Old Herborn University Seminar Monograph

33. THE FLOWERING OF THE PLANT MICROBIOME AND THE HUMAN CONNECTION

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SOIL-BORNE LEGACIES OF PLANT DISEASE

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THE ROOT MICROBIOME

There is a mesmerizing number and diversity of microbes associated with plants, especially in and around the roots. Collectively the microbes associated with the plant root form the root microbiome and its composition largely determines plant health (*Berendsen* et al., 2012). Although some pathogenic microbes are very harmful to plants, most members of the root microbiome do not cause disease in plants and some of these microbes even protect and promote plant growth (*Glick* et al., 2007; *Berendsen* et al., 2012; *Philippot* et al., 2013; *Bakker* et al., 2018). Plants shape the composition of root microbiome by secreting mixtures of microbe-stimulatory or impeding chemicals (*Badri* and *Vivanco*, 2009; *Sasse* et al., 2018; *Zhalnina* et al., 2018). Genetic differences between plants determine the composition of root exudates and the dynamics by which they are produced. Root microbiomes can consequently differ substantially between plant species and even between genotypes within a species (*Lundberg* et al., 2012; *Edwards* et al., 2015). This implies that plant genetics can contribute to the selection of a protective plant microbiome.

DISEASE-SUPPRESSIVE SOILS

The protective effect of root-associated microbial communities is best evidenced by disease-suppressive soils, in which susceptible host plants do not develop disease despite the presence of a virulent pathogen (*Schlatter* et al., 2017). Disease-suppressive soils have been identified for different plant species and plant pathogens. In most cases, the disease suppression is related to the presence of one, or a few, microbial species that can antagonize the pathogen either directly or indirectly by priming the plant's immune system. Arguably, the best-studied disease-suppressive soils are soils that have become suppressive to take-all disease of wheat (*Weller* et al., 2002; *Schlatter* et al., 2017). Take-all suppressive soils have been found around the world and similar antifungal-producing Pseudomonads have been connected to disease suppressiveness in different fields. This implies that these specific microbes are present in all of these fields and the plant is able to select and enrich these specific bacteria from the enormous diversity of microbes that each soil comprises. It implies not only a very strict and long co-evolution of the plant with certain beneficial microbes, but also that these microbes are ubiquitously present. Take-all suppressive soils typically develop after a major disease

outbreak, implying that upon attack plants cry out for help and recruit specific microbes to come to their aid.

CRY FOR HELP

Recently, we tested this 'cry for help' hypothesis and found that, when leaves of the model plant species *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) were inoculated with the downy-mildew pathogen *Hyaloperonospora arabidopsidis* (Hpa), a subsequent population of plants growing in the soil conditioned by the infected plants was more resistant to this pathogen (*Berendsen* et al., 2018). Moreover, foliar infection by this pathogen resulted in the specific promotion of a consortium of three bacterial species on the Arabidopsis roots. These bacterial strains became highly abundant in the rhizosphere of Hpa-infected Arabidopsis plants, and were isolated. Upon application to soil, these bacteria separately did not significantly affect plant performance. However, when applied together, this consortium of three strains protected Arabidopsis against Hpa infection and it promoted plant growth. Similarly, infections of Arabidopsis by the bacterial leaf pathogen *Pseudomonas syringae* gave rise to a root microbiome that could protect subsequent populations of plants against this pathogen (*Yuan* et al., 2018). Also in pepper and wheat plants, defence activation and subsequent systemic signalling led to changes in the root microbiome that were suggested to benefit the plant (*Dudenhöffer* et al., 2016; *Kong* et al., 2016). Together, these studies illustrate that plants can rapidly adjust their root microbiome in response to disease and recruit disease suppressing microbes, giving rise to a 'soil-borne legacy' that protects the next generation of plants growing in the same soil (*Bakker* et al., 2018).

INDUCED SYSTEMIC RESISTANCE

It is known that colonization of Arabidopsis roots by specific microbes can trigger induced systemic resistance (ISR) (*Pieterse* et al., 1996; *van Wees* et al., 1997). Using the ISR-model strain *Pseudomonas simiae* WCS417, the root-specific transcription factor MYB72 was identified as an essential regulator of ISR (*van der Ent* et al., 2008). MYB72 is rapidly activated upon root colonization by diverse ISR-inducing microbes and controls a large gene-regulatory network that is required for the establishment of ISR in foliar tissues. Transcriptional analyses of mutant and overexpressing genotypes revealed that MYB72 upregulates genes in the roots involved in the phenylpropanoid pathway (*Zamioudis* et al., 2014). The final products of this pathway are coumarins, phenolic compounds that have known antimicrobial effects. Recently, it was discovered that MYB72 indeed controls the biosynthesis and secretion of numerous compounds, including coumarins. Moreover, the rhizosphere microbiome composition of Arabidopsis wild-type plant is very different from mutant plants that are strongly impaired in coumarin production and secretion (*Stringlis* et al., 2018). This implies that MYB72 has a dual role in ISR, where it not only functions in

generating the systemic ISR signal, but may also play a critical role in shaping a root microbiome that elicits ISR. Preliminary results indicate that MYB72 is also required for the creation of a soilborne legacy of *Hpa*-infection (Gilles Vismans, unpublished results). This highlights the role of this transcription factor as a key regulator of the plant's communication with its microbiome.

PERSPECTIVE

Plants are able to manage the composition of their root microbiomes in response to attack in a way that benefits the survival of their offspring. In nature, the build-up of specialized pathogen inoculum is thought to cause negative soil feedback that most strongly affects the performance of dominant plant species and changes the demography of wild plants (*Klironomos*, 2002; *Mommer* et al., 2018). In agriculture, pathogen build-up causes reduced yields in soils that have not developed disease suppressiveness (*Cesarano* et al., 2017) and this is why most annual crops are planted in rotation. A 'cry for help' to the root microbiome could prove to be an integral part by which the plant immune system counteracts negative soil feedback. A fundamental understanding of these mechanisms could open up new ways to breed or engineer crop plants that are better able create beneficial microbiomes and improve agricultural yields in a sustainable manner.

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SYNTHETIC SPIKES METHOD FOR PLANT MICROBIOTA ABSOLUTE QUANTITATION

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THE IMPORTANCE OF PLANT SCIENCES AS A FIELD OF METHOD DEVELOPMENT

Plant science aims to improve plant yield through breeding and genetic manipulations especially focusing on plant nutrient acquisition and resistance against pathogens and abiotic factors as the weather. Plants are perfect models for studying eukaryotic genetics and hence many discoveries were first made here. To mention a few, an early genetic study on maize gave evidence that genes are physically positioned on chromosomes (*Creighton* and *McClintock*, 1931), which later allowed to find anomalies from this rule in the form of transposable elements, pieces of DNA that "jump" across genomic locations (*McClintock*, 1950). These moving DNA fragments are responsible for gene expression and phenotypic differences. It is estimated that 44% of the human genome consists of these transposable elements mostly in the form of non-coding repetitive sequences. Although approximately less than 0.05% of them are active, they cause or contribute to diseases as haemophilia and cancer (*Mills* et al., 2007). Corn, with its coloured kernels due to altered gene expression as a consequence of genetic "jumps" was a perfect study model for these discoveries. Plants allow for easy observation of their development and morphology. In the early 18th century Jean de Marian observed that *Mimosa pudica* expresses a daily leave movement even in the absence of light. This led to a further study in fruit flies that also showed a daily pattern now called circadian oscillations (*Robertson McClung*, 2006). These oscillations influence our sleep, cognitive and muscular abilities and hormone regulation. Another scientific area where plant science plays a critical role is host-microbe interactions field. In comparison to animals, plants lack defender mobile cells or adaptive immune system and hence had to develop a sophisticated innate immune and systemic signalling system to cope with the bacterial, fungal, oomycetes and insect attacks. As pathogens are responsible for a substantial crop loss (*Savary* et al., 2019), research into the plant immunity are our priority in securing food for the increasing human population. In a nutshell, plant immune system can be divided into two branches. The first branch of the immune system acts on pathogen- or microbial-associated molecular patterns (known as PAMP and MAMP) that activate immune response upon detection of well-conserved microbial proteins such as flg22, a 22 amino acid part of N-terminal part of flagellin. However, as shown by *Buscaill* et al. (2019), it is a plant role to first cleave the flagellin polymer using β-galactosidase 1 to trigger the immune response. Upon recognition, plants are not yet invaded and use salicylic acid triggered pathways to stimulate callose production and deposition to the enforcement of their cell wall.

The other immune branch acts intracellularly where pathogens release "effector" proteins to induce virulence. These effectors can be recognised by the plant host triggering a response (often cell death). At this point, the co-evolution between host and the microbe is especially pronounced as pathogens effectors are constantly evolving to escape the immune system recognition while evolving plants that can recognise these new effectors have a substantial advantage over the infected part of their population (*Jones* and *Dangl*, 2006).

Plant sciences made substantial progress with host-microbiota studies and development of methods used in this science field. In contrast to animal and especially mammalian study objects, work with plants benefit from a lack of ethical issues and the convenience and ease of propagation, crossings and seed storage as a method for preserving the host genomics population. The important species for plant scientists is *Arabidopsis thaliana*. This plant has no economic significance, however, belongs to Brassicaceae family of oilseed, cabbage and mustard. The major advantage of *A. thaliana* over other plants in genetic and host-microbiota studies is its relatively small nonrepetitive diploid genome which can be easily modified using chemical or X-ray mutagenesis. Moreover, a large pool of natural accessions (often called ecotypes) allows for studying the variations of plant response to various biotic and abiotic influence, including its interactions with environmental microbiota. A comprehensive study using two *A. thaliana* ecotypes grown in two different soils unravelled that bulk soil bacterial community is different from the rhizosphere, which in turn is different from the root compartment (*Bulgarelli* et al., 2012). Root microbiota is enriched with Proteobacteria and Actinobacteria. The study found many Streptomycetaceae (Actinobacteria) to be genuine root inhabitants, while some of the Proteobacteria being attracted purely by cellulose source as identified using wood splinters controls. This and other studies brought a new interest in plant microbiota studies. However, the methods of amplicon sequencing often employed in this science field are only able to provide a community profile snapshot without being able to even approximate their abundance. Due to the DNA isolation, PCR amplification and sequencing process any differences in the samples microbial load are completely lost. Unfortunately, standard methods as colony counting on agar media are not very useful as only a small proportion of soil and plant-associated bacteria and other microorganisms are able to grow in such conditions. There may be various reasons behind this: inadequate media nutrient status, obligate symbiosis with other organism, or simply very slow growth and the danger of being overgrown by other fast-growing species. Other methods of estimating microbial presence in environmental samples include ATP and phospholipid-derived fatty acid concentration measure, flow cytometry and qPCR (*Zhang* et al., 2017). However, they are laborious, variable and at least for now of low throughput and high cost. The importance of identifying the microbial load was clearly shown with studying gut microbial communities of Crohn's disease patients. This study, using flow cytometry unravelled that the main difference between healthy and Crohn's patient gut is the bacterial load and not the community structure (*Vandeputte* et al., 2017). This relation may be true for many other human gut diseases. The microbial load may also be a predominant factor controlling antibiotic treatment efficiency, microbial colonization and recolonization patterns and the

community structure stability. Hence a new method allowing for community profiling and load measurement is needed. In this monograph, I will present my authorship method answering these issues.

SYNTHETIC SPIKING METHOD JUSTIFICATION AND COMPARISON TO THE EXISTING METHODS

There are a few different methods to measure microbial load in environmental samples. This chapter will briefly summarize them and lists their advantages and limitations. The most common method used to measure microbial gene presence is qPCR (quantitative PCR). This method was used for example to establish microbial 16S rRNA and nifH gene (coding for nitrogenase enzyme, a key enzyme in atmospheric nitrogen conversion to ammonia) presence in the wheat rhizosphere (*Rilling* et al., 2018). However, this method can only be used on already isolated environmental DNA and hence assumes that all the DNA present in a sample will be isolated. Moreover, this method does not allow for any taxonomical identification of the microbiota. A method of flow cytometry can be used to count the microbial cells as shown in *Vandeputte* et al. (2017). Microbial cells from an environmental sample after suspension in a buffer and staining with a fluorescent dye are run through the flow cytometer machine. This method allows for very accurate measurement of the number and even the shape of the cells. The limitations are a need for laborious sample preparation including filtering samples from any debris and staining. The other problem is a need for separate flow cytometer machines to measure cells of an order of magnitude different sizes (prokaryotic vs. eukaryotic). For rich samples as soil or stool, it is not possible to taxonomically assign detected cells and hence a separated metagenomic analysis is needed. *Stämmler* et al. (2016) presented a relatively easy method to combine metagenomic with gene quantitation by adding a defined amount of exogenous bacterial cells into environmental samples. By sequencing the DNA isolated from such samples, a ratio of the number of genes detected from exogenous cells (in case of Stämmler et al., thermo- and halophilic strains) to the number of genes detected from the *in situ* microbiota (gut). The limitations are the need to *a priori* knowledge which strains are not present in the samples of interest, as the exogenous species must be different from the *in situ* microbiota, the need to culture strains of unusual growth requirements and the need to control the spiking cells number through optical density and/or colony forming units counting. Moreover, unless the cells are dead or starving they may have a variable number of 16S rRNA as cells are constantly reproducing and duplicate their DNA during mitosis before they split into two separate cells. Another problem is the lack of fungal and or other eukaryotic spiking cells. This method was shown to work well with gut samples, however, more complicated environments as soil may be challenging.

The below presented method of synthetic spiking (*Tkacz* et al., 2018) bypasses problems identified above. By adding a defined amount of synthetic taxonomical genes, the absolute amount of genes of interest as a proxy of a microbial load can be measured (16S rRNA, 18S rRNA and fungal ITS will be presented; however, the method

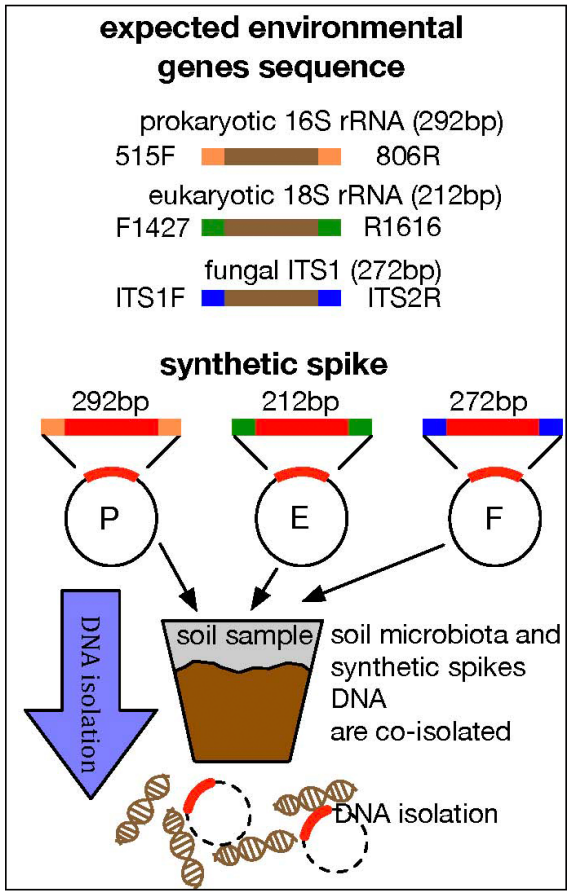


Figure 1: Synthetic spike design. Prokaryotic, eukaryotic and fungal (P, E and F) synthetic spikes in the form of plasmids (here presented as circles) were designed using primer binding site sequences, together with the length and GC content of amplicons from prokaryotic 16S rRNA (P), eukaryotic 18S rRNA (E) and fungal ITS1 (F), respectively. For P synthetic spikes the primer binding sites shown in orange, for E in green, and for F in blue (adapted from *Tkacz* et al., 2018).

can be easily expanded for the need of other genes). The spikes are added to the original sample rather than to already isolated DNA, so as the spike and the environmental DNA are co-isolated, co-amplified using a standard 16S, 18S and ITS-specific PCR (PCR where specific pairs of primers are used) and co-sequenced using high-throughput sequencing method. The method was tested using Illumina Miseq 300PE, however, any other next-generation sequencing method is suitable. The advantage of this method is that quantitation is coupled with metagenomic sequencing and hence there is no need for separate sample preparation and analysis as in the case of qPCR and flow cytometry. Moreover, the synthetic spiking method can be used to measure total prokaryotic and eukaryotic load or specific groups of microorganisms based on a selected taxonomic gene (i.e. 16S rRNA specific fragment for a given phylum) or a functional gene (i.e. nifH to measure nitrogen fixers community diversity and load). The synthetic spikes, in

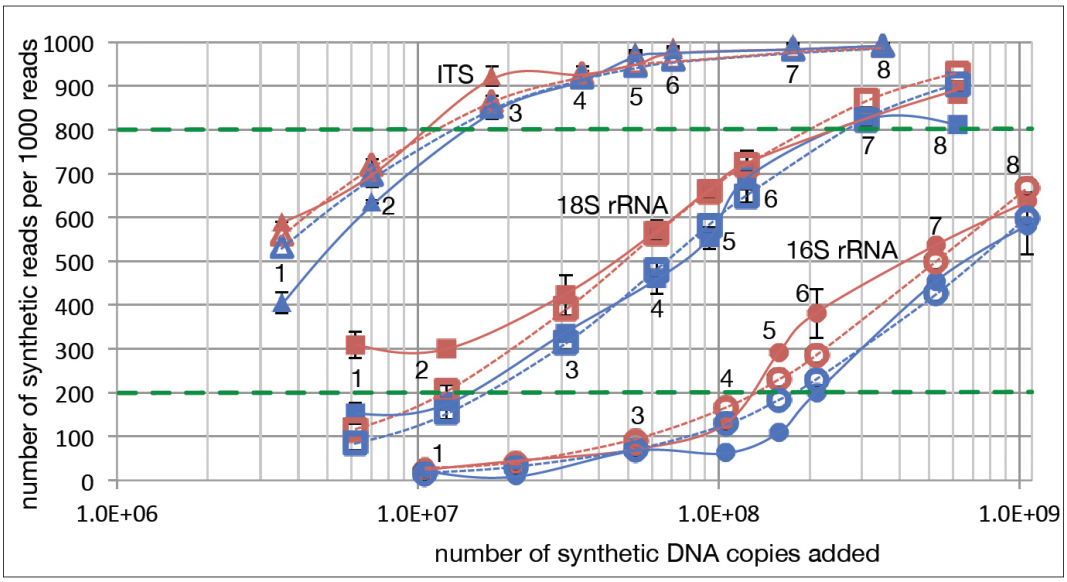


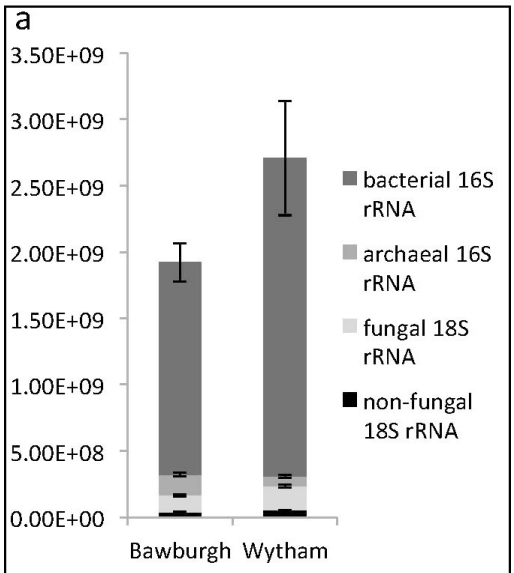
Figure 2: Model of synthetic spike addition and the number of synthetic spikes origin reads per 1000 total reads obtained in high-throughput sequencing. The number of sequencing reads of synthetic spike per 1000 total reads (Y-axis) from 16S rRNA, 18S rRNA and ITS is shown in relation to gradient levels addition to soil of prokaryotic, eukaryotic and fungal synthetic spikes (X-axis). Experimental results are shown by solid symbols and solid lines where two colours represent two soil types used as the environmental samples and model data is presented with hollow symbols and dashed lines of corresponding colours. The model shows the expected spike contribution in the sequencing output for each spike level using the averaged gene abundance for a specific soil type. Dotted green lines indicate the region with 200-800 synthetic reads per 1000 reads, where the experimental results match the model the best (adapted from *Tkacz* et al., 2018).

contrast to exogenous spiking bacterial cells, are easy to store, their amount can be easily measured using DNA quantitation methods (i.e. qubit fluorescence or nanodrop spectrophotometry) and the amount standardized between experiments (i.e. frozen synthetic spike aliquots).

