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Bacterial and Fungal Microbiota of Flower Pollen and Potential Impact on Pollen-related Allergies

Cumulative Dissertation

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Submitted by

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The present work was carried out at the Institute for Applied Microbiology, Faculty of Agricultural Sciences, Nutritional Sciences and Environmental Management, Justus-Liebig-University, Gießen during the period from April 2015 to September 2018 under the guidance of **Prof. Dr. Sylvia Schnell**.

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Declaration

I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "Statutes of the Justus Liebig University Gießen for the Safeguarding of Good Scientific Practice".

Gießen, 24th September, 2018 Binoy Ambika Manirajan

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List of abbreviations

ANOVA	Analysis of Variance
AU	Autumn crocus
B	Birch
BLAST	Basic Local Alignment Search Tool
BT	Blackthorn
CFU	Colony Forming Unit
CLSM	Confocal Laser Scanning Microscopy
CP	Cherry plum
DNA	Deoxyribonucleic Acid
ECARF	European Centre for Allergy Research Foundation
ECRHS	European Community Respiratory Health Survey in Adults
ELISA	Enzyme-Linked Immunosorbent Assay
FISH	Fluorescent *In Situ* Hybridization
HA	Hazel
HA	High Allergenic
HM	Hemp
IP	Insect-pollinated
ISAAC	International Study of Asthma and Allergies in Childhood
LA	Low Allergenic
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MG	Mugwort
NMDS	Non-metric Multidimensional Scaling
OTU	Operational Taxonomic Unit
QIIME	Quantitative Insights Into Microbial Ecology
RA	Winter rapeseed
rRNA	Ribosomal Ribonucleic Acid
RY	Winter rye
SEM	Scanning Electron Microscopy
WHO	World Health Organization
WP	Wind-pollinated

Summary

Flower pollen is a plant microhabitat which was overseen for microbial analyses compared to the intensively studied leaf and root habitats. Pollen is important for plant reproduction and provides nutrients for insects e.g. for honeybees and humans such as honey and propolis. Pollen of wind-pollinated plants is a major airborne allergen all over the world, 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.

A core microbiome consisting of 12 bacterial and 33 fungal genera was found in the pollen of the nine plant species investigated. The most abundant core genera found were *Pseudomonas* and *Rosenbergiella* (bacteria), and *Cladosporium* and *Aureobasidium* (fungi). Co-occurrence analysis highlighted significant inter- and intra-kingdom interactions, and the interaction network was shaped by four bacterial hub taxa: *Methylobacterium* (two OTUs), *Friedmanniella* and *Rosenbergiella*. *Methylobacterium* prevailed in wind-pollinated high

allergenic pollen and *Rosenbergiella* in the insect-pollinated low allergenic pollen; the latter was negatively correlated with the other three hubs, indicating habitat preference.

For evaluation of the allergic potential of the bacterial isolates and pollen, the bacterial endotoxins level were assessed. In high allergenic pollens endotoxin concentrations were higher than low allergenic ones. Interestingly, the lipopolysaccharide concentrations of Gram-negative bacteria isolated from high allergenic pollen were also significantly higher than those of low allergic pollen isolates. The levels of endotoxins in the pollen and in the corresponding bacterial isolates were highly correlated which supports our hypothesis that pollen microorganisms may play a role in pollen allergy.

In total 157 morphologically different bacterial strains, belonging to 27 bacterial families, were isolated from the nine different pollens. Among them, a new species from the genus Spirosoma was isolated from common hazel, characterized by phenotypic, phylogenetic and genotypic (draft genome sequence) variations and described as the new species *Spirosoma pollinicola* HA7^{T}.

This study enhances our basic knowledge of the pollen microbiome, provide insights on the role of pollen-associated microbes in pollen allergy, and poses the basis for further inter- and intra-kingdom interaction studies of the plant reproductive organs.

Zusammenfassung

Blütenpollen wurden bisher als Objekt für mikrobiologischen Untersuchungen übersehen, obwohl andere Pflanzenhabitate wie Blätter und Wurzeln sehr intensiv mikrobiologisch analysiert wurden. Pollen ist essentiell für die pflanzliche Reproduktion, er stellt jedoch auch Nährstoffe für viele Insekten bspw. Bienen und den Menschen als Honig oder Propolis zur Verfügung. Auf der anderen Seite wirkt der Pollen von windbestäubten Pflanzen weltweit als windgetragenes Allergen und kann schwere allergische Rhinitis verursachen.

In dieser Arbeit wurde die Abundanz, Struktur und Diversität der pollenassoziierten Mikrobiota von neun verschiedenen Pflanzen analysiert: vier windbestäubte, hoch-allergene Pollen (Birke, Roggen, Hasel und Beifuß), vier insektenbestäubte, niedrig-allergene Pollen (Herbstzeitlose, Raps, Schlehe und Kirschpflaume) sowie der Hanf als eine weitere windbestäubte Art mit geringer Allergiewirkung. Für die Mikrobiota wurde die 16S rRNA Gensequenz und für die pilzliche Mikrobiota die ITS2 Sequenz mittels Hochdurchsatzsequenzierung analysiert. Zusätzlich wurde mit klassischen Kultivierungsmethoden der kultivierbare Anteil der Mikrobiota erfasst und mittels verschiedener Mikroskopie Methoden wurde die Besiedlung der Pollen mit Bakterien visualisiert. Der Endotoxingehalt (Lipopolysaccharide und Lipoteichonsäuren) von Pollen und Bakterienisolaten wurde mit enzymgebundenen immunologischen Tests (ELISA) bestimmt

Proteobacterien (Bakterien) und *Ascomycota* (Pilze) sind die häufigsten Phyla und *Pseudomonas* (Bakterium) und *Cladosporium* (Pilz) sind die häufigsten Gattungen der analysierten Pollen. *Archaea* Sequenzen wurden nicht gefunden. Die bakteriellen und pilzlichen alpha- Diversitätsindexe waren signifikant niedriger in niedrig-allergenen Pollen und auch im Hanf im Vergleich zu hoch-allergenen Pollen. Der am höchsten signifikant beeinflussende Faktor der bakteriellen und pilzlichen Mikrobiota war das Allergiepotential des Pollens, gefolgt von der Pflanzenart und der Bestäubungsart (wind- oder insektenbestäubt). Bemerkenswert ähnlich zu den anderen niedrig-allergenen Pollen verhielten sich Hanfpollen.

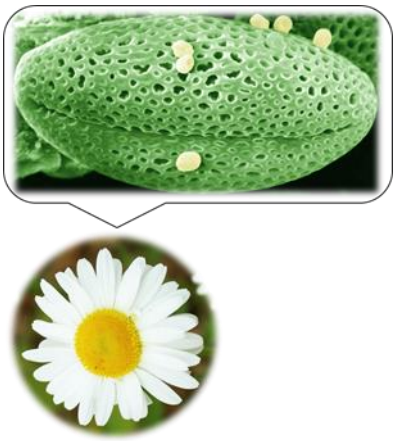
Die Mikrobiota, die in allen der neun Pflanzenarten zu finden sind („core microbiota“) bildeten 12 bakterielle und 33 pilzliche Gattungen. Die häufigsten Gattungen waren *Pseudomonas* und *Rosenbergiella* (Bakterien), sowie *Cladosporium* und *Aureobasidium* (Pilz). Die Analyse des gemeinsamen Auftretens („co-occurrence analysis“) von Arten

ermöglicht Inter- und Intrainteraktionen zwischen bakteriellen und pilzlichen Arten zu erkennen. Das Interaktionsnetzwerk wurde dominiert von vier Bakteriengattungen: *Methylobacterium* (zwei Gattungen), *Friedmanniella* und *Rosenbergiella*. *Methylobacterium* überwog in windbestäubten, hoch-allergenen Pollen, während *Rosenbergiella* in insektenbestäubten, niedrig-allergenen Pollen überwog. *Rosenbergiella* war negativ korreliert mit den anderen drei dominierenden Gattungen des Netzwerkes und zeigt die Präferenz fürs Habitat an.

Zur Beurteilung des allergenen Potential der Bakterienisolate und des Pollens wurde der bakterielle Endotoxingehalt analysiert. In hoch-allergenen Pollen war der Endotoxingehalt höher als in niedrig-allergenen Pollen. Interessanterweise waren die Lipopolysaccharidkonzentrationen der Gram-negativen Bakterienisolate von hoch-allergenen Pollen ebenfalls signifikant erhöht im Vergleich zu denjenigen von niedrig-allergenen Pollen. Der Gehalt an Endotoxin im Pollen und der davon isolierten Bakterienisolaten war hoch korreliert, was die Hypothese unterstützt, dass die Pollenmikrobiota eine wichtige Rolle bei der Pollenallergie spielt.

Es wurden insgesamt 157 morphologisch unterschiedliche Bakterienisolate (27 Familien) von neun verschiedenen Pollen isoliert. Darunter war eine neue Art der Gattung *Spirosoma*, welche von Haselpollen isoliert wurde. Dieses Isolat wurde phenotypisch, phylogenetisch und genotypisch charakterisiert und als neue Bakterienart *Spirosoma pollinicola* HA7^{T} beschrieben.

Mit dieser Arbeit wurde das Wissen zum Mikrobiom von Pollen verbessert und erste Hinweise für die Rolle der pollenassoziierten Mikroorganismen erhalten, welche eine Grundlage für weitere Studien zur Interaktion zwischen den Königreichen Pflanzen, Pilzen und Bakterien auf reproduktiven Pflanzenorganen darstellen.



Chapter 1

Introduction

1. Introduction

Plant organs are normally colonized by a complex and diverse microbiota which include bacteria, fungi, yeast and archaea (Whitaker *et al*., 2017; Berg *et al*., 2014; Whipps *et al*., 2008; Lindow and Brandl, 2003; Leben, 1972). They are inhabited as either by epiphytic or endophytic microorganisms (Mazinani *et al*., 2017; Nongkhlaw and Joshi, 2015), in different niches of the plant organs, such as root (Bodenhausen *et al*., 2013), shoot (Okubo *et al.* 2014), leaf (Yu *et al*., 2015; Bodenhausen *et al*., 2013; Hirano and Upper, 2000), fruit (Leff and Fierer, 2013), seed (Truyens *et al*., 2014) and flower (Aleklett *et al*., 2014) etc. There are many culture-dependent and independent studies which report about the bacterial inhabitance associated with plant and its micro niches. These microbial colonizers have commensal, pathogenic and mutualistic interaction with the plant host (Scortichini and Katsy, 2014) and the major members of these colonizers are bacterial communities (Meyer and Leveanu, 2012; Vorholt, 2012). Few studies recently reported on the bacterial biome inhabiting in seed endosphere and its interactions with the host plant (Alibrandi *et al*., 2017; Truyens *et al*., 2014) as well as the predicted mechanism of its transmission. The transmission of plant-associated bacterial inhabitance happens naturally either by vertical or horizontal way (Shade *et al*., 2017; Barret *et al*., 2015). However, the mechanism of vertical transmission from generation to generation is still unclear. Numerous biotic and abiotic factors such as insect occurrence, contact to other plants, radiation, pollution, temperature, wind and humidity drive the plant inhabiting microbiome structure (Obersteiner *et al*., 2016; Rastogi *et al*., 2013; Vorholt, 2012). Several studies have reported that plant genotype is an important factor which influences the bacterial community structure and composition (Kumar *et al*., 2017; Knief *et al*., 2010). As a plant microhabitat, pollen is also colonized by diverse microorganisms including bacteria and fungi. Nevertheless, very few studies have aimed to unravel the total bacterial diversity associated with pollen and the role of bacteria in pollen allergy. Colldahl

and Carlsson (1968) have isolated and cultivated different bacteria from birch and timothy pollen. They observed that bacterial isolates from different pollen provoke allergic reaction in pollen-sensitive patients by both skin titration and nasal provocation, and thus concluded the possibility of clinical effect of bacteria in pollen allergy. Zasloff (2017) has proposed that microbes on pollen grains may contribute to human allergen potential of flower pollen. He brought up the question that whether immunizing with pollen-associated microbes may prevent pollen specific allergy or not.

The basic goal of this doctoral study was to analyse the bacterial and fungal community structure, its abundance, diversity and colonization pattern between plant pollen species as well as high allergenic and less/non allergenic pollen species, from a restricted geographical area, by cultivation-dependent and -independent methods. In order to understand the niches of colonization pattern, localization of bacteria into the pollen habitat was visualized by using Scanning Electron Microscopy (SEM) as well as Fluorescent *in situ* Hybridization and Confocal Microscopy. Furthermore, to estimate the extent of cultivable bacterial fraction, different culture media from different plant pollen species were used. From the bacterial isolates the immune modulatory compounds (endotoxin) produced by Gram-negative (lipopolysaccharide) and Gram-positive (lipoteichoic acid) bacterial isolates from high allergic and less/non- allergic pollen habitat were quantified.

1.1. Pollen

Pollen grains are the haploid male microgametophyte produced within the anthers of flowering plants and it acts as a carrier of male sperm. It is essential that pollen must contact with the female reproductive organ for the fertilization of plants. The adhesive outer wall of pollen attaches on the female flower stigma and grows as a tiny tube towards the ovule in the ovary. This makes the female eggs to fertilize and finally ends in endosperm production (Edlund *et al*., 2004; Friedman and Williams, 2003). Pollen usually dispersed by wind, water,

insects or small birds. Many species of these pollen producing plants emit large quantities of pollen during the flowering season and it spreads throughout the surrounding environment.

1.2. Commercial value of pollen

As a plant product, pollen collected either by hand or from bee bread has been in trade for a wide range of nutritional and therapeutic purposes. Pollen has been used by humans for medicinal purposes, as a supplementary food, dietary compound and food for insects (Linskens and Jorde, 1997). Many studies report that pollen contains different amount and type of proteins, amino-acids, vitamins, lipids, ash (Nicolson and Human, 2013; Human and Nicolson, 2006), fibre, carbohydrate and minerals (Clark and Lintas, 1992). Pollen has been used as natural remedy for prostatitis, bleeding stomach ulcers and for certain infections (Linskens and Jorde, 1997). It provides cosmetic therapeutic effect to reduce oxidative stress and hyperpigmentation (Kim *et al*., 2015) and acts as an antioxidant too (Almaraz-Abarca, 2004). Because of these nutritional and medicinal values, a large quantity of pollen is marketed all over the world (Belhadj *et al*., 2014) in different forms. Moreover, pollen and nectar offer food source for flower visitors. The increasing demand for pollen products is probably because of the belief that the consumption of plant products is healthier than man-made medicines. Hence, the microbiological quality of the pollen and pollen products would need to be thoroughly studied.

1.3. Structure of pollen

Pollen contains an exine (outer layer), intine (inner cellulosic wall), cytoplasm and nucleoli. Pollen grain cytoplasm is responsible for the development of the pollen tube and the delivery of the sperm cells to the embryo sac. The exine is structurally more complex and provides distinctive characteristic for pollen grains (Blackmore, 2007). The main role of exine is to provide protection for the microspore cytoplasm containing sperm from hostile climatic conditions, such as prolonged desiccation, unusual temperatures, ultraviolet light exposure

and microbial damage etc. (Ariizumi and Toriyama, 2011). Moreover, it helps to facilitate pollination. The exine of pollen consists of sporopollenin, a hydrophobic complex mixture of biopolymers with long chain fatty acid, phenylpropanoids, phenolics and carotenoids (Dominguez *et al*., 1999; Kawase and Takahashi, 1995). Sporopollenin is extremely resistant to non-oxidative physical, biological and chemical degradation (Erdtman, 1960). The intine is composed of cellulose and pectin, and it covers the nutrient-rich cytoplasm (Roulston and Cane, 2000). Depending on the plant species, pollen has species-specific size, shape, structure, pattern and composition (Ariizumi and Toriyama, 2011; Kosenko1999). However, the surprising diversity of pollen morphology is mostly related to the exine structure. Due to its structure and nutritive composition, pollen provides a unique microhabitat for microorganisms.

1.4. Type of pollen and morphological difference based on dispersal

Based on the dispersal of pollen via different vectors, pollen can be classified as anemophilous (dispersal by wind), zoophilous (dispersal by insect and small birds) and hydrophilous (dispersal by water) (Tanaka *et al*., 2004; Dafni *et al*., 2000; Cook, 1982). Anemophilous flowers are less attractive, mostly small in size and bunchy with versatile and freely swinging anthers in the air. These plants release large quantities of dusty pollen into the air, to increase the pollination success rate. Anemophilous pollens are usually dry, smooth walled and small in diameter (exception is large anemophilous pollen has very low density). It can stay longer in the air because of its low settling rate and long-time viability for long distance distribution. The exine structure of the pollen also differs depending upon the delivery mechanisms. Anemophilous pollen consists of aerodynamic exine structure with non-sticky limited pollen coat (Schwendemann *et al*., 2007; Shukla *et al*., 1998; Heslop-Harrison, 1979). Zoophilous flowers are usually pollinated by insect, small birds and bats. These flowers are more attractive, scented, contain nectar, and have appealing shapes, colour

and patterns to attract pollinators. Zoophilous pollens are moist, sticky and rough, with abundant pollen coat (Dickinson *et al*., 2000; Shukla *et al*., 1998; Pacini and Franchi, 1996). Zoophilous pollen's exine contains volatile lipids to attract pollinators (Hopkins *et al*., 1969). Hydrophilous pollen grains, on the other hand, are distributed through water streams. These pollen grains are large, spherical and filamentous. Hydrophilous pollen coat consists of sticky surface with less or no exine at all (Ackerman, 2000).

1.5. Relation between wind-pollinated pollen and implications on allergenic potential

Most of the allergenic pollens are produced by wind-pollinated plant species, while the less/non- allergenic pollens are normally produced by insect-pollinated plant species. Wind-pollinated plants usually produce massive amount of very small pollen grains that stay very long time in the air and hence, these will be transported to very long distance. The high allergenic wind-pollinated pollens come from grass, tree, weeds, conifers and also ornamental plants (Songnuan, 2013; D'Amato *et al*., 2007; D'Amato, 2001). These pollen grains produce airborne-induced respiratory allergy in people sensitive to pollen allergies. The water content in the wind-pollinated pollen grains is very low and, therefore, easily hydrate when it is exposed to moist condition (Franklin-Tong, 1999). When the pollen grains get exposed to more moisture content, it causes osmotic imbalance which results in their bursting. Consequently, the pollen grains are released into the atmosphere and this may increase the chance to trigger allergy in pollen allergic people. The pollen allergy symptoms appear to change with weather and this is due to the production, dispersal and quantity of pollen. Environmental factors also play an important role in increasing pollen allergy problem in the urbanized area. Pollution is a major factor that affects the quality of wind-pollinated high allergenic pollen grains. Many studies have reported that the air pollution is one of the major reasons for raising the rate of pollen-induced allergy (Obersteiner *et al*., 2016; Majd *et al*., 2004; Emberlin, 1998; Behrendt *et al*., 1997). Even though wind-pollinated

plants are high allergenic, some of them are found to be less/non- allergenic. *Cannabaceae* plants are normally wind pollinated herbs with non- allergenic pollen (Cascini and Boschi, 2017).

1.6. Pollen allergy and human health

Millions of people worldwide suffer from pollen triggered allergy with different type of plant pollen and the number is found to be increasing every year. Pollen allergy is one of the major respiratory allergies and it has had remarkable clinical impact in Europe in past decades (ECRHS, 1996; ISAAC, 1998). Studies show that more than 20% of the German population is sensitive to pollen induced allergy (Bergmann *et al*., 2016; Estrella *et al*., 2006). Hazel, birch, alder, ragweed, rye and mugwort are considered as the most important clinically relevant allergenic pollen producing plants in Germany (Lozano-Vega *et al*., 2014; Bergmann *et al*., 2012; Estrella *et al*., 2006). The economic threat of allergic diseases for the European Union was estimated from 55 to 151 billion Euro per year (Zuberbier *et al*., 2014). It is found that the longer the pollen season, the higher the pollen concentration in the atmosphere. Moreover, cultivation of more exotic plants such as ragweed, ginkgo trees etc. increases the pollen sensitization every year. Climate change may also be one of the major reasons for increasing pollen allergy (Singer *et al*., 2005).

Pollen allergy has a significant clinical impact in Germany, and the prevalence of pollen allergy is estimated to be more than 40%. Pollen allergy is caused by hypersensitivity reaction of the human body induced by airborne pollen. Pollen carrying allergens affect the eyes, skin, throat, lungs, ears and mucous membrane of the nose. As a result, nasal congestion, sneezing, nasal discharge, itchy and watery eyes, itchy nose, itchy skin and asthma could occur (Gehrig *et al*., 2015; de Weger *et al*., 2011; Robert and Naclerio, 1991). These are the symptoms of pollen-induced seasonal allergic rhinitis (http://www.ecarf.org). Socio-economic impact of the pollen allergic rhinitis has become a serious issue because of the impaired quality of life,

cost of treatment, lesser working hours, associated clinical problems and cost of associated medical issues. The prevalence of pollen allergic rhinitis rises with increased pollution, family history of pollen allergy, life style of higher socio-economic classes and increased age of allergic patients (Skoner, 2001).

1.6.1. Pollen contain allergens

It is found that birch, hazel, mugwort and rye are the major and common allergenic pollen in Europe which trigger allergic rhinitis (http://www.ecarf.org/en/information-portal/allergies-overview/pollen-allergy/). Certain compounds produced by allergic plant pollen prime immune responses in pollen allergic individuals. Pollen allergens identified are low molecular weight proteins and glycoproteins. The main allergens detected in birch pollen are *Bet v* allergens, among which the major and dominant allergen is *Bet v1* (Marth *et al*., 2014; Grönlund and Gafvelin, 2010). Hazel pollen consists of different *Cor a* allergens and *Cor a1* is the major hazel pollen allergen (Hirschwehr *et al.*, 1992). The allergens detected in mugwort pollen are *Art v* allergens and *Art v1* is the major clinically relevant allergen detected in that category (Pablos *et al*., 2016; Knapp *et al*., 2012). *Lol p* allergens are the allergens found in rye, *Lol p1* and *Lol p5* are the major allergens in rye (Spangenberg *et al*., 2006; Griffith *et al*., 1991).

1.7. Pollen inhabiting bacteria and its prospective in allergic rhinitis

Being a biological material, pollen contains nutritive composition which is complimentary for microorganisms to grow, colonize and thereby forms a secure habitat, especially for bacteria. The sources of bacterial colonization of pollen were reported to plant materials, honey bees, other insects, birds, animals, human activities, weather, pollution (Hani *et al*., 2012) and seeds. These are either through vertical or horizontal transmission (Rodríguez *et al*., 2017; Shade *et al*., 2017; Barret *et al*., 2015). Pollen, inhabited by diverse bacteria, is scattered in the environment and carried away by wind, thereby transporting these bacteria

many miles. Colldahl and Carlsson, 1968 had initially reported the presence of bacteria in the pollen habitat and discussed the possible clinical effects of pollen inhabiting bacteria. Later, the presence of bacterial cells on the pollen surface was confirmed using scanning electron microscopy by Colldahl and Nilsson, 1973. Thereafter, a few bacteria were isolated from allergic pollen types (Spiewak *et al*., 1996).

Most of the studies relevant to clinical aspect of pollen allergy were aimed only at the allergens associated with pollen grains (Gilles-Stein *et al*., 2016; Oldenburg *et al*., 2011; Gumowski *et al*., 2000; Jahn-Schmid *et al*., 1997). However, a few studies reported the presence of lipopolysaccharide (LPS) also in pollen extract and discussed the possible role of bacterial endotoxin in the pollen triggered allergy (Hosoki *et al*., 2014; Varga *et al*., 2012). Heydenreich *et al*., 2012 isolated some Gram-positive bacteria from grass pollen, which induces inflammatory T cell response *in vitro* and summarized that pollen carrying bacteria may have adjuvant activity in pollen allergy. McKenna *et al*., 2017 isolated a few bacterial species from birch pollen and found that they have proteolytic activity and thus concluded the possibility of proteolytic activity in the development of pollen allergy. There are a few more studies that could be relevant for the safety of pollen-derived foods, such as bee-collected pollen and honey carrying a reasonable number of bacteria (Brindza *et al*., 2010; Olaitan *et al*., 2007). However, very few studies have aimed to unravel the total microbial diversity associated with pollen microhabitat. Even though, some research works exposed the presence of different bacterial species on pollen and its adjuvant effect in allergic response, little is known about the microhabitat, the role of inhabiting bacterial biome in pollen, differences of bacterial biome inhabiting in high allergenic and less/non- allergic plant pollen species and its role in pollen- induced allergy.

1.8. Significance of samples used in this study

1.8.1. Wind-pollinated high allergenic pollen plants

Birch (*Betula pendula* Roth.), winter rye (*Secale cereal* L.), common hazel (*Corylus avellanae* L.) and common mugwort (*Artemisia vulgaris* L.) (Fig 1) were the wind-pollinated allergenic pollen producing plant species selected for the pollen sample collection during the flowering season (2015 & 2016).

Among allergenic pollen producing plants, birch is one of the major pollen allergen producing trees in Europe and the pollen has the potential to travel long range through wind (WHO, 2003). Birch pollen has high clinical relevance for sensitization and thus produces allergic rhinitis in Germany (Bergmann *et al*., 2012). Birch produces a large amount of pollen and the pollination starts from March and continues until May (Žiarovská and Zeleňáková, 2016). In recent years, numbers of birch trees have increased in Europe because of its popularity as ornamental tree and this is the major reason for increasing the birch pollen load in the atmosphere. Hazel is another major pollen allergen producing tree species in Europe and it produces a large quantity of small pollen grains. The pollen season of hazel starts from December and continues up to March (D'Amato *et al*., 2007; Ipsen *et al*., 1985). Hazel pollen acts as primers of allergic sensitization in pollen allergic persons because of early flowering. Hazel is considered to be an important aeroallergen in early spring in Europe (Grewling *et al.*, 2014).

Cultivated rye is one of the major pollen allergen producing grass plants and the flowering season starts from May which continues up to July. A higher amount of atmospheric pollen concentration can be found in the succeeding two months after the flowering (WHO, 2003; D'Amato *et al*., 1998). Rye pollen allergen is also one of the prominent allergen in Germany (Hirsh *et al.*, 2000). Mugwort is another relevant source of allergenic pollen producing weed in Europe. It is a perennial weed growing on roadsides and wastelands and the pollen season

starts from July which lasts until October. Mugwort produces a large number of small pollen grains which float in the air for a longer period (Pawankar *et al*., 2013; Wopfner *et al*., 2005; Spieksma *et al*., 1980). Mugwort pollen allergen is also one of the common aeroallergens in Germany (Hirsh *et al.,* 2000)

Due to the clinical relevance of these four plant pollen species, these are registered by German Pollen Information Service Foundation and displayed in the daily pollen report (http://www.pollenstiftung.de). Among the allergy-producing pollen species in Germany, these four plant pollen species have the highest significance in the issue of triggering pollen allergies and thereby enhancing allergic rhinitis (Bergmann *et al*., 2012).

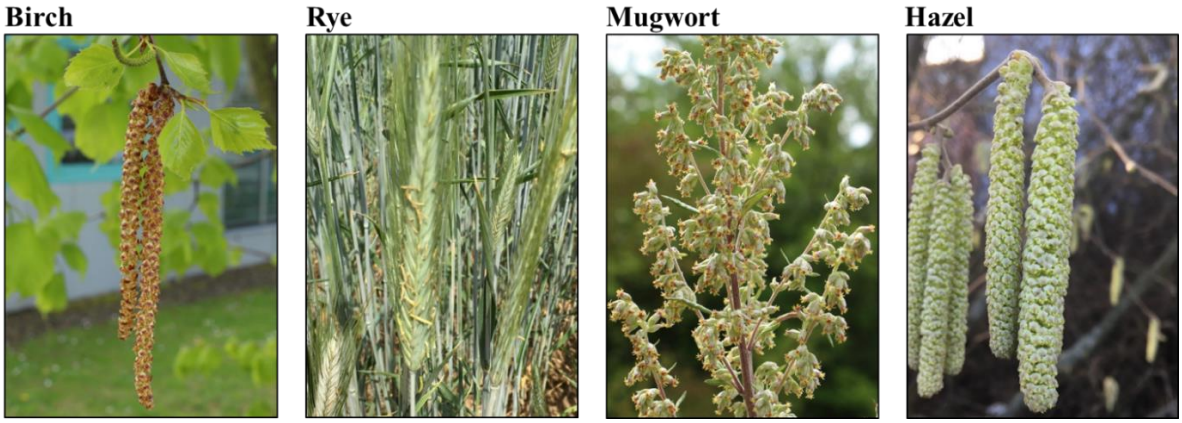


Fig 1. Wind-pollinated allergenic pollen plants flowers used in this study for the pollen collection.

1.8.2. Insect-pollinated less/non allergenic pollen plants

Autumn crocus (*Colchicum autumnale* L.), winter rapeseed (*Brassica napus* L.), blackthorn (*Prunus spinose* L.) and common cherryplum (*Prunus cerasifera* Ehrh.) (Fig 2) were the insect-pollinated less/non allergenic pollen producing plant species selected for the pollen sample collection during the flowering season (2015 & 2016).

Rape is one of the major oil seed producing annual crop plants in Europe which is used as edible soil, animal feed and also as raw material for biodiesel production. The flowering period of rape fields in Germany is from April until May (Wang *et al*., 2011). Rape produces

a large number of flowers and, therefore, holds a large quantity of pollen. Autumn crocus is a perennial herb species, widely grown on woodlands and damp meadows in Europe. The flowering of autumn crocus starts in September and lasts till October (Kupper *et al*., 2010). Blackthorn is a large deciduous shrub or small tree native to Europe. It produces large quantities of tiny flowers and the pollen season is from May to the middle of June (Siegmund *et al*., 2015; Eimer *et al*., 2012). Common cherry plum is a small wild tree species of plum which produces a large quantity of small flowers in the spring season. It is one of the popular ornamental trees in Europe. The flowering season starts from May and ends in June (Szymajda and Urghini, 2017; Roversi and Ughini, 1996). These are common flowering plants in Germany and producing a large quantity of flowers in every season. It is known that, pollen of these plants are non/low aeroallergen producing and these plants are not listed in Stiftung Deutscher Polleninformationsdienst and European Centre for Allergy Research Foundation as allergy triggering pollen producing plant.

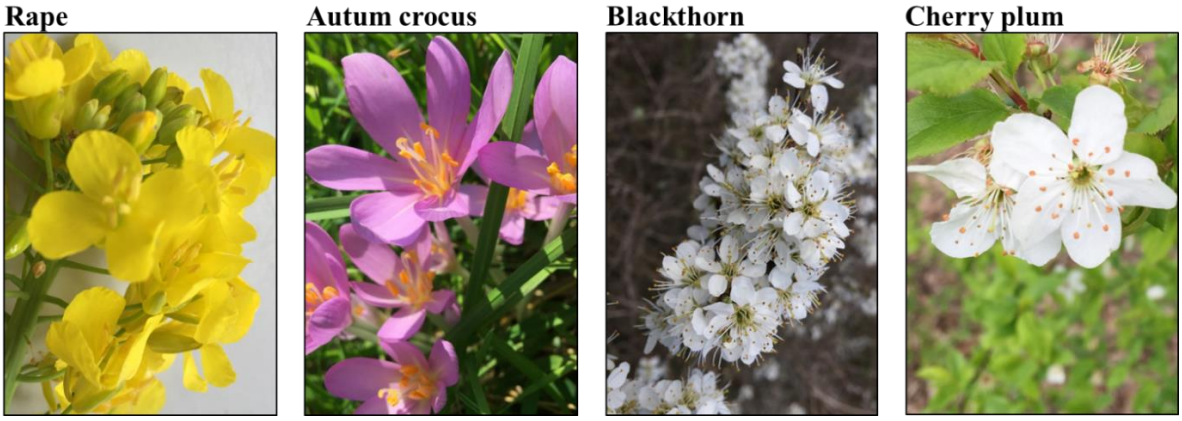


Fig 2. Insect-pollinated less/non allergenic pollen plants flowers used in this study for the pollen collection.

1.8.3. Wind-pollinated less/non allergenic pollen plants

Two varieties of hemp (*Cannabis sativa* L.) (var. finola and var. felina) (Fig 3) were the wind-pollinated less/non allergenic pollen producing plant species selected for the pollen sample collection during the flowering season (2017).

Hemp is seen to grow in many countries in Central Asia, Europe, Finno-Ugric and Russia and is used mainly for fibre and oil. Though varieties of hemp are cultivated for producing tetrahydrocannabinol, it has been generally forbidden in Germany under the narcotic law of July 28, 1981. So the hemp varieties used in this studies (finola and feline) are free of tetrahydrocannabinol and are cultivated for oil and fibre only. Hemp is an annual, flowering herb crop which produces large quantities of flowers and pollen. The flowering of hemp starts from June (Höppner and Menge-Hartmann, 1995; Pahkala *et al*., 2008).

Hemp- variety *Finola*

Hemp- variety *Felina*

Fig 3. Wind-pollinated less/non allergenic pollen plants flowers used in this study for the pollen collection.

1.9. Importance of methods used in this study

1.9.1. Cultivation-dependent methods

Traditional isolation techniques for microbial community analysis are essential to acquire a number of cultivable isolates for the further analysis. Nevertheless, only a small fraction of microbial species could be isolated in the current laboratory culture techniques and conditions were called “plate count anomaly” (Staley and Konopka, 1985). This is the only available

method to study about microbial species and its characteristics. Viable plate count (colony forming unit - CFU) method has been used for quantification of active cells from environmental samples (Amann *et al*., 1995; Staley and Konopka, 1985). Accurate identification of microbial pure cultures is an essential task for microbial molecular biology research. Bacterial 16S rRNA gene and fungal ITS sequencing are the most reliable and common method used for the identification of bacterial and fungal genus and species as well as for the phylogenetic studies (Drancourt *et al*., 2000; Weisburg *et al*., 1991). Cultivation-dependent study provides a rough information about the differences in bacterial abundance in different pollen species.

1.9.2. Cultivation-independent methods

The majority of microbial communities in the natural environment cannot be cultured in laboratory conditions. Therefore, cultivation-independent methods are essential for microbial molecular ecology for understanding about the non-cultivable major microbial fraction in the environmental samples. Next-Generation sequencing technologies (454 Pyrosequencing, Illumina Sequencing and Ion Torrent) are the most advanced technologies to study, evaluate and screen diverse microbial community from a complex environment for ecological and environmental research (Boughner and Singh, 2016; Su *et al*., 2012). Ion semiconductor-based sequencing method (Ion Torrent PGM) is one of the reliable and cost-effective tools among these. The advantages of Ion Torrent are that the time per run is less than 2 hrs and read length is about 200 bp, (Diaz-Sanchez *et al*., 2013; Merriman *et al*., 2012). Developments in Next-Generation sequencing has revolutionized the field of studies in microbial ecology. The Ion Torrent sequencing provides knowledge about microbial (Bacterial and Fungal) abundance, community structure, diversity, hub taxa, inter-kingdom co-occurrence pattern etc. from complex microbial habitat.

1.9.3. Microscopy methods

Direct visualization of microbes in the habitat is complementary to understand the colonization pattern of microbes in the specific habitat and dynamics of host-microbe interactions. Therefore, studies in microbial ecology and plant microbiology without visualization by microscopy are incomplete. Localization at microscale, colonization pattern and cell-cell interactions are detected by using microscopy only (Cardinale and Berg, 2015). Fluorescent *in situ* hybridization, coupled with confocal laser scanning microscopy (FISH-CLSM) is a well established method and have long been used for localization and visualization of target microbial cells (from domain to species level) in their host system. Scanning electron microscopy (SEM) is a powerful magnification tool and it provides three dimension and high resolution images, which visualize the colonization pattern of microbes in its habitat. FISH-CLSM and SEM have provided the images of niches of colonization and colonization pattern of bacteria on pollen in this research work.

1.9.4. Enzyme-Linked Immunosorbent Assay

The enzyme-linked immunosorbent assay (ELISA) is a good tool for clinical microbiology to detect antigen and antibody. This method is widely using to target both pathogen and pathogenic molecule (Meurmann, 1991) in research laboratories as well as on a commercial basis. ELISA was the method of choice for determining and quantifies the differences of endotoxins (Lipopolysaccharides- Gram-negative bacteria and Lipoteichoic acid- Gram-positive bacteria) present in different bacterial isolates retrieved from the pollen habitat of different plant pollen species.

1.10. Aim of the study

The bacterial microbiota associated with the pollen of nine plant species [four high allergenic pollen species (wind-pollinated), four less/non- allergenic pollen species (insect-pollinated) and a control plant pollen species (less/non allergenic wind pollinated)] from a restricted

geographical area has been analyzed by cultivation-dependent and –independent methods. The aims of the study were to (i) compare bacterial and fungal abundance, structure and diversity of pollen microbiota of the nine plant species, (ii) identify the "core" pollen microbiotas, (iii) assess inter- and intra- kingdom correlations in the bacterial–fungal microbiota, (iv) identify the "hub" taxa, (v) assess the contribution of the pollination type to the variability of the pollen microbiotas, (vi) analyze the cultivable bacterial fraction by using commercial AC medium ("all culture" medium) and a pollen-enriched mineral medium, (vii) study the niches of colonization of pollen bacteria by scanning electron microscopy as well as fluorescent in-situ hybridization and confocal microscopy, (viii) investigate bacterial endotoxin quantity and activity against endotoxin specific antibody of different pollen samples as well as bacterial isolates from pollen samples, and (ix) investigate the impact of microbiome in the high allergenic and less/non- allergenic pollen habitat.

1.11. References

Ackerman, D.J. (2000) Abiotic pollen and pollination: ecological, functional, and evolutionary perspectives. *Plant Syst Evol* **222:** 167-185.

Aleklett, K., Hart, M., and Shade, A. (2014) The microbial ecology of flowers: an emerging frontier in phyllosphere research. *Botany* **92:** 253-266.

Alibrandi, P., Cardinale, M., Rahman, M.M., Strati, F., Ciná, P., de Viana, M.L., Giamminol, E.M., Gallo, G., Schnell, S., De Filippo, C., Ciaccio., Puglia, A.M. (2017) The seed endosphere of *Anadenanthera colubrina* is inhabited by a complex microbiota, including *Methylobacterium* spp. and *Staphylococcus* spp. with potential plant-growth promoting activities. *Plant Soil*. 1–19.

Almaraz-Abarca, N., Campos, M.G., Avila-Reyes, J., Naranjo-Jimenez, N., Herrera-Corral, J., Gonzalez-Valdez. (2004) Variability of antioxidant activity among honey-bee collected pollen of different botanical origin. *Interciencia* **29:** 574–578.

Amann RI, Ludwig W, Schleifer K-H (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev* **59:** 143-169.

Andrews, J.H., Harris, R.F. (2000) The ecology and biogeography of microorganisms on plant surfaces. *Annu. Rev Phytopathol* **38:** 145-180.

Ariizumi, T., Toriyama, K (2011) Genetic regulation of sporopollenin synthesis and pollen exine development. *Annu Rev Plant Biol* **62:** 437-460.

Barret, M., Briand, M., Bonneau, S., Préveaux, A., Valière, S., Bouchez, O., Hunault, G., Simoneau, P., Jacques, M-A. (2015) Emergence shapes the structure of the seed microbiota. *Appl Environ Microbiol* **81:** 1257–1266.

Barret, M., Briand, M., Bonneau, S., Préveaux, A., Valière, S., Bouchez, O., Hunault, G., Simoneau, P., Jacgues, M-A. (2015) Emergence shapes the structure of the seed microbiota. *Appl Environ Microbiol* **81:** 1257–1266.

Behrendt, H., Becker, W.M., Friedrich, K.H., Sliwa-Tomczok,W., Tomczok, J., Friedrichs, K.H., Ring, J. (1997) Air pollution and allergy: experimental studies on modulation of allergen release from pollen by air pollutants. *J Int Arch Allergy Immunol* **113**: 69–74.

Belhadj, H., Harzallah, D., Dahamna, S., Khennouf, S. (2014) Microbiological quality control of marketed pollen. *Pharm Lett* **6:** 37-42.

Berg, G., Grube, M., Schloter, M., Smalla, K. (2014) Unraveling the plant microbiome: Looking back and future perspectives. *Front Microbiol* **7:** 148.

Bergmann, K.-C., Heinrich, J., Niemann, H. (2016) Current status of allergy prevalence in Germany: Position paper of the Environmental Medicine Commission of the Robert Koch Institute. *Allergo J Int* **25:** 6–10.

Bergmann, K.-C., Zuberbier, T., Augustin, J., Mücke, H.-G., Straff, W. (2012) Climate change and pollen allergy: cities and municipalities should take people suffering from pollen allergy into account when planting in public spaces. *Allergo J* **21:** 103–107.

Blackmore, S. (2007) Pollen and spores: Microscopic keys to understanding the earth's biodiversity. *Plant Syst Evol* **263:** 3-12.

Bodenhausen, N., Horton, M.W., Bergelson, J. (2013) Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. *PLoS ONE* **8:** e56329.

Boughner, L.A., Singh, P. (2016) Microbial Ecology: Where are we now? *Postdoc J* **4:** 3–17.

Brindza, J., Bacigálová, K., Feriane, P., Tóth, D. (2010) Pollen microbial colonization and food safety. *Acta Chim Slov* **3:** 95–102.

Brodschneider, R., Crailsheim, K. (2010) Nutrition and health in honey bees. *Apidologie* **41:** 278-294. DOI:*10.1051*/apido/2010012.

Cardinale, M., Berg, G. (2015) Visualization of plant-microbe interactions. In Lugtenberg, B (ed.), Principles of plant-microbe interactions. *Springer International Publishing*, Cham, Switzerland. pp 299–306.

Cascini, F., Boschi, I. (2017) Tetrahydrocannabinol concentration and genetic characterization of Cannabis. In: Preedy, V (ed) Handbook of Cannabis and related pathologies. *Academic Press*, UK. p e1–e10.

Clark, C.J., Lintas, C. (1992) Chemical composition of pollen from kiwifruit vines. *N Z J Crop Hort* **20:** 337–344.

Colldahl, H., Carlsson, G. (1968) Allergens in pollen. *Acta Allergologia* **XXIII:** 387-397.

Colldahl, H., Nilsson, L. (1973) Possible relationship between some allergens (pollens, mites) and certain microorganisms (bacteria and fungi). *Acta Allergologica* **28:** 283-295.

Cook, C.D.K. (1982) Pollination mechanisms in the *Hydrocharitaceae*. In: Symoens, J.J., Hooper, S.S., Compere, P. (eds) Studies on aquatic vascular plants. *Royal Botanical Society of Belgium*, Brussels, pp 1–15.

D'Amato, G. (2000) Allergenic pollen in Europe. In: D'Amato, G., Bonini, S., Bousquet, J., Durham, S., Platts-Mills, T., (eds). Pollenosis: A global approach. *JGC Int*. Publisher. 2001.

D'Amato, G., Cecchi, L., Bonini, S., Nunes, C., Annesi-Maesano, I., Behrendt, H., Liccardi, G., Popov, T., Van Cauwenberge, P. (2007) Allergenic pollen and pollen allergy in Europe. *Allergy* **62:** 976–990.

D'Amato, G., Spieksma, F., Liccardi, G., Jaeger, S., Russo, M., Kontou-Fili, K., Nikkels, H., Wutrich, B., Bonini, S. (1998) Pollen related allergy in Europe. *Allergy* **53:** 567–578.

