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ABSTRACT

Globally, about 45% of all produced fruits and vegetables are lost on the way to the consumer. Especially postharvest food loss, caused by microbial contamination, provokes major shortfalls of fruits and vegetables along the supply chain. Apart from physical and chemical treatments biological control measures were studied for decades as an environmentally friendly alternative to harmful substances. Sugar beets (*Beta vulgaris* L.), the main sugar source in temperate regions, are stored for up to 60 days after harvest, without any protective measures in beet clamps. Due to the high moisture and sugar content, microbial infections cause severe sucrose inversion and therefore reduced sugar yield. For the development of biological control measures against postharvest disease in sugar beet the aim of this thesis was a holistic assessment of microbial communities of the sugar beet during the plant's postharvest life cycle. A special focus was put on the differentiation of the postharvest microbiome of healthy and diseased sugar beets.

To picture the whole bacterial and fungal community during the storage and investigate related microbe-plant interactions along with disease-associated microbial signatures, a next-generation sequencing approach combined with classical cultivation dependent and qPCR methods was conceived. Studying the microbial community on the field and in storage transmission of pathogenic and saprotrophic fungi from the harvested to the stored sugar beets was found.

In general, all conducted studies indicated a loss in microbial diversity in decaying sugar beets when compared to the healthy samples. The diversity loss was accompanied by severe taxonomic changes due to on-field and postharvest disease. Functional predictions showed a decrease in symbiotrophic and simultaneous increase in saprotrophic fungi with disease. Based on the high similarities of field and storage samples universal markers indicating disease were defined. While *Flavobacteria*, *Plectosphaerella* and *Vishniacozyma* were found increased in healthy samples, *Lactobacillus*, *Leuconostoc*, *Gluconobacter*, *Penicillium*, *Candida* and *Fusarium* were found increased with disease. Additionally, a substantial transfer of fungi as well as bacteria from the field into the storage was found.

Based on the obtained results a holistic picture of the sugar beet microbiome changes due to disease was drawn. The identified microbial signatures improve our understanding of postharvest microbial dynamics and can be utilized to increase the efficiency of biocontrol measures in the future. This study provides the first steps into a microbiome-driven biocontrol strategy for stored fruits and vegetables and the basis for a new generation of microbial products.

ZUSAMMENFASSUNG

Weltweit gehen rund 45% aller produzierten Früchte und Gemüse auf dem Weg zum Verbraucher verloren. Insbesondere mikrobielle Kontamination nach der Ernte verursacht erhebliche Verluste von Obst und Gemüse im Lager und entlang der Lieferkette. Neben physikalischen und chemischen Behandlungen wurden jahrzehntelang biologische Maßnahmen als umweltfreundliche Alternativen untersucht. Zuckerrüben (*Beta vulgaris* L.), die Hauptzuckerquelle in gemäßigten Regionen, werden nach der Ernte bis zu 60 Tage offen am Feld gelagert. Ungeschützt und den Umwelteinflüssen ausgesetzt, kommt es zu mikrobiellen Infektionen und starken Saccharoseinversionen und damit später zu einer verminderten Zuckerausbeute. Für die Entwicklung biologischer Schutzmaßnahmen für Zuckerrüben nach der Ernte war das Ziel dieser Arbeit eine eingehende Untersuchung des Nacherernte-Mikrobioms von gelagerten Zuckerrüben, um neue Bekämpfungsstrategien zu entwickeln.
Um die gesamte Bakterien- und Pilzgemeinschaft während der Lagerung und auf diese Weise die Wechselwirkungen zwischen Mikroorganismen und Pflanze zusammen mit krankheitsassoziierten mikrobiellen Veränderungen abzubilden, wurde ein Sequenzierungsansatz der nächsten Generation in Kombination mit klassischen kultivierungsabhängigen und qPCR-Methoden entwickelt. Durch die Untersuchung der mikrobiellen Gemeinschaft auf dem Feld und in der Lagerung konnten die Spuren von pathogenen und saprotrophen Pilzen von den geernteten hin zu den eingelagerten Zuckerrüben verfolgt werden.

Mit den vorliegenden Studien in dieser Arbeit wurde ein Verlust an mikrobieller Vielfalt in verrottenden Zuckerrüben im Vergleich zu gesunden Proben gezeigt. Die Diversitätsverluste gingen mit gravierenden taxonomischen Veränderungen aufgrund von Krankheit auf dem Feld und nach der Ernte einher. Funktionelle Vorhersagen zeigten eine Abnahme der symbiotrophen und gleichzeitig eine Zunahme der saprotrophen Pilze im Laufe der Krankheit. Basierend auf den hohen Ähnlichkeiten von Feld- und Lagerproben wurden universelle Marker definiert, die auf eine Krankheit hinweisen. Während *Flavobakterien*, *Plectosphaerella* und *Vishniacozyma* in gesunden Proben vermehrt gefunden wurden, waren *Lactobacillus*, *Leuconostoc*, *Gluconobacter*, *Penicillium*, *Candida* und *Fusarium* in kranken Proben erhöht. Zusätzlich wurde ein erheblicher Transfer von Pilzen als auch Bakterien vom Feld in das Lager festgestellt.

Basierend auf den vorgestellten Ergebnissen wurde ein ganzheitliches Bild der krankheitsbedingten mikrobiellen Veränderungen in Zuckerrüben erstellt. Die identifizierten mikrobielle Signaturen verbessern unser Verständnis der mikrobiellen Dynamik nach der Ernte und können dazu verwendet werden, die Effizienz von Maßnahmen zur biologischen Kontrolle in Zukunft zu verbessern. Diese

Arbeit liefert die ersten Schritte zu einer Mikrobiom-getriebenen Biokontrollstrategie für eingelagertes Obst und Gemüse und wirkt wegweisend für eine nächste Generation mikrobieller Produkte.

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INTRODUCTION

Postharvest decay of fruits and vegetables

Food loss is still a major problem in the global fruit and vegetable supply chain. In total, one third of all produced food is lost on the way to the consumer and especially a high amount of fruits and vegetables (45%; the equivalent of 3.7 trillion apples) is wasted. Apart from losses during agricultural production, processing, and transport, food decay in postharvest storage is a tremendous problem worldwide (FAO, 2011). The main causes for postharvest decay and spoilage are fungi belonging mostly to the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Monilinia*, *Penicillium* and *Rhizopus* (Snowdon, 1990). Therefore, appropriate countermeasures are crucial to maintain quality and taste of the produce. The public pressure on the industry increased during the past decade due to the withdrawal of widely used fungicides based on human health considerations. Therefore, sustainable, but most importantly equally efficient alternatives are eagerly searched to replace those harmful chemicals (Droby et al., 2016).

Popular chemical alternatives represent essential oils, that show antimicrobial activity and low mammalian toxicity (Burt, 2004; Isman, 2000). These oils are naturally occurring antioxidants, often volatiles and applicable in low concentrations (Sivakumar and Bautista-Baños, 2014). However, the high volatility, the price per application and most importantly the effect on the produce taste are the main difficulties for future applications. Additionally, microbial volatile organic compounds (mVOCs), also intensively studied for their antimicrobial effects on phytopathogenic fungi, provide a rich toolbox for new antimicrobial molecules (Cernava, 2012). Their identification, properties and possible industrial application is discussed in a Springer book chapter, part of the book "**Bacterial volatile compounds as mediators of airborne interactions**", written by the candidate and colleagues (**Chapter 6: Manuscript IV: 'Using bacteria-derived volatile organic compounds (VOCs) for industrial processes'**). Even though VOCs were shown to have great potential substituting other harmful chemicals in industrial processes, including postharvest treatment of fruits and vegetables, the main challenges for VOCs applicability remain the costs and the influence on the taste (Mari et al., 2016).

Sugar beets are stored in beet clamps

Beta vulgaris L. (sugar beet), a herbaceous dicotyledonous plant, is the main crop for sugar production in temperate regions around the world (Trebbi and McGrath, 2004). A number of pathogens including *Pythium ultimum* Trow, *Rhizoctonia solani* Kühn and *Cercospora beticola* Sacc. cause severe harvest

shortfalls due to seedling rot or late root rot (Zachow et al., 2010). In Europe, after the harvest in October and November, sugar beets undergo open storage on the field sites in so called beet clamps for a maximum of 60 days before being processed (Liebe et al., 2016). High sugar (18%) and water (76%) content as well as fresh wounds on the surface increase microbial contamination and sugar degradation during this time (Jaggard et al., 1997; Liebe et al., 2016). Saprophytic fungi such as *Fusarium* and *Penicillium* spp. invert the sucrose to glucose and fructose and therefore cause substantial sugar yield losses (Klotz and Finger, 2004). Overall, total losses up to 50- 60% caused by respiration, microbial degradation, synthesis of raffinose etc. are possible (Hoffmann, 2012; Kenter and Hoffmann, 2009). Currently, no treatment against microbial contamination is applied, however, microbial antagonists on the beet surface were found to carry potential disease preventing properties (Zachow et al., 2008). Hence, the study of the sugar beet microbiome after storage is crucial for future biocontrol application with such antagonists (Droby and Wisniewski, 2018).

Biocontrol application as sustainable alternative control measures

Microorganisms ubiquitously inhabit most parts of the world and the surface of fruits and vegetables is no exception. Most of these bacteria are not pathogenic, however, their contribution to fruit health, disease resistance and quality has not been fully explored (Berg et al., 2014a; Droby and Wisniewski, 2018). Microorganisms, isolated from the environment, were studied for decades for their antagonistic effects against fungal pathogens. This activity was soon discovered to have technological application in agriculture, e.g. plants inoculated with antagonistic strains were significantly more resistant to several diseases (Berg, 2009; Waksman and Woodruff, 1940). The applicability was not only given for growth or preharvest application but also after the harvest (Pusey and Wilson, 1988). However, the highly efficient biocontrol alternatives, even though they showed high activity, never achieved a major fraction of the postharvest pesticide market (Droby et al., 2009). Nevertheless, microbial biocontrol products are believed to have an increasing market in the future due to the withdrawal of chemical products (Glare et al., 2012). Apart from being environmentally sustainable, biocontrol products have additional advantages over chemicals and before mentioned VOCs applications. The application of living organisms, capable of producing VOCs and other antimicrobials, could reduce the number of treatments necessary to achieve the same effect (Pal and Gardener, 2006).

Technological advances through next-generation sequencing (NGS)

In recent years, technological advances based on next-generation sequencing (NGS) such as amplicon, metagenomics, or metatranscriptomics sequencing widened the understanding of the close interactions of microbes with their host (Knief, 2014). Plants were found to be colonized by thousands of different taxa, closely interacting with the plant and potential pathogens (Berg et al., 2014b; Bulgarelli et al., 2013). Plant associated bacteria and fungi do not only contribute to plant health during growth, but play also a major role after the harvest of fruits and vegetables (Droby and Wisniewski, 2018). Therefore, the biocontrol potential for postharvest applications, as previously discussed, is tremendous (Korsten et al., 1993). Even though the microbiome response to biocontrol and potential of microbiome-assisted biocontrol is broadly discussed in recent reviews (Droby and Wisniewski, 2018; Wisniewski and Droby, 2019), so far most studies are done with cultivation dependent methods (Massart et al., 2015). However, the interaction of antagonistic bacteria with the host microbiome contributes to the biocontrol efficiency and is therefore crucial for our understanding and future biocontrol strategies (Droby and Wisniewski, 2018; Massart et al., 2015).

More details related to this introduction were also described in a review article (**Chapter 1: Manuscript I: 'Microbiome-level approaches provide the key to naturally extend the postharvest storability of fruits and vegetables in the future')** authored by the candidate and colleagues. The focus of this chapter was on the increased potential for postharvest biological control treatments integrating novel technologies shown on the example of recently published studies on a variety of stored fruits and vegetables.

OBJECTIVES AND SUMMARY OF THE THESIS

The knowledge of the postharvest microbiome in fruits and vegetables is generally very limited, even though it is crucial for the storage stability. Therefore, a comprehensive approach studying the microbiome of sugar beets from the harvest to the storage was designed. The influence of disease on the microbiome of sugar beets on the field and in storage was explored using current NGS tools. By implementing complementary cultivation-dependent methods and real time qPCR, a holistic approach to capture the dynamics of the microbial community was conceived. Moreover, by comparing the two health statuses as well as the dynamics of the microbiome during storage, the studies present in this doctoral thesis provide new directions for biocontrol strategies including microbiome-assisted application of biocontrol agents for the future (Fig. 1). The main objectives of the thesis are shown in the box below.

Main objectives:

- Exploration of the on-field and postharvest microbiome of sugar beets.
- Identification of microbial indicators for disease in the field and in stored sugar beets.
- Identification of microbial signatures which are transferred from the field to storage.
- Exploration of microbiome-assisted application for storability increase and biocontrol.

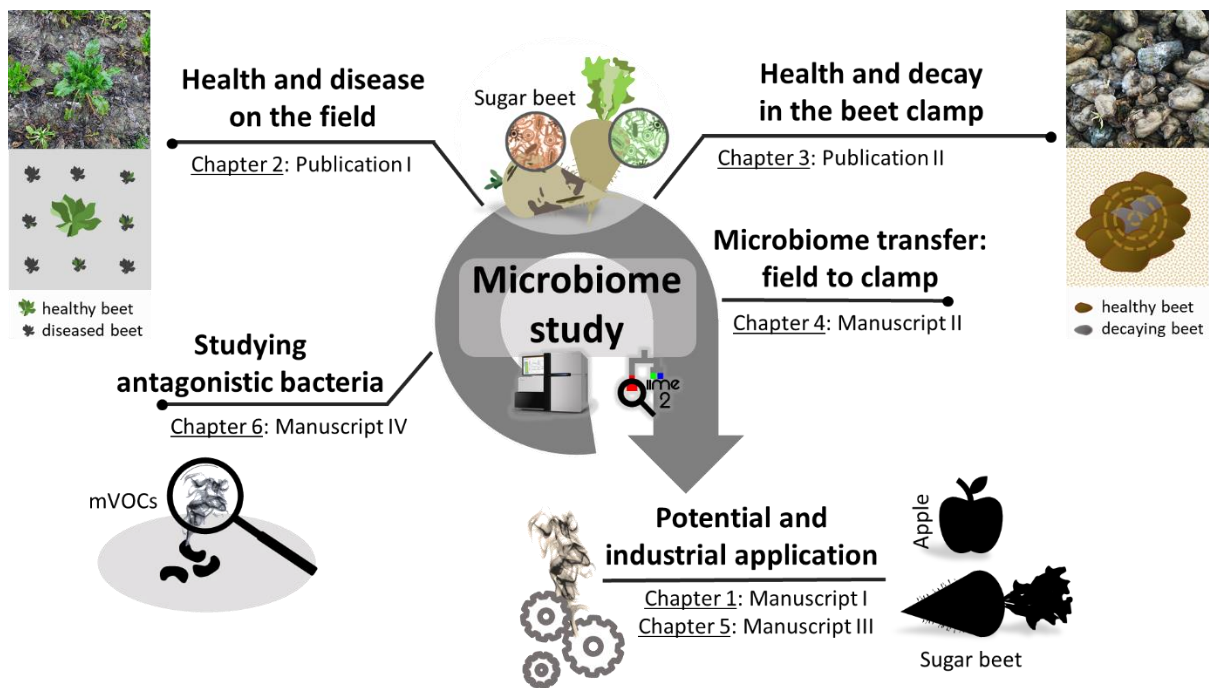

Figure 1: Graphical summary and connections of the different studies carried out in the frame of this doctoral thesis. With a comprehensive approach the potential of microbiome-assisted postharvest research for the development of biotechnological applications was shown.

Bacterial and fungal diversity is connected to disease on the field

The first aim of the thesis was to study the bacterial and fungal microbiome in sugar beet fields before harvest to gain insights into microbiome changes induces by disease. **Chapter 2: 'Publication I: Disease incidence in sugar beet fields is correlated with microbial diversity and distinct biological markers'** describes the microbial signatures associated with heath and diseased sugar beets on the field. Moreover, bacterial antagonism on the field was linked to the vulnerability to microbial shifts. Analyzing the data of over 70 sugar beets from 13 fields in Germany and Austria, microbial diversity changes as well as distinct microbial signatures were linked to the two health statuses. Healthy sugar beets from the field carried a significantly higher microbial diversity (bacterial and fungal microbiome) whereas disease induced microbial diversity loss. Severe taxonomic changes comparing healthy and diseased sugar beets, which were located spatially close in the field, were observed. Interestingly, low antagonism in healthy fields had no influence on the bacterial microbiome while the fungal microbiome was changed. Here, changes in the beta diversity as well as minor taxonomic changes were observed. In addition to the taxonomic changes observed when comparing the three groups (healthy with high antagonism, healthy with low antagonism, and diseased) functional predictions showed changes in trophic modes of the fungal microbiome. A lower proportion of symbiotrophic fungi as well as a significant increase of the saprotrophic group was found in diseased sugar beets on the field.

Based on the results of this study microbial signatures linked to a certain health status were defined. On the one hand, healthy sugar beets, carried a significantly higher abundance of *Plectosphaerella*, *Vishniacozyma*, *Flavobacteria*, *Sphingomonadales* and *Rhizobiales* in addition to a higher microbial diversity. Diseased sugar beets on the other hand carried a significantly higher abundance of *Candida*, *Fusarium*, *Penicillium*, *Lactobacillales* and *Enterobacteriales*. These indicator taxa could be used in the future for targeted disease control on the sugar beet field.

Characterization of microbial traits in the storage

The microbiome changes of sugar beets during storage dependent on different storage conditions were studied before (Liebe et al., 2016). However, the focus of this study was only on the fungal microbiome and did not connect microbiome changes to postharvest disease development. Therefore, in an amplicon sequencing approach investigating 120 sugar beets from six storage piles (beet clamps) in Austria and Germany was implemented and the microbial community changes induced by postharvest disease were observed. **Chapter 3: 'Publication II: Microbiome-driven identification of microbial indicators for postharvest diseases of sugar beets'** discusses the diversity and microbial trait

changes found. Additionally, a gradual disease development during storage was traced measuring microbial signatures using real-time qPCR.

By comparing cultivation dependent and independent methods the diverse fungal community of decaying sugar beets in beet clamps was explored. While *Penicillium* and *Fusarium* were the main fungal pathogens identified using cultivation dependent methods, a more diverse fungal community was observed using amplicon sequencing. *Penicillium*, as well as taxa not identified with cultivation dependent methods such as *Candida*, *Plectosphaerella*, *Vishniacozyma*, *Guehomyces* and *Mrakia* were found.

Overall, taxonomic differences between the samples were observed to be mostly due to health differences and to a lesser extent caused by regional differences induced by sampling location. Comparing healthy and decaying beets, a distinct microbial diversity decrease from healthy to decaying, as well as distinct taxonomic changes were observed. While *Flavobacterium*, *Pseudarthrobacter*, *Plectosphaerella* and *Vishniacozyma* were the predominant taxa in healthy sugar beets, decaying sugar beets showed increased levels of *Lactobacillus*, *Gluconobacter*, *Leuconostoc*, *Candida* and *Penicillium*. The introduced taxonomic groups within the fungal microbiome also showed a functional change with increased saprotrophic functions.

Specific primers designed to track changed taxa between the two groups (health and disease indicators) were used in real-time qPCR measurements to observe the gradual trend of the indicators during storage. A significant increase of disease indicators during storage of sugar beets with simultaneously sugar loss, analyzed using the standard sugar measurement techniques, was observed. The health indicators did not substantially decrease in spite of the microbiome study results, nevertheless a slight decrease was observed for *Vishniacozyma*. Therefor a strong correlation between sugar loss and the found disease indicators is suggested by the results of this study.

Connecting the dots: From field to beet clamp

Based on the data presented in **chapter 2 and 3** strong similarities in the microbiome data of field and storage samples were observed. The analogous diversity and taxonomic changes between the two groups in the field and the beet clamp were discussed in **Chapter 4: 'Manuscript II: On-field microbial community influences postharvest root rot in sugar beets'**. Healthy sugar beets generally had a higher microbial diversity compared to diseased beets. Microbiome shifts in the bacterial community from a healthy to a diseased/decaying microbiome were almost identical in the field and in the beet clamp. While *Flavobacteria* were significantly increased in healthy sugar beets, *Lactobacillus*, *Leuconostoc*, the family *Enterobacteriaceae* and *Gluconobacter* were found to be significantly increased in diseased

sugar beets. In the fungal microbiome, a higher degree of differences between the field and the stored sugar beets were found, however, the indicator species mentioned before: *Penicillium*, *Candida*, *Fusarium* and *Plectosphaerella*, and *Vishniacozyma* were found increased in diseased or healthy samples respectively.

Additionally, using bioinformatic predictions the microbiome transfer from the field to the beet clamp was calculated. Due to taxonomic similarities about 80% of the field microbiome can be found later on in the beet clamp microbiome. This way the field microbiome has a substantial influence on the storage microbiome. Therefore, it was suggested based on the results of this study, to use the observation of the disease indicators on the field beforehand to predict storability in the beet clamp.

Application of microbiome research in postharvest protection

Microbiome research using NGS tools had a tremendous impact on the performed research on plants, fruits and vegetables, giving a deep look into microbe structures in their natural environment (Abdelfattah et al., 2018a). Plants were found to closely interact with their inhabiting microbes and build a crucial protective shield for plant resistance against pathogens (Berg et al., 2016). This close interactions do not only play a role during growth of the plant but also after harvest of the fruits or vegetables in storage (Droby and Wisniewski, 2018).

Applicability to improve sugar beet storability

The discovered connection of sugar beet health with distinctive microbial signatures in this doctoral thesis were shown to be true for sugar beets in fields as well as in storage piles (**Chapter 2 and 3**). Health associated shifts were indicated by microbial taxonomic changes and could be quantified using microbial markers. Postharvest disease in sugar beets is connected to substantial sugar loss (Klotz and Finger, 2004). **Chapter 3** demonstrated that sugar loss can also be tracked by measuring the disease associated taxonomic markers. Moreover, the results shown in **chapter 4** showed the microbiome transfer from the fields into the storage. Therefore, the microbiome on the field has a tremendous impact on the beet storability and sugar yield. The analysis of disease indicators beforehand in the field could predict the storability of the beets and possible sugar loss during long storage periods. Moreover, the generated knowledge of this disease indicators could be utilized in the development of biocontrol agents. By screening for antagonistic strains against the main pathogens as well as the found disease indicators, postharvest disease could be potentially delayed. Nevertheless, further experimental data is needed to fully understand all connections and evaluate the proposed hypothesis.

Applicability with stored apples

A similar concept to the performed studies on sugar beet was also applied on stored apples. **Chapter 5: 'Manuscript III: Microbiome response to hot water treatment and potential synergy with biological control on stored apples'** discusses the microbiome changes in apples caused by postharvest hot water treatment (HWT) and disease. Apples were treated and stored for six months. After storage the microbiome of treated and untreated as well as healthy and diseased apples was compared. HWT induced only slight changes in the fungal community, however, fully protected the apples from postharvest disease. Healthy and diseased apples showed, similarly to the studies on sugar beets, substantial diversity and taxonomical changes. Microbial diversity loss as well as taxonomic shifts, especially in the fungal community, were associated with postharvest disease. While healthy apples showed a complex fungal microbiome mainly consisting of *Vishniacozyma*, *Cladosporium*, the order *Hypocreales* and the family *Didymellaceae*, over 80% of the diseased apple microbiome consisted of *Penicillium expansum* and *Neofabraea alba*. Additionally, based on qPCR quantifications the bacteria to fungi ratio shifted from a balance in healthy apples to a 1/99 ratio in diseased apples.

Lab-scale storage experiments confirmed the high efficiency of postharvest HWT, however, combined biocontrol strategies also using antagonistic bacteria showed an increased efficiency. Bacterial antagonists screened especially against *Penicillium* and *Neofabraea* were even more efficient applied as bacterial consortia. This way the incorporation of NGS and qPCR was shown to lead to a novel integrated biocontrol strategy. The results of this study suggest, that a combined approach of HWT with biocontrol, using control consortia, could provide an increased storability of apples.

1 Manuscript I: Microbiome-level approaches provide the key to naturally extend the postharvest storability of fruits and vegetables in the future

Peter Kusstatscher[ab], Tomislav Cernava[b] and Gabriele Berg[b]

[a] Austrian Centre of Industrial Biotechnology, Petersgasse 14, A-8010 Graz, Austria

[b] Institute of Environmental Biotechnology, Petersgasse 12, A-8010 Graz, Austria

Manuscript

ABSTRACT

The plant microbiota play an important role in plant health and productivity and have therefore become an interesting target for biotechnological applications. During the last decades, various beneficial microbes were isolated and shown to control not only preharvest diseases but also to be applicable for postharvest treatments. This has provided the basis to develop classical biocontrol agents (BCAs) and to gradually decipher their modes of actions. It became evident that complex interactions of BCAs with the native plant microbiota can contribute to the efficiency of the applied strains. Recent studies have shown that also indigenous microbes on harvested plant parts and fruits contribute to postharvest stability by interfering with pathogen infections. Next-generation sequencing-based techniques provide valuable tools for deepening investigations of fruit and vegetable microbiomes and their role in postharvest applications. The generated knowledge provides a new basis for postharvest treatments and initiates a new era of microbiome integrated strategies to overcome postharvest losses in fruits and vegetables. The traditionally used single strain BCAs might soon be replaced by community approaches to improve the biological control efficiency and to establish a healthy microbiome.

Keywords: Biocontrol; postharvest decay; NGS; increased storability

Introduction

Food loss is a major problem in modern society. According to the Food and Agriculture Organization of the United Nations (FAO) up to 45% of all fruits and vegetables produced are lost on their way to the consumer(FAO, 2011) (Fig. 1.1). Postharvest food loss can either be the direct quantitative loss e.g. during harvest, or more specifically result from losses in food quality e.g. water content loss or undesired sprouting (Aulakh and Regmi, 2013). Despite the substantial losses during processing and transport, a major loss in fruits and vegetables occurs also during storage. Here, the main causes are mold and bacterial contaminations (Bourne, 1977; Buchholz et al., 2018). Various fungi, mainly belonging to the genera *Alternaria*, *Aspergillus*, *Botrytis*, *Fusarium*, *Monilinia*, *Penicillium* and *Rhizopus* were found to cause the highest proportion of spoilage (Snowdon, 1990). Currently, these problems are faced by either increasing the production in order to account for losses, or by the usage of chemical fungicides. Even though numerous biological approaches have been developed during the past 30 years, biological products have never reached broad usage (Droby et al., 2009; Sharma et al., 2009). The public attention only increased in the past few years, after the withdrawal of key fungicides and the increasing need for alternative disease management technologies, that are both safe and effective (Droby et al., 2016).

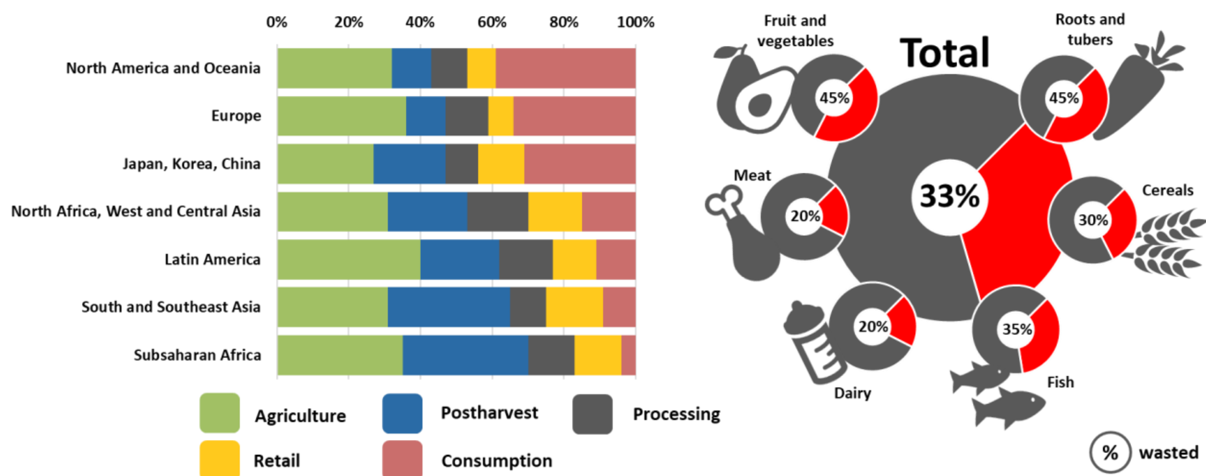

Figure 1.1: Analysis of global food loss for different regions and products. Data provided by the Food and Agriculture Organisation (FAO) of the United Nations (FAO, 2011).

