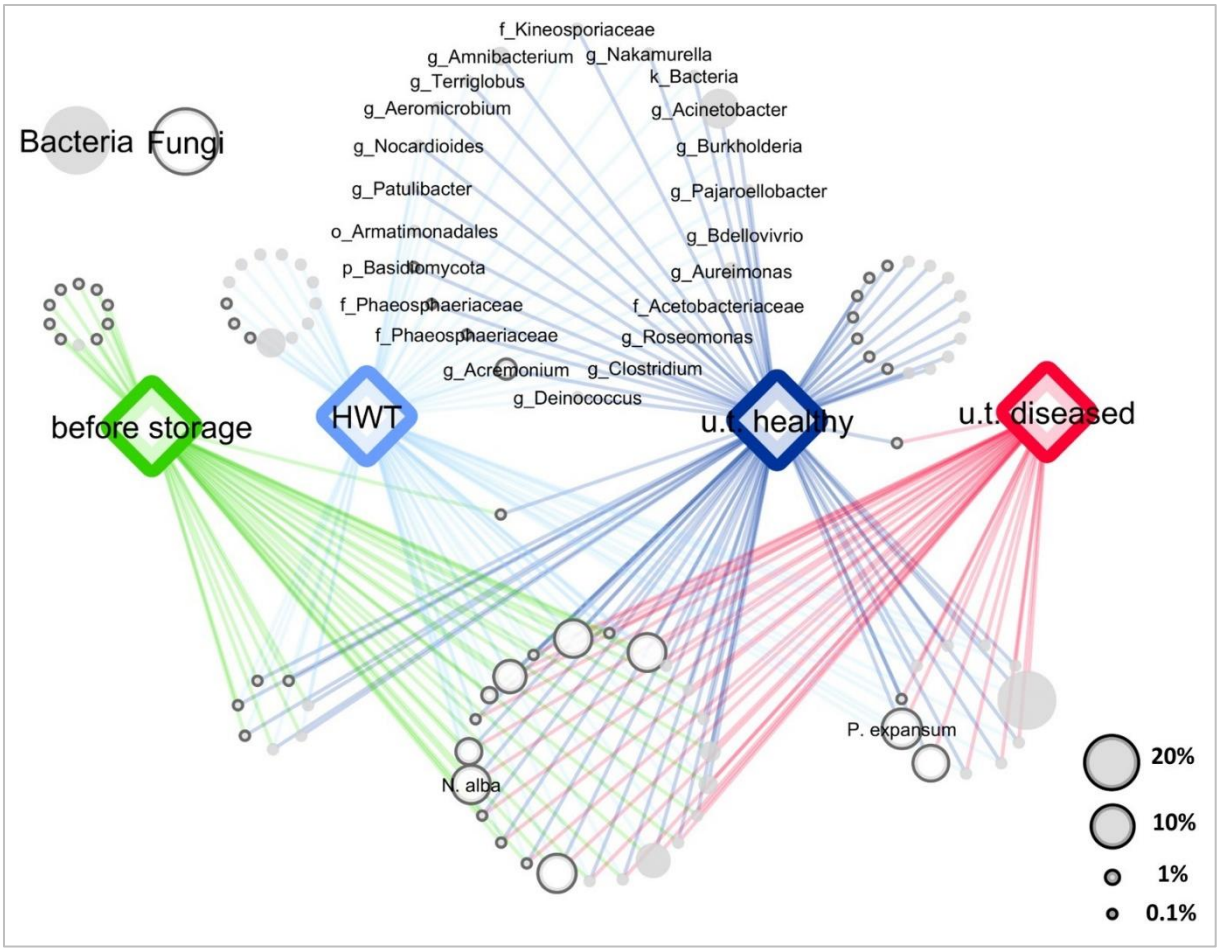


Figure 6.1: Core and specific microbiota for the four apple groups. Core bacterial and fungal microbiota on genus level (taxa occurring in 50% of all replicates) of the four groups 'before storage', 'HWT', 'untreated healthy' (u.t. healthy) and 'untreated diseased' (u.t. diseased) were combined for network analysis. Node size corresponds to relative abundance in the dataset as described in the legend on the lower right. Node color indicates bacteria (filled light grey) and fungi (outlined dark grey), as shown in the legend on the upper left. Nodes of taxa shared by healthy stored apples, indicating the healthy postharvest microbiota, are labelled as well as the two postharvest pathogens *N. alba* and *P. expansum* whose taxonomy was assigned on species level using the NCBI BLAST alignment tool.

Taxonomic changes induced by storage and disease

In order to compare taxonomic composition of the four groups, Figure 6.2 was constructed for the bacterial (Figure 6.2, A) and fungal (Figure 6.2, B) core microbiota of each group on genus level, where genera with less than 1% abundance are clustered as 'Other'. The microbiota within the four different groups showed great taxonomic variability, especially when apples

before storage were contrasted to stored apples. The bacterial microbiota within all samples was highly dominated by *Proteobacteria*, ranging from 65% in 'before storage' samples up to 80% in 'untreated healthy' apples. Apples 'before storage' had additionally a high abundance of *Bacteroidetes* (32%) compared to the other groups (3-8%), whereas all stored apple samples prevailed in *Actinobacteria* abundance (9-20%) over 'before storage' samples (1%). *Sphingomonas* was the most abundant genus in all groups (35-46%). *Hymenobacter* (31%) and *Massilia* (13%) were furthermore highly abundant in apples before storage. *Pseudomonas* (7-11%) and *Methylobacterium* (7%) were abundant in healthy apples after storage, whereas diseased apples after storage showed high abundances of *Methylobacterium* (12%) and *Frondihabitans* (11%) (Figure 6.2, A). In total, the core microbiota of the four groups 'before storage', 'HWT', 'untreated healthy' and 'untreated diseased' contained 15, 50, 49 and 18 bacterial genera, respectively.