SYNTHETIC SPIKES DESIGN AND APPLICATION

Synthetic spikes were designed to mimic fragments of the microbial genes of 16S rRNA, 18S rRNA and fungal ITS. The synthetic and microbial genes have the same highly conserved flanking regions to which a set of PCR primers bind, while for the synthetic spikes the region between these fragments is essentially randomly generated DNA sequence of a similar GC content as the microbial counterpart (Figure 1). Naturally, this random DNA is known, and its sequence is used to count the spikes-origin reads in the final sequencing output. The rest of the plasmid is of little importance; however, its length and sequence are used to control the accurate addition level of spikes (i.e. 1 ng of the 2666bp plasmid of a specified sequence consists of 365,572,814 copies).

Tkacz et al. (2018) have verified the synthetic spikes method accuracy by quantifying the number of bacterial



b

	Bawburgh	Wytham
bacterial 16S rRNA	83.3%	88.6%
archaeal 16S rRNA	8.1%	2.7%
fungal 18S rRNA	6.6%	6.8%
non-fungal 18S rRNA	2.0%	1.9%

Figure 3: Total soil microbial community profile obtained using synthetic spikes quantitation. a) absolute bacterial and archaeal 16S rRNA and fungal and non-fungal 18S rRNA gene abundance for each soil type (Bawburgh and Wytham) and **(b)** their relative abundance (adapted from *Tkacz* et al., 2018).

16S rRNA genes in a defined bacterial culture. A set of spikes (a 5-step gradient with 25-fold concentration levels differences) was added to *Rhizobium leguminosarum* culture, DNA isolated, PCR targeting bacterial 16S rRNA and Miseq sequencing performed. Based on the ratio of synthetic-origin to *Rhizobium*-origin sequencing reads, the number of bacterial 16S rRNA and subsequently bacterial cells was calculated. The method accuracy was calculated to be 99.3%.

Next, the spikes were added in a gradient concentration (with a difference of 100-fold between the top and the bottom gradient step) to soil samples. Tkacz and co-workers have chosen two soil types of a similar pH, but different organic carbon and nutrient content expecting differences in the microbiota community structure and the abundance. As soil is a habitat for bacteria, archaea, fungi, protists and many other eukaryotic organisms three different genes were targeted: 16S rRNA, 18S rRNA and a fungal-specific ITS region. As expected an increase in the spike addition resulted in an increase in the number of synthetic-origin sequencing reads (Figure 2). Most of the results values are close to

the expected (modelled) value further validating the method.

Absolute quantitation of soil samples allowed for an abundance comparison of prokaryotes to eukaryotes. Prokaryotic genes are about 10 times more abundant than the eukaryotes ones (Figure 3). The reason behind this is unknown. There were numerous soil microbial community studies however the real relation between these main domains of life is a yet untouched scientific field. What dictates that bacteria are more abundant than eukaryotes? Possible explanations range from their fast reproduction, small size, competitiveness to a better niche exploration. Hopefully, the future soil microbiologists will be able to shed more light on this topic.

POTENTIAL FUTURE METHOD DEVELOPMENT

Synthetic spikes as a method of a stable DNA addition to complex samples as gut or soil could be used to measure DNA isolation efficiency. There are two main DNA isolation methods used: organic extraction and solid phase extraction. Organic extraction requires lysis, phenol-chloroform separation of proteins from nucleic acids and ethanol nucleic acids precipitation. Solid phase extraction is based on silica filter DNA binding (so-called minicolumn method), its subsequent washing from any remaining contaminants (proteins, lipids) and elution using water (or buffer). Both these methods have their advantages and limitations with waste production, costs and time consumption factors. However, none of this method can isolate 100% of the DNA, especially from complex samples. Hence, ultimately it is not known what is the DNA quantity in any given sample. Synthetic spiking method allows to estimate a specific gene or genes in a sample, however, cannot establish the total DNA content. Theoretically, qPCR method could be used to measure the spike content in the isolated environmental DNA sample (synthetic spikes would need to be added to the samples prior to DNA isolation) and by comparing it to a well-defined standard of synthetic spikes DNA (qPCR amplification curve) one could establish how many copies of synthetic spikes are present in a sample after environmental DNA isolation. By a comparison of the number of synthetic spikes added to the sample to the number of them being isolated it is possible to measure the DNA isolation efficiency. The limitation is that this approach would actually be measuring the synthetic spikes isolation efficiency rather than the total DNA isolation efficiency. However, it can be assumed that samples with efficient synthetic spikes isolation rates have also their environmental DNA efficiently isolated. For the standard metagenomic PCR-based method it does not really matter what percentage of the environmental DNA has been isolated, as it can be assumed that the dominant microbial species detected in the sequencing output are actually dominant in the original environmental sample. However, if there is a focus on the so-called “rare microbiota” which involves a deep sequencing it is crucial to isolate as much of the environmental DNA as possible. A combination of synthetic spikes with qPCR would enable screening DNA samples for their usefulness in the “rare microbiota” studies.

CONCLUSIONS

Environmental ecology of microbial communities focuses on analysing prokaryotic and microbial eukaryotic profiles in complex samples as gut and soil. Due to the advance in the next generation sequencing methods, it is possible to obtain a truly deep community profile of any sample. However, a cross-samples comparison is hindered by the fact that the sequencing methods can only uncover the relative abundance of each species in comparison to the whole community. The size of the whole community is unknown. Microbial communities of a similar structure but of a different microbial load may vary in their population community stability and resistance to invasions and alterations. For example, a pathogenic communites may be more or less resistant to antibiotic treatment depending on their total abundance. Synthetic spiking method presented in this review allows measuring the microbial load using existing sequencing method without laborious and expensive additional steps. Moreover, it is theoretically possible to couple qPCR with the synthetic spiking method to measure the DNA isolation efficiency.

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PLANT SECONDARY METABOLITES AND THEIR DERIVATIVES IN MICROBIOTA – CROSS-KINGDOM INTERACTIONS

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SUMMARY

In this mini-review, we provide a short impression of a few of the plant secondary metabolites that emerged as root-microbiome modifying compounds. We focus on selected metabolite degradation pathways and derivatives occurring during catabolism since these compounds could be crucial for shaping the microbiota. We discuss similarities and differences between some of the known degradation pathways in animals and plants, and highlight cross-bioactivities of a few catabolites in the different kingdoms.

INTRODUCTION

During their evolution, land plants were always associated with microorganisms, i.e. the plant microbiota which, together with the host, is designated as the holobiont. The plant microbiota is composed of microbial communities inhabiting the root (rhizosphere, rhizoplane), the leaf (phyllosphere), or living within tissues as endophytes (endosphere), and of the communities associated with flowers, pollen, fruits and seeds. (*Berg* and *Raaijmakers*, 2018; *Droby* and *Wisniews,* 2018; *Durán* et al., 2018; *Manirajan* et al., 2018; *Shade* et al., 2013; *Compant* et al., 2019*).* The core microbiota represents microbes characteristically associated with a plant species, subspecies or even a cultivar (*Adam* et al., 2018; *Chaluvadi* and *Bennetzen*, 2018). In contrast, the variable part of the microbiota is established by collecting organisms from soil, splash water, air, and it depends on exuded compounds, transfer by vector organisms, for instance insects, and environmental conditions. Plant age and developmental stage influence species diversity of the microbiota (*Aleti* et al., 2017). Domestication of plants seems to alter root specific microbial communities. Indeed, wild plant species have a different microbiota with higher species diversity than domesticated plants (*Wagner* et al., 2016; *Pérez-Jaramillo* et al., 2018). Plants evolutionary divergence had a strong influence on the microbiota composition, with greater effects on the endosphere (*Fitzpatrick* et al., 2018). Recent insights in the interactions between plants and microorganisms unclose new views of co-evolution and ecology with regard to the organisms' interaction and communication dynamics. The coexistence is thought to influence and to modulate all levels of interaction including the genetics of the partners, to strengthen plant health, modulate nitrogen fixation and facilitate mineral uptake. It furthermore triggers adaption to environmental conditions by modifying metabolism, physiology, growth and reproduction.

PLANTS SECONDARY METABOLITES

The rhizosphere seems to be especially suitable for accelerated (horizontal) gene transfer, driving microbial and plant evolution. As hypothesized by *Emiliani* et al., (2009), early during conquering of land, five hundred million years ago, horizontal gene transfer of the phenylalanine ammonia lyase (PAL) gene during symbiosis with soil microorganisms enabled plants to produce phenylpropanoids and lignin, both essential to withstand the altered environmental conditions outside the aquatic habitat. According to *de Vries* et al. (2017), genes for the phenylpropanoid synthesis and lignin precursors were already present in streptophyte algae, where land plant are assumed to have evolved from. However, convergent evolution allows the generation of new enzymes from a shared pool of related enzymes with similar but not identical functions. Anyway, plants acquired new secondary metabolites, which enriched the already existing core set of secondary compounds, such as terpenoids. Additional pathways were established resulting in an enormous manifoldness of secondary metabolites, among them new terpenoids, alkaloids, heterocyclic compounds, simple phenolics, flavonoids, stilbenes, betalains and others. Many of them fulfil diverse functions including communication in new ecological cross-kingdom interactions with animals, plants, fungi, bacteria, other microorganisms, and also with other plants. Many of the compounds are highly toxic, others can be beneficial for the health and survival of target organisms, but effects can be reversed depending on the concentration, on accompanying reactants and on organisms that convert one compound to another with an altered bioactivity. Microorganisms themselves produce thousands of secondary products which can interact and influence the plant and plant secondary metabolite biosynthesis. This aspect however is not addressed further here. Just to mention one example, N-acyl-homoserine lactones, which are bacterial quorum sensing molecules, evoke multiple responses in plants, and often resistance responses are elicited (*Hartmann* et al., 2014).

Endophytes can alter the secondary metabolism of their plant host or establish alliances with the plant to complete the biosynthesis of secondary metabolites that finally accumulate in the plant. The biosynthesis of maytansine by *Maytenus serrata* and its endophytes presents an example for cross-species interaction in secondary metabolite production (*Kursari* et al., 2016). It is hypothesized that qualitative and quantitative variations of pharmaceutically important secondary metabolites of medicinal plants are strongly influenced by the microbiome (*Köberl* et al., 2013; *Schmidt* et al., 2014; *Huang* et al., 2018). The evolution of the chemical diversity of plant secondary metabolites is still in progress (*Moghe* and *Last*, 2015); thus, in evolutionary terms old molecules, to which pathogens might have adapted to, can turn again during co-evolution from an inactive to a bioactive compound by core structure modifications or substituent exchanges. A considerable number of the presently identified 300,000 secondary metabolites are formed by decorating the molecules' core structures with substituents such as sugars or position-specific functional groups that change the reactivity of the molecules dramatically, from almost inactive to highly bioactive compounds.

EXUDATION OF SECONDARY METABOLITES

Root exudation can release up to 20-30% of the carbon fixed by photosynthesis (*Haichar* et al., 2008; *Voges* et al., 2019). The root exudates, composed of primary and secondary metabolites, are essential for the establishment of plant-microbial interactions (*Philippot* et al., 2013; *Haichar* et al., 2014; *Sugiyama*, 2019; *Voges* et al., 2019). Transport proteins of the ABC type are presumed to be involved in secondary metabolite export (*Bertin* et al., 2003; *Yazaki*, 2005; *Sasse* et al., 2018). In contrast to primary metabolites and mucilage, which serve predominantly as nutrients for microorganisms, secondary metabolites are often times specific for certain plant species. Many exudates contain characteristic metabolites, which are found only in certain taxonomic groups of plants, e.g. in families, genera or single species, which can be regarded as a younger event during evolution. Other compounds are widely distributed, such as numerous simple phenolics and flavonoids. As shown by *Zwetsloot* et al. (2018), secondary metabolites can act as nutrients, toxins, interactors with membranes, inhibitors of functional proteins, chelating agents of metals and intercalators with nucleic acids, all depending on the chemical nature of the molecules. Secondary metabolites have therefore the higher potential to modulate microbial communities than any primary metabolite in a plant species dependent manner, especially when acting in concert. The composition of exuded secondary metabolites differs, depending on the developmental stage of the plant, age of the root, root zone, root hairs, abiotic and biotic stress conditions (*Bais* et al., 2006; *Weston* et al., 2012; *Selmar* and *Kleinwächter*, 2013; *Massalha* et al., 2017). In line with this scenario, drought stress alters the microbiota and profiles of secondary metabolite exudation. For instance, the root metabolome of *Quercus ilex* was found to be changed without considerable recovery under severe drought conditions (*Gargallo-Garriga* et al., 2018). The metabolome was then dominated by secondary metabolites, while after mild drought when recovery was possible, a shift to mainly primary metabolites in the exudate was observed. *Edwards* et al. (2018) assessed changes in context with the developmental stage of the plant and concluded that drought stress leads to an immature endosphere microbiota. A similar conclusion was drawn by *Xu* et al. (2018), who report a drought-dependent delay in the formation of the early microbiome of *Sorghum* roots. Drought-dependent shifts in the microbiome species composition favouring the relative abundance of the Actinobacteria together with host-specific changes seem to be a common feature among angiosperms (*Fitzpatrik* et al., 2018; *Naylor* and *Coleman-Derr*, 2018).

DEGRADATION AND DETOXIFICATION OF SECONDARY METABOLITES

Enormous amounts of secondary metabolites are released by living plants and by rotting of dead plant material. Therefore, the degradation of these compounds is important to avoid their accumulation in the environment in toxic concentrations and triggering long-lasting alterations of the soil microbiome. Presently, the effects of the products derived from the original

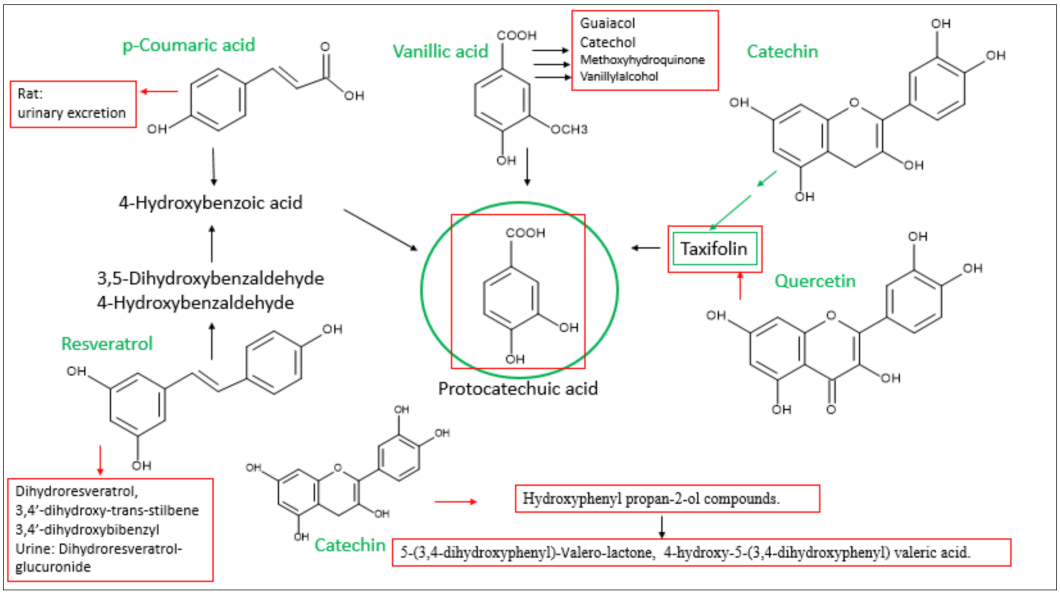

Figure 1: Protocatechuic acid is one of the pivotal catabolites occurring during the degradation of p-coumaric acid (by soil organisms and plant associated bacteria), of vanillic acid (soil bacteria, plant associated bacteria), of resveratrol (*Agrobacterium oleivorans* strain JS678 from peanut rhizosphere), of quercetin (soil bacteria, human gut bacteria) and of catechin (soil bacteria), (simplified scheme). Guaiacol is a catabolite of vanillic acid, formed in the desert locust gut by *Pantoea agglomerans.* Guaiacol is degraded via catechol. Red markers: original compounds found in animals, green markers: in plants.

secondary metabolites by degradation or conversion on plant metabolism and microbiota are poorly understood, although these molecules may have extraordinary properties and special, perhaps unexpected, biological activities. In animals for instance, isovanillic acid 3-*O*-sulfate, a product derived from cyanidin 3-*O*-glucoside degradation by the human gut microbiome, stimulates glucose uptake by muscle cells (*Houghton* et al., 2019). Short-lived, often highly reactive derivatives and catabolic intermediates may be the intrinsic key players, for instance in remodelling microbiota, influencing epigenetics and physiology in target organisms by eliciting signalling cascades that allow adaption to changed environments, or cause death of pro- and eukaryotes. The toxicity is always dose dependent. High concentrations can overexert the degradation and detoxification capacities of organisms. On the other hand, molecules can be per se toxic at very low concentrations by interfering with biomolecules, with signalling pathways, by altering gene expression or enzyme activities. The ability to detoxify and to degrade bioactive and inhibitory molecules is pivotal, since it decides on toxicity or non-toxicity for a given organism. Accumulation of secondary metabolites and relatively stable degradation intermediates in phytotoxic amounts in soil contribute to allelopathic interactions and soil sickness which reduces yields of subsequent crop cultures and soil fertility, sometimes for years.

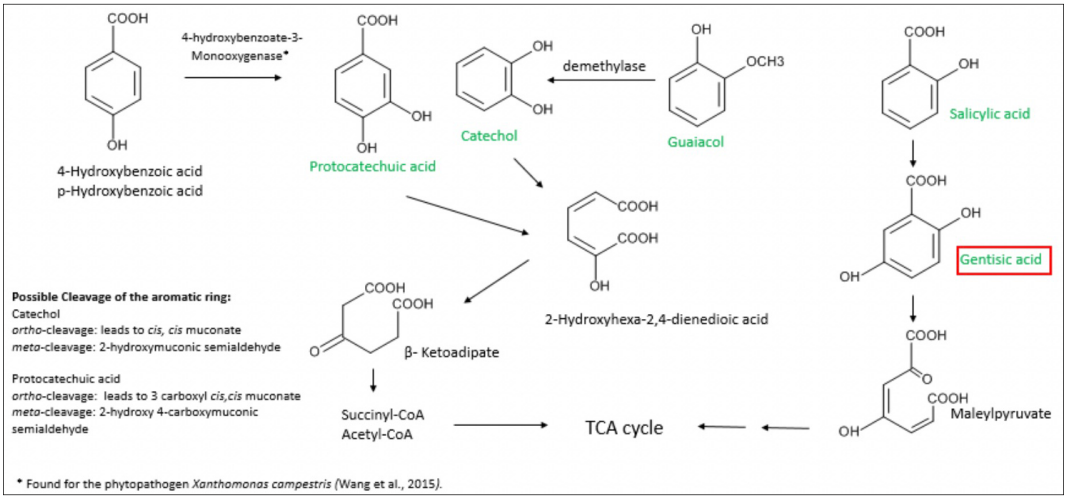

Figure 2: Degradation of the aromatic system of protocatechuic acid, catechol, guaiacol, salicylic acid, gentisic acid and p-hydroxybenzoic acid under aerobic conditions (simplified scheme). *Ortho*-cleavage: aromatic ring fission between carbon atoms with OH substituents; *meta*-cleavage: aromatic ring fission between a carbon atom with OH substituent and unsubstituted carbon-atom. Red markers: Intermediate compounds found in animals, green markers: in plants.

PLANT SECONDARY METABOLITES AS MODIFIERS OF THE MICROBIOTA

Increasing evidence suggests that plant secondary metabolites remodel and shape the species diversity and abundance of the microbiome in favour of microorganisms with beneficial functions for the plant. On the other hand, secondary metabolites released from the plant can act as allelochemicals. By reducing or shifting the microbial species diversity in soil and on root surfaces of neighbouring plants, they influence the microbiota of competitive individuals negatively. Thus, secondary metabolites can modify microbial community structure with different consequences for plants, as exemplified by the following.

p-Coumaric acid

p-Coumaric acid is a constituent in exudates of many species (Figure 1). In higher concentrations, p-coumaric acid is an allelochemical and harmful to the microbiota, for instance of cucumber. It also interferes with the lignin synthesis leading to cell wall stiffening, due to premature lignification (*Lima* et al., 2013; *Zhou* et al., 2018). Both effects result in a reduced growth of the plants. This compound has a negative effect on bacterial community composition of cucumber but promotes the fungal diversity, including phytopathogenic *Fusarium* species. The study of *Zhou* et al. (2018) shows that fungal species able to degrade phenolic compounds have an advantage. In contrast, bacteria with plant growth promoting and pathogen-antagonistic properties were found to be suppressed by p-coumaric acid. Several Gram-positive bacteria (*Bacillus subtilis, Bacillus pumilus*, *Pediococcus pentosaceus*, *Lactobacillus plantarum),* (*Licandro-Seraut* et al., 2013 and references therein) are able to convert p-coumaric acid and other phenolic acids into vinyl phenol derivatives. 4-Vinylphenol derived from

p-coumaric acid showed a stronger inhibitory effect to several *Erwinia*, *Pseudomonas*, and *Enterobacter* species than the parent compound. *Acinetobacter calcoaceticus* strain DSM 586 degrades p-coumaric acid to protocatechuic acid via 4-hydroxybenzoic acid. Following *Egland* et al. (1996), 4-hydroxybenzoate is further degraded to CO_2 by enzymes encoded by a cluster of 24 genes. In bacteria, genes for the *meta*- or *ortho*-pathways for simple phenolics degradation are often clustered into operons (*Suenaga* et al., 2009; see Figure 2). In several bacteria, a gene cluster is responsible for the degradation of protocatechuic acid via the β-ketoadipate pathway, for instance in *Streptomyces* sp. strain 2065; *Acinetobacter* sp. strain ADP1 or in *Agrobacterium tumefaciens* (*Iwagami* et al., 2000). As shown in Figure 1, many aromatic secondary metabolites are degraded via protocatechuic acid. In animals, p-coumaric acid and the precursor ferulic acid are absorbed from the stomach and intestine (*Alam* et al., 2016). Free p-coumaric acid, and especially the conjugated forms, have diverse beneficial effects (*Pei* et al., 2015). Since p-coumaric acid was found in high amounts in urine of rats fed with p-coumaric acid, *Garrait* et al. (2006) hypothesized a low metabolization of the compound.