Naturally occurring microorganisms, that are found as epiphytes on the surface or as endophytes in the tissue of fruits and vegetables, are highly diverse and comprise a great variety of microbial lineages. However, only a small fraction of them is known to be plant pathogenic (Berg et al., 2014a; Leff and Fierer, 2013). Despite their more evident role in several diseases, microbes are thought

to be key players in fruit health, quality and disease resistance (Droby and Wisniewski, 2018). This gave rise to the first biocontrol approaches that were developed in the late 1980s (Pusey and Wilson, 1988; Wilson and Wisniewski, 1989). Since then, relatively few changes in the general development of new biocontrol agents were made and adapted selection methods first described by Wilson and colleagues (Wilson et al., 1993) are still state of the art. The commonly used approach involves the isolation and screening of antagonistic single strains that carry the ability to establish quickly in wounded fruit tissue to prevent pathogenic infections. However, the impact of the introduction of these biocontrol strains into a versatile host microbiome is often neglected (Droby et al., 2016; Droby and Wisniewski, 2018). The technological advances in the past years starting from next-generation sequencing methods (NGS) to meta-omics technologies provide tools for a deeper understanding of environmental interactions with various plant hosts, diversity changes due to diseases, and biocontrol-microbiome interactions (Abdelfattah et al., 2018a; Berg et al., 2016). Novel studies track changes in the microbiome during storage of different fruits and provide this way a deeper understanding of microbial interplay in disease development (Abdelfattah et al., 2016).

This review will provide a brief history from the beginnings of microorganism-based biocontrol to the currently integrated approach of biocontrol with the host microbiome. However, the main focus is on the advances brought by modern technology exploring the postharvest microbiome and their compatibility with classical methods.

Developments and products in classical microbe-based biocontrol

The research on microbial antagonists as biocontrol agents has a long history and started with the first few studies published in the late 70s and 80s (Pusey and Wilson, 1988; Tronsmo and Raa, 1977). However, after the prohibition of several existing fungicides in the past years, the research interest in new biological alternatives gradually increased and hundreds of research papers and reviews were published (Droby et al., 2009; Sharma et al., 2009). Despite the potential of microbial antagonists to control several postharvest pests, the market for biological products is still small and only a few products found their niche whereas others were discontinued after a short period (Wisniewski et al., 2016). Facilitated by new regulations and an increased consumer awareness, the market is estimated to grow constantly in the next years (Glare et al., 2012).

Biocontrol strains are usually isolated from the natural epiphytic microflora of fruits and vegetables (Droby et al., 2009; Liu et al., 2013). Some of the best-studied biocontrol bacteria are *Bacillus subtilis* (Wilson, 1985), *B. amyloliquefaciens* (Arrebola et al., 2010), *B. megaterium* (Kong et al., 2010), *Pseudomonas syringae* (Bull et al., 1997), *Erwinia herbicola* (Bryk et al., 1998) and *Pantoea*

agglomerans (Usall et al., 2008). Several products using these strains came on the market such as BioSafe® (Jet Harvest Solutions, Longwood, FL, USA) based on *Pseudomonas syringae* for the control of potato and sweet potato diseases; Serenade® (Bayer, Leverkusen, Germany), using *B. subtilis* against diseases in stone and pome fruit, tomato and strawberry; Pantovital® (Domca, Granada, Spain), containing *P. agglomerans* and used on different fruits; and Amylo-X® (Biograd CBC, Grassobbio, Italy) using *B. amyloliquefaciens* also used on different fruit varieties.

Numerous strains of yeast were also studied for their disease control ability (Droby et al., 2009; Liu et al., 2013). Investigated strains including *Pichia guilliermondii* (Wilson et al., 1993), *Cryptococcus laurentii* (Filonow, 1998), *Candida oleophila* (Lima et al., 1997), *Candida sake* (Nunes et al., 2001), *Metschinkowia fructicola* (Kurtzman and Droby, 2001) and *Aureobasidium pullulans* (Lima et al., 1997) showed great biocontrol potential. Several product using those strains were commercialized: Candifruit® (IRTA, Leida, Spain) using *Candida sake*; Nexy® (BioNext, Paris, France) based on *Candida oleophilia*, BoniProtect® (Bio-ferm, Tulln, Austria) based on *Aureobasidium pullulans* and Shemer® (Koppert, The Netherlands) containing *Metschinkowia fructicola*.

Despite the limited market and often short lifespan of the developed products, microbial biocontrol approaches were shown to have a great potential in substituting chemical fungicides currently used, also in the prospect of many currently used fungicides facing a withdrawal in the next years due to new regulations (Wisniewski et al., 2016).

Approaches of combining biocontrol agents with classical treatments was also shown to be of great potential (Droby et al., 2009; Lima et al., 2008). Lima and colleagues showed the potential of integrating biocontrol yeasts with pesticides to lower the levels of fungicide residues with simultaneous comparable control of disease on apples (Lima et al., 2011). Similarly, the combination of hot water treatment and biocontrol was also effective on peaches (Zhang et al., 2010) and on apples (Wassermann et al., n.d.). The elucidation of the modes of action of biological treatments increased especially the research on microbial volatile organic compounds (mVOCs). Induced systemic resistance as well as the use of mVOCs for biocontrol applications are therefore gaining more interest in the last years (Bailly and Weisskopf, 2017). New concepts, combining microbial with classical treatments and the use of synthetic consortia will be of great research interest in the future (Droby et al., 2016).

Next-generation sequencing as a powerful tool for postharvest monitoring

In the past years several next-generation sequencing (NGS) approaches were developed to study plant-microbe interactions (Knief, 2014). Currently, the most used technology, barcoded amplicon sequencing or metabarcoding, involves the amplification of specific marker gene regions (e.g. 16S &

18S rRNA gene fragments, ITS region) to study the microbial community associated with the samples. Other techniques, based on shotgun sequencing, assess the metagenome or the metatranscriptome of the microbial community. For this metagenomics approaches the total community DNA of the samples is extracted and fragmented. After sequencing common workflows include reassembly of contigs, taxonomic binning, and annotation with reference databases (Kuczynski et al., 2011). Metatranscriptomics approaches (RNAseq) are based on similar workflows on the mRNA level. Following in vitro or in silico rRNA removal, the remaining RNA is re-transcribed to cDNA and sequenced on an NGS platform. This provides information about genes that are transcribed in the microbial community under certain conditions (Massart et al., 2015). Additional information can be gained using other "omic"-techniques such as metaproteomics, studying the proteins (Schneider and Riedel, 2010) or metabolomics, studying the metabolites in the microbial community and the plant (Cevallos-Cevallos et al., 2009).

Microbiome research, enabled by modern NGS and –omics tools, highlighted the importance of microorganisms not only in the human body but also in our environment. Microorganisms were found to play an important role for both plant health and productivity (Berg et al., 2014b; Bulgarelli et al., 2013). Additionally, the plant microbiome was also shown to contribute to phenotypic and epigenetic plasticity as well as evolution (Partida-Martinez and Heil, 2011). The plant microbiome, especially in the rhizosphere, is highly plant-specific (Smalla et al., 2001). Plant root exudates, which play an important role, both as chemo-attractants as well as repellants, also attract specific bacterial species from the surrounding soil (Berg et al., 2014b). Plant-associated bacteria not only contribute to the health of a plant during growth, but are also involved in fruit and plant part stability after harvest (Droby and Wisniewski, 2018). Although numerous microorganism-based products were developed over the past years (Droby et al., 2009), only a few studies investigated the establishment and impact of the antagonistic strains on the plant microbiome. Moreover, most of the available studies were conducted with classic cultivation-dependent methods (Massart et al., 2015). So far, we know that the interplay of antagonistic bacteria with the host microbiome, in addition to environmental conditions, plant species and physiological state, plays an important role in biocontrol efficiency (Massart et al., 2015).

The available NGS and „omic"-tools provide a deeper look into the numerous mechanisms during postharvest storage of fruits and vegetables and assist in the development and monitoring of postharvest treatments. Studying the community dynamics and shifts over the storage period as well as the establishment of biocontrol agents within the community could not only gain deeper insights into the microbial community but also increase the efficiency of future biocontrol treatments. Moreover, the knowledge about community dynamics combined with external factors such as

temperature, moisture etc. could increase the understanding of the most crucial parameters during postharvest storage.

The current knowledge base in postharvest microbiome research

So far, the importance of the postharvest microbiome was highlighted in several reviews (e.g. (Droby and Wisniewski, 2018; Massart et al., 2015; Wisniewski and Droby, 2019)) however, there is only a handful of studies implementing NGS methods in the development of novel postharvest solutions. Nevertheless, the pioneering studies have indicated a high potential and exploitability of this technology for novel approaches to increase postharvest storability. One of the major findings relates to microbial diversity, which is also an important indicator for plant and human health (Berendsen et al., 2012; Hooper and Gordon, 2001). The analysis of microbial communities associated with stored onions showed changes in the microbiome between healthy and diseased onions. While only a few unique taxa were identified for each health status, the diversity and abundance of the microbial community drastically shifted when the onions were affected by storage diseases (Yurgel et al., 2018). A study on mango fruit indicated substantial microbiome changes of the fruit during cold storage and subsequent storage on the shelf (Diskin et al., 2017). Additionally, the prevalence of specific fungal and bacterial taxa were associated with the occurrence of mango stem end rot. In a recent approach that included large NGS libraries from multiple locations the severe differences between the health status of sugar beets on the field and in storage was shown. Microbial diversity as well as composition was completely changed comparing a healthy and a decaying sugar beet (Kusstatscher et al., 2019a, 2019b). Abdelfattah and colleagues showed the microbiome changes induced by fruit flies in olive (Abdelfattah et al., 2018b). Fruit fly infection caused diversity reduction and taxonomical shifts within the community. Solanki and colleagues were the first to describe microbial changes induced by pesticide fumigation of wheat (Solanki et al., 2019). Phosphine treatment reduced the bacterial diversity, however did not affect the fungal community.

Additional studies investigating multiple fruits and vegetables highlighted the uniqueness of each plant microbiome. Studying the microbial taxa associated with different foods such as cucumbers, cilantro and mung bean sprouts, showed the unique composition of each foods microbiome as well as the impact of disease associated taxa (Jarvis et al., 2018). Wassermann and colleagues investigated the microbiome of raw eaten *Brassica* vegetables and showed that different genetically related vegetable types carry distinct microbiomes (Wassermann et al., 2017). Postharvest microbiome studies done on apple showed that the microbiome differs not only between fruit type but also between sampling

locations on the fruit. Additionally, the influence of managing practices was shown for the fungal and the bacterial community (Abdelfattah et al., 2016; Wassermann et al., 2019).

All these studies increase the understanding of postharvest microbial community dynamics under different conditions. They also indicate that storage rot is often not caused by one species, but is an interplay of several different factors such as microbial diversity and species pathogenicity. Moreover, postharvest disease is not predictable by the occurrence of pathogenic strains within the community, it rather seems that indigenous pathogens can become prevalent following decreases in community diversity or compositional changes (Fig. 1.2).

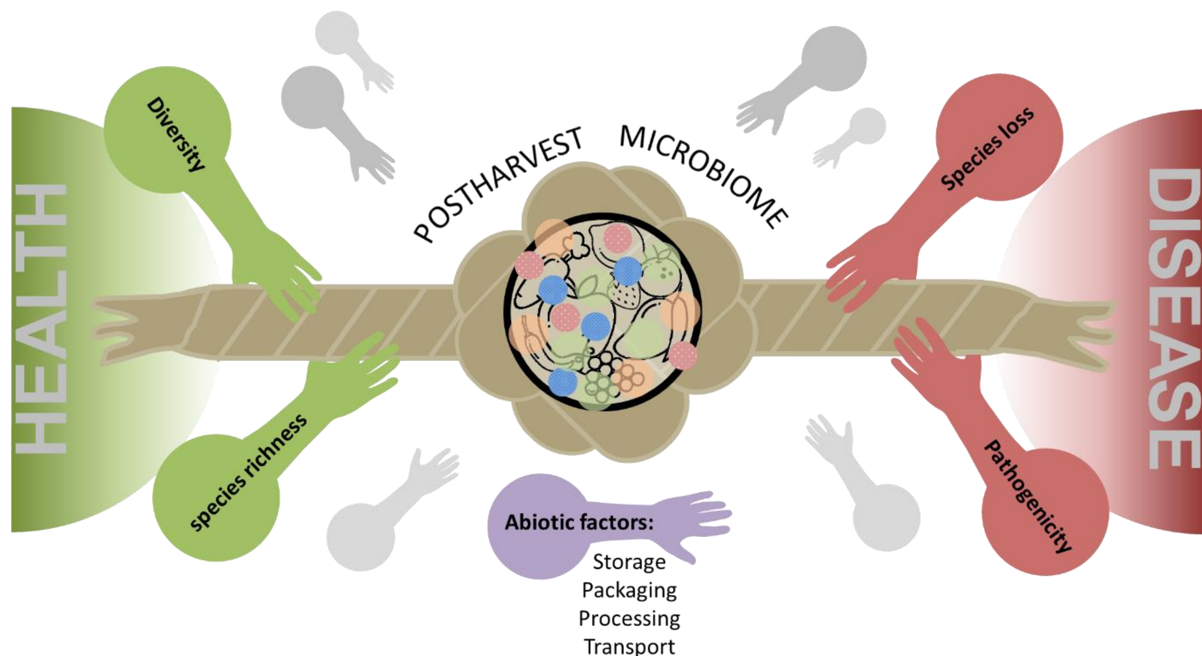

Figure 1.2: Schematic representation of the impact of different biotic and abiotic factors on the postharvest fruit and vegetables microbiome. Factors that are generally considered as beneficial for postharvest storability are shown in green, while those with negative impacts are shown in red. Important abiotic factors that can induce shifts in either direction are shown in purple.

Conclusion

The study of the postharvest microbiome just recently became topic of several researchers worldwide. The latest developments in NGS methods as well as the challenging changes in postharvest technologies over the last years drove the progress of integrating classical postharvest biocontrol with state-of-the-art bioinformatics tools. Some pioneering studies have shown the importance and opportunities of these developments and we are gradually learning more and more about the close interactions of fruits and vegetables with microorganisms even after the harvest. This new knowledge will be likely integrated in future developments of biological control applications to fully understand the interactions of biocontrol agents with the indigenous microbiome.

2 Publication I: Disease incidence in sugar beet fields is correlated with microbial diversity and distinct biological markers

Peter Kusstatscher[ab], Tomislav Cernava[bc], Karsten Harms[d], Johann Maier[d], Herbert Eigner[e], Gabriele Berg[b] and Christin Zachow[a]

[a] Austrian Centre of Industrial Biotechnology, Petersgasse 14, A-8010 Graz, Austria

[b] Institute of Environmental Biotechnology, Petersgasse 12, A-8010 Graz, Austria

[c] Roombiotic GmbH, c/o: SciencePark, Stremayrgasse 16/IV, 8010 Graz, Austria

[d] Südzucker AG, Maximilianstraße 10, 68165 Mannheim, Germany

[e] Agrana Research & Innovation Center, Josef-Reither-Straße 21 – 23, 3430 Tulln, Austria

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ABSTRACT

Sugar beets (*Beta vulgaris* L.), which are one of the major sources for sugar, alternative energy and fuel, are affected by several fungal pathogens at harvest time. In order to identify correlations between the microbiome of field-grown sugar beets and their health status before harvest, we studied 2,200 antifungal antagonists together with 73 amplicon datasets obtained with 16S rRNA gene fragments as well as the fungal ITS region in samples from 13 different field sites in Austria and Germany. Overall a substantial loss of microbial diversity (bacteriome H': 8 vs. 6.5; mycobiome H': 4.5 vs. 3.5) as well as a substantially different taxonomic composition was observed in root rot-affected sugar beets when compared to healthy beets. The Gram-positive *Lactobacillales* as well as distinct fungal taxa such as *Candida*, *Penicillium* and *Fusarium* were identified as indicators of root rot on microbiome level. In contrast, higher microbial diversity as well as distinct fungal genera assigned to *Vishniacozyma* and *Plectosphaerella* were associated with the microbiome of healthy plants. The taxonomic shifts in the fungal microbiome were accompanied by trophic specialization; pathotrophic and symbiotrophic fungi were replaced by saprotrophic fungi in diseased sugar beets. Moreover, samples with high proportions of antagonistic bacteria were not vulnerable to shifts in the fungal microbiome. The overall findings show implications between microbial antagonists and plant health as well as key taxa that are indicative for the health status in beets. They provide the basis for the development of improved disease management systems, and preventive counteractions.

Keywords: Agriculture; Microbiome; Microorganism

Introduction

Plants live in close relationships with their microbiota and are thus often considered as holobionts (Berg et al., 2016; Vandenkoornhuyse et al., 2015). They offer different colonization niches for microorganisms that established mutualistic, neutral or parasitic interaction with the plant (Compant et al., 2010; Raaijmakers et al., 2009). These associations were formed as early as during the Devonian and as evidenced by fossil records (Krings et al., 2012). In these co-evolved symbioses, microbes colonizing the plant rhizo- and endosphere are plant-specific (Berg and Smalla, 2009) and can fulfill different roles to support their host's health and productivity (Berg, 2009; Lugtenberg et al., 2002). Not only the prevalence of microbial pathogens but especially microbial diversity in general was found to be a key factor for plant health and prevention of pathogen outbreaks (Mendes et al., 2011; van Elsas et al., 2012; Yan et al., 2017). Detailed functional understanding of beneficial, plant-colonizing microorganisms and their modes of action in the large microbial community is crucial for the development of biological applications in agriculture (Berg et al., 2017, 2014b).

Sugar beet (*Beta vulgaris* L.) is a herbaceous dicotyledonous plant, mainly grown for the production of sugar (sucrose content up to 18%) in temperate regions (Trebbi and McGrath, 2004). In the last years, its importance for biotechnological processes and as alternative energy source has increased (Maung and Gustafson, 2011). The seasonal yields in the main growing regions (Northern Europe and North America) are around 55 tons of sugar beets or 8 tons of white sugar per hectare and mainly depend on climatic factors and crop rotation strategy (Götze et al., 2017; Kenter et al., 2006; Pervin and Islam, 2015). However, also various fungal pathogens can diminish the yearly harvest and cause substantial economic losses at all plant stages. While *Aphanomyces cochlioides* Drechsler (Drechsler, 1928) and *Pythium ultimum* Trow (Osburn et al., 1989) cause root and seedling rot in an early stage of plant development, *Sclerotium rolfsii* Sacc., *Rhizoctonia solani* Kühn (Kiewnick et al., 2001) and *Cercospora beticola* Sacc. (Weiland and Koch, 2004) can cause severe harvest losses during later growth stages (Zachow et al., 2010). After harvest, Fusarium spp., often already present in the mycobiome of the harvested beets, can cause storage rot and lead to potentially high sugar yield losses (Liebe et al., 2016). In order to reduce disease incidence, various chemical fungicides are currently used, while biological treatments only play a minor role (Bartholomäus et al., 2017; Georgakopoulos et al., 2002). Natural antagonists of plant-pathogenic fungi, however, are a promising alternative for plant protection (Berg, 2009). The indigenous microbiome of sugar beets and their ancestors was shown to carry microbial antagonists with the potential of averting fungal infection (Zachow et al., 2014, 2008). When grown in fields, individual sugar beets are not equally affected by prevalent pathogens and some remain unaffected even under high pathogen pressure. This varying disease

severity on the field most likely results from varying pathogen pressure or protection by beneficial microorganisms present in the rhizosphere of single sugar beet roots (Büttner et al., 2008; Zachow et al., 2014).

While it is already known that plants interact closely with their colonizing microbiota, knowledge related to how whole microbial communities influence plant health is still scarce and requires evaluations at microbial community level (Berendsen et al., 2012). We hypothesized that the health status of the plants will not only depend on the occurrence of specific beneficial species but be reflected by the whole prevalent bacteriome as well as the mycobiome. Therefore, we selected healthy and diseased sugar beets from infected fields in Austria and Germany before harvest and assessed specific signatures in their microbiomes. The antagonistic potential of bacterial isolates, obtained from healthy beets, was analyzed in order to assess differences in the microbial community composition related to the frequency of antagonistic bacteria.

Material and Methods

Sugar beet sampling and isolation of bacteria

Healthy (n= 46) and diseased (n= 27) sugar beets were obtained from different fields in Austria (Upper Austria) and Germany (Bavaria). The exact location of the sampling sites is provided in Table 2.1. Fields were selected for their disease (beet rot) frequency and sampled shortly before harvest. Healthy sugar beets that were surrounded by diseased plants were targeted (Fig. 2.S1). In addition, diseased sugar beets from the surrounding areas were sampled and used as comparison in the microbiome study. After arrival in the laboratory within 24 hours, 20 g of the healthy sugar beet skin was peeled and washed with 50 mL of sodium chloride solution (0.85%) in a stomacher (Bagmixer; Intersciences, St. Nom, France) for 3 min. The obtained solution was used for total community DNA extractions and simultaneously plated in dilutions on nutrient agar (Sifin, Berlin, Germany) after heat treatment (90°C, 30 min) to screen for spore forming bacteria. A total of 2,200 bacterial strains (48 per sugar beet) were picked after two days of incubation and preserved in 96 well plate glycerol stocks (30% glycerol) at -80°C. For the diseased sugar beets, 20 g of peel was washed with 50 mL of sodium chloride solution (0.85%) by homogenizing for 3 min. The obtained solution was used for total community DNA extractions.

Antagonistic activity tests against plant pathogens

The bacterial strains from the isolation approach were tested for their antagonistic activity against a saprophytic fungal isolate *Fusarium oxysporum* 108_10C (strain collection of the Institute of

Environmental Biotechnology - Graz University of Technology), previously isolated from diseased sugar beets. The dual culture assays were conducted on Waksman agar (pH 6.8) containing 5 g/L sodium chloride (Carl-Roth, Karlsruhe, Germany), 5 g/L pepton (Carl-Roth), 10 g/L glucose (Carl-Roth), 3 g/L yeast extract (Carl-Roth) and 18 g/L agarose (Carl-Roth), as described by Berg and colleagues (2002) (Berg et al., 2002). Growth inhibition was documented by measuring the inhibition zone and this way the antagonistic potential from each field was determined.

DNA extraction and amplicon library construction

A total of 4 mL homogenate obtained in the initial samples preparations steps (described above) was centrifuged at 13,000 g, 20 min and 4 °C. Pellets were stored at -70 °C until further use. Total community DNA was extracted from the samples using the FastDNA® Kit for Soil (MP Biomedicals/USA). In the first step, the pellet was re-suspended in 978 µL Sodium Phosphate buffer. All other steps were conducted according to the manufacturer's instructions. The DNA extracts were used for 16S rRNA gene and ITS (internal transcribed spacer) amplicon amplifications. The primer pair 515f (GTGYCAGCMGCCGCGGTAA) and 926r (CCGYCAATTYMTTTRAGTTT) targeting the complete hypervariable region 4 of the 16S rRNA gene and the ITS1 region primer pair ITS1f (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC) were used. Both primer pairs were modified with specific primer pads (TATGGTAATT/AGTCAGCCAG) and linkers (GT/GG) for the attachment of sample-specific Golay barcode sequences, as described in the protocols and standards section of the Earth microbiome project (Walters et al., 2015).

The PCR was conducted in two steps. In the first PCR step the above mentioned primer pairs were used. In a secondary PCR step, barcode sequences were attached to the modified primer pads and linkers for multiplexing. All PCR reactions were conducted in triplicates. The first PCR (amplification of the V4 region or ITS region) was performed in a total volume of 10 µL (1 µL DNA, 2 µL Taq&Go, 0.1 µL of each primer, 0.15 µL of mPNA and pPNA and 6.5 µL of water). The amplification blockers mPNA and pPNA were added to prevent the amplification of mitochondrial and chloroplast DNA (Lundberg et al., 2013). The reactions were performed on a Whatman Biometra® Tpersonal and Tgradient thermocycler (Biometra GmbH, Göttingen, Germany) with the following settings: 95 °C for 45 s, 78 °C 5 s, 55 °C 45 s, 72 °C 90 s (35x), including an initial denaturation of 5 min at 95 °C and a final extension of 5 min at 72 °C. In the second PCR (multiplexing with Golay barcodes) a total volume of 30 µL (2 µL of the first PCR (template), 6 µL Taq&Go, 1.2 µL of barcode-primers and 19.6 µL of water) run at the following settings: 95 °C for 30 s, 53 °C 30 s, 72 °C 30 s (15×), including an initial denaturation of 5 min at 95 °C and a final extension of 5 min at 72 °C. After each PCR amplification step, the quality was checked by gel electrophoresis. All three technical replicates of quality checked PCRs from each sample

were pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) according to the manufacturer's protocol. Equimolar DNA concentrations of each barcoded amplicon sample were sent to GATC Biotech AG, Konstanz, Germany. After entry quality control and adapter ligation, 16S rRNA gene fragment and ITS region amplicons were sequenced on an Illumina HiSeq instrument with paired-end sequencing (2 × 300 bp).

Initial bioinformatic data analysis within the QIIME pipeline

Paired-end amplicon sequences obtained on the Illumina HiSeq platform were analyzed in a combined approach with QIIME 2 (2018.6 release) and QIIME 1.9.1 (Caporaso et al., 2010) (Table 2.S1). After joining reads and barcode extraction in QIIME 1.9.1 the data was transferred to QIIME 2. The data was demultiplexed and the DADA2 algorithm (Callahan et al., 2016) was applied to denoise truncated reads and to generate sequence variants (SVs), which were then summarized in a feature table. To increase the quality chimeric data as well as mitochondria and chloroplast reads (for 16S data) or bacteria and archaea reads (for ITS data) were excluded from the dataset by filtering. A total of 6,619,417 ITS and 1,762,913 16S reads were assigned to 3,233 and 9,480 SVs respectively (Table 2.S2). Alpha and beta diversity analysis were performed using the QIIME 2 core diversity metrics and group significance tests the dataset was rarefied to a depth of 2,400 reads (16S data) and 11,000 reads (ITS data) and rarefaction analysis indicated this was sufficient sequencing depth to capture the species diversity in all samples (Fig. 2.S2). For taxonomic assignment a Naïve-Bayes classifier was trained on the SILVA v123 (Quast et al., 2013) at 99% similarity as well as the UNITE v7 database (Kõljalg et al., 2013). The confidence threshold for feature classification was set to the default value of 0.7.

Bioinformatic comparison of different sample groups. Feature tables were split into three groups according to their health status (high, low and diseased) and each group filtered for the core microbiome (features present in 50% of the samples), which revealed in total 223 bacterial and 243 fungal core features. After remerging the respective core microbiomes networks based on the identified SVs were generated within QIIME 1.9.1. with the 'make_OTU_network' script using collapsed feature tables generated in QIIME 2 and visualized in Cytoscape 3.6.1 (Shannon et al., 2003). Significant taxonomic differences between the groups were observed with the ANCOM test in QIIME 2. Venn Diagrams were calculated with a web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). Functional analysis of fungal feature tables was performed using the FUNGuild online tool (http://www.stbates.org/guilds/app.php).

Statistical analysis of bioinformatics data. Alpha and beta diversity were analyzed within the QIIME 2 pipeline. In order to assess their significance, the implemented Kruskal-Wallis (alpha diversity)

and the anosim test (beta diversity) were used. Significant taxonomic differences between the groups were assessed with the ANCOM test in QIIME 2.