The fungal microbiota was dominated by *Ascomycota*, ranging from 72% in 'untreated healthy' samples up to 97% in 'untreated diseased' apples. *Basidiomycota* were more abundant in healthy apples before (19%) and after (11-26%) storage, compared to 'untreated diseased' apples (3.5%). On genus level, *Mycosphaerella* dominated 'before storage' samples (30%), followed by *Alternaria* (19%), *Vishniacozyma* (12%), *Cladosporium* (8%) and *Aureobasidium* (7%). Stored 'HWT' samples were dominated by a not further assigned taxon of *Hypocreales* (20%), followed by *Cladosporium* (15%), *P. expansum* (11%), *Acremonium* and *Didymellacae* sp. (each 10%) and *Vishniacozyma* (9%). Almost the same fungal genera were highly abundant in stored 'untreated healthy' samples, with *Vishniacozyma* (21%) being the main representative, except *P. expansum* featuring only 1% abundance. Stored 'untreated diseased' apples were almost exclusively composed of the two postharvest pathogens *P. expansum* (45%) and *N. alba* (42%) (Figure 6.2, B). Both fungi were present in 'HWT' and 'untreated healthy' apples, although with less relative abundance. 'before storage' apples contained 0.1% *N. alba*, while *P. expansum* was absent. The samples 'before storage', 'HWT', 'untreated healthy' and 'untreated diseased' contained 28, 27, 33 and 18 fungal core genera, respectively.

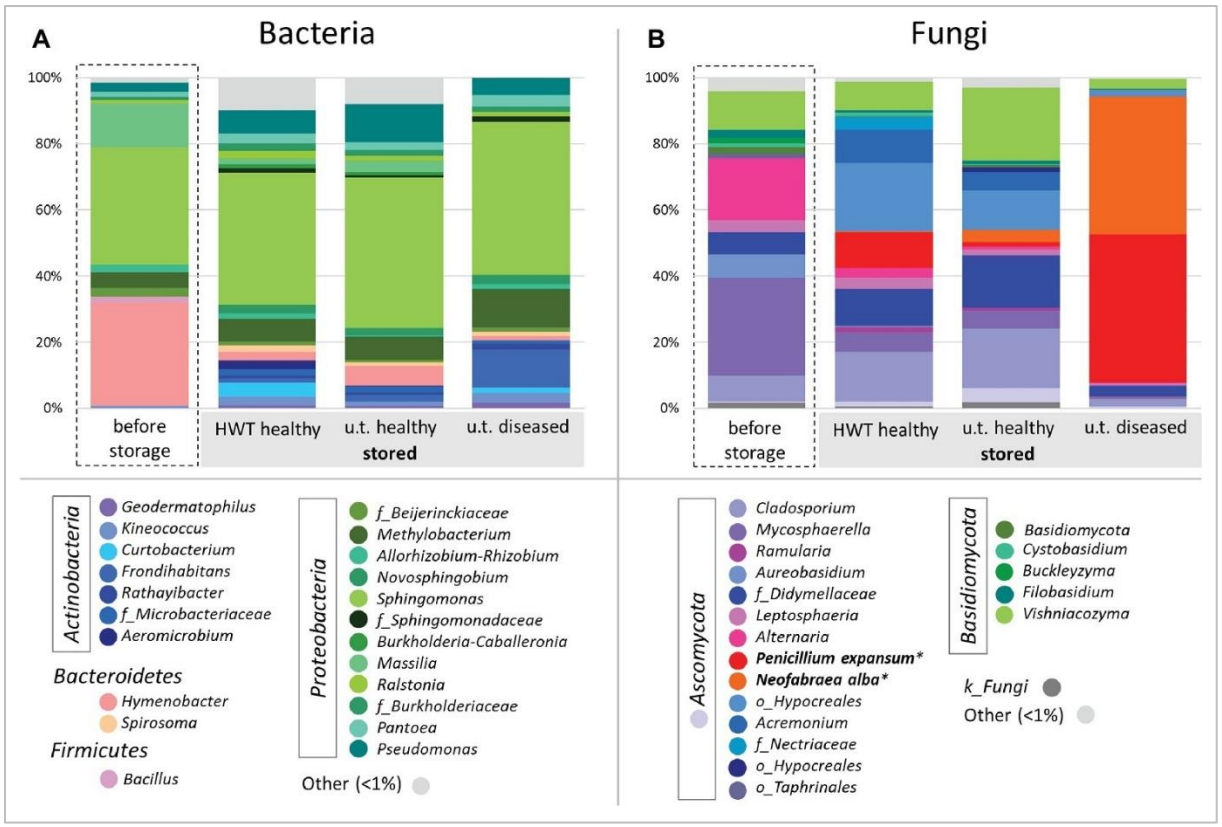


Figure 6.2: Bacterial and fungal taxonomy of apples investigated. Core microbiomes were defined for taxa occurring in 50% of the replicates in the respective groups. Color-coded bacterial (A) and fungal (B) taxa are indicated in the bottom legend and are shown on genus level and grouped by phylum. Sequences of storage pathogens highlighted in bold were further identified on species level using NCBI BLAST alignment tool. Taxa occurring with less than 1% are shown as 'Other'.

Diversity changes induced by storage and disease

The bacterial and fungal diversity within the apple samples was assessed by Shannon diversity index. Apples from the category 'before storage' showed significantly the lowest bacterial diversity (H'=5.19±0.8), followed by stored apples from the category 'untreated diseased' (H'=5.72±0.3). Both were significantly less diverse than stored 'untreated healthy' (H'=6.46±0.6) and 'HWT' samples featuring highest bacterial diversity (H'=6,68±0.4) (Figure 6.3, A). Fungal diversity was highly decreased in stored 'untreated diseased' apples (H'=1.93±0.8), being significantly lower compared to all healthy apples: 'before storage': H'=3.77±0.5, 'HWT': H'=3,87±0.6 and 'untreated healthy': H'=4.31±0.1 (Figure 6.3, B).