Vanillic acid

Vanillic acid is a compound derived from the bacterial degradation of exuded ferulic acid, produced via 4-hydroxy-3-methoxyphenyl-hydroxy-propionic acid and vanillin. Vanillic acid alters the cucumber rhizosphere microbial communities and inhibits the growth of the seedlings. 0.2 μmol g^{-1} soil vanillic acid decreased the absolute abundance of *Bacillus* and *Pseudomonas* species, but increased the relative abundances of *Arenimonas, Gemmatimonas, Haliangium, Opitutus, Pseudolabrys, Steroidobacter* and *Rhodanobacter* species, while *Nitrospira spec*. was reduced (*Zhou* and *Wu*, 2018). The authors assumed an increase of species with denitrification capabilities and a decrease of nitrifying bacteria; thus, the nitrogen supply of the plant might be negatively affected. Vanillic acid can be transformed into protocatechuic acid by *O*-methyltransferase which is expressed, for instance, in strains of *Agrobacterium fabrum* (*Campillo* et al., 2014). The basidiomycete *Sporotrichum pulverulentum* decarboxylates vanillic acid to methoxy hydroquinone and reduces vanillin to vanillyl alcohol (*Ander* et al., 1980). When vanillic acid is non-oxidatively decarboxylated, guaiacol is produced. The vanillate decarboxylase enzymatic complex is obviously frequent in bacteria and yeasts, for example in *Bacillus megaterium*, *Streptomyces* strains, *Rhodotorula rubra*, and in *Nocardia* and *Streptomyces* species (*Álvarez-Rodríguez* et al., 2003). Thus, the different degradation pathways for vanillic acid result in a number of bioactive intermediates, such as guaiacol, catechol, protocatechuic acid and vanillin. Thus, protocatechuic acid, presents a catabolite of many secondary metabolites: Flavonoids, p-coumaric acid, resveratrol, vanillic acid and many others. The compound has a very high pharmacological potential (*Kakkar* and *Bais,* 2014).

The microbial degradation of plant derived phenolics with phytotoxic properties have been addressed by *Blum* and *co-workers* in the 1990ies (*Blum*, 1998; *Blum* et al., 2000). They found a reduction of the growth inhibitory effect of phenolic acid mixtures on cucumber seedlings by bacteria isolated from the rhizosphere and bulk soil. The microorganisms produced benzoic acids such as vanillic acid and

p-hydroxybenzoic acid from cinnamic acids, which were subsequently metabolized. Microbial pathways to cleave aromatic compounds are known from simple phenolics and flavonoid degradation pathways (Figure 2).

In mammalian species, vanillic acid seems to have beneficial effects due to its anti-inflammatory properties (*Calixto-Campos* et al., 2015). Studies with isolated rat hearts led to the assumption that vanillic acid may have a cardioprotective effect (*Radmanesh* et al., 2017). *In vitro* experiments with human cell lines point to antimicrobial, anti-inflammatory, anticancer, and liver-protective effects of vanillic acid (*Gong* et al., 2019). One of the catabolites, guaiacol, promotes the aggregation of the desert locust *Schistocerca gregaria*. *Pantoea agglomerans,* a member of the locust gut microbiota, is assumed to be mainly responsible for guaiacol production (*Dillon* et al., 2000). Guaiacol can be transformed into catechol and further degraded to compounds able to enter the TCA cycle.

Salicylic acid

Salicylic acid, commonly found in plants, has functions during the entire life cycle of plants, during seed germination, growth and development, flowering and senescence. Often, salicylate is stored in the form of a precursor, such as salicin in the bark of willow trees (*Salix* species). Salicylic acid (SA) is released into the soil by root exudation and by rotting plant material. In high concentrations, salicylic acid acts as an allelochemical (*Manthe* et al., 1992). In the plant, SA can function as a phytohormone involved in abiotic stress responses. It serves as a signal in local and nonspecific systemic defence against pathogens (*Rivas-San Vicente* and *Plasencia*, 2011). *Lebeis* et al. (2015) described the impact of SA on the species composition of the root microbiota, favouring bacterial strains able to use of SA either as a growth signal or as a carbon source. Interestingly, certain microorganisms of the root microbiota prime the induced systemic resistance in leaves (*Pieterse* et al., 2014). Most of the Gram-negative bacteria, such as *Burkholderia* species, can degrade salicylic acid (*Chowdhury* et al., 2014), (Figure 2). Several plant pathogens have developed methods to eliminate SA signalling in plant defence responses. One strategy is the degradation of SA by salicylate hydrogenase yielding catechol as a catabolic intermediate, or by the Nag pathway via gentisic acid (*Chowdhury* et al., 2014; *Lowe-Power* et al., 2016; *Qi* et al., 2018).

The pharmaceutical effects of salicylic acid and derivatives in animals are well known and are not discussed here. Surprisingly, the effects of acetylsalicylate (aspirin) and salicylic acid on human gut microorganisms are almost completely unknown, except for microbial transformation of aspirin to salicylic acid. In 2018, a clinical trial was started to elucidate effects of aspirin on the human gut microbiota composition and metabolome (ClinicalTrials.gov identifier [NCT number]: NCT03450317). Salicylic acid consumption leads to a number of detoxification products which are excreted. In human urine, salicylurate, glucuronide conjugates and gentisic acid have been identified (*Cham* et al., 1980). Gentisic acid is a bioactive intermediate with anti-inflammatory, antirheumatic and antioxidant properties.

Trans-resveratrol

Trans-resveratrol, the aglycon of *trans*-piceid, is another phytoalexin, which is synthesized by a small number of plant species, for instance, peanut. The active compound is released, similar to all

phytoalexins, only upon severe stress exposure or pathogenic attack. The compound acts anti-pathogenic, possesses antimicrobial activity and is also an allelochemical. The general effects on the rhizobiome are not yet known, but resveratrol might be a good candidate for modulating microbiota, perhaps also indirectly. In wheat cultivars infected with *Blumeria graminis*, resveratrol stimulates the synthesis of phenolic compounds and the efficiency of photosynthesis (*Pociecha* et al., 2014). *Acinetobacter oleivorans* strain JS678, isolated from the peanut rhizosphere, is able to metabolize resveratrol completely via 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde. Subsequent compounds of the catabolic sequence are 4-hydroxybenzoate and protocatechuic acid. The latter is cleaved by bacterial protochatechuate 3,4-dioxygenase resulting in 3-carboxy-*cis,cis*-muconate (*Kurt* et al., 2018), (Figure 1). The aldehyde intermediates did not only promote growth of strain JS678, but also the growth of all other strains isolated from the rhizosphere which were unable to perform the first degradation steps. In mammals, *trans*-resveratrol is well absorbed in the gut. Most of it is conjugated in the liver with glucuronic acid or sulfate, but the conversions are reversible. Resveratrol was found to modulate the gut microbiota of rats and humans. It might possess positive effects on metabolic syndrome and has many other beneficial effects on human health, but results of such studies are often contradictory (*Salehi* et al., 2018). Human gut bacteria convert resveratrol to dihydroresveratrol, 3,4'-dihydroxy-trans-stilbene and 3,4'-dihydroxybibenzyl (lunularin), but there are differences between individuals (*Chaplin* et al., 2018; *Pallau* et al., 2019). Dihydroresveratrol is also conjugated to glucuronic acid. Since the glucuronide is found in high amounts in urine, the compound seems not be metabolized further.

DEGRADATION OF FLAVONOIDS LEADS TO SIMPLE PHENOLIC INTERMEDIATES

Flavonoids have multiple functions in the rhizosphere and interact with microorganisms (*Hassan* and *Matthesius*, 2012) Flavonoids contribute to microbiota sculpting, also by their degradation products. Microorganisms of the human gut and bacteria isolated from rhizosphere and bulk soil can degrade flavonoids, but the catabolism can differ (Figure 1), and depends on aerobic or anaerobic conditions.

Rhododendron formosanum accumulates catechin in the leaves, which is released from leaf litter and enriched in the soil (*Wang et al*., 2013). In the rhizosphere, *Pseudomonas*, *Herbaspirillum*, and *Burkholderia* species were the dominant genera. *Pseudomonas* used catechin as a carbon source by conversion of (-)-catechin into protocatechuic acid followed by degradation into glycerol. Taxifolin was identified as a first intermediate, thus the introduction of a keto group in position 4 of the C-ring prior to C-ring cleavage is a prerequisite for subsequent catabolic steps. Although not further investigated, ketone formation is most likely catalysed by catechol oxygenase, the key enzyme enabling the β-ketoadipate pathway for catechin degradation. Catechol oxidase was identified in *Acinetobacter calcoaceticus* and in some fungi (*Arunachalam* et al., 2003). Protocatechuic acid is assumed to intensify the phytotoxic effect of catechin dramatically (*Wang*

et al., 2013). Catechin itself acts as a bacteriostatic compound (*Pollock* et al., 2011). In humans however, the compound has several positive properties, including cytoprotective effects, induction of detoxifying enzymes, improvement of cognitive dysfunctions and many others (*Kakkar* and *Bais*, 2014).

At present, it seems that gut microorganisms do not perform the β-ketoadipate pathway for catechin degradation. Instead, intestinal bacteria isolated from human (*Eggerthella lenta* rK3 and *Flavonifractor plautii* aK2) and rat (*Adlercreutzia equolifaciens* MT4s-5 and *Flavonifractor plautii* MT42) performed C-ring cleavage yielding (2R)-1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl) propan-2-ol. *Flavonifractor plautii* aK2 converts 1-(3,4-dihydroxyphenyl)-3-(2,4,6-trihydroxyphenyl) propan-2-ol subsequently to 5-(3,4-dihydroxyphenyl)-valerolactone and 4-hydroxy-5-(3,4-dihydroxyphenyl) valeric acid. The final degradation products of catechin are phenylpropionic acids, compounds with anti-inflammatory properties (*Kutschera* et al., 2011; *Takagaki* and *Nanjo*, 2016).

The flavanol quercetin is a lipophilic compound which is converted in the liver by *O*-methylation, glucuronidation, and sulphation, yielding quercetin-3-glucuronide, quercetin-3′-sulfate, and isorhamnetin-3-glucuronide (*Suganthy* et al., 2016). Quercetin-3-O-glucuronide and isorhamnetin-3-O-glucuronide (methylquercetin-3-O-glucuronide) are found in the brain, where the compounds are supposed to develop neuroprotective properties. Quercetin and also the conjugates can be attached to or immersed in the lipid bilayer of membranes, but depth of immersion depends highly on charge and hydrophilic substitutions (*Košinová* et al., 2012). The interaction alters fluidity and other properties of the membrane which is thought to be correlated with the radical scavenger function and anti-inflammatory effects of quercetin (*Tsuchiya*, 2015). In plants and microorganisms, membrane interactions of the aglycon are attributed to the antimicrobial and allelopathic effects resulting in membrane leakage, one of the most harmful injuries caused by allelochemicals (*Schulz* et al., 2013).

Many microorganisms are, however, able to degrade quercetin. A fungal pathway starts with C-ring cleavage by quercetin 2,3-dioxygenase, producing 2-protocatechoylphloroglucinol carboxylate. In the anaerobic degradation pathway as performed in the human gut by bacteria, for instance by *Eubacterium ramulus*, quercetin is reduced to taxifolin and then converted to alphitonin. Final products are 3,4-dihydroxyphenylacetic acid and phloroglucinol (*Braune* et al., 2001). 3,4-Dihydroxyphenylacetic acid is also a known metabolite of the neurotransmitter dopamine in animals. A number of soil bacteria degrade quercetin via taxifolin and protocatechuic acid, thus this pathway is widely distributed among bacteria (*Mansuroglu*, *Schütz* and *Schulz*, unpublished).

Terpenoids

In *Arabidopsis thaliana* roots, triterpenes, such as thalianin and arabidin, have an important function in shaping the root microbiome. Several isolated bacterial strains were able to convert these compounds and resulting metabolites have differential influences on the growth of the microorganisms (*Huang* et al., 2019). The authors assume a role of the triterpene biosynthetic network in enriching the rhizosphere especially with Proteobacteria species. *Chen* et al. (2019) report a sesterterpenoid-induced modulation of the root microbiota composition of *Arabidopsis thaliana,*

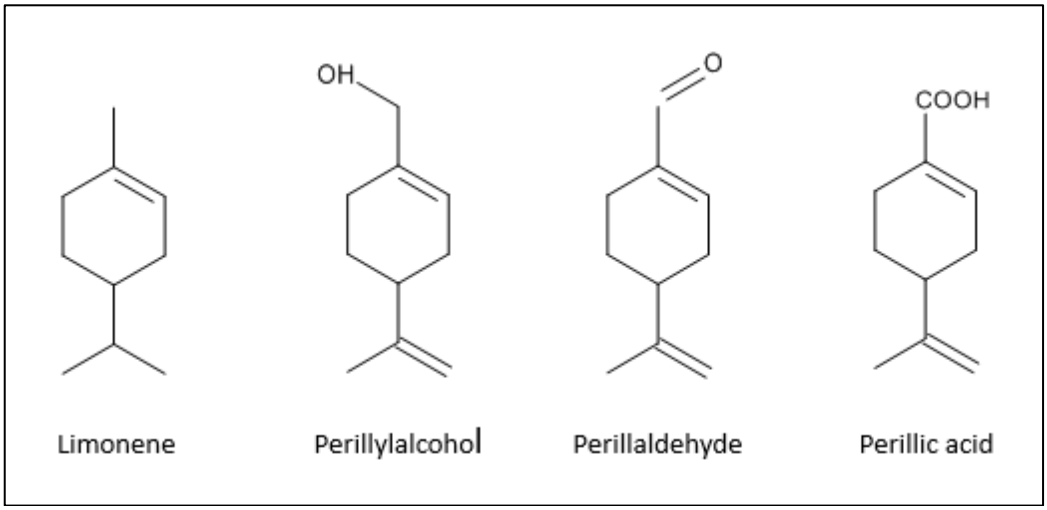

Figure 3: *Fusarium verticillioides, Pseudomonas putida* and some yeasts convert limonene into perillylalcohol, perillaldehyde and perillic acid. Perillic acid is also a secondary metabolite of many plants.

ascertained by the use of loss-of-function mutations in the sesterterpenoid biosynthesis genes. The recently duplicated prenyltransferase-terpene synthase (PT-TPS) gene cluster allows the biosynthesis of these, in evolutionary terms young compounds, due to the late introduction of the prenylation step.

Terpenoids can be converted by microorganisms, leading to hundreds of derivatives (*Marmulla* and *Harder*, 2014; *Parshikov*, 2015), which cannot be considered here. It is presently unclear if plants themselves perform terpenoid degradation, or whether some degradation products and derivatives result intrinsically from activities of endophytes or other members of the microbiota. For instance, the maize pathogen and endophyte *Fusarium verticillioides* converts R-(+)-limonene to R-(+)-perillyl alcohol, which can be oxidized to perillyl aldehyde and perillic acid (Figure 3). This ability is not restricted to *F. verticillioides*. *Pseudomonas putida* and several yeasts perform the same conversions. Perillic acid is found in many plants, sometimes in high amounts. The limonene derivatives are pharmaceutically interesting because of their anticancer and anti-inflammatory properties in humans.

In herbivory animals, the microbiotas are involved in terpenenoid degradation. The mountain pine beetle, *Dendroctonus ponderosae*, feeds on terpene rich conifers without suffering from intoxification, due to its microbiota that take over detoxification and degradation work (*Adams* et al., 2013). Species of the rumen microbiota of goats have capabilities to degrade monoterpene hydrocarbons, oxygenated monoterpenes and the sesquiterpene cedrene, at least in part (*Maleckyl* et al., 2011).

Glucosinolate

Glucosinolate break down products (Figure 4), in particular isothiocyanates (ITCs), are a class of degradation products well known for herbicidal and mostly negative impacts on microorganisms. In agriculture, the compounds are used for biofumigation to suppress weeds and pathogens. These compounds have microbiome modulating properties (*Hu* et al., 2015; *Siebers*

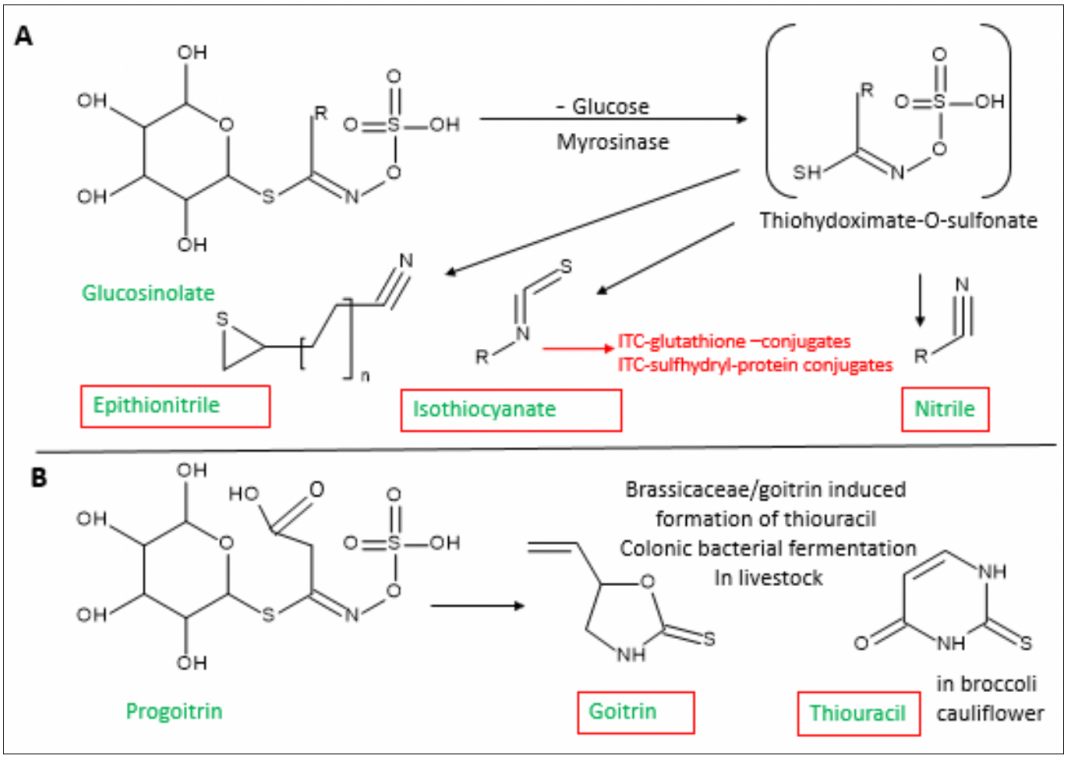

Figure 4: A: Glucosinolate break down is initialized by myrosinase or enzymes possessing similar activities (bacteria). The deglucosylated product is unstable, resulting in the main break down products epithionitriles, isothiocyanates (ITCs) and nitriles. These break down products are found in plants and animals (Angelino et al. 2015; Witzel et al., 2015). In animals, ITC-glutathione- and other sulfhydryl-conjugates can be formed. B: Progoitrin is the precursor of goitrin, which induced thiouracil synthesis in plants and animals. Red markers: Compounds found in animals, green markers: in plants. (Simplified scheme).

et al., 2018). Glucosinolates, typical for Brassicacea and related families, strongly suppress particularly fungi but also many bacteria. Not only pathogenic fungi but also beneficial ones are affected. In agricultural and in natural ecosystems, mycorrhizal fungi can be destroyed with negative long-term effects on several trees and crops (*Lankau*, 2010; *Hilton* et al., 2013; *Hansen* et al., 2019). The findings under field conditions are in agreement with those obtained from exposure of soil samples to mixtures and single glucosinolates and *Brassica* seed extracts. Microorganisms able to cope with glucosinolate break down products are favoured whereas others almost disappeared without recovery. Some cultured bacteria and fungi, isolated from rape seed extract treated soil, eliminated progoitrin derived cyclic ITC goitrin up to 60% within five days (*Siebers* et al., 2018).