Deposition of sequence data. Raw data for each sample used in this study was deposited at the European Nucleotide Archive (ENA; https://www.ebi.ac.uk/ena) in the fasta-file format and is available under the Bioproject accession number PRJEB28861.

Results

Antagonistic potential on different sugar beet fields

In order to screen for bacterial antagonists against the fungal pathogen *Fusarium oxysporum*, bacteria were sampled from 13 fields in Austria and Germany. The antagonistic potential was evaluated with dual culture assays conducted with 2,200 bacterial strains obtained from healthy sugar beets within highly infected sugar beet fields (Table 2.1). A total of 257 bacterial antagonists, which occurred in distinct fields (Dierneder Staffling (118), Moarfeld (51), Gilsenöd-Blindmühl (33), Wirtsacker (18) and Berg am Spitz (10)), showed a high antagonistic potential, while there were no or lower proportions of antagonistic bacteria in other fields (Table 2.1).

Table 2.1: Antagonistic potential of bacteria isolated from different fields. High occurrence of antagonistic isolates (>6% of tested isolates) is highlighted in gray.

Field	Country	GPS	Samples	Antagonists [% of isolates]	Antagonists [#]
Dierneder Staffling	AT	48.202976, 14.627077	5	49.17	118
Moarfeld	AT	48.206228, 14.625774	5	21.25	51
Gilsenöd-Blindmühl	DE	48.763281, 12.836881	6	11.46	33
Wirtsacker	DE	48.765223, 12.783715	5	7.5	18
Berg am Spitz	AT	48.198215, 14.627236	3	6.94	10
Neuer Schlag	DE	48.818079, 12.922237	1	6.25	3
Vierhofener Feld	DE	48.675090, 12.997164	5	3.33	8
Haidfeld	DE	48.818879, 12.919264	2	3.13	3
Rettenbach	DE	48.812911, 12.887295	6	3.13	9
Waldweg	DE	48.810239, 12.906380	5	1.67	4
Dorffeld	AT	48.200278, 14.624533	2	0	0
Straßk. Moosweg	DE	48.827327, 12.752367	1	0	0
Kiefelfeld	DE	48.812026, 12.908140	1	0	0

Microbial diversity is significantly decreased in diseased sugar beets. Amplicon data obtained from 73 samples revealed 223 bacterial and 243 fungal core OTUs. The comparison of healthy and diseased sugar beets showed overall a lower microbial diversity (alpha diversity) in diseased samples (Shannon index: 6.5 in 16S samples and 3.5 in ITS samples) compared to the microbiome of healthy sugar beets (Shannon index: 8.0 in 16S samples and 4.5 in ITS samples). The calculated beta diversity showed significant differences (p-value = 0.001) in the composition of the microbiomes of the two groups. All samples of diseased sugar beets (n= 27) clustered significantly (p-value= 0.001) different from samples of healthy sugar beets (n= 46) (Fig. 2.1A).

Samples from the healthy sugar beets were further split into two groups, representing high (n=23) and low (n=23) proportions of antagonists (according to Table 2.1). The threshold for clustering was set to 6% antagonist incidence. The Shannon indices of the three sub-groups were compared in order to further assess differences in alpha diversity (Fig. 2.1B). There was no significant difference between the two healthy groups in the 16S rRNA gene amplicons, however there was a significant difference between all three groups in the ITS region amplicons.

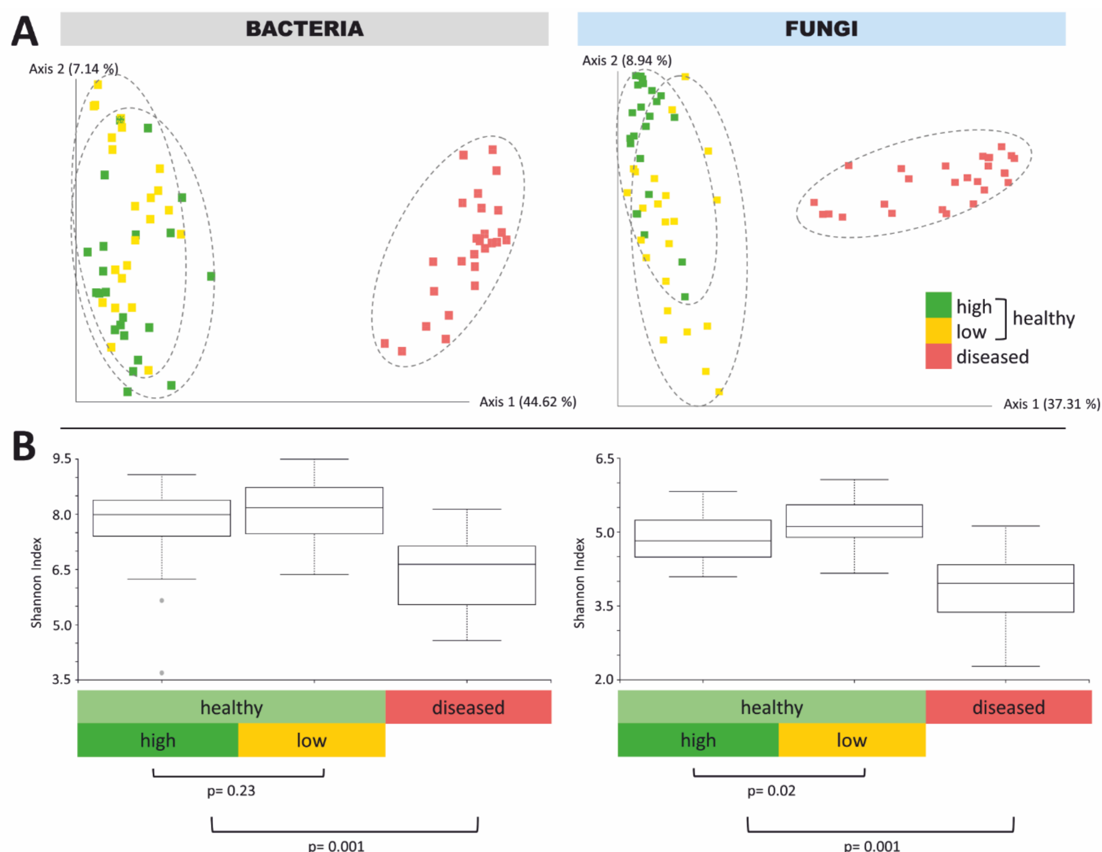

Figure 2.1: Microbial diversity of sugar beets grown in fields of Austria and Germany. (A) PCoA plots show the bacterial and fungal samples indicated with dots. Significant differences (p≤ 0.05) can be obtained from a total of 46 healthy and 27 diseased samples. Distances shown in the plot are based on the Bray Curtis diversity metrics. (B) Shannon indices of healthy (high (>6% antagonists) and low (<6% antagonists)) and diseased sugar beets are shown in boxplots. The significance of the differences between different samples is indicated through p-values obtained with pairwise Kruskal-Wallis tests.

The core microbiome composition differs between healthy and diseased sugar beets

Bioinformatics analysis of the core microbiomes from healthy and diseased sugar beets showed a clear difference in the community compositions. This was observed with the bacterial as well as the fungal data when comparing healthy and diseased groups (Fig. 2.2). *Proteobacteria* (36%), mostly from the class *Alphaproteobacteria* (14- 22%), made up the biggest fraction of taxa in both, healthy and diseased, samples. *Bacteroidetes* (16- 20%) and *Actinobacteria* (10- 14%) were also among the predominant phyla in healthy as well as diseased samples. The phylum of *Firmicutes*, with an average abundance of 2% in the healthy compared to 37% in the diseased samples, showed the biggest difference between those two groups. While at the order level *Lactobacillales* (average abundance: 35%) and *Enterobacteriales* (10%) were the predominant taxonomic groups in diseased sugar beet roots, healthy samples, on the other hand, where *Flavobacteriales* (15%), *Rhizobiales* (12%) and *Sphingomonadales* (8%) were the most abundant taxonomic groups, showed a more balanced distribution between all of the taxa.

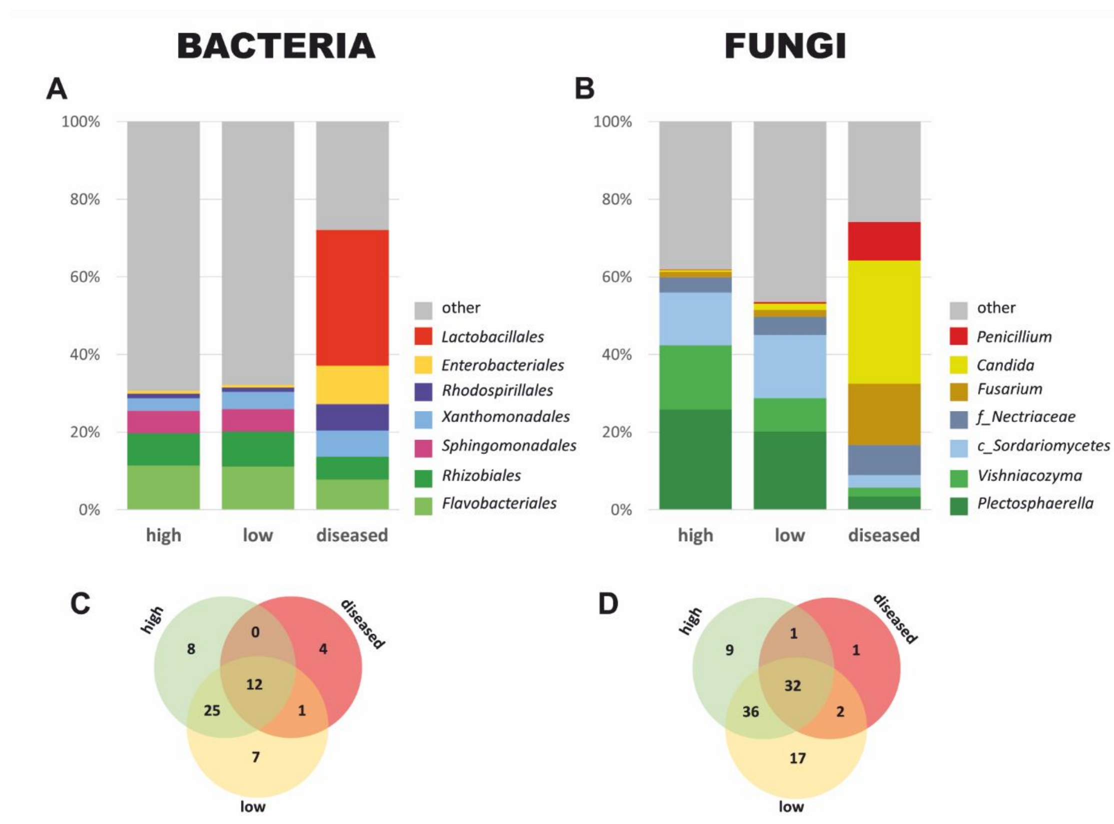

Figure 2.2: Relative composition of the bacterial and fungal core microbiome in different sugar beet samples. Healthy sugar beet samples are clustered into “high” and “low” according to their antagonistic potential in Table 1. Bacterial taxa are shown on the left, fungi on the right side. The most abundant (>5%) bacterial taxa are shown at order level (A), (>7%) fungal taxa at genus level, or when specifically indicated at f_: family level and c_: class level (B). Venn diagrams show the shared and unique number of taxa for each sample group.

In the fungal dataset, *Ascomycota* with an average abundance of 80% in healthy and 90% in diseased samples were the main feature fraction of all samples. *Basidiomycota* (17% in healthy, 4.5% in diseased samples), *Mortierellomycota* (1.5% in healthy, 0% in diseased samples) and unassigned phyla (3% in healthy, 7% in diseased samples) highlighted the first differences between the two groups. At the class level *Sordariomycetes* (53%), *Tremellomycetes* (15%) and *Dothideomycetes* (12%) were the most prominent OTUs in healthy samples, while *Sordariomycetes* (38%), *Saccharomycetes* (34%) and *Eurotiomycetes* (8%) were prominent in diseased sugar beets. On genus level, the distinct change from a healthy to a diseased mycobiome is the most visible. A change in taxonomic groups such as *Plectosphaerella* (22%) and *Vishniacozyma* (12%) in the healthy beet samples to *Candida* (31%), *Fusarium* (16%) and *Penicillium* (8%) in the diseased samples was observed. Both healthy groups (high and low antagonism), however, were shown to be very similar in the bacteriome. Only the fungal microbiome showed a decrease of *Vishniacozyma* (16% down to 8%) as well as *Plectosphaerella* (25% down to 20%) species from high to low antagonism. To highlight the less evident differences in the fungal microbiome, a network analysis was performed. A lower diversity as well as taxa such as *Candida*, *Fusarium* and *Penicillium* species had a higher prevalence in the microbiome of diseased sugar beets, whereas a higher total abundance as well as taxa such as *Plectosphaerella* or *Vishniacozyma* species were mostly found in both of the healthy groups (Fig. 2.3). *Plectosphaerella* (-5 percent points) and *Vishniacozyma* (-8 percent points) species were decreased in low antagonistic samples while *Candida* (+1.5 percent points) as well as most of the shared taxa with the high antagonistic group were increased.

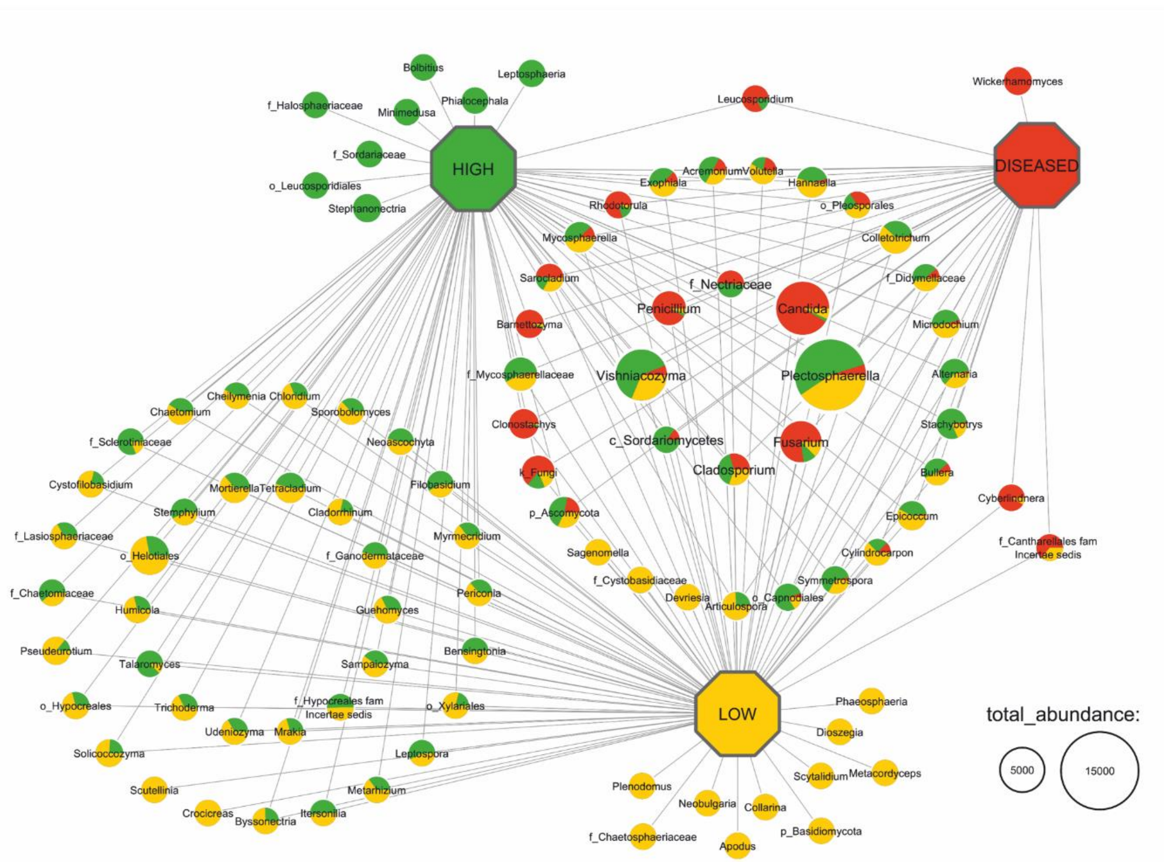

Figure 2.3: The shared fungal taxa between high (>6 % antagonists), low (<6 % antagonists) and diseased groups are shown in an OTU-network. Pie charts in the nodes indicate the fraction of the taxa coming from each group. Node size correlates with the total taxa abundance. The taxonomic level of the features is indicated by k_: kingdom; f_: family; c_: class or o_: order.

A total of three predominant bacterial and six fungal indicator organisms were identified for healthy and diseased sugar beets. The bacterial order *Lactobacillales*, as well as the fungal genera *Candida*, *Fusarium*, *Penicillium* indicate a diseased status reflected by the microbiome. On the other hand, the bacterial order *Flavobacteriales* and the class *Cyanobacteria* as well as the fungal genera *Plectosphaerella*, *Vishniacozyma* and the class *Sordariomycetes* reflect a healthy status (Fig. 2.4).

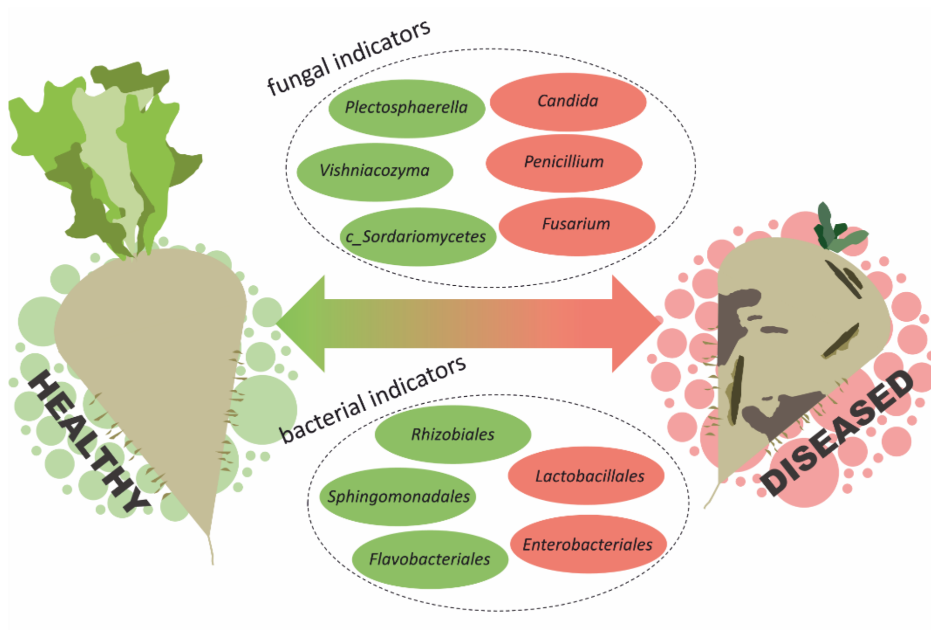

Figure 2.4: Identified indicator taxa based on observations of differences in microbiomes of healthy (green) and diseased (red) field-grown sugar beets. Fungal genera (or classes (c_)) are listed on the top, bacterial orders on the bottom of the illustration.

Taxonomic changes are accompanied by functional specification in the fungal microbiome

The differences on a taxonomic level between the two groups were further verified by analyzing the trophic modes of the found core OTUs (Fig. 2.5). The analysis revealed a higher abundance of saprotrophic fungi in diseased samples (57% compared to 20- 21% in healthy samples). Interestingly, symbiotrophic fungal taxa almost exclusively occurred in healthy samples (5% in "high" samples, 8% in "low" samples compared to 0.3% in diseased samples). Overall, the fraction of pathotrophic fungi was found to be higher in healthy samples, however, in diseased samples saprotrophic fungi were dominant in the mycobiome.

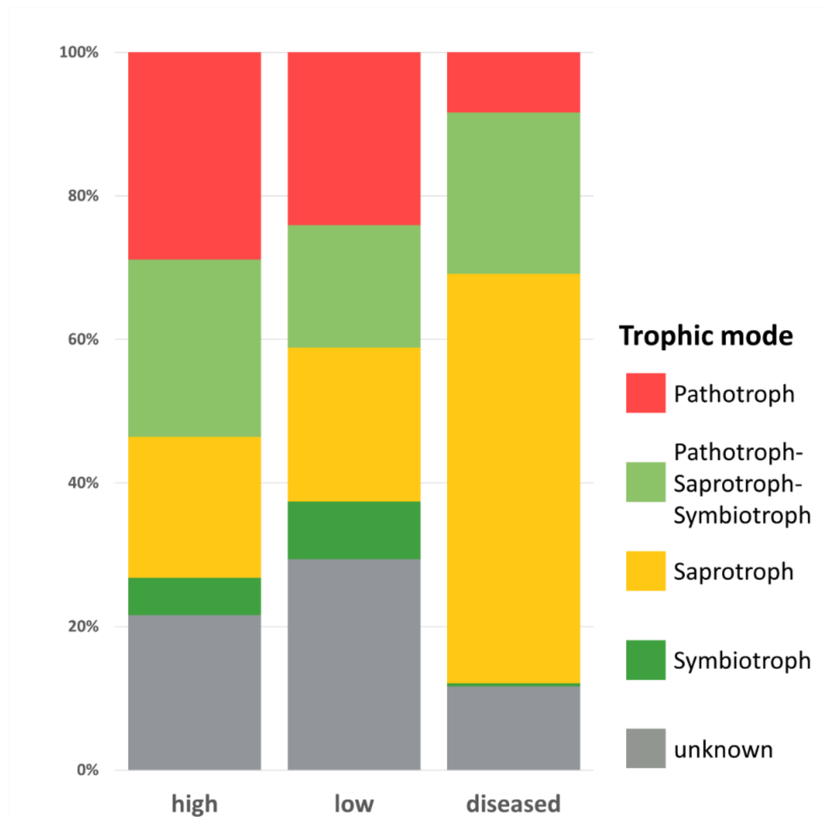

Figure 2.5: Relative abundances of fungal OTUs in the three groups classified by trophic mode. Functions were assigned using the FUNGuild database and the ITS core feature table.

Discussion

Microbial diversity decrease is linked to disease incidence

Within the present study we could show that field-grown healthy and diseased sugar beets harbor a significantly distinct microbiome as well as microbial indicators. Sugar beets derived from different fields in Austria and Germany showed substantial changes in microbial diversity and composition, when spatially close healthy and diseased samples were compared. Diseased plants showed a significant decrease in microbial diversity. Microbial diversity was previously shown to be an important factor for plant health and suggested as a health indicator (Berg et al., 2017; Yan et al., 2017). Keesing and colleagues (2010) suggested that reduced microbial diversity is not only an indicator for disease in plants but also in animals and humans (Keesing et al., 2010). This follows the hypothesis that diverse microbial communities are able to regulate the abundance of pathogenic microbes and prevent the invasion of alien species (van Elsas et al., 2012).

The evaluated antagonistic potential towards fungal pathogens of isolated bacteria from sampled sugar beets displayed a higher frequency of antagonistic bacteria on some fields. In

complementary studies, Zachow and colleagues (2014, 2008) already reported the high antagonistic potential of sugar beet-associated bacteria. Especially highly infected sugar beet fields were shown to harbor a higher number of antagonists in their microbiota. However, the differences between high and low antagonistic sugar beet microbiomes were less pronounced. No significant changes in the bacteriome and only small changes in the abundance of specific fungal taxa such as *Vishniacozyma* (-7% points) or *Plectosphaerella* (-5% points) were observed. This could be indicative for the importance of those taxa for a healthy microbiome. While the bacterial community was rather similar between the two microbiomes with different antagonistic level, the minor shift in the fungal community could have resulted from the higher proportion of antagonistic bacteria. Due to unspecific interactions with pathogenic and non-pathogenic fungi, antagonistic bacteria within the community of healthy beet roots could be a key factor to avert the prevalence of disease-inducing as well as indicating taxa. This once more highlights the tight interplay between different microbes (bacteria and fungi) in the plant associated community with the plant as already reported in previous studies (Artursson et al., 2005; Berendsen et al., 2012).

Changes in mycobiome functioning in healthy and diseased sugar beets

Predictions of trophic specialization based on the ITS region amplicons, revealed a balanced fungal microbiome in both healthy groups, however, an increased fraction of saprotrophic fungi in diseased samples. Higher proportions of *Candida* and *Penicillium* sp. in those samples resulted in this higher abundance of saprotrophic fungi. The high proportion of pathotrophic fungi in both healthy samples is due to the increased abundance of *Plectosphaerella* sp., which was the most abundant taxonomic group in those samples. Several *Plectosphaerella* species are associated with root rot in melon (*Cucumis melo*) (Carlucci et al., 2012). Symbiotrophic fungi only occurred in healthy groups in a higher proportion (>5%), due to an increased fraction of the fungal genera *Colletotrichum* and *Mortierella* in those samples. Despite the observed severe changes between healthy and diseased samples, only minor changes can be observed between both healthy (high and low antagonism) groups. The decrease of the group "pathotroph-saprotroph-symbiotroph" in the samples with low antagonism resulted from the lower abundance of *Vishniacozyma* in those samples. Additionally, the decrease of *Plectosphaerella* sp. went along with the decrease of pathotrophic fungi. Overall, the findings suggest a change from a fungal microbiome balanced in functions (both healthy groups) to a more saprotrophic dominated microbiome in diseased samples. The pathotrophic fungi in the healthy samples were replaced by saprotrophic fungi in diseased samples, more involved in degradation processes, as already observed in other studies (Cooke and Rayner, 1984). In the samples of low antagonistic potential, this decrease of pathotrophic fungi is already indicated through taxonomic changes.

Indicator taxa can be defined to investigate disease development

The data reported in this study showed a highly pronounced difference between a healthy sugar beet and its infected counterpart. Those differences in the microbiome of sugar beets can be used to define key taxa which indicate a healthy and a diseased sugar beet microbiome. Some of the taxa indicative for a healthy microbiome were shown to have beneficial effects on plant growth or disease control. *Plectosphaerella cucumerina*, to which species all of the found *Plectosphaerella* species belong, is a necrotrophic fungus from the class *Sordariomycetes*, which was previously described as a potential biological control agent against potato cyst nematodes (Atkins et al., 2003) as well as a potential bioherbicide in a recent study (Bailey et al., 2017). Moreover, beneficial effects on sugar beets growth was previously described (Ying-Wu et al., 2009). *Flavobacteria* are typical rhizosphere bacteria and known for their root colonization (Bulgarelli et al., 2012). They are currently in discussion for their involvement in plant defense mechanisms (Kolton et al., 2014). Taxa associated with the diseased microbiome such as *Fusarium* and *Penicillium* are typical found on sugar beets during storage and are also associated with sugar reduction after harvest (Liebe et al., 2016; Liebe and Varrelmann, 2015). Additionally, *Lactobacillales*, known for the fermentation of sugar in food products, can potentially reduce the sugar content when colonizing sugar beets after the harvest and are therefore unwanted.

Microbial diversity as well as the presence of specific, plant-beneficial microbes is a key factor for plant health. In the present study, potentially beneficial as well as harmful bacterial and fungal taxa were found to interplay in a highly complex microbial community. Changes in the microbial composition could affect the plant's sensibility to different diseases during plant development and indicator species represent a potential early detection system for disease development during growth or storage after harvest. We could show that taxonomic changes were accompanied by changes in the trophic specialization of the mycobiome. Moreover, the antagonistic potential within the bacterial community negatively correlates with the prevalence of saprophytic fungi. This might be indicative for health maintenance by the indigenous microbial community. The overall findings provide the basis for targeted disease control in sugar beet cultivation as well as the development of disease preventing field management.

Supplementary Material

Table 2.S1. QIIME 1.9.1 and QIIME2 scripts used for bioinformatic analyses of the amplicon datasets.