Beta diversity analyses, applied on the whole bacterial and fungal dataset and based on Bray Curtis distance matrix, indicated clear clustering between apples before and after storage in all cases (Figure 6.3, C and D). Statistical significance in bacterial composition, assessed via pairwise ANOSIM (Table 6.1), revealed significant differences between all groups, except for the comparison of 'HWT' and 'untreated healthy' samples. Highest variability was found when 'before storage' samples were compared to the remaining groups. The fungal composition was significantly different between all four groups, while difference between 'HWT' and 'untreated healthy' samples was lowest.

In order to identify bacterial and fungal taxa that potentially contribute to pathogen resistance in 'untreated healthy' apples, significant differences in taxa abundance between 'untreated healthy' and 'untreated diseased' samples were calculated (Supplementary Table 6.2). A total of 42 bacterial and 28 fungal taxa were found significantly increased in 'untreated healthy' apples as well as 2 fungal taxa (*P. expansum* and *N. alba*) being significantly increased in 'untreated diseased' apples. Increased in 'untreated healthy' apples were, among others e.g. *Sphingomonas*, *Pseudomonas* and *Methylobacterium* as well as *Vishniacozyma*, *Cladosporium* and *Acremonium*.

Additionally, the impact of HWT on the apple postharvest microbiota was evaluated as well, by calculating significant differences in taxa abundance between 'HWT' and 'untreated healthy' apples (Supplementary Table 6.3). A total of 25 bacterial and 22 fungal genera were found to be significantly different abundant between the two groups. Significantly increased in 'HWT' were e.g. *Hymenobacter*, *Rathayibacter* as well as *Filobasidium*; increased in 'untreated healthy' were e.g. *Curtobacterium*, *Rhodococcus* as well as *Penicillium* and *Alternaria*. However, as previous stated, the overall bacterial microbiome and diversity was not significantly different between the two groups only the fungal microbial composition was slightly changed.

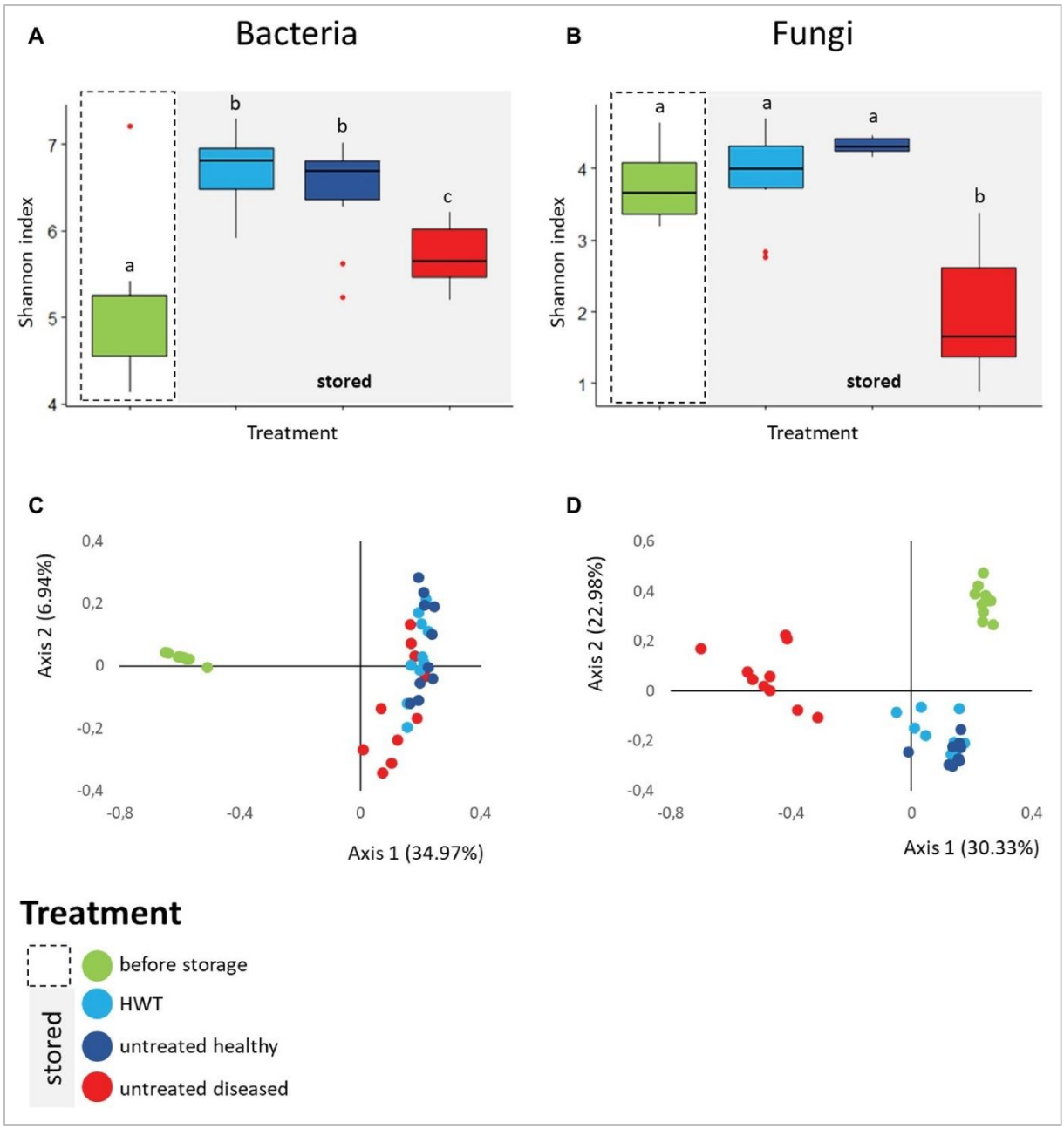


Figure 6.3: Alpha- and beta-diversity analyses on apple-associated bacterial and fungal structure. Box-and-Whiskers-plots visualize Shannon diversity index of the four different apple groups for bacteria (A) and fungi (B). Significant differences ($p \leq 0.05$) were assessed by Kruskal Wallis test and are indicated by different lower case letters. Community clustering of bacterial (C) and fungal (D) composition of the samples is indicated by color-coded two dimensional Bray Curtis PCoA plots. Color code for the differentially treated apple samples is explained in the legend on the bottom left. Significant differences in bacterial and fungal composition was tested using ANOSIM pairwise test and can be looked up in Table 6.1.