The isothiocyanate goitrin has antithyroid effects in mammals by inhibiting the formation of thyroxin, whereas several thiocyanates have a negative influence on iodine uptake. Thiouracil, thought to be derived from goitrin, occurs naturally in some *Brassica* vegetables such as broccoli and cauliflower (*Vanden Bussche* et al., 2011), and is also produced by intestinal bacteria present in the gut of vertebrates. The pathway how thiouracil is synthesized

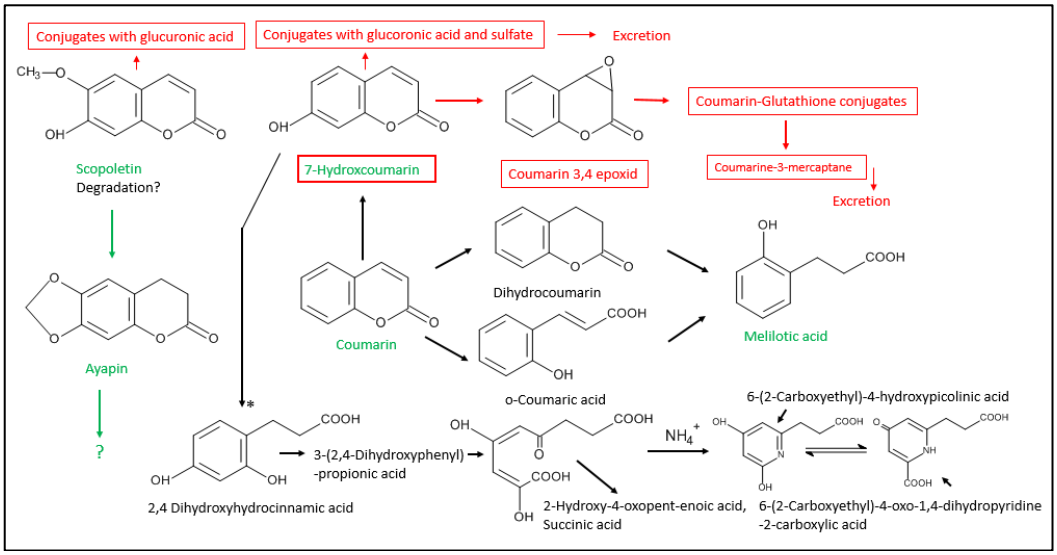

Figure 5: Coumarin degradation and detoxification differs strongly in plants and animals (simplified scheme). Presently only 7-coumarin was identified in animal and plant organisms.
*: Degradation pathway of *Pseudomonas mandelii* 7HK4.

from isothiocyanate is unknown. Thiouracil is a much stronger goitrogen than goitrin (*Kiebooms* et al., 2014). Nitriles, another class of compounds derived from glucosinolate break down, have hepatotoxic effects in cattle. On the other hand, glucosinolate break down products have tumour prevention properties. Benzyl isothiocyanate degradation by *Enterobacter cloacae* led to benzylamine and hydrogen sulphide (*Tang* et al., 1972), but otherwise ITC degradation can occur rather fast and intermediates are hardly identifiable.

Coumarins

The coumarin scopoletin, exuded by roots of the model plant *Arabidopsis thaliana* after inoculation with *Pseudomonas simiae* WCS417, has a modulating effect on the root microbiome. Scopoletin, a compound with antifungal properties, suppressed the pathogens *Fusarium oxysporum* and *Verticillium dahliae*, but was stimulating on ISR-inducing rhizobacteria *Pseudomonas simiae* WCS417 and *Pseudomonas capeferrum* WCS358 (*Stringlis* et al., 2018; 2019). Scopoletin and its 7-β-D-glucoside scopolin are widely distributed in higher plant species (*Gnonlonfin* et al., 2012). It can be speculated therefore that microbiome modulation by scopoletin might be a more general feature. This compound has additional functions, for instance it is a well-known phytoalexin and supports iron uptake. *Voges* et al. (2019) found community shifts in absence of flavonoids and coumarins using a reduced synthetic community (SynCom) of *Arabidopsis thaliana* root-isolated bacteria. *Arabidopsis* f6'h1 mutant lines, unable to synthesize coumarin, showed community shifts under iron deficiency conditions. Application of coumarins causes a partial recovery of the community similar to the wild type.

Ayapin presents a possible degradation product of scopoletin, but the further degradation pathway is not known, whereas the one of coumarin was studied in a few microorganisms: *Arthrobacter* spec., *Bacillus cereus*, *Pseudomonas* spec, *Pseudomonas orientalis*,

various *Saccharomyces cerevisiae* strains and *Fusarium solani* (*Häser* et al., 2006 and references therein). The major pathway in all of them starts with the reduction to dihydrocoumarin and subsequent lactone opening yielding melilotic acid [3-(2-hydroxyphenyl)propionic acid]. In *Arthrobacter,* another pathway yields first o-coumaric acid which is reduced to melilotic acid. In higher plants the same degradation products were found, but the pathways are not sufficiently studied. The further degradation of melilotic acid remains to be elucidated (Figure 5).

The degradation pathway in human and rodents seems to be different, and some of the intermediates could explain the hepatotoxic effects of coumarin in animals. In a first step coumarin is hydroxylated. 7-Hydroxycoumarin (umbelliferone) can be conjugated with glucuronic acid or sulfate in the liver; both conjugates and the hydroxylated coumarin are not toxic. Meanwhile, several degradation pathways and new detoxification products are known (*Leonart* et al., 2017). The most toxic intermediate represents coumarin 3,4-epoxide, which can be conjugated with glutathione. The glutathionylated intermediate is finally converted to coumarin-3-mercapturic acid. The toxicity in animals seems to be species, even individual dependent.

7-Hydroxycoumarin, found in many plants, is toxic for *Ralstonia solanacearum, Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus,* and *Pseudomonas aeruginosa*, but other *Pseudomonas* species and species of *Arthrobacter, Aspergillus, Penicillium*, and *Fusarium* spp. can use it as a C-source. 7-Hydroxycoumarin can be completely degraded by *Pseudomonas mandelii* 7HK4, due to the hcdABC operon (*Krikštaponis* and *Meškys*, 2018; Figure 5). The operon encompasses genes for a flavin-binding hydroxylase (HcdA), an extradiol dioxygenase (HcdB), and a putative hydroxymuconic semialdehyde hydrolase (HcdC).

Benzoxazinones

Benzoxazinones are a special group of secondary products exhibiting a dispersed distribution within the angiosperms. Compounds belonging to this class of heterocycles are present in several wild and domesticated Poaceae and in some dicotyledonous plants (*Sicker* and *Schulz*, 2002; *Schulz* et al., 2013; *Niculaes* et al., 2018). Bezoxazinones have antimicrobial, allelopathic and anti-herbivory functions. They are unstable in the deglucosylated from. Their spontaneous degradation products, the benzoxazolinones BOA (benzoxazolinone) and MBOA (6-methoxybenzoxazolinone) have a modulating activity on the maize root microbiome composition and microorganisms in bulk soil, but different causative mechanisms for the modulation are discussed (*Hu* et al., 2018; *Kudjordjie* et al., 2019). *Cotton* et al. (2019) suggest a function for benzoxazinoids in the control of root flavonoid biosynthesis and a microbiome shaping activity that is also triggered by the root type. In a former study, DIMBOA [2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one], the main benxoxazinone in maize, was found to attract *Pseudomonas putida*. This compound affects the expression of the bacterial genes associated with chemotaxis and benzoate catabolism (*Neal* et al., 2012). Benzoxazinoids can be detoxified by plants and degraded by microorganisms using different pathways which are described in more detail in the following paragraph. In plasma and urine of rats, benzoxazinone [2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one] glucuronides have been identified (*Adhikari* et al., 2012).

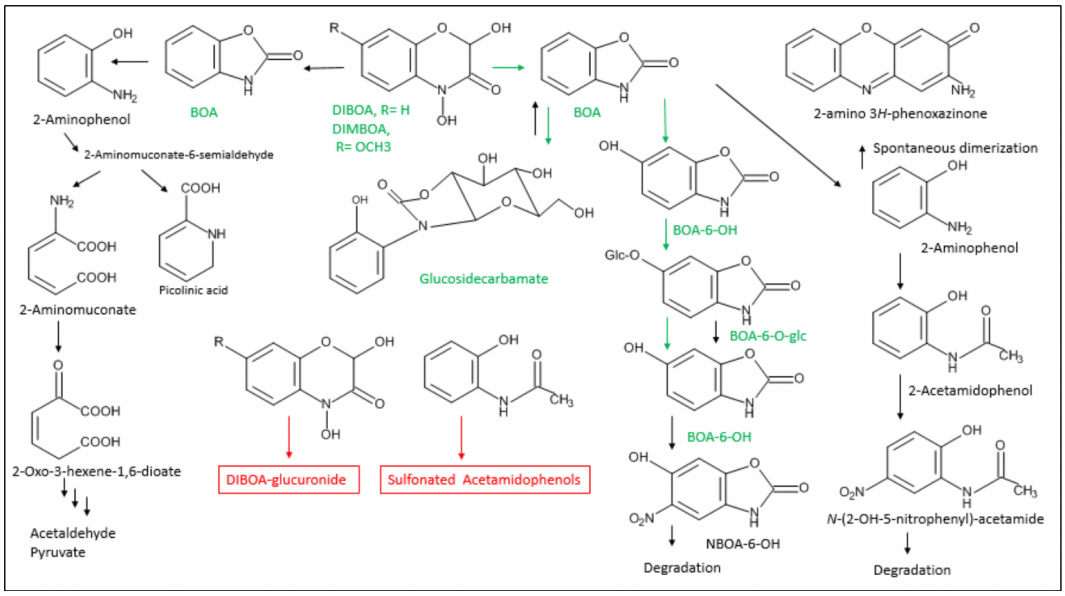

Figure 6: Bacterial pathways for BOA degradation via 2-aminophenol, either by ring cleavage yielding 2-aminomucconate with picolinic acid as a spontaneous byproduct (Arora et al. 2015), or by acetylation of 2-aminophenol /hydroxylation of BOA and subsequent nitration, yielding degradable nitro aromatic compounds (Simplified scheme). Names of plant products are in green. The only detoxification products found in mammals are DIBOA-glucuronide and sulfonated acetamidophenol (red).

PLANT AND MICROBIAL CONVERSIONS OF BENZOXAZOLINONES

Many plants are able to detoxify benzoxazolinones either by hydroxylation or by demethylation of methoxylated benzoxazolinone and subsequent glucosylation (most dicotyledoneous plants). *N*-glucosylation, and rearrangement to glucoside carbamates is more common in wild and cultured Poaceae (Figure 6). The detoxification products are stored in the vacuole, are immobilized in the cell walls or are released by exudation. Deglucosylation of BOA-O-glucosides regenerates the hydroxylated detoxification intermediates BOA-6-OH, BOA-5-OH and the rare BOA-4-OH. Fungal release of the glucose moiety from glucoside carbamate and regeneration of benzoxazolinone is also possible. Thus, these detoxification products meliorate the phytotoxicity of BOA and of the hydroxylated intermediates only temporarily since the compounds are not degraded. As far as investigated, the dominant detoxification product found in maize, glucoside carbamate, promotes the growth of numerous fungi and bacteria (*Schütz* et al., 2019).

A limited number of fungi are able to cleave the benzoxazolinone heterocycle yielding 2-aminophenol, which can be malonylated, probably concomitantly with the cleavage of the heterocycle, to N-(2-hydroxyphenyl) malonamic acid (HPMA), (*Friebe* et al., 1998; *Glenn* et al., 2016). *Acinetobacter calcoaceticus* is presently the only known bacterium able to degrade benzoxazolinone to 2-aminophenol, whereas conversion of 2-aminophenol to acetamidophenol is performed by many fungi and bacteria. In humans, conversion products of acetamidophenol have been found. After consumption of

Figure 7: *Abutilon theophrasti* seedlings (7-day-old) were incubated overnight with 0.5 mM BOA-4-OH without rhamnolipid, 1, 2 und 3mg rhamnolipid in 30ml water (A). B: Roots were heavily covered with a polymer coat (0 mg) but less with increasing amounts of rhamnolipid (left to right side). C. Addition of *Papiliotrema baii* prevent the development of polymer coats almost completely.

benzoxazinoid-rich bread, human postprandial plasma contained two sulfonated acetamidophenols (hydroxy-N-(2-hydroxyphenyl) acetamide and N-(2-hydroxyphenyl) acetamide), (*Hanhineva* et al., 2014). Presently it is unclear whether the gut microbiota is able to degrade benzoxazolinone and how acetamidophenols are generated. While sulphatation and glucuronylation reactions are performed in the liver, *Hanhineva* et al. (2014) assumed that 2-acetamidophenol results from sourdough fermentation since the compound is already present in the bread.

Released 2-aminophenol is otherwise oxidatively dimerized to 2-aminophenoxazinone or degraded, for instance by *Pseudomonas* and *Burkholderia* species (*Arora* et al., 2015). One pathway starts with ring opening to 2-aminomuconic-6-semialdehyde which is further oxidatively degraded to fragments entering the TCA cycle (Figure 6). A spontaneous by-product formed from the semialdehyde is picolinic acid, a bioactive compound with neuroprotective and immuno-modulating properties. In another pathway, presently only found in *Burkholderia* species, 2-aminophenol is hydroxylated via 1,4 benzenediol to 1,2,4 benzenetriol and then cleaved to maleylacetic acid.

2-Aminophenoxazinone has a higher phytotoxicity compared to benzoxazolinones. Phenoxazinone and benzoxazolinones can be extracted from soil after wheat and rye culture. Thus, the compounds are relatively stable and cannot easily eliminated. As a consequence, decontamination of arable soil from benzoxazolinones and phenoxazinones takes several months (*Schulz* et al., 2013 and references therein).

MICROBIAL SUPPORT OF BOA-OH ELIMINATION VIA POLYMERIZATION ON THE ROOT SURFACE

Abutilon theophrasti, a weed found in maize and beet fields, does not accumulate considerable amounts of BOA-6-O-glucoside or glucoside carbamate. Instead, the plant polymerizes hydroxylated BOA in high amounts at the root surface, presenting a completely different way to eliminate toxic compounds. Hydroxylation of benzoxazolinones, a prerequisite for polymerization reactions, is performed by the plant. Root colonizing microorganisms originated from soil, are involved in the process. Depending on the cultivation site,

Abutilon theophrasti can be colonized by a microbial consortium with *Actinomucor elegans* as the dominant species. The fungus is associated with several bacteria, among them *Pantoea ananatis* and *Stenotrophomonas maltophilia*, and the yeast *Papiliotrema baii*. The consortium is a stable microcommunity and the zygomycete cannot be cured from the partners by the addition of antibiotics (*Haghikia* et al., 2014). *Abutilon theophrasti*, inoculated with the *A. elegans* consortium developed dark brown roots within a few hours when incubated with BOA-6-OH or BOA-4-OH. The yeast and *Pantoea ananatis* react with high H_2O_2 production, a substrate for peroxidase catalysed polymerization. Interestingly, polymer coat formation at the root surface can be meliorated or removed by the addition of rhamnolipid (Figure 7, *Schulz* et al., unpublished). The glycolipid with antimicrobial properties is synthesized by many *Pseudomonas, Pantoea, Enterobacter, Burkholderia* species and others. Since *P. baii* not only produces high amounts of H_2O_2 but also the glycolipid 2-O-(β-D-galactopyranosyl-(1->6)-β-D-galacto-pyranosyl) 2S-hydroxynonanoic acid (*Schilasky*, *Siebers*, *Schulz* and *Dörmann*, unpublished), polymer formation and the glycolipid may interact, perhaps in radical scavenging activities. Although speculative, certain microorganisms may use glycolipids to clean roots from polymers.

BACTERIAL NITRATION OF HYDROXYLATED BENZOXAZOLINONE AND ACETAMIDOPHENOL FOR A FAST DEGRADATION

We detected a new compound in the wash liquid obtained from root surfaces of *Abutilon* seedlings inoculated with the *A. elegans* consortium after incubation with BOA-6-OH.

The compound is produced in high amounts by *Pantoea ananatis* and by the complete *A. elegans* consortium when incubated with BOA-6-OH in Czapek medium (Figure 8). It was identified as 6-hydroxy-5-nitro-benzo[*d*]oxazol-2(3*H*)-one (NBOA-6-OH, Figure 6) (*Schulz* et al., 2017, 2018). NBOA-6-OH can be produced by several soil bacteria, but obviously not by fungi. This compound is rapidly degraded without accumulation of identifiable degradation products, also by fungi (*Schütz* et al., 2019). Methoxylated benzoxazolinone (MBOA) is another substrate for nitration by the complete *A. elegans* community, yielding 6-methoxy-4-nitro-benzoxazolin-2(3*H*)-one (NMBOA). Bacterial nitration activates benzoxazolinones for subsequent rapid degradation. Since no intermediates accumulate, it is presently unclear how the degradation pathway is constructed and which enzymes are involved. Several reaction sequences are possible, depending on oxygen availability (*Ju* and *Parales*, 2010).

2-Acetamidophenol can be nitrated by strains of *Paenibacills polymyxa* and *Aminobacter aminovorans* (*Schütz* et al., 2019*)*. The product, *N*-(2-OH-5-nitrophenyl)-acetamide, accumulates in the culture medium, and is also biodegradable. It was described first as a fungal product by *Zikmundova* et al., (2002) derived from acetamindophenol. Nitration may shorten the life time of molecules, as found with BOA-6-OH and 2-acetamidophenol. Nevertheless, nitro aromatic compounds can have fatal effects in animals and are also toxic for numerous microorganisms (*Ju* and *Parales*, 2010). We assume that bacterial nitration of suitable molecules may be more widespread that thought and occurs with different classes of secondary metabolites. For instance,

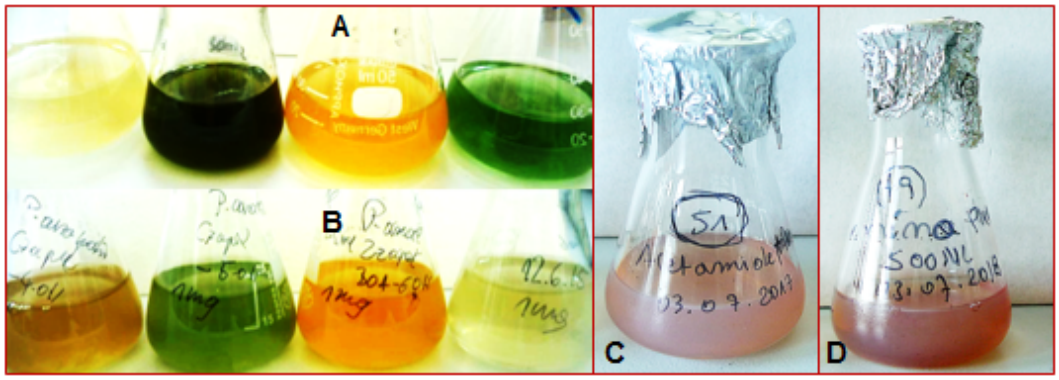

Figure 8: Incubations of the *Actinomucor elegans* consortium in Czapek medium supplemented with 0.5 mM BOA-6-OH lead to yellow compound (A), which is also formed with *Pantoea ananatis*, isolated from the consortium (B). The compound was identified as 6-hydroxy-5-nitrobenzo[*d*]oxazol-2(3*H*)-one. Incubation of *Paenibacills polymyxa* (C) and *Aminobacter aminovorans* (D) with 2-acetamidophenol in Czapek medium for 2 days results in colored cultures containing different products, one of them was identified as *N*-(2-OH-5-nitrophenyl)-acetamide.

nitration of catechin to 6,8-dinitrosocatechin which happens in the stomach, was described by *Veljovic-Jovanovic* et al., (2014). Quercetin partly suppresses the nitration and also prevents further oxidation of 6,8-dinitrosocatechin to the quinone form by nitrous acid. Required nitrite, necessary for nitration, results from the activity of bacteria in the oral cavity of animals including humans, which reduce nitrate present in high concentrations in vegetables.

CONCLUDING REMARKS

Hacquard et al., (2015) ascertained no overlap of the abundant bacterial organisms of mammal and plant root microbiotas. Analyses of the barley, *Arabidopsis,* maize, rice and grapevine microbiotas revealed a high abundance of members of the Chloroflexi, Actinomycetales, Verrucomicrobiota, Bacterioidales, Saprospirales, Burkholderiales, Xanthomonadales, Pseudomonadales, Rhizobiales, and Sphingomonadales. All of them are almost not present in the microbiotas of mammals, including humans. The latter microbiotas are dominated by Bacteriodales, Clostridiales and Lactobacilliales, which are, in turn, not or rarely found in the plant microbiotas. For fungi, a comparable study is presently not available. Nevertheless, pathways for secondary metabolite degradation have sometimes the same catabolic reaction sequences and the same intermediates transiently accumulate, as found for quercetin and some simple phenolics. Others, such as coumarin, differ notably in their detoxification and catabolism by soil, plant and animal microbiotas. For benzoxazinoids, degradation pathways are presently only known from soil and plant associated microorganisms. To cite *Chen* et al. (2019), the plant microbiome acts as intermediary between human and natural microbiomes and opens therefore a possible way of transferring antibiotic resistance. One can speculate that transfer of bacterial genes involved in secondary metabolite degradation and perhaps transfer of entire gene clusters into members of the different microbiotas mirrors co-evolutionary events.