Process step	Script	Pipeline, plugins and parameters	Comment
Join reads	join_paired_ends.py	QIIME 1.9.1 (SeqPrep)	Performed on raw data
Remove barcodes from sequences	extract_barcodes.py	QIIME 1.9.1	Performed on joined reads; After this step export and import into QIIME2
Demultiplexing	demux emp-single	QIIME 2- 2018.6	Assign sequences to samples
Filtering, length adjustment	dada2 denoise-single	QIIME 2- 2018.6	trim length according to quality (230 bp for 16S data, 200 bp for ITS data)
Identify additional chimeric sequences	vsearch uchime-denovo	QIIME 2- 2018.6	
Remove chimeric sequences	feature-table filter-features feature-table filter-seqs	QIIME 2- 2018.6	
Classify features	feature-classifier classify-sklearn	QIIME 2- 2018.6 (99% cutoff level) DB: SILVA release 128 / UNITE v7	confidence threshold 0.7
Filter data	taxa filter-table taxa filter-seqs	QIIME 2- 2018.6	Filter mitochondria, chloroplast in 16S dataset and bacteria, archaea in ITS dataset
Core diversity	diversity core-metrics	QIIME 2- 2018.6	Performed on filtered and quality checked data; Rarefied data to 2,400 reads (16S) and 11,000 reads (ITS)
Alpha diversity	diversity alpha-rarefaction alpha-group-significance	QIIME 2- 2018.6	Performed on filtered and quality checked data; significance tested with Kruskal-Wallis
Beta diversity	beta-group-significance	QIIME 2- 2018.6	Performed on filtered and quality checked data; significance tested with anosim
Split data into groups	feature-table filter-samples	QIIME 2- 2018.6	Data was split into groups according to health (high antagonism, low antagonism, diseased)

Core microbiome	feature-table filter-features	QIIME 2- 2018.6	Filter all features occurring in at least 50% of the samples in a specific group; subsequent merging the core-tables for comparisons of the core microbiomes of different groups
Make OTU-network	make_out_network.py	QIIME 1.9.1	OTU network for rendering in Cytoscape

Table 2.S2. General overview of sequencing data. Number of reads, assigned SVs using the DADA2 algorithm, and Shannon index of each group is provided.

Sample	No. samples	No. ITS reads	No. 16S reads	SVs ITS	SVs 16S	Shannon Index ITS	Shannon Index 16S
Diseased	27	1,975,233	451,916	585	1,939	3.5	6.5
Healthy high	23	2,377,553	566,601	1,996	4,561	4.3	8
Healthy low	23	2,266,631	744,396	2,050	6,057	4.7	8.2
Total	73	6,619,417	1,762,913	3,233	9,480	4.1	7.5

Figure 2.S1. Picture and schematic representation of the sampling strategy. Healthy sugar beets as well as diseased beets from the surrounding area were sampled from fields in Austria and Germany. For healthy samples, healthy sugar beet plants within areas with high disease occurrence were selected.

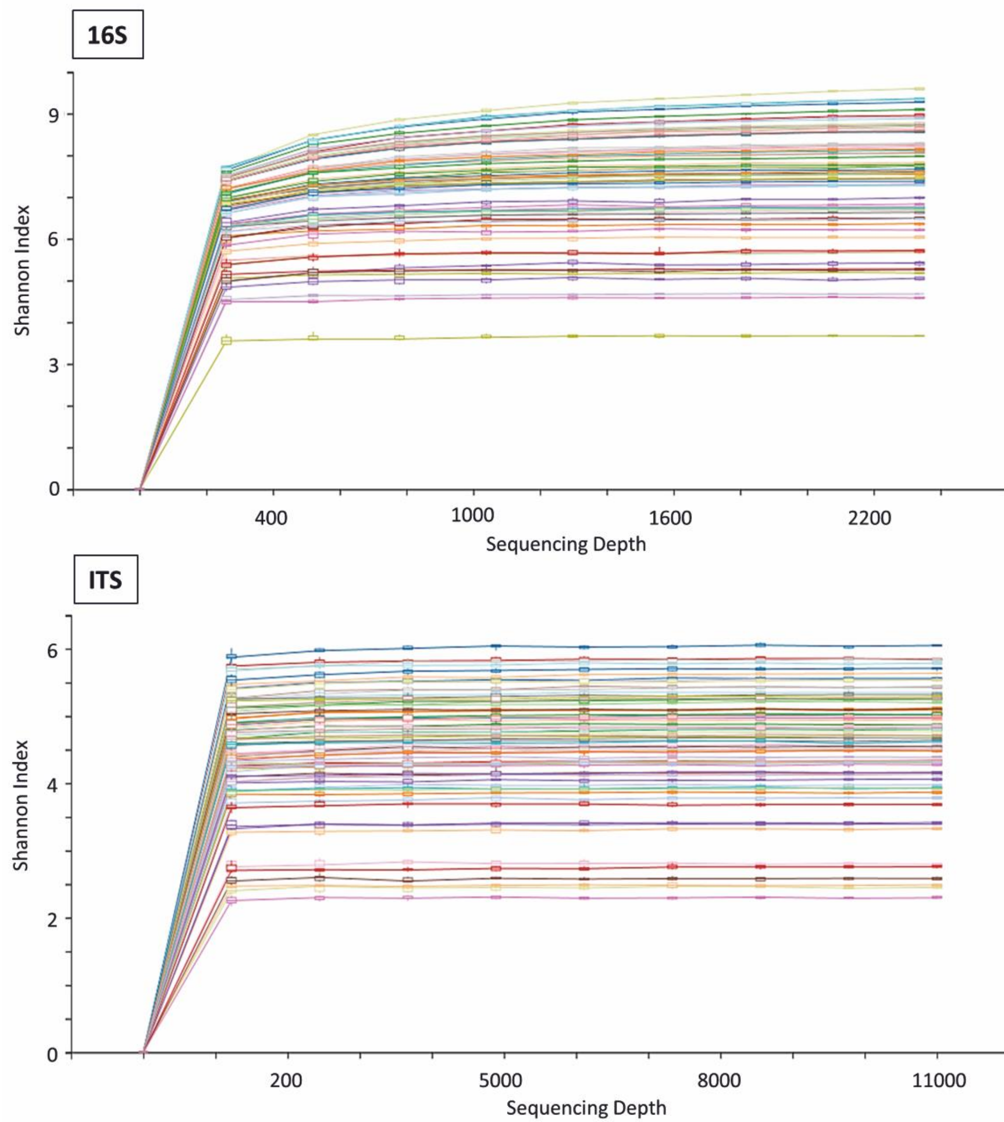

Figure 2.S2. Alpha rarefaction plots at a sampling depth of 2,400 for 16S gene fragment sequences and a sampling depth of 11,000 for ITS fragments.

3 Publication II: Microbiome-driven identification of microbial indicators for postharvest diseases of sugar beets

Peter Kusstatscher[ab], Christin Zachow[a], Karsten Harms[c], Johann Maier[c], Herbert Eigner[d], Gabriele Berg[b] and Tomislav Cernava[be]

[a] Austrian Centre of Industrial Biotechnology, Petersgasse 14, A-8010 Graz, Austria

[b] Institute of Environmental Biotechnology, Petersgasse 12, A-8010 Graz, Austria

[c] Südzucker AG, Maximilianstraße 10, 68165 Mannheim, Germany

[d] Agrana Research & Innovation Center, Josef-Reither-Straße 21 – 23, 3430 Tulln, Austria

[e] Roombiotic GmbH, c/o: SciencePark, Stremayrgasse 16/IV, 8010 Graz, Austria

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ABSTRACT

Background: Sugar loss due to storage rot has a substantial economic impact on the sugar industry. The gradual spread of saprophytic fungi such as *Fusarium* and *Penicillium* spp. during storage in beet clamps is an ongoing challenge for postharvest processing. Early detection of shifts in microbial communities in beet clamps is a promising approach for the initiation of targeted countermeasures during developing storage rot. In a combined approach, high-throughput sequencing of bacterial and fungal genetic markers was complemented with cultivation dependent methods and provided detailed insights into microbial communities colonizing stored roots. These data were used to develop a multi-target qPCR technique for early detection of postharvest diseases.

Results: The comparison of beet microbiomes from six clamps in Austria and Germany highlighted regional differences; nevertheless, universal indicators of the health status were identified. Apart from a significant decrease in microbial diversity in decaying sugar beets ($p \leq 0.01$), a distinctive shift in the taxonomic composition of the overall microbiome was found. Fungal taxa such as *Candida* and *Penicillium* together with the gram-positive *Lactobacillus* were the main disease indicators in the microbiome of decaying sugar beets. In contrast, the genera *Plectosphaerella* and *Vishniacozyma* as well as a higher microbial diversity in general were found to reflect the microbiome of healthy beets. Based on these findings, a qPCR-based early detection technique was developed and confirmed a twofold decrease of health indicators and an up to 10,000 fold increase of disease indicators in beet clamps. This was further verified with analyses of the sugar content in storage samples.

Conclusion: By conducting a detailed assessment of temporal microbiome changes during the storage of sugar beets distinct indicator species were identified that reflect progressing rot and losses in sugar content. The insights generated in this study provide a novel basis to improve current or develop next-generation postharvest management techniques by tracking disease indicators during storage.

Keywords: *Beta vulgaris*; storage rot; indicator species; phytopathogens; bacterial microbiome; fungal microbiome

Background

Plant colonizing microorganisms live in close relationship with their host and are a crucial factor for plant growth and health (Berendsen et al., 2012; Berg et al., 2014b; Vandenkoornhuyse et al., 2015). For various crop plants, this was observed along the entire value-chain including the postharvest period (Droby et al., 2016). The exploration of plant-microbe interactions, plant-beneficial bacteria and fungi including yeasts, their functions and modes of action is a key for advanced developments related to biotechnological applications in agriculture (Berg et al., 2017, 2014b). The development of postharvest applications based on biologicals is challenging due to the great diversity of postharvest pathogens as well as the often highly challenging postharvest treatments and storage conditions (Castoria et al., 2001; Tzortzakis and Economakis, 2007). The herbaceous dicotyledonous plant, *Beta vulgaris* L. (sugar beet) is the main crop for sugar production (sucrose content up to 18%) in temperate regions all over the world (Trebbi and McGrath, 2004). A number of plant pathogens such as *Pythium ultimum* Trow (Osburn et al., 1989), *Rhizoctonia solani* Kühn (Kiewnick et al., 2001) and *Cercospora beticola* Sacc. (Weiland and Koch, 2004) cause severe harvest shortfalls due to seedling rot or late root rot (Zachow et al., 2010). After harvest, starting from late October, sugar beets are stored in Europe directly on the fields for a maximum of 60 days due to limited process capacities and increased economic viability of sugar refineries. High water (76%) and sugar content (18%) in the unprocessed beets (Jaggard et al., 1997) provide perfect conditions for microbial colonization, especially when cracks, root tip breakage and fresh wounds on the surface provide easy entry points (Liebe et al., 2016). Microbial colonization, mainly by pathogenic or saprophytic fungi such as *Fusarium, Penicillium*, and *Botrytis* spp., leads to substantial sugar yield losses. A major observation is microbial inversion of sucrose into unwanted glucose and fructose molecules (Klotz and Finger, 2004). The combined occurrence of microbial degradation, respiration of the beet root, synthesis of raffinose and other causes can yield sugar losses of up to 50-60% during storage (Hoffmann, 2012; Kenter and Hoffmann, 2009).

Natural antagonists that are part of the indigenous beet microbiome, previously studied by Zachow and colleagues (2008) (Zachow et al., 2008), carry the potential for alternative plant protection applications during growth and postharvest (Berg, 2009; Janisiewicz and Korsten, 2002). In our previous study we found correlations between the disease incidence in sugar beet fields and the antagonistic potential of the prevalent microbiota (Kusstatscher et al., 2019a). These observations provide the basis for sustainable methods to prevent high sugar yield losses, caused by fungal infection with a targeted use of antagonistic microorganisms that could also provide postharvest protection (Schillinger et al., 1996). However, in order to develop targeted and sustainable countermeasures, it is

crucial to identify key players in the rot onset and to improve early detection strategies of rot causing pathogens for beet clamps. Moreover, when biological control is employed, it is important to understand to which natural counterparts beneficial microorganisms will be exposed. Although rot-causing fungal pathogens were previously identified (Liebe et al., 2016), the health-related dynamics of bacteria and fungi in stored sugar beets remained unexplored.

The aim of this study was to analyze temporal community changes in the microbiome of stored roots, correlate them to sugar beet health, and finally integrate the generated knowledge into a novel disease detection technique. Therefore, we investigated the bacterial and fungal microbiome of stored sugar beets in different beet clamps located in important cultivation areas of Austria and Germany. By implementing a detailed assessment of the beet clamp microbiome, specific biological markers indicating disease development in stored beets were found. These observations were thereafter confirmed with sugar beets stored under controlled conditions to verify the applicability of the identified markers. The overall findings provide a basis for novel postharvest management techniques that implement microbial and molecular markers for targeted countermeasures.

Results

Identification of fungal taxa from decaying sugar beets

In order to identify fungal taxa in infected sugar beets from clamps in Austria and Germany, two complementary methods were applied. The community structure was reconstructed with Sanger sequencing of 18S rRNA gene fragments from fungal isolates and ITS Illumina amplicon sequencing of total community DNA (Fig. 3.1). The 18S rRNA gene sequencing-based community reconstruction with 120 fungal strains indicated a fungal community structure with 11 different genera, which was dominated by *Penicillium* (37%) and *Fusarium* (22%) species, while ITS amplicon sequencing indicated a more diverse composition. A total of 80 amplicon datasets revealed more than 50 different fungal genera. The most prominent genera were assigned to *Plectosphaerella* (11%), *Guehomyces* (10%), *Penicillium* (10%), *Candida* (10%), *Mrakia* (8%), *Vishniacozyma* (8%) and *Tetracladium* (4%). While *Penicillium* was abundant in both approaches, *Fusarium* was only predominant in the isolate-based community reconstruction. Moreover, the highest proportion of fungal strains (86%) was recovered from the beet surface; however, a substantial fraction of the identified *Fusarium* species (39%) originated from the sugar beet endosphere.

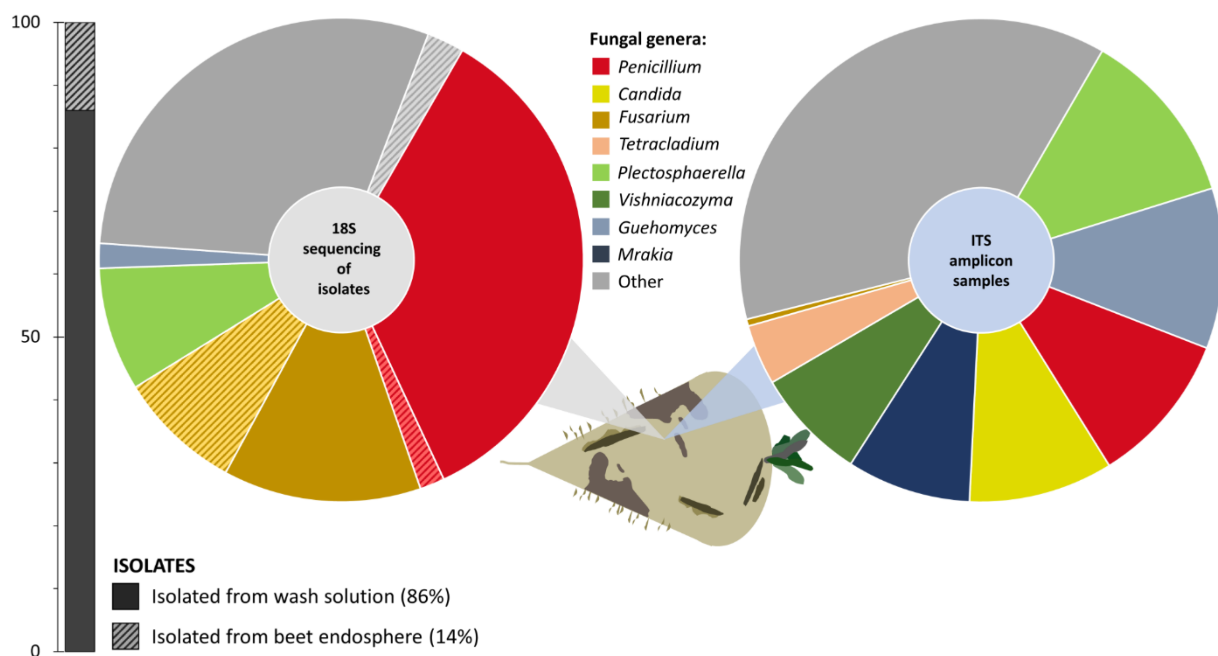

Figure 3.1: Comparison of 18S rRNA gene fragment sequencing results of fungal strains isolated from beet clamps in Austria and Germany and ITS sequencing of amplicon samples. The obtained sequences were assigned up to genus level. Color-coded segments indicate different genera in both datasets. Shaded areas represent the fraction of fungal isolates obtained from the beet endosphere.

Microbial diversity was significantly decreased in decaying sugar beets

The comparison of amplicon data obtained from 120 samples of healthy and decaying sugar beets showed a significantly lower bacterial diversity in infected samples (Shannon index: 4.5 (16S) and 3.5 (ITS)) compared to the microbiome of healthy sugar beets (Shannon index 5.5 (16S) and 4.5 (ITS)) (Fig. 3.2B). The calculated Bray Curtis distances showed significant differences in the composition of the microbiomes of the two groups. When a group-wise comparison was conducted, samples of decaying sugar beets (n= 80) clustered significantly (p-value ≤ 0.01) different from samples of healthy sugar beets (n= 40). The variation within the infected group was found to be higher, compared to the healthy samples, which clustered more closely together (Fig. 3.2A).

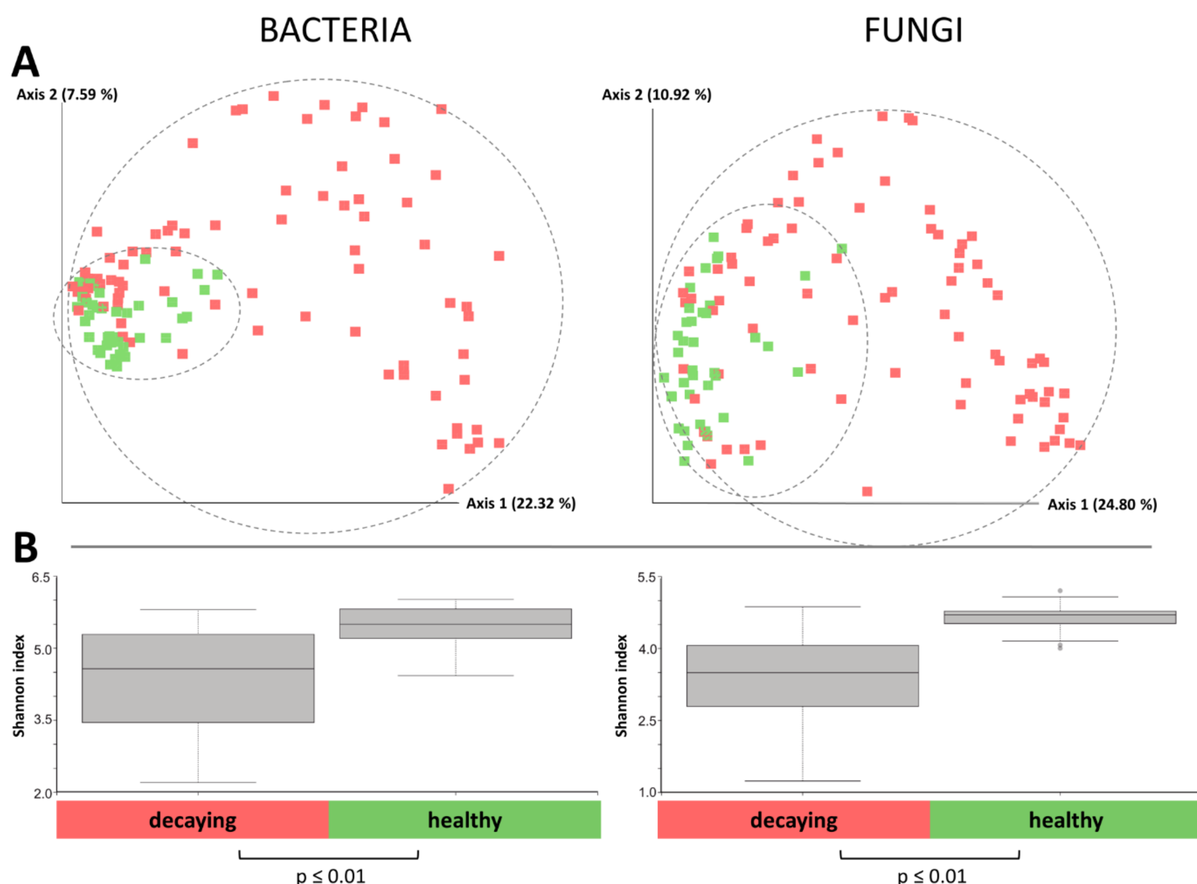

Figure 3.2: Alpha and beta diversity comparison of healthy and decaying sugar beet microbiome samples. The bacterial and fungal microbiome of each sample is indicated with one dot (A). Highly significant differences in the diversity were obtained from a total of 40 healthy and 80 decaying samples (B). Distances shown in the PCoA plot are based on the Bray Curtis diversity metrics.

The core microbiome composition was altered in decaying sugar beets

Taxonomic assignments of the identified features indicated a decay-specific microbiome of the analyzed sugar beets. The comparison of healthy and decaying samples showed a clearly distinguishable composition of taxa in both bacterial and fungal amplicon reads. *Proteobacteria* with an average relative abundance of 41% (healthy samples) and 51% (decaying samples) were the most abundant taxa on phylum level. *Bacteriodetes* (27% and 12.5%) and *Actinobacteria* (28% and 11%) were also highly abundant in both groups. The main difference between both groups was due to the phylum *Firmicutes* (0.4% in healthy and 25% in decaying samples). A major fraction of *Firmicutes* in the decaying samples belonged to the order of *Lactobacillales* (24%). The predominant *Proteobacteria* in healthy samples were mainly members of the orders *Pseudomonadales* (10%), *Sphingomonadales* (9%), *Rhizobiales* (8.5%), *Xanthomonadales* (6.5%) and *Enterobacteriales* (2.5%). In contrast, the 51% *Proteobacteria* found in decaying samples belonged to the orders *Rhodospirillales* (20%),

Enterobacteriales (8%), *Pseudomonadales* (8%), *Xanthomonadales* (5%), *Sphingomonadales* (4%) and *Rhizobiales* (4%). At order level the most abundant taxa of healthy sugar beets were *Flavobacteriales* (21%), *Micrococcales* (21%) and *Pseudomonadales* (10%); whereas, the predominant taxa of decaying sugar beets were *Lactobacillales* (24%), *Rhodospirillales* (20%) and *Flavobacteriales* (9%). At genus level *Lactobacillus* (18.4%), *Gluconobacter* (16%) and *Leuconostoc* (11.3%) were the most abundant taxa in decaying samples, whereas, *Flavobacterium* (20,6%), *Pseudarthrobacter* (13,5%) and *Pseudomonas* (9%) were the most abundant taxa in healthy samples. (Fig. 3.3A).

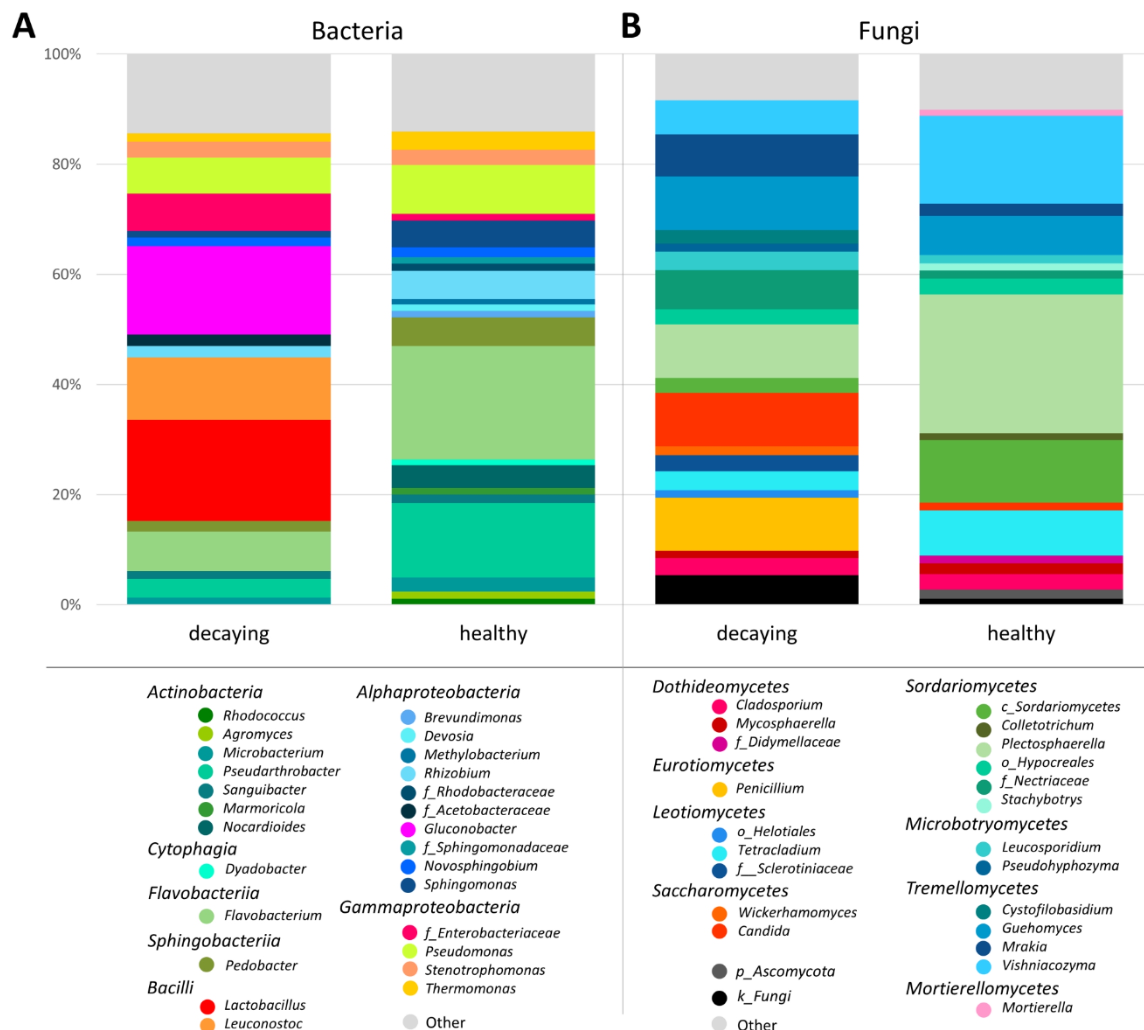

Figure 3.3: The core microbiome of healthy and decaying sugar beets from beet clamps in Austria and Germany. Relative abundances of prevalent bacterial (A) and fungal taxa (B) are shown. All taxa with an abundance ≥ 1% were identified on genus level if the resolution was sufficient. The grouping was conducted based on assignments at class level and taxa that were not assignable at genus level were additionally labeled: f_: family, o_: order, c_: class, p_: phylum, k_: kingdom.

The ITS dataset showed diversified fungal microbiomes in both healthy and decaying sugar beets. When the structure of the whole dataset was assessed, a total of 60- 62% *Ascomycota* and 33% *Basidiomycota* were observed within the fungal community. At class level, an increased fraction of

Saccharomycetes (+ 10 percent points; 12% total) and *Eurotiomycetes* (+ 9 percent points; 10% total) as well as a decreased fraction of *Sordariomycetes* (-16 percent points; 24% total) was found in the decaying samples. At order level, an increased abundance of *Cystofilobasidiales* (+ 11 percent points; 21% total), *Saccharomycetales* (+ 10 percent points; 12% total) and *Eurotiales* (+ 9.5 percent points; 10% total) was observed. At genus level this resulted an increased number of *Candida* (+ 7.5%; 9.5 total)), *Penicillium* (+ 9.5%; 10% total)), *Guehomyces* (+ 5%; 10% total) and *Mrakia* (+ 5%; 8% total). Healthy samples by contrast showed an increased amount of the genera *Plectosphaerella* (+ 10%; 21% total) as well as *Vishniacozyma* (+ 12%; 18% in total). This was already shown in an increased abundance of the classes *Sordariomycetes* (+ 16%; 40% in total) as well as *Tremellomycetes* (+ 2%; 30% in total). In comparison, at genus level, the most abundant genera in decaying samples were *Plectosphaerella*, *Guehomyces*, *Candida* and *Penicillium* (all 10%); whereas, in healthy samples the genera *Plectosphaerella* (21%) and *Vishniacozyma* (18%) dominated (Fig. 3.3B).