Table 6.1: Pairwise ANOSIM results calculating significant differences in bacterial and fungal composition associated with differentially treated apple groups.

		Bacteria		Fungi	
Group 1	**Group 2**	**R**	**p-value**	**R**	**p-value**
HWT	untreated diseased	0.21	0.002	0.79	0.001
HWT	untreated healthy	0.06	0.136	0.41	0.001
HWT	before storage	1.00	0.001	0.95	0.001
untreated diseased	untreated healthy	0.26	0.001	0.81	0.001
untreated diseased	before storage	1.00	0.001	0.85	0.001
untreated healthy	before storage	1.00	0.001	1.00	0.001

Quantification of bacteria, fungi, *P. expansum* and *Neofabraea* sp. during storage and disease

A real time PCR was performed to quantify total bacterial 16S rRNA and fungal ITS gene copy numbers. Bull´s eye rot-causing *Neofabraea* strains and *P. expansum* were specifically quantified as well (Figure 6.4). No significant differences in 16S rRNA gene copy abundance was observed between the four different apple groups; neither between apple 'before storage' and all stored apples, nor within the stored groups (Figure 6.4, A). Pathogen infestation as well as HWT did accordingly not affect the bacterial abundance in apples. Regarding the total fungal ITS genes we found significantly higher abundances within 'untreated diseased' apples compared to all other groups (Figure 6.4, B), due to significant increase of both storage pathogens *Neofabraea* and *P. expansum* (Figure 6.4, C and D, respectively). *Neofabraea* was already present in 'before storage' apples in similar abundances as in 'HWT' and 'untreated healthy' apples while *P. expansum* was almost absent in apples 'before storage'. Overall, fungi were found to proliferate more efficiently compared to bacteria in stored apples, as showed via calculating the prokaryote to eukaryote ratio (Figure 6.4, E). Whereas the ratio was almost balanced in apples before storage (58% bacteria and 42% fungi), fungal genes increased up to the two-fold in stored, healthy apples. A dramatic increase of fungal genes was however observed within stored, diseased apples; 99.4% of total microbial genes detected were fungal.