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WHEAT GLUTEN, COELIAC DISEASE AND THE MICROBIOME: EXPLORING THE CONNECTIONS

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SUMMARY

Coeliac Disease (CD) is the most common food-induced immune mediated enteropathy in humans with an estimated prevalence of approximately 1% in worldwide human populations. A related condition known as non-coeliac gluten sensitivity (NCGS) appears to be increasing in prevalence, and may be even more common in the general population. These diseases are induced by the consumption of gluten, the common term for a family of digestion resistant, proline and glutamine-rich seed storage proteins found in the cereals wheat, barley and rye. The only current therapy for CD and NCGS is the maintenance of a life-long gluten free diet. Here, I present a brief review of these diseases as well as the role that study of the gut microbiome in humans and a non-human primate (NHP) model of CD can play in identifying treatments to address these conditions. Additionally, I present a brief overview of my research to develop a non-GMO (genetically modified organism), decreased gluten wheat and its relevance and potential applications in the context of CD, NCGS and human nutrition.

INTRODUCTION

The most well-known symptoms due to gluten ingestion in coeliac disease sufferers, including diarrhoea and stomach upset, were already described in antiquity about 2000 years ago: these disease manifestations were noted by the Greek physician Aretaeus of Cappadocia in the second century BCE (cited in: (*Freeman*, 2015). A derivation of the term he used "koiliakos", meaning "suffering in the bowels" gave coeliac disease its name (*Fasano* and *Flaherty*, 2014).

Remarkably, however, it wasn't until the middle of the 20th century that the definitive association between wheat consumption and the disease was established. In the 1930's the Dutch paediatrician, Dr. Willem-Karel Dicke, formulated the hypothesis that cereals such as wheat, rye, barley and (sometimes) oats were harmful to his paediatric coeliac patients. However, it was only during, and in the aftermath of the 2nd World War that he was able to definitively establish the connection between cereal consumption and deleterious symptoms in his patients. He noted that some of his patients improved when wheat flour was not available due to the deprivations of the war and that their condition worsened again after the war when wheat once again became available and his patients resumed eating wheat as a component of their diets. This, and subsequent careful studies of faecal fat content in his patients, led Dr. Dicke to publish the now generally accepted view that these cereals are the causative factor of coeliac disease (*van*

Berge-Henegouwen and *Mulder*, 1993).

In the years since this groundbreaking work there has been an increasing interest in coeliac disease as clinicians gradually became aware that the condition is not as rare as once thought, and as patient groups lobbied for new treatments. Coeliac disease was once thought to be primarily a paediatric condition, but it is now known that it can arise at any age. It is an autoimmune response triggered by the consumption of gluten in genetically susceptible individuals: principally individuals expressing human leukocyte antigen (HLA) haplotypes DQ2 and DQ8 (for a recent review see: *Lindfors* et al., 2019). In susceptible individuals, the consequence of consumption of wheat, barley, or rye is a chronic intestinal inflammation and flattening of the nutrient-absorbing villi of the small intestine. The clinical presentation of the disease, however, can vary drastically from very severe to no symptoms, to extra-intestinal symptoms such as dermatitis herpetiformis (skin rash) or neurological symptoms (*Jackson* et al., 2012). Because its symptoms vary so widely, there can oftentimes be a long interval between initial presentation and diagnosis, and the disease is still under-reported.

Nowadays, the disease is identified by screening for auto-antibodies such as transglutaminase 2 antibodies (TG2As), and endomysial antibodies (EmAs), as well as antibodies to gliadin. Definitive diagnosis follows with a small intestinal mucosal biopsy while the patient is still on a gluten-containing diet to confirm villous flattening. Once diagnosed, the only treatment is strict adherence to a lifelong gluten free diet. This can be quite challenging since wheat gluten is present in many processed foods, and it imposes an economic burden because most gluten-free substitutes for normally gluten-containing products are over 200% more expensive than their gluten-containing counterparts (*Lee* et al., 2007).

In recent years a number of drug candidates have emerged that are undergoing testing as potential treatments for coeliac disease. These include a modulator of intestinal tight junction permeability (*Kelly* et al., 2013), a polymer that sequesters gluten (*Alhassan* et al., 2019), and several competing gluten-degrading enzymes (*Sollid* and *Khosla*, 2011; *Wolf* et al., 2015; *Wungjiranirun* et al., 2016). There were also high hopes for a therapeutic vaccine (Nexvax2) under development by the company ImmusanT that was in Phase II trials (*Di Sabatino* et al., 2018). These hopes, however, were dashed with the very recent announcement (June 25, 2019) that ImmusanT was suspending the Phase II trial because interim analysis of the data showed that the vaccine was no better than placebo in providing protection from gluten exposure to patients (http://www.immusant.com/ImmusanT%20Nexvax2%20P2%20-%2025Jun19%20Final.pdf).

With the exception of the now discontinued Nexvax2, the CD therapies in development are not intended to allow CD sufferers to resume an unrestricted diet, rather they are meant to provide protection to patients from small amounts of inadvertently consumed gluten while they are following a gluten free diet.

In addition to coeliac disease, and wheat allergy (not discussed here), a recently identified condition known as non-coeliac gluten sensitivity (NCGS) has emerged. The key similarity with CD is that symptoms in patients resolve with a gluten free diet, but this syndrome is not associated with villous flattening, and the lack of clear biomarkers of disease makes this a still

mysterious condition (*Dale* et al., 2019). Besides gluten, there is evidence that so-called FODMAPS (fermentable oligo-, di-, monosaccharides and polyols) are responsible for symptoms in some NCGS patients. This has led *Dale* et al., (2019) to suggest that a more accurate term for the condition might be non-coeliac wheat sensitivity (NCWS) rather than NCGS, to acknowledge the role that other wheat constituents play in this syndrome, and it might also be considered a subtype of irritable bowel syndrome (IBS). The prevalence of NCGS is unclear, as many individuals are self-diagnosed and avoid gluten and wheat products out of a belief that such a diet is healthier. Estimates range widely, from less than 1% to greater than 10% of the population (*Aziz* et al., 2016). Until specific biomarkers and other diagnostic criteria are developed, this syndrome will remain somewhat undefined.

COELIAC DISEASE AND THE MICROBIOME

Considering that the study of the human microbiome is a young field, which was stimulated by the development of the inexpensive "next generation" sequencing technologies that arose out of technology developed for the sequencing of the human genome, the first human microbiome studies were, in effect, exercises in charting the territory and establishing a catalogue of the existing microbial diversity living in the diverse ecological niches present on and in healthy human bodies (*Human Microbiome Project Consortium*, 2012).

Likewise, these are still early days in the study of the human gut microbiome in relation to coeliac disease, and the studies reported to date might be regarded as being in the "cataloguing" phase of identifying differences in the gut microbiomes of coeliac patients compared to matched controls (*Sanz* et al., 2011). Such studies provide a valuable service in beginning to unravel the interactions between the intestinal pathology of coeliac disease and the attendant gut microbial community. The composition of the intestinal microbiome in effect may serve as a surrogate biomarker of the health of the individual. In addition, changes in the microbiome over time, for example in response to dietary or other interventions, may serve to document the effect of those interventions (*Lindfors* et al., 2019). Whether identified differences in the microbiomes of coeliac patients compared to healthy controls are a cause or consequence of the disease is not completely clear. Nevertheless, there is evidence that the HLA alleles, which predispose the individuals carrying them to develop coeliac disease, may influence their carriers to develop a disease-predisposing microbiome composition (*Olivares* et al., 2015, 2018; *Sanz*, 2015).

In general, the studies reported to date frequently show microbiome differences between CD individuals and controls but are somewhat inconclusive with regard to functional consequences (*Cheng* et al., 2013). In many cases, a decreased diversity in the gut microbiomes of coeliac patients compared to healthy controls has been found, frequently with differences in the types of gluten-degrading species present between the two groups (*Caminero* et al., 2014, 2016). In addition, gut dysbiosis in patients compared to healthy subjects is associated with a decrease in the ratio of putatively harmless bacteria *(Lactobacillus – Bifidobacterium)* compared to potentially harmful bacteria *(Bacteroides*/*Prevotella – E. coli*) (*Sanz* et

al., 2011; *Sánchez* et al., 2013).

Interestingly, one study found that early antibiotic use, which affects the gut microbiome, was associated with the development of coeliac disease (*Mårild* et al., 2013), however, a later study did not find such an association (*Kemppainen* et al., 2017). A recent review of the landscape of environmental and genetic factors that influence the development of coeliac disease highlights the role of microbiome composition as a causative factor in the loss of tolerance to gluten in CD patients (*Serena* et al., 2019).

In our own studies testing the effects of different animal feeds in a non-human primate (rhesus macaque) model of coeliac disease, we found that the faecal microbiome of gluten sensitive animals on a gluten containing diet displayed an overabundance of Streptococcaceae and Lactobacillaceae and a depletion of Coriobacteriaceae compared to healthy controls. In addition, the GS animals exhibited an upregulation of pro-inflammatory miRNAs (*Mohan* et al., 2016). This is the first assessment of faecal microbiomes in gluten sensitive rhesus macaques and the results strengthen the case for the use of this NHP model of CD to test experimental therapies (*Bethune* et al., 2008). Experiments to assess the effects on the faecal microbiomes in the NHP model of other dietary interventions, such as decreased gluten wheat, in combination with gluten degrading enzymes, are warranted. Our initial studies in the NHP model using a decreased gluten barley (*lys3a* mutant) animal feed pave the way for follow-up studies with decreased gluten wheat (*Sestak* et al., 2015; *Sestak* et al., 2016).

CEREAL SEED PROTEIN MUTATIONS: RELEVANCE TO WHEAT GLUTEN, NUTRITION AND CD

The seeds of grasses like rice, wheat and maize directly and indirectly provide most of the calories that feed humanity, thus the mechanisms that control the accumulation of the major storage compounds in seeds, namely starch, proteins and oils, have been of longstanding interest to scientists. In addition, because the storage proteins of many cereal crops such as maize and wheat are deficient in amino acids essential in the human diet and the diets of monogastric animals, considerable efforts have been devoted to identifying genetic variants that increase the amounts of these amino acids, principally lysine, threonine, tryptophan and methionine, in the seeds of cereal crops (*Azevedo* and *Arruda*, 2010). One of the earliest known genetic variants whose effect is to increase essential amino acids in cereal seeds is the opaque2 (*o2*) mutation in maize (*Mertz* et al., 1964), which leads to reductions in the accumulation of the 22 kilodalton size class of zein seed storage proteins (known as the α zeins) as well as to increased lysine content in maize seeds. When the gene underlying this mutation was finally isolated over 50 years after the mutant was first identified, it was found to encode a bZIP (basic leucine zipper) transcription factor (TF) that activates transcription of seed storage protein genes by binding to an upstream element found in the promoters of many of these genes (*Schmidt* et al., 1987) (*Schmidt* et al., 1990; 1992). Subsequently this mutation, together with genetic modifiers that ameliorate its negative pleiotropic effects, formed the basis for the

development of QPM (quality protein maize) varieties (*Prasanna* et al., 2001) containing increased lysine with demonstrated nutritional benefits in humans (*Gunaratna* et al., 2010; *Nuss* and *Tanumihardjo*, 2011).

Since the identification of the transcription factor underlying the *o2* mutation, which contains a basic DNA-binding region followed by a protein-protein interaction motif known as a leucine zipper (*Schmidt* et al., 1990), multiple additional transcription factors belonging to different structural classes have been determined to play roles regulating processes of seed nutritional reserve accumulation in maize and other cereals. These include transcription factors belonging to the DOF (DNA-binding one zinc finger) family, which interact with the O2-family transcription factors in binding to the promoters of seed storage protein genes (*Vicente-Carbajosa* et al., 1997; *Mena* et al., 1998; *Zhang* et al., 2015).

The success of increasing the content of essential amino acids such as lysine in *o2* maize motivated the search for similar mutants in related cereals. A program of barley mutagenesis and screening for increased lysine using a dye binding technique at the Risø agricultural experiment station in Roskilde, Denmark in the 1960s led to the identification of numerous enhanced lysine mutants of which the best characterized is the *lys3a* mutant (*Køie* and *Doll*, 1979; *Miflin* and *Shewry*, 1979). This mutant is also referred to as Risø 1508 or sex3c (shrunken endosperm xenia) (*Ullrich* and *Eslick*, 1977); it was induced by ethylenimine mutagenesis of the two-rowed spring malting variety, Bomi (*Doll* et al., 1974). The *lys3a* mutant is almost completely lacking in class C hordeins, and accumulates considerably reduced amounts of several B class hordeins, while having a 45% increase in the accumulation of free and protein bound lysine in the seed compared to the parental variety (*Shewry* et al., 1977; *Shewry* et al., 1978; *Munck* et al., 2001). These phenotypes are the result of a single recessive allele (*Doll*, 1973) that was mapped to barley chromosome 5H (*Karlsson*, 1977; *Ullrich* and *Eslick*, 1977; *Jensen*, 1979), although more recent research asserts that this map position is erroneous and that the mutant is located on chromosome 1H (*Druka* et al., 2011; *Rustgi* and *von Wettstein*, 2015). The underlying lesion responsible for the *lys3a* mutant's pleiotropic effects was hypothesized to be in a 5-methylcytosine DNA glycosylase gene (DEMETER, DNA demethylase) (*Gehring* et al., 2009) responsible for removing the methyl groups from methylated DNA since it was found that the methylation state of the promoters of several seed storage protein genes was altered in the mutant (*Sørensen*, 1992; *Sørensen* et al., 1996) (*Wen* et al., 2012).

The original *lys3a* mutant suffered from negative pleiotropic effects including lower yield and shrunken seeds containing reduced total starch. Nevertheless, concerted breeding efforts minimized these effects (*Eggum* et al., 1995; *Jørgensen* et al., 1999). Barley varieties containing the *lys3a* allele, however, were still lower in starch content than conventional cultivars and for this reason they were never widely grown (*Munck* and *Jespersen*, 2009). In recent years, there has been a resurgence of interest in this mutant, not because of its higher lysine content, but due to its lower levels of several classes of hordeins. The *lys3a* mutant, in combination with several other mutations that reduce the accumulation of hordeins, has been used to create ultra-low gluten barley (ULG) (*Tanner* et al., 2016). In addition, we tested the *lys3a* mutant in the rhesus macaque model of coeliac disease to determine if it, alone

or in combination with a gluten-degrading enzyme supplement, can ameliorate the symptoms of gluten sensitivity in this NHP model of coeliac disease (*Sestak* et al., 2015; 2016). In our 2016 paper, we reported that a diet of this *lys3a* mutant barley feed, combined with a gluten-degrading enzyme (Tolerase G, manufactured by DSM), appeared to eliminate symptoms due to gluten sensitivity in this animal model of coeliac disease.

We subsequently determined that a missense mutation in the Barley Prolamin-box Binding Factor (BPBF) represents the molecular lesion underlying the *lys3a* mutation (*Moehs* et al., 2019). This barley transcription factor is homologous to a domain of one finger (DOF) zinc finger transcription factor found in maize that has been shown to interact with O2 to control the expression of maize zein seed storage proteins (*Zhang* et al., 2015). A wheat homolog of BPBF had also been identified (*Ravel* et al., 2006).

This allowed us to use the non-transgenic, reverse genetic technique known as TILLING (Targeting Induced Local Lesions in Genomes; *Colbert* et al., 2001), a variation on mutation breeding, to identify 488 novel wheat lines that contain induced variants in the A, B and D genome copies of the WPBF genes in hexaploid bread wheat. Combining inactivating (recessive) alleles in all three homoeologous copies of the wheat PBF gene in hexaploid bread wheat led to a reduction in the accumulation of several classes of wheat gliadins and low molecular weight glutenins and to an increase in the accumulation of free and protein-bound lysine in wheat endosperm. These lines, which we refer to as decreased gluten wheat (DGW), have lower amounts of the epitopes that are detrimental to coeliac patients, but they are not low enough in gluten to be safe to consume for CD patients. However, they are novel in that they contain levels of gluten that are lower and outside of the range of the available genetic variation in existing wheat germplasm, including such "ancient" wheats as einkorn and spelt. In addition, there are ongoing efforts to decrease the gluten even further.

At present, the alleles are being introgressed into elite varieties and functional tests are being conducted to assess the types of products (cookies, cake, bread, noodles, tortillas, etc.) for which the DGW is best suited. Based on the outcomes of these tests, combined with other factors, will influence which market class(es) of wheat the alleles will be introgressed into. There are at least 6 market classes of wheat, including spring and winter wheats, as well as hard red and white wheats, in addition to durum pasta wheat. These different market classes all have particular functionality and uses.

A number of other groups are pursuing the development of decreased gluten wheat by other methods including RNAi (*Gil-Humanes* et al., 2010), and CRISPR (*Sánchez-León* et al., 2017). These methods have been very effective at creating transcriptional shutdowns in the expression of gliadins in the case of RNAi, or gene deletions of gliadins in the case of CRISPR, but these wheats face additional hurdles that come with being classified as transgenic and thus will face additional public scrutiny and a costlier path to market (*Laursen* 2016; *Jouanin* et al., 2018; *Hundleby* and *Harwood*, 2019).

CONCLUSION

The incidence of coeliac disease and non-coeliac gluten (wheat) sensitivity has been on a slow upward trajectory in recent decades and this is not simply due to increased testing and awareness (*Rubio-Tapia* and *Murray*, 2010). During the Swedish paediatric coeliac disease epidemic of the 1980s and 1990s, for example, the incidence reached about 3% of tested 12- year-old children, well above previously known rates of the disease (*Myléus* et al., 2009). Given that the predisposing HLA alleles are quite common in most human populations, but only a small subset of individuals carrying these alleles lose tolerance to gluten and develop coeliac disease, considerable effort has been devoted to identifying possible environmental variables that may contribute to loss of gluten tolerance (reviewed in: *Lindfors* et al., 2019)). Among the possible identified factors include infections with intestinal viruses (*Bouziat* et al., 2017; *Kahrs* et al., 2019), and a recent study suggested that vaccination against rotavirus may decrease paediatric coeliac disease cases (*Hemming-Harlo* et al., 2019). The seasonality of intestinal virus infections in children may also play a role in the link identified between seasonality of birth and the development of coeliac disease (*Daniel* et al., 2019). In the case of the Swedish CD epidemic, it was proposed that changes in the amount and timing of the introduction of wheat into infants' diets, as well as whether wheat was introduced while infants were still breastfeeding, played a role in the subsequent development of coeliac disease (*Ivarsson* et al., 2002). A recent meta-analysis, however, did not support a role for breastfeeding in subsequent development of CD (*Szajewska* et al., 2015) nor did adjusting the timing of gluten introduction into the diet of genetically predisposed infants prevent the development of CD in a well-designed, double-blind, randomized, placebo-controlled intervention study (*Vriezinga* et al., 2014).

Against the backdrop of clinical disease there is also a cultural shift occurring in many western nations with a dramatic increase in, and availability of, gluten free products, the self-diagnosis of "gluten sensitivity" and adoption of a gluten free and/or a low carbohydrate diet by a significant fraction of the public even in the absence of disease. This has been stimulated in part by a spate of best-selling, non-scientific books with sensationalistic titles such as "Wheat belly: lose the wheat, lose the weight, and find your path back to health," and "Grain brain: the surprising truth about wheat, carbs, and sugar--your brain's silent killers" that hold the consumption of wheat responsible for a host of current western societal ills, from "brain fog", to fatigue to obesity (*Davis*, 2014; *Perlmutter* and *Loberg*, 2018). In the current climate, it is not surprising that sensationalistic claims received more notice than the sober, scientific rebuttals (*Jones*, 2012; *Brouns* et al., 2013).

The "DIY" approach to gluten sensitive self-diagnosis even in the absence of a clinical diagnosis has also laudable aspects in the sense of the desire to control one's own health. One example of this is the growth in genetic self-testing companies such as 23andMe and its rivals. Self-testing of one's own microbiome is not far behind; there are already companies that offer this testing as a service, and individual accounts of dietary self-experiments and effects on the individual's own microbiome have been published (*Sprague*, 2017). Although there is no

"microbiome pill" to cure CD or NCGS, a number of microbiome companies (Second Genome, LNC Therapeutics) are dissecting the human microbiome with the objective to develop probiotics or pharmaceutical compounds to modulate disease states such as inflammatory bowel disease (IBD). Nature Publishing Group has just published a special collection of papers from the NIH funded Human Microbiome Project (https://www.nature.com/collections/fiabfcjbfj), and this is a research area that will likely continue to produce exciting insights and innovations in human health care in the years ahead (*Proctor*, 2019).