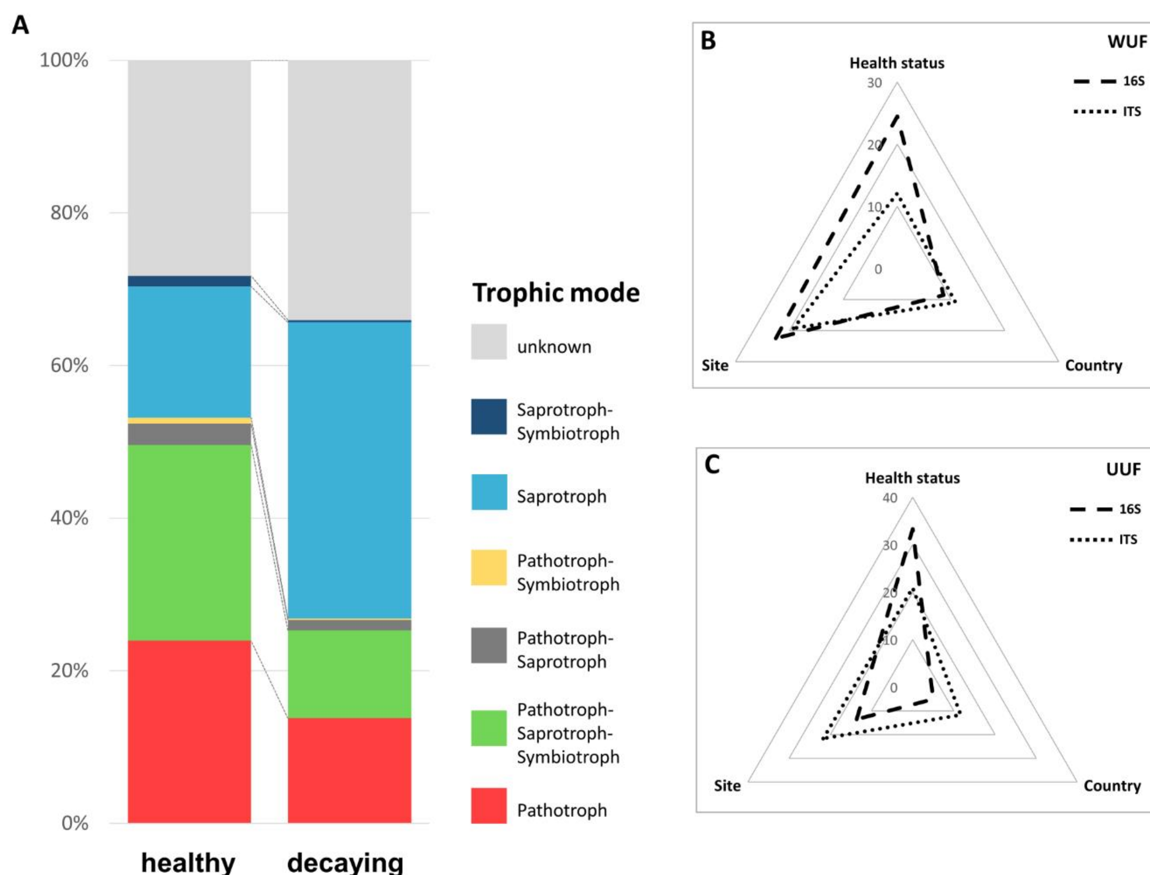

Figure 3.4: (A) Trophic modes in the fungal microbiome depending on sugar beet health status and (B, C) explained variance between samples by distinct parameters. The trophic modes were assigned according to identified core features of the samples and classifications stored in the FUNGuild database. A PERMANOVA analysis was performed by using weighted (WUF) as well as unweighted UniFrac (UUF) distance metrics.

Trophic specialization in the fungal microbiome

Taxonomic differences between healthy and decaying sugar beets were found to be accompanied by changes in the trophic modes of the identified core features. Healthy samples were mainly colonized by pathotrophic (24%) and pathotrophic-saprotrophic-symbiotrophic (26%) fungi. The trophic distribution in the decaying samples, however, was dominated by saprotrophic fungi (39%) with a decreased fraction of pathotrophic (14%) and pathotrophic-saprotrophic-symbiotrophic (12%) fungi. Overall, a decrease in pathotrophic and symbiotrophic functions and an increase in saprotrophic functions from the microbiome in healthy to the microbiome in decaying sugar beets was observed (Fig. 3.4A).

The health status of beets was identified as the major driver for microbial community composition

The comparison of six different beet clamps in Austria and Germany showed significant differences in diversity as well as taxonomic composition. Health status explained the largest proportion of variance of the beets (33.3% variation in 16S dataset and 20.9% for ITS, $p \leq 0.001$). Different beet clamp sampling sites also explained 13.6% variation in the 16S and 21.7% variation in the ITS dataset ($p \leq 0.001$), however, variances within the groups were higher (F statistic = 3.43 (16S) and 6.25 (ITS) compared to 56.36 (16S) and 30.91 (ITS) between health statuses). The country that sugar beet samples originated from accounted for the least variance (5% in 16S data and 11.7% in ITS data, $p \leq 0.001$) (Fig. 3.4 B,C; Table 3.S1). These findings were also reflected in β-diversity PCoA plots, where sample were separated by health status (Fig. 3.S1, 3.S2).

Samples obtained from the storage in Grossmugl (Austria) showed clear differences in the microbial composition when compared to the sampling spots located in lower Germany (Mittich, Kleinweichs and Osterhofen). Sampling locations that were geographically located closer to each other (Fig. 3.S3 C), however, showed less significant differences. Overall, a change from relatively balanced abundances of bacterial taxa (microbiome of healthy sugar beets) to a predominance of *Lactobacillales*, as well as *Rhodospirillales* (decaying sugar beets) was evident for every sampling spot. The fungal community changed from a microbiome dominated by *Vishniacozyma* and *Plectospaerella* to an increasing number of *Penicillium* and *Candida* species (Fig. 3.5).

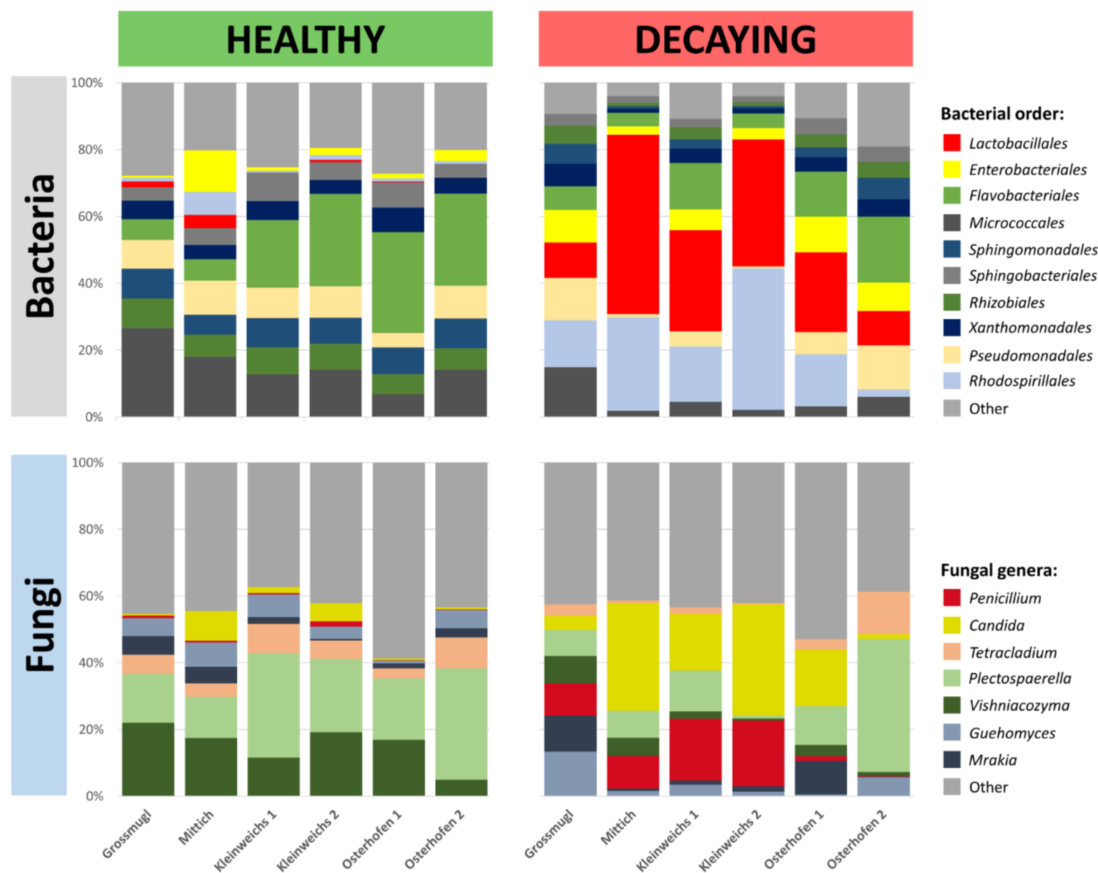

Figure 3.5: Relative abundance of the most abundant (≥ 5%) bacterial and fungal taxa in healthy and decaying sugar beet samples. Stored roots from six sugar beet clamps in Austria and Germany were analyzed by amplicon sequencing of the 16S rRNA gene fragment and the ITS region. The results were grouped according to the health status and the sampling site of the beets.

Identification of disease indicators and correlation to sugar content in stored sugar beets

Specific taxa, indicative either for the microbiome of healthy or decaying sugar beets, were selected based on the differences in their abundance in the representative samples (Fig. 3.3 and 3.5). *Flavobacterium* and *Pseudarthrobacter* within the bacterial community as well as *Plectospaerella* and *Vishniacozyma* within the fungal community were found to be dominant in healthy sugar beets. In contrast, *Lactobacillus* and *Gluconobacter* as well as *Candida* and *Penicillium* were prevalent in decaying sugar beets. By implementing a real-time qPCR analysis with specific primers targeting microbial indicators in stored sugar beets, the gradual increase of disease indicators and simultaneous loss of health indicators was shown. During a three-month storage trial, an increase of *Candida* (10^5 to 5×10^6 copies/g), *Fusarium* (2×10^3 to 10^4 copies/g) and *Penicillium* (0 to 10^4 copies/g) and simultaneous decrease of *Vishniacozyma* (10^5 to 5×10^4 copies/g) was observed (Figure 3.6 A). In case of

Plectosphaerella an initial decrease in abundance (2×10^5 to 10^5 copies/g), but overall constant abundances (10^5 copies/g) throughout the storage period were found.

In order to verify the disease progress in the samples that were used for qPCR primer evaluations, complementary analytical analyses of beet carbohydrates were conducted with stored samples. The sugar content of sugar beets stored under controlled conditions showed a decreasing concentration of sucrose (-3 percent points) during the storage period of three months. At the same time, an increase of inverted sugars (glucose (2 to 14 g/kg) and fructose (1.5 to 14 g/kg)) was observed (Fig. 3.6 B).

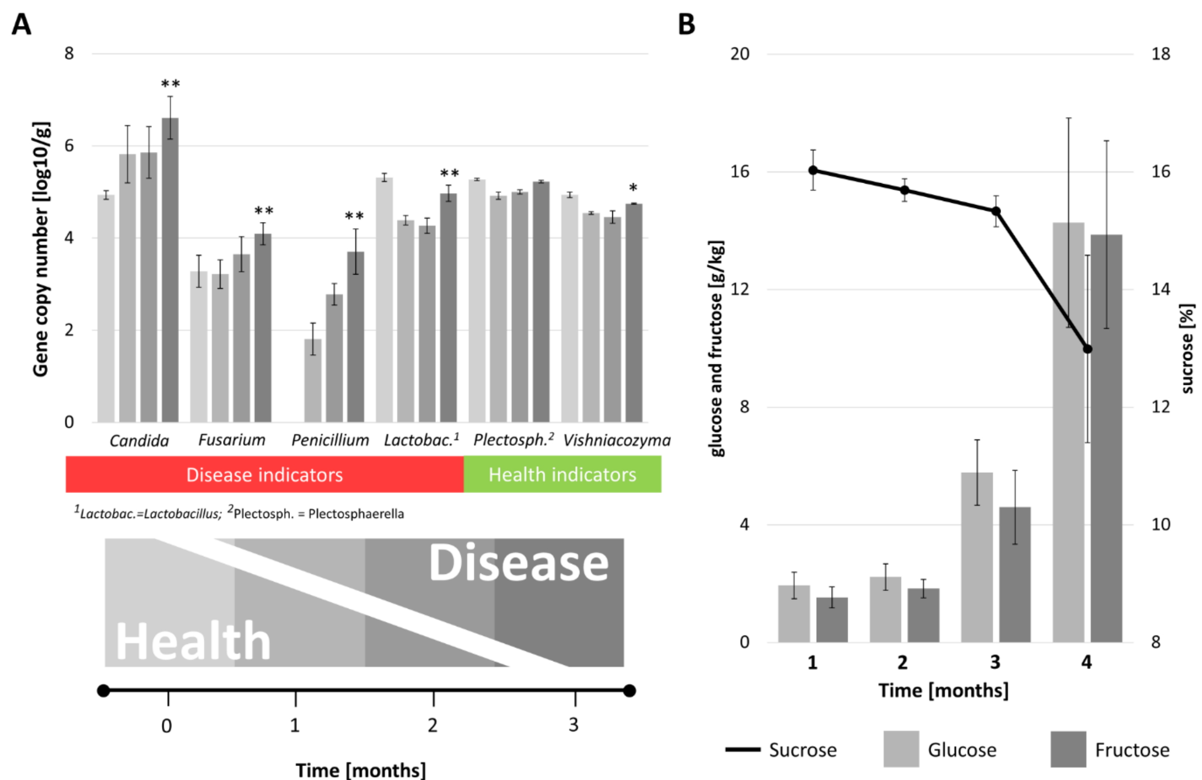

Figure 3.6: Real-time qPCR analysis of bacterial and fungal indicator taxa in stored sugar beets (A) and analyzed sucrose, glucose and fructose contents in sugar beets (B). Gene copy numbers per gram sugar beet surface showed distinct tendencies related to accumulations of health and disease indicators during three months of controlled storage (color gradient). Statistical significance between the first and last measurement was tested using the students t-test: p-value < 0.01 **, p < 0.05 *.

Discussion

Fungal pathogens prevail among isolates from decaying sugar beets

The obtained results of the present study provide the first detailed microbiome characterization of conventionally stored sugar beets in an industrially scaled, uncontrolled environment. By combining different methods, a holistic assessment of the fungal microbiome in decay-affected sugar beet was constructed. 18S gene sequencing data of 120 fungal isolates was compared to ITS next-generation

amplicon data. In comparison, 86% of isolated fungi identified on genus level were also found in the amplicon libraries. The cultivation-dependent identification of fungal isolates, showed a prevalence of certain taxa such as *Penicillium* and *Fusarium*, when compared to the amplicon sequencing dataset. This likely resulted from the specific procedure during the isolation process that could have affected the frequency of isolated strains. While only homogenized peel was used for the total DNA extraction, also surface sterilized fragments of infected sugar beets were placed on agar plates during isolation. This could have facilitated the isolation of *Fusarium* species, since this pathogen primarily colonizes the plant endosphere (Zhang et al., 2012). In case of *Penicillium*, its high spore production allows it to overgrow slow-growing fungal taxa and likely lead to its isolation in higher proportions. The fungal ITS library obtained with high-throughput sequencing showed overall a higher diversity of different fungal taxa, which is partially due to cultivability limitations of certain taxa on standard isolation media (Müller and Ruppel, 2014; Wu et al., 2000).

Bacterial diversity decrease was accompanied by an emergence of several highly abundant lineages

Microbial diversity as well as distinct changes in the microbial community were previously shown to be linked to disease incidence (Berg et al., 2017; Yu et al., 2012). The data obtained in this study supports the hypothesis, that lower diversity in the bacterial as well as fungal community is connected to a higher sensitivity to microbiome shifts that substantially alter the community structure. The lower diversity in decaying samples was reflected by a significant decrease in diversity indices in both the bacterial and the fungal dataset. In analogy to our findings, changed microbial diversity was found in stored onions when comparing healthy and diseased ones and fungal diversity was found to be higher in roots of healthy winter wheat plants (Lemanczyk and Sadowski, 2002; Yurgel et al., 2018). Moreover, a decrease in diversity was shown to facilitate invasion of pathogenic species into communities (van Elsas et al., 2012).

Even though amplicon based sequencing can be affected by certain biases (Schirmer et al., 2015), the taxonomic composition of the bacterial as well as fungal beet microbiome, obtained with this dataset, was primarily linked to the health status of the sampled sugar beets. The geographic location of the beet clamps played a less significant role for the observed variability. Similarly, also Yurgel and colleagues (2018) observed taxonomic changes based on health status in stored onions (Yurgel et al., 2018). Additionally, Liebe et al. (2016) already observed a similar effect in sugar beets when stored at different temperatures (Liebe et al., 2016). Depending on the storage conditions, the analyzed beets harbored specific fungal taxa; whereas, the originating environment was less influential. In this study, sugar beets, stored under representative conditions without any protection from adverse environmental factors (moisture, temperature fluctuations, frost, etc.), showed a fungal

community dominated by *Candida*, *Penicillium*, *Guehomyces* and *Plectosphaerella* sp. in decaying sugar beets. The fungal microbiome of sampled healthy beet roots was, interestingly, comparable with the analyzed reference sugar beets in Liebe et al. (2016) harnessing mostly *Plectosphaerella* sp. (Liebe et al., 2016). The observed taxonomic changes were also reflected by trophic modes within the fungal community. Dominant pathotrophic and pathotrophic-saprotrophic-symbiotrophic functions in healthy samples were replaced by saprotrophic functions in decaying sugar beets. Similar findings were also made by Yu and colleagues (2012) linking the prevalence of saprotrophic fungi mostly to diseased pea plants, the abundance of pathogenic fungi, however, not to a specific health status (Yu et al., 2012).

Identification of health indicators in the microbiome of sugar beets

Different potential biological markers were identified by contrasting healthy and diseased samples of stored sugar beets. Distinct taxa were shown to be highly abundant in samples representing each disease condition. The necrotrophic fungal lineage *Plectosphaerella*, found in healthy beets, was previously shown to be a growth promoting microbe in sugar beets (Ying-Wu et al., 2009). Moreover, it was reported as a potential biological control agent against potato cyst nematodes as well as a potential bioherbicide (Atkins et al., 2003; Bailey et al., 2017). Previous studies on sugar beet storage observed this taxon mostly in sugar beets before storage (Liebe et al., 2016). Other health-related taxa, such as *Flavobacterium* and *Pseudarthrobacter* were often reported in the rhizosphere of different plants as well as their involvement in plant defense mechanisms or growth promotion (Bulgarelli et al., 2012; Kolton et al., 2014; Krishnamoorthy et al., 2018; Wei et al., 2018). Other taxa, associated with decaying sugar beets, such as *Penicillium*, are typical saprophytic fungi and postharvest pathogens and were observed previously in rotting sugar beet after harvest (Bugbee, 1975; Liebe et al., 2016; Snowdon, 1990). *Lactobacillus* as well as the fungal genus *Candida* were predominantly detected in decaying sugar beets and are associated with sugar fermentation to acid or alcohol compounds and are unwanted in stored sugar beets because of this activity (Calabia and Tokiwa, 2007; du Preez et al., 1986). We hypothesize that such taxa occur on decaying sugar beets primarily due to increased free monosaccharides originating from the hydrolyzation processes of sucrose by fungal extracellular proteins.

Real-time qPCR analyses conducted on the basis of the identified health and disease indicators in stored sugar beets provided a first evidence for the applicability of such indicators for agricultural management strategies. The data was obtained within small-scale experiments and must be further expanded in upcoming approaches to confirm the reliability of the indicators for industry-scale applications. During the representative storage period of three months, health related indicators were either decreasing or remained constant. In contrast, disease-related indicators increased substantially

over the storage period. The quantitative analysis of these taxa indicated a gradual disease development that is linked to microbial sucrose concentration loss and simultaneously increase in inverted sugars during storage (Liebe and Varrelmann, 2015), which was confirmed by targeted analyses in the present study.

Conclusion

Storage rot in stored sugar beets was shown to be accompanied by a change in microbial abundances. The present study highlighted substantial shifts within the bacterial as well as fungal community that correlated to decay incidence in stored roots. Changes in the prevalence of certain taxa can potentially indicate decay development at an early stage and facilitate an implementation of targeted countermeasures. Taxonomic changes were shown to be accompanied by trophic specialization in the fungal community. For upcoming postharvest applications, the novel insights provide a basis to design suitable biocontrol agents maintaining the balance of taxa associated with the microbiome of healthy sugar beets and preventing the establishment of degrading microorganisms. Furthermore, the identification of diseases indicators can be used as decision tool and supports the prioritization of beet processing of harvested beets during storage management. Additional studies are needed to confirm the implementability of the obtained results and to assign levels of quantitative measurements, which will allow to indicate the degree of disease.

Methods

Sampling of sugar beets and isolation of fungi

Healthy (n= 40) and decaying (n= 80) sugar beets were obtained from beet clamps in Austria (Upper Austria) and Germany (Bavaria). The detailed sampling locations are provided in Table 3.S2 and Figure 3.S3 C. Decaying sugar beets were obtained from nests of fungal mycelia in the beet clamps (Fig. 3.S3 A,B). Samples with severe and intermediate fungal infection were selected. Healthy sugar beets were collected from the non-infected, symptoms-free surrounding area of infected beet clamps. Following the sampling, 20 g of the sugar beet skin (surface of tap root and stem end) was peeled and washed with 50 mL of 0.85% sodium chloride solution in a stomacher (BagMixer; St. Nom, France) for 3 min. The obtained solution was prepared for total community DNA extraction as described later. A total of 100 µL of the solution obtained from decaying sugar beets was plated on SNA plates (Nirenberg, 1976) containing penicillin G (100 µg/mL), dihydrostreptomycinsulfate (50 µg/mL) and chlortetracycline (10 µg/mL) in serial 1/10 dilutions until a final dilution of 10-10 was reached. In addition, surface sterilized (submerged in 4% sodium hypochlorite, 5 min) and washed (two times sterile distilled water) beet

sections from diseased beets were placed on a SNA plate to obtain fungal isolates growing in the beet endosphere. A total of 10 fungal strains per sugar beet were randomly picked based on morphology from the plates and further subcultured on PDA, SNA and water agar plates (tap water + 18 g/L agar). The strains were further grouped using morphologic clustering after inspecting the single isolates on the different plates. Several strains of each morphologic cluster (120 strains in total) were subjected to 18S rRNA gene fragment Sanger sequencing (LGC Genomics, Berlin, Germany). Quality checked sequences were blasted against the NCBI database as well as the UNITE v7 database (Kõljalg et al., 2013).

Storage of sugar beets under controlled conditions

A total of 20 untreated and undamaged sugar beets harvested from a single field in Germany (Rhenish Hesse, Rhineland-Palatinate; 49° 35' 54.388''N, 8° 12' 48.823''E) were stored directly after harvest under controlled condition at 8 °C and 75% relative humidity for 3 months. Sampling of five sugar beets at the beginning (T0) and every 30 days (T1, T2, and T3) was performed as described above. A total of 20 g of sugar beet peel was washed in a stomacher with 50 mL of sodium chloride (0.85%). A total of 4 mL of the solution was centrifuged into a pellet and further used for community DNA extraction. Sugar content in the sugar beet flesh was measured using standardized ICUMSA (International Commission for Uniform Methods of Sugar Analysis) methods for the determination of glucose and fructose by enzymatic assays and the polarization of sugar (sucrose) by the cold aqueous digestion method (ICUMSA, 2007, 1994).

Total community DNA extraction and construction of amplicon library

A total of 4 mL of the obtained washing solution from the sampling step was centrifuged (13000g, 20 min, 4 °C) and the pellet was stored at -70 °C until further use. Using the FastDNA® Kit for Soil (MP Biomedicals/USA) genomic DNA was extracted from all samples. All steps were conducted as stated in the manufacturer's protocol. Following DNA extraction, the 16S rRNA primers 514f and 926r (GTGYCAGCMGCCGCGGTAA; CCGYCAATTYMTTTRAGTTT) and the ITS primer pair ITS1f and ITS2r (CTTGGTCATTTAGAGGAAGTAA; GCTGCGTTCTTCATCGATGC) were used in PCR for amplicon library construction. As described in the protocols and standards section of the Earth microbiome project (Walters et al., 2015), both primer pairs were modified with specific primer pads (TATGGTAATT/AGTCAGCCAG) and linker (GT/GG) for the attachment of a Golay barcode sequences. Two consecutive PCR reactions were performed and all PCR reactions, conducted in triplicates were pooled after the second PCR. The first PCR (amplification of the V4 and V5 region or ITS1 region) was performed in a total volume of 10 µL (1 µL DNA, 2 µL Taq&Go, 0.1 µL of each Primer, 0.15 µL of mPNA

and pPNA and 6.5 µL of water). Added blocking primers mPNA and pPNA prevented the amplification of mitochondrial and chloroplast DNA (Lundberg et al., 2013). The reactions were performed on a Whatman Biometra® Tpersonal and Tgradient thermocycler (Biometra GmbH, Göttingen, Germany) with the following settings: 95 °C for 45 s, 78 °C 5 s, 55 °C 45 s, 72 °C 90 s (35×), including an initial denaturation of 5 min at 95 °C and a final extension of 5 min at 72 °C. A second PCR step (multiplexing with Golay barcodes) a total volume of 30 µL (2 µL of the first PCR (template), 6 µL Taq&Go, 1.2 µL of barcode-primers and 19.6 µL of water) run at the following settings: 95 °C for 30 s, 53 °C 30 s, 72 °C 30 s (15×), including an initial denaturation of 5 min at 95 °C and a final extension of 5 min at 72 °C. After each PCR amplification step, the quality was checked by gel electrophoresis. All tree replicates of quality checked PCRs from each sample were pooled and purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) according to the protocol. Equimolar DNA concentrations of each barcoded amplicon sample were sent to GATC Biotech AG, Konstanz, Germany. After entry quality control and adapter ligation, 16S rRNA and ITS gene amplicons were sequenced on an Illumina HiSeq instrument.

Data evaluation using bioinformatics tools

Data obtained with Illumina HiSeq amplicon sequencing was analyzed with QIIME 2 (2018.6 release) and QIIME 1.9.1 (Caporaso et al., 2010) according to tutorials provided by the QIIME developers. After joining forward and reversed reads and barcode extraction in QIIME 1.9.1 the data was imported into QIIME 2 for further analysis. After demultiplexing, the DADA2 algorithm (Callahan et al., 2016) was applied to denoise and truncate the reads and summarize sequence variants (SVs) in a feature table. To increase the quality, chimeric data was filtered as well as mitochondria and chloroplast reads (for 16S data) or bacteria and archaea reads (for ITS data) were discarded. A total of 3,489 ITS and 8,935 16S SVs were assigned for a total of 16,155,698 ITS and 4,036,955 16S reads (Table 3.S3). Alpha diversity, beta diversity as well as statistical analysis was performed using the QIIME2 core diversity metrics. Naïve-Bayes classifier were trained on the SILVA v128 (Quast et al., 2013) at 99% similarity as well as the UNITE v7.2 (Kõljalg et al., 2013) database for taxonomic assignment. Subsequently, core microbiomes (features present in at least 50% of the samples) were calculated for each group (healthy and decaying) and exported for display in bar charts. Functional analysis of fungal feature tables was performed using the FUNGuild online tool (Nguyen et al., 2016).