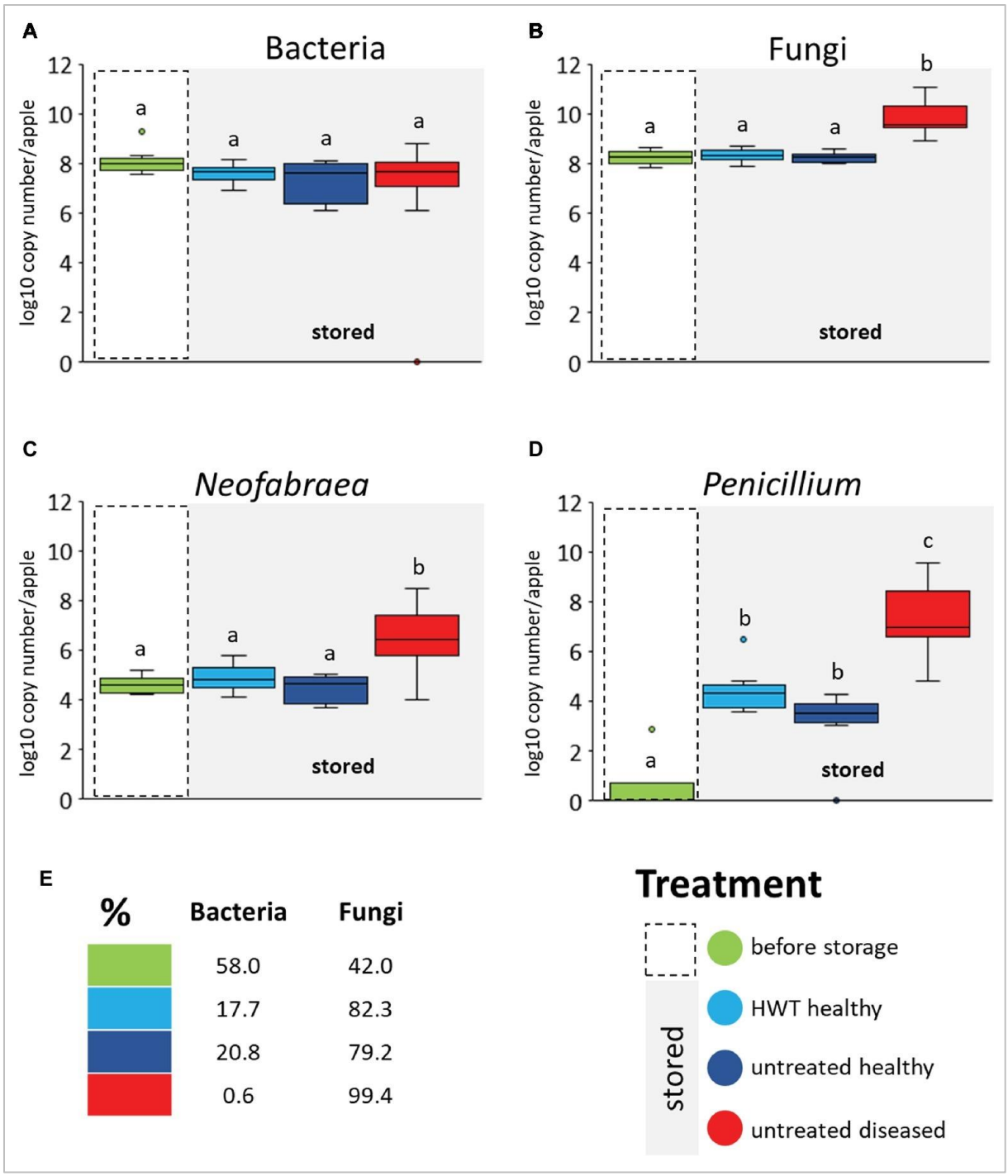


Figure 6.4: Microbial gene copy numbers in apple groups determined by qPCR. Values are given by primers targeting bacterial 16S rRNA genes (A), fungal ITS region (B) and genes of *N. alba* (C) and *P. expansum* (D). Gene copy numbers are calculated per apple used for the microbiome analysis. Significant differences ($p \leq 0.05$) were assessed by Wilcox test (Bonferroni correction) and are indicated by different lower case letters. The prokaryote to eukaryote ratio within the total microbial gene copies detected in apples of the respective groups is shown (E). Color code for apple groups is depicted in the legend on the bottom right.

Efficiency of HWT and biological control application against postharvest diseases determined in small-scale storage experiments

The efficacy of potential biocontrol strains (*P. vagans* 14E4, *B. amyloliquefaciens* 14C9 and *P. paralactis* 6F3) identified using antagonistic screening methods was tested in small-scale storage experiments with or without combined HWT against *N. malicorticis* and *P. expansum*. *P. vagans* E14 was applied as single agent as well as combined with the other potential biocontrol strains in form of a consortium. Negative control apples that were wounded artificially but not infected with fungal pathogens appeared to be unaffected after two as well as after five weeks of storage. Positive control apples that were inoculated with the fungal pathogens and untreated showed 100% infection rate for *N. malicorticis* and 96% for *P. expansum* (Figure 6.5, A). Treatment using biocontrol strains slightly decreased infection rates, however, still up to 88% of apples were infected. HWT reduced infection rates of *N. malicorticis* and *P. expansum* to 58% and 75%, respectively. Overall, combining HWT and the biocontrol consortium reduced the total infection rates the most (up to 42%). Similar results were shown when the infection diameter was measured (Figure 6.5, B). Here, no significant differences in infection diameter were found between positive control samples and apples treated with biocontrol strains that were not subjected to HWT. In contrast, HWT approved to be efficient in reducing pathogen infection rates, while the combined treatment of HWT and potential biocontrol strains resulted in even less infection.

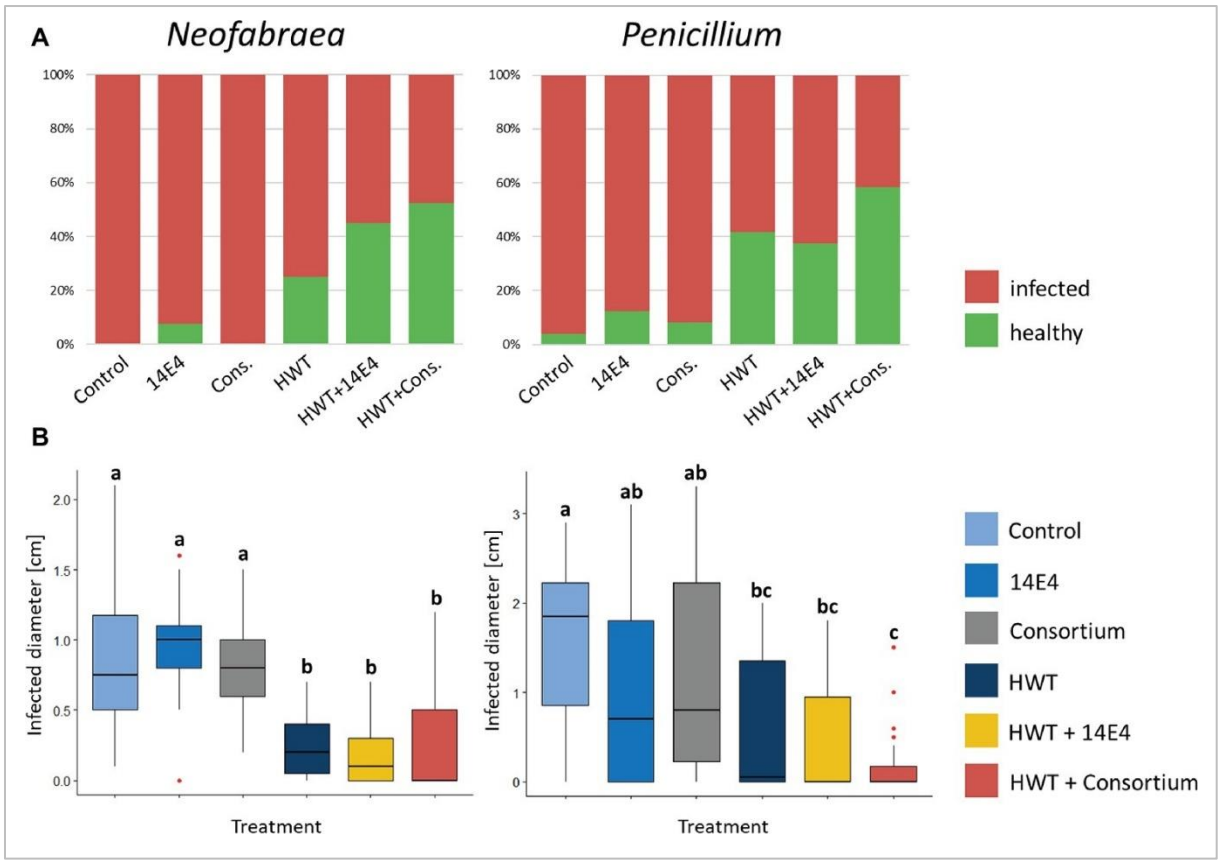


Figure 6.5: Fraction of infected apples after storage (A) and analysis of infected diameter (B). Apples were treated with fungal spores or conidia as well as bacterial strain *P. vagans* 14E4, a bacterial consortium and/or HWT. Control samples were only inoculated with fungal spores and stored. Statistical differences between differentially treated apple samples was assessed by Wilcox test (Bonferroni correction) and are indicated by lower case letters.