The decreased hordein (*lys3a*) barley that we used as a model in our studies during the course of the development of decreased gluten wheat appeared to positively impact the gut microbiomes of gluten sensitive rhesus macaques (*Mohan* et al., 2016), and it is to be hoped that similar follow-up studies will be conducted with decreased gluten wheat in the future. Along these lines, a recent report claims that a diet of the ancient diploid wheat, einkorn, induced beneficial changes in the gut microbiomes of pigs compared to a diet of conventional wheat (*Barone* et al., 2018), including increased abundance of the putatively health-associated species *Oscillospira* (*Konikoff* and *Gophna*, 2016). These microbiome changes, however, are unlikely to be related to the (minor) differences in the gluten composition between einkorn and conventional wheat used in the study, but more likely to be related to other compositional differences between these different wheats.

It may be that the increased awareness of gluten sensitivity in the public consciousness, an interest in low carbohydrate diets, as well as a desire to purchase local, organically grown food, has contributed to the rapid recent rise in the demand for and marketing of the so-called ancient wheats: einkorn, hulled spelt, and Kamut®. Although it has not been substantiated that these still niche types of wheat are lower in gluten and thus healthier for individuals with gluten sensitivity (*Dinu* et al., 2018), their market rise is a signal of consumer demand. Any decreased gluten wheat that ultimately comes to market might follow a similar market trajectory. The story of Quality Protein Maize, likewise, offers an encouraging parallel with DGW, in that QPM ultimately emerged as an agronomically and functionally beneficial variety with proven health benefits. While DGW must still surmount agronomic, functional and health and nutritional tests before it has any impact, the field ahead is full of promise.

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FLOWER POWER? THE PLANT MICROBIOME AND HUMAN HEALTH ISSUES

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SUMMARY

The microbiome is crucial for plant growth and health. Recent studies reveal an unexpected microbial diversity and abundance associated with plants. Plant genotype, soil type, climate, geography as well as pest and pathogens were identified as main drivers of the plant microbiota. The plant-associated microbial diversity seems to be impressive but their dimension is not yet understood. During evolution, microbiomes have secretly co-evolved with their host plants. On the other side, within millennia of domestication, crops underwent traceably many different adaptive trends, allowing rapid speciation and divergence that lead to phenotypic and genotypic distinction to their wild ancestors. Together, domestication and intensive agricultural management shifted the crop microbiota. This resulted in diversity loss, which has consequences for human and *one* health issues. The plant microbiome, which is on one hand specifically adapted to the diverse microhabitats of plants – from the seed to the flower – is on the other hand connected to the whole biosphere. First insights reveal inter-connected microbiomes of plants, e.g. with the built environment, food and humans. These connections need to be better understand for sustainable agriculture as well as plant, human and environmental health and functioning.

INTRODUCTION

Plants and their associated microbes have been interacting with each other for a long time, forming assemblages of species that is referred to as a holobiont (*Vandenkoornhuyse* et al., 2016). The plant-associated microbiota has the ability to contribute multiple aspects to the functioning of the plant holobiont, such as (i) germination and growth support, (ii) supply of nutrients and minerals, (iii) resistance against biotic stress factors (pathogen defense), (iv) resistance against abiotic factors, and (v) production of bioactive metabolites involved in multifold interactions (*Berg* et al., 2017). Plants harbour distinct habitat-specific microbial signatures, which are shaped by a long list of abiotic (soil type, climate, geography) and biotic factors (plant genotype, pest and pathogens). *Whipps* et al., (1988) where the first who implemented the microbiome concept for plant-associated microbial communities. They defined "*the microbiome as a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their theatre of activity*".

The rhizosphere, which was introduced for the below-ground, root-associated part of the plant by Lorenz Hiltner already in 1901, is one of the best-studied microbiomes at all (*Philli-pot* et al., 2013). The phyllosphere microbiome colonize all above-ground organs, which are exposed to the air and permanently changing abiotic factors such as ultraviolet (UV) radiation, temperature and water, and a general low nutrient availability (*Remus-Emsermann* and *Schlechter*, 2018). The phyllosphere can be further subdivided into the caulosphere (stems), phylloplane (leaves), anthosphere (flowers), and carposphere (fruits). Ectophytic microbial communities acting in the interface with soil and air, while endophytic communities represent an intimate core of the plant microbiota and connecting the different plant microhabitats and development stages are of special importance for health issues (*Hardoim* et al., 2015). A reservoir for plant's endophytes is the rhizosphere, which represents the below-ground interface with the highly diverse soil microbiota (*Berg* et al., 2005). The flower and seed microbiome are both understudied microhabitats of the plant. The spermosphere is the zone surrounding seeds where interactions between the soil, microbial communities and germinating seeds take place (*Schiltz* et al., 2012). This microenvironment links the above and below-ground microbiome of plants.

Despite more than 100 years of research and deep knowledge about single plant microbiomes, less is known about transmission and interactions of the plant microbiomes. Therefore, this review will summarize our work about microbiome internal and external interactions – within the plants themselves and with its environment in context with the state of current knowledge. Moreover, these interactions will be discussed in view of plant and human health issues.

INTERNAL RELATIONSHIPS: THE MICROBIOME DURING PLANT'S LIFE CYCLE

The seed microbiome and germination

Plant's life cycles are connected with sexual reproduction and characterized by two distinct stages in their life cycle: the gametophyte stage (haploid) and the sporophyte stage (diploid). The haploid gametophyte produces the male and female gametes by mitosis in distinct multicellular structures, after fusion of the diploid zygote develops into the sporophyte. After reaching maturity, the diploid sporophyte produces spores by meiosis, which in turn divide by mitosis to produce the haploid gametophyte. The new gametophyte produces gametes, and the cycle continues. Interestingly, the life cycle of higher plants is dominated by the sporophyte stage, with the gametophyte borne on the sporophyte while in mosses, the haploid gametophyte is more developed than the sporophyte. For a long time, it was assumed that plants recruit the majority of microorganisms from surrounding soil. Recently it was discovered that all plants transmit a core microbiome from one generation to the other, which is strongly specific for each plant genotype (*Berg* and *Raaijmakers,* 2018).

Mosses, which were the first land plants and which in the past formed the main vegetation, have a long time of co-evolution with their associated microbiobta. This resulted in a highly

specific and specialized microbiome. For example, *Sphagnum* species have, independently of their origin (Norway, Russia, Germany, Austria) a highly similar microbiome (*Opelt* et al., 2007; *Bragina* et al., 2013, 2014). To explain this similarity, the gametophyte as well as the sporophyte was studied. Inside of the sporophyte, a well-defined microbial community was identified, which colonized the gametophyte presenting the moss plant (*Bragina* et al., 2012). Detailed studies showed that specific microorganisms are essential for their germination procedure (*Hornschuh* and *Kutschera,* 2001). Seeds of plants from natural ecosystems are less studied than crop seeds. They have to feature high adaptations in dispersal, persistence and germinative ability under diverse environmental conditions (*Fenner* and *Thompson*, 2005). Seeds and seedlings are exposed to a range of hazards like drought, resource limitation, herbivores and eukaryotic or prokaryotic pathogens (*Bever* et al., 2015). We studied seeds of native alpine plant species growing together for centuries under the same environmental conditions in an Alpine meadow (Hochschwab, Austria). They showed highly unique microbiome signatures and an exceptionally small core microbiome (*Wassermann* et al., 2019). The seeds harboured a unique composition of bacteria, arachea and fungi in abundances with approximately 10^{10} gene copy numbers per gram seed. The plant genotype was clearly identified as the main driver, while different life styles had less, and seed morphology no significant impact.

There exist a lot of knowledge on seed-borne pathogens as well as international surveillance system (ISTA, International Seed Testing Association, www.seedtest.org). In the last decade, crop seeds have been studied as source transmitting a plant-specific core microbiota with neutral or beneficial plant-interaction (*Johnston-Monje* et al., 2011, 2016; *Adam* et al., 2016; *Glassner* et al., 2018; *Gloria* et al., 2018). All of these studies reveal also an unexpectedly high diversity and abundance of the seed-associated microbiota (*Berg* and *Raijmakers*, 2018). They showed up to 20,000 microbial species and up to two billion of bacterial cells in one seed. In general, the seed microbiota consists of bacteria, archaea and fungi. The presented studies focus also on the main drivers of the seed microbiota. Some recent studies on seed-associated microbiomes describe microbial compositions to vary between different geographical sites (*Klaedtke* et al., 2016), and soil types and microbiomes (*Hardoim* et al., 2012; *Liu* et al., 2013). In addition, chemical and microbial seed treatments shaped the microbiota (*Mormile*, 2016; *Rezki* et al., 2016). However, a plant genotype and even cultivar specificity of the seed microbiome has been described frequently (*Barett* et al., 2015; *Adam* et al., 2016; *Rybakova* et al., 2017; *Wassermann*, et al., 2019). Seed endophytes can even be highly conserved across generations of a plant species (*Links* et al., 2014). Besides the horizontal transfer of microbiota from diverse environmental sources, thus, vertical transfer of microbiota to the next generation via seeds plays a key role in adjusting the seed microbiome (*Truyens* et al., 2014).

Assembly and stability of the microbiome in mature plants

Colonization of emerging seedling is controlled by the plant through different strategies, such as the specific profile of root exudates and its immune system (*Doornbos* et al., 2012; *Truyens* et al., 2014; *Sánchez-Cañizares* et al., 2017). Microorganism's from seeds and soil, both colonize the plant

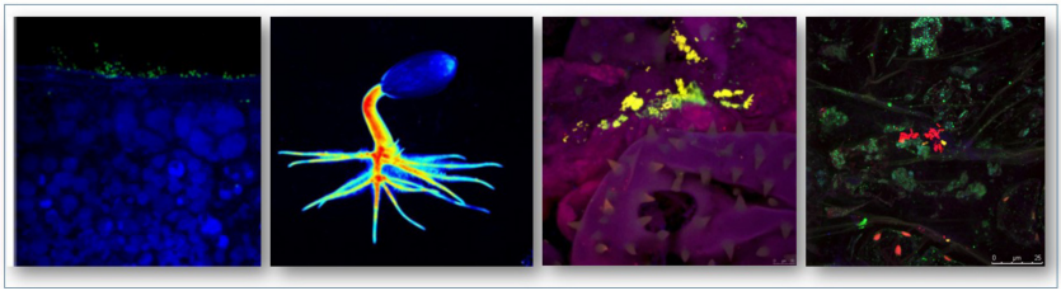

Figure 1: Vizualization of bacterial communities in pumkin microenvironments during its life cycle (seed, seedling, pistal, and petals) by fluorescence *in situ* hybridisation (FISH) in combination with confocal laser scanning microscopy (CLSM) studies

rhizosphere (*Adam* et al., 2016). From the rhizosphere, only a certain subset of microorganisms is able to invade the endosphere (*Berg* et al., 2005; *Hardoim* et al., 2010). Again, plant secondary metabolites and its immune system are the crucial factors for the selection procedure (*Huang* et al., 2019). In early stages of plant's life cycle, the microbiome is characterized by high diversity and fluctuation. Mature plants are characterized by a very stable microbiome, while in the senescent bacterial phyla which are associated with degradation, the microbiome is more abundant (*Smalla* et al., 2001). Microbiome stability and equilibriums are essential for plant functioning during the whole life cycle of a plant (*Berg* et al., 2017).

The flower microbiome

Interestingly, flowering is a plant stage, which is characterized by a highly specific microbiome, which was highly visible in microbial fingerprints of different plant species (*Smalla* et al., 2001). The flower is an underexplored microenvironment. Here, we present some data about pumpkin flowers (*Lukesch*, 2011): petals, pistils and epigynous ovaries of flowers at different maturation stages as well as fruits were analyzed. All parts of the flowers were colonized with bacterial communities at a similar level (2.7×10^6 to 1.4×10^7 16S rRNA gene copies ng^{-1} DNA). Generally, flower parts at withered maturation stage showed higher abundances than at flourish or youngest stage. Functional analysis showed that a high proportion of the bacterial community had antagonistic traits to a broad-spectrum of phytopathogens; they belong to the classes Alphaproteobacteria and Gammaproteobacteria and the phylum Firmicutes. Deep sequencing of Gammaproteobacteria-specific amplicons revealed highest relative abundances of Pseudomonadaceae and Enterobacteriaceae. The analyses at species level showed a predominance of *Pseudomonas viridiflava*, one of the key pathogens of Styrian oil pumpkin, in pistils and petals. Our results were confirmed by fluorescence *in situ* hybridisation (FISH) in combination with confocal laser scanning microscopy (CLSM) studies. Using the FISH-CLSM approach, the colonization of pistils and petals by diverse bacterial communities could be detected (Figure 1). The highest bacterial density was found on pollen grains attached to pistils, which gives rise to the assumption that pollen act as a vector for bacteria between pumpkin plants. This study suggests the oil pumpkin flower as an interesting niche for plant-associated bacteria, which should be further considered as reservoir for biocontrol agents. It is also in contrast to the general opinion that the mother

plant is highly suggested to be responsible for the recruitment of the seed microbiota (*Nelson*, 2018). Recent results by *Manirajan* et al. (2018) confirmed a high microbial diversity influenced by plant species and pollination type associated with pollen. The knowledge about the anthosphere microbiome was even successfully used for manipulation by introducing beneficial bacteria at flowering into progeny seeds *(Mitter* et al., 2018).

Domestication and biodiversity loss

Cultivation of crop plants started 13,000 years ago and today´s divergence of domesticated plants to their wild ancestors emerged as a consequence of selecting wild plants that were gathered and cultivated by hunter-gatherers in early domestication periods. Domestication have impacted plant microbiome assembly and functions via habitat expansion and via changes in crop management practices, root exudation, root architecture, and plant litter quality (*Pérez-Jaramillo* et al., 2016). The authors proposed a "back to the roots" framework that comprises the exploration of the microbiome of indigenous plants and their native habitats for the identification of plant and microbial traits with the ultimate goal to reinstate beneficial associations that may have been undermined during plant domestication. Seeds transmit the footprint of domestication (*Berg* and *Raaijmakers*, 2018), and especially their altered morphology over time is therefore frequently studied by archeobotanic and genomic research.

Hence, threats of plant extinction, driven by modern human culture, affect the whole genomic entirety of the holobiont. Studying the impact of domestication on crop seed microbiomes, the seed microbiomes of plant from natural ecosystems are especially of interest, as solely undisturbed environments are appropriate to explain indigenous plant-microbe-interactions.

In conclusion, the plant microbiome is definitely inter-connect during plant's life cycle. Together, domestication and intensive agricultural management shifted the crop microbiota. This resulted in diversity loss, which has consequences for human and *one* health issues. More research is needed to discover these internal relationships, and use that knowledge for nature conservation and sustainable agriculture.

EXTERNAL RELATIONSHIPS: THE PLANT MICROBIOME AND HUMAN HEALTH ISSUES

The plant - indoor microbiome connection

The built indoor microbiome has importance for human health because buildings are complex ecosystems that house not only people, but also trillions of microorganisms interacting with each other (*Kembel* et al., 2012). Residents leave their microbial fingerprint mainly from their skin (*Grice* and *Segre*, 2011) but less is known about the transfer from plants. Our hypothesis that indoor plants contribute substantially to the microbial abundance and diversity in the built environment was developed after studying the surface microbiome in intensive care units (ICU) of the Graz university hospital (*Oberauner* et al., 2012). Interestingly, the beneficial part of the ICU microbiome was similar to the phyllosphere microbiome (*Berg* et al., 2014a). We explained that by window ventilation, and in fact we found evidence for a transfer of pollen und their associated microbiome. The

importance of window ventilation for hospitals was already described by *Kembel* et al. (2012), who showed that the phylogenetic diversity of airborne bacterial communities was lower indoors than outdoors, and mechanically ventilated rooms contained less diverse microbial communities than did window-ventilated rooms. In a next step, we experimentally confirmed a proof of principle by analyzing the microbiome of the spider plant *Chlorophytum comosum* in relation to their surrounding environment *(Mahnert* et al., 2014). The abundance of Archaea, Bacteria, and Eukaryota (fungi) increased on surrounding floor and wall surfaces within six months of plant isolation in a cleaned indoor environment, whereas the microbial abundance on plant leaves and indoor air remained stable. We observed a microbiome shift: the bacterial diversity on surfaces increased significantly but fungal diversity decreased. The majority of cells were intact at the time of samplings and thus most probably alive including diverse Archaea as yet unknown phyllosphere inhabitants. The next question we had was to which extent plant-specific diversity contribute to the indoor diversity. To understand the microbiota of indoor phyllospheres and its driving factors in built environments, we used an experiment design under controlled conditions by analyzing 14 phylogenetically diverse plant species grown in the greenhouses of the Botanical Garden in Graz (Austria) demonstrating different climate zones (*Ortega* et al., 2016; *Mahnert* et al., 2018). Statistical analysis showed a significantly higher correlation of community composition - for bacteria as well as for fungi - to plant genotype in comparison to the ambient climatic variables. Finally, we could show that man-made shifts of the microbiome as well as the resistome can be influenced and compensated by the plant microbiome (*Mahnert* et al., 2019).

The food - gut microbiome connection

Recently, the importance of the plant microbiota for human health was evidenced (*David* et al., 2014). The plant-associated microbial diversity can be transferred to the gut microbiome because fruits and vegetables are the major component of a healthy diet (*Berg* et al., 2014b). One prominent example, which was already studied, are Brassicaceae (*Lebeis*, 2015). All family members are characterized by glucosinolates (GLSs) that are part of the effective defense mechanisms of the plant (*Lüthy* and *Matile*, 1984). Moreover, *Brassica* species are known for a bacteria-dominated composition of the microbiome and harbour no mycorrhiza. The hydrolysis of GLSs into highly active breakdown products, mostly isothiocyanates (ITC) and nitriles, is caused by myrosinase activity. Those volatile breakdown products are utilized in biofumigation processes, where *Brassica* residues are incorporated into soil as they provide suppressive or control effect against nematodes and soilborne fungal pathogens like *Verticillium longisporum (Witzel* et al., 2013). Interestingly, GLSs are also involved in human health issues; the GLS metabolism has become increasingly important over the past decade due to the exploration of anti-cancer activity of ITCs (*Halkier* and *Gershenzon*, 2006). Since humans consume their vegetables often cooked, the GLS-metabolizing ability of bacteria (*Tani* et al., 1974) has recently aroused scientific interest. Some authors consider the addition of myrosinase-active bacteria to a *Brassica* rich diet to supplement inactivated plant myrosinases (*Mullaney* et al., 2013). While the majority of bacterial strains known to exhibit myrosinase activity are ubiquitous inhabitants of

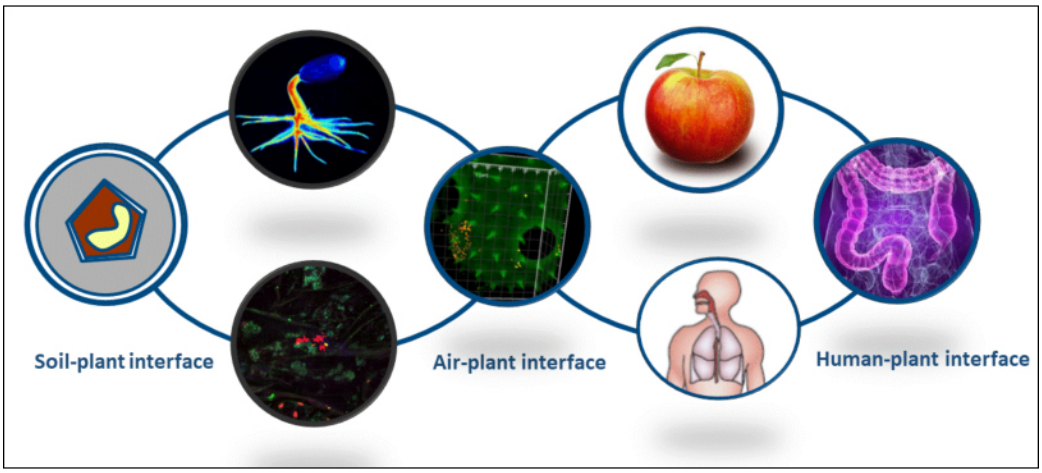

Figure. 2: Inter-connected microbiomes and their interfaces.

the human intestinal tract (*Mullaney* et al., 2013), still little is known about myrosinase-active bacteria colonizing edible plants tissues. We showed that due to their GLS content *Brassica* harbour a very specific microbiota containing also myrosinase-active bacteria (*Wassermann* et al., 2017).