Statistical analysis of bioinformatics data

Alpha and beta diversity was tested in QIIME 2. Therefore, the Kruskal-Wallis (alpha) and the anosim test (beta) were used. Variance explained by parameters was analyzed with a PERMANOVA test in

QIIME. Significant taxonomic differences between the groups were observed with the ANCOM test in QIIME 2.

Real-time qPCR measurement targeting microbial indicators

Following the community DNA extraction from stored sugar beet samples obtained under controlled conditions, qPCR amplifications using specific primers, were conducted in order to quantify distinct taxonomic groups that were selected as disease indicators. Specific primers targeting *Candida*, *Fusarium*, *Penicillium*, *Lactobacillus*, as found in previous literature were implemented. Primers for *Vishniacozyma* and *Plectosphaerella* were designed using the Primer-BLAST tool (Ye et al., 2012) and deposited sequences in the NCBI database (Tab. 3.1). The quantification was performed with a Corbett Research TM thermocycler (Rotor-Gene 6000, Corbett Research, United Kingdom) and SYBR Green PCR master mix TM (KAPA Biosystems, USA). The standard curves were obtained using a single isolate gene fragment with known copy numbers and further 1:10 dilutions. Three replicates of each standard dilution were prepared to calculate mean values. The standards were employed to determine the gene copy numbers in the analyzed samples. Negative controls (using pure dH_2O) were implemented and further subtracted from the analyzed samples to reduce quantification inaccuracies.

Table 3.1: Sequences, annealing temperatures, fragment length and sources of the implemented qPCR primers. The primers for *Vishniacozyma* and *Plectospaerella* were designed with deposited sequences (accession numbers provided) in the NCBI database and the Primer-BLAST tool (Ye et al., 2012).

Taxonomic group	Forward primer	Reverse primer	Length [bp]	Annealing Temp [°C]	Reference/ Source
Vishniacozyma	CGCATCGATGAAGAACGCAG	AAAACCCAAGTGGGGTGAGG	151	64.6	NR_073260.1, this study
Plectospaerella	ATCTCTTGGCTCCAGCATCG	GATACTGGAAGGCGCCATGT	112	65	GU724980.1, this study
Candida	TCTAACGTCTATGCGAGTG	ATACCCAAATTCGACGATCG	244	59.4	(Ogata et al., 2015)
Fusarium	CAACTCCCAAACCCCTGTGA	GCGACGATTACCAGTAACGA	398	58	(Abd-Elsalam et al., 2003)
Lactobacillus	GCAGCAGTAGGGAATCTTCCA	GCATTYCACCGCTACACATG	342	62.1	(Walter et al., 2001)
Penicillium	ATGAAATCCTCCCTGTGGGTTAGT	GAAGGATAATTTCCGGGGTAGTCATT	92	65	(Tannous et al., 2015)

Supplementary Material

Table 3.S1: Summary of performed PERMANOVA test. Pairwise comparison of categories using the unweighted (UUF) and weighted (WUF) UniFrac distance metrics for both, the 16S and ITS, datasets.

UUF

16S

Group 1	Group 2	Df	Permutations	SumOfSqs	MeanSqs	p-value	F.Model	R2sqared	%Influence
healthy	**diseased**	1	999	2.846	2.846	0.001	56.358	0.333	33.3
Austria	**Germany**	1	999	0.425	0.425	0.001	5.911	0.050	5.0
6 beet clamp locations		5	999	1.160	0.232	0.001	3.420	0.136	13.6

ITS

Group 1	Group 2	Df	Permutations	SumOfSqs	MeanSqs	p-value	F.Model	R2sqared	%Influence
healthy	**diseased**	1	999	1.805	1.805	0.001	30.912	0.209	20.9
Austria	**Germany**	1	999	1.009	1.009	0.001	15.477	0.117	11.7
6 beet clamp locations		5	999	1.871	0.374	0.001	6.251	0.217	21.7

WUF

16S

Group 1	Group 2	Df	Permutations	SumOfSqs	MeanSqs	p-value	F.Model	R2sqared	%Influence
healthy	**diseased**	1	999	1.229	1.229	0.001	36.742	0.245	24.5
Austria	**Germany**	1	999	0.432	0.432	0.001	10.658	0.086	8.6
6 beet clamp locations		5	999	1.125	0.225	0.001	6.314	0.225	22.5

ITS

Group 1	Group 2	Df	Permutations	SumOfSqs	MeanSqs	p-value	F.Model	R2sqared	%Influence
healthy	**diseased**	1	999	3.968	3.968	0.001	16.044	0.121	12.1
Austria	**Germany**	1	999	3.543	3.543	0.001	14.120	0.108	10.8
6 beet clamp locations		5	999	6.322	1.264	0.001	5.375	0.192	19.2

Table 3.S2: Sampling locations and sample conditions of the implemented sugar beets. Healthy and decaying beets were sampled from beet clamps in Austria (AT) and Germany (DE). At the locations Kleinweichs and Osterhofen, two neighboring beet clamps were sampled (1 and 2).

Beet clamp	Country	Location	healthy samples	decaying samples
Grossmugl	AT	48° 29' 33.065''N, 16° 14' 11.77''E	15	40
Kleinweichs 1	DE	48° 45' 44.896''N, 12° 50' 30.328''E	5	13
Kleinweichs 2	DE	48° 45' 59.35''N, 12° 49' 50.905''E	5	9
Mittich	DE	48° 26' 18.139''N, 13° 23' 1.755''E	5	8

Osterhofen 1	DE	48° 42' 58.522''N, 13° 1' 1.293''E	5	6
Osterhofen 2	DE	48° 42' 39.42''N, 13° 1' 21.604''E	5	4

Table 3.S3: Overview of sequencing data. Number of reads, assigned **sequence variants (SVs)** using the DADA2 algorithm and Shannon Index of each group is given.

Sample	No. samples	No. ITS reads	No. 16S reads	SVs ITS	SVs 16S	Shannon Index ITS	Shannon Index 16S
Decaying	80	12,704,534	3,140,874	1,862	5,024	3.5	4.5
Healthy	40	3,451,164	886,081	1,627	3,911	4.5	5.5
Total	120	16,155,698	4,036,955	3,489	8,935	3.8	4.8

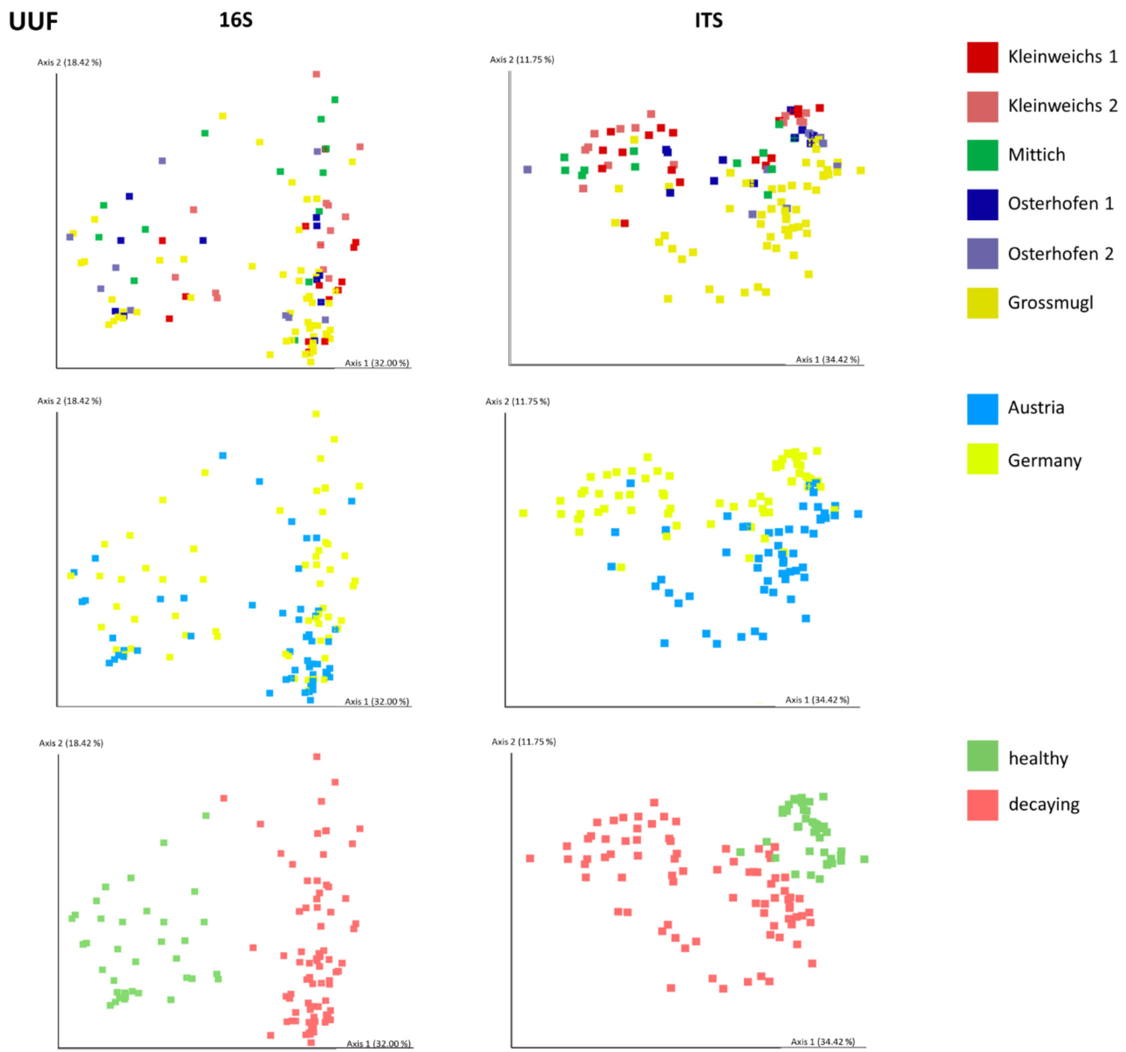

Figure 3.S1: Principal component analysis of bacterial and fungal communities from different beet clamps. PCoA using the unweighted UniFrac (UUF) distance metric. Samples are color-coded based on their geographic origin or health status.

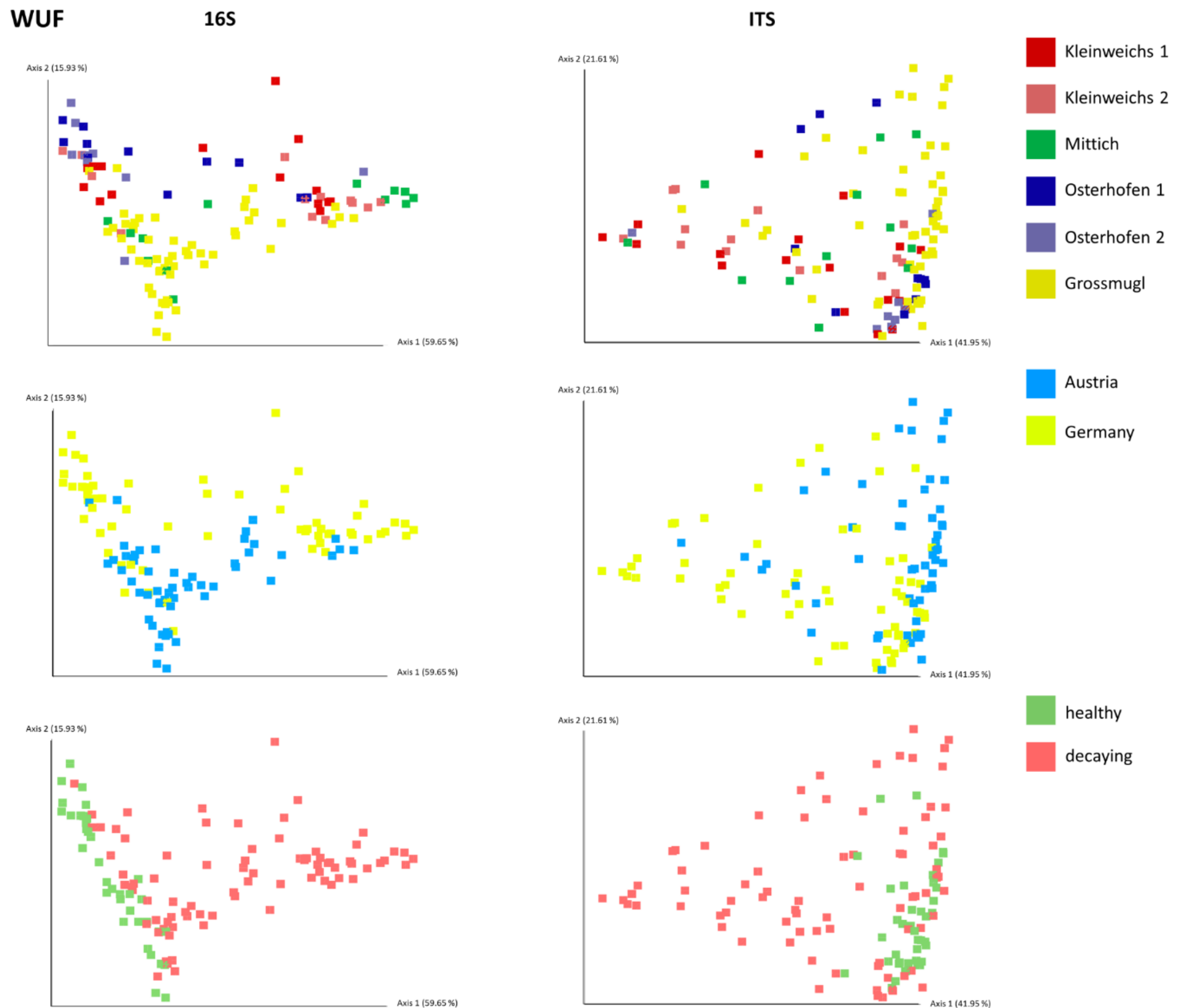

Figure 3.S2: Principal component analysis of bacterial and fungal communities from different beet clamps. PCoA using the weighted UniFrac (WUF) distance metric. Samples are color-coded based on their geographic origin or health status.

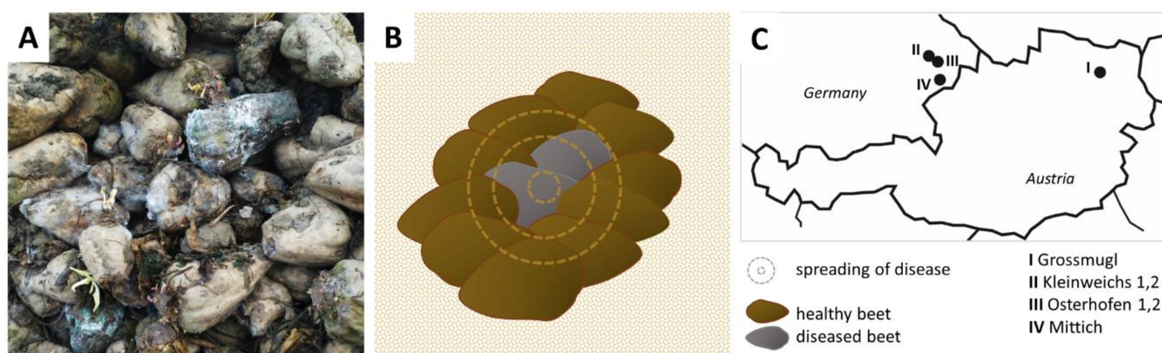

Figure 3.S3: Sample visualization, schematic representation of fungal growth in the beet clamps, and geographic locations of the sampling sites. Fungal nests start within the clamp and spread to the surrounding beets (**A, B**). Healthy, uninfected beets, as well as decaying sugar beets within the same beet clamp were sampled from six different beet clamps in Austria and Germany (**C**).

4 Manuscript II: On-field microbial community influences postharvest root rot in sugar beets

P. Kusstatscher[1,2], T. Cernava[2,3], K. Harms[4], J. Maier[4], H. Eigner[5], G. Berg[2] and C. Zachow[1]

[1] ACIB GmbH, Graz, Austria

[2] Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria

[3] Roombiotic GmbH, Graz, Austria

[4] Südzucker AG, Mannheim, Germany

[5] Agrana Research & Innovation Center, Tulln, Austria

Manuscript

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ABSTRACT

Sugar beets (*Beta vulgaris* L.) are grown in temperate regions and primarily used for sugar production. Due to limited capacities of the sugar refineries, sugar beets in Europe are stored after harvest for up to 60 days. Microbial degradation leads to sugar decrease during this time. To investigate disease impact on microbiome level, the bacterial and fungal community of field-grown as well as stored sugar beets was compared. Using a barcoded amplicon sequencing approach targeting bacteria and fungi, microbiome dynamics, differences and potential functions were accessed. Moreover, microbial transmission from field-grown to stored sugar beets was investigated. Interestingly, the microbiome of beets affected by root rot in the field as well as in storage showed broad overlaps and bioinformatics-based predictions indicated a transmission of field microbiomes to the storage clamps. Root rot was accompanied by a loss in microbial diversity (Shannon index decrease of 5.5 to 4.5 (bacteria) and 4.5 to 3.5 (fungi)) as well as the replacement of *Plectosphaerella* and *Vishniacozyma*, as predominant species in healthy roots, with *Penicillium*, *Candida* and *Fusarium* sp. in rotting beets. Furthermore, the Gram-positive *Lactobacillus* was the predominant bacterial genus in rotting beets. Along with taxonomic changes also a trophic specialization of the fungal community was observed. The overall findings can be implemented in new postharvest strategies following a microbiome-driven approach for biological treatments.

Keywords: *Beta vulgaris*; storage rot; sugar beet microbiome; health indicator species

Introduction

Microorganisms colonizing plants live in close relationship with their hosts (Berendsen et al., 2012; Vandenkoornhuyse et al., 2015). Recent studies have shown that the plant microbiome is not only important for the plant growth and preharvest health, but also plays a big part in postharvest stability of fruits and vegetables (Berg, 2009; Droby and Wisniewski, 2018). The herbaceous dicotyledonous plant, *Beta vulgaris* L. (sugar beet) is the main crop for sugar production (sucrose content up to 18%) in temperate regions all over the world (Trebbi and McGrath, 2004). Pathogen pressure before harvest caused by fungal phytopathogens like *Phythium ultimum* Trow and *Rhizoctonia solani* Kühn (Zachow et al., 2008), but especially decay after harvest is diminishing sugar yield. With extended storage periods of 45-60 days in Europe, sugar beets, containing high water and sugar concentrations, provide ideal conditions for postharvest microbial colonization (Liebe et al., 2016).

Interactions and dynamics of microbes before and during storage are especially important for the development of biocontrol application. This study investigates the sugar beet microbiome before harvest, on the field, and during storage in beet clamps and its implications in health and storability of the plant. Bioinformatic predictions were applied to understand the connections between the field and storage clamp microbiome and the microbial diversity. Overall diversity and taxonomic differences were assessed in detail to facilitate future developments of pathogen management technologies based on biological applications.

Material and Methods

Sampling and DNA extraction

Healthy (N= 86) and diseased (N= 97) sugar beets were sampled from fields (N= 13) and beet clamps (N= 6) in Austria and Germany. During sampling their health status was inspected visually. Upon arrival in the laboratory, 20 g of sugar beet peel was homogenized with 50 mL of NaCl solution (0.85%) in a stomacher (BagMixer, St. Nom, France) for 3 min. A total of 4 mL of the obtained solution was centrifuged (13,000 × g, 20 min) to a pellet and frozen at -70°C for further use. Total DNA extraction was performed using the FastDNA® Kit for Soil (MP Biomedicals, USA) according to the manufacturer's protocol.

Construction of barcoded amplicon libraries

Barcoded amplicon libraries were constructed targeting the V4-V5 region of the 16S rRNA gene as well as the ITS1 region according to the Earth Microbiome Project protocol (Parada et al., 2016; Walters et al., 2015). The primers were barcoded using linker sequences. All PCR steps were conducted in triplicates and according to Kusstatscher and colleagues (Kusstatscher et al., 2019a). All PCRs were

quality checked via gel electrophoresis and purified up using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA). Equimolar DNA concentrations of each barcoded amplicon sample were sent to GATC Biotech AG, Konstanz, Germany. After entry quality control and adapter ligation, 16S rRNA and ITS gene amplicons were sequenced on an Illumina HiSeq2500 instrument (2x 250 bp).

Data processing using bioinformatics tools

Data was evaluated with a combined approach in QIIME 1.9.1 and QIIME 2 (2018.6 release) (Caporaso et al., 2010). After demultiplexing and quality filtering sequence variants (SVs) were summarized in a feature table using the DADA2 algorithm. Features were classified using a Naïve Bayes classifier trained on the SILVA 128 release and UNITE v7.2. Alpha and beta diversity was analyzed in QIIME 2. Core features, present in 50% of the samples were generated for each health status (healthy and diseased) and used for taxonomic comparison. Source tracking was performed using the sourcetracker2 software (Knights et al., 2011). Trophic modes of fungal features were analyzed using the online-tool FUNGuild (Nguyen et al., 2016).

Statistical analysis

Alpha and beta diversity was analyzed in QIIME 2. Therefore, the Kruskal-Wallis (alpha) and the anosim test (beta) were used. Variance explained by parameters was analyzed with a PERMANOVA test and significant taxonomic differences were tested using the Kruskal-Wallis test with multiple testing correction (FDR) in QIIME 1.9.1.

Results

Diversity analysis

The diversity comparison of the 193 samples from healthy and diseased sugar beets from fields and clamps in Austria and Germany showed significant lower diversity in diseased samples (Shannon index 4.5 compared to 5.5 (bacterial community) and 3.5 compared to 4.5 (fungal community)) (Fig. 4.1 A,B). The calculated weighed unifrac distances (WUF) showed significant ($p \leq 0.05$) grouping of the samples within the two groups (healthy and diseased). Diseased samples showed generally a higher diversity within the group (Fig. 4.1 C,D).

Further statistical analysis obtaining the proportion of variance explained by a certain factor using the weighted unifrac distance matrix (WUF) showed a high influence of the sampling site (explaining 22%-46% variance) as well as of the health status (explaining 14-52% variance) in the samples (Fig. 4.1 E,F). The sampling country (geographic influence) on the other hand was less influential (explaining 8-14% variance).

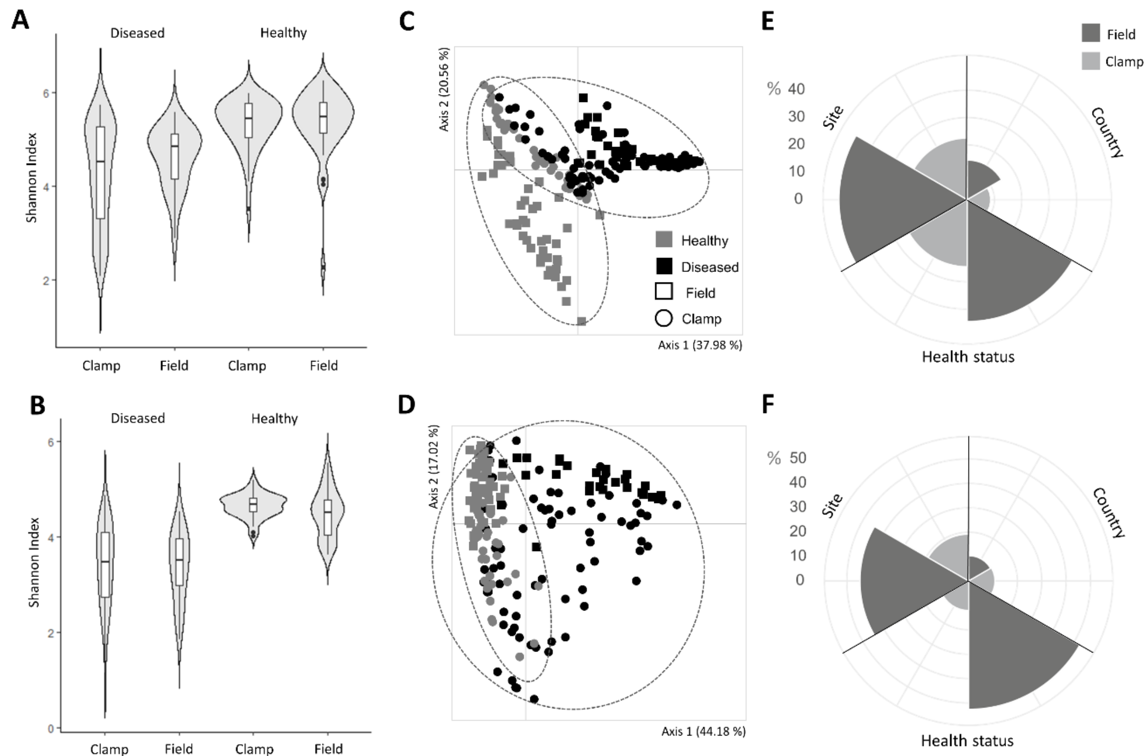

Figure 4.1: Diversity of analyzed samples and explained variance by different parameters. Shannon indices for bacterial (A) and fungal (B) data were calculated using the Kruskal-Wallis test. The weighted unifrac distances (WUF) between samples are shown for bacterial (C) and fungal data (D). The explained variance tested with PERMANOVA by three parameters (sampling site, health status and sampling country) is shown for the bacterial (E) and fungal data (F).

Taxonomic comparison and microbial functions

Core microbiomes (features present in 50% of the samples) of both groups (healthy and diseased) showed a distinct colonization of the beets with specific taxa. While in the bacterial microbiome, *Flavobacterium* was the most abundant taxon for healthy samples (12-21%), diseased sugar beets in comparison showed high abundance in *Lactobacillus* (18-26%), *Leuconostoc* (7-11%) and *Gluconobater* (6-16%) (Tab. 4.1). In the fungal microbiome, similar taxonomic changes can be observed. *Plectosphaerella* (24-25%) and *Vishniacozyma* (13-16%) were by far the most abundant taxa in healthy samples, *Candida* (10-33 %), *Penicillium* (10%) and *Plectosphaerella* (3.5-10%) were the most abundant taxa in diseased samples. In the fungal microbiome also some unique taxa were found for distinct fields, especially members of *Basidiomycota* such as *Mrakia* (2-8%), *Guehomyces* (7-10%) and *Leucosporidium* (2-3%). These yeast taxa were always found in higher relative abundances in decaying beets (Tab. 4.2).

The consequence of the taxonomic differences between healthy and decaying sugar beets for the fungal microbiome became evident when assigning taxonomic groups to trophic modes (detailed data not shown). While in the healthy samples (field and clamp) both pathotrophic and saprotrophic

groups were in a relatively balanced abundance (15-35%), the saprotrophic group in diseased samples was highly increased (+21 percent points).

Table 4.1: Taxonomic composition of sugar beet samples in core microbiomes grouped relating according their health status. Bacterial core features (present in 50% of samples and rel. abundance ≥ 2%) of field samples and beet clamp samples are shown. Features (grouped by their phylum) are listed on genus level, or where feature classification was not applicable on genus level, they were identified on family level (f_). Taxa significantly increased in field or clamp samples, increased in healthy or diseased samples were tested using the Kruskal-Wallis test ($p \leq 0.001$).