Dafni, A., Hesse, M., Pacini, E. (2000) Pollen and pollination. *Springer-Verlag Wien*, New York. 1-12.

de Weger, L. A., Beerthuizen, T., Gast-Strookman, J. M., van der Plas, D. T., Terreehorst, I., Hiemstra, P. S., Sont J. K. (2011). Difference in symptom severity between early and late grass pollen season in patients with seasonal allergic rhinitis. *Clin Transl Allergy* **1:** 18.

Diaz-Sanchez, S., Hanning, I., Pendleton, S., D'Souza, D. (2013) Next-generation sequencing: The future of molecular genetics in poultry production and food safety. *Poult Sci* **92:** 562–572.

Dickinson, H.G., Elleman, C.J., Doughty, J. (2000) Pollen coatings: Chimaeric genetics and new functions. *Sex. Plant Reprod* **12:** 302–309.

Dominguez, E., Mercado, J.A., Quesada, M.A., Heredia, A. (1999) Pollen sporopollenin: Degradation and structural elucidation. *Sex Plant Reprod* **12:** 171–178.

Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J.-P., Raoult D. (2000) 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J Clin Microbiol* **38:** 3623-3630.

Edlund, A.F., Swanson, R., Preuss, D. (2004) Pollen and stigma structure and function: The role of diversity in pollination. *Plant Cell* **16:** S84–S97.

Eimert, K., Rückert, F.E., Schröder, M-B. (2012) Genetic diversity within and between seedstock populations of several German autochthonous provenances and

conventionally propagated nursery material of blackthorn (Prunus spinosa L.). *Plant Syst Evol* **298:** 609–618.

Emberlin, J. (1998) The effect of air pollution on allergic pollen. *J Europian Resir Rev* **53:** 164–167.

Erdtman, G (1960) The acetolysis method- A revised description. *Svensk Bot Tidskr* **54:** 561-564.

Estrella, N., Menzel, A., Krämer, U., Behrendt, H. (2006) Integration of flowering dates in phenology and pollen counts in aerobiology: analysis of their spatial and temporal coherence in Germany (1992–1999). *Int J Biometeorol* **51:** 49.

Estrella, N., Menzel, A., Krämer, U., Behrendt, H. (2006) Integration of flowering dates in phenology and pollen counts in aerobiology: analysis of their spatial and temporal coherence in Germany (1992–1999). *Int J Biometeorol* **51:** pp 49–59

European Community Respiratory Health Survey. Variations in the prevalence of respiratory symptoms, selfreported asthma attacks and the use of asthma medications in the European Community Respiratory Health Survey (ECRHS). *Eur Respir J* 1996. **9:** 687–695.

Franklin-Tong, V.E. (1999) Signaling and the modulation of pollen tube growth. *Plant cell* 11:727-38.

Friedman, W.E., Williams, J.H. (2003) Modularity of the angiosperm female gametophyte and its bearing on the early evolution of endosperm in flowering plants. *Evolution* **57:** 216–230.

Gehrig, R., Gassner, M., Schmid-Grendelmeier, P. (2015) Alnus × spaethii pollen can cause allergies already at Christmas. *Aerobiologia* **31:** 239–247. DOI:*10.1007*/s10453-014-9360-4.

Gilles-Stein, S., Beck, I., Chake,r A., Bas, M., McIntyre, M., Cifuentes, L., Petersen, A., Gutermuth, J., Schmidt-Weber, C., Behrendt, H., Traidl-Hoffmann. (2016) Pollen derived low molecular compounds enhance the human allergen specific immune response *in vivo*. *Clin Exp Allergy* **46:** 1355–1365.

Grewling, L., Jenerowicz, D., Nowak, M., Polańska, A., Jackowiak, B., Czarnecka-Operacz, M., Smith M. (2014) Clinical relevance of *Corylus* pollen in Poznań, western Poland. *Ann Agric Environ Med* **21:** 64–9

Griffith, I.J., Smith, P.M., Pollock, J., Theerakulpisut, P., Avjioglu, A., Davies, S., Hough, T., Singh, M.B., Simpson, R.J., Ward, L.D., Knox, R.B. (1991) Cloning and sequencing of Lol p I, the major allergenic protein of rye-grass pollen. *FEBS Lett* **279:** 210–215.

Grönlund, H., Gafvelin, G. (2010) Recombinant Bet v 1 vaccine for treatment of allergy to birch pollen. *Hum Vaccines* **6:** 970–977.

Gumowski, P.I., Clot, B., Davet, A. Saad, S., Hassler, H., Dunoyer-Geindre, S. (2000) The importance of hornbeam (Carpinus sp.) pollen hypersensitivity in spring allergies *Aerobiologia* **16:** 83.

Hani, B., Dalila, B., Saliha, D., Daoud, H., Mouloud, G., Seddik, K. (2012) Microbiological sanitary aspects of pollen. *Adv Environ Biol* **6:**1415–1420.

Heslop-Harrison, J. (1979) Aspects of the structure, cytochemistry and germination of the pollen of rye. *Ann Bot* 44: 1–47.

Heydenreich, B., Bellinghausen, I., König, B., Becker, W.-M., Grabbe, S., Petersen, A., Saloga, J. (2012) Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses. *Clin Exp Allergy* **42:** 76–84.

Hirano, S.S., Upper, C.D. (2000) Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae*—a pathogen, ice nucleus, and epiphyte. *Microbiol Mol Biol Rev* **64:** 624–653.

Hirsch, T., Neumeister, V., Weiland, S.K, von Mutius, E., Hirsch, D., Grafe, H., Duhme, H., Leupold, W. (2000) Traffic exposure and allergic sensitization against latex in children. *J Allergy Clin Immunol* **106:** 573-8.

Hirschwehr, R., Valenta, R., Ebner, C., Ferreira, F., Sperr, W.R., Valent, P., Rohac, M., Rumpold, H., Scheiner, O., Kraft, D. (1992) Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J Allergy Clin Immunol* 90: 927-36.

Hopkins, C.Y., Jevans, A.W., Bock, R. (1969) Occurrence of octadecatrans-2,cis-9,cis-12 trienoic acid in pollen attractive to the honey bee. *Can J Biochem* 47: 433–436.

Höppner, F., Menge-Hartmann, U. (1995) Cultivation experiments with two fibre hemp varieties. *JIHA* **2:** 18-22.

Hosoki, K., Aguilera-Aguirre, L., Boldogh, I., Sun, Q., Sur, S. (2014) Ragweed Pollen Extract (RWPE)-Induces TLR4-Dependent Neutrophil Recruitment That Augments Allergic Airway Inflammation. *J Allergy Clin Immunol* **133:** AB59.

Human, H., Nicolson, S.W. (2006) Nutritional content of fresh, bee-collected and stored pollen of *Aloe greatheadii* var. davyana (*Asphodelaceae*). *Phytochemistry* **67:** 1486–1492.

Ipsen, H., Bøwadt, H., Janniche, H., Nüchel Petersen, B., Munch, E. P., Wihl, J.-A., Løwenstein, H. (1985) Immunochemical characterization of reference alder (*Alnus glutinosa*) and hazel (*Corylus avellana*) pollen extracts and the partial immunochemical identity between the major allergens of alder, birch and hazel pollens. *Allergy* **40:** 510–518.

Jahn-Schmid, B., Siemann, U., Zenker, A., Bohle, B., Messner, P., Unger, F.M., Sleytr, U.B., Scheiner, O., Kraft, D., Ebner, C. (1997) Bet v 1, the major birch pollen allergen, conjugated to crystalline bacterial cell surface proteins, expands allergen-specific T cells of the Th1/Th0 phenotype in vitro by induction of IL-12. *Int Immunol* **9:** 1867-74.

Kawase, M., Takahashi, M. (1995) Chemical composition of sporopollenin in *Magnolia grandiflora* (*Magnoliaceae*) and *Hibiscus syriacus* (*Malvaceae*). *Grana* **34:** 242-245.

Kim, S.B., Jo, Y.H., Liu, Q., Ahn, J.H., Hong, I.P., Han, S.M., Hwang, B.Y., Lee, M.K. (2015) Optimization of extraction condition of bee pollen using response surface methodology: correlation between anti-melanogenesis, antioxidant activity, and phenolic content. *Molecules* **20:** 19764–19774.

Knapp, B., Fischer, G., Van Hemelen, D., Fae, I., Maillere, B., Ebner, C., Schreiner, W., Bohle, B., Jahn-Schmid, B. (2012) Association of HLA-DR1 with the allergic response to the major mugwort pollen allergen: molecular background. *BMC Immunol* 13: 43.

Knief, C., Ramette, A., Frances, L., Alonso-Blanco, C., Vorholt, J.A. (2010) Site and plant species are important determinants of the *Methylobacterium* community composition in the plant phyllosphere. *ISME J* **4:** 719-728.

Kosenko, V.N. (1999) Contributions to the pollen morphology and taxonomy of the *Liliaceae*. Grana 38: 20–30.

Kumar, Manoj., Brader, G., Sessitsch, A., Mäki, A., van Elsas, J.D., Nissinen, R. (2017) Plants assemble species specific bacterial communities from common core taxa in three Arcto-Alpine climate zones. *Front Microbiol* **8:** 12.

Kupper, J., Rentsch, K., Mittelholzer, A., Artho, R., Meyer, S., Kupferschmidt, H., Naegeli, H. (2010) A fatal case of Autumn crocus (*Colchicum autumnale*) poisoning in a heifer: confirmation by mass-spectrometric colchicine detection. *J Vet Diagn Invest* **22:** 119–122.

Leben, C. (1972) Micro-organisms associated with plant buds. *J Gen Microbiol* **71:** 327-331.

Leff, J.W., Fierer, N. (2013) Bacterial communities associated with the surfaces of fresh fruits and vegetables. *PLoS ONE* **8:** e59310.

Lindow, S.E., Brandl, M.T. (2003) Microbiology of the phyllosphere. *Appl Environ Microbiol* **69:** 1875–1883.

Linskens, H.E., Jorde, W. (1997) Pollen as food and medicine--a review. *Econ Bot* **51:** 78-87.

Linskens, H.F., Jorde, W. (1997) Pollen as food and medicine-a review. *Econ Bot* **51:** 78–87.

Lozano-Vega, G., Benezeth, Y., Marzani, F., Boochs, F. (2014) Modular method of detection, localization, and counting of multiple-taxon pollen apertures using bag-of-words. *J Electron Imaging* **23:** pp053025.

Majd, A., Chehregani, A., Moin, M., Gholami, M., Kohno, S., Nabe, T., Shariatzade, M.A. (2004) The effects of air pollution on structures, proteins and allergenicity of pollen grains. *Aerobiologia* **20:** 111-118.

Marth, K., Germatuik, T., Swoboda, I., Valenta, R. (2014) Tree pollen allergens. In Lockey, R.F., Ledford, D.K (Eds). Allergens and Allergen Immunotherapy: Subcutaneous, Sublingual, and Oral, 5th ed. *CRC Press*, Boca Raton, FL, USA.

Mazinani, Z., Zamani, M., Sardari, S. (2017) Isolation and identification of phyllospheric bacteria possessing antimicrobial activity from *Astragalus obtusifolius*, *Prosopis juliflora*, *Xanthium strumarium* and *Hippocrepis unisiliqousa*. *Avicenna J Med Biotechnol* **9:** 31–37.

Merriman, B., R&D Team, I.T. and Rothberg, J.M. (2012) Progress in Ion Torrent semiconductor chip based sequencing. *ELECTROPHORESIS* **33:** 3397–3417.

Meurmann, O. (1991) ELISA in the clinical microbiology laboratory. In Wreghitt, T.G., Morgan-Capner, P (eds), Colindale: Public health laboratory service. *Cambridge University Press*, Great Britain. p 693.

Meyer, K.M., Leveau, J.H. (2012) Microbiology of the phyllosphere: a playground for testing ecological concepts. *Oecologia* **168:** 621–629.

Newman, D. J., Cragg, G.M. (2015) Endophytic and epiphytic microbes as "sources" of bioactive agents. *Front Chem* **3:** 34.

Nicolson, S.W., Human, H. (2013) Chemical composition of the 'low quality' pollen of sunflower (*Helianthus annuus, Asteraceae*). *Apidologie* **44:** 144–152.

Nongkhlaw, F.M., Joshi, S.R. (2015) Investigation on the bioactivity of culturable endophytic and epiphytic bacteria associated with ethnomedicinal plants. *J Infect Dev Ctries* **9:** 954–61.

Obersteiner, A., Gilles, S., Frank, U., Beck, I., H€aring, F., Erns, D., Rothballer, M., Hartmann, A., Traidl-Hoffmann, C., Schmid, M. (2016) Pollen-associated microbiome correlates with pollution parameters and the allergenicity of pollen. *Plos One* **11:** e0149545.

Okubo, T., Ikeda, S., Sasaki, K., Ohshima, K., Hattori, M., Sato, T., Minamisawa, K. (2014) Phylogeny and functions of bacterial communities associated with field-grown rice shoots. *Microbes Environ* **29:** 329–332.

Olaitan, P. B., Adeleke, O. E., and Ola, I. O. (2007) Honey: a reservoir for microorganisms and an inhibitory agent for microbes. *Afr Health Sci* **7:** 159–165.

Oldenburg, M., Petersen, A., Baur, X. (2011) Maize pollen is an important allergen in occupationally exposed workers. *J Occup Med Toxicol* **6:** 32.

Pablos, I., Wildner, S., Asam, C., Wallner, M., Gadermaier, G. (2016) Pollen Allergens for Molecular Diagnosis. *Curr Allergy Asthma Rep* 16: 31.

Pacini, E., Franchi, G.G. (1996) Some cytological, ecological and evolutionary aspects of pollination. *Acta Soc Bot Pol* **65:** 11–16.

Pahkala, K., Pahkala, E., Syrjälä, H. (2008) Northern limits to fiber Hemp production in Europe. *JIH* **13:** 104-116.

Pawankar, R., Canonica, G.W., Holgate, S.T., Lockey, R.F., Blaiss. M. (2013) Section 3.5 in WAO white book on allergy update. D'Amato, G., Rottem, M. (Eds). *World Allergy Organization* pp 95-100.

Phenology and human health: Allergic disorders. Report on a WHO meeting, Rome, Italy, 16-17. January 2003. www.euro.who.int/__data/assets/pdf_file/0013/240232/e79129.pdf

Rastogi, G., Coaker, G.L., Leveau J.H.J. (2013) New insights into the structure and function of phyllosphere microbiota through high-throughput molecular approaches. *FEMS Microbiol Lett* **348:** 1–10.

Robert, M., Naclerio, M.D. (1991) Allergic Rhinitis. *N Engl J Med* **325:** 860-869.

Rodríguez, C.E., Mitter, B., Barret, M., Sessitsch, A., and Compant, S. (2017) Commentary: seed bacterial inhabitants and their routes of colonization. *Plant Soil.* pp1–6.

Roulston, T.H., Cane, J.H. (2000) Pollen nutritional content and digestibility for animals. *Plant syst evol* **222:** 187-209.

Roversi, A., Ughini, V. (1996) nfluence of weather conditions of the flowering period on sweet cherry fruit set. *Acta Hortic* **410:** 427-434.

Schwendemann, A.B., Wang, G., Mertz, M.L., McWilliams, R.T., Thatcher, S.L., Osborn, J.M. (2007) Aerodynamics of saccate pollen and its implications for wind pollination. *Am J Bot* **94:** 1371-81.

Scortichini M., Katsy E.I. (2014) Common themes and specific features in the genomes of phytopathogenic and plant-beneficial bacteria. In: Katsy, E. (ed) Plasticity in plant-growth-promoting and phytopathogenic bacteria. *Springer*, New York, NY.

Shade, A., Jacques, M-A., and Barret, M. (2017) Ecological patterns of seed microbiome diversity, transmission, and assembly. *Curr Opin Microbiol* **37:** 15–22.

Shade, A., Jacques, M-A., Barret, M. (2017) Ecological patterns of seed microbiome diversity, transmission, and assembly. *Curr Opin Microbiol* **37:** 15–22.

Shukla, A.K., Vijayaraghavan, M.R.., Chaudhry, B. (1998). "Abiotic pollination". Biology of pollen. *APH Publishing*, New Delhi. pp. 67–69.

Siegmund, J.F., Wiedermann, M., Donges, J.F., Donner, R.V. (2015) Impact of climate extremes on wildlife plant flowering over Germany. *Biogeosciences Discuss* **12:** 18389–18423.

Singer, B.D., Ziska, L.H., Frenz, D.A., Gebhard, D.E., Straka, J.G. (2005) Increasing Amb a 1 content in common ragweed (Ambrosia artemisiifolia) pollen as a function of rising atmospheric CO2 concentration. *Funct Plant Biol* **32:** 667–670.

Skjøth, C. A., Sommer, J., Stach, A., Smith, M., Brandt, J. (2007) The long-range transport of birch (*Betula*) pollen from Poland and Germany causes significant pre-season concentrations in Denmark. *Clin Exp Allergy* **37:** 1204–1212.

Skoner, D.P. (2001) Allergic rhinitis: definition, epidemiology, pathophysiology, detection, and diagnosis. *J Allergy Clin Immunol* **108:** S2–S8. DOI:*10.1067*/mai.2001.115569.

Songnuan, W. (2013) Wind-pollination and the roles of pollen allergenic proteins. *Asian Pac J Allergy Immunol* **31:** 261-70.

Spangenberg, G., Petrovska, N., Kearney, G.A., Smith, K.F. (2006) Low-pollen-allergen ryegrasses: Towards a continent free of hay fever? In Gilissen, L.J.E.J., Wichers, H.J., Savelkoul, H.F.J. Bogers, R.J. (eds) Allergy Matters: New Approaches to Allergy Prevention and Management, vol. 10. *Wageningen*, Wageningen UR. pp.121–28.

Spieksma, F.T., Charpin, H., Nolard, N., Stix, E. (1980) City spore concentrations in the European Economic Community (EEC). IV. Summer weed pollen (rumex, plantago, *Chenopodiaceae*, artemisia), 1976 and 1977. *Clin Exp Allergy* **10:** 319–329.

Staley, J.T., Konopka, A. (1985) Measurement of in situ activities of non-photosynthetic microorganisms in aquatic and terrestrial habitats. *Ann Rev Microbiol* **39:** 321-346.

Su, C., Lei, L., Duan, Y. Zhang, K-Q., Yang, J. (2012) Culture-independent methods for studying environmental microorganisms: methods, application, and perspective. *Appl Microbiol Biotechnol* **93:** 993.

Szymajda, M., Zurawicz, E. (2017) 'Kalipso' European Plum. *Hortscience* **52:** 316–319.

Tanaka, N., Uehara, K., Murata, J. (2004) Correlation between pollen morphology and pollination mechanisms in *Hydrochariataceae*. *J Plant Res* **117:** 265–276.

The International Study of Asthma and Allergy in Childhood (ISAAC). Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis and atopic eczema. *Lancet* 1998. **351**: 1225–1232.

Truyens, S., Weyens, N., Cuypers, A., Vangronsveld, J. (2015) Bacterial seed endophytes: genera, vertical transmission and interaction with plants. Environ Microbiol Rep **7:** 40–50.

Varga, A., Budai, M. M., Milesz, S., Bácsi, A., Tőzsér, J. and Benkő, S. (2013) Ragweed pollen extract intensifies lipopolysaccharide-induced priming of NLRP3 inflammasome in human macrophages. *Immunology* **138:** 392–401.

Vorholt, J.A. (2012) Microbial life in the phyllosphere. *Nat Rev Microbiol* **10:** 828–840.

Wang, N., Qian, W., Suppanz, I., Wei, L., Mao, B., Long, Y., Meng, J., Muller, A.E., Jung, C. (2011) Flowering time variation in oilseed rape (*Brassica napus* L.) is associated with allelic variation in the FRIGIDA homologue BnaA.FRI.a. *J Exp Bot* **62:** 5641–5658.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173:** 697–703.

Whipps, J.M., Hand, P., Pink, D. Bending, G.D. (2008) Phyllosphere microbiology with special reference to diversity and plant genotype. *J Appl Microbiol* **105:** 1744–1755.

Whipps, J.M., Hand, P., Pink, D., Bending, G.D. (2008) Phyllosphere microbiology with special reference to diversity and plant genotype. *J App Microbiol* **105:** 1744–1755.

Whitaker, B. K., Bauer, J. T., Bever, J. D., Clay, K. (2017) Negative plant-phyllosphere feedbacks in native Asteraceae hosts – a novel extension of the plant-soil feedback framework. *Ecol Lett* **20:** 1064–1073.

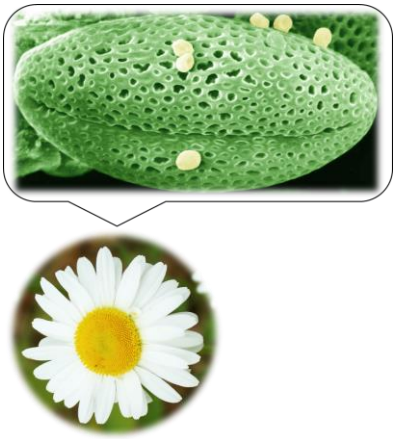
Wopfner, N., Gadermaier, G., Egger, M., Asero, R., Ebner, C., Jahn-Schmid, B., Ferreira, F. (2005) The spectrum of allergens in ragweed and mugwort pollen. *Int Arch Allergy Immunol* **138:** 337-346.

Yu, X., Yang, J., Wang, E., Li, B., Yuan, H. (2015) Effects of growth stage and fulvic acid on the diversity and dynamics of endophytic bacterial community in *Stevia rebaudiana* Bertoni leaves. *Front Microbiol* **6:** 867.

Zasloff, M. (2017) Pollen has a microbiome: implications for plant reproduction, insect pollination and human allergies. *Environ Microbiol* **19:** 1–2.

Žiarovská, J., Zeleňáková, L. (2016) Central and Eastern European spring pollen allergens and their expression analysis—state of the art. *Diversity* **8:** 19.

Zuberbier, T., Lötvall, J., Simoens, S., Subramanian ,S.V., Church, M.K. (2014) Economic burden of inadequate management of allergic diseases in the European Union: a GA2LEN review. *Allergy* **69:** 1275–1279.



Chapter 2

Extended summary

2.1. Short introduction

Pollen allergy is a major public health problem which has been found to further increase every year (Platts-Mills *et al.*, 2015). In Europe, the prevalence of pollen allergy was estimated around forty percentage of the population (D'Amato *et al.*, 2007) while the economic impact of allergic diseases is between 55 and 151 billion euro per annum (Zuberbier *et al.*, 2014). Plant pollen is a microhabitat for diverse bacterial biota and this includes a number of Gram-positive and negative cells (Ambika Manirajan *et al.*, 2016). Spiewak *et al.*, (1996) suggested that the endotoxins of pollen associated bacterial cells may impair pollinosis.

Endotoxin has been associated in the pathogenesis of a variety of different clinical conditions. The study of endotoxin started since 19th century by Richard Pfeiffer and given the term 'endotoxin' for the heat stable toxin found from heat inactivated *Vibrio cholera* (Bayston and Cochen, 1990). Lipoteichoic acid (LTA) and lipopolysaccharide (LPS) are the major endotoxins produced by Gram-positive and Gram-negative bacterial species which induce host immune responses (Dai *et al.*, 2014; Parolia *et al.*, 2014; Rosenfeld *et al.*, 2006). Moreover, lipoproteins derived from Gram-positive bacteria also stimulate host immune response (Hashimoto *et al.*, 2006). They are major class of surface proteins which can modulate allergen-specific effector cells in an allergic response (Revets *et al.*, 2005). Endotoxin of Gram-negative bacteria (LPS) consists of a hydrophilic heteropolysaccharide part and a covalently linked hydrophobic lipid portion anchored in the outer membrane. The LPS molecule commonly consists of three structural components: lipid A, a non-repeating core oligosaccharide and O-antigen (Raetz and Whitfield, 2002; Bayston and Cochen, 1990; Rietschel *et al.*, 1982). The hydrophobic region consists of lipid A portion which is responsible for the toxic biological effect (Steimle *et al.*, 2016; Watson and Kim, 1963) and the O-antigen which is associated with the virulence property (Mäkelä *et al.*, 1973).

Lipoteichoic acid is an amphiphilic glycopolymer with a hydrophilic chain of glycerol-phosphate unit linked to glycolipid anchor. Lipoteichoic acids are commonly composed of hydrophilic repetitive glycerophosphate units and D-alanine or hexose substituents as well as a lipophilic glycolipid anchor (Schneewind and Missiakas, 2014; Morath *et al.*, 2005). The glycolipid anchors of LTA are the crucial molecules that trigger the immunity (Jang *et al.*, 2011).

2.2. Results

2.2.1. Cultivation-dependent analysis of bacterial microbiota

2.2.1.1. CFU determination, isolation and identification of bacteria from plant pollen species

The total colony count of pollen bacterial populations ranged average from 3.8×10^5 CFU g^{-1} (in hazel) to 8.5×10^8 CFU g^{-1} (in blackthorn). The trend of CFU shows that the allergenic pollen producing plants (birch, winter rye, mugwort and hazel) have lower CFU numbers than low allergenic plants (winter rapeseed, autumn crocus, cherry plum, blackthorn and hemp). The numbers of CFUs in different plant pollen species were found significantly different from each other (Kruskal-Wallis test $p<0.05$). Moreover, the abundance of cultivable bacterial taxa also showed significant differences (Student's t-test $p=0.02$) between high allergenic and less/non allergenic plant pollen species (Fig 1A). Colony morphologies and colony counts were found similar in both AC medium and pollen enriched medium. A total of 157 morphologically different bacterial colonies were isolated from nine different plant pollen species (16 from birch, 18 from winter rye, 21 from mugwort, 20 from hazel, 15 from winter rapeseed, 12 from autumn crocus, 20 from cherry plum, 15 from blackthorn and 20 from hemp) on the two different agar media (All Culture agar media and pollen enriched agar media) (Table S1, Chapter 6). These isolates were members of 27 different bacterial family. Among these, the family *Microbacteriaceae* was found in all the plant pollen species while *Flavobacteriaceae* and *Rhizobiaceae* were found only in birch. *Nocardioidaceae* and *Xanthomonadaceae* were found only in winter rye and *Erwiniaceae* and *Morganellaceae*

were found only in hemp. *Paenibacillaceae*, *Enterococcaceae*, *Brevibacteriaceae* and *Kineosporiaceae* were found only in hazel, winter rapeseed, blackthorn and cherry plum respectively. Allergenic pollen isolates showed a higher number of different families than low allergenic pollen species except in cherry plum (Fig 1B).

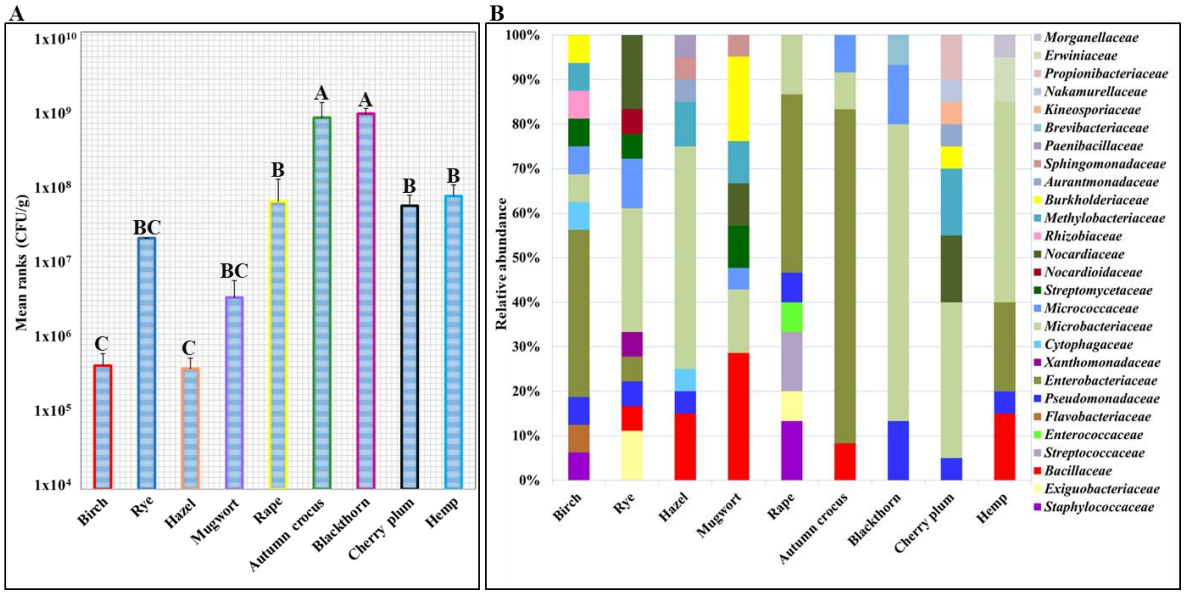


Fig. 1. (A) Numbers of cultivable bacteria on flower pollen (CFUs per Gram of dry pollen weight). The values are means of three samples per pollen species (n=3±SE). Kruskal–Wallis test, p<0.003. Different letters indicate significantly different means (Tukey test, p<0.05). (B) Relative abundance of cultivated bacterial families from birch, winter rye, hazel, mugwort, winter rapeseed, autumn crocus, blackthorn, cherry plum and hemp pollen.

2.2.2. Cultivation-independent analysis of bacterial microbiota

2.2.2.1. Bacterial community composition

Taxonomic affiliation was assigned using UCLUST with the aligned representative set of sequences of the SILVA 128 database (Quast *et al*., 2013). *Proteobacteria* was found as the major phyla in plant pollen habitat followed by *Firmicutes* and *Actinobacteria*. Moreover, *Acidobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Gemmatimonadetes*, *Chloroflexi* and a few percentages of unclassified OTUs were also found in a low percentage (Fig 2).

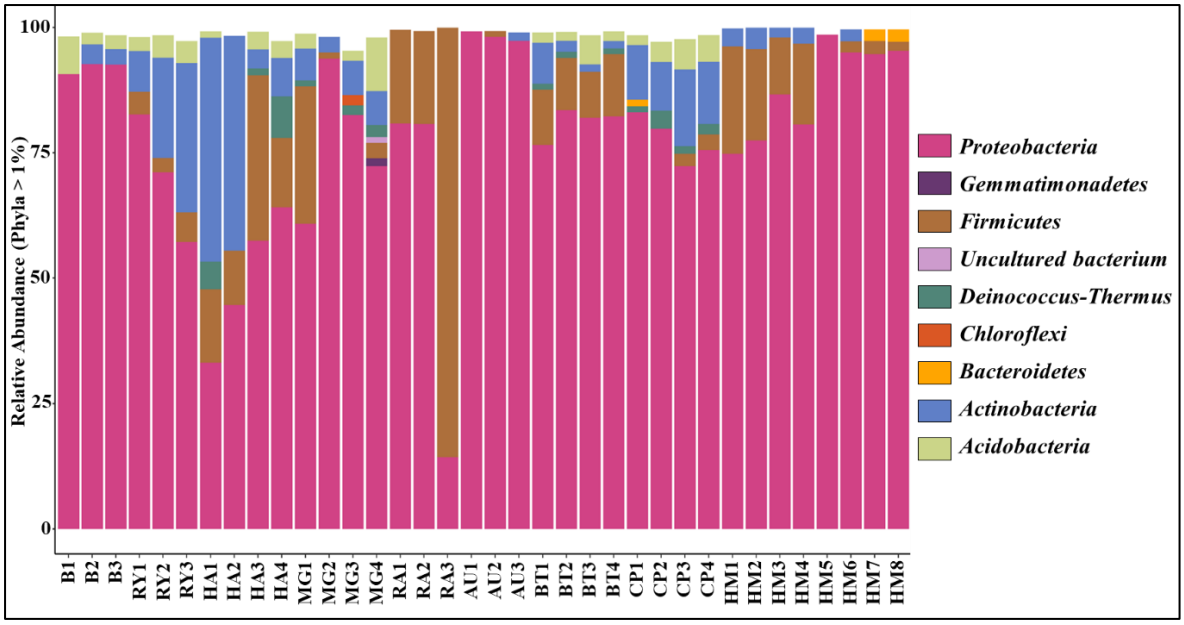


Fig 2. Bacterial phylum level profile in the pollen samples, according to 16S rRNA gene amplicon library sequencing; only phylum with a relative abundance of >1% are shown. B-birch, RY-winter rye, HA-hazel, MG- mugwort, RA-winter rapeseed, AU-autumn crocus, BT-blackthorn, CP-cherry plum and HM-hemp (HM1-4 variety *felina* and HM5-8 variety *finola*) (including replicates).

Enterobacteraceae and *Pseudomonadaceae* were the abundant bacterial families in low allergenic plants. When considering the hemp pollen habitat, *Enterobacteraceae*, *Pseudomonadaceae* and *Streptococcaceae* were found in the variety *felina*. *Pseudomonadaceae*, *Enterobacteraceae* and *Acetobacteraceae* were found in the variety *finola*. Furthermore, the diversity of bacterial family was low in low allergenic plant pollen habitat. *Acetobacteraceae*, *Beijerinckiaceae* and *Enterobacteraceae* were the most abundant bacterial families in birch while *Oxalobacteraceae* and *Xanthomonadaceae* were found in winter rye. Moreover, *Bacillaceae*, *Methylobacteriaceae*, *Acetobacteraceae* and *Pseudomonadaceae* were found abundant in hazel pollen. In mugwort pollen, *Acetobacteraceae* and *Bradyrhizobiaceae* were the dominant bacterial families. (Fig 3).

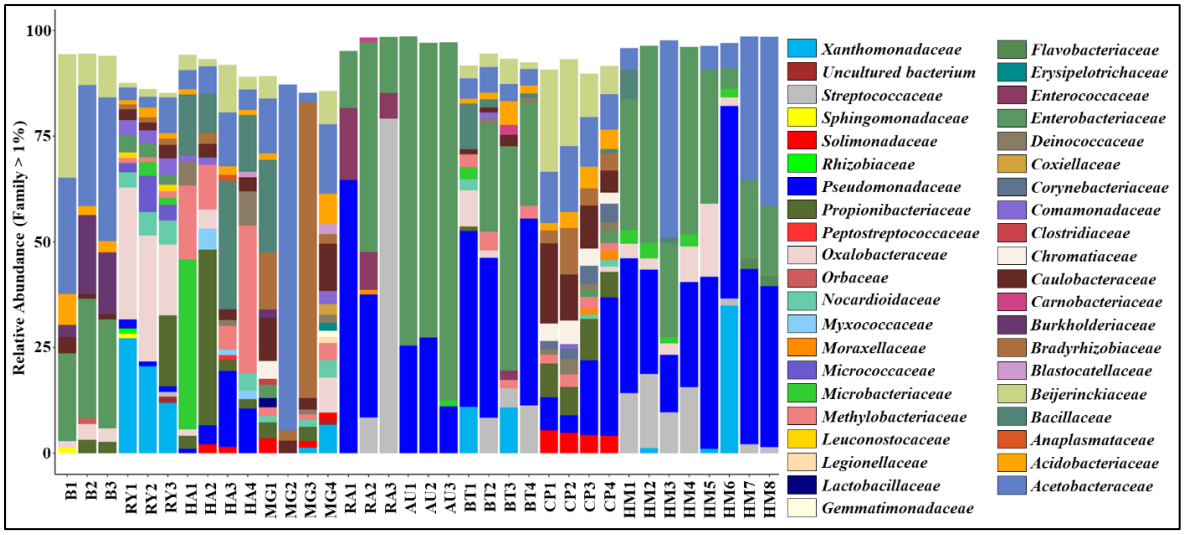


Fig 3. Bacterial family level profile in the pollen samples, according to 16S rRNA gene amplicon library sequencing; only family with a relative abundance of >1% are shown. B-birch, RY-winter rye, HA-hazel, MG- mugwort, RA-winter rapeseed, AU-autumn crocus, BT-blackthorn, CP-cherry plum and HM-hemp (HM1-4 variety *felina* and HM5-8 variety *finola*) (including replicates).

2.2.2.2. Alpha-diversity

All the three calculated alpha diversity values (Shannon's diversity index, phylogenetic diversity and observed species) were significantly different between plant pollen species (ANOVA, $p<0.05$). The calculated values of observed richness, Shannon index and phylogenetic diversity were significantly higher in mugwort and lower in autumn crocus (Fig 4). High allergenic pollen species led to a significant upturn in alpha diversity based on Shannon's diversity index, phylogenetic diversity and observed species (*t*-test, $p<0.05$) (Fig 5).

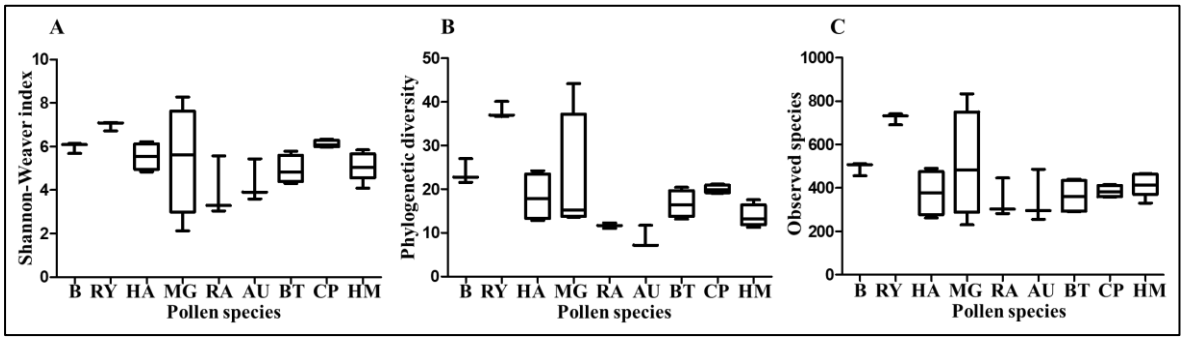


Fig 4. Alpha diversity indices of bacterial microbiota based on OTU_{97} (A) Shannon–Weaver (B) phylogenetic diversity and (B) Observed species, according to plant pollen species

(ANOVA, p<0.05). B=birch; RY=winter rye; HA=hazel; MG=mugwort; RA=winter rapeseed; AU=autumn crocus; BT=blackthorn; CP=cherry plum; HM=hemp.

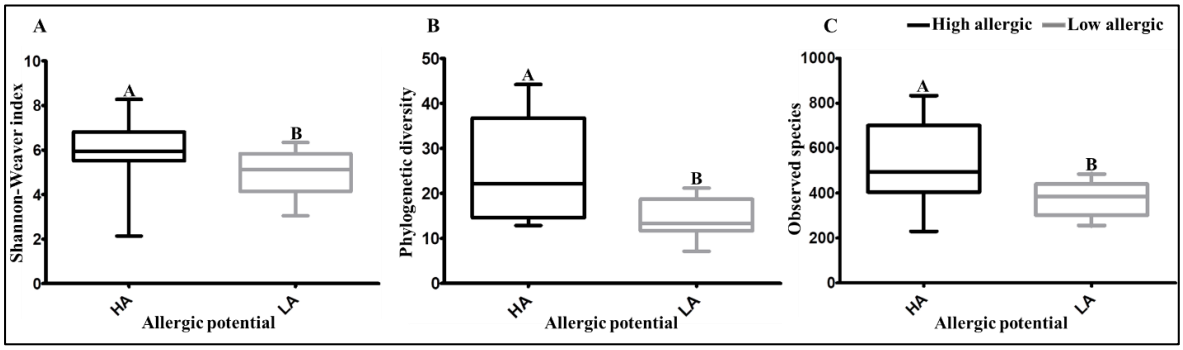


Fig 5. Alpha diversity indices of bacterial microbiota based on OTU_{97} (A) Shannon–Weaver (B) phylogenetic diversity and (B) Observed species, according to allergenic potential. Different letters indicate significantly different means (Tukey test, p<0.05). HA= high allergenic (black); LA= less/non Allergenic (grey).

2.2.2.3. Beta-diversity

The results of beta diversity (Bray-Curtis distances) using non-metric multidimensional scaling (NMDS) were significantly affected by the factor "pollen species" (ADONIS, R^2=0.630, *p*=0.001) (Fig 6) and the factor "allergenic potential" (ADONIS, R^2=0.155, *p*=0.002) (Fig 6A). The factor "collection site" (ADONIS, R^2=0.169, *p*=0.208) was not affected the beta-diversity metrics of pollen bacterial biota (Fig 6B). Moreover, the hemp (wind pollinated low allergenic pollen species) were grouped with less/non- allergenic insect pollinated pollen species.

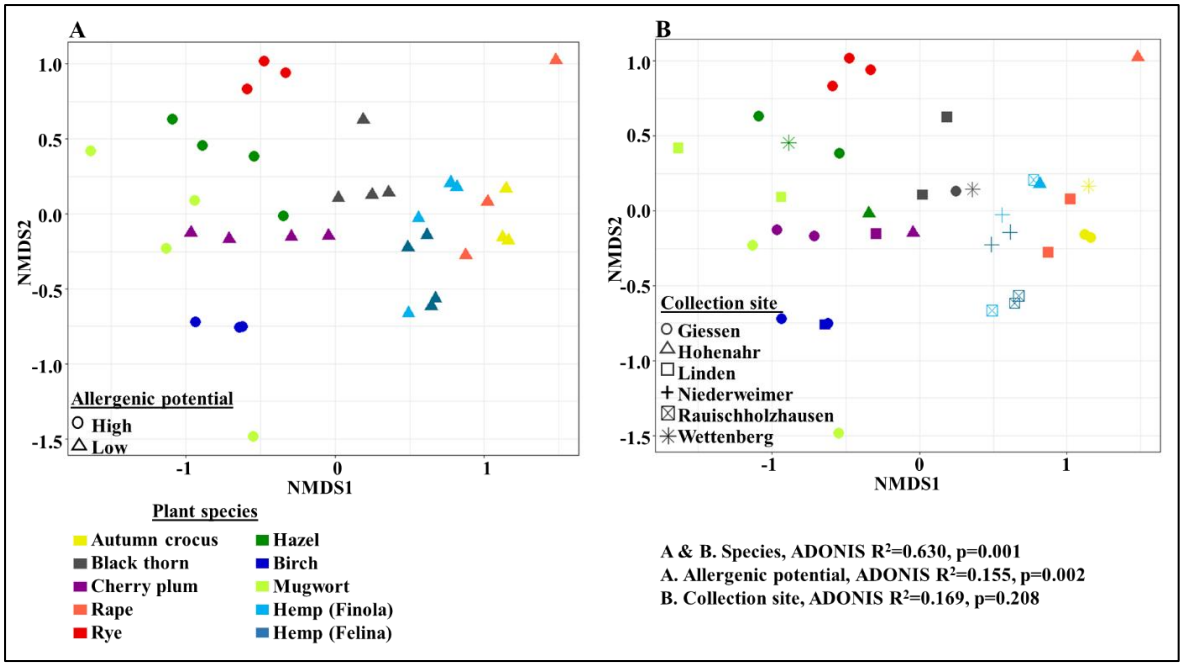


Fig 6. NMDS plots for bacterial microbiota structure based on Bray-Curtis distance. Samples are coloured by plant species. Shapes represent (A) Allergenic potential and (B) Collection site.

2.2.2.4. Scanning electron microscopy (SEM).

Scanning electron microscopic images of allergenic and low allergenic pollen revealed the evidence of surface colonization of bacteria in the tectum of plant pollen. The bacteria were found in the form of single cells, clusters and biofilm on the tectum of pollen. The size of the allergenic pollen species was smaller than low allergenic pollen. The size and shape, as well as pattern of tectum of different pollen species, exhibited drastic differences (Fig 7).