Why is that plant-human microbiome connection so important? The loss of microbial diversity in the gut is associated with acute outbreaks as well as with chronic disease, e.g. allergies, obesity, mental diseases (*Turnbaugh* et al., 2006). *Hanski* et al. (2012) found first answers about the global question why biodiversity is so important. They showed a correlation between allergies in children and surrounding microbial biodiversity. Increasing chronic diseases in children were explained by the "missing microbe theory", which was published by *Blaser* (2014). In 2017, this was further developed into the "theory of disappearing microbiota and the epidemics of chronic diseases", which postulate that losses of particular bacterial species of our ancestral microbiota have altered the context in which immunological, metabolic and cognitive development occur in early life, which results in increased disease. Structural and especially functional microbial diversity is already established as a key factor in preventing human diseases, and is suggested as biomarker for plant health as well (*Berg* et al., 2017). However, despite many indications, this is not well understood and especially mechanistic studies understanding microbial diversity are missing. To our opinion, to study the plant - human microbiome connection offers an enormous potential to solve human health problems in future.

The microbiome of soil and plants plays a crucial role in plant and ecosystem health (*Berg* et al., 2017; *Laforest-Lapointe* et al., 2017). However, overlapping compositions, and interconnected microbiomes of human, animal and plant in connection with health should be considered, and used to expand the version of "One health" that includes environmental health and its relation to human cultures and habits (*Flandroy* et al., 2018). The interlinked microbiomes are shown in Figure 2, but the links and transmission routes have to be studied much more in detail.

CONCLUSIONS

Microbial biodiversity associated with plants is important for plant health; the balance between the microbiota and the host is crucial during the whole life cycle. Diversity loss associated with plants cause plant diseases, outbreaks of human pathogens of plants-origin as well as human health problems. Knowledge on the plant microbiota and their inter-connection can provide solutions to face health problems, e.g. to fight against multi-resistant pathogens and outbreaks. However, it can also provide solutions for crop production under climate change conditions. Plant microbiome engineering and biotechnology open novel options to develop microbials, which fulfil important functions for the plant host, e.g. nutrient, mineral and vitamin supply, and protection against biotic and abiotic stresses. Altogether, the plant microbiome will be the key to the next green revolution (Science Breakthroughs by 2030; http://nas-sites.org).

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THE MICROBIOME OF FLOWER POLLEN AND ITS POTENTIAL IMPACT ON POLLEN-RELATED ALLERGIES

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SUMMARY

Flower pollen are a plant microhabitat which was overseen for microbial analyses compared to the intensively studied leaf and root habitats. Pollen are important for plant reproduction and provides nutrients for insects and humans such as honey and propolis. Pollen of wind-pollinated plants are a major airborne allergen causing severe allergic rhinitis.

In this work the abundance, structure and diversity of the microbiota associated with the pollen of nine different plants, including four wind-pollinated, high allergenic species (birch, winter rye, common hazel and common mugwort), four insect-pollinated, low allergenic species (autumn crocus, winter rapeseed, blackthorn and cherry plum) and one wind-pollinated but low allergic species (hemp) were compared. The microbiota was analysed by high-throughput sequencing approach based on bacterial 16S rRNA gene and fungal ITS2 region. In parallel, culture-dependent methods were used to estimate the extent of the cultivable bacterial fraction, and microscopic methods were used to visualise the colonization of bacteria on pollen grains. Furthermore, bacterial endotoxin levels (lipopolysaccharides and lipoteichoic acids) of pollen were compared with those of the bacterial isolates, by using enzyme-linked immunosorbent assay.

Proteobacteria (bacteria) and Ascomycota (fungi) were the most abundant phyla, while *Pseudomonas* (bacteria) and *Cladosporium* (fungi) were the most abundant genera found in the pollen microhabitat. Archaea sequences were not detected. Furthermore, the bacterial and fungal alpha diversity indices were significantly lower in the low allergenic pollen and in hemp, compared to the high allergenic pollen. The most significant influencing factors in bacterial and fungal microbiotas were 'allergenic potential' followed by 'plant species' and 'pollination type' (wind- and insect-pollinating) of the pollen. Notably, the hemp clustered closer to the other low allergenic pollen species.

This study enhances our basic knowledge of the pollen microbiome and provides insights on the role of pollen-associated microbes in pollen allergy.

INTRODUCTION

Microorganisms are ubiquitous on earth and very numerous in many habitats. In microbial ecology we aim at understanding why microorganisms are in their respective habitat and what their function is in the ecosystem. We also try to understand their adaptation mechanism to environmental parameters and their interaction among each other and with higher organisms such

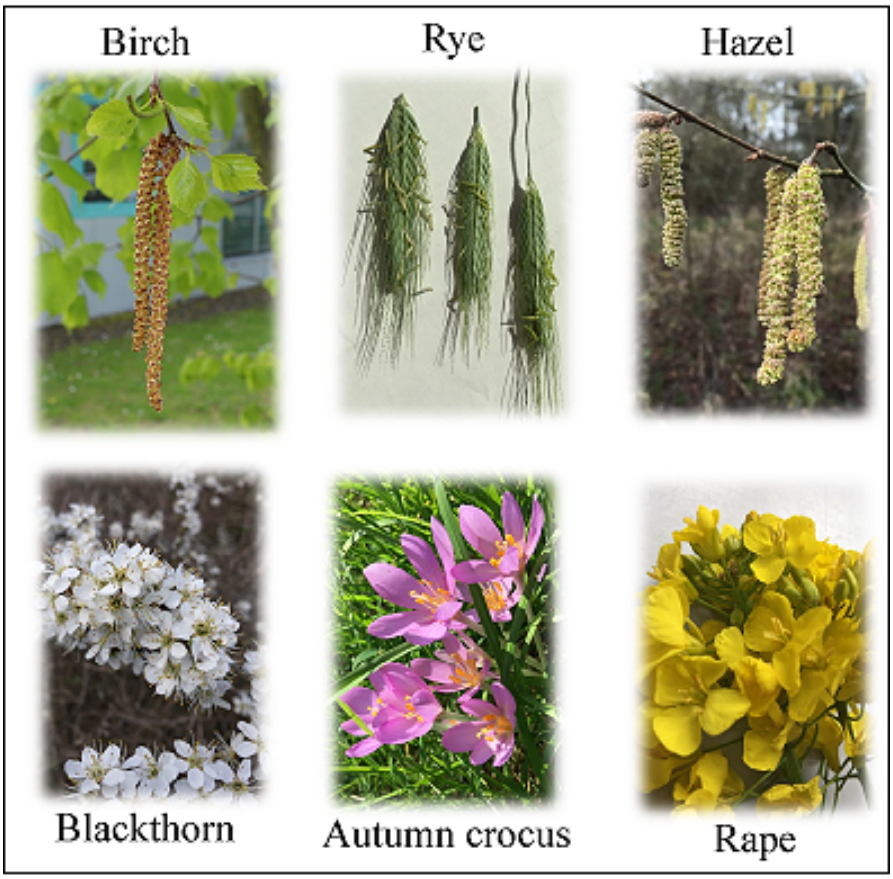

Figure 1: Flowers of the plants sampled. Upper row wind-pollinated plants (*Betula pendula, Secale cerale* L., *Corylus avellanae*); lower row insect-pollinated plants (*Prunus spinose* L., *Colchicum autumnale* L., *Brassica napus* L.). Photos taken by B. Ambika Manirajan.

as plants and animals. The 33rd Old Herborn University Seminar focused on the inter-kingdom interaction between the plant microbiome and the human connection. One aspect of the plant microbiome and its potential impact on human health is seen in the pollen microbiome and allergy related diseases. Pollen of wind-pollinated plants are air-born and therefore a typical air-microbiome could have been expected. However, similar to other plant habitats like the rhizosphere, leaf surface, or fruit, also pollen have a very unique microbial structure which is highly plant specific. The plant selects environmental microorganisms and favours plant surface colonization e.g. by providing surface structures for attachment or exudates supporting microbial growth. Some microorganisms are even taken up in plant tissue for more intensive interactions and nutrient exchange, e.g. mycorrhiza forming arbuscular structures within the plant cell. Recent reports document a specific seed endosphere microbiome (*Truyens* et al., 2015, *Alibrandi* et al., 2018) which enables the plant to vertically transmit specific microorganisms to the next generation.

In this study the bacterial and fungal community of both wind-pollinated (allergic) pollen and insect-pollinated

(non or less allergic) pollen was analysed. The size, surface structure and chemical composition of wind- and insect-pollinated pollen are different and affect colonization of bacteria and fungi. The goal was to elucidate the bacterial and fungal structure and diversity of allergic and non-allergic pollen using culture-depending methods, molecular methods and microscopy. Bacterial isolates from pollen are still under study to estimate their immune modulatory compounds (endotoxins) and their impact on allergy related diseases.

HIGH BACTERIAL DENSITY ON POLLEN GRAINS

The flowers of the plants that were sampled are shown in Figure 1. Bacterial cell numbers on pollen grains were analysed using two different methods:
(**i**) Classical microbiological techniques have been used to determine the number of cultivable bacteria. Fresh pollen samples were suspended in buffer and serial dilutions were made for plate counting using an agar medium which reflects the nutrients on pollen. For this a pollen medium was prepared with the pollen of the respective plant species added as substrate in mineral agar medium. In addition, also 1:10 diluted AC agar medium was used (*Ambika Manirajan* et al., 2016). The number of colony-forming units (CFU) were between 3.8×10^5 g^{-1} (hazel) and 8.5×10^8 g^{-1} (blackthorn). Pollen of autumn crocus had similar CFU as blackthorn and birch CFU were almost the same as hazel. Pollen of rye, mugwort, rape, cherry plum and hemp were between the above given numbers. The number of CFU was lower in allergic pollen (birch, rye, mugwort and hazel) compared to the non-allergic pollen (rape, autumn crocus, cherry plum, blackthorn and hemp). Colony morphology and colony numbers were similar on both agar media. Based on colony and cell morphology bacteria have been further cultured to retrieve in total 157 pure strains. The isolates belonged to 27 different bacterial families. Pollen of each plant showed a unique pattern of cultivable bacteria. Only one family the Microbacteriaceae were found in all plant pollen species whereas Flavobacteriaceae and Rhizobiaceae were only present on birch pollen. Nocardioidaceae and Xanthomonadaceae only occur on rye pollen. The families Paenibacillaceae, Enterococcaceae, Brevibacteriaceae and Kineoporiaceae were only found in pollen samples of hazel, rape, blackthorn and cherry plum, respectively. From hazel pollen a novel bacterial species was isolated and described as *Spirosoma pollinicola* (*Ambika Manirajan* et al., 2018a) which was the first bacterium that has be isolated from pollen.
(**ii**) A second method used for visualization of bacteria on pollen grains was microscopy. Fluorescent *in situ* hybridization and confocal laser scanning microscopy and scanning electron microscopy showed surface attached bacteria on the pollen grains. Different cell morphologies of bacteria were seen as single cells, small groups and also small colonies. Beside the surface attached bacteria also the shape and structure of the different pollen grains were visualized (*Ambika Manirajan* et al., 2016). Similar observations were documented by *Kesseler* and *Harley* (2014) who aimed to present primarily the beauty of the different pollen structure of many plants growing in the London Royal Botanical Garden.

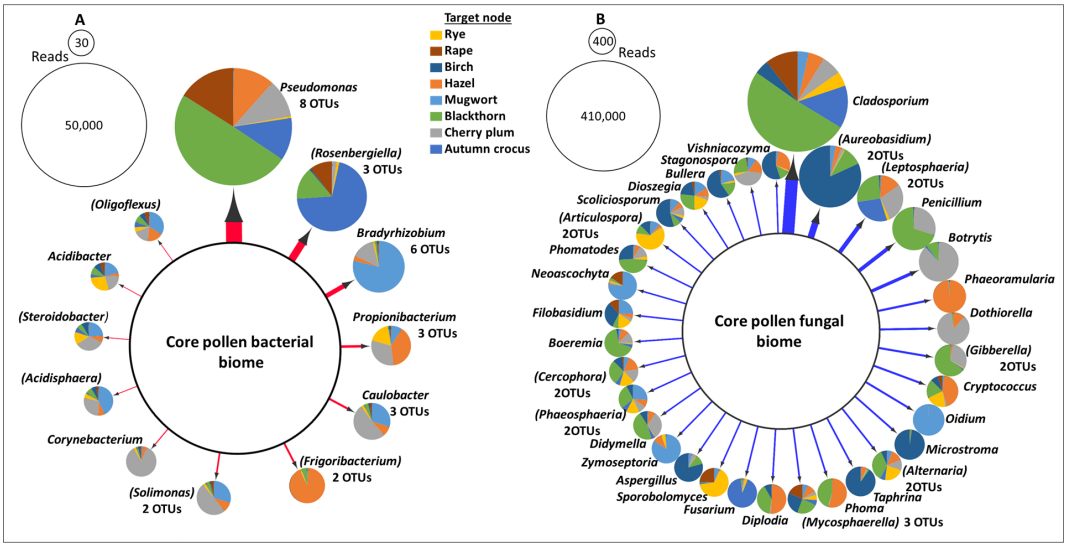

Figure 2: Structure and abundance of the pollen bacterial (A) and fungal (B) core microbiome, defined as the OTUs (97% similarity level) detected in all pollen species, grouped by genus. Pies are coloured by pollen species and show the distribution of the respective core genera. Pie size and edge width indicate the absolute abundance (number of reads) of the respective OTUs, according to the legend.

UNEXPECTED DIVERSITY OF BACTERIA AND FUNGI ON POLLEN GRAINS

Compared to other plant habitats like the root or leave surface the pollen surface seems to represent only little space for epiphytic microorganisms. Already the cultivation dependent approach showed a unique and taxonomically divers bacterial structure. Using high-throughput sequencing of the 16S rRNA gene fragment (bacteria) and the internal transcribed space 2 (ITS2, fungi) an unexpected high diversity was found. In total 39 bacterial families have been retrieved plus sequences which cannot be assigned to a known family. Thirteen of the bacterial families have been found with both the cultivation-dependent and molecular approach (Propionibacteriaceae, Sphingomonadaceae, Burkholderiaceae, Methylobacteriaceae, Rhizobiaceae, Nocardioidaceae, Micrococcaceae, Xanthomonadaceae, Enterobacteriaceae, Pseudomonadaceae, Enterococcaceae, Streptococcaceae, Bacillaceae). Summarizing, by both approaches a total number of 53 bacterial families have been detected on 8 plant pollen species. Most abundant on phylum level were the Proteobacteria followed by Firmicutes, Actinobacteria, Acidobacteria and Deinococcus-Thermus. A high specificity of bacterial community structure of each plant pollen species has been also described by *Obersteiner* et al. (2016) for pollen of timothy grass and birch.

The fungal community was dominated by the major phylum Ascomycota and Basidiomycota. The family Davidiellaceae was found in all pollen samples analysed. In blackthorn, rape and rye it was the dominant family.

Leptosphaeriaceae were present in pollen of autumn crocus, blackthorn, cherry plum, hazel and rye. The family Dothioraceae was observed on birch, hazel and mugwort pollen. Some families were very specific for single plant pollen species: Mycosphaerellaceae on hazel pollen and Erysiphaceae on mugwort pollen. Metschnikowiaceae were found on autumn crocus and rape. Tremellaceae occurred on birch, hazel, rye and mugwort which are all wind-pollinated and belong to the allergic pollen. *Hutchinson* and *Barron* (1997) found mostly fungal species affiliated to Basidiomycota and Ascomycota on pollen of *Pinus nigra*. In a metagenomics study on the phyllosphere and carposhere of olive (*Abdelfattah* et al., 2015) and strawberry (*Abdelfattah* et al., 2016) similar results were reported. The family Davidiellaceae was found to be the most abundant family in almond nectar (*Schaeffer* et al., 2017).

For diversity measurements several indices can be calculated e.g. Shannon-Weaver index, phylogenetic diversity or observed species. The bacterial diversity of each plant pollen species can be compared and was significantly different between the analysed pollen. Mugwort and rye showed the highest diversity indices, on autumn crocus and rape pollen the diversity indices were lowest. Comparing the diversity indices of all pollen with allergic potential with those pollen without allergic potential a significant higher diversity of bacteria was found in the allergic pollen (all wind-pollinated).

Analysis of beta diversity enables to recognize the effect on the bacterial community structure of collection site and plant pollen species. Bray-Curtis distances were calculated using non-metric multidimension scaling analysis and demonstrate a significant effect of the factor plant species (envfit R^2=0.8685, P<0.001, ADONIS R^2=0.5198, P<0.001) and pollination type (envfit R^2=0.3903, P<0.001, ADONIS R^2=0.1744, P<0.001). The factor collection site did not show any significant effect (envfit R^2=0.0992, P<0.515, ADONIS R^2=0.0381, P<0.786).

The fungal diversity was similarly high as the bacterial diversity. Again, the pollen of wind pollinated plants (with allergic potential) showed significant higher diversity indices compared to insect-pollinated plants. Beta diversity of fungal community was significantly affected by the factor plant species and not by the collection site.

Pollen of all plants sampled contained a core biome of shared genera (Figure 2). The core bacteria on pollen consisted of 12 genera with *Pseudomonas*, *Rosenbergiella* and *Bradyrhizobium* as the most abundant genera. The fungal core biome was almost three times bigger and contained 33 fungal genera. The genera *Cladosporium*, *Aureobasidium* and *Leptosphaeria* were the most abundant.

INTERACTIONS OF POLLEN MICROBIOTA

Bacterial and fungal sequencing data were analysed for potential inter- and intro-kingdom microbe-microbe interactions using co-occurrence pattern of OTUs. This information was used for network analysis depicting the interactions with a method which was developed by *Barberan* et al. (2015) for microbiome data. Positive correlations among taxa describe a co-occurrence, while negative interactions describe a high abundance of one taxon

and a low abundance of the other taxa. Hub taxa are very strongly interconnected with other taxa and therefore affect the whole community strongly. As hub taxa three bacterial genera were recognized: *Methylobacterium* (two taxa), *Friedmanniella* and *Rosenbergiella*. Members of the genera *Methylobacterium* have been detected on the phyllospere of many plants and are known for their potential beneficial effect for the plants (*Agler*, 2016). *Methylobacterium* metabolize methanol which is thought to be provided on the plant leaf surface. *Friedmanniella* was found in air samples and on plants and also as plant endophyte (*Alibrandi* et al., 2018). *Rosenbergiella* has been isolated from flowers (*Bartlewicz* et al., 2016) and was described as core bacterium associated with the bee Ceratina (*Graystock* et al., 2017). All of these bacterial hub taxa interconnect with the fungal hub taxa *Cladosporium* which was also determined as the most abundant core taxa.

POTENTIAL IMPACT OF POLLEN BACTERIA ON ALLERGY-RELATED DISEASES

For infections by pathogenic bacteria, the endotoxin quality and quantity of the respective bacterium is important for inflammation reaction. Inhalation of bacterial endotoxin has been reported to cause lung inflammatory reactions, fever and shaking chills (*Rylander* et al., 1989; *Sandstorm* et al., 1992; *Michel* et al., 1997). Therefore, our working hypothesis is that pollen bacteria may contribute to allergy related diseases due to their endotoxin. Lipopolysaccharides (LPS) are bacterial endotoxins and have been quantified using an LPS-EndoLISA test. Pollen samples were used directly for determination of the LPS-concentration and all pollen of allergic plants (birch, rye, hazel, mugwort) showed significant higher concentrations than pollen of non-allergic plants (rape, autumn crocus, blackthorn, cherry plume and hemp) which matched to the result of a significant different bacterial structure of pollen of allergic plants and non-allergic plants. Bacterial isolates from pollen were grown on standard medium and LPS-concentrations were determined from pure cultures. A high variation of LPS concentration between 4 to 260 ng was measured between bacterial strains. However, the mean LPS concentration of all bacterial strains isolated from allergic pollen was significantly higher than the LPS concentration of all bacterial isolates from non-allergic plants. LPS extracted from *Artemisia vulgaris* (mugwort) pollen caused an inflammation of the lung and an allergic sensitization (*Oteros* et al., 2018). Already in 1968 Colldahl and Carlsson cultured pollen bacteria and extracts from pure cultures induced skin, nasal and eye symptoms in pollen-sensitive patients (*Colldahl* and *Carlsson*, 1968). Later, *Spiewak* et al. (1996) measured endotoxin concentrations of rye, mugwort, hazel and alder and simultaneously determined the number of colony-forming units which both did not correlate. Nevertheless, the authors discussed a potential contribution of Gram-negative bacteria on pollen to pollinosis. A potential contribution of Gram-positive bacteria on grass pollen was indicated by the work of *Heydenreich* et al. (2011). A high number of e.g. *Bacillus cereus* and *Bacillus subtilis* was determined on grass pollen and homogenized bacteria brought in contact with immune cells caused upregulation of CD80, CD83,

and CD86 and other immune responses. Therefor *Heydenreich* et al. (2011) postulated that Gram-positive bacteria on grass pollen produce adjuvant activity inducing inflammatory T cell response.

Environmental parameters for the plant have been discussed to affect the allergic reactions of their pollen. The ozone level of the sampling location of birch pollen correlated to the immune response evaluated by neutrophil migration assay and response of dendritic cell interleukin (*Beck* et al., 2013). Pollen of birch and timothy grass were collected in rural and urban sites and analysed for bacterial composition and correlated to air pollution parameter NO_2, NH_3 and O_3 (*Obersteiner* et al., 2016). The authors found a different microbial community composition of birch pollen depending on the urbanization index and mostly the air NO_2 concentrations (*Obersteiner* et al., 2016).