Discussion

The present study is the first to provide deeper insights into the taxonomic, diversity and abundance changes induced by currently in-use HWT at industrial scale. The efficacy of HWT in reducing postharvest pathogens was demonstrated by metabarcoding analysis and microbial quantification via qPCR. In addition, specific heat-resistant and indigenous bacterial microorganisms seem to contribute to disease resistance. Small-scale storage experiments furthermore suggest the combination of highly effective HWT and a biological control consortium to be a promising approach to prevent postharvest loss of apples.

HWT at industrial scale was proven to be highly efficient as during long-term storage for six months, not a single among 100 HW-treated apples was decayed. Among untreated and stored apples, 10% were infected by storage pathogens. We studied the induced changes in the microbiome comparing 'HWT' and 'untreated healthy' apples. The difference between the two groups was insignificant for bacteria on any level measured; alpha and beta diversity matrixes, as well as gene quantification revealed no significant differences between the two groups. The fungal composition was, however, slightly influenced. Accordingly, we hypothesize that the apple is protected by the previously studied HWT-initiated transcription and translation of heat-shock proteins in the plant, where a subset of which comprise pathogenesis-related proteins (Fallik et al. 2001; Pavoncello et al. 2001). The plant response affects the present bacteria to a lesser extent that the fungi. However, still few bacterial and fungal taxa were found to be significantly different abundant between HW-treated and untreated healthy apples, which are therefore suggested to be directly affected by HWT. Whether this microbiota is heat-sensitive or diminished by HWT-induced plant response remains, however, unclear. Among others, also *Penicillium* was significantly reduced in HW-treated apples.

Overall, healthy apples (HWT or untreated) showed a distinct microbiome compared to diseased apples. A total of 18 bacterial and 4 fungal taxa were shared between HW-treated and untreated but healthy apples, while being absent in diseased apples. Explicitly selecting taxa from the healthy postharvest microbiome might provide promising opportunities for future applications to reduce postharvest decay of apples and other fruits.

The impact of pathogen infestation on the bacterial and especially on the fungal microbiota of stored apples was severe. Microbial diversity was significantly reduced and the composition was clearly shifted. Almost 90% of all fungal sequences detected in diseased apples were composed by co-occurring *N. alba* (42% rel.) and *P. expansum* (45% rel.) and

especially the low abundant taxa were almost outcompeted during pathogen infection. Observing apples before storage, the ratio between bacteria and fungi was almost balanced (58% to 42% for bacteria and fungi, respectively). The ratio shifted towards 20% bacteria and 80% fungi in stored but healthy apples (both HW-treated and untreated samples) and climaxed in 99.4% fungal genes, out of all microbial genes detected, in diseased apples. This percentage was almost exclusively covered by pathogenic *Neofabrea* species and *P. expansum* as detected via specific gene quantification, coinciding significantly with the observations in microbiota taxonomy. Even though the infected spots on diseased apples reached a maximum of only 4 cm in diameter on one apple, this emphasizes even more the fast impact of pathogen infestation on the overall microbial composition. The results of this study suggest that the two pathogens are highly co-occurring; moreover, a mutualistic effect is suggested. Outbreaks of pathogenic *Neofabraea* species, known to infect the apple fruit already in the field (Snowdon 1990), most likely facilitates infestation of rapidly proliferating *P. expansum,* which attacks the fruit through damaged tissues and wounds during storage (Amiri and Bompeix 2005). After a six-months storage period this results in a disease outbreak induced by both pathogens to an equal extent. For a significant reduction of *P. expansum* in stored fruits, prevention of *Neofabraea* infection might therefore be essential. The infectious cycles of the two pathogens was confirmed in the present study as well, as *N. alba* was detected already in apples before storage, whereas *P. expansum* was present only in apples stored for six months.

Overall, among stored apples, HWT and pathogen infestation influenced the bacterial community to a lesser extent than the fungal. Surprisingly, the greatest effect on the bacterial microbiota was mediated by long-term storage. Apples before storage exhibited significantly lower bacterial diversity compared to all stored samples, including diseased apples. The bacterial microbiota was furthermore significantly shifted during storage, whereas bacterial abundance was unchanged across all samples investigated. Storage, therefore, seems to exhibit an even higher effect on the bacterial microbiota than pathogen infestation, whereas the opposite was observed for the fungal community. During storage significant shifts in fungal composition and slight, but not significant increase in diversity was observed. Especially the bacterial genera *Hymenobacter* and *Massilia* and the fungi *Mycosphaerella*, *Alternaria* and *Aureobasidium,* featuring high abundances in apples before storage, were significantly reduced after the six-months storage period; probably due to cold-sensitivity of those taxa.

Small-scale experiments demonstrated a significant reduction of symptoms caused by postharvest pathogens *N. malicorticis* and *P. expansum* when fruits were subjected to HWT