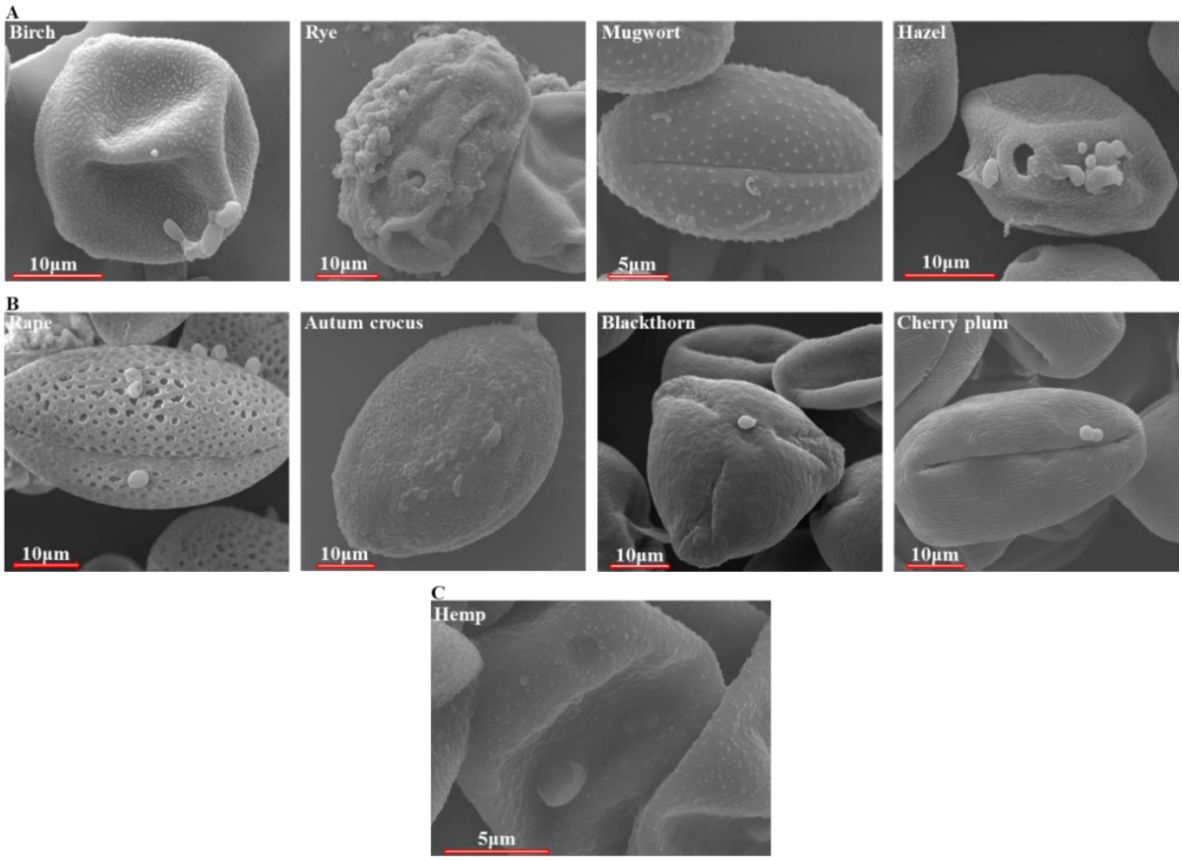


Fig 7. Scanning electron microscopy images showing the size difference between pollen species, pollination type and bacterial colonization pattern. (A) High allergenic pollen species (Wind-pollinated) (B) Less/non allergenic pollen species (Insect-pollinated) (C) Less/non allergenic pollen species (Wind-pollinated).

2.2.3. Cultivation-independent analysis of fungal microbiota

2.2.3.1. Fungal community composition

Ascomycota and *Davidiellaceae* were the major phylum and family found in pollen microhabitat respectively (Fig 8&9). *Tremellaceae* was found in all the high allergic pollen species while *Dothioraceae* was found in birch, winter rye, hazel and mugwort as well as a few in blackthorn. *Leptosphaeriaceae* was observed in hazel, winter rye, autumn crocus, blackthorn, and cherry plum. *Mycosphaerellaceae* and *Erysiphaceae* were found only in hazel, and mugwort respectively. *Metschnikowiaceae* was found in autumn crocus and winter rapeseed (Fig 9).

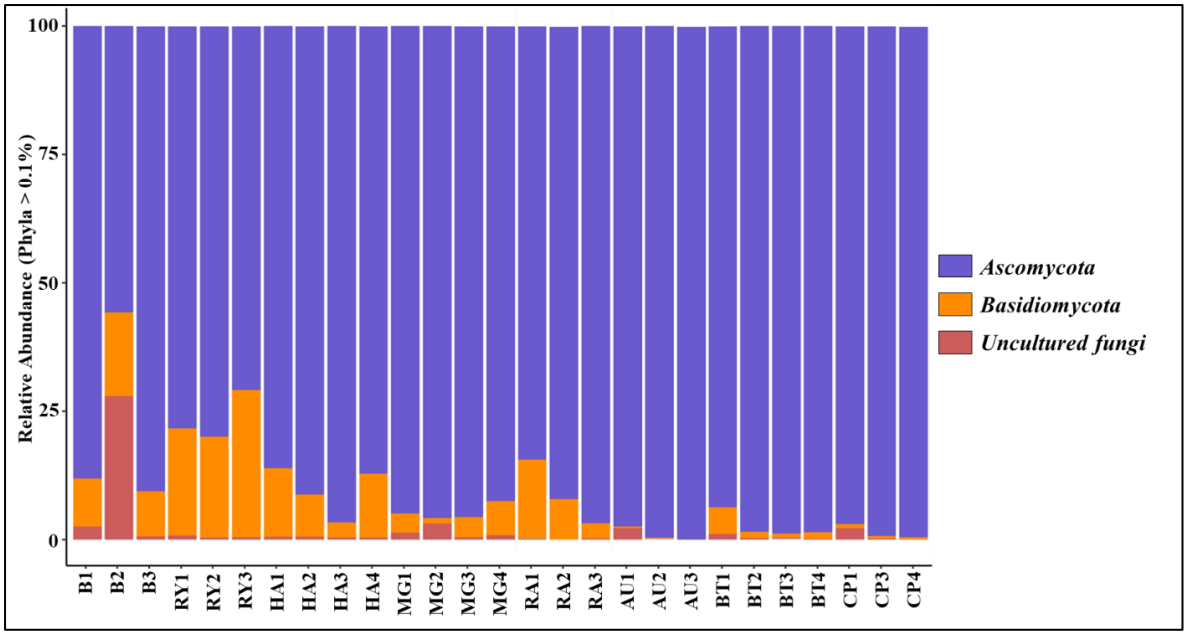


Fig 8. Fungal phylum level profile in the pollen samples, according to 16S rRNA gene amplicon library sequencing; only phylum with a relative abundance of >1% are shown. B-birch, RY-winter rye, HA-hazel, MG- mugwort, RA-winter rapeseed, AU-autumn crocus, BT-blackthorn, CP-cherry plum and HM-hemp (HM1-4 variety *felina* and HM5-8 variety *finola*) (including replicates).

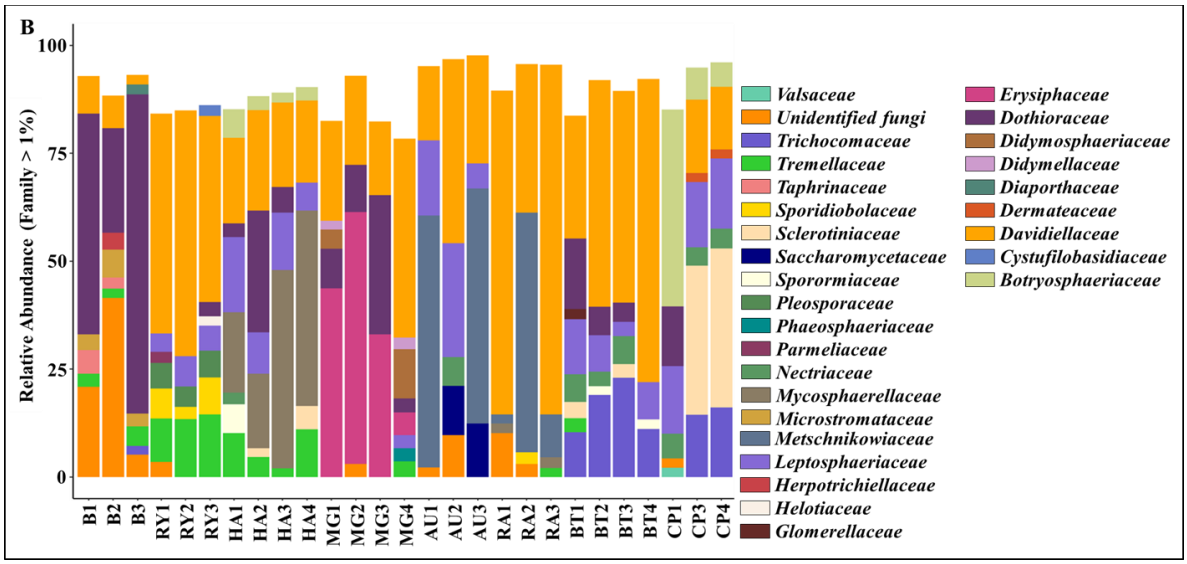


Fig 9. Fungal family level profile in the pollen samples, according to ITS2 gene amplicon library sequencing; only families with a relative abundance of >1% are shown. B-birch, RY-winter rye, HA-hazel, MG- mugwort, RA-winter rapeseed, AU-autumn crocus, BT-blackthorn and CP-cherry.

2.2.3.2. Alpha-diversity

All the three calculated alpha diversity values (Shannon's diversity index, phylogenetic diversity and observed species) were significantly different between plant pollen species

(ANOVA, p<0.05). The calculated values of observed richness, Shannon index and phylogenetic diversity were significantly higher in winter rye and lower in autumn crocus (Fig 10). High allergenic pollen species led to a significant upturn in alpha diversity based on Shannon's diversity index, phylogenetic diversity and observed species (t-test, p<0.05) (Fig 11).

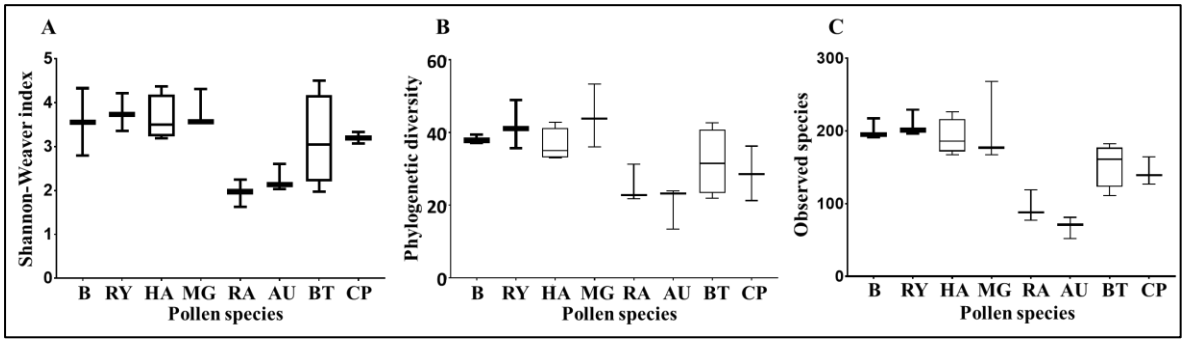


Fig 10. Alpha diversity indices of fungal microbiota based on OTU_{97} (A) Shannon–Weaver (B) phylogenetic diversity and (B) Observed species, according to plant pollen species (ANOVA, p<0.05). B=birch; RY=winter rye; HA=hazel; MG=mugwort; RA=winter rapeseed; AU=autumn crocus; BT=blackthorn; CP=cherry plum.

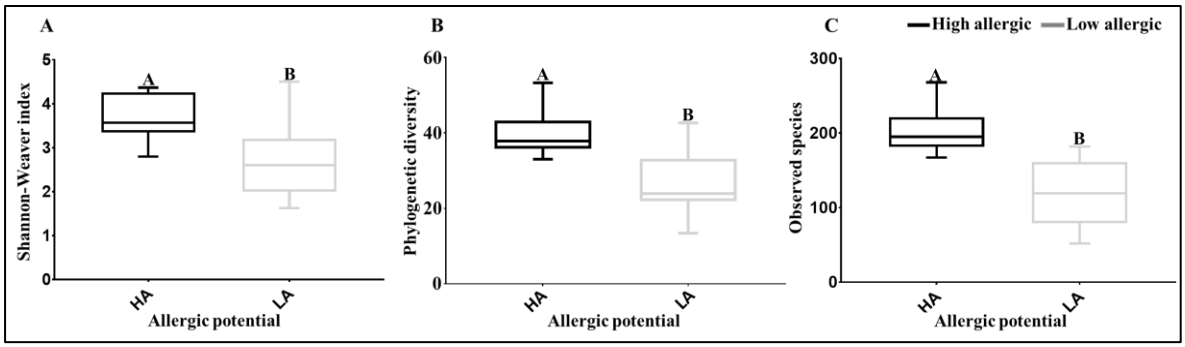


Fig 11. Alpha diversity indices of fungal microbiota based on OTU_{97} (A) Shannon–Weaver (B) phylogenetic diversity and (B) Observed species, according to allergenic potential. Different letters indicate significantly different means (Tukey test, p<0.05). HA= high allergenic (black); LA= less/non allergenic (grey).

2.2.3.3. Beta-diversity

The results of beta diversity (Bray-Curtis distances) using non-metric multidimensional scaling (NMDS) were significantly affected by the factor "pollen species" (ADONIS, R^2=0.575, p=0.001) (Fig 12) and the factor "allergenic potential" (ADONIS, R^2=0.143,

p=0.003) (Fig 12A). The factor "collection site" (ADONIS, R^2=0.037, *p*=0.734) was not affected to the beta-diversity metrics of pollen bacterial biota (Fig 12B).

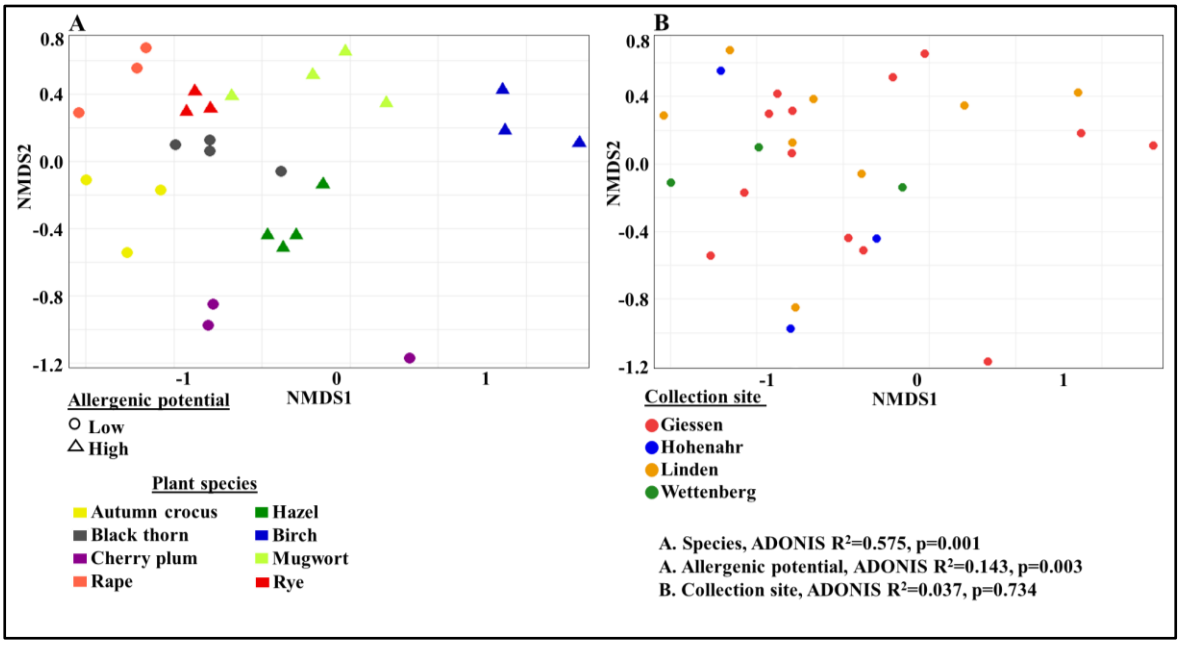


Fig 12. NMDS plots for fungal microbiota structure based on Bray-Curtis distance. Samples are coloured by plant species. (A) Shapes represent - Allergenic potential and (B) Different colour represent - Collection site.

2.2.4. Core-microbiome

The pollen microhabitate of nine pollen species contained a core bacterial biome including 12 bacterial genera. The most abundant core genera were Pseudomonas and Rosenbergiella. Most of the core bacterial community patterns were distributed extremely different between the plant pollen species (Fig 13).

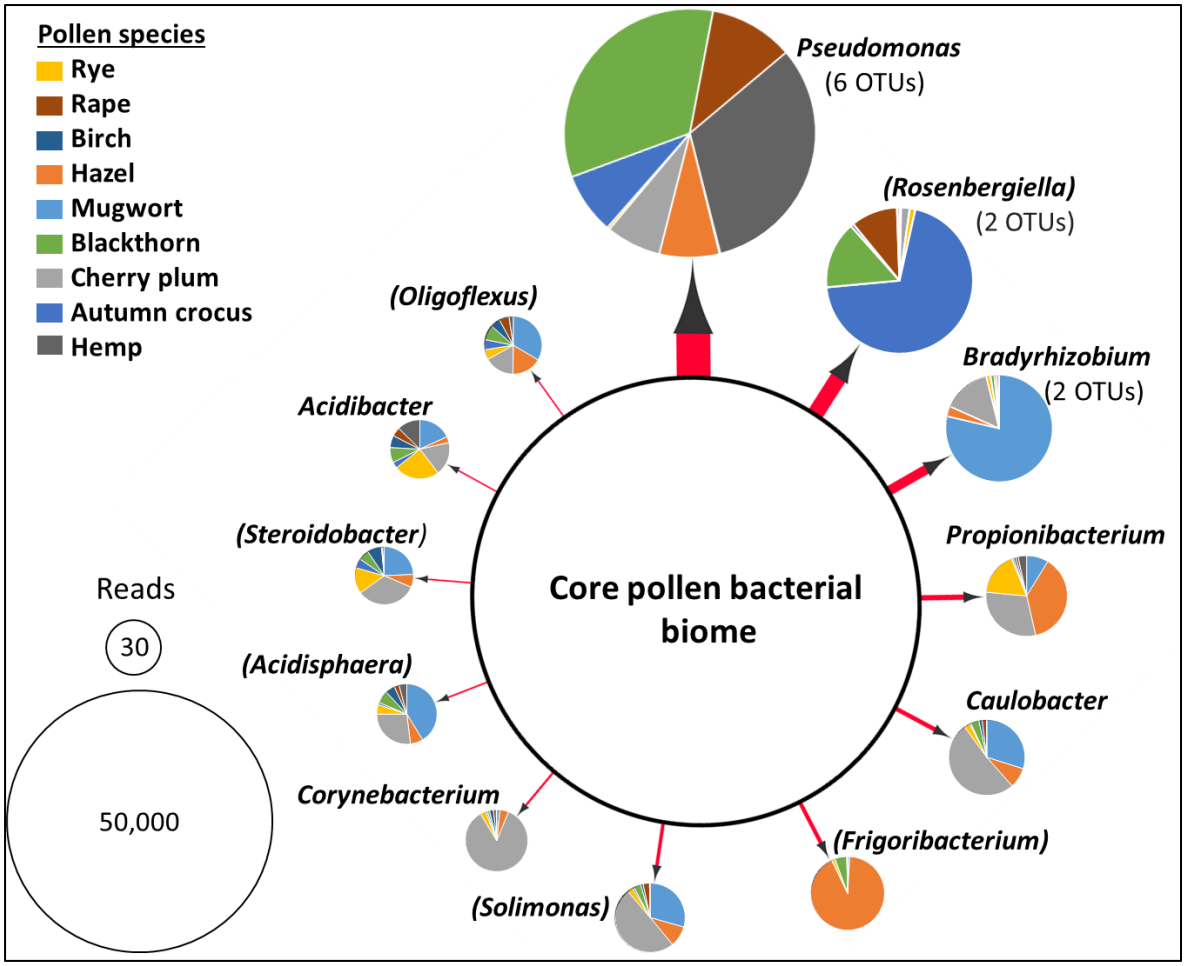


Fig 14. Structure and abundance of the pollen bacterial core microbiome, defined as the OTUs (97% similarity level) detected in all nine pollen species, grouped by genus. Pies are coloured by pollen species and show the distribution of the respective core genera. Pie size and edge width indicate the absolute abundance (number of reads) of the respective OTUs, according to the legend.

2.2.5. Endotoxin quantitative determination assay

2.2.5.1. Lipopolysaccharide ELISA (LPS-ELISA)

The results of LPS-ELISA quantitative determination assay of high allergenic and low allergenic pollen samples showed significantly high lipopolysaccharide concentration in high allergenic pollen samples (*t*-test, P<0.0001). Mugwort pollen samples exhibited the highest LPS quantities while the autumn crocus showed the lowest. Moreover, hemp pollen samples had a medium level of LPS concentration (Fig 14A). The result of LPS-ELISA quantitative determination assay of the different bacterial species, isolated from high allergenic and low

allergenic pollen samples, revealed significantly high lipopolysaccharide concentrations in bacterial species, isolated from high allergenic pollen samples (*t*-test, P<0.0001) (Fig 14B).

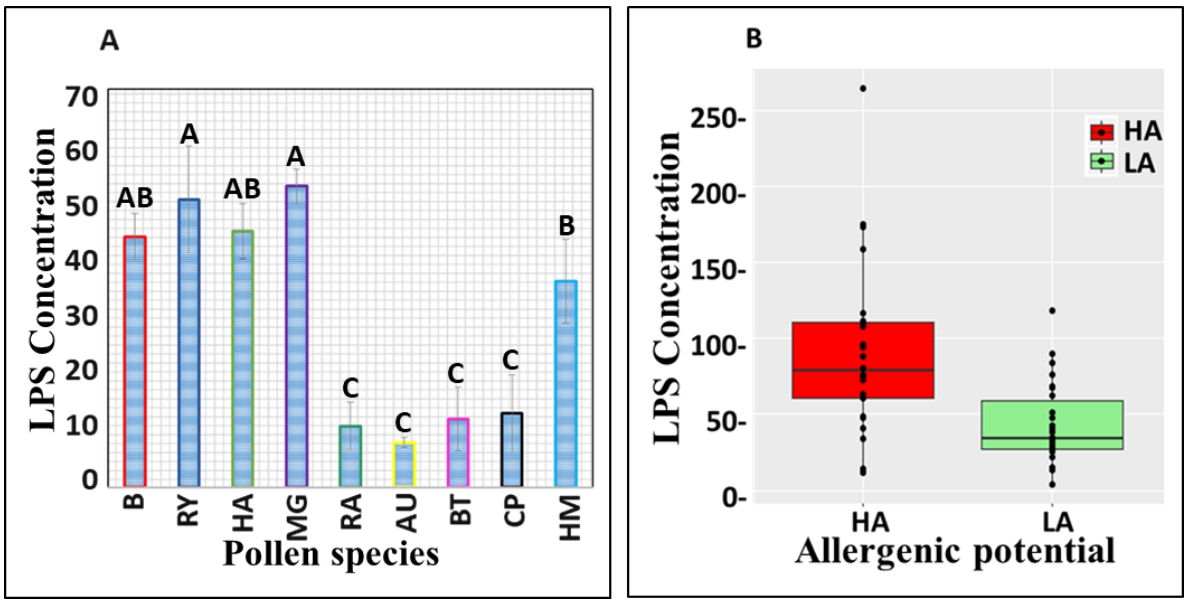


Fig 14. (A) LPS-ELISA quantitative determination assay of different pollen species (between allergenic potential: *t*-test, P<0.0001). Different letters indicate significantly different means (Tukey test, $p<0.05$). (B) Mean of LPS concnetrations of all bacterial strains isolated from high allergenic and low allergenic pollen samples (ng/ml) (*t*-test, P<0.0001). B=birch; RY=winter rye; HA=hazel; MG=mugwort; RA=winter rapeseed; AU=autumn crocus; BT=blackthorn; CP=cherry plum; HM=hemp. HA= high allergenic pollen; LA= low allergenic pollen.

2.2.5.2. Lipoteichoic acid ELISA (LTA-ELISA)

The results of LTA-ELISA quantitative determination assay of high allergenic and low allergenic pollen samples showed significantly high lipoteichoic acid concentration in high allergenic pollen samples (*t*-test, P<0.0001). Nevertheless, birch pollen samples accounted for the highest LTA quantity and the cherry plum pollen samples with the lowest LTA quantity. Moreover, hemp pollen samples had a medium level of LPS concentration (Fig 15A). The result of LTA-ELISA quantitative determination assay of the different bacterial strains isolated from high allergenic and low allergenic pollen samples show significantly high lipoteichoic acid concentrations in bacterial species, isolated from high allergenic pollen samples (*t*-test, P=0.06) (Fig 15B). Even though, the five isolates (*Clavibacter michiganensis*, *Brevibacterium frigoritolerans*, *Agreia pratensis*, *Amnibacterium soli* and

Frondihabitans sucicola) from LA pollen samples showed higher value in LTA-ELISA, they showed very little reads in high throughput sequencing in the specific pollen species. Moreover, out of the ten isolates from HA pollen samples, eight isolates [*Arthrobacter oryzae*, *Bacillus simplex*, *Clavibacter michiganensis*, *Rathayibacter festucae*, *Amnibacterium kyonggiense*, *Bacillus aerophilus* and *Bacillus safensis* (two isolates)] exhibited a comparatively high number of reads in high-throughput sequencing in specific pollen species.

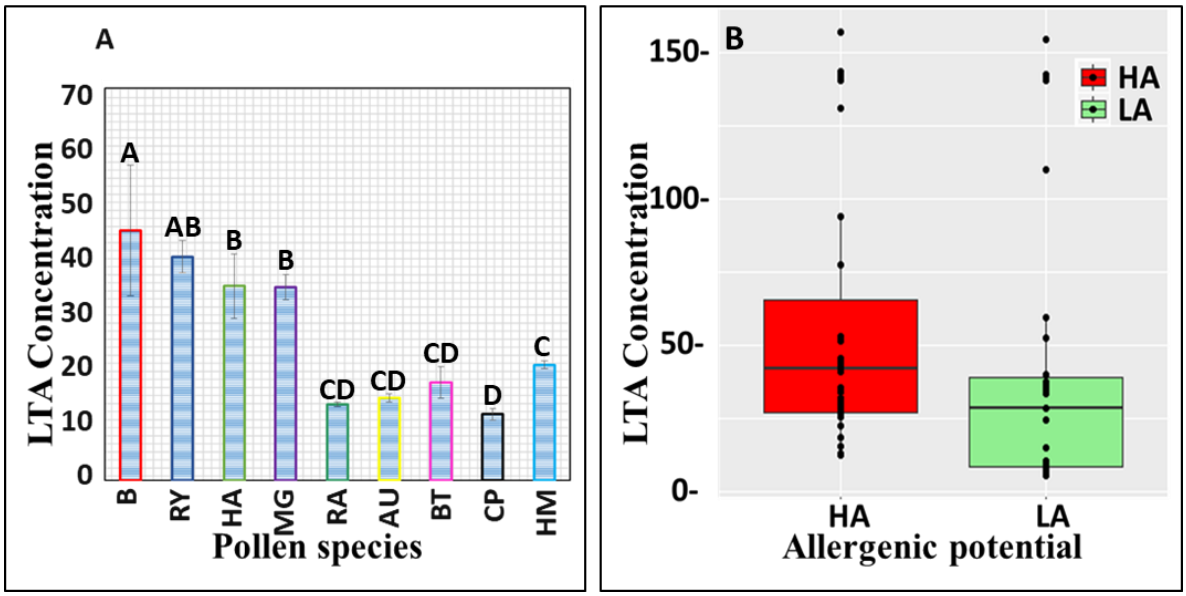


Fig 15. (A) LTA-ELISA quantitative determination assay of different pollen species (between allergenic potential: *t*-test, P<0.0001). Different letters indicate significantly different means (Tukey test, $p<0.05$). (B) Mean of LTA concentration of all bacterial strains isolated from high allergenic and low allergenic pollen samples (ng/ml) (*t*-test, P=0.06). B=birch; RY=winter rye; HA=hazel; MG=mugwort; RA=winter rapeseed; AU=autumn crocus; BT=blackthorn; CP=cherry plum; HM=hemp. HA= high allergenic pollen (red); LA= low allergenic pollen (green).

2.3. Discussion

In the cultivation-dependent study, *Microbacteriaceae* was the only family found in all the pollen species. *Microbacteriaceae* was also one abundant bacterial families previously isolated from birch pollen (McKenna *et al*., 2017), leaves and flower petals (Junker *et al*., 2011), flower nectar (Jacquemyn *et al*., 2013), phyllosphere and litter (Behrendt *et al*., 2002). A significant variation of CFU numbers between nine different pollen species indicates that the species-specific structure and allergenic potential of pollen could affect the number of the

bacteria inhabiting pollen. The cultivable fraction (CFU) of bacterial abundance in allergenic pollen species was significantly less compared to less/non allergenic plants.

To overcome the limitations of the cultivation-dependent method, the cultivation-independent metagenomic study was conducted to recover the actual bacterial fraction. Metagenomic and bioinformatics studies provide more knowledge about uncultivable and hidden microbial communities from environmental samples (Hiraoka *et al*., 2016; Xu, 2006). *Enterobacteriaceae* and *Pseudomonadaceae* were the most abundant bacterial families found in the pollen habitat. Different metagenomic studies revealed that *Enterobacteriaceae* and *Pseudomonadaceae* were members in flower nectar microbiota (Schaeffer *et al*. 2017; Mortazavi *et al*. 2015; Jacquemyn *et al*. 2013; Shade *et al*. 2013; Fridman *et al*. 2012) while *Enterobacteriaceae* and *Pseudomonadaceae* were found abundant in cannabis flowers (McKernan *et al.,* 2016). The major fungal phylum observed in the pollen microhabitat was *Ascomycota,* followed by *Davidiellaceae* and *Basidiomycota*. Colldahl and Nilsson (1973) initially reported the presence of fungi like structure on the pollen surface. The fungal species isolated from *Pinus nigra* were from *Basidiomycota* and *Ascomycota* families (Hutchison and Barron 1997). Metagenomic study related to different plant microbial habitat such as carposphere and phyllosphere of olive (Abdelfattah *et al*., 2015) and strawberry (Abdelfattah *et al*., 2016) reported that *Ascomycota* and *Basidiomycota* were the most abundant phyla. Schaeffer *et al*. (2017) found *Davidiellaceae* was abundant in almond nectar.

The results of alpha diversity put forward the message that the two factors, viz. plant pollen species and allergenic potential were significantly affecting bacterial and fungal species richness, diversity and evenness. Beta diversity revealed that the plant pollen species and allergenic potential were the major factors shaping the pollen inhabiting bacterial and fungal community composition. Pollen has its own species-specific size, structure, composition (Ariizumi and Toriyama, 2011; Kosenko, 1999) and biochemical makeup (Schulte *et al*., 2008). The exine structure of wind-pollinated pollen grains are non-sticky with limited pollen

outer coat (Schwendemann *et al*., 2007; Shukla *et al*., 1998). The insect-pollinated pollen consist of sticky, hydrophobic and abundant pollen coat (Dickinson *et al*., 2000; Shukla *et al*., 1998; Pacini and Franchi, 1996). These pollen coat difference between pollination types indicates that the wind-pollinated pollen contain less sporopollenin outer layer and insect-pollinated pollen grains covered with a thick layer of sporopollenin outer layer. Sporopollenin is a hydrophobic complex mixture of biopolymers with long chain fatty acid, phenylpropanoids, phenolics and carotenoids (Dominguez *et al*., 1999; Kawase and Takahashi, 1995). Sporopollenin is extremely resistant to non-oxidative physical, biological and chemical degradation (Erdtman, 1960). This might be the reason for the selection parameter or less advantageous factor for various bacteria to grow in insect-pollinated less/non allergic pollen habitat. Moreover, dry wind-pollinated allergic pollen easily hydrate when it is exposed to moist condition (Franklin-Tong, 1999).

A core microbiome analysis is important for understanding the complex microbial assemblage across ecologically similar habitats (Shade and Handel, 2012). The core microbiome of pollen microhabitate consists of 12 bacterial genera, includes most abundant were *Pseudomonas*, *Rosenbergiella* and *Bradyrhizobium*. Moreover, intra-kingdom microbe-microbe interactions were observed using co-occurrence pattern correlation and network analysis (Barberán *et al*., 2012) and identified the hub taxa from this pollen microbiome interaction network (Agler *et al*., 2016). *Methylobacterium* (2 OTUs), *Friedmanniella* and *Rosenbergiella* were the four hubs that strongly inter connected and showed a significant effect in shaping the pollen microbiota interaction network. *Methylobacterium* OTUs were found significantly higher in wind-pollinated allergenic pollen species while *Rosenbergiella* in insect-pollinated less/non allergenic pollen species. But *Friedmanniella* did not show any significant deferences (Fig 3, Chapter 4). *Methylobacterium* is a known inhabitant in different plant habitat such as root, leaf, fruit and flower (Ottesen *et al.*, 2013), seed endophyte in *Crotalaria pumila* (Sánchez-López *et al.*, 2018), and has positive relationships with plants

and other bacteria (Iguchi *et al*., 2015). *Rosenbergiella* was the most frequently found bacterium in flower habitats (Bartlewicz *et al*., 2016). *Friedmanniella* was found in hybrid poplar endophytic habitat (Ulrich *et al*., 2008), seed endophyte in *Anadenanthera colubrina* (Alibrandi *et al*., 2017), mangroves (Tuo *et al*., 2016), apple phyllosphere (Yashiro *et al*., 2011) and in spiders that are hiding in flowers (Iwai *et al*., 2010). The hub microbes play an important role (suppressing or inducing the development of other populations) in shaping its own microbial community and thus strongly interact with other community members (Agler *et al*., 2016). The hub taxa are more ecologically relevant than other microbial populations in the same habitat because the removal of these hub taxa would affect the general community assemblage (Faust and Raes, 2012). The hub microbes might have beneficial relationships with the host and the other microorganisms inhabiting in the same habitat (Agler *et al*., 2016). The SEM images of different plant pollen species reveal the evidence of colonization of morphologically different bacteria on the tectum of pollen grains. Moreover, the differences in shape, size and tectum structure, as well as the species-specific morphology were visualized. The images reveal that the allergenic pollen species are smaller than low allergenic pollen species. Colldahl and Nilsson (1973) initially reported the presence of bacteria on the tectum of birch, timothy and pine pollen using SEM images. Different studies reported that the pollen grain of different plant species has species-specific morphological differences (Komai *et al.*, 2014; Güneş, 2012). Majority of wind-pollinated pollen grains are smaller compared to other pollination type pollen grains (Whitehead, 1969). The SEM images are the visual evidence to support the results of cultivation-dependent and independent results in this study.

Quantification of endotoxin from the pollen samples and the bacterial isolates will provide basic knowledge about the clinical relevance of high allergenic and low allergenic pollen as well as the role of bacterial species isolated from allergenic pollen samples in pollen allergy. Endotoxins are one major factor for the pathogenesis of bacterial infection and are known as

airborne immunotoxicant for human (Michel *et al.*, 1991; Michel *et al.*, 1989). Inhalation of bacterial endotoxin causes blood and lung inflammatory reaction, fever and shaking chills (Michel *et al.*, 1997; Sandstorm *et al.*, 1992; Rylander *et al.*, 1989). So far, the influence of environmental pollution parameters such as nitrogen dioxide, ammonia, ozone (Obersteiner *et al.*, 2016), sulfur oxides and heavy metals (Helander *et al.*, 1997) for the pollen allergy has been studied. However, only a little attention has been given to the potential role of pollen bacterial inhabitance in pollen allergy.

The LPS-ELISA using samples from different pollen species (including HA and LA) demonstrated the presence of high level of LPS in high allergenic pollen species. Thus the results of LPS-ELISA using the Gram-negative bacterial species isolated from different pollen species (including HA and LA), confirmed the previous result, which revealed that the bacterial isolates from high allergenic pollen samples had a higher amount of LPS quantity than the isolates from low allergenic pollen species. Colldahl and Carlsson (1968) reported that the extracts of bacterial species isolates from allergenic pollen induced clinical symptoms in pollen-sensitive patients. Later Spiewak *et al*. (1996) showed that the allergenic plant pollen, as well as Gram-negative bacterial species isolated from these pollen, contain high LPS level. The LPS exposure triggers immune responses in bronchial epithelial cells and thus might influence the development of allergic asthma (Eisenbarth *et al.*, 2002; Braun-Fahrlander *et al.*, 2002). LPS associated with *Artemisia* pollen was essential for inducing inflammation of the lung and allergic sensitization (Oteros *et al.*, 2018).

The LTA-ELISA results of pollen samples (including HA and LA) revealed the presence of high level of LTA concentration in HA pollen samples while low level in low allergenic pollen samples. Even though, five isolates from LA pollen exhibited higher LTA concentration, the numbers of reads of these bacteria were found too low from the respective pollen samples. Gram-positive bacteria can also trigger the immune response similar to Gram-negative bacteria (Georgiev, 2009). Gram-positive bacteria associated with the grass

pollen influences the allergic immune responses during skin prick test in human as well as in cell culture (Heydenreich *et al*., 2012).

These results determine the presence of high concentration of endotoxins in the pollen of high allergenic plant pollen species. Allergens are immunomodulatory compounds generally associated with pollen grains, food, matrices and fecal particles. Moreover, bacterial endotoxins from organic and cotton dust are the major causative agents for the development of immune modulatory reactions like bronchial reactivity including fever, asthma and wheezing etc (Williams *et al.*, 2005; Rylander *et al.*, 1989). Bacterial compounds together with other allergens (Traidl-Hoffmann *et al.*, 2009) as lipids (Bublin *et al* 2014) and proteins (Hales *et al.*, 2008; Renz and Herz, 2002) contribute a major role in allergic immune response.

2.4. References

Abdelfattah, A., Li Destri Nicosia, M.G., Cacciola, S.O., Droby, S., Schena, L. (2015) Metabarcoding analysis of fungal diversity in the phyllosphere and carposphere of olive (*Olea europaea*). *PLoS One* **97:** e0131069.

Abdelfattah, A., Wisniewski, M., Li Destri Nicosi, M.G., Cacciola, S.O., Schena, L. (2016) Metagenomic analysis of fungal diversity on strawberry plants and the effect of management practices on the fungal community structure of aerial organs. *PLoS One* **97:** e0160470.

Agler, M.T., Ruhe, J., Kroll, S., Morhenn, C., Kim, S-T., Weigel, D., Kemen, E.M. (2016) Microbial Hub Taxa Link Host and Abiotic Factors to Plant Microbiome Variation. *PLoS Biol* **14:** e1002352.

Alibrandi, P., Cardinale, M., Rahman, M.M., Strati, F., Ciná, P., de Viana, M.L., et al. (2017) The seed endosphere of *Anadenanthera colubrina* is inhabited by a complex microbiota, including *Methylobacterium* spp. and *Staphylococcus* spp. with potential plant-growth promoting activities. *Plant Soil* **422:** 81–99.

Ariizumi, T., Toriyama, K. (2011) Genetic regulation of sporopollenin synthesis and pollen exine development. *Annu Rev Plant Biol* **62:** 437–460.

Barber´an, A., Bates, S.T., Casamayor, E.O., Fierer, N. (2012) Using network analysis to explore co-occurrence patterns in soil microbial communities. *ISME J* **97:** 343–51.

Bartlewicz, J., Lievens, B., Honnay, O., Jacquemyn, H. (2016) Microbial diversity in the floral nectar of *Linaria vulgaris* along an urbanization gradient. *BMC Ecol* **16:** 18.

Bayston, K.F., Cohen, J. (1990) Bacterial endotoxin and current concepts in the diagnosis and treatment of endotoxaemia. *J Med Microbiol* **31:** 73–83. DOI:*10.1099*/00222615-31-2-73.

Behrendt, U., Ulrich, A., Schumann, P., Naumann, D., and Suzuki, K. (2002) Diversity of grass-associated *Microbacteriaceae* isolated from the phyllosphere and litter layer after mulching the sward; polyphasic characterization of *Subtercola pratensis* sp. nov., *Curtobacterium herbarum* sp. nov. and *Plantibacter flavus* gen. nov., sp. nov. *Int J Syst Evol Microbiol* **52:** 1441–1454.

Braun-Fahrlander, C., Riedler, J., Herz, U., Eder, W., Waser, M., Grize, L., Maisch, S., Carr, D., Gerlach, G., Bufe, A., Lauener, R.P., Schierl, R., *et al*. (2002) Environmental

exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* **347:** 869–877.

Bublin, M., Eiwegger, T., Breiteneder, H. (2014) Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol* **134:** 521–529.

Colldahl, H., Carlsson, G. (1968) Allergens in pollen. *Acta Allergologia* **XXIII:** 387–397.

D'Amato, G., Cecchi, L., Bonini, S., Nunes, C., Annesi-Maesano, I., Behrendt, H., Liccardi, G., Popov, T., Van Cauwenberge, P. (2007) Allergenic pollen and pollen allergy in Europe. *Allergy* **62:** 976–990.

Dai, L., DeFee, M.R., Cao, Y., Wen, J., Wen, X., Noverr, M.C., Qin, Z. (2014) Lipoteichoic acid (LTA) and lipopolysaccharides (LPS) from periodontal pathogenic bacteria facilitate oncogenic herpesvirus infection within primary oral cells. *PLoS One* **9:** e101326.

Dickinson, H.G., Elleman, C.J., Doughty, J. (2000) Pollen coatings: Chimaeric genetics and new functions. *Sex Plant Reprod* **12:** 302–309.

Dominguez, E., Mercado, J.A., Quesada, M.A., Heredia, A. (1999) Pollen sporopollenin: Degradation and structural elucidation. *Sex Plant Reprod* **12:** 171–178.

Eisenbarth, S.C., Piggot, D.A., Huleatt, J.W., Visintin, I., Herrick, C.A., Bottomly, K. (2002) Lipopolysaccharide-enhanced, Toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* **196:** 1645–1651.

Erdtman, G (1960) The acetolysis method- A revised description. *Svensk Bot Tidskr* **54:** 561-564.

Faust, K., Raes, J. (2012) Microbial interactions: from networks to models. *Nat Rev Microbiol* **10:** 538-550.

Franklin-Tong, V.E. (1999) Signaling and the modulation of pollen tube growth. *Plant cell* 11:727-38.

Fridman, S., Izhaki, I., Gerchman, Y., Halpern, M. (2012) Bacterial communities in floral nectar. *Environ Microbiol Rep* **4:** 97–104.

Georgiev V.S. (2009) *Mammalian Host Defenses: Innate and Adaptive Immunity*. In: Georgiev V.S. (eds) National Institute of Allergy and Infectious Diseases, NIH. Infectious Disease. Humana Press. pp 577–626.