Phylum	Genus/Taxa	Healthy Field (%)	Diseased Field (%)	Healthy Clamp (%)	Diseased Clamp (%)
Actinobacteria	*Rhodococcus*	0.47[1]	2.30[1]	0.00	0.00
	Microbacterium	1.64	2.60	2.58	1.25
	Pseudarthrobacter	2.24	0.86	13.51[1]	3.44[1]
	Marmoricola	2.25[1]	1.32[1]	0.00	0.00
	Nocardioides	2.38	0.00	4.13	0.99
Bacteroidetes	*Dysgonomonas*	0.00	3.26	0.00	0.00
	Flavobacterium	11.81[2]	6.35	20.62[2]	7.14
	Pedobacter	2.40	1.38	5.22	1.95
	Sphingobacterium	0.89[1]	3.05[1]	0.00	0.00
Cyanobacteria	*Microcoleus*	2.07	0.00	0.00	0.00
Firmicutes	*Lactobacillus*	0.00	26.30[3]	0.00	18.37[3]
	Leuconostoc	0.00	6.60[3]	0.00	11.29[3]
Proteobacteria	*Ochrobactrum*	0.00	2.25	0.00	0.00
	Rhizobium	1.44	2.36	5.14	2.09
	f_Acetobacteraceae	0.00	0.00	0.00	2.10
	Gluconobacter	0.00	5.52[3]	0.67	16.04[3]
	f_Sphingomonadaceae	2.19	0.00	0.00	0.00
	Novosphingobium	3.03	0.00	0.00	0.00
	Sphingomonas	0.00	0.00	4.80[1]	1.16[1]
	f_Enterobacteriaceae	0.79	7.29[3]	1.22	6.80[3]
	Pantoea	0.00	2.57	0.00	0.00
	Pseudomonas	1.94	2.66	8.90[1]	6.53[1]
	Stenotrophomonas	0.90	3.60	2.76	2.88
	Thermomonas	2.22	2.40	3.27	1.53
Other	Other	61.31	17.31	27.19	16.41

Significance: [1] increased in field or clamp samples; [2] increased in healthy samples; [3] increased in diseased samples

Table 4.2: Taxonomic composition of sugar beet samples in core microbiomes grouped according to their health status. Fungal core features (present in 50% of samples and rel. abundance ≥ 2%) of field samples and beet clamp samples are shown. Features (grouped by their phylum) are listed on genus level, or where feature classification was not applicable on genus level, on family (f_), order (o_), class (c_) or kingdom level (k_). Taxa significantly increased in field or clamp samples, increased in healthy or diseased samples were tested using the Kruskal-Wallis test ($p \leq 0.001$).

Phylum	Genus/Taxa	Healthy Field (%)	Diseased Field (%)	Healthy Clamp (%)	Diseased Clamp (%)
Ascomycota	*Cladosporium*	2.07	2.48	2.76	3.15
	f_Mycosphaerellaceae	3.89[1]	0.19[1]	0.00	0.00
	Mycosphaerella	0.00	0.00	2.01[1]	1.26[1]
	Penicillium	0.31	10.27[3]	0.85	9.68[3]
	o_Helotiales	4.52	0.00	0.00	0.00
	Tetracladium	0.00	0.00	8.18[1]	3.43[1]
	f_Sclerotiniaceae	0.00	0.00	0.04	2.93
	Barnettozyma	0.05[1]	2.37[1]	0.00	0.00
	Candida	1.10	32.88[3]	1.46	9.72[3]
	c_Sordariomycetes	15.73[2]	3.36	11.38[2]	2.69
	Colletotrichum	3.53[1]	0.17[1]	0.00	0.00
	Plectosphaerella	24.18[2]	3.48	25.18[2]	9.76
	o_Hypocreales	0.00	0.00	2.89[1]	2.76[1]
	Clonostachys	0.06[1]	4.05[1]	0.00	0.00
	f_Nectriaceae	4.45	8.03[3]	1.46	7.08[3]
	Fusarium	1.71	16.34[3]	0.53	0.48
Basidiomycota	*Leucosporidium*	0.00	0.00	1.53[1]	3.36[1]
	Cystofilobasidium	0.00	0.00	0.59[1]	2.45[1]
	Guehomyces	0.00	0.00	7.10[1]	9.70[1]
	Mrakia	0.00	0.00	2.20[1]	7.67[1]
	Hannaella	2.25[1]	0.23[1]	0.00	0.00
	Vishniacozyma	13.27[2]	2.40	15.96[2]	6.19
Other	*k_Fungi*	1.29	5.97[3]	1.10	5.37[3]
	Other	21.60	7.78	14.79	12.31

Significance: [1] increased in field or clamp samples; [2] increased in healthy samples; [3] increased in diseased samples

Microbial transmission of field microbiomes into storage

By using specific bioinformatic tools, microbiome origins were tracked in different sample groups. The analysis of the clamp microbiomes and its origin in the microbiomes of the sugar beet field showed that a high percentage (59-87%) originates from the field. Interestingly, diseased field samples were mostly identified as the microbiome origin of diseased clamps (31-50%) and healthy field microbiomes were mostly the origin of healthy clamps (60-80%) (Fig. 4.2).

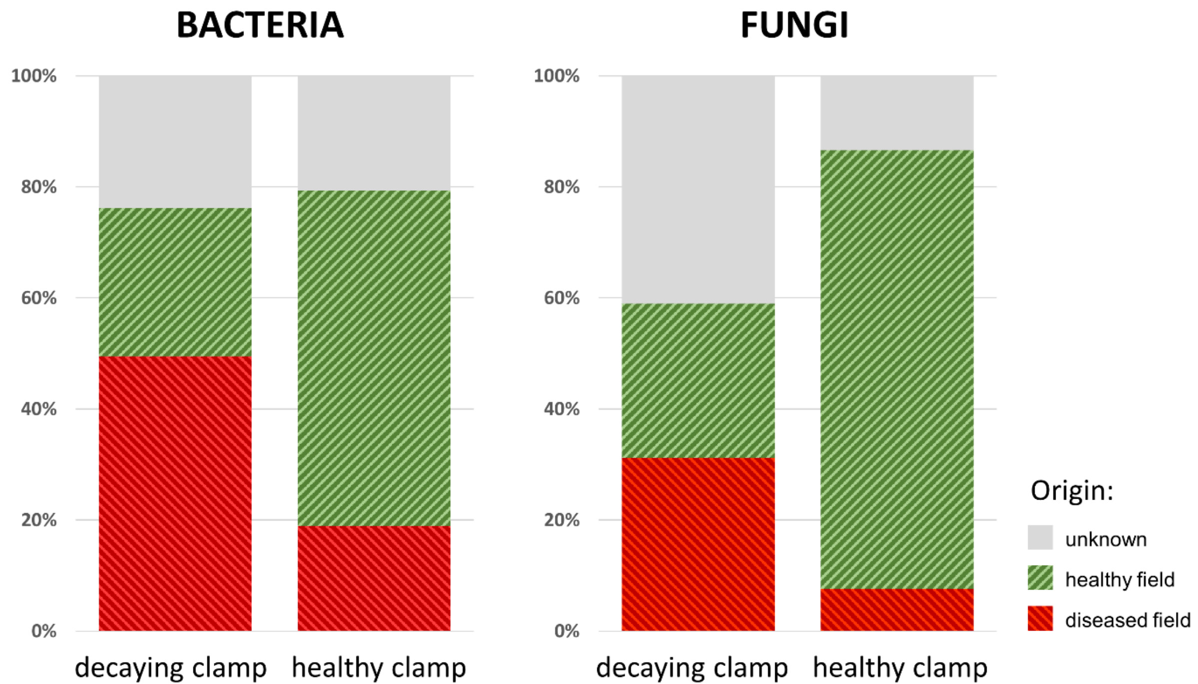

Figure 4.2: Predicted microbial transmission from the field to beet clamps. The source of the decaying or healthy microbiome in the clamp was calculated for the bacterial and fungal data using the Sourcetracker2 software.

Discussion

In this study, detailed insights into the bacterial as well as fungal community of healthy and diseased sugar beets on fields as well as in the beet clamps were obtained. Moreover, the similarities of the analyzed microbiomes of sugar beet fields with the clamp microbiomes were highlighted. The alpha and beta diversity in healthy and diseased sugar beets was shown to be fundamentally different. Healthy sugar beets usually showed a higher microbial diversity compared to decaying beets. These findings support the hypotheses provided by Berg and colleagues (2017), where plant health is linked to microbial balance and higher diversity (Berg et al., 2017).

Not only diversity changes between the two health states were found, but also substantial differences in taxonomic composition. While, in the bacterial community, the healthy samples showed a high taxonomic diversity with only *Flavobacterium* as a higher abundant, group-specific taxon, especially *Lactobacillales* (*Lactobacillus*, *Leuconostoc*) were highly increased in decaying samples. In the fungal community, the shift from *Plectosphaerella* and *Vishniacozyma* as the main taxa to *Penicillium* and *Candida* accompanied the transition from a healthy to a diseased sugar beet microbiome. Similar taxonomic changes in stored sugar beets were observed by Liebe et al. (2016) were *Plectosphaerella* was mostly found in reference sugar beets at the beginning of storage, while rotting sugar beets showed a high proportion of *Penicillium* species (Liebe et al., 2016).

Interestingly, the observed taxonomic changes had a high influence also in trophic functions of the fungal microbiome. While pathotrophic symbiotrophic and saprotrophic groups were found balanced in healthy sugar beets, decaying sugar beets inhabited a highly increased proportion of saprotrophic fungi.

Additionally, bioinformatic tools targeting microbial transmission of field microbiomes showed its influence on beet microbiomes during storage. A high proportion of the microbiome found on the fields was transferred into beet clamps and influenced the storability. While microbial changes during storage were already known, the new findings could further facilitate the development for innovative postharvest storage technologies optimized for sugar beets.

Conclusion

Sugar beet rot on the field and during storage was shown to be accompanied by a change in microbial diversity as well as abundance. Substantial shifts in the bacterial and fungal community were analogous in field and clamp samples and microbiome traces that were transmitted from the fields to the clamps were found. Changes in the prevalence of certain taxonomic groups could be used as early indicators for decay and facilitate an early implementation of countermeasures. Taxonomic changes were always accompanied by trophic specialization in the fungal community. Future postharvest biocontrol applications might be improved if the maintenance of microbial diversity and antagonism towards specific taxa is ensured.

5 Manuscript III: Microbiome response to hot water treatment and potential synergy with biological control on stored apples

Birgit Wassermann[#], Peter Kusstatscher[#] and Gabriele Berg

Institute of Environmental Biotechnology, Graz University of Technology, Graz, Austria

[#] both authors contributed equally

Manuscript

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, 2011). 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 (FAO-STAT, 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., 2010). *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., 2004; 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; Maxin et al., 2014; Pavoncello et al., 2001). 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 5.S1. 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 an 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 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 5.S1 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.

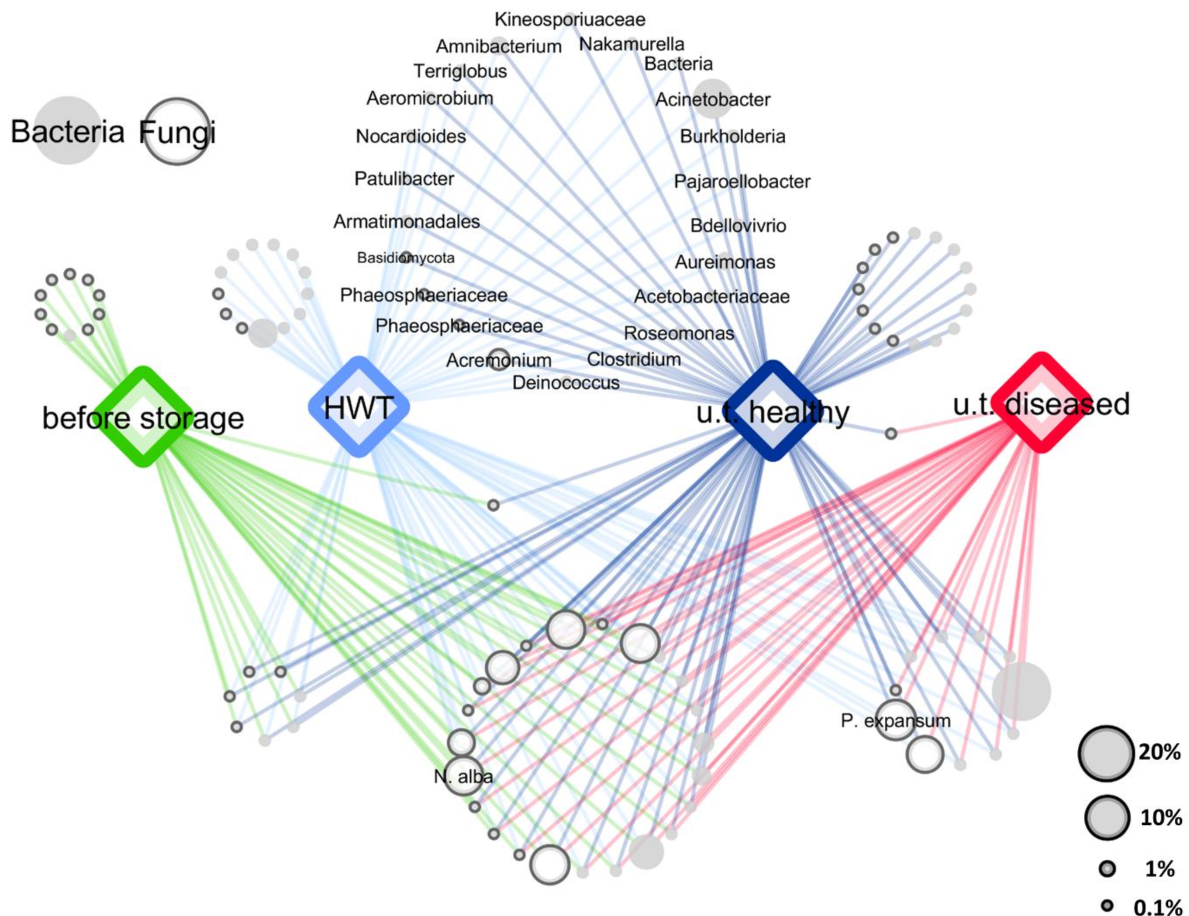

Figure 5.1: Core and specific microbiota for the four apple groups. Core bacterial and fungal microbiota on genus level (taxa occurring in 50% of all replicates) of the four groups 'before storage', 'HWT', 'untreated healthy' (u.t. healthy) and 'untreated diseased' (u.t. diseased) were combined for network 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.

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 5.1). 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.

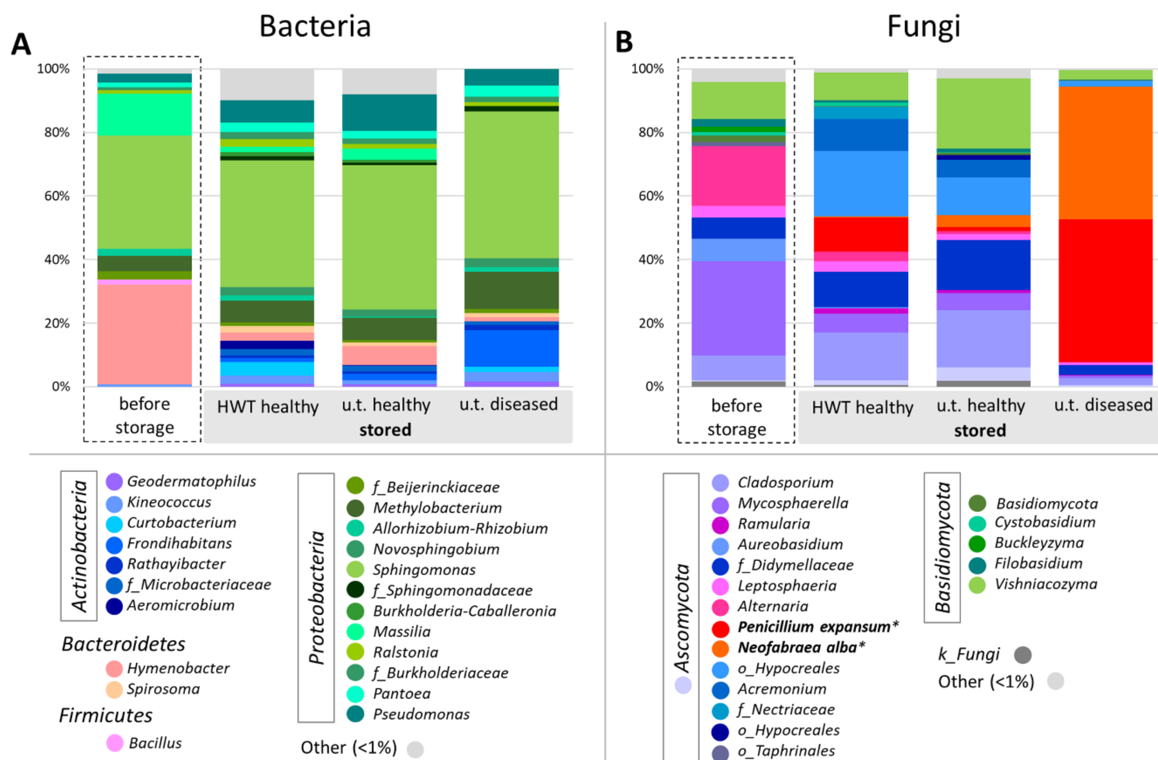

Figure 5.2: Bacterial and fungal taxonomy of apples investigated. Core microbiomes were defined for taxa occurring in 50% of the replicates in the respective groups. Color-coded bacterial (A) and fungal (B) taxa are indicated in the bottom legend and are shown on genus level and grouped by phylum. Sequences 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'.

Taxonomic changes induced by storage and disease

In order to compare taxonomic composition of the four groups, figure 2 was constructed for the bacterial (Figure 5.2, A) and fungal (Figure 5.2, 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 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 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.

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 5.3, 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 5.3, B).

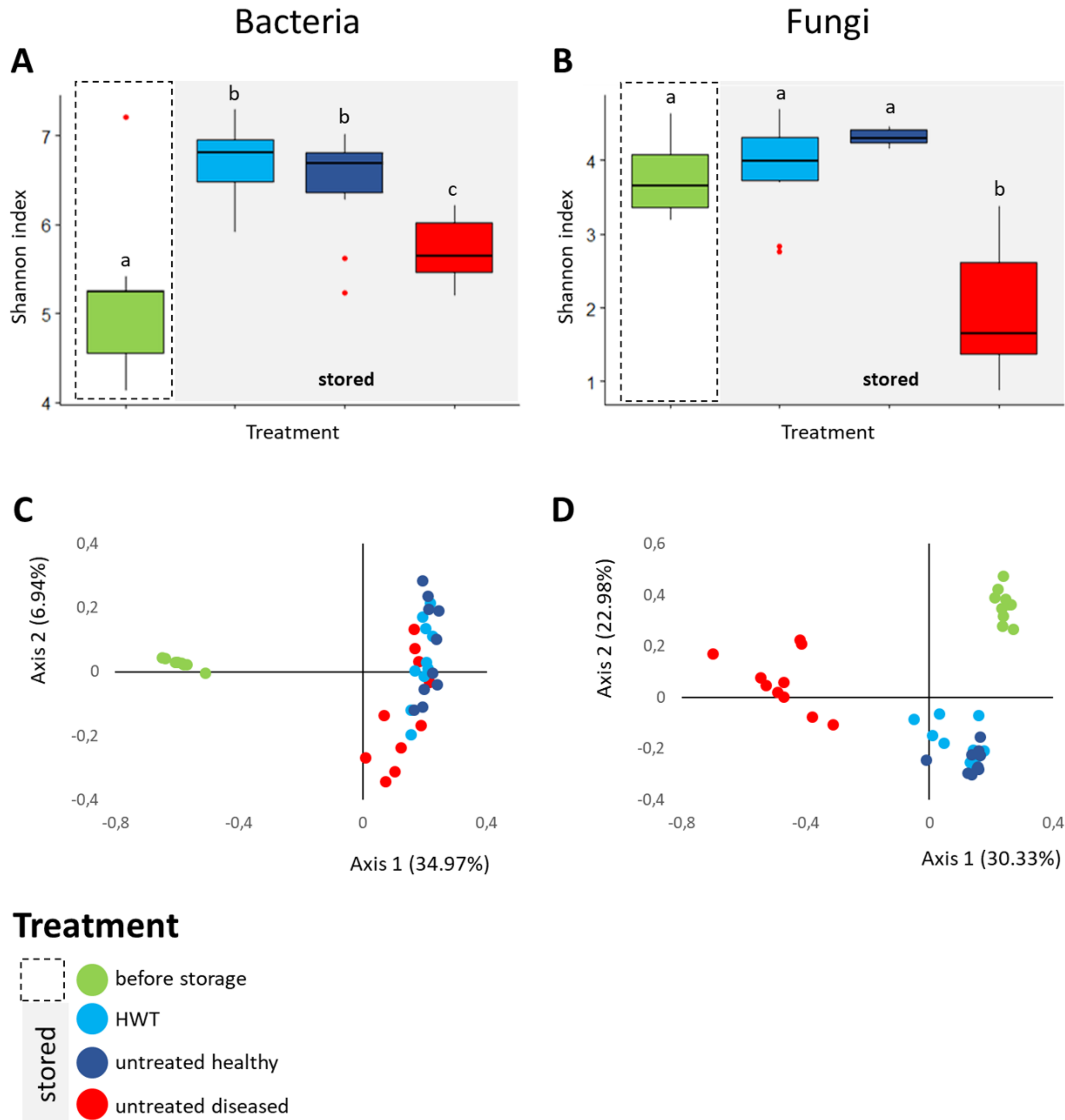

Figure 5.3: Alpha- and beta-diversity analyses on apple-associated bacterial and fungal structure. Box-and-Whiskers-plots visualize Shannon diversity index of the four different apple groups for bacteria (A) and fungi (B). Significant differences ($p \leq 0.05$) were assessed by Kruskal Wallis test and 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 5.1.

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 5.3, C and D). Statistical significance in bacterial composition, assessed via pairwise ANOSIM (Table 5.1), 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.

Table 5.1: 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

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 5.S2). 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 5.S3). 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.

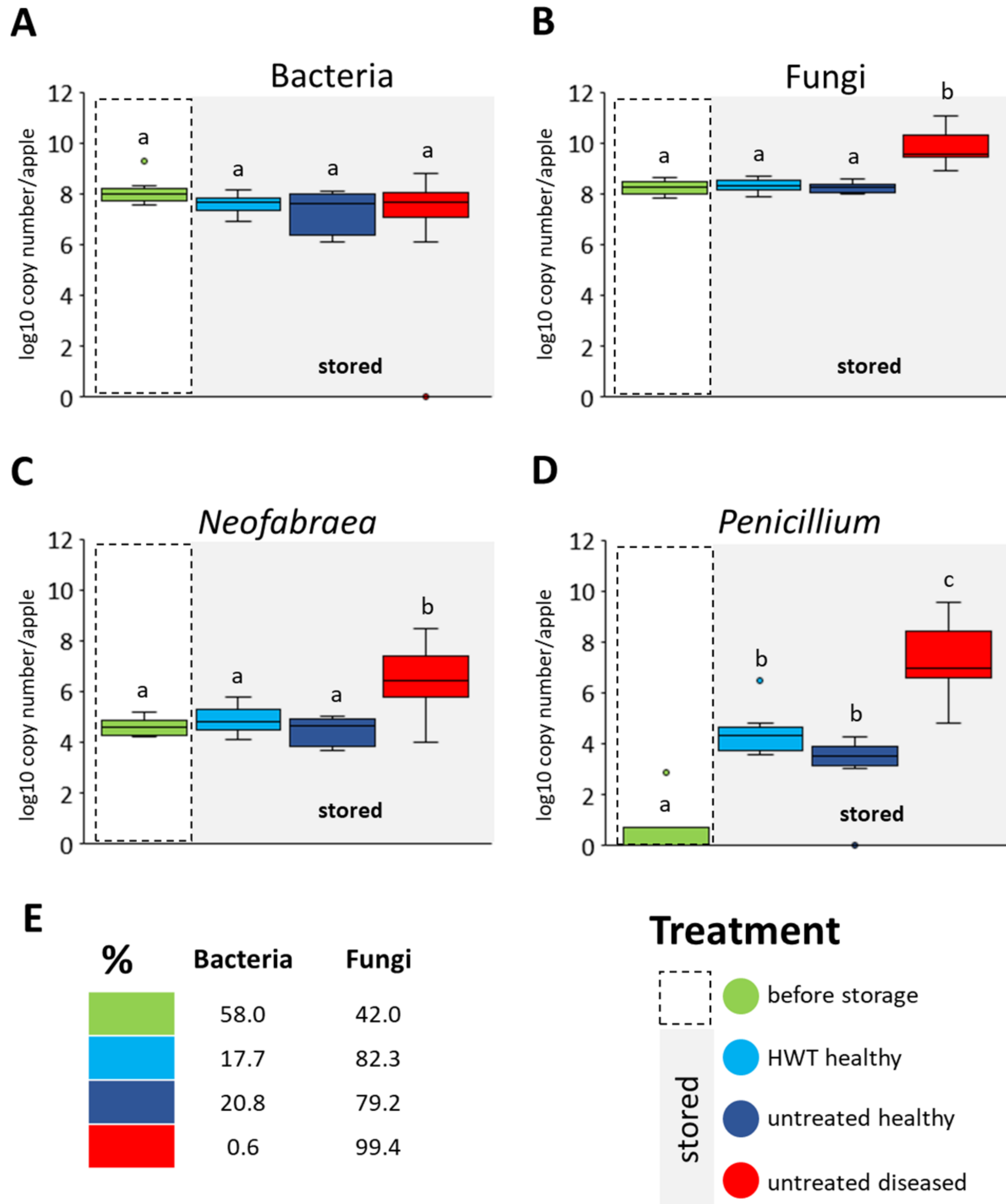

Figure 5.4: Microbial gene copy numbers in apple groups determined by qPCR. Values are given by primers targeting bacterial 16S rRNA genes (A), fungal ITS region (B) and genes of *N. alba* (C) and *P. expansum* (D). Gene copy numbers are calculated per apple used for the microbiome analysis. Significant 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.

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

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 5.4, 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 5.4, B), due to significant increase of both storage pathogens *Neofabraea* and *P. expansum* (Figure 5.4, 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 5.4, 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.

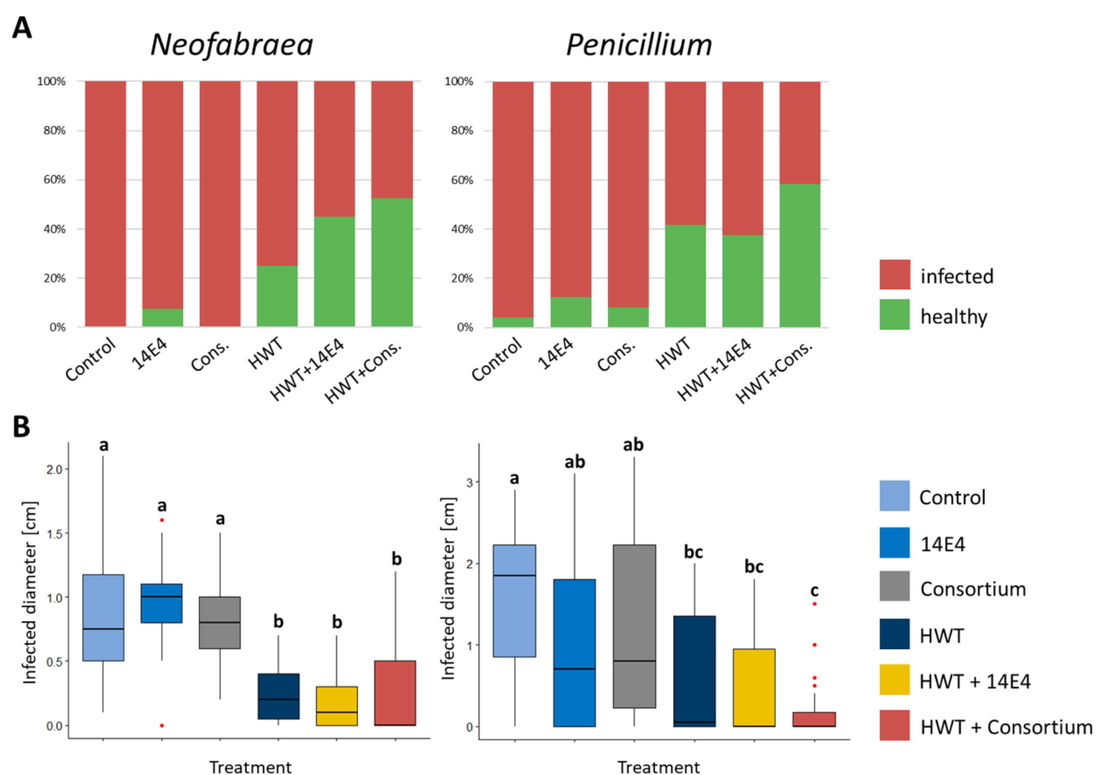

Figure 5.5: Fraction of infected apples after storage (A) and analysis of infected diameter (B). Apples were treated with fungal spores or conidia as well as bacterial strain *P. vagans* 14E4, a bacterial consortium and/or HWT. Control samples were only inoculated with fungal spores and stored. Statistical differences between differentially treated apple samples was assessed by Wilcox test (Bonferroni correction) and are indicated by lower case letters.