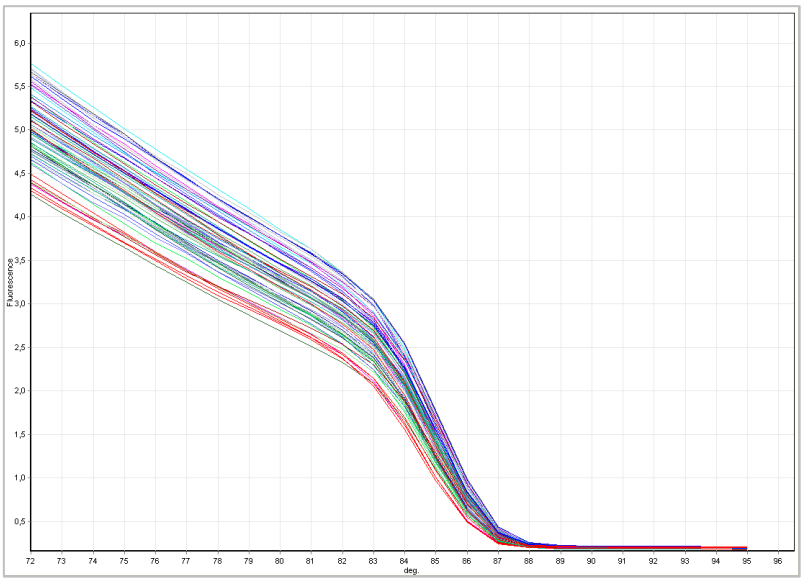
with or without additional application of a biological control consortium, while the latter even enhanced the efficacy of the treatment. The efficiency was equally pronounced against both pathogens as determined by counting infected apples and measuring diameters of infection on apples artificially wounded and infected with the pathogens. The combined method of HWT and biological control consortium, previously isolated from apples, reduced infection rates up to 42%. Our experiment showed that the fungicidal effect was stable for at least five weeks as we evaluated fruit decay after three weeks for *P. expansum* and after five weeks for slow-growing *N. malicorticis.* Efficacy of combined methods of HWT and biological control has already been proven successful for apple (Spadaro et al. 2004; Conway et al. 2004), citrus fruits (Porat et al. 2002; Obagwu and Korsten 2003), pear (H. Zhang et al. 2008), strawberry (Wszelaki 2003), mandarin fruit (Hong et al. 2014) and tomato (Zong et al. 2010). However, the present study was the first to test microbial consortia in combination with HWT. Nevertheless, the efficacy of the combined method needs however to be confirmed on industrial scale.

Until now, only few studies have assessed the microbial dynamics during storage. Investigations on the oomycete and fungal community of sugar beets infested by storage soft rot showed that the susceptibility to storage pathogens was rather conditioned by the cultivar than by the oomycete and fungal community present. Accordingly, plant-inherent but unspecific resistance mechanism were suggested to decrease the spread of pathogens, but without preventing the infection (Liebe et al. 2016). However, the bacterial microbiome, which was not investigated in this study, could potentially contribute to disease expression as well. The dynamic changes of the endophytic bacterial community associated with potato tubers in response to bacterial storage pathogens was investigated by Kõiv et al. (Kõiv et al. 2015). Here, pathogenesis of the plant is assumed to be initiated by the pathogen but complex contributions from the endophytic community are significantly involved. A crucial impact of endophytic bacteria and fungi on the development of postharvest stem-end rots was also observed for mango fruits (Diskin et al. 2017). In summary, and with reference to the present results, the severity of postharvest infestations may be rather mediated by the interactions of specific members of the total community than by one specific pathogen. High diversity in plants was already described to determine abundance of pathogens (Berg et al. 2017).

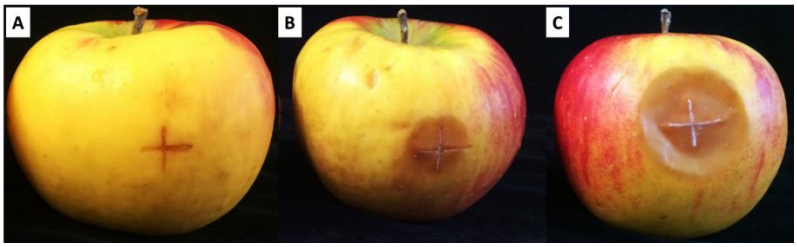
Conclusion

The indigenous apple microbiome is important for health within the postharvest period and during storage. A healthy apple microbiome is characterized by high bacterial and fungal diversity and evenness, a balanced ratio between both groups and several health indicators, while diseased apples show dysbiosis, diversity loss and dominant fungal pathogens. HWT-induced plant response diminished pathogen infection at industrial scale, and showed an impact on the fungal composition. We suggest that the apple fruit is protected by either HWT or the inherent microbiome; however, presumable it is the combination of both, mediating disease resistance. Small-scale storage experiments applying HWT together with biological control agents provide further confirmation of the considerable potential of combining methods into one control strategy to reduce postharvest decay of apples. Moreover, harnessing the indigenous microbiota of fruits for a biological control approach is a promising and sustainable future strategy to prevent postharvest decay of fresh and stored produce.

Supplementary Material



Supplementary Figure 6.1: Analyzation of the melting curve of qPCR samples after amplification. Melting was performed after each run using a heat gradient from 72 to 95°C.



Supplementary Figure 6.2: Pathosystem of *P. expansum* on Topaz apples directly (A), one week (B) and three weeks (C) after artificial wounding over the course of small scale storage experiments. Diameters of infected areas were measured after three weeks of storage.

Supplementary Table 6.1: Primers used in the present study.

Primer name	Primer sequence 5'-3'	Reference
515f 927r	GTGYCAGCMGCCGCGGTAA CCGYCAATTYMTTTRAGTTT	(Caporaso et al. 2010)
ITS 1f ITS 2r	CTTGGTCATTTAGAGGAAGTAA GCTGCGTTCTTCATCGATGC	(T. J. White et al. 1990)
Pexp_patF_F Pexp_patF_R	ATGAAATCCTCCCTGTGGGTTAGT GAAGGATAATTTCCGGGGTAGTCATT	(Tannous et al. 2015)
NeoF NeoR	CTTTCTCCGTTGTCCCATCC GAACATTGCGCATCTGGTCC	(Cao et al. 2013)

Supplementary Table 6.2: Bacterial and fungal genera with significantly different abundance in 'untreated healthy' and 'untreated diseased' apples.