Güneş, F. (2012) Pollen morphology of *Lathyrus* (*Leguminosae*) taxa belonging to *Lathyrus*, *Orobastrum* and *Cicercula* sections from Turkey. *Plant Syst Evol* **298:** 1777.

Hales, B.J., Pearce, L.J., Kusel, M.M.H., Holt, P.G., Sly, P.D, Thomas, W.R. (2008) Differences in the antibody response to a mucosal bacterial antigen between allergic and non-allergic subjectsSmoke-free legislation reduces exposure in children Thorax **63:** 221–227.

Hani, B., Dalila, B., Saliha, D., Daoud, H., Mouloud, G., Seddik, K. (2012) Microbiological sanitary aspects of pollen. *Adv Environ Biol* **6:**1415–1420.

Hashimoto, M., Tawaratsumida, K., Kariya, H., Kiyohara, A., Suda, Y., Krikae, F., Kirikae, T., Götz, F. (2006) Not lipoteichoic acid but lipoproteins appear to be the dominant immunobiologically active compounds in *Staphylococcus aureus. J Immunol* **177:** 3162–3169.

Helander, M.L., Savolainen, J., Ahlhotm, J. (1997) Effects of air pollution and other environmental factors on birch pollen allergens. *Allergy* **52:** 1207–1214.

Heydenreich, B., Bellinghausen, I., König, B., Becker, W.-M., Grabbe, S., Petersen, A., Saloga, J. (2012) Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses. *Clin Exp Allergy* **42:** 76–84.

Hiraoka, S., Yang, C., Iwasaki, W. (2016) Metagenomics and bioinformatics in microbial ecology: Current status and beyond. *Microbes Environ* **31:** 204–212.

Hutchison, L.J., Barron, G.L. (1997) Parasitism of pollen as a nutritional source for lignicolous *Basidiomycota* and other fungi. *Mycol Res* **101:** 191–4.

Iguchi, H., Yurimoto, H., Sakai, Y. (2015) Interactions of Methylotrophs with Plants and Other Heterotrophic Bacteria. *Microorganisms* **3:** 137–151.

Iwai, K, Aisaka, K., Suzuki, M. (2010) *Friedmanniella luteola* sp. nov., *Friedmanniella lucida* sp. nov., *Friedmanniella okinawensis* sp. nov. and *Friedmaniella sagamiharensis* sp. nov., isolated from spiders. *Int J Syst Evol Microbiol* **60:** 113–120.

Jacquemyn, H., Lenaerts, M., Tyteca, D., Lievens, B. (2013) Microbial diversity in the floral nectar of seven *Epipactis* (*Orchidaceae*) species. *Microbiol Open* **2:** 644–658.

Jang, K.S., Baik, J.E., Han, S.H., Chung, D.K., Kim, B. G. (2011) Multi-spectrometric analyses of lipoteichoic acids isolated from *Lactobacillus plantarum*. Biochem. Biophys. Res. Commun. 407, 823–830.

Junker, R.R., Loewel, C., Gross, R., Dötterl, S., Keller, A. and Blüthgen, N. (2011) Composition of epiphytic bacterial communities differs on petals and leaves. *Plant Biol* **13:** 918–924.

Kawase, M., Takahashi, M. (1995) Chemical composition of sporopollenin in *Magnolia grandiflora* (*Magnoliaceae*) and *Hibiscus syriacus* (*Malvaceae*). *Grana* **34:** 242-245.

Komai, F., Okada, K., Inoue, Y., Yada, M., Tanaka, O., Kuwabata, S. (2014) SEM observation of wet lily pollen grains pretreated with ionic liquid. *J Japanese Soc Horticultural Sci* **83:** 317–21.

Kosenko, V.N. (1999) Contributions to the pollen morphology and taxonomy of the *Liliaceae*. *Grana* **38:** 20–30.

Liu, Z.-G., Song, J.-J., Kong, X.-L. (2010) Study on pollen allergens in China. *Biomed Environ Sci* **23:** 319–322.

Mäkelä, P.H., Valtonen,V.V., Valtonen, M. (1973) Role of O-antigen (Lipopolysaccharide) factors in the virulence of *Salmonella*. *J Infect Dis* **128:** 81–85.

McKenna, O., Posselt, G., Briza, P., Lackner, P., Schmitt, A.O., Gadermaier, G., Wessler, S., Ferreira, F. (2017) Multi-approach analysis for the identification of proteases within birch pollen. *Int J Mol Sci* **18:**1433.

McKernan, K., Spangler, J., Helbert, Y., Lynch, R.C., Devitt-Lee, A., Zhang, L., Orphe, W., Warner, J., Foss, T., Hudalla, C.J., Silva, M., Smith, D.R. (2016) Metagenomic analysis of medicinal cannabis samples; pathogenic bacteria, toxigenic fungi, and beneficial microbes grow in culture-based yeast and mold tests. *F1000Research* **5:** 2471.

Michel, O., Duchateau, J., Sergysels, R. (1989) Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. *J Appl Physiol* **66:** 1059–1064.

Michel, O., Ginanni, R., Le Bon, B., Duchateau, J. (1991) Effect of endotoxin contamination on the antigenic skin test response. *Ann Allergy* **66:** 39–42.

Michel, O., Nagy, A-M., Schroeven, M., Duchateau, J., Neve, J., Fondu, P., Sergysel, R. (1997) Dose-response relationship to inhaled endotoxin in normal subjects. *Am J Respir Crit Care Med* **156:** 1157–1164.

Morath, S., von Aulock, S., Hartung, T. (2005) Structure/function relationships of lipoteichoic acids. *J Endotoxin Res* **11:** 348–356.

Mortazavi, R., Attiya, S., Ariya, P.A. (2015) Arctic microbial and next-generation sequencing approach for bacteria in snow and frost flowers: Selected identification, abundance and freezing nucleation. *Atmos Chem Phys* **15:** 6183–6204.

Obersteiner, A., Gilles, S., Frank, U., Beck, I., H€aring, F., Erns, D., Rothballer, M., Hartmann, A., Traidl-Hoffmann, C., Schmid, M. (2016) Pollen-associated microbiome correlates with pollution parameters and the allergenicity of pollen. *Plos One* **11:** e0149545.

Oteros, J., Bartusel, E., Alessandrini, F., Núñez, A., Moreno, D.A., Behrendt, H., Schmidt-Weber, C., Traidl-Hoffmann, C., Buters J. (2018) Artemisia pollen is the main vector for airborne endotoxin. *J Allergy Clin Immunol* **pii:** S0091-6749(18)30999-0.

Ottesen, A.R., Peña, A.G., White, J.R., Pettengill, J.B., Li, C., Allard, S., Rideout, S., Allard, M., *et al.* (2013) Baseline survey of the anatomical microbial ecology of an important food plant: *Solanum lycopersicum* (tomato). *BMC Microbiol* **13:** 114.

Pacini, E., Franchi, G.G. (1996) Some cytological, ecological and evolutionary aspects of pollination. *Acta Soc Bot Pol* **65:** 11–16.

Parolia, A., Gee, L.S., de Moraes Porto, ICC, Mohan, M. (2014) Role of cytokines, endotoxins (LPS), and lipoteichoic acid (LTA) in endodontic infection. *J Dent Oral Disord Ther* **2:** 1–5.

Platts-Mills, A.E., Thomas, A.E. (2015). The Allergy Epidemics: 1870–2010. *J Allergy Clin Immunol* **136:** 3-13.

Raetz,C.R., Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71:** 635–700.

Renz, H., Herz, U. (2002) The bidirectional capacity of bacterial antigens to modulate allergy and asthma. *Eur Respir J.* **19:** 158–171.

Revets, H., Pynaert, G., Grooten, J., De Baetselier, P. (2005) Lipoprotein I, a TLR2/4 ligand modulates Th2-driven allergic immune responses. *J Immunol* **174:** 1097–1103.

Rietschel, E.T., Schade, U., Jensen, M., Wollenweber, H-W., Luderitz, O., Greisman, S.G. (1982) Bacterial endotoxins: chemical structure, biological activity and role in septicaemia. *Scand J Infect Dis* (Suppl) **31:** 8–21.

Rosenfeld, Y., Shai, Y. (2006) Lipopolysaccharide (endotoxin)-host defense antibacterial peptides interactions: role in bacterial resistance and prevention of sepsis. *Biochim Biophys Acta* **1758:** 1513–1522.

Rylander, R., Bake, B., Fisher, J.J., Helander, I.M. (1989) Pulmonary function and symptoms after inhalation of endotoxin. *Am Rev Respir Dis* **140:** 981–986.

Sánchez-López, A.S., Pintelon, I., Stevens, V., Imperato, V., Timmermans, J.P., González-Chávez, C., Carrillo-González, R., Van Hamme, J., Vangronsveld, J., Thijs, S. (2018) Seed endophyte microbiome of *Crotalaria pumila* unpeeled: identification of plant-beneficial *Methylobacteria*. *Int J Mol Sci* **19:** pii: E291.

Sandstrom, T., Bjermer, L., Rylander, R. (1992) Lipopolysaccharide (LPS) inhalation in healthy subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid. *Eur Respir J* **5:** 992–996.

Schaeffer, R.N., Vannette, R.L., Brittain, C., Williams, N.M., Fukami, T. (2017) Non-target effects of fungicides on nectar-inhabiting fungi of almond flowers. *Environ Microbiol Rep* **9:** 79–84.

Schneewind, O., Missiakas, D. (2014) Lipoteichoic acids, phosphate-containing polymers in the envelope of Gram-positive bacteria. *J Bacteriol* **196:** 1133–1142.

Schulte, F., Lingott, J., Panne, U., Kneipp, J. (2008) Chemical characterization and classification of pollen. *Anal Chem* **80:** 9551–56.

Shade, A., McManus, P.S., Handelsman, J. (2013) Unexpected diversity during community succession in the apple flower microbiome. *mBio* **4:** e00602–12.

Shade, A., and Handelsman, J. (2012) Beyond the Venn diagram: the hunt for a core microbiome. *Environ Microbiol* **14:** 4–12.

Shukla, A.K., Vijayaraghavan, M.R.., Chaudhry, B. (1998). "Abiotic pollination". Biology of pollen. *APH Publishing*, New Delhi. pp. 67–69.

Spiewak, R., Skorska, C., Prazmo, Z., Dutkiewicz, J. (1996) Bacterial endotoxin associated with pollen as a potential factor aggravating pollinosis. *Ann Agric Environ Med* **3:** 63–66.

Steimle, A., Autenrieth, I.B., Frick, J-S. (2016) Structure and function: Lipid A modifications in commensals and pathogens. *Int J Med Microbiol* **306:** 290–301.

Traidl-Hoffmann, C., Jakob, T., Behrendt, H. (2009) Determinants of allergenicity. *J Allergy Clin. Immunol* **123:** 558–566.

Tuo, L., Pan, Z., Li, F.N., Lou, I., Guo, M., Lee, S. M., Chen, L., HU, L., Sun, C.-H. (2016) *Friedmanniella endophytica* sp. nov., an endophytic *Actinobacterium* isolated from bark of *Kandelia candel. Int J Syst Evol Microbiol* **66:** 3057-3062.

Ulrich, K., Ulrich, A., Ewald, D. (2008) Diversity of endophytic bacterial communities in poplar grown under field conditions. *FEMS Microbiol Ecol* **63:** 169–180.

Ushio, M., Yamasaki, E., Takasu, H., Nagano, A. J., Fujinaga, S., Honjo, M. N., Ikemoto, M., Sakai, S., Kudoh, H. (2015) Microbial communities on flower surfaces act as signatures of pollinator visitation. *Sci Rep* **5:** 8695.

Watson, D.W., Kim, Y.B. (1963) Modification of host responses to bacterial endotoxins. I. Specificity of pyrogenic tolerance and the role of hypersensitivity in pyrogenicity, lethality, and skin reactivity. *J Exp Med* **118:** 425–446.

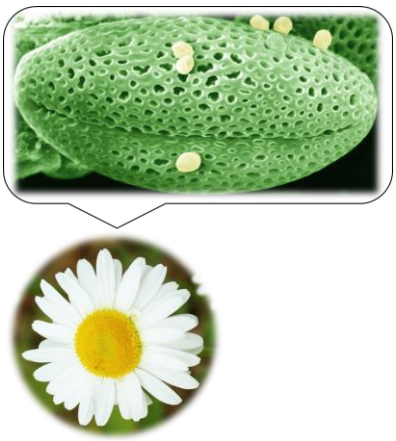
Whitehead, D.R. (1969) Wind pollination in the angiosperms: evolutionary and environmental implications. *Evolution* **23:** 28–35.

Williams, L.K., Ownby, D.R., Maliarik, M.J., Johnson, C.C. (2005) The role of endotoxin and its receptors in allergic disease. *Ann Allergy Asthma Immunol* **94:** 323–332.

Xu, J. (2006) Microbial ecology in the age of genomics and metagenomics: concepts, tools, and recent advances. *Mol Ecol* **15:** 1713–1731.

Yashiro, E., Spear, R.N., McManus, P.S. (2011) Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere. *J Appl Microbiol* **110:** 1284–1296.

Zuberbier, T., Lotvall ,J., Simoens, S., Subramanian, S.V., Church, M.K. (2014) Economic burden of inadequate management of allergic diseases in the European Union: a GALEN review. *Allergy* **69:** 1275–1279.



Chapter 3

Ambika Manirajan, B., Ratering, S., Rusch, V., Schwiertz, A., Geissler-Plaum, R., Cardinale, M., Schnell, S. (2016) Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity. *Environ Microbiol* **18:** 5161–5174. DOI:*10.1111*/1462-2920.13524.

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Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity

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Summary

Diverse microorganisms colonise the different plant-microhabitats, such as rhizosphere and phyllosphere, and play key roles for the host. However, bacteria associated with pollen are poorly investigated, despite its ecological, commercial and medical relevance. Due to structure and nutritive composition, pollen provides a unique microhabitat. Here the bacterial abundance, community structure, diversity and colonization pattern of birch, rye, rapes and autumn crocus pollens were examined, by using cultivation, high-throughput sequencing and microscopy. Cultivated bacteria belonged to *Proteobacteria*, *Actinobacteria* and *Firmicutes*, with remarkable differences at species level between pollen species. High-throughput sequencing of 16S rRNA gene amplicon libraries showed *Proteobacteria* as the dominant phylum in all pollen species, followed by *Actinobacteria*, *Acidobacteria* and *Firmicutes*. Both plant species and pollination type significant influenced structure and diversity of the pollen microbiota. The insect-pollinated species possessed a more similar microbiota in comparison to the wind-pollinated ones, suggesting a levelling effect by insect vectors. Scanning electron microscopy as well as fluorescent *in situ* hybridisation coupled with confocal laser scanning microscopy (FISH-CLSM) indicated the tectum surface as the preferred niche of bacterial colonisation. This work is the most comprehensive study of pollen microbiology, and strongly increases our knowledge on one of the less investigated plant-microhabitats.

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Introduction

Plant-associated microbiome studies have increased enormously because of their recognised importance. The majority of such studies focused on the plant microhabitats corresponding to rhizosphere, phyllosphere and endosphere (Ryan *et al*., 2008; Turner *et al*., 2013; Aleklett *et al*., 2014), while other niches, such as anthosphere, carposphere and spermosphere remained less investigated so far (Vandenkoornhuyse *et al*., 2015). It has been shown that plants usually exhibit a high species-specificity level of associated bacteria (Redford *et al*., 2010; Berg *et al*., 2014). A large microbiome spreads on the surface of plants and most abundant members of this are bacteria (Meyer and Leveau, 2012). Plant organs carry number of microorganisms, especially bacterial communities, and they can be found in and on various organs, including pollen (Junker and Keller 2015). Certain bacterial species could be isolated and cultivated from pollen extracts by Colldahl and Carlsson (1968). This observation was later confirmed with scanning electron microscopy by Colldahl and Nilsson (1973). The sources of bacterial colonisation of pollen were reported to be honey bees, weather, plant materials, other insects, animals and human activities (Hani *et al*., 2012). Although some research indicated the presence of various bacterial species on pollen, little is known on this microhabitat (Gilliam, 1979; Hani *et al*., 2012).

Many plants are emitting large quantity of pollen during spring to autumn and several types of plant pollen may cause serious pollen-related diseases (Carinanos and Casares-Porcel, 2011; Oldenburg *et al*., 2011). Therefore, pollen-associated bacteria may have a potential ecological and medicinal impact. In addition, they may also enter the

plant reproduction processes and be directly transmitted to the next generation as seed endophytes.

Two recent studies indicated that flower organs harbour a unique microbiota different from that of the leaves of the same plant species and independent from environmental conditions (Junker and Keller, 2015). Recently, it could be shown for birch and timothy grass that the microbial community associated with pollen is plant species-specific (Obersteiner *et al.*, 2016). Furthermore, pollen are a promising source of unknown bacteria, as a new genus was isolated from pollen (Jojima *et al.*, 2004). Since the bacterial community of pollen is mainly unknown, well-structured hypothesis-driven studies on pollen microbial communities are necessary. This will allow to disclose the structure, composition, dynamics as well as specificity and functions with respect to various types of plants and pollens.

Given the most recent evidence, we hypothesised (i) that the influence of the specific characteristics of flowers is stronger than that of the geographic location and (ii) that pollination will influence the pollen microbiota. Hence, insect-pollinated plants will differ from wind-pollinated ones, due to the spreading of insect-carried microbes across plant species boundaries.

The aim of this study was to compare the bacterial microbiotas associated with the pollen of four plant species (two wind-pollinated and two insect-pollinated respectively; Supporting Information Fig. S1) from a restricted geographical area by cultivation-dependent and -independent methods. And thus to allow to (i) compare bacterial abundance, structure and diversity between the four species, (ii) identify the "core" pollen microbiotas, (iii) assess the contribution of the pollination type to the variability of the pollen microbiotas and (iv) to estimate the extent of the culturable bacterial fraction by using commercial AC medium ("all culture" medium) and a pollen-enriched mineral medium. In addition, we studied the niches of colonisation of pollen bacteria by scanning electron microscopy as well as fluorescent *in-situ* hybridisation and confocal microscopy.

This work is the first comprehensive report on bacterial microbiota associated with pollen, which integrates the results of cultivation-dependent, cultivation-independent and microscopy analysis of different pollen species simultaneously.

Results

Cultivation-dependent analysis of bacterial microbiota

Isolation of bacteria from pollen. Total of 61 (18 from rye, 16 from birch, 15 from rape and 12 from autumn crocus) morphologically different bacterial colonies were isolated from the pollen species on the two different agar media. Colony morphologies and colony numbers were similar between the AC medium and pollen medium. Total numbers of CFUs of the different pollen species (birch, rye, rape and autumn crocus) were significantly different from each other on AC medium (Kruskal–Wallis test $p = 0.024$; Fig. 1A). The highest number was found in autumn crocus ($7.5 \pm 7.4 \times 10^8$), while the lowest in birch ($4.1 \pm 3.1 \times 10^5$). Pollen medium gave similar results (data not shown).

Identification of the bacterial isolates by 16S rRNA gene sequencing. All 16S rRNA gene sequences of the bacterial isolates were identified by the EzTaxon database (Kim *et al.* 2012). A total of 44 bacterial species were identified from the morphologically different types of bacterial colony isolates (15 species from rye, 15 from birch, 10 from rape and 9 from autumn crocus respectively) (Supporting Information Table S1). Four major phyla were identified from the pollen isolates: *Proteobacteria* (45.5%), *Actinobacteria* (31.8%),

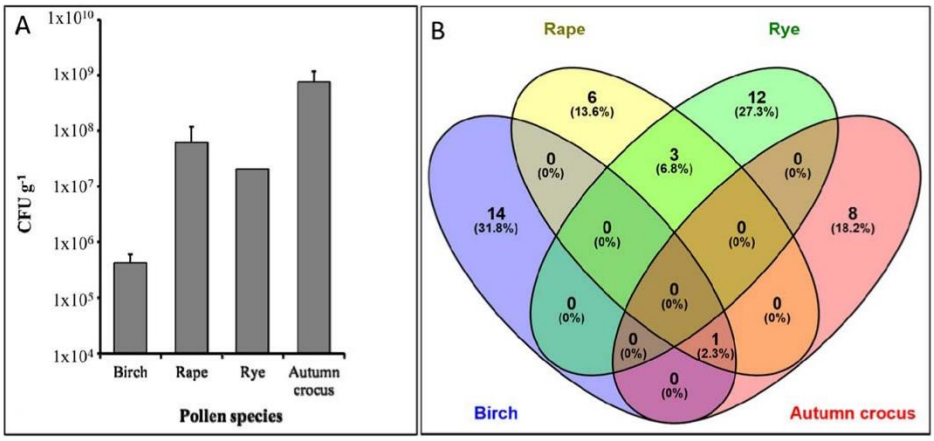

Fig. 1. (A) Numbers of cultivable bacteria on flower pollen (CFUs per gram of dry pollen weight). The values are means of three samples per pollen species ($n = 3 \pm$ SE). Kruskal–Wallis test, $p = 0.024$. (B) Venn diagram shows the isolated bacterial species shared by the analysed pollens.

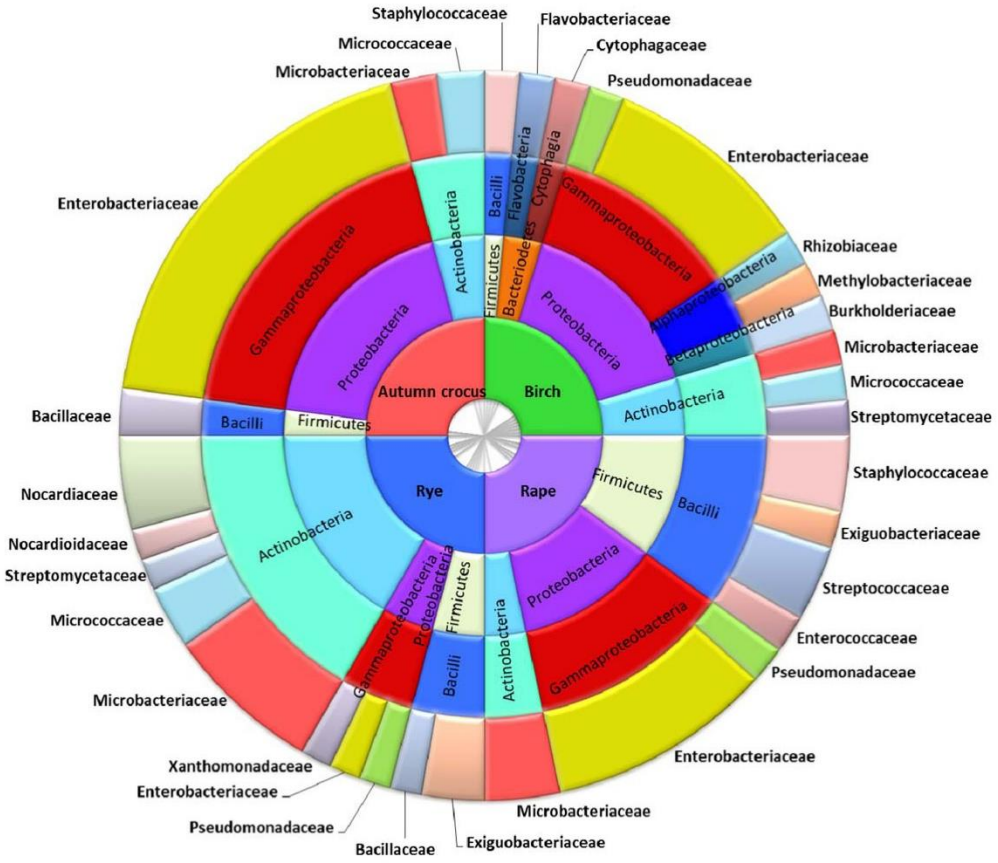

Fig. 2. Multilevel doughnut chart shows the relative abundance of cultivated bacterial taxa from birch, rape, rye and autumn crocus pollen.

Firmicutes (18.2%) and *Bacteroidetes* (4.5%) (Fig. 2). The bacterial richness at the species level of birch and rye pollen was significantly higher than in rape and autumn crocus (Kruskal–Wallis test $p = 0.026$). The isolates belonging to *Proteobacteria* (especially *Enterobacteriaceae*) were more abundant in birch, rape and autumn crocus, while *Actinobacteria* (especially *Microbacteriaceae* and *Nocardiaceae*) were found to be more abundant in rye (Fig. 2). At the bacterial species level, each pollen hosted a very unique community, while only three species were shared by rape and rye. *Rosenbergiella nectarea* was found on all pollens except rye (Fig. 1B and Supporting Information Table S1).

Cultivation-independent analysis of the bacterial microbiota

Ion torrent sequencing. In total 1,140,212 raw sequences were obtained from two independent runs. After removal of technical sequences (primer/barcode/linker), length trimming (range: 350–450 nucleotides) and quality filtering (threshold = 20, calculated on 50-nucleotides sliding windows, option -w and -g in QIIME: as soon as a window is below the quality threshold, the whole sequence is eliminated), 361,518 remaining sequences were grouped into OTUs at a 97% similarity (hereafter OTU_{97}). After removal of 111,692 plastidic OTUs, 147,282 mitochondrial OTUs, 181 chimeric OTUs and 1076 singletons, 92,880 sequences remained (14,420 from rye, 28,604 from birch, 38,188 from rape and 11,668 from autumn crocus, with an average of 8444 ± 5344 reads per pollen sample), grouped into 1056 bacterial OTUs (532 in rye, 441 in birch, 377 in rape and 133 in autumn crocus). In order to keep a higher number of sequences, one sample of autumn crocus was eliminated because it had a notably lower number of sequences (611). This would have sensibly reduced the coverage of the sequencing. However, an additional analysis of the data including this sample showed no variation in the output of any of the alpha- and beta-diversity metrics (data not shown).

Analysis of ion torrent sequences. Proteobacteria was the dominant phylum in most of the samples except in one

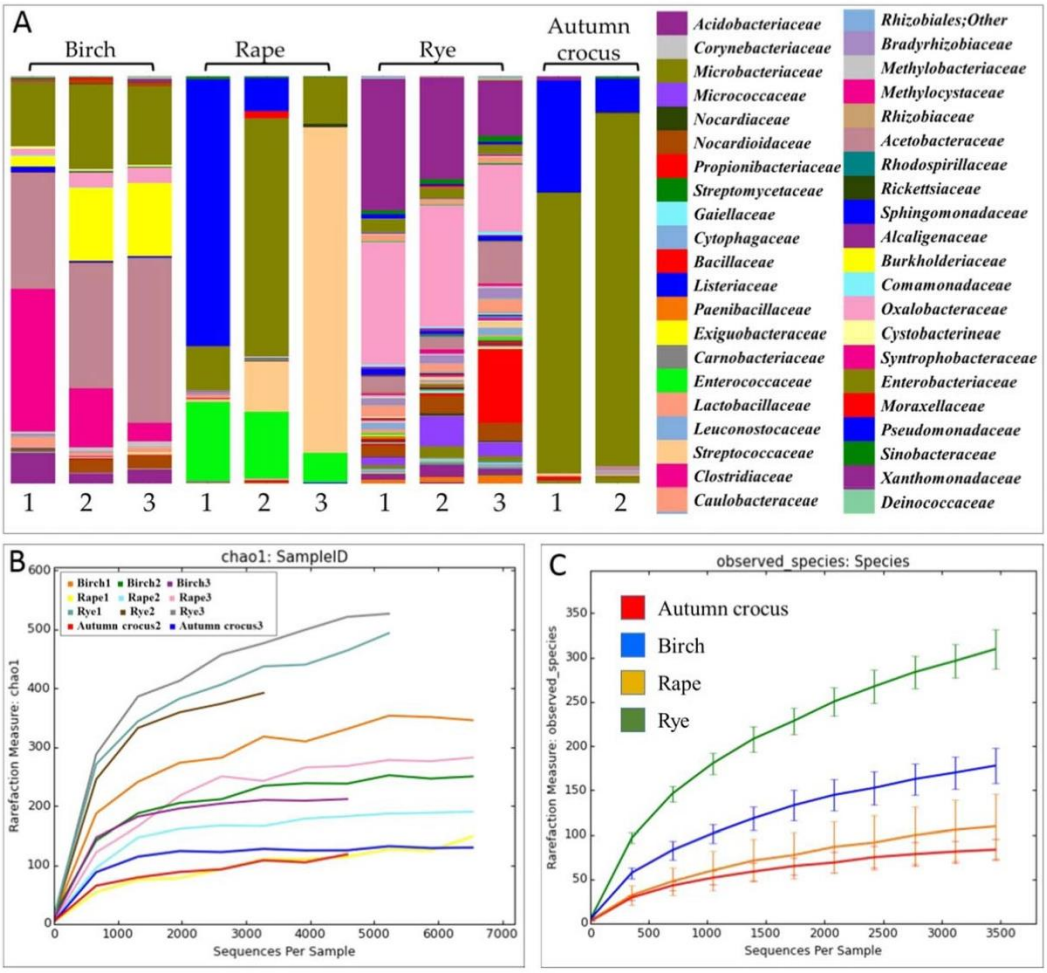

Fig. 3. (A) Occurrence of bacterial families in the pollen samples, retrieved by 16S rRNA gene amplicon library sequencing (Ion Torrent); for better visibility, only families with a relative abundance of $\geq$ 0.5% in any sample were included in the bar graph legend. (B) Chao1-rarefraction curves of individual samples based on $OTUs_{97}$. (C) Rarefaction curve describing the observed bacterial richness (number of $OTUs_{97}$) among pollen species. Means of three replicates (n = 3 = SD).

pollen sample of rape. *Actinobacteria* and *Acidobacteria* were the next dominant phyla in both birch and rye pollen samples, while *Actinobacteria* and *Firmicutes* dominated in autumn crocus and *Firmicutes* in rapes. At the family level, *Acetobacteraceae*, *Enterobacteriaceae*, *Methylocystaceae*, *Burkholderiaceae* and *Acidobacteriaceae* were the most abundant in birch pollen; *Enterobacteriaceae*, *Streptococcaceae*, *Pseudomonadaceae* and *Enterococcaceae* in rape; *Oxalobacteraceae*, *Xanthomonadaceae*, *Micrococcaceae* and *Acetobacteraceae* in rye; *Enterobacteriaceae* and *Pseudomonadaceae* in autumn crocus (Fig. 3A).

The rarefaction curves of observed species and Chao1 richness estimator showed clear differences between pollen species (Fig. 3B and C).

For alpha- and beta-diversity analysis, the dataset was rarefied to 3468 sequences in order to eliminate the biases due to the different sequence depths across samples.

Eight different alpha diversity measures were calculated on the rarefied dataset and all of them were found to be significantly different between pollen species (ANOVA, $p < 0.01$). Birch and rye pollen showed higher values than rape and autumn crocus (Table 1).

The ten most abundant OTUs (relative abundance $>$ 1% of the total rarified dataset) were significantly different between pollen species (ANOVA, FDR-corrected $p < 0.05$) and included 60.63% of the total reads (rarified dataset). Among those, the OTU identified as *Enterobacteriaceae* was more abundant in autumn crocus and to a minor extent in rape, *Xanthomonadaceae* was more abundant in rye, three

Table 1. Alpha diversity indices based on $OTUs_{97}$ (mean ± standard deviation) and statistical comparison between different pollen species.

Species	Shannon	Shannon-equitability	Dominance	Simpson	Simpson reciprocal	PD whole tree	Chao1	Observed species
Birch	4.35 ± 0.06 a	0.58 ± 0.01 a	0.11 ± 0.02 b	0.89 ± 0.02 a	9.32 ± 1.32 a	13.9 ± 0.98 a	251 ± 44.1 a	185 ± 21.6 a
Rye	5.37 ± 0.37 a	0.65 ± 0.04 a	0.08 ± 0.02 b	0.92 ± 0.02 a	12.6 ± 3.90 a	24.4 ± 3.57 b	428 ± 31.5 b	308 ± 26.2 b
Rape	2.51 ± 0.70 b	0.37 ± 0.08 b	0.37 ± 0.12 a	0.63 ± 0.12 b	2.88 ± 1.03 b	6.91 ± 0.25 c	183 ± 83.0 b	112 ± 45.5 bc
Autumn crocus	2.25 ± 0.13 b	0.35 ± 0.00 b	0.42 ± 0.01 a	0.58 ± 0.01 b	2.36 ± 0.06 b	5.70 ± 2.47 c	121 ± 20.6 b	86 ± 22.6 c

Different letters indicate statistically significant differences between means across pollen species and within diversity indices (ANOVA $p < 0.05$, Tukey test).

Oxalobacteraceae OTUs more abundant in rye and to a minor extent in birch, *Buchnera* was only found abundant in birch, *Enterococcaceae* was abundant in rape and two *Acetobacteraceae* OTUs were more abundant in birch (Fig. 4).

The BLAST similarity search of representative sequences from these ten most abundant OTUs gave more precise hints about their taxonomic affiliation (Supporting Information Table S2): the *Enterobacteriaceae* OTU was most closely related to *Rosenbergiella* spp. (100%); the *Xanthomonadaceae* OTU *Stenotrophomonas rhizophila* (100%); the three *Oxalobacteriaceae* OTUs to *Massilia* sp. (99.4%), *Noviherbaspirillum suwonense* (100%) and *Duganella* sp. (100%) respectively; the *Enterococcaceae* OTU to *Enterococcus* spp. (100%) and the *Buchnera* OTU as *Buchnera aphidicola* (98.2%). The identification of the three *Acetobacteraceae* OTUs did not improve, since several genera, such as *Acetobacter*, *Gluconacetobacter*, *Asaia*, *Neoasaia* and *Kozakia*, gave the same similarity score (94.8%–96.4%). BLAST search of the representative sequence of one of these *Acetobacteraceae* OTUs (denovo2199, Supporting Information Table S2), against sequences of type strains only, resulted in a best match with *Gluconacetobacter tumulisoli* (NR114383) with a similarity of just 94.8%. This suggests the occurrence of potentially new bacterial species or even new genera associated with pollen. Interestingly, when aligned with sequences of uncultured material, it showed a best match with a clone obtained from indoor dust (AM697168, Rintala *et al.*, 2008).

Beta diversity analysis by PCoA-plot of jackknifed-weighted Unifrac distances at OTU_{97} level showed that pollen species samples were grouped into very significant discrete clusters (Adonis $R^2 = 0.764$, $p < 0.001$). Rye and birch being the most clearly differentiated as well as rape and autumn crocus closely segregated (Fig. 5A). Interestingly, the pollination type was highly significant (Adonis $R^2 = 0.418$, $p < 0.001$) (Fig. 5A). Sampling site did not affect the structure of the microbiota ($p = 0.096$ and 0.133 for adonis and anosim tests respectively) (Fig. 5A).

Profile clustering network analysis indicated the shared as well as the exclusive $OTUs_{97}$ between pollen species (Fig. 5B). Birch and rye shared more $OTUs_{97}$, with *Oxalobacteraceae* (*Massilia* sp.), *Methylocystaceae* and *Acetobacteraceae* being the most abundant. *Xanthomonadaceae* (*Stenotrophomonas rhizophila*) was found abundant in birch rye and autumn crocus, but was absent in rape, while *Enterococcus*, found abundant in rye, rape and autumn crocus, was absent in birch (Fig. 5B). Rape-birch and rye-autumn crocus, respectively, shared very few $OTUs_{97}$, which is coherent with their relative position in the beta-diversity plot (Fig. 5A). However, rape and autumn crocus shared abundant $OTUs_{97}$ [*Enterobacteriaceae* (*Rosenbergiella* sp.) and *Pseudomonas*], which explains the reduced Unifrac distances (Fig. 5A).

The core microbiome, defined as all $OTUs_{97}$ detected in all analysed species, comprised only 15 $OTUs_{97}$ belonging to the *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Actinobacteria* and *Firmicutes*. Moreover, only four core-OTUs were found in all eleven pollen samples analysed (Fig. 5C). Most of these core OTUs were differentially distributed across pollen species, showing characteristic patterns (Fig. 5C). One of the core OTUs was identified by QIIME as *Sinobacteriaceae* (Fig. 5C); further BLAST and EzTaxon alignment of the representative sequence

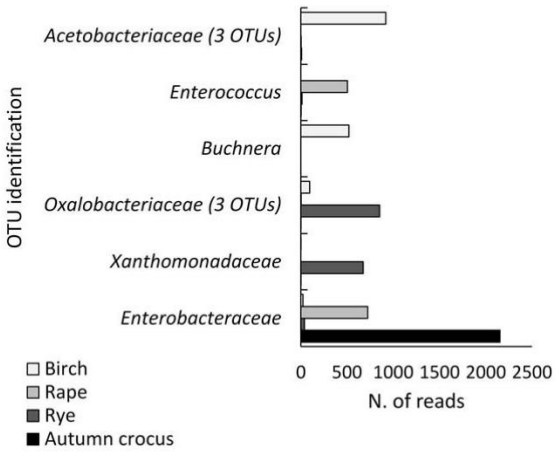

Fig. 4. Abundance of the 10 biggest OTUs was significantly different between pollen of the four plant species (ANOVA, FDR-adjusted $p < 0.05$).

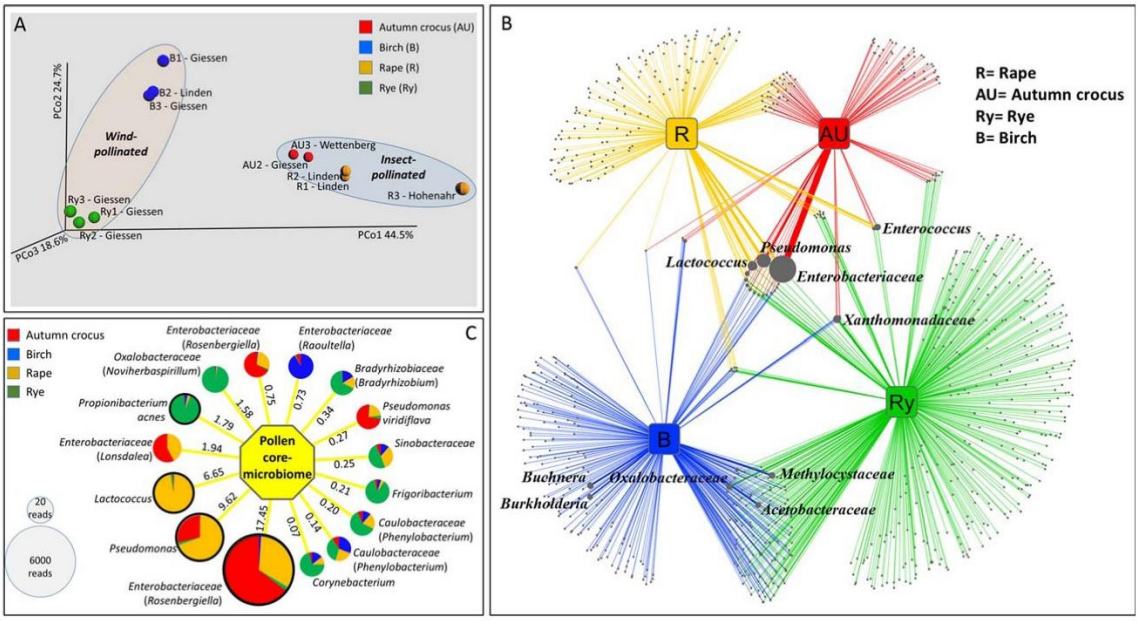

Fig. 5. (A) Principal Components Analysis (PCoA) of weighted jackknifed UniFrac distances, comparing all pollen samples at OTU_{97} level. Samples (spheres) are coloured by species and grouped by pollination type. Adonis significance test: $R^2 = 0.764$, $p < 0.001$ for the factor "species"; $R^2 = 0.418$, $p < 0.001$ for the factor "pollination type." (B) Profile clustering network showing occurrence and relative abundance of all $OTUs_{97}$ across the analysed pollen species. Abundant OTUs are labelled with the original QIIME identification of the OTUs. Edge size proportional to the mean OTUs abundance per pollen species. Node sizes defines total OTU abundance. Colour of each cluster represents the different pollen species. (C) Structure and abundance of the pollen core microbiome, defined as the $OTUs_{97}$ detected in all pollen species. Pies are coloured by pollen species and show the distribution of the respective core OTU. Pie size indicates the absolute abundance (number of reads) of the respective OTU, while numbers at the edges indicate the relative abundance (percentage of total reads). Black border around the pie indicates that the OTU was found in all pollen samples. Labels indicate the original QIIME identification of the OTU and, in brackets, the next relative (unambiguous) genus as retrieved by BLAST alignment (in some cases, BLAST alignment did not result in closer unambiguous relatives than the QIIME identification).

(against sequences of type strains only) resulted in a best match with the species *Hydrocarboniphaga daqingensis* with a similarity of 91%, indicating that potential new bacterial genera or even new families occur ubiquitously in pollens. BLAST alignment against uncultured material gave a match of 100% with a clone from freshwater habitats of the Amazon River (JX673475, unpublished).

Comparison of cultivation-dependent and -independent results. We detected 18 families by cultivation, while by IonTorrent 104 families were annotated, according to the QIIME classification. So, the cultured fraction accounted for about 17% of the total bacterial diversity on the analysed pollen, at family level. One family (*Flavobacteriaceae*) was detected by isolation (isolate Bd-AC-1 from birch pollen, Supporting Information Table S1) but not by IonTorrent sequencing (Supporting Information Table S3).

Scanning electron microscopy (SEM)

Microscopic observations revealed the surface colonisation on the tectum (outer surface) of the pollen. The bacterial cells were found adhering to the outer surface of the pollen grains as single cells or clusters or as a thin biofilm. Different bacterial morphotypes were found in the four pollen species (Fig. 6A–D).

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

Fluorescent signals were detected with the universal bacterial probe and with the FISH probes specific to *Actinobacteria* (Fig. 6E–F). Bacterial cells were mainly detected as single cells on the *tectum*. No signal was obtained in the negative controls stained with the nonsense FISH probes (Fig. 6G).