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 5.5, 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 5.5, 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.

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 (Conway et al., 2004; Spadaro et al., 2004), citrus fruits (Obagwu and Korsten, 2003; Porat et al., 2002), pear (Zhang et al., 2008), strawberry (Wszelaki and Mitcham, 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

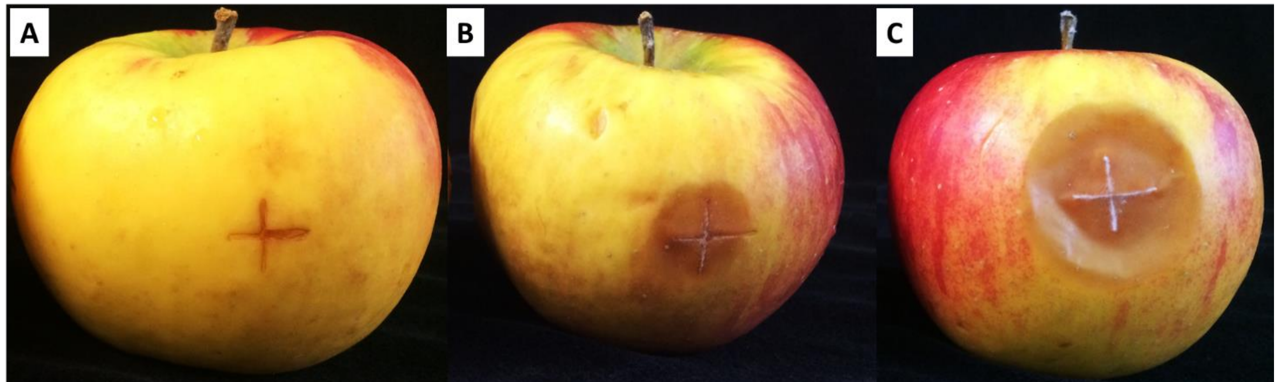

Figure 5.S1: Pathosystem of *P. expansum* on Topaz apples directly (A), one week (B) and three weeks (C) after artificial wounding over the course of small scale storage experiments. Diameters of infected areas were measured after three weeks of storage.

Table 5.S1: Primers used in the present study.

Primer name	Primer sequence 5'-3'	Reference
515f	GTGYCAGCMGCCGCGGTAA	(Caporaso et al., 2010)
927r	CCGYCAATTYMTTTRAGTTT	
ITS 1f	CTTGGTCATTTAGAGGAAGTAA	(White et al., 1990)
ITS 2r	GCTGCGTTCTTCATCGATGC	
Pexp_patF_F	ATGAAATCCTCCCTGTGGGTTAGT	(Tannous et al., 2015)
Pexp_patF_R	GAAGGATAATTTCCGGGGTAGTCATT	
NeoF	CTTTCTCCGTTGTCCCATCC	(Cao et al., 2013)
NeoR	GAACATTGCGCATCTGGTCC	

Table 5.S2: Bacterial and fungal genera with significantly different abundance in 'untreated healthy' and 'untreated diseased' apples.

	Taxonomy	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).

Table 5.S3: 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).

6 Manuscript IV: Using bacteria-derived volatile organic compounds (VOCs) for industrial processes

Peter Kusstatscher, Tomislav Cernava and Gabriele Berg

Institute of Environmental Biotechnology, Petersgasse 12, A-8010 Graz, Austria

Book chapter written for the Springer book:

Bacterial volatile compounds as mediators of airborne interactions

ABSTRACT

Microbial volatiles harbour an extensive spectrum of chemical compounds. Bacteria-derived volatile organic compounds (VOCs) were studied for decades in order to uncover their role in microbial interactions and to decipher their importance in the communication with other organisms. In the frame of extensive screening approaches, various VOCs with growth inhibiting effects against pathogenic bacteria and fungi were found in the recent years. These discoveries have not only a great importance for the general scientific knowledge, but also open the way for many technological applications of those molecules. The application of the discovered bacterial volatiles in industrial decontamination processes provides new alternatives for conventional chemicals. Moreover, they might facilitate the reduction of harmful, toxic and cancerogenic chemicals and widen the toolbox for a broader spectrum of biological decontamination agents. In addition, VOCs have a great potential for microbiome management and control, and can be applied as bio-preservatives, -pesticides, and fumigants.

Introduction

Microbial communities live in close relation with each other and can colonize higher organisms. Many hosts were shown to harbour well-defined and often highly diverse communities, which include bacteria, fungi as well as archaea. Such host-associated microbiota fulfil important functions such as growth promotion or specific defence mechanisms against pathogens (Berg, 2009; Mendes and Raaijmakers, 2015). Our knowledge related to the interactions of bacteria within the community and their hosts gradually improved during the last years. Studies have shown that prevalent defence mechanisms rely on competition for nutrients, antibiosis through soluble molecules and microbial volatile organic compounds (VOCs) (Berg, 2009; Mulero-Aparicio et al., 2019; Raaijmakers et al., 2009). Especially VOCs were found to be mediators of various interactions between microorganisms such as bacteria and fungi but also across kingdoms, e.g. between microorganisms and plants (Effmert et al., 2012; Kanchiswamy et al., 2015). Microbial VOCs typically occur as complex mixtures of relatively low-weight lipophilic compounds and are collectively often described as the "volatilome" (Kanchiswamy et al., 2015; Maffei et al., 2011). The potential of reaching greater distances within the soil or surrounding environment makes them the ideal candidates for signal transduction (Maffei et al., 2011). The functions of volatiles are numerous, e.g. they influence physiological processes (e.g. nitrification), they support metabolic reaction (e.g. as electron acceptors or donors), they are quorum sensing/quenching molecules, they serve as food source within the food chain when integrated in organic matter, or they provide other so far unknown functions (Effmert et al., 2012).

A broad spectrum of different volatile molecules can be released by microorganisms; small inorganic and organic compounds (<120 Da) or more complex VOCs (120-300 Da). While smaller molecules were studied for a long time, larger compounds received more attention just in the last years (Effmert et al., 2012). More than 800 different volatiles were identified so far and it was shown that volatile spectra of single microbial strains can be rather simple (<10 VOCs) or extremely complex (>50 VOCs) (Effmert et al., 2012; Kai et al., 2010, 2007). By applying modern technologies such as solid phase microextraction (SPME), gas chromatography, in combination with mass spectrometry (GC/MS) or other trapping methods such as proton transfer reaction, secondary electron spray ionization an increasing number of volatiles can be detected (Wenke et al., 2012). These detection methods can be coupled with efficient screening assays in order to target volatiles with specific characteristics, e.g. antimicrobial effects against defined phytopathogens (Cernava et al., 2015).

The most dominant classes of molecules emitted by fungi are alcohols, ketones, hydrocarbons, terpenes, alkanes, and alkenes (Chiron, 2005). In contrast, bacteria prominently release alcohols, alkanes, alkenes, ketones but also esters, pyrazines, sulphides and lactones (Wenke et al., 2012).

Terpenoids, short chain-alcohols and acids can especially be found in the volatilome of various *Streptomyces* species (Citron et al., 2012), *Pseudomonas* species emit particularly C9-C16 alkanes and alkenes (Effmert et al., 2012).

The effects of these highly diverse molecules as well as detailed modes of action are largely unknown although their potential for various applications has been discovered a long time ago. Already over 60 years ago Dobbs and Hinson (1953) described the fungistatic effect of bacterial volatiles on soil-borne fungi (Dobbs and Hinson, 1953). In addition, Strobel and colleagues showed the great applicability of fungal VOCs from *Muscodor albus* and brought it for mycofumigation applications into the market (Strobel, 2006). Volatiles produced by *Streptomyces griseus* were shown to induce an early sclerotium formation in *Rhizoctonia solani* and *Sclerotium cepivorum* as well as reduce sporulation in *Gloesporium aridum* (McCain, 1966). Soon single molecules such as but-3-en-2-one from *Streptomyces griseoruber* were found to have a strong growth inhibiting effect on fungi (Herrington et al., 1987). Additionally, acetamide, benzaldehyde, benzothiazole, 1-butanamine, 1-decene, methanamine and phenylacetaldehyde produced by several different genera of bacteria were found to have fungistatic effects (Zou et al., 2007). The discovery of these molecules is not only interesting from the scientific point of view, but also provides the basis for novel, untapped industrial applications. However, the development of competitive products requires a targeted exploration of natural resources (Fig. 6.1). Specific examples for the high potential of microbial volatiles to replace conventionally used disinfectants in the industry will be presented in this chapter. In addition, various applications based on VOCs for the food, animal feed or other biotechnological sectors that were developed in the recent years will be shown. This brief overview is meant to serve as an inspiration for further developments that will be based on these highly promising molecules.

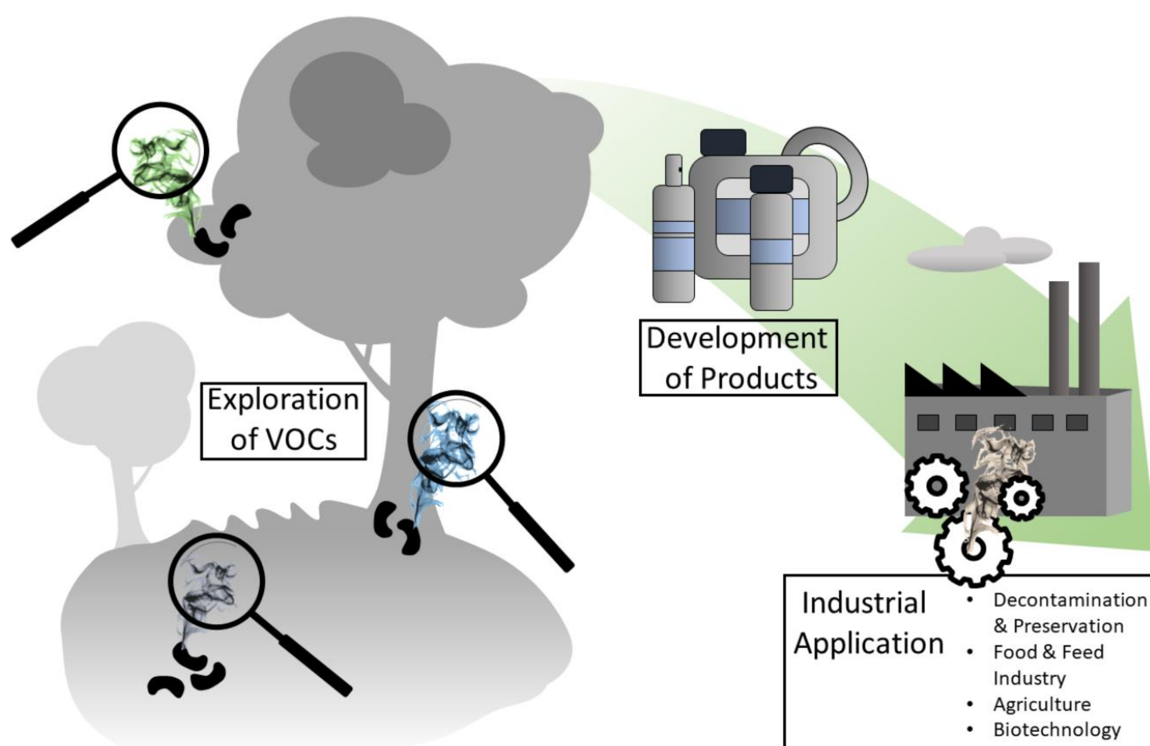

Figure 6.1: Schematic representation of the exploration of natural VOCs from environmental samples for product development in industrial processes. Natural environments often harbour highly diverse microbial communities that include members with useful traits. The identification of beneficial microorganisms and their secondary metabolites, including VOCs, is a crucial step for the development of improved products for various industrial applications.

Potential of VOCs for industrial applications

Food production industry

Nowadays the food industry faces serious problems with providing sufficient food products for the growing market. Moreover, the products should meet not only the quality demands of the consumer but also the safety standards of the legislation. Therefore, food research groups all around the world investigate new techniques to develop environmentally friendly and safe methods for reducing food contaminations. Consumer protection legislations restrict the usage of harmful chemicals used in the past, but also provide the basis to introduce novel, improved methods into the market. Apart from advanced physical treatments such as cold plasma treatments, UV-light treatments or high-voltage pulsed electric field treatments (Boziaris, 2014; Niemira, 2012), bacterial volatiles

provide a broad spectrum of promising decontamination agents with high efficiency and low environmental impact.

Recently alkyl-substituted pyrazines, VOCs found in the spectrum of e.g. *Paenibacillus polymyxa*, were identified as mediators of antimicrobial effects, reducing the growth of plant and human pathogens (Cernava, 2012; Rybakova et al., 2016). The low mammalian toxicity, and the activity against a broad spectrum of pathogens at low concentrations are the two major factors making pyrazines, especially 2,5-bis(1-methylethyl)-pyrazine, and 5-isobuthyl-2,3-dimethyl-pyrazine potential bio-based fumigants (Janssens et al., 2019).

For meat products, in particular fresh meat, efficient preservation is key to guarantee a long shelf life. While physical parameters such as temperature, atmospheric oxygen, moisture, or light can be controlled, microbial colonisation can still be a challenging factor (Zhou et al., 2010). Techniques like refrigeration, chemical preservation, active packaging, and high hydrostatic pressure are state of the art methods in the industry (Dave and Ghaly, 2011; Zhou et al., 2010). However, natural bio-preservatives, providing the requirements for green label products which attract more customers, are interesting for industrial applications. Schöck and colleagues (2018) investigated the applicability of 5-isobuthyl-2,3-dimethyl-pyrazine and 2-isobutyl-3-methyl-pyrazine in a maltodextrin carrier on processed meat. Following the application of an optimized formulation, the microbial pressure was decreased up to 95% and therefor the shelf life increased substantially (Schöck et al., 2018).

In a similar approach the antimicrobial effect of 5-isobutyl-2,3-dimethyl-pyrazine utilizing its fumigation potential on hatching eggs was evaluated (Kusstatscher et al., 2017). Industrialized production of fertilized eggs requires a high level of sterility to provide a good chicken development. Therefore, egg shells are disinfected using fumigated formaldehyde (Williams, 1970). Formaldehyde was linked to harmful health effects and therefor handling is challenging (Wartew, 1983). In the recent study, the researchers were able to achieve comparable decontamination rates of egg shells using fumigated pyrazine compounds. Successful decontamination was also confirmed using micrographic observations (Kusstatscher et al., 2017).

In addition to the aforementioned applications, postharvest fruit treatment harbors an increasing market of sustainable treatments replacing harmful chemicals. Fruit availability all around the year requires a prolonged storability after harvest, which was so far mostly achievable with either high energy consumption or the application of various preservatives. However, consumers demands for low residues restrict the industry in potential chemical use (Sivakumar and Bautista-Baños, 2014). Therefore, a wide range of natural compounds including microbial volatiles are continuously explored for their application potential. Volatiles are especially applicable, because their application reduces required volumes and artificial atmosphere packaging can provide protection along the marketing

chain (Mari et al., 2016). In a targeted approach, different compounds produced by two *Bacillus* strains were shown to reduce *Penicillium crustosum* on citrus fruit to an extent comparable to modified atmospheric packaging (Arrebola et al., 2010). Additionally, volatiles of *Streptomyces platensis* F-1 reduced disease incidence of gray mold in strawberries by 73% (Wan et al., 2008) and volatiles of *Streptomyces globisporus* JK-1 were explored to control *Botrytis cinerea* in tomato (Q. Li et al., 2012).

Animal feed treatments

Microbial contaminations of animal feed are not only an economic problem, but also an important threat for animal health. Once such contaminants enter the food chain, they can also become serious risk factors for humans. The main sources of animal feed can be contaminated by fungi (*Penicillium, Aspergillus, Fusarium* and *Alternaria*) as well as bacteria (*Campylobacter* and *Salmonella*) and often require the destruction of large quantities of soy, corn or wheat (Kabak et al., 2006; Malorny et al., 2008; Rodrigues and Naehrer, 2012). Researchers found that 30 up to 100% of the 475 million tons of animal feed consumed in the European Union each year are affected by microbial contaminations to some extent (Pinotti et al., 2016; Rodrigues and Naehrer, 2012). While fungal contaminants primarily produce various mycotoxins, *Salmonella* pathovars can cause large disease outbreaks in animal farming. One aggravating factor is the extended persistence of these microorganisms in different substrates, including animal feed (Jones and Richardson, 2004; Magossi et al., 2019). Moreover, a total of 380 deaths and 19,000 hospitalizations are caused by *Salmonella* infections each year in the US and additional cost of $2.3 billion are related to *Salmonella* for the food and feed industry (CDC, 2011). Animal feed, apart from slaughter houses, were identified as one major sources of infections and therefor surveillance programs have been set up around the globe (X. Li et al., 2012; Magossi et al., 2019).

Currently, animal feed that is potentially contaminated with *Salmonella* is treated with formaldehyde or various organic acids (Carrique-Mas et al., 2007; Jones, 2011). While formaldehyde is toxic and an environmental hazard, organic acids employed for the decontamination are mostly safe alternatives, but substantial decrease the palatability of animal feed (Wales et al., 2010). Microbial volatiles that naturally deactivate the relevant pathogens are a promising replacement for current treatments. In a recent study conducted by the authors it was shown that the application of distinct alkylpyrazine compounds that are often produced by *Bacillus* and *Paenibacillus* species can be used to remove contaminating *Salmonella* from animal feed (Fig. 6.2). Diatomite was employed for the first time to deliver the antimicrobial 5-isobutyl-2,3-dimethylpyrazine to contaminated soy feed. The number of contaminated samples was substantially reduced when higher proportions of the alkylpyrazine-enriched diatomite were added. This example provides evidence for the applicability of

VOCs for animal feed treatments. Further research is however required to optimize the dosage and application of the active ingredients.

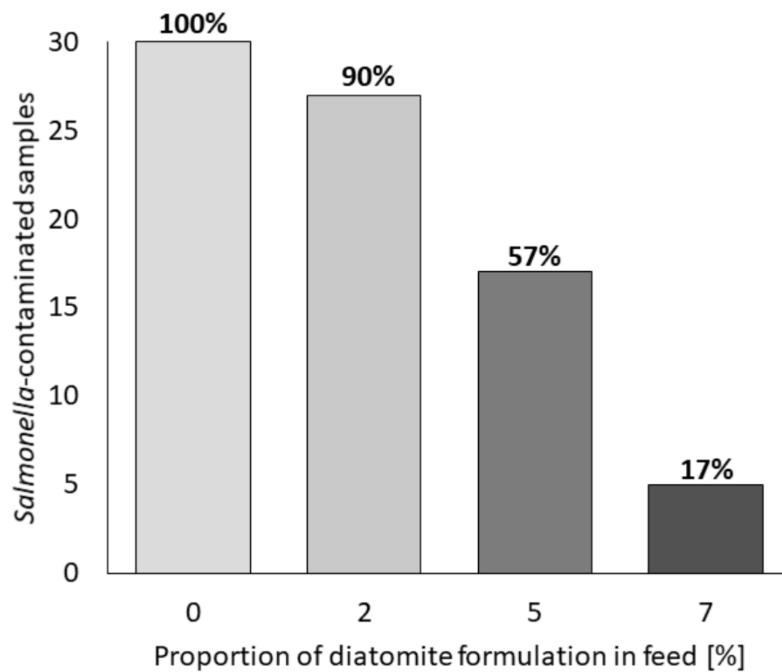

Figure 6.2: Removal of *Salmonella* in contaminated soy with bacterial VOCs. Diatomite enriched with 5-isobutyl-2,3-dimethylpyrazine (30% w/w content) was used to treat 30 contaminated samples with three different concentrations respectively. Viable *Salmonella* were recovered after seven days of incubation at representative storage conditions. Contaminated proportions are indicated above the columns.

Decontamination in industrial production facilities

Many industrial sectors face problems related to bacterial and fungal contaminations at their production facilities. Such contaminations affect industrial sectors beyond food and animal feed production. Especially bioreactors are prone to contaminations because they not only provide suitable environments for the cultivated microorganisms, but also for undesired contaminants. In this context, large-scale photobioreactors represent a production environment with aggravating process conditions. Under the current cultivation conditions for microalgae unwanted, co-occurring microorganisms such as bacteria, fungi, zooplankton or other weedy microalgae potentially disturb mass cultivations and can lead to a complete collapse of the cultures and thus rise production costs (Benemann and Oswald, 1996; Kim et al., 2014). Currently, the most common decontamination procedures include rinsing of the reactors with sodium hypochlorite or the application of hydrogen peroxide. However, it was found that the low stability and the high reactivity of the disinfectant are often disadvantageous for various process environments. Novel, efficient alternatives could improve

industrial-scale microalgae cultivations by providing more reliable methods for large-scale photobioreactor decontaminations. In a recent study, the applicability of VOCs was assessed for this purpose (Krug et al., 2019). Alkylpyrazines that were also found to be also applicable for other agricultural and industrial processes (Kusstatscher et al., 2017; Mülner et al., 2019; Schöck et al., 2018) were assessed in terms for their employability in photobioreactor decontaminations. It was show that the application of 5-isobutyl-2,3-dimethylpyrazine in microalgae cultures was highly efficient and led to significant reductions of cell viability of common eukaryotic contaminants. This preliminary study provides the basis to further explore the applicability of highly efficient VOCs for decontaminations of industrial process environments. They could provide and unlock, environmentally friendly alternative for broad scope of applications including algae production.

The potential of VOCs for future developments

Due to the manifold exploitable characteristics of VOCs, their implementation in industrial processes will likely gain momentum and find more applications in various production processes. Several promising examples of VOCs applicability were provided in this chapter. It can be expected that many more applications will likely be developed in the future. For example, VOCs can be used to manage desired functional properties of food products regarding, safety and preservation issues, organoleptic or health properties (Cocolin and Ercolini, 2015). Especially postharvest applications provide a near-natural environment to deploy VOCs-based treatments. Their employment can reach efficiencies that are in the range of conventional treatments that are currently employed (Mari et al., 2016). Microbial volatiles have a great potential for medicinal applications or the treatment of medical facilities. The increasing occurrence of antibiotic-resistant microorganisms is globally a serious threat for mankind (Andersson, 2003; Knapp et al., 2009). For several decades it seemed that the battle against many infectious diseases was won. This was disproved when various bacteria-caused diseases reappeared as a leading cause of death worldwide. Recently, the world health organization (WHO) released an updated list of threatening microorganisms, which have become multi-resistant towards available antibiotics (Tacconelli et al., 2017). It was generally found that important drivers for the development of antibiotic resistance are the misuse and overuse of antibiotics in humans and animals (Conly, 1998; Moore, 2019; Ventola, 2015). Furthermore, current decontamination methods facilitate the emergence of resistant microorganisms and can thus aggravate the situation in hospitals (Vandini et al., 2014). The transfer of natural regulation processes to critical, man-made environments like hospitals could be a viable strategy to improve the current situation. In this context, the employment of natural mixtures of microbial volatiles might provide a vital strategy to counteract resistance

formation. First studies have shown that volatiles of plant-associated bacteria harbour the potential to counteract important human pathogenic bacteria including important health care-associated infections, e.g. caused by *Stenotrophomonas maltophilia* (Cernava, 2012). However, the employment of such substances would require novel methods of delivery, especially in environments that are inhabited by humans. The design of efficient formulations should consider to the composition of natural volatilomes that are found in healthy environments, e.g. the phyllosphere of plants. This could facilitate a prolonged efficacy of novel, nature-based decontamination methods.

Apart from decontamination applications, VOCs also harbour potential for novel applications in agriculture. Due to their importance in interspecies communication and defence strategies of microorganisms, studies suggest their potential for microbiome management and control in the rhizosphere of plants. Targeted application of VOCs could promote the formation of a desired microbial community (Fierer, 2017; Kanchiswamy et al., 2015; Liu and Brettell, 2019). Additionally, VOCs produced by plant endophytes were found to enhance plant resistance to pathogens and protect against herbivores. Therefore, novel biocontrol strategies are possible (Fierer, 2017; Strobel, 2006). Furthermore the study of pathogen associated VOCs could be utilized as an early detection tool for plant associated diseases and has also great potential for medical applications (Bos et al., 2013; Sankaran et al., 2010).

Conclusion

Microbial VOCs provide a broad spectrum of novel, bioactive molecules and are therefore an important source of new decontamination strategies for industrial applications. The exploration of VOCs is not only important to increase our understanding of microbial interplay, but also provides the potential to transfer identified mechanisms of this powerful molecules to new application fields. In the past years, detailed studies of microbial interaction compounds led to the discovery of newly, so far unnoticed, antimicrobial molecules. This is not only a leap for the scientific community but has also an impact on possible technological implementations of those molecules. During the last decade several new processes relying on the intensive research put into VOCs discovery were developed. They are often considered as environmentally friendly and safe alternatives to traditional chemical compounds. This provides a basic rationale to further explore the applicability of microbial volatiles in industrial processes.

7 Curriculum vitae - Peter Kusstatscher

Address: Keplerstraße 59
8020 Graz
Austria

Date of birth, place: 3rd December 1991, Bolzano (Italy)

Education:

2016-2019: Doctoral Programme in Technical Sciences, Graz University of Technology

2014-2016: Master's degree in Biotechnology, Graz University of Technology

2011-2014: Bachelor's degree in Molecular Biology, University of Graz

Additional qualifications:

2019: Certified Project Manager, University of Graz

2017: SAP ERP course, University of Duisburg-Essen

Business management for students of natural sciences, University of Graz

Work experience:

2017-2019: Junior Scientist, ACIB GmbH

2016: Project Assistant, ACIB GmbH

2014: Student Assistant, ACIB GmbH

Publications:

2019: Kusstatscher, P., Zachow, C., Harms, K., Maier, J., Eigner, H., Berg, G., & Cernava, T. (2019). Microbiome-driven identification of microbial indicators for postharvest diseases of sugar beets. *Microbiome*, 7(1), 1-12.

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2017: Kusstatscher, P., Cernava, T., Liebminger, S., & Berg, G. (2017). Replacing conventional decontamination of hatching eggs with a natural defense strategy based on antimicrobial, volatile pyrazines. *Scientific reports*, *7*(1), 13253.

Talks:

2019: "Postharvest and on-field microbial community changes caused by root rot in sugar beets" -V International Symposium on Postharvest Pathology

"Storability of sugar beets is indicated by signature taxa" - 21st DocDay Graz

2018: „Verbesserung der Lagerstabilität von Zuckerrüben durch mikrobielle Konsortien" - DPG AK Biologischer Pflanzenschutz

2017: "Biological control of post-harvest losses in sugar beet" - ESIB 2017

Posters:

2019: "Signature taxa indicate the storability of sugar beets before harvest" - FEMS 2019

2018: "Biological postharvest control in sugar beet" - IOBC-WPRS meeting of the Working Group "Biological and integrated control of plant pathogens"

2017: "A new way to control post-harvest losses in sugar beets" - ESIB 2017

2016: "Community changes during the decontamination of hatching eggs using diazine derivatives" – 8th ÖGMBT Annual Meeting

"Diazine derivate induced community changes during the decontamination of natural products" - 3rd Theodor Escherich Symposium

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