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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

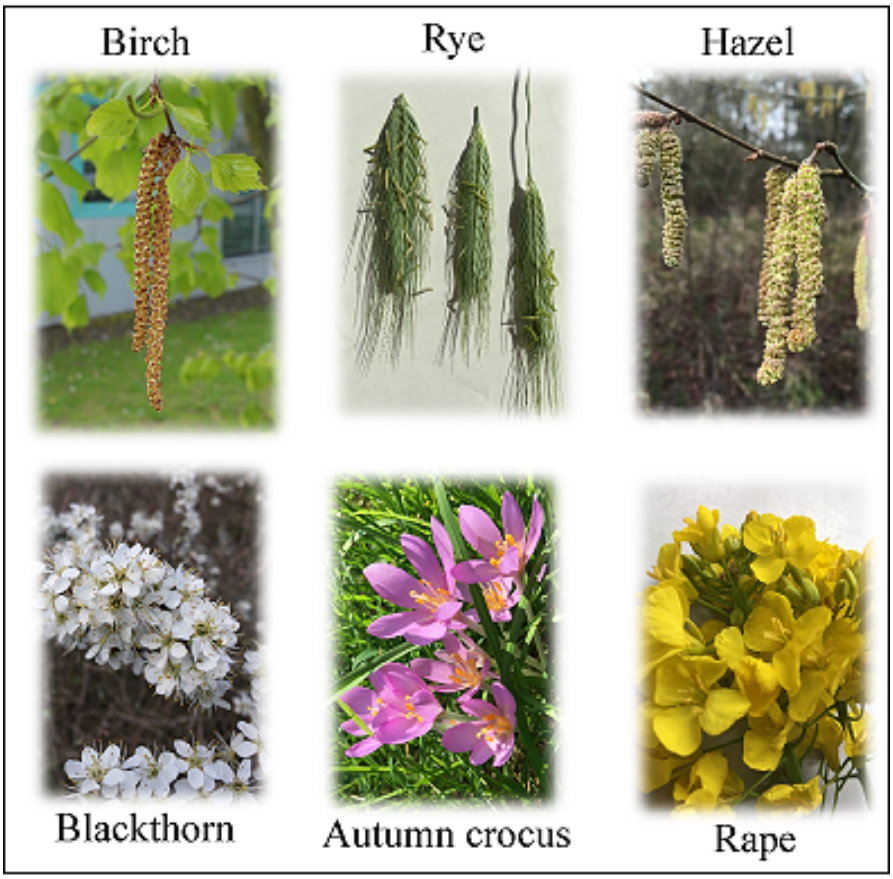

Figure 1: Flowers of the plants sampled. Upper row wind-pollinated plants (*Betula pendula, Secale cerale* L., *Corylus avellanae*); lower row insect-pollinated plants (*Prunus spinose* L., *Colchicum autumnale* L., *Brassica napus* L.). Photos taken by B. Ambika Manirajan.

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.

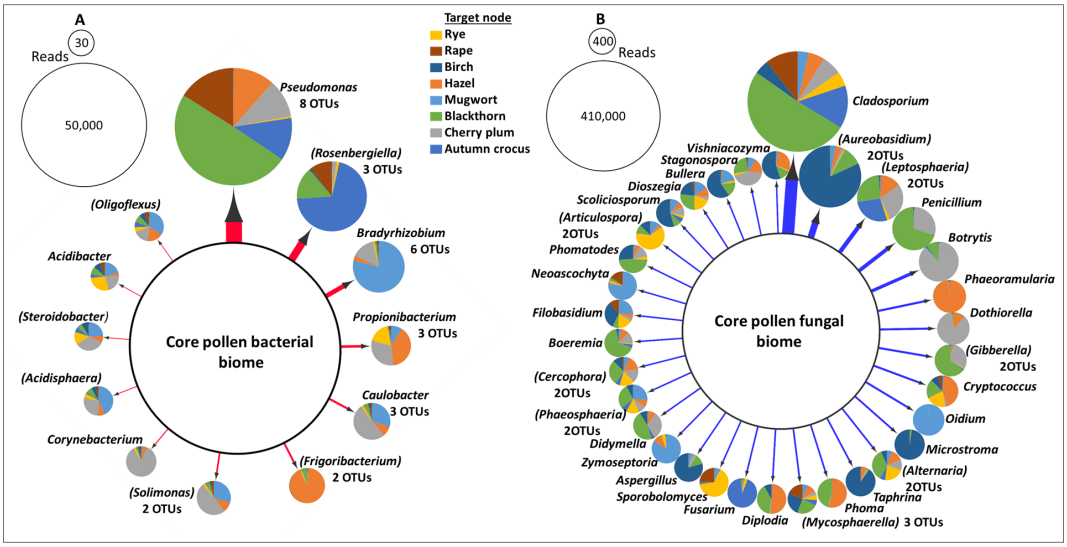

Figure 2: Structure and abundance of the pollen bacterial (A) and fungal (B) core microbiome, defined as the OTUs (97% similarity level) detected in all pollen species, grouped by genus. Pies are coloured by pollen species and show the distribution of the respective core genera. Pie size and edge width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