Discussion

This study compared the structure, diversity and colonisation pattern of the bacterial microbiotas associated with pollen of four different plant species from the same geographic region, but with different pollination strategies. Flower pollen is one of the less known plant micro-

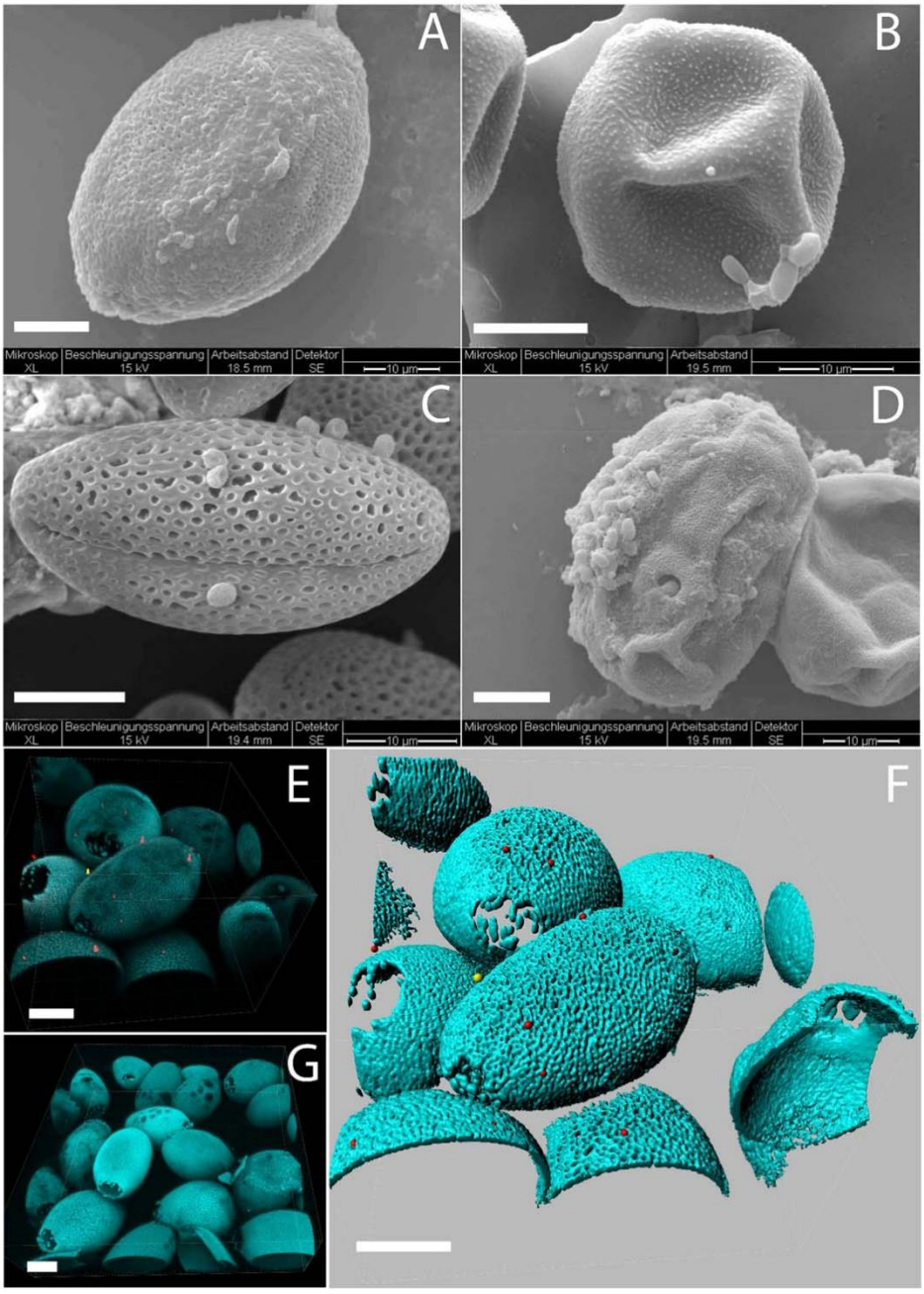

Fig. 6. Scanning electron microscopy images showing the bacterial colonization of autumn crocus (A), birch (B), rape (C) and rye (D) pollen. **(**E) Volume rendering of a confocal laser scanning microscopy image-series, showing FISH-stained bacteria on autumn crocus (*Colchicum autumnale* L.) pollen grains. Yellow: *Actinobacteria* (double stained by both probe HGC236 and universal probe EUB338-MIX); red: other bacteria (stained by the universal-probe only). Cyan: Pollen autofluorescence. **(**F) Three-dimensional model of panel E: bacteria were localized on the *tectum*, the outer surface of pollen grains. (G) Negative control stained with a mix of non-sense FISH-probes labelled with all the fluorochromes used in E. Scale bars in all panels represent 10 µm.

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habitats. While pollen-associated fungi were extensively investigated, only a few studies focused on bacteria. However, these studies relied only on the cultivated fraction (Colldahl and Nilsson, 1973), amplicon libraries sequencing (Junker and Keller, 2015) or molecular fingerprinting (Obersteiner *et al.*, 2016). Here, for the first time, cultivation-dependent, cultivation-independent and microscopy analysis of four different pollen species were performed, thus achieving the most comprehensive bacterial microbiota analysis of pollen.

Cultivation-dependent analysis of bacterial microbiota

Comparison of CFU numbers showed that the population size of cultivable bacteria was significantly different between pollen species. Autumn crocus and rape had a higher bacterial load, followed by rye and birch (Fig. 1A). It is known, that various chemical and physical factors limit bacterial growth and survival in and around the plant surface (Lindow and Brandl, 2003). The ultrastructure examination of 34 species of plant pollen showed that the structure and pattern of the pollen outer layer (exine) differed between plant species and genera (Kosenko, 1999). The pollen showed a species-specific structure (Ariizumi and Toriyama, 2011), which influences the occurrence and abundance of microbes (Blackmore *et al.*, 2007). Furthermore, the bacterial population size on the plant surface depends on the level and availability of nutrition (Remus-Emsermann *et al.*, 2012). Nutritional composition studies of pollen revealed that hand-collected, bee-collected and stored pollen contained different amounts and types of proteins, lipids, ash and carbohydrates (Clark and Lintas, 1992; Human and Nicolson, 2006; Nicolson and Human, 2013). *Proteobacteria* (especially *Gammaproteobacteria*, family *Enterobacteriaceae*) were predominant among the isolates, followed by *Actinobacteria, Firmicutes* and *Bacteroidetes* in all the pollen species except for rye, were *Actinobacteria* dominated, followed by *Proteobacteria* (Fig. 2). Floral nectar-related major bacterial phyla from different plant species were *Proteobacteria*, *Actinobacteria* and *Firmicutes* (Álvarez-Pérez *et al.*, 2012; Mortazavi *et al.*, 2015). The extreme low overlap of bacterial species between the investigated pollens (Fig. 1B) demonstrated, that, the culturable fraction, of the pollen microbiota had a surprisingly high level of species-specificity. Only *Rosenbergiella nectarea* (Halpern *et al.*, 2013) was isolated from three of the four investigated pollen species, thus confirming that flower organs are the preferred habitat of this genus (Lenaerts *et al.*, 2014).

Cultivation-independent analysis of bacterial microbiota

Our culture independent approach showed that a highly diverse bacterial community is inhabiting the pollen. The taxa inventory obtained by high-throughput sequencing corroborated the results of cultivation-dependent analysis: *Proteobacteria* was the most abundant phylum and *Gammaproteobacteria* was one of the most abundant classes. At the family level, *Enterobacteriaceae* was one of the most abundant family in all pollen except rye (Fig. 3A). The bacterial species richness in wind-pollinating pollen species was significantly higher than in insect-pollinating pollen species (Fig. 3C). According to the alpha diversity indices, bacterial diversity significantly differed across the pollen species (Fig. 3B; Table 1). Beta diversity clearly indicated that bacterial communities were significantly different between pollen species, with the additional factor "pollination type" being also highly significant (Figs. 4 and 5A). These differences might be related to the different pollen coat structure between wind-pollinated and insect-pollinated species. It could be shown that insect-pollinated pollen has a more lipid-rich coat than wind-pollinated pollen (Edlund *et al.*, 2004). Bacterial and fungal patterns on different pollen species (birch and timothy grass) were found to be species-specific, and the birch pollen showed higher microbial diversity than timothy grass (Obersteiner *et al.*, 2016). However, these two pollen species have been collected from different regions, while in our work we analysed samples originating from four sampling sites within a restricted geographic area, with different pollen species sampled from the same sites. Beta-diversity analysis demonstrated that the site of origin is not a significant factor shaping the composition of pollen microbiota. The statistical significance of "pollination type" we found, based on two species per category, should be taken as good evidence rather than as prove, and the analysis of more pollen species would be suitable for further confirmation.

The network analysis indicated that there were more species-specific bacterial taxa than shared ones (Fig. 5B), which is in agreement with bacterial diversity analyses of floral nectar (Álvarez-Pérez *et al.*, 2012). It also revealed that there were more common bacterial taxa between wind-pollinating species than between insect-pollinated ones. This suggests an impact of the pollen characteristics, similarly to the microorganisms in the floral nectar, which were influenced by chemistry of the nectar (Fridman *et al.*, 2012), and impacted by plant pollinator interactions (Ushio *et al.*, 2015; Aizenberg-Gershtein et al., 2013) and wind pollination (Glushakova and Chernov, 2007). The identified core-microbiota (Fig. 5C) included only a few OTUs, both abundant (including *Rosenbergiella*, *Pseudomonas* and *Lactococcus*) and rare ones. *Rosenbergiella sp.* was isolated from floral nectar (Halpern *et al.*, 2013), while previous studies reported *Lactococcus sp.* detected in bee-collected pollen and *Pseudomonas sp.* on pollen samples (Vanneste *et al.*, 2011; Basim *et al.*, 2006). It can be speculated that by increasing the sequencing effort additional minor members shared by all pollen species

would be revealed, thus increasing the diversity. However, this would probably not significantly affect the size of the core bacterial microbiota of pollen. Considering the high level of specificity encountered, we suppose that adding other plant species would further reduce the size of the core-microbiome, although this remains to be verified by future studies.

Hitherto, there are only a few examples of habitat-related core-microbiomes. Neotropical plant phyllospheres consisted of a core bacterial biome of 32 bacterial species (Kembel *et al.*, 2014). A study of three different wild plants showed more than ten common root-associated bacterial families (Aleklett *et al.*, 2015). Thus, the bacterial core-microbiome of pollen appears to be smaller than that of other plant parts. More research is needed to better assess the habitat-related core-microbiomes next to the single-species core-microbiomes, since same plant organs play the same roles for the plants and might then be colonized by functionally similar microbes.

Comparison of cultivation-dependent and -independent results

The comparison of the bacterial families detected by isolation and by high-throughput sequencing showed the typical bias related to the culturability of most environmental microbes: about 17% of all the families detected by the cultivation-independent approach were also isolated. Only the family *Flavobacteriaceae* was isolated (one isolate of birch pollen) but not present in the total microbiota. The reason might be that the shorter sequences delivered by the IonTorrent method did not allow the annotation of the corresponding OTU at family level, although even the higher taxonomic ranges including this missing family (order Flavobacteriales, class Flavobacteriia) were not identified by the QIIME pipeline. Overall, we found a good correspondence between isolated families and total families for the analysed pollen species: as an example, the family *Enterobacteriaceae* was one of the most abundant by cultivation-independent approach for all the pollens except rye; interestingly we got several isolates of *Enterobacteriaceae*, but only one isolate from rye (Supporting Information Table S3). Further examples are the families *Bacillaceae* and "*Exiguobacteriaceae*," which were only detected in two pollen species by cultivation-independent approach and, accordingly, were only isolated from the same pollen species (Supporting Information Table S3). Both *Burkholderiaceae* and *Enterococcaceae* families were abundant only in one pollen species (birch and rape respectively), and the two only isolates found belonging to these families came from the same pollen species (Supporting Information Table S3). As a final example, the family *Microbacteriaceae* occurred in all pollen species by cultivation-independent analysis and was isolated from all pollen species, too.

SEM and FISH-CLSM microscopy were used to investigate the colonisation pattern of bacteria on pollen, in order to gain insight into the pollen–bacteria interactions (Gamalero *et al.*, 2003; Moter and Göbel, 2000). With both microscopic methods morphologically and taxonomically different bacteria were detected colonising the pollen surface (tectum). Furthermore, the difference of the tectum structure between pollen species was visualised (Fig. 6). Microscopic analysis thus supported the results of both culture-dependent and -independent methods. We hypothesise that the adhered bacteria colonise, multiply and eventually form biofilms on the sticky suitable habitat of pollen tectum. Bacterial adhesion is the preliminary stage of colony/biofilm formation, and SEM or confocal microscopy are key tools to evaluate this (El Abed *et al.*, 2012; Cardinale, 2014). Colldahl and Nilsson (1973) observed with SEM that the surface of birch and timothy pollens carried microorganisms-like particles. FISH-CLSM analysis of the pumpkin anthosphere showed the presence of *Alphaproteobacteria*, *Gammaproteobacteria* and *Firmicutes* on pollen grains, densely colonised by micro-colonies (Fürnkranz *et al.*, 2012). Our microscopy observations clearly indicated that bacteria colonise ubiquitously the pollen micro-habitat as single cells, colonies as well as biofilm-like structures. The multilayered pollen walls might be impermeable to FISH probes, which would hinder the detection of bacterial cells inside them; however we did not find any internal bacterial cell even in the broken pollen grains that we often found in our confocal microscopy observations. The comparison with the electron microscopy images let us argue that there might be the possibility that some bacterial cells get detached and lost during the washing steps of FISH: in fact, no washing step is performed for the SEM analysis, and more bacterial cells were eventually detected there on the pollen grains.

Our results highlighted the complexity of the pollen microbiota and provided for the first time its comprehensive characterization, including clear identification of cultivated and molecularly detected bacteria and identification of drivers of community structure and diversity. More research is needed to understand the implications of our discoveries on plant ecology and reproduction, which should focus on the function(s) of pollen-inhabiting microbes and on their horizontal as well as vertical transmission. Plant microbiome is not a single biological unit but a puzzle of complementary parts: our study on pollen microbiota significantly contributed to reveal one additional piece of this mosaic of microhabitats.

Experimental procedures

Sample collection

Birch (*Betula pendula* Roth., "B" when abbreviated), rape (*Brassica napus* L., "R" when abbreviated), rye (*Secale cereale*

L., "Ry" when abbreviated) and autumn crocus (*Colchicum autumnale* L., "Au" when abbreviated) were the selected plant species to collect the pollen samples. Birch and rye are wind-pollinated species, while rape and autumn crocus are insect-pollinated. Flowers of each plant species were collected in sterile separate DNA-free sterile plastic tubes from four locations in a restricted geographical region (within Giessen and in the 50 km surrounding, Supporting Information Table S4) during the flowering season (B1 – 2015-04-28, B2 and B3 – 2015-05-06 R1 and R2 – 2015-05-27, R3 – 2015-01-06; Ry1, Ry2 and Ry3 – 2015-06-10; Au1 – 2015-09-07, Au2 – 2015-09-11 and Au3 – 2015-09-14). For each species, three biological samples (for birch, rape and rye about 200–1000 flowers from the same plant or from closely growing plants, for autumn crocus about 30 flowers each) were collected. Anthers were immediately dissected sterile from the flowers into separate plastic tubes, kept in silica desiccator for one day to dry out. Then pollen grains were collected and extracted by shaking the tubes gently with a vortex mixer for 20–30 sec. A subsample was immediately frozen at −20°C for DNA extraction and other two subsamples were fixed for fluorescence *in-situ* hybridisation (FISH; see below for details). The remaining sample was used for bacterial isolation on agar medium plates.

Cultivation-dependent analysis of bacterial biome

Isolation of bacteria from pollen. The pollen samples (Birch: 23, 32 and 36 mg; Rape: 28, 33 and 89 mg; Rye: 20, 39 and 74 mg; Autumn crocus: 100, 110 and 250 mg) were mixed each with 5 ml of shaking solution (0.05% Tween 80 and 0.18% $Na_4P_2O_7$; Musovic *et al.*, 2006) and agitated for 30 min. This was followed by serial dilutions with 0.02% Tween 80 + 0.085% NaCl to a dilution of 10^{-5}. About 100 µl of each dilution was plated in triplicate onto 1:10 diluted AC agar medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and pollen medium; the latter consisted of minimal salt medium (Widdel and Bak, 1992) amended with commercial flower pollens (Blüten pollen, Bergland Pharma GmbH, Heimertingen, Germany) and 10 ml of pollen extract obtained from the same pollen sample used for isolation (10 g of autoclaved flowers in 500 ml sterile water for 30 min and filter-sterilized) (Supporting Information Table S5). Cycloheximide (200 mg l^{-1}) was added to the media to avoid growth of fungi. The plates were incubated aerobically for five days at 25°C. Colony counts were determined for both AC agar and pollen medium. The number of colonies of the three replicates of each dilution were averaged, and the total colony forming units (CFUs) per gram of sample were calculated and compared between pollen species by the Kruskal–Wallis test (Kruskal and Wallis, 1952).

Morphologically different colonies were selected and subcultured from single colonies for pure culture isolation. Each culture was grown in liquid AC medium to confirm purity by microscopic observation of the cellular morphology. For conservation of the pure culture it was grown in liquid AC (1:10) medium until an optical density of 0.8 to 1.2 at 600 nm was reached, and a 20% glycerol stab was stored at −80°C.

Identification of the bacterial isolate by 16S rRNA gene sequencing. Genomic DNA was isolated from the pure cultures with the NucleoSpin DNA isolation kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). The isolated genomic DNA was used as template for the polymerase chain reaction (PCR) using a MyCycler™ (Bio-Rad, München, Germany) to amplify the 16S rRNA gene with primers EUB9F (5′-GAGTTTGATCMTGGCTCAG-3′) and EUB1492R (5′-ACGGYTACCTTGTTACGACTT-3′) (Lane, 1991). The PCR products were cleaned using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) and sequenced by LGC genomics (Berlin, Germany). The high-quality region of the sequences was manually selected by visualising the electropherograms with the software MEGA version 6.0 (Tamura *et al.*, 2011) and then compared with the reference sequences by BLAST (Zhang *et al.*, 2000) and Ez Taxon (Kim *et al.*, 2012) alignment. Final figures were assembled with Photoshop CS6 (Adobe Systems Inc, San Jose). Venn diagram was drawn by Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/).

The 16S rRNA gene sequences obtained were submitted to NCBI gene library with the following accession numbers: KX450414–KX450474.

Cultivation-independent analysis of bacterial microbiota

Isolation of total DNA from pollen. Metagenomic DNA from different pollen species was obtained by a modified DNA extraction procedure (Burgmann *et al.*, 2001). For each pollen sample, 1 ml of extraction buffer (2.5 g l^{-1} SDS, 0.2 M sodium phosphate buffer (pH 8), 50 mM EDTA and 0.1 M NaCl, pH 8) was added to the reaction tube containing 40–500 mg of pollen grains and 100 mg sterile zirconium beads were added into the same tube. Cells were then disrupted for 2 min at maximum speed in a cell mill MM200 (Retsch, Haan, Germany). Then the samples were centrifuged at 4°C and 10,000 × *g* for 6 min in a microcentrifuge (Heraeus Fresco, Thermo Fisher Scientific Inc., Waltham). The supernatant was transferred into a new 2 ml microcentrifuge tube (Laborhaus Scheller GmbH & Co KG, Euerbach, Germany).

The supernatant was incubated with 10 µl RNase A (10 mg ml^{-1}) at 37°C for 30 min, then 850 µl of phenol/chloroform/isoamyl alcohol (25:24:1) were added and mixed with gentle swirling. The tubes were then centrifuged again at 10,000 × *g* for 5 min at 4°C, the aqueous phase was collected in a new sterile tube and chloroform/isoamyl alcohol (24:1) was added, mixed by gentle swirling and centrifuged 10,000 × *g* for 5 min at 4°C. Aqueous phase were transferred into new tube, DNA was precipitated with 1 ml of precipitation buffer [20% Poly (ethylene glycol) and 2.5 M NaCl] at 4°C for 1 h and centrifuged again at 10,000 × *g* for 5 min at 4°C. The DNA was washed with ice cold 75% ethanol, dried out, dissolved in nuclease free water. An aliquot of the DNA was visualized in 1% agarose gel stained with gel red.

Ion torrent sequencing. The partial sequence of the hypervariable regions (V4&V5) of the 16S rRNA gene of each DNA samples was PCR amplified using the primer 520 F (5′-AYTGGGYDTAAAGNG-3′) (Claesson *et al.*, 2009) and 907 R comp (5′-CCGTCAATTCMTTTRAGTTT-3′) (Engelbrektson *et al.*, 2010). PCR was carried out at a final volume of 15 µl contain 10 ng of genomic DNA, 3 µl of 5× KAPAHiFi (KAPA Biosystems, Wodurn) buffer, KAPA dNTP mix 200 µM each, primer 5 pmol each and KAPAHiFi polymerase 0.3 units. The PCR was performed with MyCycler™ (Bio-Rad) for 25 cycles

with the initial denaturation for 3 min at 95°C, cyclic denaturation for 20 sec at 98°C, annealing for 30 sec at 55°C and extension for 30 sec at 72°C with final extension for 5 min. The amplified PCR product was confirmed with agarose gel electrophoresis and this DNA fragments used as the template of second PCR reaction. The second PCR was done with primer 520 F and 907 R comp, adapter (5′-CCATCTCATCCCT GCGTGTCTCCGACTCAG-3′) and barcodes (Supporting Information Table S6) with KAPA2G Robust HotStart Ready-Mix (KAPA Biosystems). The final volume of 50 μl contain 2 μl of template, 10 μl of 5× KAPAHiFi buffer, KAPA dNTP mix 600 μM, primer 5 pmol and KAPAHiFi polymerase 1 unit. The PCR was performed with MyCycler™ (Bio-Rad) for 8 cycles with the initial denaturation for 3 min at 95°C, cyclic denaturation for 20 sec at 98°C, annealing for 30 sec at 55°C and extension for 30 sec at 72°C with final extension for 7 min. The PCR products were purified using QIAquick PCR purification kit (QIAGEN GmbH) and further purified with DNA purification beads NucleoMag® NGS clean-up kit (MACHEREY-NAGEL GmbH & Co. KG) to remove primer-dimers. Concentration of the amplified DNA was estimated with Qubit dsDNA HS assay kit by Qubit® 3.0 fluorometer (Life Technologies, Carlsbad). Concentration of each sample was adjusted to 1 μM, the samples were pooled to the final concentration of 26 pM and used for emulsion PCR with Ion PGM Template OT2 400 kit (Life Technologies) by using Ion OneTouch 2 (Life Technologies). Further the enrichment was done with Ion PGM Hi-Q OT2 (Life Technologies) kit by using Ion OneTouch ES (Life Technologies). After the enrichment, samples were loaded on a 314 chip for posterior sequencing (Ion PGM Hi-Q Sequencing kit & Ion 314v2 chip) (Life Technologies) by using Ion PGM Sequencer (Life Technologies).

PCR and sequencing was repeated two times (with the same metagenomic DNAs) and the sequences were merged in a unique dataset after checking that both delivered very similar outputs in terms of taxonomical composition of the microbiotas.

Sequences were submitted to EMBL under the project numbers PRJEB14477 and PRJEB14487.

Analysis of ion torrent sequences. The fastq file from Ion Torrent was analysed using QIIME, version 1.9 (Caporaso *et al.*, 2010a, b). The sequences were quality (threshold: 20) and length (350–450 bp) filtered, maximum homopolymer run < 6. The sequences were dereplicated and assigned to specific samples by corresponding barcodes. Operational taxonomic units were generated with a sequence similarity $\geq 97\%$ using the uclust method (Edgar, 2010). Chimera slayer (Haas *et al.*, 2011) was used to remove chimeric OTUs previously aligned to the Greengenes core reference alignment (DeSantis *et al.*, 2006) by pyNAST method (Caporaso *et al.*, 2010a, b). Taxonomy was assigned on the Greengenes database (McDonald *et al.*, 2012). Profile clustering networks was constructed using Cytoscape, version 3.3.0 (Shannon *et al.*, 2003) based on node and edge table created from QIIME with the script *make_otu_network.py*. Alpha diversity indices (Shannon-Wiener, Shannon-equitability, Dominance, Simpson, Simpson-reciprocal, PD-whole tree, Chao1 and observed species) were statistically compared between pollen species by ANOVA and Tukey HSD post-hoc test, using the software Statistica (Statsoft Inc., Tulsa). For Beta diversity analysis, jackknifed-weighted Unifrac distances (Lozupone and Knight, 2005) were used to assess the similarity of the community structure between pollen samples, and were visualized by Principal Component Analysis (PCoA)-plot (Jost, 2007; Milani *et al.*, 2013; Seedorf *et al.*, 2014; Schneider *et al.*, 2015) using Emperor (Vazquez-Baeza *et al.*, 2013). Statistical significance of the factor "species," "pollination type" and "sampling site" was tested by permutational multivariate analysis of variance, as implemented in QIIME (script *compare.categories.py*, method "Adonis," 999 permutations). Final figures were assembled with Photoshop CS6 (Adobe Systems Inc, San Jose).

Scanning electron microscopy (SEM)

Freshly collected pollen samples (10–100 mg) were dried out in silica desiccator for 24 hours and stored at −80°C until scanning electron microscopy examination. The tip of a small laboratory spoon of each pollen sample was hydrated 1 h at room temperature, then mounted on metal stubs and then coated with a thin layer of gold. They were observed with scanning electron microscope XL30 (Philips, Amsterdam, The Netherlands).

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

For FISH staining of Gram-negative bacteria, one pooled sample per pollen species consisting of 10–100 mg of pollen grains was fixed with a 3:1 mixture of 4% paraformaldehyde and 1× PBS, by incubation at 4°C for 6 hours. The samples were then washed three times with 1× PBS for increasing times, and then stored in 250–750 μl of 99.8% ethanol:1X PBS (1:1) at −20°C. Additionally, one pooled sample per pollen species was also fixed directly in 99.8% ethanol: 1× PBS (1:1) and stored at −20°C, for FISH staining of Gram-positive bacteria. For FISH staining, 25 μl of each sample were dried onto a poly-L-lysine-coated slide and treated with 1 mg ml^{-1} lysozyme for 10 min at room temperature, in order to increase the permeability of the cell walls to the FISH probes. Then, the slides were dipped stepwise into 50%, 80% and 96% ethanol, respectively, for 3 min each. About 15 μl of hybridisation buffer (180 μl 5 M NaCl, 20 μl 1 M Tris HCl, 5 μl 2% SDS, 300 μl formamide and 495 μl H_2O) containing 2.5 ng ml^{-1} of each Cy3-labeled EUB338MIX FISH probe and one of the Cy5-labeled specific probes (either LGC 354-MIX for *Firmicutes* or HGC236 for *Actinobacteria*; Table 2) were added to each sample and incubated at 45°C for 2 hours in the dark in a humid chamber saturated with hybridisation buffer. After removing the hybridisation buffer and washing with pre-warmed (46°C) washing buffer (1.02 μl 5 M NaCl, 1 μl 1 M Tris HCl, 0.5 μl 0.5 M EDTA and 47.48 μl ddH_2O), the slides were dipped for 5 sec into ice-cold water, dried out with soft compressed air, immediately mounted with antifade reagent and finally covered with a coverslip and sealed with transparent nail polish. The occurrence of false positive signals derived from a specific adhesion of FISH probes or fluorochromes to pollen structures was checked by staining a sample of each pollen species with ATTO488-, Cy3- and Cy5-labeled NONEUB probes (non-

Table 2. FISH probes used in this work.

Probe name	Sequence (5'–3')	Fluorochrome	Target	% Formamide (at 42°C)	Reference
EUB338*	GCTGCCTCCCGTAGGAGT	Cy3	Most bacteria	15	Amann et al. (1990)
EUB338II*	GCAGCCACCCGTAGGTGT	Cy3	*Planctomycetales*	15	Daims et al. (1999)
EUB338III*	GCTGCCACCCGTAGGTGT	Cy3	*Verrucomicrobiales*	15	Daims et al. (1999)
NONEUB**	ACTCCTACGGGAGGCAGC	Cy3	/	15	Wallner et al. (1993)
NONEUB**	ACTCCTACGGGAGGCAGC	Cy5	/	15	Wallner et al. (1993)
HGC236	AACAAGCTGATAGGCCGC	Cy5	*Actinobacteria*	15	Erhart et al. (1997)

* and ** used mixed in equimolar concentration.

sense probes with sequence complementary to that of EUB338 probe).

FISH-stained pollen samples were observed with a confocal laser scanning system Leica SP8 (Leica Microsystems GmbH, Mannheim, Germany), equipped with a microscope DM 6000CS and solid state Ar and HeNe lasers. Cy3 signal emitted by the universal bacterial probe EUB338MIX was excited with the 561 nm laser light and detected in the range 563–610, while Cy5 signal emitted by the phylum-specific probes was excited with the 633 nm laser light and detected in the range 645–690. Confocal stacks were acquired with a Z-step of 0.5–0.8 μm using the objective Leica HC PL APO CS2 63×/1.20 W. Volume-rendering and three-dimensional models of the confocal stacks were created with the software Imaris 8.2 (Bitplane AG, Zürich, Switzerland). Final figures were assembled with Photoshop CS6.

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References

Aizenberg-Gershtein, Y., Izhaki, I., and Halpern, M. (2013) Do honeybees shape the bacterial community composition in floral nectar? *Plos One* **8:** e67556.

Aleklett, K., Hart, M., and Shade, A. (2014) The microbial ecology of flowers: An emerging frontier in phyllosphere research. *Botany* **92:** 253–266.

Aleklett, K., Leff, J.W., Fierer, N., and Hart, M. (2015) Wild plant species growing closely connected in a sualpine meadow host distinct root-associated bacterial communities. *PeerJ* **3:** e804.

Álvarez-Pérez, S., Herrera, C.M., and de Vega, C. (2012) Zooming-in on floral nectar: A first exploration of nectar-associated bacteria in wild plant communities. *FEMS Microbiol Ecol* **80:** 591–602.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56:** 1919–1925.

Ariizumi, T., and Toriyama, K. (2011) Genetic regulation of sporopollenin synthesis and pollen exine development. *Annu Rev Plant Biol* **62:** 437–460.

Basim, E., Basim, H., and Özcan, M. (2006) Antibacterial activities of Turkish pollen and propolis extracts against plant bacterial pathogens. *J Food Process Eng* **77:** 992–996.

Berg, G., Grube, M., Schloter, M., and Smalla, K. (2014) Unraveling the plant microbiome: Looking back and future perspectives. *Front Microbiol* **7:** 148.

Blackmore, S., Wortly, A.H., Skvarla, J.J., and Rowley, J.R. (2007) Pollen wall development in flowering plants. *New Phytol* **2007:** 483–498.

Burgmann, H., Pesaro, M., Widmer, F., and Zeyer, J. (2001) A strategy for optimizing quality and quantity of DNA extracted from soil. *J Microbiol Methods* **45:** 7–20.

Caporaso, J.G., Bittinger, K., Bushman, F.D., DeSantis, T.Z., Andersen, G.L., and Knight, R. (2010a) PyNAST: A flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26:** 266–267.

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

Cardinale, M. (2014) Scanning a microhabitat: Plant-microbe interactions revealed by confocal laser microscopy. *Front Microbiol* **5:** 94.

Carinanos, P., and Casares-Porcel, M. (2011) Urban green zones and related pollen allergy: A review. Some guidelines for designing spaces with low allergy impact. *Landscape Urban Plan* **101:** 205–214.

Claesson, M.J., O'sullivan, O., Wang, Q., Nikkilä, J., Marchesi, J.R., Smidt, H., *et al.* (2009) Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. *Plos One* **4:** e6669.

Clark, C.J., and Lintas, C. (1992) Chemical composition of pollen from kiwifruit vines. *N Z J Crop Hort* **20:** 337–344.

Colldahl, H., and Carlsson, G. (1968) Allergens in pollen. *Acta Allergol* **28:** 387–397.

Colldahl, H., and Nilsson, L. (1973) Possible relationship between some allergens (pollens, mites) and certain microorganisms (bacteria and fungi). *Acta Allergol* **28:** 283–295.

Daims, H., Brühl, A., Amann, R., Schleifer, K.H., and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: Development and

evaluation of a more comprehensive probe set. *Syst Appl Microbiol* **22:** 434––444.
DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., *et al.* (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microb* **72:** 5069–5072.
Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26:** 2460–2461.
Edlund, A.F., Swanson, R., and Preuss, D. (2004) Pollen and stigma structure and function: The role of diversity in pollination. *Plant Cell* **16:** S84–S97.
El Abed, S., Hamadi, F., Latrache, H., and Ibnsouda, S.K. (2012) Scanning electron microscopy (SEM) and environmental SEM: Suitable tools for study of adhesion stage and biofilm formation. In: *Scanning Electron Microscopy.* Kazmiruk, V. (ed). New York: INTECH Open Access Publisher.
Engelbrektson, A., Kunin, V., Wrighton, K.C., Zvenigorodsky, N., Chen, F., Ochman, H., *et al.* (2010) Experimental factors affecting PCR-based estimates of microbial species richness and evenness. *ISME J* **4:** 642–647.
Erhart, R., Bradford, D., Seviour, R.J., Amann, R., and Blackall, L.L. (1997) Development and use of fluorescent *in situ* hybridization probes for the detection and identification of *Microthrix parvicella* in activated sludge. *Syst Appl Microbiol* **20:** 310–318.
Fridman, S., Izhaki, I., Gerchman, Y., and Halpern, M. (2012) Bacterial communities in floral nectar. *Environ Microbiol Rep* **4:** 97–104.
Fürnkranz, M., Lukesch, B., Müller, H., Huss, H., Grube, M., and Berg, G. (2012) Microbial diversity inside Pumpkin: Microhabitat-specific communities display a high antagonistic potential against phytopathogens. *Microb Ecol* **63:** 418–428.
Gamalero, E., Lingua, G., Berta, G., and Lemanceau, P. (2003) Methods for studying root colonization by introduced beneficial bacteria. *Agronomie* **23:** 407–418.
Gilliam, M. (1979) Microbiology of pollen and bee bread: The yeast. *Apidologie* **10:** 43–53.
Glushakova, A.M., and Chernov, I.Y. (2007) Seasonal dynamic of the numbers of epiphytic yeasts. *Microbiologiya* **76:** 668–674.
Haas, B.J., Gevers, D., Earl, A.M., Feldgarden, M., Ward, D.V., Giannoukos, G., *et al.* (2011) Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res* **21:** 494–504.
Halpern, M., Fridman, S., Atamna-Ismaeel, N., and Izhaki, I. (2013) *Rosenbergiella nectarea* gen. nov., sp. nov., in the family *Enterobacteriaceae*, isolated from floral nectar. *Int J Syst Evol Microbiol* **63:** 4259–4265.
Hani, B., Dalila, B., Saliha, D., Daoud, H., Mouloud, G., and Seddik, K. (2012) Microbiological sanitary aspects of pollen. *Adv Environ Biol* **6:** 1415–1420.
Human, H., and Nicolson, S.W. (2006) Nutritional content of fresh, bee-collected and stored pollen of *Aloe greatheadii* var. davyana (*Asphodelaceae*). *Phytochemistry* **67:** 1486–1492.
Jojima, Y., Mihara, Y., Suzuki, S., Yokozeki, K., Yamanaka, S., and Fudou, R. (2004) *Saccharibacter floricola* gen. nov., sp. nov., a novel osmophilic acetic acid bacterium isolated from pollen. *Int J Syst Evol Microbiol* **6:** 2263–2267.
Jost, L. (2007) Partitioning diversity into independent alpha and beta components. *Ecology* **88:** 2427–2439.
Junker, R.R., and Keller, A. (2015) Microhabitat heterogeneity across leaves and flower organs promotes bacterial diversity. *FEMS Microbiol Ecol* **91:** fiv097.
Kembel, S.W., O'conner, T.K., Arnold, H.K., Hubbell, S.P., Wright, S.J., and Green, J.L. (2014) Relationship between phyllosphere bacterial communities and plant functional traits in a neotropical forest. *pnas* **111:** 13715–13720.
Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., *et al.* J., (2012) Introducing EzTaxon-e: A prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* **62:** 716–721.
Kosenko, V.N. (1999) Contributions to the pollen morphology and taxonomy of the *Liliaceae*. *Grana* **38:** 20–30.
Kruskal, W.H., and Wallis, W.A. (1952) Use of ranks in one-criterion variance analysis. *J Am Stat Assoc* **47:** 583–621.
Lane, D.J. (1991) 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics.* Stackebrandt, E., and Goodfellow, M. (eds). New York: John Wiley and Sons Inc, pp. 115–175.
Lenaerts, M., Álvarez-Pérez, S., De Vega, S., Van Assche, A., Johnson, S.D., Willems, K.A., *et al.* (2014) *Rosenbergiella australoborealis* sp. nov., *Rosenbergiella collisarenosi* sp. nov. and *Rosenbergiella epipactidis* sp. nov., three novel bacterial species isolated from floral nectar. *Syst Appl Microbiol* **37:** 402–411.
Lindow, S.E., and Brandl, M.T. (2003) Microbiology of the phyllosphere. *Appl Environ Microb* **69:** 1875–1883.
Lozupone, C., and Knight, R. (2005) UniFrac: A new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* **71:** 8228–8235.
McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A.P., *et al.* (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *Isme J* **6:** 610–618.
Meyer, K.M., and Leveau, J.H.J. (2012) Microbiology of the phyllosphere: A playground for testing ecological concepts. *Oecologia* **168:** 621–629.
Milani, C., Hevia, A., Foroni, E., Duranti, S., Turroni, F., Lugli, P., *et al.* (2013) Assessing the fecal microbiota: An optimized ion torrent 16S rRNA gene-based analysis protocol. *Plos One* **8:** e68739.
Mortazavi, R., Attiya, S., and Ariya, P.A. (2015) Arctic microbial and next-generation sequencing approach for bacteria in snow and frost flowers: Selected identification, abundance and freezing nucleation. *Atmos Chem Phys* **15:** 6183–6204.
Moter, A., and Göbel, U.B. (2000) Fluorescence *in situ* hybridization (FISH) for direct visualization of microorganisms. *J Microbiol Meth* **41:** 85–112.
Musovic, S., Oregaard, G., Kroer, N., and Sorensen, S.J. (2006) Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among gram-positive and gram-negative bacteria indigenous to the barley rhizosphere. *Appl Environ Microb* **72:** 6687–6692.
Nicolson, S.W., and Human, H. (2013) Chemical composition of the 'low quality' pollen of sunflower (*Helianthus annuus*, Asteraceae). *Apidologie* **44:** 144–152.
Obersteiner, A., Gilles, S., Frank, U., Beck, I., Häring, F., Erns, D., *et al.* (2016) Pollen-associated microbiome correlates

with pollution parameters and the allergenicity of pollen. *Plos One* **11:** e0149545.
Oldenburg, M., Petersen, A., and Baur, X. (2011) Maize pollen is an important allergen in occupationally exposed workers. *J Occup Med Toxicol* **6:** 32.
Redford, A.J., Bowers, R.M., Knight, R., Linhart, Y., and Fierer, N. (2010) The ecology of the phyllosphere: Geographic and phylogenetic variability in the distribution of bacteria on tree leaves. *Environ Microbiol* **12:** 2885–2893.
Remus-Emsermann, M.N., Tecon, R., Kowalchuk, R., and Leveau, J.H. (2012) Variation in local carrying capacity and the individual fate of bacterial colonizers in the phyllosphere. *ISME J* **6:** 756–765.
Rintala, H., Pitkäranta, M., Toivola, M., Paulin, L., and Nevalainen, A. (2008) Diversity and seasonal dynamics of bacterial community in indoor environment. *BMC Microbiol* **8:** 56.
Ryan, R.P., Germaine, K., Franks, A., Ryan, D.J., and Dowling, D.N. (2008) Bacterial endophytes: Recent developments and applications. *FEMS Microbiol Lett* **278:** 1–9.
Schneider, D., Engelhaupt, M., Allen, K., Kurniawan, S., Krashevska, V., Heinemann, M., *et al.* (2015) Impact of lowland rainforest transformation on diversity and composition of soil prokaryotic communities in Sumatra (Indonesia). *Front Microbiol* **6:** 1339.
Seedorf, H., Kittelmann, S., Henderson, G., and Janssen, P.H. (2014) RIM-DB: A taxonomic framework for community structure analysis of methanogenic archaea from the rumen and other intestinal environments. *Peer J* **2:** e494.
Shannon, P., Markie, A., Ozier, O., Baliqa, N.S., Wang, J.T., Ramage, D., *et al.* (2003) Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res* **13:** 2498–2504.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28:** 2731–2739.
Turner, T.R., James, E.K., and Poole, P.S. (2013) The plant microbiome. *Genome Biol* **14:** 10–1186.
Ushio, M., Yamasaki, E., Takasu, H., Nagano, A.J., Fujinaga, S., Honjo, M.N., *et al.* (2015) Microbial communities on flower surfaces act as signatures of pollinator visitation. *Sci Rep-UK* **5:** 8695.
Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015) The importance of the microbiome of the plant holobiont. *New Phytol* **206:** 1196–1206.
Vanneste, J.L., Giovanardi, D., Yu, J., Cornish, A.D., Kay, C., Spinelli, F., *et al.* (2011) Detection of *Pseudomonas syringae* pv. Actinidiae in kiwifruit pollen samples. *N Z Plant Prot* **64:** 246–251.
Vazquez-Baeza, Y., Pirrung, M., Gonzalez, A., and Knight, R. (2013) Emperor: A tool for visualizing high-throughput microbial community data. *Gigascience* **2:** 16.
Wallner, G., Amann, R., and Beisker, W. (1993) Optimizing fluorescent *in situ* hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. *Cytometry* **14:** 136–143.
Widdel, F., and Bak, F. (1992) Gram-negative mesophile sulfate-reducing bacteria. In *The Prokaryotes*. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer, pp. 3352–3378.
Zhang, Z., Schwartz, S., Wagner, L., and Miller, W. (2000) A greedy algorithm for aligning DNA sequences. *J Comput Biol* **7:** 203–214.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Table S1. Cultured bacteria associated with four different flower pollen species, identified using the EzTaxon server on basis of 16S rRNA gene sequence data.
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Fig. S1. Flower samples used in this work for the pollen collection: rape (A), rye (B), birch (C) and autumn crocus (D).

Supplementary Material:-

Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity

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Table S1 Cultured bacteria associated with four different flower pollen species, identified using the EzTaxon server on basis of 16S rRNA gene sequence data.