		untreated healthy	untreated diseased	FDR_P
Bacterial genera	*Sphingomonas*	18875	3009	0.00
	Pseudomonas	4839	343	0.00
	Methylobacterium	2945	762	0.00
	Hymenobacter	2456	92	0.00
	Massilia	1464	0	0.00
	Novosphingobium	911	187	0.00
	f_Microbacteriaceae	722	69	0.00
	f_Burkholderiaceae	704	103	0.00
	Ralstonia	621	94	0.00
	Kineococcus	523	188	0.00
	Spirosoma	504	82	0.00
	Mucilaginibacter	390	0	0.00
	Burkholderia	363	0	0.00
	Roseomonas	323	0	0.00
	Rathayibacter	303	116	0.01
	Amnibacterium	302	0	0.00
	f_Sphingomonadaceae	301	99	0.01
	Geodermatophilus	285	112	0.00
	f_Beijerinckiaceae	277	86	0.00
	Clostridium sensu stricto 1	230	0	0.00
	Nocardioides	156	0	0.00
	k_Bacteria	145	0	0.00
	Acidiphilium	134	0	0.00
	Deinococcus	133	0	0.00
	Basidiomycota	130	0	0.00
	f_Kineosporiaceae	125	0	0.00
	Aureimonas	125	0	0.00
	Patulibacter	116	0	0.00
	o_Armatimonadales	115	0	0.00
	f_Nocardioidaceae	109	0	0.00
	Aeromicrobium	108	0	0.01
	Bdellovibrio	96	0	0.00
	Pedobacter	94	0	0.02
	Pajaroellobacter	82	0	0.00
	Nakamurella	77	0	0.01
	uncultured bacterium	74	0	0.00
	uncultured	74	0	0.02
	Terriglobus	73	0	0.01
	Acinetobacter	62	0	0.01
	k_Bacteria	62	0	0.01
	Terrisporobacter	59	0	0.00
	Belnapia	50	0	0.00
	Jatrophihabitans	34	0	0.02
	f_Fimbriimonadaceae	28	0	0.02
	f_Acetobacteraceae	25	0	0.02
Fungal genera	*Vishniacozyma*	4810	588	0.00
	Cladosporium	3948	454	0.00
	f_Didymellaceae	3233	574	0.00
	o_Hypocreales	2622	382	0.01
	Acremonium	1222	0	0.00
	Mycosphaerella	1183	121	0.00
	p_Ascomycota	931	125	0.00
	Leptosphaeria	438	175	0.01
	k_Fungi	419	0	0.00
	o_Hypocreales	294	0	0.00
	Filobasidium	274	39	0.00
	Alternaria	203	45	0.00
	f_Didymellaceae	200	71	0.02
	Ramularia	196	40	0.00
	p_Basidiomycota	180	24	0.00

	unidentified	156	0	0.00
	Symmetrospora	106	15	0.00
	Uncobasidium	68	0	0.02
	Bullera	30	0	0.00
	f_Phaeosphaeriaceae	23	0	0.00
	Cystobasidium	23	0	0.00
	Bensingtonia	22	0	0.00
	f_Mycosphaerellaceae	19	0	0.01
	f_Cystobasidiaceae	15	0	0.00
	f_Sporidiobolaceae	11	0	0.01
	Sporobolomyces	10	0	0.01
	Aureobasidium	9	0	0.02
	Kurtzmanomyces	8	0	0.02
	P. expansum	284	9122	0.02
	N. alba	804	8512	0.02

*Abundance in absolute hits that was significantly higher in the respective apple group, is highlighted in bold. **Significances were calculated by applying non-parametric Kruskal-Wallis/FDR-P (alpha=0.05).

Supplementary Table 6.3: Bacterial and fungal genera with significantly different abundance in 'HWT' and 'untreated healthy' apples.

	Taxonomy	**HWT***	**untreated healthy***	**FDR_P****
Bacterial genera	*Hymenobacter*	**2456**	801	0.02
	Rathayibacter	**303**	200	0.04
	Amnibacterium	**302**	104	0.02
	k_Bacteria	**145**	30	0.03
	Basidiomycota	**130**	10	0.01
	f_Solirubrobacteraceae	**109**	0	0.01
	Pedobacter	**94**	0	0.03
	o_Myxococcales	**74**	0	0.01
	f_Beijerinckiaceae	**74**	0	0.03
	k_Bacteria	**62**	0	0.02
	Terrisporobacter	**59**	0	0.01
	Belnapia	**50**	0	0.01
	Jatrophihabitans	**34**	0	0.03
	f_Fimbriimonadaceae	**28**	0	0.03
	Curtobacterium	66	**1268**	0.01
	Rhodococcus	0	**286**	0.01
	Meiothermus	0	**174**	0.01
	Flavisolibacter	0	**132**	0.01
	Marmoricola	0	**107**	0.02
	Turicibacter	0	**55**	0.02
	Gemmata	0	**54**	0.01
	f_Blastocatellaceae	0	**50**	0.02
	Lacibacter	0	**39**	0.03
	f_Nocardioidaceae	0	**37**	0.03
	p_Armatimonadetes	0	**18**	0.03
Fungal genera	*f_Didymellaceae*	**3233**	1766	0.04
	p_Ascomycota	**931**	286	0.01
	k_Fungi	**419**	95	0.02
	o_Hypocreales	**294**	0	0.00
	Filobasidium	**274**	154	0.04
	p_Basidiomycota	**180**	39	0.01
	o_Entylomatales	**177**	0	0.01
	Symmetrospora	**106**	41	0.02
	Uncobasidium	**68**	0	0.03
	Bullera	**30**	0	0.00

	Bensingtonia	**22**	0	0.00
	f_Mycosphaerellaceae	**19**	0	0.01
	o_Capnodiales	**15**	0	0.01
	f_Cystobasidiaceae	**11**	0	0.01
	Kurtzmanomyces	**8**	0	0.03
	Penicillium	284	**1976**	0.01
	f_Nectriaceae	0	**718**	0.00
	Alternaria	203	**571**	0.01
	Cystobasidium	23	**186**	0.00
	Aureobasidium	9	**54**	0.01
	f_Apiosporaceae	0	**18**	0.01
	Leptospora	0	**9**	0.01

*Abundance in absolute hits that was significantly higher in the respective apple group, is highlighted in bold. **Significances were calculated by applying non-parametric Kruskal-Wallis/FDR-P (alpha=0.05).

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