CONCLUSIONS

The microbiome of pollen is highly plant specific and very divers. Comparison of the microbiome of pollen from allergic and non-allergic plants together with endotoxin analysis of pollen isolates provide some evidence that the allergic potential of pollen seems to depend on both the bacterial composition and the plant allergen (pollen-associated lipid mediator and protein). Environmental parameters affect the diversity and structure of pollen bacteria and pollen derived allergens. Future research is required to resolve questions like:

- what portion of the immune reaction of allergic persons is due to pollen bacteria and pollen derived allergens?
- how regulates the plant microbial colonization of its pollen and what is the relevance of pollen bacteria to plant fertilization?
- what is the impact of environmental changes (climatic changes, ozone level, agricultural pesticide/herbicide/fungicide) on pollen derived allergen and the pollen microbiome?
- and finally, are insects negatively affected by an environmentally caused alteration of the pollen microbiome?

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PLANTS AND MICROBES TOGETHER – A SUMMARY OF OLD HERBORN UNIVERSITY SEMINAR 33

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INTRODUCTION – PLANT AS HOST

During the past 3-plus decades of the **Old Herborn University Seminar**, prior seminars focused on mammalian and vertebrate animals as hosts for microbial communities with resident microbiomes affecting healthy physiology and disease states. In this **Old Herborn University Seminar 33 (OHUS 33)**, we explored the Kingdom Plantae as hosts in order to provide a broader perspective on microbial ecology, microbiome science and environmental health. This seminar focused on land plants with elaborate roots in soil below ground and complex above-ground architecture. This edition did not include any detailed examination of aquatic plants, algae, or more ancient plant types.

Land plants first appeared approximately 500 million years ago and created new "homes" for many environmental microbes. Terrestrial ecosystems have rich soil environments that provide a fertile substrate for microbial proliferation and development of intricate microbial communities. Elaborate rhizobial (root) systems embedded in soil provide a large surface area of direct interactions between soil microbes and plants. The intricate plant anatomy extending from roots to trunks and stems to leaves and flowers permit investigations of microbial communities in different plant habitats. Consideration of microbiomes in pollen and seeds provide opportunities to explore horizontal and vertical transmission of microbes between plants. Beyond identification of specific microbes, microbial metabolites provide signals that deliver insights into microbe:host and microbe:environment communication patterns. Transmission of signals above ground via volatile organic compounds (VOCs) and below ground in soils surrounding root structures provide obvious opportunities to understand communication between plant and microbial species within intact ecosystems. Such lessons may provide clues about optimal strategies for promoting biodiversity and considerations about plants as food for humans and as sources of medicinal compounds.

Volker Rusch started the Seminar with a compelling history of plant:microbiome science and general concepts for Seminar 33. Plant-associated microbial habitats include the gemmisphere (buds), anthosphere (flowers), carposphere (fruit), and phyllosphere (leaves) above the ground surface. Below-ground, intra-soil habitats include the rhizosphere (soil adjacent to roots) and rhizoplane (root surfaces). Combined with other plant-associated habitats, one can readily appreciate that, like animals, plants accommodate diverse microbial communities that may differ in terms of composition and function based on anatomic location. As an example, the plant pathogen *Pseudomonas syringae* pv. *glycinea* colonizes soybean plant buds and can be found in healthy seedlings and

healthy parts of diseased seedlings. The question arises as to whether and how plants develop resistance to micro-organisms and the contributions of plant-associated microbial communities to disease resistance. As new concepts have emerged, the view of the land plant as a holobiont with a complex anatomy and a multitude of plant:microbial interactions provide a fresh perspective on plant ecosystems and implications for human and environmental health.

FOUNDATIONS OF THE PLANT MICROBIOME BELOW GROUND: THE SOIL AND THE ROOTS

Some people talk about "grass roots" efforts as a strategy to galvanize popular support for human initiatives. In terms of plant microbiome science, the 33rd OHUS began with a detailed examination of plant roots (not grass) and the soil. *Roeland Berendsen* from Utrecht (**Chapter I**) explored the concept of soil-borne legacies and how soil-borne microbial communities promote plant health in successive generations (*Bakker* et al., 2018). Plant pathogens infecting leaves (above ground) or seedlings may alter microbial composition in the rhizosphere, and consortia of soil bacterial species following this "cry for help" may alter microbial composition in the rhizosphere or areas of soil adjacent to plant roots. Experiments by *Roeland Berendsen* and colleagues were performed with sugar beet seedlings, and specific soil microbes were identified that, when combined, were able to confer disease-suppression in subsequent plant generations. These concepts of induced systemic resistance (ISR) and disease-suppressive soils highlights the ability of plant-associated microbial communities to profoundly affect plant health and vertically transmit disease resistance.

An intriguing comparison of a land plant root system with the mammalian intestine emphasizes common features such as nutrient/water absorption, nutrient trafficking, and the presence of rich, complex microbial communities adjacent to eukaryotic host cells. Colonization resistance in the mammalian gastrointestinal tract refers to the abilities of health-associated, diverse gut microbiomes to resist penetration and infection by enteric pathogens in animals. Soil microbes in the rhizosphere and rhizoplane may similarly suppress proliferation and colonization on root surfaces (rhizoplane) by plant pathogens, effectively resulting in patterns of "colonization resistance" in soil. The intestinal microbiome may have a profound impact on mammalian host immunity by conferring signals stimulating development and maturation of innate and adaptive immunity. Similarly, the soil microbiome may confer ISR by providing metabolites as signals stimulating plant cells to release key antimicrobial compounds such as coumarins, glucosinolates, and terpenoids. *Margot Schulz* (**Chapter III**) eloquently described these and other classes of plant microbial metabolites that may be modified by plant microbes. Compounds such as coumarins (p-coumaric acid) may suppress soil bacteria while enhancing soil fungal populations. These same compounds may suppress inflammation and stimulate anti-oxidant activity in mammals.

Andrzej Tkacz (**Chapter II**) described techniques to explore microbial counts and total microbial load in plants and the environment, instead of

simply relying on ratios or percent of total reads. By applying quantitative PCR methodologies, the rhizosphere contains vastly richer microbial communities than the bulk soil and rhizoplane. Additional insights include findings demonstrating that Proteobacteria (Gram-negative bacteria) are dominant in soil environments. Plant scientists are continuing to amass data regarding the complexity, composition and function of microbial communities in different plant tissues and soil locations. Soil associated with richer plant growth yielded greater total microbial counts than soil associated with poorer plant growth. "Rich" soil yielded greater quantities of bacteria and may explain the relative resistance to plant pathogen colonization and greater nutrient bioavailability from "plant-friendly" microbes in fertile ground. In *Margot Schulz'* presentation (**Chapter III**), we confronted the relative abundances of soil bacterial and fungal populations. Notably, the vast majority of plant pathogens and infectious disease phytopathology are caused by fungi. So, it is important to consider the relative impact of soil-resident microbes and microbial metabolites on soil fungal communities. *Margot Schulz* described the relative diminution of fungal and Gram-negative bacterial pathogens in soils inoculated with rapeseed extract. Rapeseed extract containing goitrin compounds derived from plants may have a dramatic impact on compositional shifts in soil microbial communities. Interestingly, rapeseed extract inoculations resulted in relative population shifts from a predominance of ascomycota (leading source of plant pathogens) to basidiomycota in inoculated soils. Perhaps plant metabolites undergo biochemical conversions to secondary metabolites by plant- or soil-associated microbes (progoitrin to goitrin and derivative metabolites) and modulate changes in microbial communities that benefit or compromise plant survival. One can easily appreciate the potential impact on plant communities as a result of more fertile soil ecosystems, and the deleterious impact on rival plant species by resisting invasion of resident ecosystems.

MICROBIAL METABOLITES – CONVERSATIONS BETWEEN PLANTS AND MICROBES

Margot Schulz (**Chapter III**) eloquently described a variety of plant-associated and microbial metabolites in plant ecosystems and emphasized that land plants produce a plethora of secondary metabolites in collaboration with plant-associated microbial communities. By modifying "older" primary metabolites through evolving enzymatic bioconversions, secondary metabolites with new biochemical functions were generated during millions of years of plant evolution. Such biochemical diversification by plant-microbiome collaboration provided ample opportunities for coevolution with significant impacts on the development of different ecosystems containing land plants. Accumulation of specific secondary metabolites represented a later step in the evolution of land plants since their initial appearance approximately 500 million years ago. Importantly, **Seminar 33** invited parallel discussions and consideration of similar phenomena in the development of terrestrial microbiomes with their resident animal-associated microbial communities and microbial metabolites.

Various classes of plant-associated metabolites were described in **OHUS**

33, and these compounds (and classes) included coumarins (p-coumaric acid, scopoletin), salicylates (salicylic acid), glucosinolates, benzoxazinoids, terpenoids and goitrins. Coumarins or simple phenolic compounds such as p-coumaric acid in soil diminished the relative abundance of soil bacteria while simultaneously enhancing relative abundances of soil fungi. Thus, these secondary metabolites may yield dramatic shifts in soil microbial community composition and function with important consequences for plant communities. Interestingly, the same compound (p-coumaric acid) enhances the proliferation of *Lactobacillus plantarum* in the mammalian intestine. Similar metabolites can affect both land plant- and animal-associated microbial communities. Via at least two enzymatic conversions, p-coumaric acid can be converted to the secondary microbial metabolite, protocatechuic acid, and this compound has general antioxidant properties as well as serving as a precursor compound for the TCA cycle (aerobic respiration). *Roeland Berendsen* (**Chapter I**) told the story of another coumarin compound, scopoletin, produced in root exudates by plants as a response to iron deficiency. Scopoletin enriched in root exudates then plays a key role in soil iron mobilization and enhancement of proliferation of beneficial microbes fostering induced systemic disease resistance (ISR) in plants (*Stringlis* et al., 2018).

Another fascinating class of metabolites are the glucosinolates that are produced by various plants including the class of vegetable sources known as *Brassica* plants. *Brassica* plants include human food plants such as broccoli, cauliflower, cabbage and turnip, in addition to the seed plants yielding mustard and canola cooking oil. These plants yield elevated concentrations of glucosinolates (thought to have originated approximately 90 million years ago) which can be converted to isothiocyanate (ITC) compounds. These metabolites can be toxic to plant-associated microbes, especially fungi, and these metabolites may protect such plants due to the prominence of fungal phytopathogens. Glucosinolates are powerful microbiome modulating agents and have been extensively used for biofumigation purposes targeting weeds and phytopathogens. Extracts with elevated concentrations of glucosinolates such as rapeseed extract suppress soil mycorrhizal fungi and Gram-negative bacteria, but the microbial survivors seem to enhance growth of other plants such as *Arabidopsis thaliana*. Perhaps the data shared by *Margot Schulz* (**Chapter III**) provide clues regarding microbial metabolites and pathways for suppressing infection and promoting beneficial microbes in soil environments.

Benzoxazinoids are indole-derived plant metabolites enriched in root exudates of *Poaceae* species such as maize, rye and wheat. These compounds are converted to benzoxazinones such as DIMBOA (maize) and DIBOA (rye), and these metabolites, in turn, can be converted to soil-toxic compounds, MBOA and BOA. MBOA can suppress soil microbes when administered directly, and soil microbes may detoxify the soil environment by providing microbial enzymes adept at covalently modifying these compounds. Various soil microbes may detoxify soil by converting benzoxazinones to less toxic or inert compounds via hydroxylation, glucosylation and nitration pathways. These findings emphasize the potentially beneficial role of plant-associated and soil microbes in detoxifying and revitalizing land to support healthy plant ecosystems and sustainable agriculture.

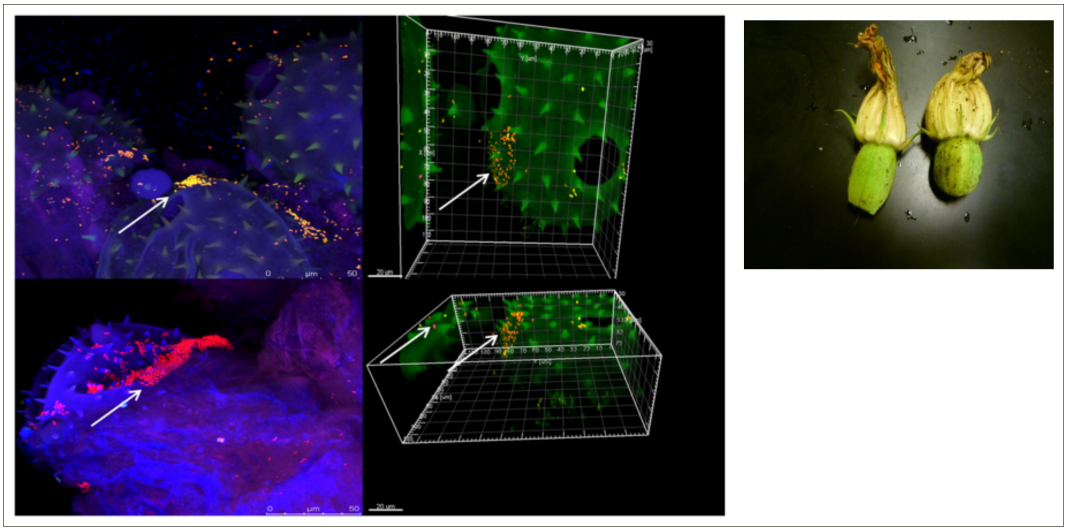

Figure 1: The oilseed pumpkin microbiome.
Bacteria on the pollen grains of the pistil of withered female oilseed pumpkin flower by CLSM and FISH (credit to G. Berg and E. Adam, Graz, Austria).

MICROBES AND PLANTS ABOVE GROUND: LEAVES, FLOWERS, POLLEN (AND SEED)

OHUS 33 considered the foliar microbiome (phyllosphere), interactions between the leaf and root microbiomes, and compositional and functional aspects of plant microbiomes above ground. Microbes can travel within plant hosts, and microbial colonization in one anatomic region (roots) may affect microbial composition remotely in plant parts that are above ground level (leaves). Such colonization patterns may be secondary to microbial proliferation and motility of microbial cells within plants. This microbe-associated communication pattern is bidirectional from root to leaf and from leaf to root. Foliar colonization and establishment of plant leaf-associated microbial communities (phyllosphere) affect the root biology by modulating root exudates in the soil (metabolite production) and the composition of root-associated microbial communities (rhizosphere).

Gabrielle Berg (**Chapter V**) followed discussions initiated by *Volker Rusch* of land plant biology above the ground surface by addressing plant microbial communities in leaves (phyllosphere) and flowers (anthosphere). The lettuce plant with its large leaves provided an elegant model system for exploring foliar colonization by plant-associated microbes. The lettuce microbiome demonstrated the existence of complex foliar microbial communities and the differences in microbial composition between different lettuce cultivars. A salient feature of the lettuce microbiome was the relatively greater abundance of Gram-negative bacterial species (Proteobacteria) in the lettuce phyllosphere when compared to the lettuce rhizosphere. The "resistome" of the lettuce leaf microbiome was another prominent feature among leaf-associated bacteria defending their niche against microbial competitors. Keeping in mind that many antimicrobial agents were originally derived from environmental fungi and bacteria (e.g. *Streptomyces*), it is not surprising

to see such an elaborate resistome contributing to successful colonization of plants by different bacterial species. From the lettuce to pumpkin, *Gabrielle Berg* transitioned to discussions of plant microbiomes in seeds, flowers and fruit. The pumpkin seed microbiome also showed antimicrobial resistance features that may be crucial in shaping the pumpkin seed and fruit microbiome. Consideration of seed microbiomes in plants raises the issue regarding the roles of microbes in vertical transmission between plant generations. *Gabrielle Berg* showed that both pumpkin flower and seed-associated microbes form structured communities as displayed via confocal laser scanning microscopy (CLSM) and fluorescence *in situ* hybridization (FISH) (Figure 1). The seed microbiome provides an explanation for vertical transmission of plant-associated microbes and possible enhancement of plant germination. Published findings regarding the existence of a placental microbiome in mammals including humans (*Aagaard* et al., 2014; *Seferovic* et al., 2019) highlights potentially common themes of vertical transmission of health-promoting microbes among land plants and terrestrial animals. *Gabrielle Berg* commented on recent efforts in the agricultural industry to fortify plant seeds via inoculation of plant microbes, and new insights about vertical transmission of microbiomes may provide opportunities to create healthier, disease-resistant crops.

Sylvia Schnell (**Chapter VI**) expanded the consideration of horizontal and vertical transmission of plant-associated microbes by describing microbial communities in plant pollen. By sharing molecular and microscopic data (CLSM and FISH), bacterial communities can be easily visualized on pollen from plants such as rye, birch and rapeseed. Such pollen can transmit plant-associated microbes between plants and provide a mechanism for horizontal transfer of microbes between flowers. Specific bacterial genera, *Pseudomonas* and *Rosenbergiella*, and fungal genera, *Cladosporium* and *Aureobasidium*, are prominent members of the core pollen microbiome across different plant species. The implications for human health are readily apparent with a more highly diverse, richer pollen microbiome in plants associated with human respiratory allergies.

PLANT MICROBIOLOGY AND HUMAN HEALTH: ONE HEALTH ON ONE PLANET

The **Old Herborn University Seminar 33** provided a timely forum to consider the plant kingdom and its associated microbial partners. Concepts such as vertical metagenomic transmission and the beneficial contributions of microbial metabolism can help expand our understanding of the holobiont in both botany and zoology. The holobiont (*Sanchez-Canizares* et al., 2017) represents combinations of plant (or animal) and microbial cells that constitute intact multicellular organisms. By probing plant biology and various plant-associated microbial habitats, we have attained a deeper comprehension of beneficial microbes, microbial pathogens, disease resistance, microbial metabolites and biochemistry.

One important topic left to the end of this summary is that of human-plant interactions and the possible roles of plant-associated microbes in such plant-mammal interspecies relationships. *Gabrielle Berg* described findings related to plants in built environments occupied by humans, and how plants in indoor environments may

promote microbial biodiversity. Such indoor biodiversity may enhance the biodiversity of the human microbiome on skin, in the respiratory tract, the oral cavity or the gastrointestinal tract. Clearly plant microbiomes in human food plants such as pumpkins and members of the *Brassica* family provide a direct connection between microbiota on plants (vegetables and fruits) and the human oral cavity and gastrointestinal tract (*Wassermann* et al., 2019). Although direct relationships between plant-based human nutrition, the human microbiome and human health were not major topics of this Seminar, such topics are ripe for discussion and considerations in future studies.

Max Moehs (**Chapter IV**) described chemical mutagenesis strategies impacting plant genetics that could yield significant results for human health and disease susceptibility. By using TILLING, mutants of grain crops such as wheat or barley could be generated that alter seed protein profiles. Since wheat and barley proteins such as gliadins and low molecular weight (lmw) glutenins are strong triggers for coeliac disease in humans, mutants with reduced gliadin and/or lmw glutenin content could serve as more "human-friendly" grain sources. Such grain crops could reduce the coeliac disease burden in human populations and may also positively affect the epidemiology of disorders such as gluten sensitivity and wheat allergy as well. *Max Moehs* described experiments with mutant barley (reduced gluten content) in a non-human primate coeliac disease model (*Mohan* et al., 2016). Findings showed that small intestinal pathology was ameliorated with the mutant barley-based diet, and detectable changes in the composition of the primate intestinal microbiome were also documented following gluten consumption. Clearly alterations in plant proteins yielded effects on human pathology and such changes in diet may modulate the composition of the human microbiome. *Sylvia Schnell* showed us that that elevated plant pollen-associated microbial diversity was associated with more highly allergenic plant species such as birch, rye and mugwort. Plant microbiome proteins may have a direct impact on the susceptibility of humans to respiratory allergies.

Plants and plant-associated microbes clearly benefit human health by providing nutritious food sources and by promoting planetary biodiversity (*Hacquard* et al., 2015). Many studies have demonstrated that human health is associated with microbial biodiversity and that human disease states often are associated with less diverse microbial communities. *Margot Schulz* (**Chapter III**) mentioned that human nitrates released into the ambient air through exhalation may benefit plant leaf growth by providing nitrogen sources via volatile organic compounds (VOCs). Perhaps humans and plants can benefit each other by sharing microbial metabolites. Plants and humans can benefit each other, and conversely harm each other. As we have discussed, plants may generate toxic metabolites and may contribute to immune-mediated diseases in humans such as coeliac disease and respiratory allergies. Plants may serve as vehicles for food-borne disease outbreaks. Humans can play a major role in destroying plant habitats and harming or reducing plant-associated biodiversity. The final lesson of **Seminar 33** may be that we must explore nature and seek to arrive at a deeper understanding of plant and animal ecosystems to save our planet. As *Gabrielle Berg* persuasively stated, we should strive for One Health on One Planet with One Future.

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