Name of the isolate	Accession number of the isolate	Rye	Rape	Birch	Autumn crocus	Length of aligned sequences (bp)	Phylum or Class	Taxonomical affiliation (Accession number) [% of pairwise sequence similarity]
RYC17	KX450460	✓				874	Proteobacteria	*Pantoea brenneri* (EU216735) [99.8]
RYA3	KX450447	✓				949	Proteobacteria	*Pseudomonas poae* (AJ492829) [100]
RYC16	KX450459	✓				913	Proteobacteria	*Stenotrophomonas rhizophila* (CP007597) [100]
RYA10	KX450454	✓				944	Firmicutes	*Bacillus simplex* (AB363738) [100]
RYA7	KX450451	✓				974	Firmicutes	*Exiguobacterium sibiricum* (CP001022) [100]
RYB14	KX450457	✓				871	Actinobacteria	*Arthrobacter niigatensis* (AB248526)[99.7]
RYA9	KX450453	✓				861	Actinobacteria	*Arthrobacter oryzae* (AB279889) [99.9]
RYB15	KX450458	✓				911	Actinobacteria	*Nocardioides alpinus* (GU784866) [97.5]
RYA2	KX450446	✓				998	Actinobacteria	*Rathayibacter festucae* (AM410683) [99.9]
RYC18	KX450461	✓				918	Actinobacteria	*Rhodococcus corynebacterioides* (AF430066) [99.7]
RYA5, RYC19	KX450449, KX450462	✓				901, 942	Actinobacteria	*Rhodococcus fascians* (JMEN01000010) [100,100]
RYA1	KX450445	✓				908	Actinobacteria	*Streptomyces anulatus* (DQ026637) [100]
RYA4, RYA6, RYA11, Rb-Pol-2	KX450448, KX450450, KX450455, KX450443	✓	✓			758-864	Actinobacteria	*Clavibacter michiganensis* (HE608962, U30254) [99.8 - 100]
RYA8, Ra-AC-3	KX450452, KX450432	✓	✓			958, 841	Actinobacteria	*Frigoribacterium faeni* (Y18807) [98.4, 99.4]

RYB12, Rb-AC-1	KX450456, KX450437	✓	✓			908, 775	Firmicutes	*Exiguobacterium artemiae* (AM072763) [100, 100]
Rb-AC-2, Rb-Pol-1	KX450438, KX450442		✓			732, 739	Proteobacteria	*Pantoea vagans* (EF688012) [100, 100]
Ra-Pol-1	KX450434		✓			835	Proteobacteria	*Pseudomonas endophytica* (LN624760) [100]
Ra-Pol-3, Rb-AC-5, Rb-Pol-3	KX450436, KX450441, KX450444		✓			770 - 778	Proteobacteria	*Rahnella woolbedingensis* (KF308409) [99.9 - 99.9]
Rb-AC-3	KX450439		✓			708	Firmicutes	*Enterococcus haemoperoxidus* (KB946316) [99.9]
Ra-AC-4, Rb-AC-4	KX450433, KX450440		✓			825, 791	Firmicutes	*Lactococcus garvieae* (AP009332) [100, 100]
Ra-Pol-2, Ra-AC-2	KX450435, KX450431		✓			835, 796	Firmicutes	*Staphylococcus xylosus* (D83374) [100, 100]
Ra-AC-1, Bb-AC-3, AU2, AU5, AU11	KX450430, KX450416, KX450463, KX450464, KX450466		✓	✓	✓	849 - 1074	Proteobacteria	*Rosenbergiella nectarea* (HQ284827) [99.7 – 100]
Bb-Pol-6	KX450422			✓		885	Proteobacteria	*Burkholderia andropogonis* (LAQU01000081) [97.9]
Bd-AC-3	KX450427			✓		802	Proteobacteria	*Erwinia billingiae* (Y13249) [99.9]
Bd-AC-4	KX450428			✓		843	Proteobacteria	*Erwinia tasmaniensis* (CU468135) [99.1]
Bb-Pol-7	KX450423			✓		971	Proteobacteria	*Methylobacterium mesophilicum* (D32225) [99.6]
Bd-AC-2	KX450426			✓		868	Proteobacteria	*Pseudomonas congelans* (AJ492828) [100]
Bd-Pol-1	KX450429			✓		854	Proteobacteria	*Rahnella aquatilis* (CP003244) [100]
Bb-Pol-4	KX450420			✓		916	Proteobacteria	*Rhizobium soli* (EF363715) [99.7]
Bb-AC-4, Bb-Pol-5	KX450417, KX450421			✓		825, 879	Proteobacteria	*Sodalis praecaptivus* (CP006569) [97.7, 97.8]
Bb-Pol-8	KX450424			✓		858	Firmicutes	*Staphylococcus cohnii* (D83361) [100]
Bd-AC-1	KX450425			✓		821	Bacteroidetes	*Chryseobacterium indoltheticum* (AY468448) [100]
Ba-AC-1	KX450414			✓		965	Bacteroidetes	*Hymenobacter roseus* (HG965772) [98.3]

Bb-AC-2	KX450415			✓		963	Actinobacteria	*Microbacterium phyllosphaerae* (AJ277840) [100]
Bb-Pol-2	KX450419			✓		857	Actinobacteria	*Micrococcus luteus* (CP001628) [100]
Bb-Pol-1	KX450418			✓		925	Actinobacteria	*Streptomyces hydrogenans* (AB184868) [100]
AU19	KX450474				✓	1024	Proteobacteria	*Erwinia persicina* (U80205) [99.2]
AU8, AU14	KX450465, KX450469				✓	1072, 1012	Proteobacteria	*Erwinia piriflorinigrans* (GQ405202) [97.9, 97.2]
AU17	KX450472				✓	1020	Proteobacteria	*Lonsdalea quercina* (JF311441) [100]
AU15	KX450470				✓	929	Actinobacteria	*Micrococcus yunnanensis* (FJ214355) [99.7]
AU12	KX450467				✓	767	Proteobacteria	*Pantoea septica* (EU216734) [98.7]
AU16	KX450471				✓	702	Proteobacteria	*Rahnella victoriana* (KF308403) [99.9]
AU13	KX450468				✓	1035	Firmicutes	*Brevibacterium frigoritolerans* (AM747813) [100]
AU18	KX450473				✓	1029	Actinobacteria	*Curtobacterium flaccumfaciens* (AJ312209) [100]

Table S2 BLAST similarity search details of representative sequence of biggest OTUs significantly different between pollen species.

OTU designation by QIIME	OTU ID	Best BLAST matches (type strains only)	Similarity (%)	Accession number
Enterobacteriaceae	denovo1070	*Rosenbergiella australoborealis*	100	NR126305
		Rosenbergiella collisarenosi	100	NR126304
		Rosenbergiella epipactidis	100	NR126303
		Rosenbergiella nectarea	99.7	NR117969
Xanthomonadaceae	denovo2355	*Stenotrophomonas rhizophila*	100	NR121739
Oxalobacteraceae	denovo1140	*Massilia suwonensis*	99.4	NR116872
		Massilia jejuensis	99.4	NR116871
		Massilia niabensis	99.4	NR044571
		Massilia varians	99.4	NR042652
		Massilia alkalitolerans	99.4	NR043094
	denovo403	*Noviherbaspirillum suwonense*	100	NR133798
	denovo2439	*Duganella phyllosphaerae*	100	NR108529
		Duganella zoogloeoides	100	NR114106
Enterococcaceae	denovo541	*Enterococcus rotai*	100	CP013655

		Enterococcus silesiacus	100	CP013614
		Enterococcus phoeniculicola	100	NR113938
		Enterococcus moraviensis	100	NR113937
		Enterococcus haemoperoxidus	100	NR113936
		Enterococcus thailandicus	100	NR114015
		Enterococcus plantarum	100	NR118050
		Enterococcus ureasiticus	100	NR117520
		Enterococcus quebecensis	100	NR117519
		Enterococcus termitis	100	NR042406
		Enterococcus caccae	100	NR043285
		Enterococcus ureilyticus	100	NR125485
Buchnera	denovo329	*Buchnera aphidicola*	98.2	JQ269547
Acetobacteraceae	denovo2322	*Acetobacter tropicalis*	94.9	NR113846
		Gluconacetobacter takamatsuzukensis	94.8	NR114384
		Gluconacetobacter aggeris	94.8	NR114382
		Gluconacetobacter asukensis	94.8	NR113364
		Gluconacetobacter tumulicola	94.8	NR113363
		Kozakia baliensis	94.8	NR113858
	Denovo509	*Neoasaia chiangmaiensis*	96.4	NR113975
		Kozakia baliensis	96.4	NR113858
		Asaia siamensis	96.4	NR113845
		Asaia lannensis	96.4	NR114144
		Asaia bogorensis	96.4	NR113849
		Asaia prunellae	96.4	NR112880
		Asaia astilbis	96.4	NR122089
	Denovo2199	*Gluconacetobacter tumulisoli*	94.8	NR114383

Table S3 Comparison of bacterial families detected by isolation (identification by sequencing of the 16S rRNA gene and EzTaxon database comparison) and by IonTorrent high-throughput sequencing (identification by Qiime pipeline annotation).

Bacterial family	**Birch**		**Rape**		**Rye**		**Autumn crocus**	
	Relative abundance in the total microbiota (%)	**Number of isolates**	**Relative abundance in the total microbiota (%)**	**Number of isolates**	**Relative abundance in the total microbiota (%)**	**Number of isolates**	**Relative abundance in the total microbiota (%)**	**Number of isolates**
Acetobacteraceae	**33.20**		**0.71**		**5.58**		**0.39**	
Acidobacteriaceae	**4.52**		**0.20**		**1.99**			
Aerococcaceae					**0.17**			
Alcaligenaceae	**0.31**		**0.15**		**0.41**			
Ardenscatenaceae					**0.18**			
Bacillaceae					**0.37**	**1**	**0.42**	**1**
Beijerinckiaceae	**0.53**				**0.64**			
Bradyrhizobiaceae	**0.24**		**0.32**		**2.44**		**0.54**	
Burkholderiaceae	**12.83**	**1**			**0.43**		**0.15**	
C111 (*Acidimicrobiales*)	**0.64**				**0.84**			
Caldicoprobacteraceae	**0.30**				**0.13**			
Carnobacteriaceae			**1.00**		**0.14**			
Caulobacteraceae	**1.42**		**0.29**		**2.58**		**0.15**	
Chitinophagaceae			**0.54**		**0.31**		**0.73**	

Clostridiaceae	**0.18**		**0.37**		**0.57**			
***Comamonadacea*e**	**0.15**		**0.19**		**0.66**			
Corynebacteriaceae	**0.70**		**0.68**		**0.27**		**0.36**	
Coxiellaceae	**0.12**				**0.62**			
Crenotrichaceae	**0.55**							
Cystobacterineae	**0.34**				**0.46**			
***Cytophagacea*e**	**0.92**	**1**			**0.22**			
Deinococcaceae	**0.22**		**0.14**		**0.28**			
EB1017 (*Acidimicrobiales*)					**0.13**			
Ellin515 (*Pedosphaerales*)					**0.19**			
Ellin5301 (*Gemmatimonadales*)					**0.83**			
Ellin6075 (Chloracidobacteria)	**0.72**				**0.49**			
Enterobacteriaceae	**18.67**	**6**	**26.88**	**6**	**2.72**	**1**	**77.81**	**9**
Enterococcaceae			**14.36**	**1**	**0.35**		**0.68**	
Erysipelotrichaceae			**0.40**					
Erythrobacteraceae	**0.30**				**0.28**			
Eubacteriaceae					**0.19**			
Frankiaceae	**0.23**							
Gaiellaceae	**0.60**		**0.14**		**0.38**			

Gemmataceae	**0.64**				**0.19**			
Gemmatimonadaceae					**0.24**			
Geodermatophilaceae	**0.55**				**0.19**			
Halomonadaceae							**0.76**	
Haloplasmataceae					**0.24**			
Hyphomicrobiaceae					**0.12**			
Intrasporangiaceae	**0.16**				**0.34**			
Koribacteraceae					**0.83**			
Lactobacillaceae	**0.14**		**0.29**		**0.67**		**0.27**	
Legionellaceae	**0.20**				**0.13**			
Leuconostocaceae			**0.12**		**1.27**		**0.35**	
Listeriaceae			**0.96**					
mb2424 (*Acidobacteria*-6)					**0.33**			
Methylobacteriaceae	**0.94**	**1**	**0.25**		**0.53**			
Methylocystaceae	**18.42**				**0.78**			
Microbacteriaceae	**0.84**	**1**	**0.57**	**2**	**1.69**	**5**	**1.16**	**1**
Micrococcaceae	**0.30**	**1**			**4.29**	**2**		**1**
Micromonosporaceae					**0.31**			
ML1228J-1 (*Natranaerobiales*)					**0.95**			
Moraxellaceae	**0.23**		**0.62**		**0.30**		**0.58**	

Mycobacteriaceae	**0.55**				**0.64**			
Nakamurellaceae	**0.27**				**0.61**			
Neisseriaceae	**0.13**				**0.73**		**0.22**	
Nitrosomonadaceae					**0.14**			
Nitrospiraceae					**0.84**			
Nocardiaceae	**0.27**				**0.34**	**3**		
Nocardioidaceae	**2.69**		**0.19**		**3.84**	**1**		
oc28 (*Anaerolineae*)					**0.19**			
Opitutaceae	**0.11**		**0.14**		**0.56**			
Oxalobacteraceae	**2.97**		**0.13**		**25.41**		**0.22**	
Paenibacillaceae					**0.22**			
Peptostreptococcaceae	**0.58**							
Phyllobacteriaceae	**0.55**				**0.12**			
Piscirickettsiaceae	**0.12**							
Planococcaceae	**0.27**				**0.61**			
Polyangiaceae					**0.12**			
Propionibacteriaceae	**0.18**		**0.25**		**6.37**		**0.80**	
Pseudomonadaceae	**0.99**	**1**	**24.57**	**1**	**0.67**	**1**	**17.87**	
Pseudonocardiaceae					**0.22**			
Rhizobiaceae	**0.30**	**1**			**0.72**			

Rhodobacteraceae	**0.14**				**0.58**			
Rhodocyclaceae	**0.78**				**0.86**			
Rhodospirillaceae	**0.61**		**0.14**		**0.27**			
Rickettsiaceae	**0.82**		**0.37**					
Ruminococcaceae					**0.13**			
SBYG_4172 (*Clostridiales*)					**0.18**			
Sinobacteraceae	**0.19**		**0.36**		**1.21**		**0.27**	
Solibacteraceae					**0.28**			
Solirubrobacteraceae	**0.64**				**0.22**			
Sphingomonadaceae	**0.71**				**1.12**			
Sporichthyaceae	**0.13**				**0.33**			
Staphylococcaceae	**0.27**	**1**	**0.80**	**2**	**0.12**			
Streptococcaceae	**0.59**		**31.12**	**2**	**0.81**		**0.34**	
Streptomycetaceae		**1**			**0.24**	**1**	**0.50**	
Streptosporangiaceae					**0.19**			
Symbiobacteriaceae					**0.43**			
Syntrophobacteraceae					**0.25**			
Trueperaceae					**0.82**			
Turicibacteraceae					**0.48**			
UD5 (*Betaproteobacteria*)					**0.28**			

Veillonellaceae					**0.24**			
Xanthomonadaceae	**0.31**				**23.63**	**1**	**0.49**	
Other (*Lactobacillales*)			**0.16**		**0.12**			
Other (*Rhizobiales*)	**0.98**				**0.12**			
Other (*Gammaproteobacteria*)	**0.75**							
[*Chthoniobacteraceae*]	**0.20**				**0.20**			
[*Cloacamonaceae*]	**0.51**							
[*Entotheonellaceae*]					**0.17**			
[*Exiguobacteraceae*]			**0.60**	**1**	**0.17**	**2**		
[*Fimbriimonadaceae*]	**0.94**				**0.19**			
[*Tissierellaceae*]	**0.19**		**0.54**		**0.48**		**0.22**	
0319-6A21 (*Nitrospirales*)					**0.45**			
0319-6G20 (*Myxococcales*)	**0.12**		**0.13**		**0.22**		**0.22**	
A1-B1 (*Gemmatimonadales*)					**0.13**			

Table S4 Details of the sampling sites were the pollen samples were collected

Pollen Sample	Species	Sampled site	Geographic coordinates
B1	Birch	Giessen	Lat: 50.569326; Long: 8.671992
B2	Birch	Linden	Lat: 50.531427; Long: 8.657827
B3	Birch	Giessen	Lat: 50.581524; Long: 8.694574
R1	Rape	Linden	Lat: 50.534488; Long: 8.676344
R2	Rape	Linden	Lat: 50.537497; Long: 8.660916
R3	Rape	Hohenahr	Lat: 50.682829; Long: 8.478091
RY1	Rye	Giessen	Lat: 50.606621; Long: 8.658561
RY2	Rrye	Giessen	Lat: 50.606878; Long: 8.658299
RY3	Rye	Giessen	Lat: 50.607171; Long: 8.657949
AU2	Autumn crocus	Giessen	Lat: 50.573791; Long: 8.696016
AU3	Autumn crocus	Wettenberg	Lat: 50.646189; Long: 8.620275

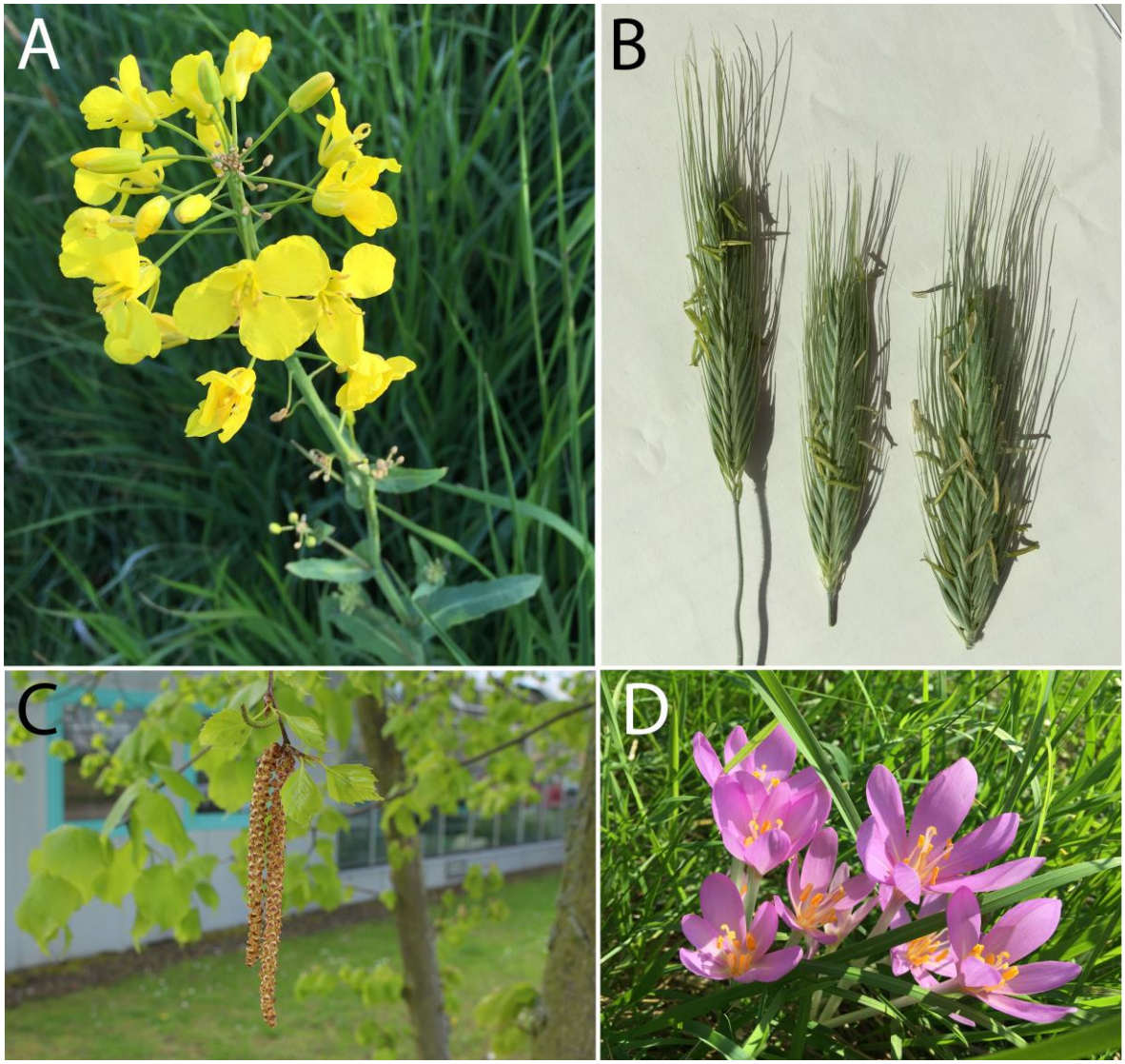


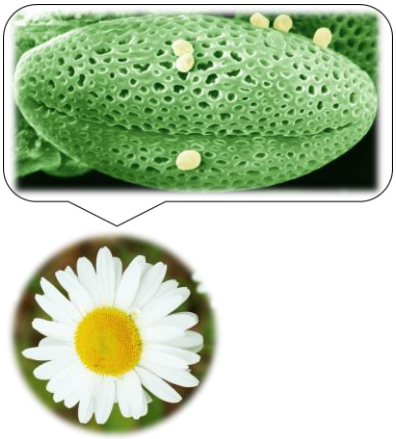
Fig. S1 Flower samples used in this work for the pollen collection: rape (A), rye (B), birch (C) and autumn crocus (D).

Table S5 Composition of pollen medium

1. Basic media salt	
$NaCl$	1.00 g
$MgCl_2\ 6H_2O$	0.40 g
$CaCl_2\ 2H_2O$	0.15 g
KCl	0.50 g
KH_2PO_4	0.20 g
NH_4Cl	0.25 g
2. Phosphate buffer 0.4 M pH7	
$Na_2HPO_4\ 2H_2O$	1.78 g
$NaH_2PO_4\ 2H_2O$	0.78 g
3. Trace elements	
$FeCl_2\ 4H_2O$	1.000 mg
$ZnCl_2$	0.070 mg
$MnCl_2\ 4H_2O$	0.100 mg
H_3BO_3	0.006 mg
$CoCl_2\ 6H_2O$	0.130 mg
$CuCl_2\ 2H_2O$	0.002mg
$NiCl_2\ 6H_2O$	0.024 mg
$Na_2MoO_4\ 2H_2O$	0.036 mg
4. $MgSO_4\ 7H_2O$	1.00 g
5. Flower pollen (commercial)	6.50 g
6. Vitamin B_{12}	0.05 mg
7. Riboflavin	0.05 mg
8. Thiamine	0.10 mg
9. Pyridoxine dihydrochloride	0.15 mg
10. Nicotinic acid	0.10 mg
11. Calcium-D(+)- Pantothenate	0.05 mg
12. 4-Aminobenzoic acid	0.04 mg
13. D(+)- biotine	0.01 mg
14. Pollen extract (of respective pollen sampled)	10 ml
14. Agar	15 g

Table S6 Details of barcode sequences, linker primer sequences and reverse primer used for Ion Torrent sequencing.

Sample ID	Barcode Sequence	Linker Primer Sequence	Reverse Primer
B1	ACGAGTGCGT	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
B2	CGTGTCTCTA	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
B3	TCTCTATGCG	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
R1	ATACGACGTA	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
R2	TGTACTACTC	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
R3	ACGCGAGTAT	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
RY1	TACACGTGAT	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
RY2	TGACGTATGT	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
RY3	TATATATACA	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
AU1	ACACATACGC	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
AU2	AGTGACACAC	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT
AU3	CGTATGCGAC	GATAYTGGGYDTAAAGNG	CCGTCAATTCMTTTRAGTTT



Chapter 4

Ambika Manirajan, B., Maisinger, C., Ratering, S., Rusch, V., Schwiertz, A., Cardinale, M., Schnell, S. (2018) Diversity, specificity, co-occurrence and hub taxa of the bacterial-fungal pollen microbiome. *FEMS Microbiol Ecol* **94:** fiy112. DOI:*10.1093*/femsec/fiy112.

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Research Article

RESEARCH ARTICLE

Diversity, specificity, co-occurrence and hub taxa of the bacterial–fungal pollen microbiome

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One sentence summary: The structure and diversity of the bacterial and fungal microbiome of eight different pollen species (four wind-pollinated and four insect-pollinated) was highly influenced by both pollen species and pollination type.

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ABSTRACT

Flower pollen represents a unique microbial habitat, however the factors driving microbial assemblages and microbe–microbe interactions remain largely unexplored. Here we compared the structure and diversity of the bacterial–fungal microbiome between eight different pollen species (four wind-pollinated and four insect-pollinated) from close geographical locations, using high-throughput sequencing of the 16S the rRNA gene fragment (bacteria) and the internal transcribed spacer 2 (ITS2, fungi). *Proteobacteria* and *Ascomycota* were the most abundant bacterial and fungal phyla, respectively. *Pseudomonas* (bacterial) and *Cladosporium* (fungal) were the most abundant genera. Both bacterial and fungal microbiota were significantly influenced by plant species and pollination type, but showed a core microbiome consisting of 12 bacterial and 33 fungal genera. Co-occurrence analysis highlighted significant inter- and intra-kingdom interactions, and the interaction network was shaped by four bacterial hub taxa: *Methylobacterium* (two OTUs), *Friedmanniella* and *Rosenbergiella*. *Rosenbergiella* prevailed in insect-pollinated pollen and was negatively correlated with the other hubs, indicating habitat complementarity. Inter-kingdom co-occurrence showed a predominant effect of fungal on bacterial taxa. This study enhances our basic knowledge of pollen microbiota, and poses the basis for further inter- and intra-kingdom interaction studies in the plant reproductive organs.

Keywords: flower pollen; microbiome; inter-kingdom diversity; intra-kingdom diversity; core microbiome; hub species

INTRODUCTION

Plants represent a mosaic of microhabitats for diverse assemblages of epiphytic and endophytic microorganisms (Junker *et al.* 2011; Junker and Keller 2015), whose establishment and distribution depend on the availability of nutrients (Andrews and Harris 2000). Plant reproductive organs are less investigated to

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this point (Vandenkoornhuyse *et al.* 2015). The nutritive composition (Nicolson and Human 2013) and complex structure of pollen grains (Blackmore *et al.* 2007) facilitate microorganisms to colonize them. Colldahl and Carlsson (1968) initially reported the presence of bacteria and fungi in the pollen microhabitat; however, the structure and function of the pollen microbiome are still unclear (Hani *et al.* 2012). Microbial populations associated with pollen seem to correlate with different parameters of environmental pollution and allergic potential (Obersteiner *et al.* 2016). Moreover, some of the pollen-inhabiting bacteria might influence the immune response of the human body (Varga *et al.* 2013; McKenna *et al.* 2017; Zasloff 2017). Thus a detailed knowledge of the microbiota associated with different pollen species is highly important because of the role of pollen in food, feed and health as well as for the plant reproduction and development by pollen-mediated transfer of symbionts and/or pathogens during fertilization. It would also provide an insight into the influence of pollen microbiota in plant–microbe interactions, plant pollination, seed quality, endophyte development, as well as its potential role in pollen allergy for future studies. In our previous work (Ambika Manirajan *et al.* 2016), we characterized the structure, diversity and colonization pattern of the bacterial microbiota associated with four different pollen species including two anemophilous (wind-pollinated) and two zoophilus (insect-pollinated) pollen species, by culture-dependent and -independent methods; the latter was based on the Greengenes reference database (DeSantis *et al.* 2006). We showed that the plant species and the pollination type clearly affected structure and diversity of the bacterial microbiota, while the fungal microbiota and the microbial network were not investigated. Here, we extended the study to a total of eight pollen species by adding two wind-pollinated and two insect-pollinated species. We investigated bacteria and fungi simultaneously by metabarcoding analysis using the recently released SILVA 128 database for bacteria (Quast *et al.* 2013) and the UNITE v7.1 (dynamic) database for fungi (Abarenkov *et al.* 2010). High-throughput metagenomic data from complex environments can provide complementary information about interactions and changes in mixed bacterial–fungal communities (Zhou *et al.* 2015). The interactions between bacteria and fungi may influence microbiome structure, niche construction, symbiosis, vitality and microbial growth (Frey-Klett *et al.* 2011; Braga, Dourado and Araujo 2016).

The objectives of this work were (i) to compare the diversity and structure of bacterial and fungal microbiota associated with eight different flower pollen species simultaneously, (ii) to define the core set of pollen-inhabiting bacterial and fungal genera, (iii) to assess inter- and intra- kingdom correlations, and (iv) to identify the hub microbial taxa and evaluate their influence on the pollen microbiome.

MATERIALS AND METHODS

Sample collection and preparation

The pollen collection from the eight selected plant species was done in the flowering season (April–October), and each pollen species was sampled from different locations around Giessen (Hessen, Germany; Table S1, Supporting Information). Pollen from birch (*Betula pendula* Roth.), rape (*Brassica napus* L.), rye (*Secale cereale* L.) and autumn crocus (*Colchicum autumnale* L.) were collected (three replicates each) during the year 2015; common hazel (*Corylus avellana* L.), blackthorn (*Prunus spinosa* L.), common mugwort (*Artemisia vulgaris* L.) and cherry plum (*Prunus cerasifera* Ehrh.) were collected (four replicates each) in the year 2016. The flowers were collected separately in sterile polypropylene vials, kept cool and transported to the lab, where they were immediately processed. The anthers of the flowers were dissected and immediately kept in silica desiccator for 24 h to dry out for avoiding the germination of pollen and to reduce the relative humidity (Ma and Khan 1976; Shivanna and Rangaswamy 1992). The dehydration helped to prevent the microbial cell activities and has been used as preservation method for many microorganisms (Grivell and Jackson 1969; Heckly 1978), thus post-collection changes of the microbiome composition were minimized. All the samples were treated using the same method to account for possible slight modification in the microbiome composition due to dehydration. The pollen grains were then extracted by shaking after drying. The extracted dry pollen grains were thoroughly observed and cleaned off the rarely found debris of anther by using sterile tweezers. This was followed by equal weighing of pollen samples, which were then mixed each with 2 ml of shaking solution (0.18% $Na_4P_2O_7$ and 0.05%Tween 80) (Musovic *et al.* 2006) and immediately stored at −80°C until total DNA extraction, which was performed according to Ambika Manirajan *et al.* (2016).

Ion Torrent sequencing of the bacterial 16S rRNA gene fragment and the fungal ITS2 region

For the Ion Torrent sequencing, the samples of the four species collected from 2016 were used for this study. The partial sequences of the hypervariable region of the 16S rRNA gene fragment (V4 and V5) were PCR amplified using the primer 520 F (5′-AYTGGGYDTAAAGNG-3′) and 907 R (5′-CCGTCAATTCMTTTRAGTTT-3′) (Claesson *et al.* 2009; Engelbrektson *et al.* 2010). The PCR was carried out according to Ambika Manirajan *et al.* (2016). The bacterial sequences of the four pollen species collected in 2015 used in this work were from Ambika Manirajan *et al.* (2016) (project number PRJEB14477 and PRJEB14487).

For the fungal ITS2 Ion Torrent sequencing, the samples of the eight species collected from 2015 as well as from 2016 were used. PCR amplification of fungal ITS2 region was performed with standard protocol of 25 cycles (95°C for 3 min, cycles with 98°C for 20 s, 56°C for 30 s, 72°C and a final extension of 72°C for 1 min), using the primers ITS3 KYO2 forward (5′-GATGAAGAACGYAGYRAA-3′; Toju *et al.* 2012) and ITS4 reverse (5′-TCCTCCGCTTATTGATATGC-3′; White *et al.* 1990). The product of the first PCR was used for a second reaction (Berry *et al.* 2011). The same PCR conditions with 8 cycles were used for the second PCR, using the primers ITS3 KYO and ITS4 with sequencing adaptors.

PCR products were eluted and purified from agarose gel (QIAquick PCR purification kit, QIAGEN GmbH, Hilden, Germany), then primer-dimers were removed with NucleoMag® beads (NGS clean-up kit, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). The concentration of the purified PCR product was estimated with Qubit dsDNA HS assay kit by Qubit® 3.0 fluorometer (Life Technologies, Carlsbad, USA) and the concentration of each product was adjusted to 1 μM. The PCR products were pooled and the final concentration was adjusted to 26 pM. The pooled product was used for emulsion PCR with the Ion PGM Hi-Q OT2 kit (Life Technologies) using Ion One Touch 2 (Life Technologies). The final product was loaded on a 314 or 318 chip for posterior sequencing (Life Technologies), using an Ion PGM sequencer (Life Technologies).

Archaea

Archaeal 16S rRNA gene fragment was amplified from all pollen samples using the primer A112 F (5′-GCTCAGTAACACGTGG-3′) and A934B R (5′-GTGCTCCCCCGCCAATTCCT-3′) (Großkopf, Janssen and Liesack 1998), along with a positive control (DNA of *Methanosarcina thermophila* DSM 1825^T). PCR was carried out for 28 cycles in Mycycler (Bio-Rad, Hercules, USA) with the initial denaturation for 4 min at 95°C, cyclic denaturation for 1 min 30 s at 94°C, annealing for 45 s at 58°C (↓0.5 touchdown) and extension for 1 min 30 s with a final extension for 7 min. The PCR products were confirmed by agarose gel electrophoresis.

Analysis of the bacterial 16S rRNA gene fragment Ion Torrent sequencing data

For the analysis of the bacterial 16S rRNA gene fragment sequencing data, we also included the sample AU1 with newly sequenced data (it was omitted in our previous study because of low number of reads). The 16S rRNA gene fragment sequence data were analyzed using QIIME, version 1.9 (Caporaso *et al.* 2010). Quality (threshold: 20) and length (250–450) filtering were applied, allowing a maximum five homopolymer run. Chimeric sequences were detected using the UCHIME algorithm implemented in VSEARCH (Rognes *et al.* 2016). OTUs were generated at a similarity level of ≥97% and taxonomy was assigned using UCLUST with the unaligned representative sequences of the QIIME version of the SILVA 128 database (Quast *et al.* 2013), released in September 2016. Sequences of plastids, mitochondria and singleton OTUs were deleted from the dataset as well as chimera. To compare the alpha diversity indices (Shannon, Shannon – equitability, Dominance, Simpson, PD whole tree, Chao1, Observed species and Good's coverage) between pollen species (ANOVA) and pollination type (Student's t-test), a normalized data set rarefied to 4200 reads per sample was used. Statistical comparisons were done with STATISTICA version 13 (Dell Inc., Tulsa, USA). Beta diversity of bacterial microbiome between pollen species, pollination type and sample collection site was calculated based on Bray–Curtis distances on the proportional data; the statistical significance of the factors was analyzed using the function envfit included in R package 'vegan' (Okasanen *et al.* 2013) and non-parametric multivariate analysis of variance using distance matrices (ADONIS) (Anderson 2001), as implemented in QIIME.

Analysis of fungal ITS2 Ion Torrent sequencing data

For the analysis of fungal ITS2 sequencing data, we solely used the sequences obtained from the present study. The ITS2 data were analyzed using QIIME 1.9 (Caporaso *et al.* 2010). Quality (threshold: 20) and length (50–450) filtering were applied, allowing a maximum of five homopolymer run. The ITS2 region was retrieved using ITSx software (Bengtsson-Palme *et al.* 2013). Chimeric sequences were detected using UCHIME algorithm implemented in VSEARCH (Rognes *et al.* 2016). OTUs were generated at a similarity level of ≥97% and taxonomy was assigned to each OTU using the representative sequences of the UNITE v7.1 (dynamic) for fungal sequences released 20.11.2016 database (Abarenkov *et al.* 2010). Protozoa, Plantae, Protista, Chromista OTUs as well as one sample with low number of reads (CP2) were deleted from the dataset. To compare the alpha diversity indices (Shannon, Shannon – equitability, Dominance, Simpson, PD whole tree, Chao1, Observed species and Goods coverage) between pollen species (ANOVA) and pollination type (Student's t-test), a normalized dataset rarefied to 2800 reads per sample was used. Statistical comparisons were done with STATISTICA 13 (Dell Inc.). Beta diversity of fungal microbiome between pollen species, pollination type and sample collection site was calculated based on Bray–Curtis distances on the proportional data; the statistical significance of the factors was analyzed using the function envfit included in R package 'vegan' (Oksanen *et al.* 2007) and non-parametric multivariate analysis of variance using distance matrices (ADONIS) (Anderson 2001), as implemented in QIIME.

The bacterial and fungal sequences were submitted to the EMBL database (www.ebi.ac.uk/ena) under the project number PRJEB23282.

Shared taxa, core-microbiome, co-occurrence analysis and "hub" taxa

The OTUs sharing between and across wind pollinated and insect pollinated species were visualized using Venny 2.1 (Oliveros 2007). The core bacterial and fungal microbiome was calculated as the respective OTUs found in all pollen species. Highly significant positive ($R > 0.5$, $P < 0.005$) and negative ($R < -0.5$, $P < 0.005$) Spearman correlations between OTU occurrence patterns were visualized as a network using Cytoscape, version 3.6.0 (Shannon *et al.* 2003). Nodes were indicated as prevalent in wind-pollinated or insect-pollinated species when the fold increase in one pollination type was > 3 times than the other. The "hub taxa" were defined as the most interactive OTUs, according to their degree, betweenness centrality and closeness centrality (Agler *et al.* 2016; van der Heijden and Hartmann 2016). Additionally, inter-kingdom correlations were visualized separately.

RESULTS

A PCR product was obtained for the bacterial 16S rRNA gene fragment and fungal ITS2. However, no archaeal 16S rRNA gene fragment was amplified from all pollen samples, while the positive control (DNA isolated from pure culture of *Methanosarcina thermophila* DSM 1825^T) was amplified.

Ion Torrent sequence analysis

Sequencing output and initial data processing

For the bacterial 16S rRNA gene fragment 2 086 637 raw sequences were obtained from 28 pollen samples with four different ion torrent sequencing runs; 364 104 high quality reads (13 004 ± 8301 reads per sample) were retained after length filtering as well as removal of chimeric, mitochondrial and plastid sequences, and grouped into 6029 OTUs representing 126 different bacterial genera. For fungal ITS2 region 1 366 347 raw sequences were obtained from the 28 pollen samples; 1 339 864 high quality reads (47 852 ± 42 525 reads per sample) were retained after length filtering as well as removal of chimeric, protozoa, protista, plantae and chromista sequences, removal of one cherry plum sample (CP2 had a notably lower number of sequences) and grouped into 6115 OTUs representing 542 different fungal genera.

Taxonomic structure

Bacteria

Proteobacteria was the most abundant phylum, followed by *Firmicutes*, *Actinobacteria*, *Acidobacteria* and *Deinococcus-Thermus*. Autumn crocus and rape pollen samples showed least number

of taxa. *Enterobacteriaceae* and *Pseudomonadaceae* were the most abundant bacterial families in autumn crocus and blackthorn; *Enterobacteriaceae*, *Streptococaceae* and *Pseudomonadaceae* in rape; *Pseudomonadaceae*, *Acetobacteraceae*, *Caulobacteraceae* and *Propionibacteriaceae* in cherry plum; *Acetobacteraceae*, *Enterobacteriaceae* and *Burkholderiaceae* in birch; *Oxalobacteraceae* and *Xanthomonadaceae* in rye; *Methylobacteriaceae* and *Bacillaceae* in hazel; *Acetobacteraceae* and *Bradyrhizobiaceae* in mugwort (Fig. 1A).

Fungi

Ascomycota was the major phylum in all the eight pollen species, followed by *Basidiomycota*. *Davidiellaceae* was found in all the eight pollen species, and was the major dominant family in blackthorn, rape and rye; *Leptosphaeriaceae* in autumn crocus, blackthorn, cherry plum, hazel and rye; *Dothioraceae* in birch, hazel and mugwort; *Mycosphaerellaceae* in hazel; *Erysiphaceae* in mugwort. *Tremellaceae* was found in all the wind pollinating pollen species. *Metschnikowiaceae* was found only in autumn crocus and rape (Fig. 1B).

Alpha diversity

All the calculated diversity metrics were significantly different between pollen species for both bacteria and fungi (ANOVA, $P < 0.05$) (Table 1). Wind pollinated pollen species had significantly higher Shannon–Weaver and phylogenetic diversity indices compared to insect pollinated pollen, for both bacteria and fungi (ANOVA, $P < 0.001$) (Fig. 1C and D). Within the pollination types, the Shannon index of bacteria was significantly higher than that of fungi, while phylogenetic diversity of fungi was higher than that of bacteria. No significant differences were found in the alpha diversity indices between collection sites (Fig. S1, Supporting Information).

Beta diversity

Bacteria

Beta diversity (Bray–Curtis distances) using non-metric multidimensional scaling (NMDS) analysis demonstrated the significant effect of the factors 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 $R^2 = 0.1744$, $P < 0.001$) (Fig. 2A). The factor collection site did not show any significant effect (envfit $R^2 = 0.0992$, $P < 0.515$, ADONIS $R^2 = 0.0381$, $P < 0.786$) (Fig. S2A, Supporting Information).

Fungi

Beta diversity (Bray–Curtis distances) using NMDS analysis demonstrated the significant effect of the factor plant species (envfit $R^2 = 0.8865$, $P < 0.001$, ADONIS $R^2 = 0.5747$, $P < 0.001$) and pollination type (envfit $R^2 = 0.2503$, $P < 0.001$, ADONIS $R^2 = 0.1429$, $P < 0.001$) (Fig. 2B). The factor collection site did not show any significant effect (envfit $R^2 = 0.0756$, $P < 0.692$, ADONIS $R^2 = 0.0372$, $P < 0.734$) (Fig. S2B, Supporting Information).

Shared taxa

The number of shared OTUs in the pollination type was notably higher within wind pollinated than within insect pollinated pollen species for bacteria [5.2% (215 OTUs) *vs*. 2.3% (87 OTUs)] but not for fungi [3.8% (164 OTUs) *vs*.4.5% (177 OTUs)]. The bacterial and fungal OTUs shared between wind and insect pollinated pollen types were respectively 29.6% (1787 OTUs) and 34.8% (2125 OTUs) (Fig. S3, Supporting Information). Interestingly plant pollen of the same genus (*Prunus*) shared the most bacterial and fungal OTUs between each other (11.7% and 16.3%, respectively) (Fig. S3, Supporting Information).

Core microbiome

The pollen microhabitat contained a core microbiome including 32 bacterial and 62 fungal OTUs, representing 12 bacterial 33 fungal genera, respectively. They represented ~27% and ~79% of the total bacterial and fungal reads, respectively. The most abundant core genera were *Pseudomonas* and *Rosenbergiella* for the bacteria, *Cladosporium* and *Aureobasidium* for the fungi (Fig. S4, Supporting Information). However, the distribution pattern of the bacterial and fungal core genera across the pollen species looked very different from each other (Fig. S4, Supporting Information).

Co-occurrence analysis and determination of "hub" taxa

The co-occurrence analysis between all OTUs showed 153 positive and 41 negative significant correlations (Fig. 3A). *Methylobacterium* (two OTUs), *Friedmanniella* and *Rosenbergiella* were found to be the hub taxa, based on network connectivity statistics (Fig. 3B). The *Methylobacterium* hub OTUs were prevalent (>3 fold increase) in wind pollinating pollen species, while there was no difference between pollination types in *Friedmanniella* hub. Moreover, *Rosenbergiella* prevailed in insect pollinated pollen species. The correlation between *Rosenbergiella* and the other hub OTUs was negative, indicating a clear habitat complementarity. The network was organized in three levels: the hub taxa, the OTUs directly influenced by the hub taxa (first level of influence, two clusters driven by the hub taxa) and the other OTUs (second level of influence) that were all correlated to the first level OTUs. In the first level of influence, the correlations between the two different clusters were almost all negative, indicating a preferential habitat colonization between pollination types. In the second level of influence, the OTUs were mostly positively correlated with both first level's network cluster, except only two negative correlations (Fig. 3A); however these OTUs were indirectly influenced by the hub taxa, since most of the positive correlations occurred with the first level's clusters driven by *Methylobacterium* and *Friedmanniella* (Fig. 3A), indicating a higher integration in the microbial network of the wind pollinating pollen.

Considering only the co-occurrence between fungal and bacterial OTUs, there were 21 positive and 4 negative strongly significant correlations (Fig. S5, Supporting Information). *Proteobacteria* and *Ascomycota* were the phyla more often involved in correlations. A total of 21 OTUs did not show any correlation. The pattern showed that several fungal OTUs correlated with more than one bacterial OTUs, indicating that, in the inter-kingdom interactions, fungi were prevalently influencing the bacteria (Fig. S5, Supporting Information). Only a few bacterial OTUs, belonging to *Beijerinckiaceae*, *Rosenbergiella* and *Deinococcus*, showed the opposite trend, being correlated to more than one fungal OTU.

DISCUSSION

This is the first microbiome study of both the pollen-associated bacteria and fungi. Here we assessed structure, diversity, core microbiome, co-occurrence interactions and hub taxa of four wind pollinated (birch, rye, common mugwort and common

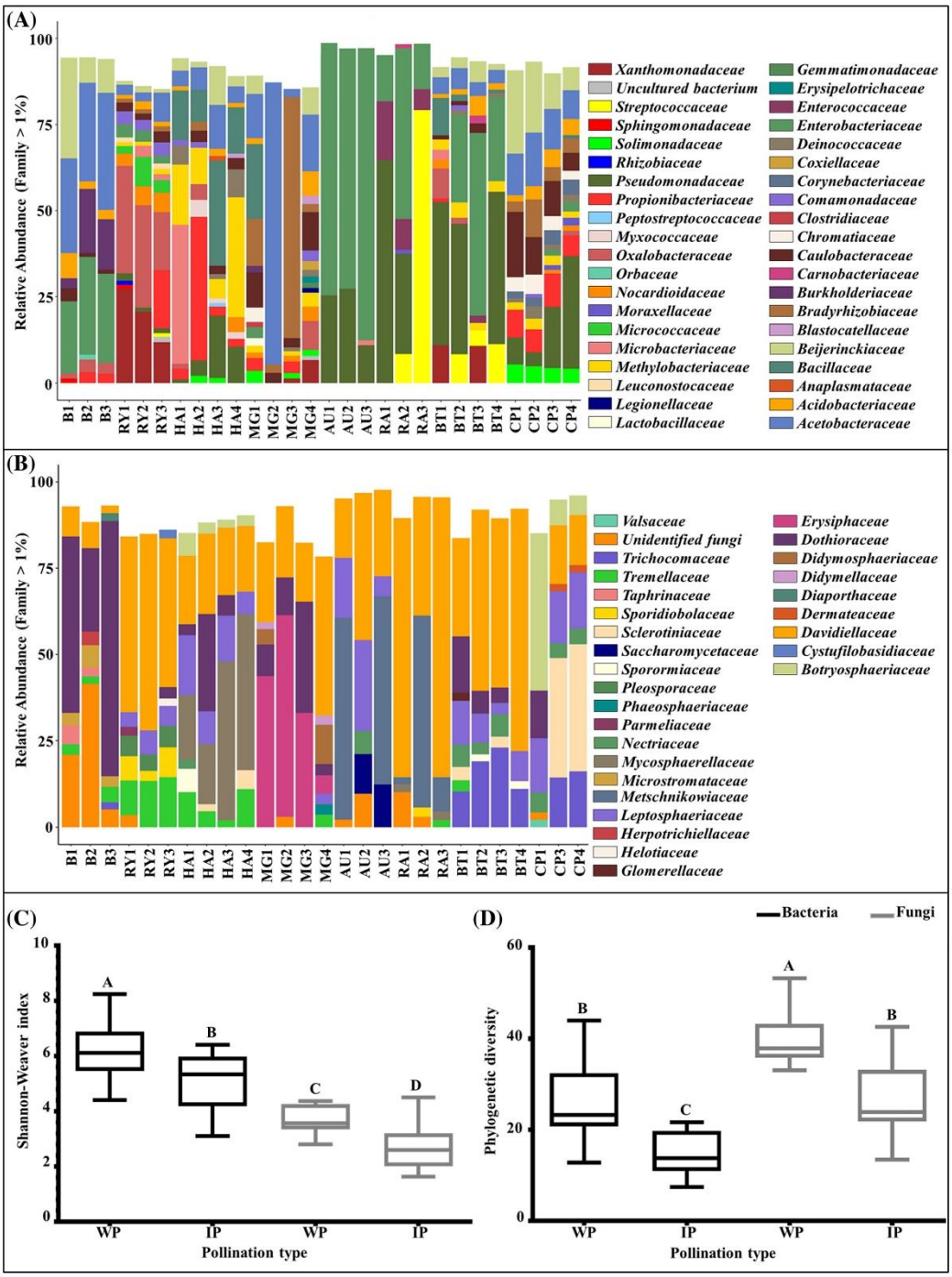

Figure 1. (A) Bacterial family level profile in the pollen samples, according to 16S rRNA gene amplicon library sequencing; only families with a relative abundance of >1% are shown. (B) Fungal family level profile in the pollen samples, according to ITS2 gene amplicon library sequencing; only families with a relative abundance of >1% are shown. AU-autumn crocus, B-birch, BT-black thorn, CP-cherry plum, HA-hazel, MG-mugwort, RA-rape and RY-rye (including replicates). (C) Shannon–Weaver and (D) phylogenetic diversity indices of bacterial (black) and fungal (grey) microbiota (OTU 97%), according to pollination type. Different letters indicate significantly different means (Tukey test, $P < 0.05$). WP = Wind-pollinated; IP = Insect-pollinated.

Table 1. Alpha diversity indices of pollen-associated bacterial (A) and fungal (B) microbiota (OTUs 97% similarity level). Different letters within diversity indices indicate significantly different means between pollen species according to Tukey test ($P < 0.05$), and ANOVA P value is also indicated (bold).

Pollen species	Shannon	Shannon - equitability	Dominance	Simpson	Phylogenetic diversity	Chao1	Observed species	Good's coverage
A. Bacteria								
Birch	6.00±0.25^{ab}	0.67±0.02^{ab}	0.05±0.01^{ab}	0.95±0.01^{ab}	22.42±01.15^{bc}	840.3±110^{ab}	488.3±41.53^{ab}	0.94±0.01^{ab}
Rye	6.95±0.19^{b}	0.73±0.02^{a}	0.03±0.00^{a}	0.97±0.00^{a}	33.62±02.06^{c}	1238.9±069^{b}	704.3±27.68^{b}	0.95±0.01^{b}
Hazel	5.49±0.59^{ab}	0.65±0.04^{ab}	0.08±0.04^{ab}	0.92±0.04^{ab}	17.72±04.97^{ab}	654.5±177^{a}	362.3±101.4^{a}	0.91±0.00^{a}
Mugwort	5.25±1.91^{ab}	0.58±0.18^{ab}	0.23±0.11^{ab}	0.77±0.11^{ab}	27.70±10.05^{c}	764.5±280^{ab}	477.5±194.7^{ab}	0.94±0.02^{ab}
Autumn crocus	4.83±1.08^{ab}	0.56±0.10^{ab}	0.14±0.10^{ab}	0.86±0.10^{ab}	9.72±02.52^{a}	762.7±193^{a}	374.7±118.5^{a}	0.95±0.01^{a}
Rape	4.16±1.13^{a}	0.49±0.12^{b}	0.24±0.16^{b}	0.76±0.16^{b}	11.93±01.80^{ab}	677.5±194^{a}	353.0±72.17^{a}	0.95±0.01^{a}
Blackthorn	4.87±0.66^{ab}	0.58±0.06^{ab}	0.11±0.03^{ab}	0.89±0.03^{ab}	15.57±04.20^{ab}	745.7±132^{a}	342.3±76.14^{a}	0.94±0.03^{a}
Cherry plum	6.12±0.20^{ab}	0.71±0.01^{ab}	0.04±0.01^{a}	0.96±0.01^{a}	19.91±01.46^{ab}	717.4±105^{a}	379.8±44.50^{a}	0.95±0.01^{a}
ANOVA *P* value	**0.015**	**0.014**	**0.039**	**0.039**	**0.000007**	**0.006**	**0.0009**	**0.001**
B. Fungi								
Birch	3.56±0.77^{ab}	0.46±0.09^{ab}	0.27±0.16^{ab}	0.73±0.16^{ab}	38.11±1.2^{abc}	478.2±55^{ab}	201±14^{a}	0.96±0.00^{ab}
Rye	3.77±0.43^{a}	0.49±0.05^{a}	0.26±0.07^{ab}	0.74±0.07^{ab}	41.90±6.7^{bc}	51.2±46^{a}	209±18^{a}	0.96±0.00^{a}
Hazel	3.64±0.52^{a}	0.48±0.06^{a}	0.19±0.07^{a}	0.81±0.07^{b}	36.45±4.5^{abc}	446.9±130^{ab}	191±25^{a}	0.96±0.01^{ab}
Mugwort	3.81±0.43^{a}	0.50±0.03^{a}	0.22±0.01^{ab}	0.78±0.01^{ab}	44.35±8.7^{c}	457.8±124^{ab}	204±56^{a}	0.96±0.01^{abc}
Autumn crocus	2.25±0.31^{ab}	0.37±0.04^{ab}	0.33±0.06^{ab}	0.67±0.06^{ab}	20.20±5.9^{a}	23.6±84^{b}	68±15^{b}	0.98±0.00^{c}
Rape	1.95±0.31^{b}	0.30±0.05^{b}	0.51±0.15^{b}	0.50±0.15^{b}	25.26±5.2^{ab}	294.4±91^{ab}	95±22^{bc}	0.98±0.00^{bc}
Blackthorn	3.14±1.05^{ab}	0.41±0.11^{ab}	0.31±0.16^{ab}	0.70±0.15^{ab}	31.89±8.9^{abc}	494.1±110^{a}	179±63^{ac}	0.96±0.01^{ab}
Cherry plum	3.20±0.13^{ab}	0.45±0.01^{ab}	0.21±0.03^{a}	0.80±0.03^{b}	28.69±7.5^{abc}	400.8±54^{ab}	143±19^{abc}	0.97±0.00^{ab}
ANOVA *P* value	**0.006**	**0.023**	**0.035**	**0.035**	**0.003**	**0.016**	**0.0004**	**0.0009**

hazel) and four insect pollinated (rape, autumn crocus, blackthorn and cherry plum) plant pollen species (Table S1, Supporting Information).

According to the 16S rRNA gene fragment and ITS2 metabarcoding analysis, the pollen is a unique and highly diverse microbial habitat (Figs. 1A, B and 2A, B), with the different pollen species having distinctive bacterial and fungal community compositions. However the plant species blackthorn and cherry plum, which belong to the same genus (*Prunus*), share more bacterial and fungal genera compared to the other plant species, thus indicating a similar selection of bacteria and fungi by related plant species (Fig. S3, Supporting Information). The species-specificity found here confirms previous results (Ambika Manirajan *et al*. 2016; Obersteiner *et al*. 2016), and can be explained by pollen-specific physical and biochemical makeup (Schulte *et al*. 2008). Furthermore, the floral nectar hosts bacterial communities specific for each plant species (Fridman *et al*. 2012). Some selected pollen-associated microbial taxa may be transmitted through the pollination process to the ovule and the seed (Rodríguez *et al*. 2017), by either vertical or horizontal transmission (Barret *et al*. 2015; Shade, Jacques and Barret 2017). Nevertheless, the transmission of flower pollen microbiota to the seed and the way in which the pollen microbes may structure the endophytic and epiphytic seed microbiomes are almost unknown. Hodgson *et al*. (2014) investigated in forbs (i.e. herbaceous eudicots) the possible mechanism of vertical transmission of fungal endophytes through pollen.

Our alpha diversity results suggest that, within pollination types, bacteria and fungi show an opposite trend of Shannon diversity and phylogenetic diversity indices (Fig. 1C and D). The hydrophobic property of the pollen coat (Fellenberg and Vogt 2015), availability of nutrients (Roulston and Cane 2000) and short life span of the pollen grains in flowers might limit the bacterial phylogenetic diversity. Aerial hyphae and conidia of many fungi are coated with hydrophobins, which are small surface active proteins that could facilitate adhesion to hydrophobic surfaces (Wösten, Schuren and Wessels 1994; Takahashi *et al*. 2005). Some fungal conidia release liquid exudates upon contact with hydrophobic surface (Kunoh *et al*. 1988) and the exudates containing cutinase can degrade the surfaces of the host cell (Pascholati *et al*. 1992). The high adhesive nature of pollen outer layer (Shukla, Vijayaraghavan and Chaudhary 1998) could in turn be an advantage for fungal spore diversity: indeed living pollen was shown to be parasitized and utilized by many fungi (Hutchison and Barron 1997).

Proteobacteria was the most abundant phylum found in pollen habitat, followed by *Firmicutes*, *Actinobacteria*, *Acidobacteria* and *Deinococcus-Thermus*. *Enterobacteriaceae* was the most abundant bacterial family in the pollen habitat. High throughput sequencing studies of flower nectar microbiota revealed that *Proteobacteria* was the most abundant phylum, as well as the dominance of *Firmicutes*, *Actinobacteria*, *Acidobacteria* and *Deinococcus-Thermus*, including the abundance of *Enterobacteriaceae* family (Fridman *et al*. 2012; Jacquemyn *et al*. 2013; Shade, McManus and Handelsman 2013; Mortazavi, Attiya and Ariya 2015). In the almond nectar, *Enterobacteriaceae* was one of the most abundant families (Schaeffer *et al*. 2017). *Ascomycota* was the most abundant fungal phylum in pollen microhabitat, followed by *Basidiomycota* and *Davidiellaceae* (Fig. 1). In *Pinus nigra*, freshly released pollen was readily parasitized by fungi and most of the fungal species isolated included members from *Basidiomycota* and *Ascomycota* (Hutchison and Barron 1997). Metagenomic studies on the phyllosphere and carposphere of olive (Abdelfattah *et al*. 2015) and strawberry (Abdelfattah *et al*. 2016) unveiled that *Ascomycota* and *Basidiomycota* were the most abundant phyla in the leaf, fruit as well as flower habitat, and *Davidiellaceae* was one of the most abundant family. Also in almond nectar *Davidiellaceae* was found to be abundant (Schaeffer *et al*. 2017). The presence of *Metschnikowia* in nectar of insect pollinating plants

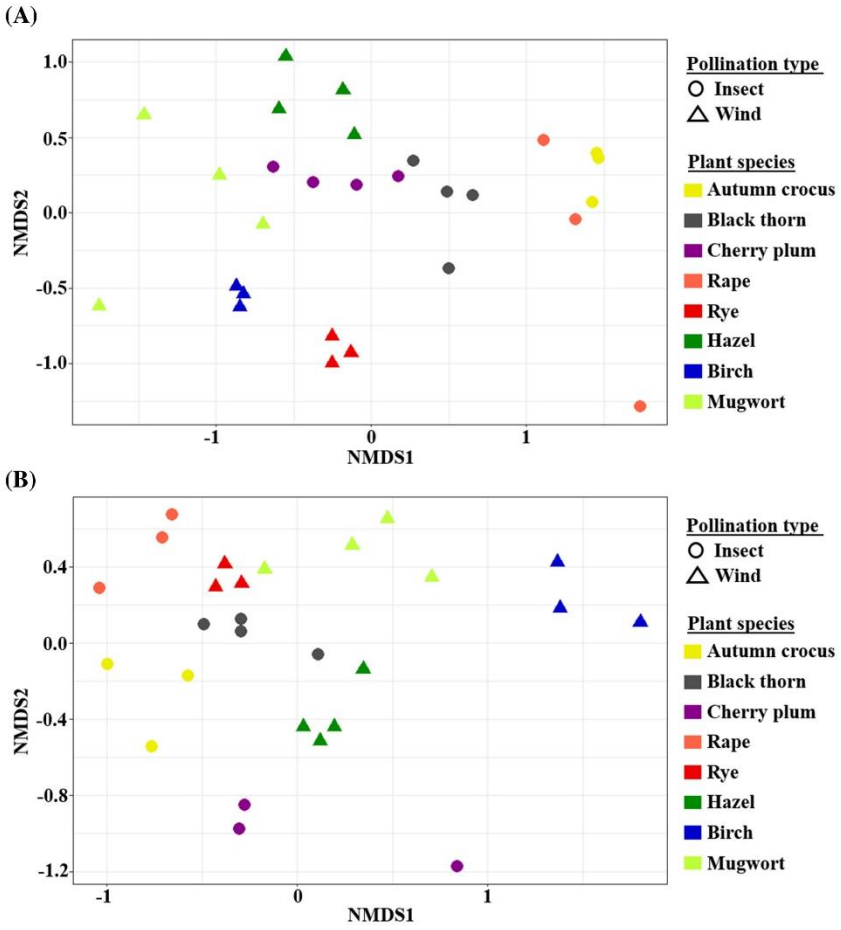

Figure 2. **(A)** Non-metric multidimensional scaling (NMDS) plot for bacterial microbiota structure based on Bray–Curtis distance. Samples are colored by plant species and shapes represent the pollination type. ADONIS significance test: $R^2 = 0.869$, $P < 0.001$ for the factor "species"; $R^2 = 0.390$, $P < 0.001$ for the factor "pollination type". **(B)** NMDS plots for fungal microbiota structure based on Bray–Curtis distance. Samples are colored by species and sample shapes represent the pollination type. ADONIS significance test: $R^2 = 0.887$, $P < 0.001$ for the factor "species"; $R^2 = 0.250$, $P < 0.001$ for the factor "pollination type".

has been reported (Lievens *et al.* 2015). High cell numbers of yeasts and bacteria in nectar might explain the spreading of these microorganisms into other flower parts such as pollen. Indeed *Rosenbergiella* was more abundant in insect-pollinating pollen than wind-pollinating pollen and was positively correlated with *Metschnikowia* (Fig. 3A). Furthermore, flower nectar is an attractant for insects in insect-pollinating plants, characterized by high sugar composition (Chalcoff, Aizen and Galetto 2006) and high osmotic pressure (Beutler 1930). These factors might provide advantage for some microbial species, thus leading to the development of a unique microbial community.

The core microbiome analysis enables a better understanding of the complex microbial assemblage across ecologically similar habitats (Shade and Handelsman 2012). The core microbiome of pollen microhabitat showed 12 bacterial and 33 fungal genera (Fig. S4, Supporting Information), of which the most abundant were *Pseudomonas, Rosenbergiella* and *Bradyrhizobium* for bacteria, and *Cladosporium, Aureobasidium* and *Leptosphaera* for fungi. De Souza *et al.* (2016) demonstrated that an organ-specific core microbiome consisting of bacteria and fungi exists in the sugarcane, and this microbiome is not affected by the plant developmental stage. *Rosenbergiella nectarea* was isolated and described from flower nectar by Halpern *et al.* (2013). However, the number of species-specific bacterial and fungal OTUs found in the pollen habitat was higher than the number of shared ones, and the same applies to the different pollination types. Pollen morphology might be interrelated with pollination vector (Hesse 2000); in fact, wind pollinated pollen grains are smaller and drier than animal pollinated ones (Shukla, Vijayaraghavan and Chaudhary 1998; Dafni, Hesse and Pacini 2000) and have differences in exine structure (Tanaka, Uehara and Murata 2004; Schulte *et al.* 2008; Ariizumi and Toriyama 2011) and composition (Edlund, Swanson and Preuss 2004). Beta diversity (Fig. 2A and B) demonstrated that both bacterial and fungal microbiota were significantly affected by pollination type, which confirms our previous results regarding the bacterial microbiota of pollen and showed a similar situation for pollen-inhabiting fungi. In this work, we used samples collected from two different years (2015 and 2016) but we have not considered this factor in our beta-diversity analysis. In fact, in order to study seasonal dynamics of the pollen microbiome, it will be necessary to conduct a study where the same pollen species are collected from different years.

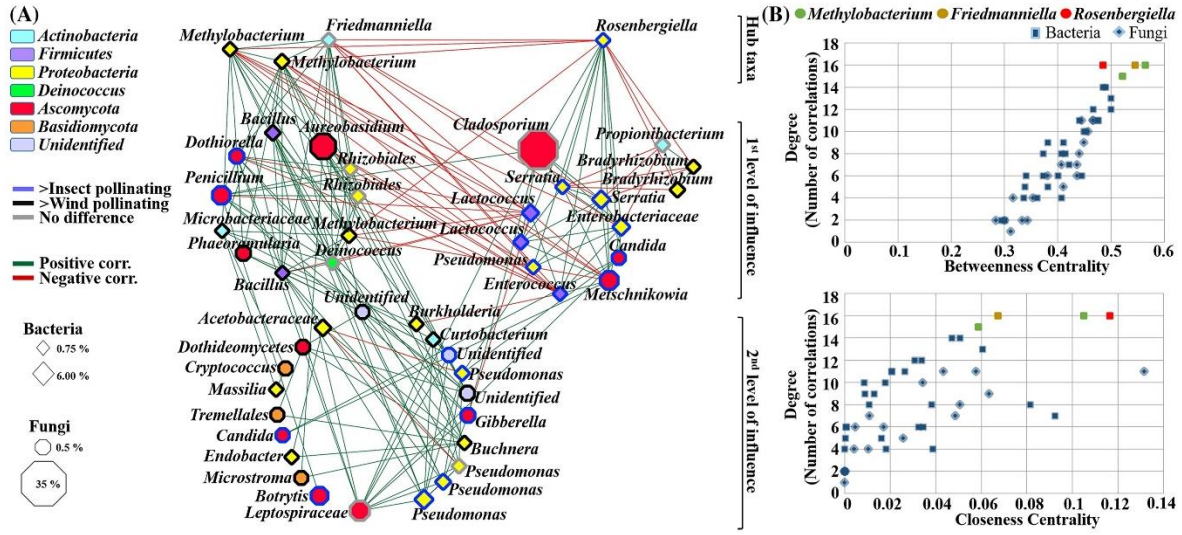

Figure 3. (A) Correlations of occurrence patterns in the bacterial–fungal microbiome of eight different plant pollen species. Nodes represent OTUs (97% similarity level) and edges represent positive ($R^2 > 0.5$, $P < 0.005$, green lines) or negative ($R^2 < -0.5$, $P < 0.005$, red lines) Spearman correlations. Node thickness indicates the OTU relative abundance, node border color indicates greater relative abundance of OTUs found in the respective pollination type (> 3 fold increase). (B) "Hub" OTUs were indicated as those which were significantly more central (measures of node importance) based on betweenness and closeness centrality.

In this study, we explored the potential inter- and intra-kingdom microbe–microbe interactions by using co-occurrence pattern correlation and network analysis, a method that was first developed for soil microbiome (Barberán *et al.* 2012) and later used also for the plant-microbiome (Cardinale *et al.* 2015; Chen *et al.* 2018). We were also able to identify the hub taxa from the pollen microbiome using the approach of Agler *et al.* (2016). In the pollen correlation network, three bacterial hub genera (*Methylobacterium* (two OTUs), *Friedmanniella* and *Rosenbergiella*) were strongly inter-connected and showed a significant effect in shaping the pollen microbial network directly and indirectly: in fact, the hub taxa affect the colonization of other microbes, which in turn interact with further microbes (Fig. 3A and B). The hub microbes might have beneficial relationships with the host plant, as well as with other microorganisms inhabiting the same environment (Agler *et al.* 2016). *Methylobacterium* represents one of the main genera associated to diverse plant habitats and has positive relationships with plants and other bacteria (Iguchi, Yurimoto and Sakai 2015). *Friedmanniella* was found to be one of the endophytes in hybrid poplar (Ulrich, Ulrich and Ewald 2008), mangroves (Tuo *et al.* 2016), as well as seed endophyte in *Anadenanthera colubrina* (Alibrandi *et al.* 2017); it was also found in the apple phyllosphere (Yashiro, Spear and McManus 2011) and in spiders that are hiding in flowers (Iwai, Aisaka and Suzuki 2010). *Rosenbergiella* was the most frequently found bacterium in flower habitats (Bartlewicz *et al.* 2016), and it was also found as a member of the core bacteria associated with *Ceratina* bees (Graystock, Rehan and McFrederick 2017). It appears now clearly that pollen is a preferred habitat. It is challenging to describe the common traits of these hub genera, especially because *Friedmanniella* and *Methylobacterium* species have been isolated from very different habitats. Both genera have frequently been isolated from air samples, contain some pigments known to protect from UV and gamma radiation, and are tolerant to grow in the presence of 2% and more NaCl which indicates their desiccation tolerance. *Friedmanniella aerolata* (Kim *et al.* 2016) whose 16S gene sequence is 100% identical to that of our hub OTU has been isolated from air samples and utilized glucose, arabinose, mannitol and maltose. *Methylobacterium mesophilicum* and *M. brachiatum* have 100% identical 16S RNA gene sequence of hub OUT 1 of *Methylobacterium* and were isolated from leaf and water samples (Kato *et al.* 2008), respectively. Beside methanol, both *Methylobacterium* spp. utilize various sugars like glucose, arabinose and xylose (Kato *et al.* 2008). In pollen various mono- and oligosaccharides in the range of 10%–20% have been determined (Conti *et al.* 2016) which can be utilized as substrates by many bacteria.

The hub taxa are more ecologically relevant than other microbes because the removal of such hub taxa would affect the general community assemblage (Faust and Raes 2012). The hub microbes can play important roles in shaping the microbial community, for example by suppressing or inducing the development of other populations (Agler *et al.* 2016; van der Heijden and Hartmann 2016). The functional role of the microbes in the pollen habitat is currently unknown, but it was suggested to contribute to the establishment of a beneficial seed microbiome. Indeed, both *Methylobacterium* and *Friedmanniella* spp. were detected and/or isolated from the seeds of *Anadenanthera colubrina* (Alibrandi *et al.* 2017). When looking at the inter-kingdom correlations only, it seems that fungi affect the bacterial taxa in the pollen habitat more than *vice-versa* (Fig. S5, Supporting Information). The associations between microbes in natural microbiomes are highly complex because many factors are involved in microbial interactions (Braga, Dourado and Araujo 2016). Bacteria and fungi exhibit both antagonism and cooperation in a mixed bacterial–fungal complex habitat (Frey-Klett *et al.* 2011), and the co-occurrence analysis between bacteria and fungi in soil showed that the bacterial genus *Burkholderia* had mostly positive correlation with fungi (Stopnisek *et al.* 2016). So far, very little is known about the output of the interactions between fungi and bacteria in natural environments. Mycorrhiza-helper bacteria are known to influence the fitness of the fungal hosts (Frey-Klett, Garbaye and Tarkka 2007), while in the case of *Piriformospora indica* (a plant-beneficial fungus), it seems that the positive effects for the host are indeed caused

by the intrahyphal bacterium *Rhizobium radiobacter* (Glaeser *et al.* 2016). Fungi secrete some molecules such as ethanol (Chen, Dolben and Okegbe 2014), antibiotics (Houbraken, Frisvad and Samson 2011), simple sugars (Heisel, Montassier and Johnson 2017), etc., which can influence the growth of bacteria in mixed habitats. Although the outputs of such interactions as well as the effect on both the host and the environment remain largely unknown, it is clear that it can lead to beneficial synergies (Labbé *et al.* 2014; Zhang *et al.* 2014; Kurth *et al.* 2015; Van Der Heijden *et al.* 2016). Here we showed a prevailing effect of the fungi over the bacteria (Fig. S5, Supporting Information), which suggests that some fungi might act as a scaffold for specific bacteria in the pollen habitat. However, the fact that no fungi appeared among the hub taxa (Fig. 3A and B) suggests that some bacterial species are more important in shaping the microbial assemblage and the interaction network. It is possible that those bacteria positively correlated to fungi e.g. use specific fungal exudates as carbon/energy source: this might explain why similar bacteria (for example *Pseudomonas*, Fig. S5, Supporting Information) associate with the same fungus. Further laboratory studies on fungal–bacteria interactions, focusing on the interacting species identified in this work, will be necessary to elucidate the nature of the inter-kingdom interactions in the pollen habitat, as well as their output and eventual effects for the plant.

Archaea do not appear to be members of pollen microbiome or they are below the detection level. The hydrophobic outer layer of pollen (Fellenberg and Vogt 2015) might be the reason for a fewer number or the absence of archaea in the pollen habitat. For instance, no archaeal sequences were found in the beebread samples, although some archaeal sequences were retrieved in the bee gut of Africanized honeybee (Saravia *et al.* 2015).

In the past, the pollen microhabitat has been a neglected area in plant microbiome ecology. Our investigations showed that the pollen microbiome is influenced by both pollen species and pollination type. A specific core microbial community inhabiting the pollen habitat exists, and significant inter- and intra-kingdom associations strongly shape the microbial assemblage. Bacterial hubs are driving the pollen microbiome interactions, and are probably the most ecologically important microbes of this tiny, yet complex microhabitat. More research is needed to understand the importance and role of pollen microbiome in pollination, seed microbiome establishment, pathogen transmission and quality of pollen-related food.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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REFERENCES

Abarenkov K, Nilsson RH, Larsson K-H *et al.* The UNITE database for molecular identification of fungi–recent updates and future perspectives. *New Phytol* 2010;**97**:281–5.

Abdelfattah A, Li Destri Nicosia MG, Cacciola SO *et al.* Metabarcoding analysis of fungal diversity in the phyllosphere and carposphere of olive (*Olea europaea*). *PLoS One* 2015;**97**:e0131069.

Abdelfattah A, Wisniewski M, Li Destri Nicosi, MG *et al.* Metagenomic analysis of fungal diversity on strawberry plants and the effect of management practices on the fungal community structure of aerial organs. *PLoS One* 2016;**97**:e0160470.

Agler MT, Ruhe J, Kroll S *et al.* Microbial hub taxa link host and abiotic factors to plant microbiome variation. *PLoS Biol* 2016;**97**:e1002352.

Alibrandi P, Cardinale M, Rahman MM *et al.* The seed endosphere of *Anadenanthera colubrina* is inhabited by a complex microbiota, including *Methylobacterium* spp. and *Staphylococcus* spp. with potential plant-growth promoting activities. *Plant Soil* 2018,**422**:81–99.

Ambika Manirajan B, Ratering S, Rusch V *et al.* Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity. *Environ Microbiol* 2016;**97**:5161–74.

Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Aust Ecol* 2001;**26**:32–46.

Andrews JH, Harris RF. The ecology and biogeography of microorganisms on plant surfaces. *Annu Rev Phytopathol* 2000;**38**:145–80.

Ariizumi T, Toriyama K. Genetic regulation of sporopollenin synthesis and pollen exine development. *Annu Rev Plant Biol* 2011;**62**:437–60.

Barberán A, Bates ST, Casamayor EO *et al.* Using network analysis to explore co-occurrence patterns in soil microbial communities. *ISME J* 2012;**97**:343–51.

Barret M, Briand M, Bonneau S *et al.* Emergence shapes the structure of the seed microbiota. *Appl Environ Microbiol* 2015;**97**:1257–66.

Bartlewicz J, Lievens B, Honnay O *et al.* Microbial diversity in the floral nectar of *Linaria vulgaris* along an urbanization gradient. *BMC Ecol* 2016;**97**:1–11.

Bengtsson-Palme J, Ryberg M, Hartmann M *et al.* Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. *Methods Ecol Evol* 2013;**97**:914–9.

Berry D, Ben Mahfoudh K, Wagner M *et al.* Barcoded primers used in multiplex amplicon pyrosequencing bias amplification. *Appl Environ Microbiol* 2011;**77**:7846–9.

Beutler R. Biologisch-Chemische Untersuchungen am Nektar von Immenblumen. *Z Vgl Physiol* 1930;**12**:72–176.

Blackmore S, Wortley AH, Skvarla JJ *et al.* Pollen wall development in flowering plants. *New Phytol* 2007;**97**:483–98.

Braga RM, Dourado MN, Araujo WL. Microbial interactions: ecology in a molecular perspective. *Braz J Microbiol* 2016;**47**:86–98.

Caporaso JG, Kuczynski J, Stombaugh J *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 2010;**7**:335–6.

Cardinale M, Grube M, Erlacher A *et al.* Bacterial networks and co-occurrence relationships in the lettuce root microbiota. *Environ Microbiol* 2015;**97**:239–52.

Chalcoff VR, Aizen MA, Galetto L. Nectar concentration and composition of 26 species from the temperate forest of South America. *Ann Bot* 2006;**97**:413–21.

Chen AI, Dolben, EF, Okegbe, C. *Candida albicans* ethanol stimulates *Pseudomonas aeruginosa* WspR-controlled biofilm formation as part of a cyclic relationship involving phenazines. *PLoS Pathog* 2014;**10**:e1004480.

Chen Y, Sun R, Sun T *et al.* Organic amendments shift the phosphorus-correlated microbial co-occurrence pattern in the peanut rhizosphere network during long-term fertilization regimes. *Appl Soil Ecol* 2018;**124**:229–39.

Claesson MJ, O'sullivan O, Wang Q *et al.* Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. *PLoS One* 2009;**97**:e6669.

Colldahl H, Carlsson G. Allergens in pollen. *Acta Allergol* 1968;**28**:387–97.

Conti I, Medrzycki P, Argenti C *et al.* Sugar and protein content in different monofloral pollen – building a database. *Bull Insectol* 2016;**69**:318–20.

Dafni A, Hesse M, Pacini E. *Pollen and Pollination*. New York: Springer-Verlag Wien, 2000, 1–12.

DeSantis TZ, Hugenholtz P, Larsen N *et al.* Greengenes, a chimerachecked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microb* 2006;**97**:5069–72.

De Souza RSC, Okura VK, Armanhi JSL *et al.* Unlocking the bacterial and fungal communities assemblages of sugarcane microbiome. *Sci Rep* 2016;**97**:28774.

Edlund AF, Swanson R, Preuss D. Pollen and stigma structure and function: the role of diversity in pollination. *Plant Cell* 2004;**16**:S84–S97.

Engelbrektson A, Kunin V, Wrighton KC *et al.* Experimental factors affecting PCR-based estimates of microbial species richness and evenness. *ISME J* 2010;**97**:642–7.

Faust K, Raes J. Microbial interactions: from networks to models. *Nat Rev Microbiol* 2012;**10**:538–50.

Fellenberg C, Vogt T. Evolutionarily conserved phenylpropanoid pattern on angiosperm pollen. *Trends Plant Sci* 2015;**20**:212–8.

Frey-Klett P, Burlinson1 P, Deveau A *et al.* Bacterial–fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists. *Microbiol Mol Biol Rev* 2011;**97**:583–609.

Frey-Klett P, Garbaye J, Tarkka M. The mycorrhiza helper bacteria revisited. *New Phytol* 2007;**176**:22–36.

Fridman S, Izhaki I, Gerchman Y *et al.* Bacterial communities in floral nectar. *Environ Microbiol Rep* 2012;**97**:97–104

Glaeser SP, Imani J, Alabid I *et al.* Non-pathogenic *Rhizobium radiobacter* F4 deploys plant beneficial activity independent of its host *Piriformospora indica*. *ISME J* 2016;**97**:871–84.

Graystock P, Rehan SM, McFrederick QS. Hunting for healthy microbiomes: determining the core microbiomes of *Ceratina*, *Megalopta*, and *Apis* bees and how they associate with microbes in bee collected pollen. *Conserv Genet* 2017;**18**:701–11.

Grivell AR, Jackson JF. Microbial culture preservation with silica gel. *J Gen Microbiol* 1969;**58**:423–5.

Großkopf R, Janssen PH, Liesack W. Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl Environ Microbiol* 1998;**64**:960–9.

Halpern M, Fridman S, Atamna-Ismaeel, N *et al.* *Rosenbergiella nectarea* gen. nov., sp. nov., in the family *Enterobacteriaceae*, isolated from floral nectar. *Int J Syst Evol Microbiol* 2013;**63**:4259–65.

Hani B, Dalila B, Saliha D *et al.* Microbiological sanitary aspects of pollen. *Adv Environ Biol* 2012;**97**:1415–20.

Heckly RJ. Preservation of microorganisms. In: Perlman D (ed). *Advances in Applied Microbiology*. London: Academic Press, 1978, 5–28.

Heisel T, Montassier E, Johnson A. High-fat changes fungal microbiomes and interkingdom relationships in murine gut. *mSphere* 2017;**5**:e00351–17.

Hesse M. Pollen wall stratification and pollination. *Plant Syst Evol* 2000;**222**:1–17.

Hodgson S, de Cates C, Hodgson J *et al.* Vertical transmission of fungal endophytes is widespread in forbs. *Ecol Evol* 2014;**97**:1199–1208.

Houbraken J, Frisvad JC, Samson RA. Fleming's penicillin producing strain is not *Penicillium chrysogenum* but *P. rubens*. *IMA Fungus* 2011;**2**:87–95.

Hutchison LJ, Barron GL. Parasitism of pollen as a nutritional source for lignicolous *Basidiomycota* and other fungi. *Mycol Res* 1997;**101**:191–4.

Iguchi H, Yurimoto H, Sakai Y. Interactions of methylotrophs with plants and other heterotrophic bacteria. *Microorganisms* 2015;**3**:137–51.

Iwai K, Aisaka K, Suzuki M. *Friedmanniella luteola* sp. nov., *Friedmanniella lucida* sp. nov., *Friedmanniella okinawensis* sp. nov. and *Friedmanniella sagamiharensis* sp. nov., isolated from spiders. *Int J Syst Evol Microbiol* 2010;**60**:113–20.

Jacquemyn H, Lenaerts M, Tyteca D *et al.* Microbial diversity in the floral nectar of seven Epipactis (*Orchidaceae*) species. *MicrobiologyOpen* 2013;**97**:644–58.

Junker RR, Keller A. Microhabitat heterogeneity across leaves and flower organs promotes bacterial diversity. *FEMS Microbiol Ecol* 2015;**91**:fiv097.

Junker RR, Loewel C, Gross R *et al.* Composition of epiphytic bacterial communities differs on petals and leaves. *Plant Biol* 2011;**97**:918–24.

Kato Y, Asahara M, Goto K *et al.* *Methylobacterium persicinium* sp. nov., *Methylobacterium komagatae* sp. nov., *Methylobacterium brachiatum* sp. nov. and *Methylobacterium gregans* sp. nov., isolated from freshwater. *Int J Syt Evol Microbiol* 2008;**58**:1134–41.

Kim S-J, Hamada M, Ahn J-H *et al.* *Friedmanniella aerolata* sp. nov., isolated from air. *Int J Syst Evol Microbiol* 2016;**66**:1970–5.

Kunoh H, Yamaoka N, Yoshioka H *et al.* Preparation of the infection court by *Erysiphe graminis*: I. Contact mediated changes in morphology of the conidium surface. *Exp Mycol* 1988;**97**:325–35

Kurth F, Feldhahn L, Bönn M *et al.* Large scale transcriptome analysis reveals interplay between development of forest trees and a beneficial mycorrhiza helper bacterium. *BMC Gnomics* 2015;**97**:658.

Labbé JL, Weston DJ, Dunkirk N *et al.* Newly identified helper bacteria stimulate ectomycorrhizal formation in *Populus*. *Front Plant Sci* 2014;**97**:579.

Lievens B, Hallsworth JE, Pozo MI *et al.* Microbiology of sugar-rich environments: diversity, ecology and system constraints. *Environ Microbiol* 2015;**97**:278–98.

Ma T-H, Khan SH. Pollen mitosis and pollen tube growth inhibition by SO_2 cultured pollen tubes of *Tradescantia*. *Environ Res* 1976;**12**:144–9.

McKenna O, Posselt G, Briza P *et al.* Multi-approach analysis for the identification of proteases within birch pollen. *Int J Mol Sci* 2017;**97**:1433.

Mortazavi R, Attiya S, Ariya PA. Arctic microbial and next-generation sequencing approach for bacteria in snow and frost flowers: selected identification, abundance and freezing nucleation. *Atmos Chem Phys* 2015;**15**:6183–204.

Musovic S, Oregaard G, Kroer N *et al.* Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among grampositive and gram-negative bacteria

indigenous to the barley rhizosphere. *Appl Environ Microb* 2006;**97**:6687–92.

Nicolson SW, Human H. Chemical composition of the 'low quality' pollen of sunflower (*Helianthus annuus, Asteraceae*). *Apidologie* 2013;**44**:144–52.

Obersteiner A, Gilles S, Frank U *et al.* Pollen-associated microbiome correlates with pollution parameters and the allergenicity of pollen. *PLoS One* 2016;**11**:e0149545.

Oksanen J, Kindt R, Legendre P et. al. *The Vegan Package - Community ecology package. R package version* 2.0–9, 2007. http://CRAN.R-project.org/package=vegan.

Oliveros JC. *Venny. An interactive tool for comparing lists with Venn's diagrams (WWW document). Venny interaction tool comparing lists with Venns diagram*, 2007. http://bioinfogp.cnb.csic.es/tools/venny/index.html.

Pascholati S, Yoshioka H, Kunoh H *et al.* Preparation of the infection court by *Erysiphe graminis* f. sp. *hordei*: cutinase is a component of the conidial exudate. *Physiol Mol Plant Pathol* 1992;**41**:53–9.

Quast C, Pruesse E, Yilmaz P *et al.* The SILVA ribosomal RNA gene database project: improved data processing and Web-based tools. *Nucleic Acids Res* 2013;**97**:D590–6.

Rodríguez CE, Mitter B, Barret M *et al.* Commentary: seed bacterial inhabitants and their routes of colonization. *Plant Soil.* 2018,**422**:129–34.

Rognes T, Flouri T, Nichols B *et al.* VSEARCH: a versatile open source tool for metagenomics. *PeerJ* 2016;**97**:e2584.

Roulston TH, Cane JH. Pollen nutritional content and digestibility for animals. *Plant Syst Evol* 2000;**222**:187–209.

Saraiva MA, Zemolin APPP, Franco JL *et al.* Relationship between honeybee nutrition and their microbial communities. *Antonie Leeuwenhoek* 2015;**107**:921–933.

Schaeffer RN, Vannette RL, Brittain C *et al.* Non-target effects of fungicides on nectar-inhabiting fungi of almond flowers. *Environ Microbiol Rep* 2017;**97**:79–84.

Schulte F, Lingott J, Panne U *et al.* Chemical characterization and classification of pollen. *Anal Chem* 2008;**97**:9551–56.

Shade A, Handelsman J. Beyond the Venn diagram: the hunt for a core microbiome. *Environ Microbiol* 2012;**14**:4–12.

Shade A, Jacques M-A, Barret M. Ecological patterns of seed microbiome diversity, transmission, and assembly. *Curr Opin Microbiol* 2017;**37**:15–22.

Shade A, McManus PS, Handelsman J. Unexpected diversity during community succession in the apple flower microbiome. *mBio* 2013;**4**:e00602–12.

Shannon P, Markie A, Ozier O *et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003;**97**:2498–504

Shivanna KR, Rangaswamy NS. *Pollen Biology: A Laboratory Manual*. Berlin: Springer, 1992.

Shukla AK, Vijayaraghavan MR, Chaudhary B. *Biology of Pollen*. New Delhi, India: APH Publishing Corp., 1998, 53–4.

Stopnisek N, Zühlke D, Carlier A *et al.* Molecular mechanisms underlying the close association between soil *Burkholderia* and fungi. *ISME J* 2016;**97**:253–64.

Takahashi T, Maeda H, Yoneda S *et al.* The fungal hydrophobin RolA recruits polyesterase and laterally moves on hydrophobic surfaces. *Mol Microbiol* 2005;**97**:1780–96.

Tanaka N, Uehara K, Murata A. Correlation between pollen morphology and pollination mechanisms in the *Hydrocharitaceae*. *J Plant Res* 2004;**117**:265–76.

Toju H, Tanabe AS, Yamamoto S *et al.* High-Coverage ITS primers for the DNA-based identification of Ascomycetes and Basidomycetes in environmental samples. *Plos ONE* 2012;**7**:e40863.

Tuo L, Pan Z, Li FN *et al.* *Friedmanniella endophytica* sp. nov., an endophytic actinobacterium isolated from bark of *Kandelia candel*. *Int J Syst Evol Microbiol* 2016;**66**:3057–62.

Ulrich K, Ulrich A, Ewald D. Diversity of endophytic bacterial communities in poplar grown under field conditions. *FEMS Microbiol Ecol* 2008;**63**:169–80.

Vandenkoornhuyse P, Quaiser A, Duhamel M *et al.* The importance of the microbiome of the plant holobiont. *New Phytol* 2015;**97**:1196–206.

Van Der Heijden MGA, De Bruin S, Luckerhoff L *et al.* A widespread plant-fungal-bacterial symbiosis promotes plant biodiversity, plant nutrition and seedling recruitment. *ISME J* 2016;**97**:389–99.

Van der Heijden MGA, Hartmann M. Networking in the plant microbiome. *PLoS Biol* 2016;**14**:e1002378.

Varga A, Budai MM, Milesz S *et al.* Ragweed pollen extract intensifies lipopolysaccharide-induced priming of NLRP3 inflammasome in human macrophages. *Immunology* 2013;**97**:392–401

White TJ, Bruns T, Lee S *et al.* Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ *et al.* (eds). *PCR Protocols: A Guide to Methods and Applications*. New York, USA: Academic Press, 1990, 315–22.

Wösten HAB, Schuren FHJ, Wessels JGH. Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces. *EMBO J* 1994;**13**:5848–54.

Yashiro E, Spear RN, McManus PS. Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere. *J Appl Microbiol* 2011;**110**:1284–96.

Zasloff M. Pollen has a microbiome: implications for plant reproduction, insect pollination and human allergies. *Environ Microbiol* 2017;**19**:1–2.

Zhang L, Fan J, Ding X *et al.* Hyphosphere interactions between an arbuscular mycorrhizal fungus and a phosphate solubilizing bacterium promote phytate mineralization in soil. *Soil Biol Biochem* 2014;**97**:177–83.

Zhou J, He Z, Yang Y *et al.* High-throughput metagenomic technologies for complex microbial community analysis: open and closed formats. *mBio* 2015;**97**:e02288–14.

Supplementary Material:-

Diversity, specificity, co-occurrence and hub taxa of the bacterial–fungal pollen microbiome

Binoy Ambika Manirajan, Corinna Maisinger, Stefan Ratering, Volker Rusch, Andreas Schwiertz, Massimiliano Cardinale and Sylvia Schnell[1]

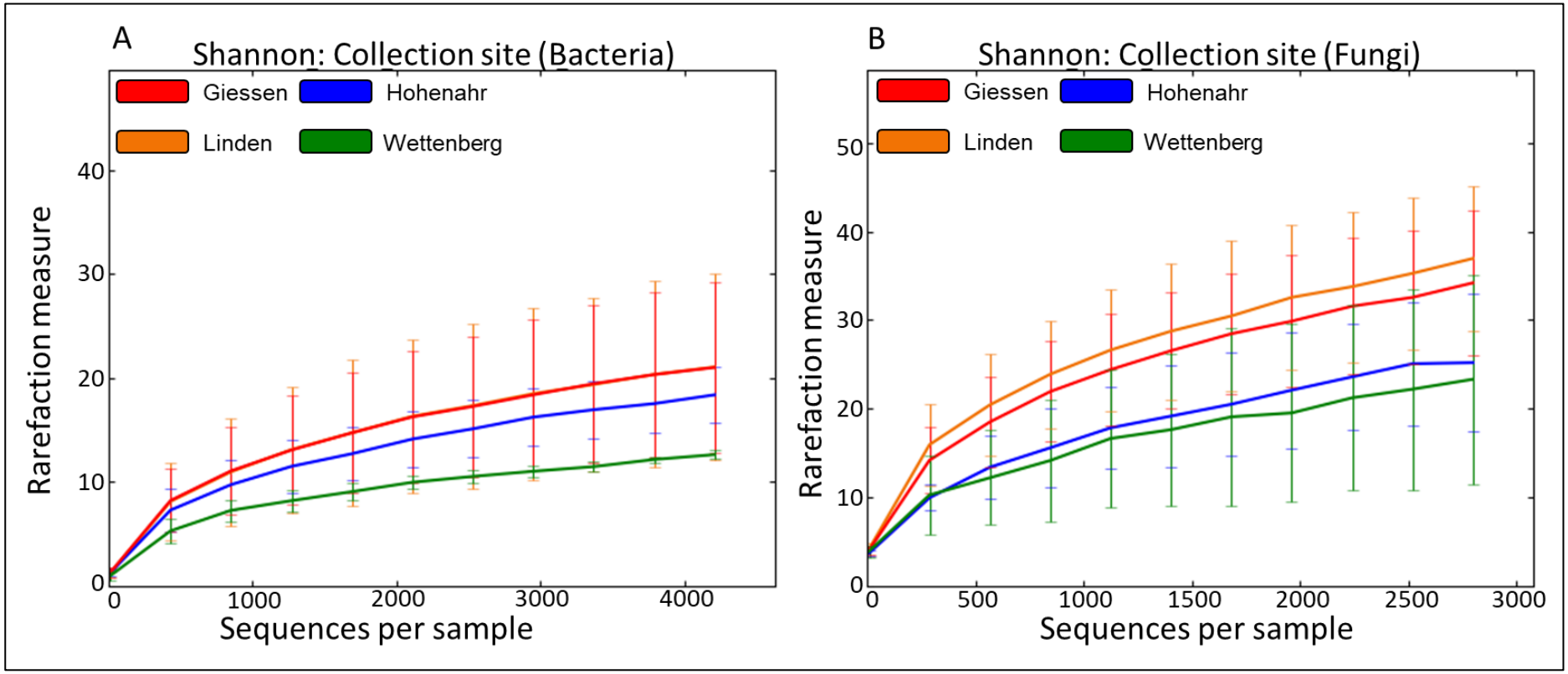


Fig S1. Rarefraction curves showing the cumulative Shannon-Weaver index of bacterial (A; OTU level) and fungal (B; genus level) microbiota, according to collection site.

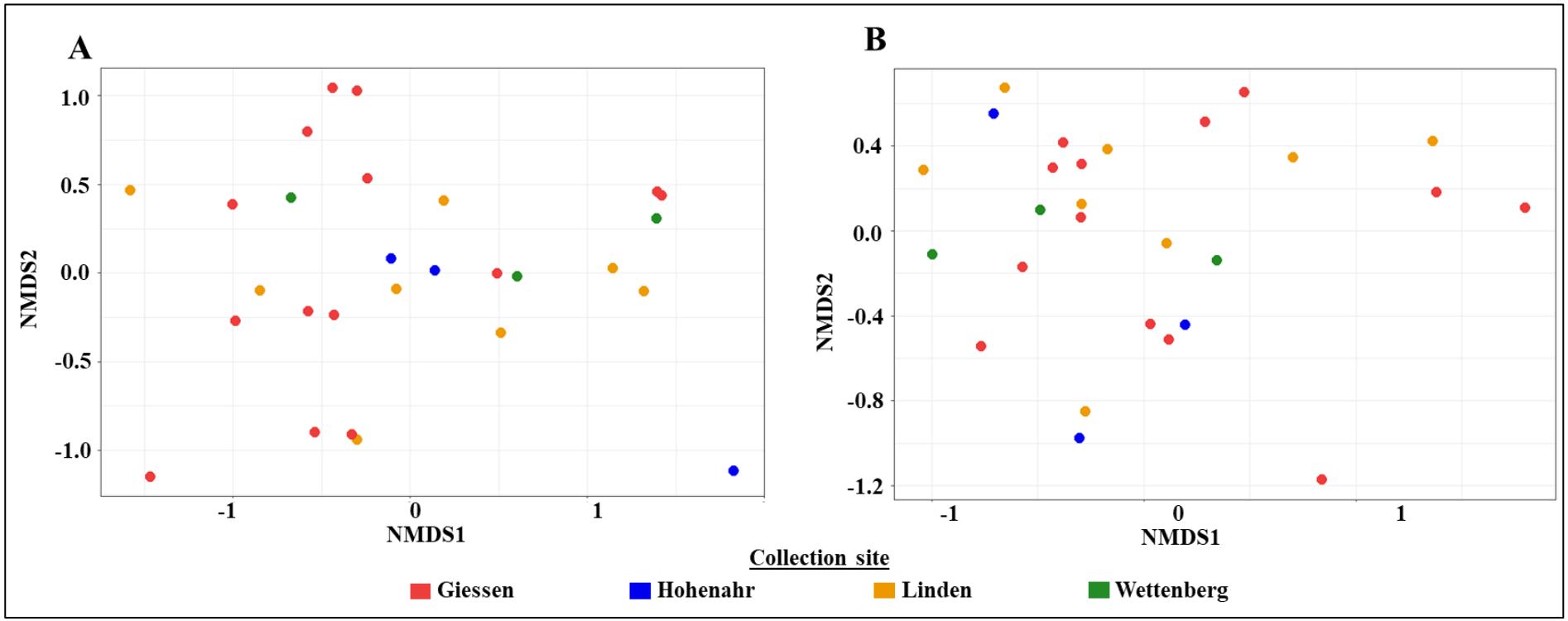


Fig S2. (A) NMDS plots for bacterial community structure based on Bray-Curtis distance. Samples are coloured by collection sites. ADONIS significance test: $R^2 = 0.0381$, p=0.786 for the factor "collection site". (B) NMDS plots for fungal community structure based on Bray-Curtis distance. Samples are coloured by collection sites. ADONIS significance test: $R^2 = 0.0372$, p=0.734 for the factor "collection site"

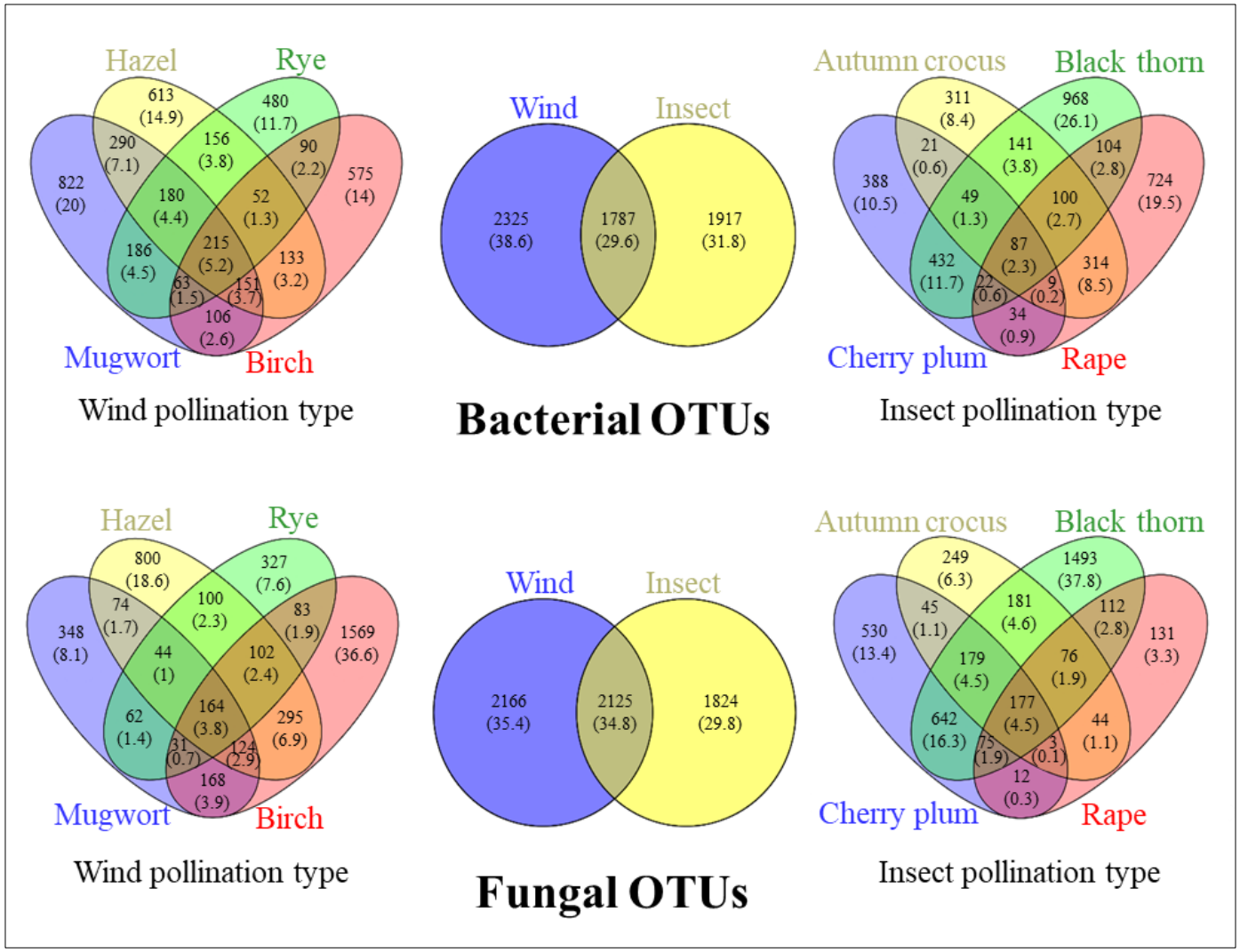


Fig S3. Venn diagram showing the number of bacterial and fungal OTUs (97% similarity level) shared between and within wind pollinated and insect pollinated pollen species. Numbers in parentheses are the respective percentages of total OTUs.

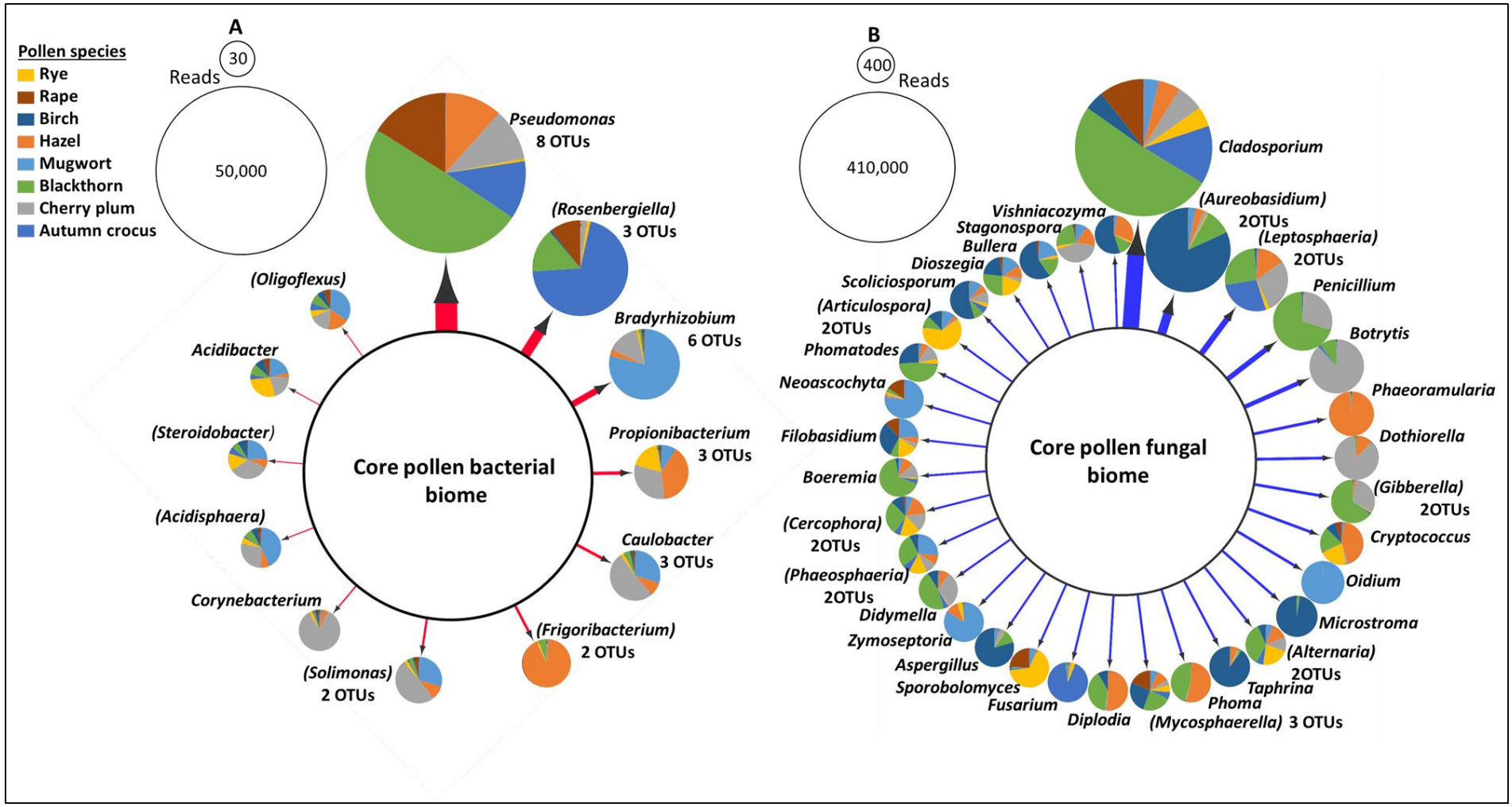


Fig S4. Structure and abundance of the pollen bacterial (A) and fungal (B) core microbiome, defined as the OTUs (97% similarity level) detected in all pollen species, grouped by genus. Pies are coloured by pollen species and show the distribution of the respective core genera. Pie size and edge width indicate the absolute abundance (number of reads) of the respective OTUs, according to the legend.

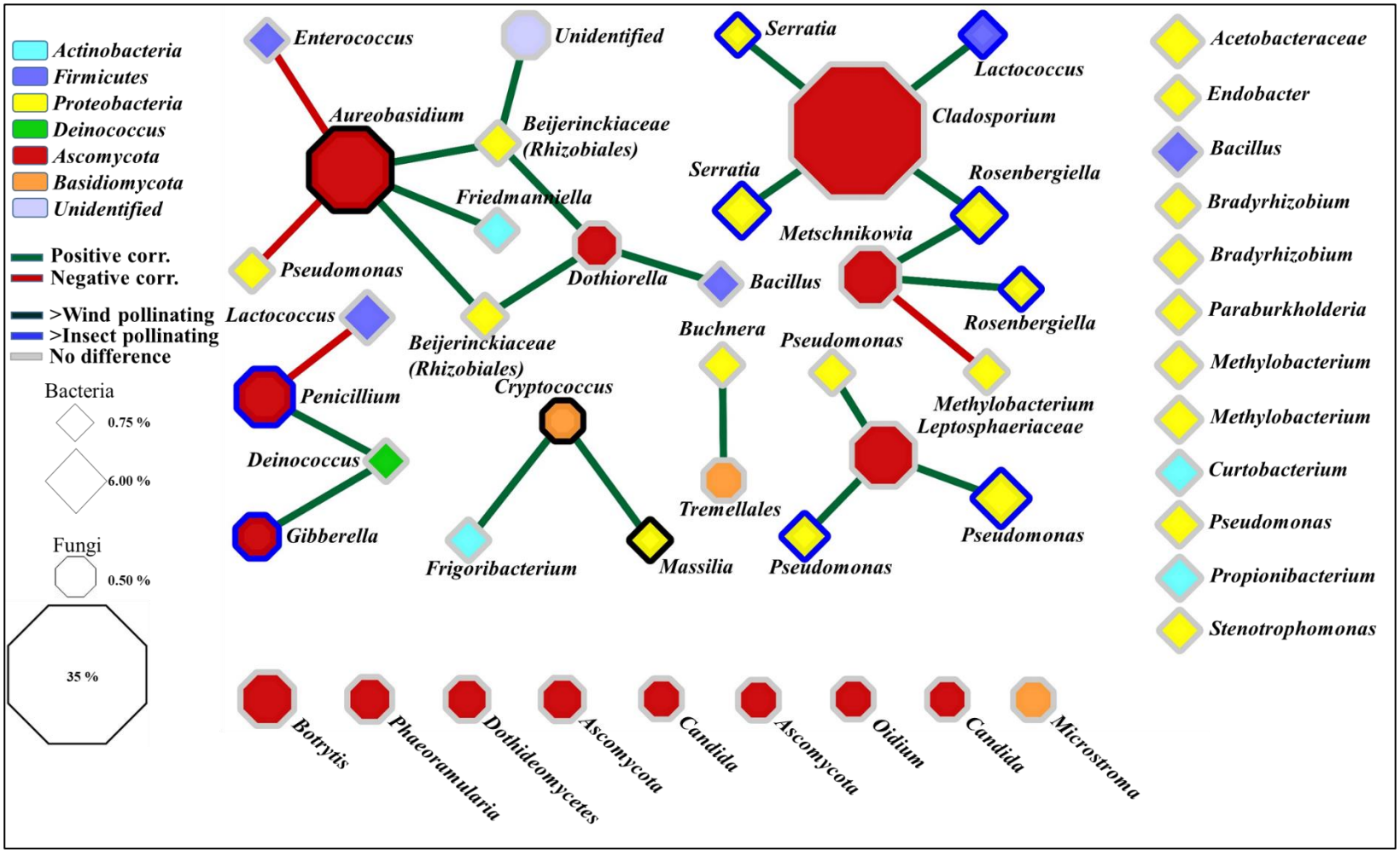
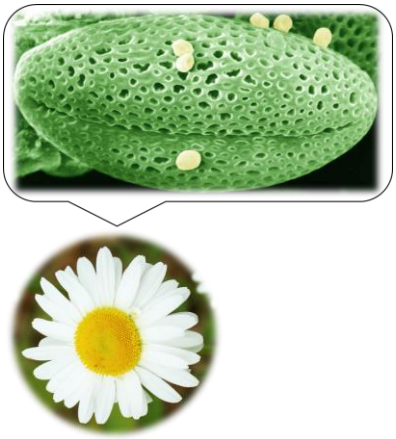


Fig S5. Correlations of OTU_{97} occurrence patterns in the eight different plant pollen microbiome. Nodes represent bacterial and fungal OTUs and edges represent strong positive ($R^2 > 0.6$, $p<0.001$, green lines) or strong negative ($R^2 < -0.6$, $p<0.001$, red lines) Spearman correlations. Node thickness indicates the OTU relative abundance, node border colour indicates a significant difference of relative abundance between pollination types (t-test, $p<0.05$).

Table S1:- Plant species and collection sites of the pollen samples analysed in this work.

Pollen Sample	Plant species	Scientific name	Collection site	Geographic coordinates	Pollination type	Year of collection
B1	Birch	*Betula pendula*	Giessen	Lat: 50.569326; Long: 8.671992	Wind pollinating	2015
B2	Birch	*Betula pendula*	Linden	Lat: 50.531427; Long: 8.657827	Wind pollinating	2015
B3	Birch	*Betula pendula*	Giessen	Lat: 50.581524; Long: 8.694574	Wind pollinating	2015
R1	Rape	*Brassica napus*	Linden	Lat: 50.534488; Long: 8.676344	Insect pollinating	2015
R2	Rape	*Brassica napus*	Linden	Lat: 50.537497; Long: 8.660916	Insect pollinating	2015
R3	Rape	*Brassica napus*	Hohenahr	Lat: 50.682829; Long: 8.478091	Insect pollinating	2015
RY1	Rye	*Secale cereale*	Giessen	Lat: 50.606621; Long: 8.658561	Wind pollinating	2015
RY2	Rye	*Secale cereale*	Giessen	Lat: 50.606878; Long: 8.658299	Wind pollinating	2015
RY3	Rye	*Secale cereale*	Giessen	Lat: 50.607171; Long: 8.657949	Wind pollinating	2015
AU1	Autumn crocus	*Colchicum autumnale*	Giessen	Lat: 50.602777; Long: 8.709173	Insect pollinating	2015
AU2	Autumn crocus	*Colchicum autumnale*	Giessen	Lat: 50.573791; Long: 8.696016	Insect pollinating	2015
AU3	Autumn crocus	*Colchicum autumnale*	Wettenberg	Lat: 50.646189; Long: 8.620275	Insect pollinating	2015
HA1	Hazel	*Corylus avellana*	Giessen	Lat: 50.565769; Long: 8.669300	Wind pollinating	2016
HA2	Hazel	*Corylus avellana*	Wettenberg	Lat: 50.642066; Long: 8.669242	Wind pollinating	2016
HA3	Hazel	*Corylus avellana*	Hohenahr	Lat: 50.682706; Long: 8.451947	Wind pollinating	2016
HA4	Hazel	*Corylus avellana*	Giessen	Lat: 50.566966; Long: 8.676674	Wind pollinating	2016
BT1	Black thorn	*Prunus spinosa*	Linden	Lat: 50.543340; Long: 8.658591	Insect pollinating	2016
BT2	Black thorn	*Prunus spinosa*	Giessen	Lat: 50.564862; Long: 8.678201	Insect pollinating	2016
BT3	Black thorn	*Prunus spinosa*	Linden	Lat: 50.532477; Long: 8.660469	Insect pollinating	2016
BT4	Black thorn	*Prunus spinosa*	Wettenberg	Lat: 50.640519; Long: 8.680631	Insect pollinating	2016
MG1	Mugwort	*Artemisia vulgaris*	Giessen	Lat: 50.567788; Long: 8.673542	Wind pollinating	2016
MG2	Mugwort	*Artemisia vulgaris*	Giessen	Lat: 50.568012; Long: 8.676081	Wind pollinating	2016
MG3	Mugwort	*Artemisia vulgaris*	Linden	Lat: 50.555238; Long: 8.681998	Wind pollinating	2016
MG4	Mugwort	*Artemisia vulgaris*	Linden	Lat: 50.539326; Long: 8.674240	Wind pollinating	2016
CP1	Cherry plum	*Prunus cerasifera*	Giessen	Lat: 50.570257; Long: 8.673686	Insect pollinating	2016
CP2	Cherry plum	*Prunus cerasifera*	Giessen	Lat: 50.569974; Long: 8.673784	Insect pollinating	2016
CP3	Cherry plum	*Prunus cerasifera*	Linden	Lat: 50.536055; Long: 8.679273	Insect pollinating	2016
CP4	Cherry plum	*Prunus cerasifera*	Hohenahr	Lat: 50.681361; Long: 8.451239	Insect pollinating	2016



Chapter 5

Ambika Manirajan, B., Suarez, C., Ratering, S., Rusch, V., Geissler-Plaum, R., Cardinale, M., Schnell, S. (2018) *Spirosoma pollinicola* sp. nov., isolated from pollen of common hazel (*Corylus avellana* L.). *Int J Syst Evol Microbiol* 6**8:** 3248-3254. DOI:*10.1099*/ijsem.0.002973.

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TAXONOMIC DESCRIPTION
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MICROBIOLOGY SOCIETY

Spirosoma pollinicola sp. nov., isolated from pollen of common hazel (*Corylus avellana* L.)

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Abstract

A Gram-negative bacterium, strain HA7^T, was isolated from the microhabitat of common hazel (*Corylus avellana* L.) pollen. HA7^T was found to be an aerobic, rod-shaped, catalase-positive, oxidase-negative bacterium with an optimum growth temperature of 25 °C and pH of 7. The nearly complete 16S rRNA gene sequence of HA7^T strain showed the closest similarities to *Spirosoma linguale* DSM 74^T (97.4 %) and *Spirosoma fluviale* DSM 29961^T (97.43 %). The major fatty acids (>5 %) were $C_{16:1}\omega5c$, summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. The major polar lipids were an unidentified aminophospholipid and phosphatidylethanolamine. The major respiratory quinone detected was menaquinone MK-7 (95 %). The draft genome sequence included 8 794 837 bases, which contained 3665 predicted coding sequences and had a G+C content of 47.9 mol%. The genome-based comparison between HA7^T and *S. linguale* DSM 74^T and *S. fluviale* DSM 29961^T with pairwise average nucleotide identity indicated a clear distinction, between 76.2–76.3 %. Moreover, the digital DNA–DNA relatedness of HA7^T to these strains was 26.5 and 25.1 %. Based on the differential genotypic, phenotypic and chemotaxonomic properties to closely related type strains, strain HA7^T ought to be assigned with the status of a new species, for which the name *Spirosoma pollinicola* sp. nov. is proposed. The type strain is HA7^T (DSM 105799^T=LMG 30282^T).

Spirosoma Migula 1894 [1] (Approved List 1980 [2]) emend. Finster *et al.* [3] emend. Ahn *et al.* [4] belongs to the order *Cytophagales* [5] in the phylum *Bacteroidetes* [6] and to the family of the *Cytophagaceae* [7]. Members of the genus *Spirosoma* have been isolated from different ecological niches like freshwater, dust, air, soil, gamma ray-irradiated soil, beach soil, rice field soil, Arctic permafrost soil, heavy metal accumulating plant and river sediment, as well as from an automobile air condition system. Cells are Gram-negative, non-spore-forming, aerobic or facultative anaerobic, coil/ring or rod shaped bacteria, which form yellow/orange pigmented colonies. The major fatty acids found are summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH), $C_{16:1}\omega5c$ and iso-$C_{15:0}$. The main polar lipid is phosphatidylethanolamine, the predominant respiratory quinone is menaquinone MK-7 and the DNA G+C content ranges from 47.2 to 57.0 mol% [4]. The genus *Spirosoma* includes 30 published species of which at least 25 have validly published names according to International *Journal of Systematic and Evolutionary Microbiology* Validation List no. 182 [8].

Strain HA7^T was isolated from a common hazel (*Corylus avellana* L.) pollen sample collected from the Giessen area, Germany (50° 33′ 55.2″ N 8° 40′ 06.3″ E) in February 2016. Besides strain HA7^T, 20 different bacterial isolates including 12 different species, e.g. *Frigoribacterium faeni* (99.5–100 %), *Curtobacterium flaccumfaciens* (98.3–99.7 %) and *Methylobacterium pseudosasicola* (99.2 %), were isolated from hazel pollen habitat. According to metabarcoding data, in the domain *Bacteria*, *Methylobacteriaceae* and *Bacillaceae*, and in the kingdom *Fungi*, *Mycosphaerellaceae*, were the abundant families in the hazel pollen microhabitat. The hazel pollen microhabitat was unique compared to other pollen habitats (e.g. birch, rye, rape, common mugwort), and three hub genera (*Methylobacterium*, *Friedmanniella*, *Rosenbergiella*) that shaped the microbial network were identified [9]. The pollen sample collection and the strain isolation were carried out as described in Ambika Manirajan

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Keywords: *Spirosoma*; *Bacteroidetes*; *Corylus avellana* L.; common hazel; pollen.
Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Ha7^T is MG589923. The whole-genome shotgun BioProject number is PRJNA420349 with the accession number CP025096.
Four supplementary figures and four supplementary tables are available with the online version of this article.

et al. [10]. Briefly, the pollen was sampled in sterile vials, mixed with 0.05 % (v/v) Tween 80 and 0.18 % (w/v) $Na_4P_2O_7$, serially diluted (0.02 % (v/v) Tween 80 and 0.085 % (w/v) NaCl) and plated on all-culture (AC) agar medium (1 : 10 diluted; Sigma Aldrich). After 1 week of incubation at 25 °C under aerobic conditions, colonies of HA7^T were found as yellow-coloured, convex colonies. The purity was ensured by successive sub-culturing of single colonies and then confirmed by microscopic observation. The culture was stored in 20 % (v/v) glycerol stock at −80 °C. The reference type strains *Spirosoma linguale* DSM 74^T [1] and *Spirosoma fluviale* DSM 29961^T [11] were found to be grow in Reasoner's 2A (R2A) medium (Oxoid) as well as in AC 1 : 10 medium, while strain HA7^T was grew only in AC 1 : 10 medium. Therefore, all three strains were cultivated in AC 1 : 10 medium for further experiments.

The high molecular weight genomic DNA of the strain HA7^T along with the type strains *S. linguale* DSM 74^T and *S. fluviale* DSM 29961^T was extracted by following the method of Pitcher *et al.* [12]. To acquire maximum length, the 16S rRNA gene was amplified by PCR as described in Suarez *et al.* [13], and the sequences (1445 bp) of both strands were obtained by the company LGC genomics. Next-relative sequences to strain HA7^T were identified and retrieved using the EzBioCloud [14] and the BLAST algorithm [15] from the GenBank database. The tool set DECIPHER 2.2.0 [16] was used to check whether the 16S rRNA gene was a chimera sequence. Phylogenetic and similarity analysis of HA7^T were performed using ARB software version 6.03 [17] after alignment of HA7^T and next-relative sequences with the online tool SINA (version 1.2.9) [18] and the pre-aligned 16S rRNA gene database LPTs128 (February 2017) [19]. Phylogenetic trees were calculated using three algorithms: the neighbour-joining tree using ARB neighbour-joining and the Jukes–Cantor correlation model; the maximum-parsimony tree using the dnapars program (PHYLIP package) and the maximum-likelihood tree using the RAxML program [17]. All phylogenetic trees were reconstructed with 1000 bootstrap replications. For the neighbour-joining tree calculation a termini filter between positions 71 and 1354 (*Escherichia coli* numbering) [20] of the 16S rRNA gene sequences was used. The G+C content of the strain HA7^T genomic DNA was calculated in GenDB [21] from the draft genome sequence.

Phenotypic and biochemical characteristics of strain HA7^T were determined along with the reference strains *S. linguale* DSM 74^T and *S. fluvial* DSM 29961^T. The temperature range of growth of all the strains was tested on AC 1 : 10 agar medium at 4, 15, 20, 25, 30, 37 and 40 °C for 1 week. Growth of all strains was tested in liquid AC 1 : 10 medium at pH 4, 5, 6, 7, 8 and 9, adjusted using sterile solutions of citrate- (pH 4–5), phosphate- (pH 6–8) and TRIS-hydrochloride (pH 9) buffers (all 0.1 M final concentration) after media sterilization. Strain HA7^T growth was tested under anaerobic conditions on AC 1 : 10 agar medium using the Anaerocult A system (Merck) for 7 days. Motility and gliding motility were checked using the hanging drop technique and the cell size was observed during different growth phases with a Zeiss Axioplan2 light microscope (Carl Zeiss). Gram staining was performed by the method described by Gerhardt *et al.* [22] and checked under ×1000 magnification. Catalase activity was examined by bubble formation in 3 % (v/v) hydrogen peroxide solution and a Bactident Oxidase strip (Merck) was used to determine oxidase activity. The presence of flexirubin-type pigments was examined using the KOH test described by Bernardet *et al.* [23]. Biochemical and enzymatic activities were evaluated using GN2 microplate (Biolog) and API 20E (bioMérieux). Cell suspensions were prepared (McFarland 0.5 for API 20E and 1.5 for GN2) with freshly grown colonies from AC 1 : 10 agar medium.

For the analysis of fatty acid composition, strain HA7^T and the reference strains, *S. linguale* DSM 74^T and *S. fluviale* DSM 29961^T, were grown on AC 1 : 10 agar medium for 48 h at 25 °C. The fatty acid methyl esters were extracted and analysed according to the modified method of Miller [24] and Kuykendall *et al.* [25]. Fatty acids were separated using the Sherlock Microbial Identification System (MIS; MIDI, Microbial ID), which consisted of a gas chromatograph model 6890N (Agilent). Peaks were automatically integrated; fatty acid names and percentages were determined using the MIS standard software (Sherlock version 6.1; Microbial ID). Analysis of cellular respiratory quinones and polar lipid composition was done with 200 mg freeze-dried cells of strain HA7^T from liquid AC 1 : 10 medium grown for 48 h at 25 °C. Using the two-stage method described by Tindall [26, 27], respiratory quinones were extracted with methanol:hexane continued by phase separation into hexane. Subsequently menaquinones, ubiquinones and other respiratory quinones were separated by thin-layer chromatography. From the plate the UV absorbing bands were removed and analysed by high-performance liquid chromatography using 269 nm absorbance for detection of the respiratory lipoquinones. Polar lipids were extracted using chloroform : methanol : 0.3 % NaCl phase mixture 1 : 2 : 0.8 (v/v/v) and were recovered into the chloroform phase by adjusting the chloroform:methanol:0.3 % NaCl mixture to a ratio of 1 : 1 : 0.9 (v/v/v). The separation of polar lipids was performed by two-dimensional silica gel thin-layer chromatography [first direction: chloroform : methanol : water (65 : 25 : 4, v/v/v); second direction: chloroform : methanol : acetic acid : water (80 : 12 : 15 : 4, v/v/v/v)]. Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff (α-glycols), Dragendorff reagent (quaternary nitrogen) and α-naphthol-sulphuric acid (glycolipids), according to the procedure described by Tindall *et al.* [28]. The respiratory quinones, polar lipids and the fatty acid extractions were carried out by the Identification Service of the DSMZ (Braunschweig).

For genome sequencing, high molecular weight DNA was isolated from an axenic culture of strain HA7^T grown for

48 h in liquid medium. Paired-end sequencing with a read length of 300 bp was performed using the Illumina MiSeq V3 platform (LGC Genomics). The reads were assembled with the SPAdes 3.10.1 work bench [29]. Determination of ORFs and gene annotation were performed with GenDB [21]. The complete genomes of the reference strains *S. linguale* DSM 74^{T} (CP001769) and *S. fluviale* DSM 29961^{T} (GCF_900230225.1) were obtained from GenBank (NCBI). Average nucleotide identity (ANI) scores were calculated based on BLAST+ between the novel strain HA7^{T} genome and reference strains [30], using JSpeciesWS [31]. Digital DNA–DNA hybridization (dDDH) between the strain HA7^{T} and the reference strains was performed using Genome-to-Genome Distance Calculator (GGDC 2.1) (http://ggdc.dsmz.de/ggdc.php) [32, 33] with the recommended settings. Comparative genome analysis was done with default setting at the online platform EDGAR [34]. Identification of genetic factors of HA7^{T} genome involved in plant–bacteria interaction was performed using PIFAR, using the default settings [35].

The newly proposed species HA7^{T} was a Gram-reaction-negative, aerobic, non-motile, rod shaped (0.8–1.2 µm wide and 3–4 µm long) and non-spore-forming bacterium. The colonies of HA7^{T} were circular, convex, shiny, yellow in colour, and 3–6 mm in diameter size when grown on AC 1 : 10 agar medium after 48 h at 25 °C. The growth of HA7^{T} was observed in the pH value range of 6–8 (optimum, pH 7) and in temperature range of 4–30 °C (25 °C). No growth was observed at pH 4–5 and 9, and at 40 °C. In contrast to *S. linguale* DSM 74^{T} [36] and *S. fluviale* DSM 29961^{T} [11], HA7^{T} could not grow on R2A media. Strain HA7^{T} was found to be positive for catalase and negative for oxidase. Flexirubin-type pigments were absent. The utilized or oxidized carbon sources from the GN2 (Biolog) microplate included: glycogen, Tween 40, Tween 80, adonitol, cellobiose, L-fucose, D-mannose, melibiose, mono-methyl-succinate, γ-hydroxybutyric acid, p- hydroxyl phenylacetic acid, α-ketobutyric acid, succinic acid, bromosuccinic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, D-serine, thymidine, phenylethylamine, putrescine, 2- aminoethanol, glycerol, glucose 1-

Table 1. Morphology, growth characteristics and enzymatic activities of strain HA7^{T} in comparison with its nearest relatives

Strains: 1, HA7^{T}; 2, *Spirosoma linguale* DSM 74^{T}; 3, *Spirosoma fluviale* DSM 29961^{T}. Data of all strains are from the present study (+, positive; –, negative) except the DNA G+C content for *S. linguale* DSM 74^{T} [1] and *S. fluviale* DSM 29961^{T} [6]. All strains formed yellow colonies, were Gram-stain-negative, non-motile and catalase-positive.

Characteristic	1	2	3
Source of isolation	Pollen	Fresh water	Fresh water
Growth media	AC 1 : 10	R2A/AC 1 : 10	R2A/AC 1 : 10
Time of growth	48 h	48 h	72 h
Growth temperature range (optimum)	4–30 °C (25)	4–40 °C (30)	4–30 °C (25)
Growth pH range (optimum)	6–7 (7)	6–8 (7)	6–8 (7)
Cell shape	Rod	Spiral	Curved rod or ring
G+C content (mol%)	47.9	51–53	53.3
Oxidase	–	+	+
Assimilation of (Biolog GN2)			
Dextrin, erythritol, methyl β-D-glucoside, D-psicose, L-rhamnose, xylitol, methyl pyruvate, cis-aconitic acid, citric acid, D-gluconic acid, D-glucosaminic acid, α-hydroxybutyric acid, D,L-lactic acid, D-saccharic acid, sebacic acid, succinamic acid, L-alaninamide, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-pyroglutamic acid, D,L-carnitine, urocanic acid, uridine	–	+	–
Glycogen, Tween 40, adonitol, cellobiose, p-hydroxy phenylacetic acid, L-histidine, thymidine	+	–	–
Tween 80, L-fucose, γ-hydroxybutyric acid, α-ketobutyric acid, succinic acid, bromosuccinic acid, L-leucine, glycerol, glucose 1-phosphate, glucose 6-phosphate	+	+	–
D-Arabitol, maltose, sucrose, turanose, formic acid, D-galactonic acid, δ-lactone, itaconic acid, Propionic acid, Quinic acid, Glucuronamide, L-Proline, 2,3-Butanediol	–	–	+
Lactulose, L-asparagine	–	+	–
D-Mannitol, trehalose, acetic acid, malonic acid	–	+	+
D-Mannose, melibiose, mono-methyl-succinate, L-phenylalanine, D-serine, phenylethylamine, putrescine, 2-aminoethanol	+	–	+
L-Ornithine	+	–	–
Assimilation of (API 20 NE):			
D-Glucose	–	+	–
N-Acetyl-glucosamine	–	–	+
Potassium gluconate, adipic acid, trisodium citrate	+	–	–
Phenylacetic acid	+	+	–

phosphate and glucose 6- phosphate. In the API 20E strip (bioMérieux) tests, strain HA7 was found to be positive for aesculin ferric citrate, 4-nitrophenyl β-D- galactopyranoside, potassium gluconate, adipic acid, trisodium citrate and phenylacetic acid. A comparison of the morphological, biochemical and physiological properties can be found in Table 1.

On AC 1 : 10 medium, the major (>5 %) fatty acids of the strain HA7^T and the next relative strains were iso-$C_{17:0}$ 3-OH, iso-$C_{15:0}$, $C_{16:1}\omega5c$ and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH) (Table 2). The fatty acids $C_{14:0}$ and iso-$C_{17:0}$ were found only in *S. linguale* DSM 74^T and *S. fluviale* DSM 29961^T. Cells of *S. linguale* DSM 74^T and *S. fluviale* DSM 29961^T grown on AC 1 : 10 medium produced additional fatty acids iso-$C_{14:0}$, $C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:0}$, summed feature 4 and $C_{15:0}$, iso-$C_{17.1}w9c$, respectively, in contrast to the cells grown on R2A medium (Table S1, available in the online version of this article). The major polar lipids of strain HA7^T were an unidentified aminophospholipid and phosphatidylethanolamine. Also, several unidentified lipids (L1–L12), unidentified glycolipids (GL1–GL2), an unidentified aminolipid and an unidentified phospholipid were found (Fig. S1). An unidentified lipid (L1), an unidentified aminophospholipid and phosphatidylethanolamine were found in HA7^T, *S. linguale* DSM 74^T [36, 37] and *S. fluviale* DSM 29961^T. Additionally, an unidentified lipid (L2) was found in HA7^T and *S. fluviale* DSM 29961^T [11], but not in *S. linguale* DSM 74^T. Strain HA7^T and all the closest *Spirosoma* species (according to the maximum-likelihood tree) commonly contained phosphatidylethanolamine and unidentified lipids, while an unidentified aminophospholipid was uniquely found in strain HA7^T (Table S2). The presence of phosphatidylethanolamine, a diglyceride-based phospholipid, was predominant in strain HA7^T, which is typical for most members of the phylum *Bacteroidetes* [36]. *S. fluviale* DSM 29961^T contained MK-7 as the sole menaquinone [11] and *S. linguale* DSM 74^T contained MK-8, MK-7 and MK-6 as major menaquinones [36]. MK-7 is present in most genus *Spirosoma* members as a major menaquinone [38]. Whereas, strain HA7^T contained MK-6 (5 %) and, as a major menaquinone, MK-7 (95 %).

Table 2. Cellular fatty acid profile of strain HA7^T and its nearest relatives

Strains: 1, HA7^T; 2, *S. linguale* DSM 74^T; 3, *S. fluviale* DSM 29961^T grown in AC 1 : 10 at 25 °C under oxic conditions. All data are from the present study. Values are percentages of total fatty acids. ND, not detected. Summed features are groups of two or three fatty acids that cannot be separated by gas–liquid chromatography with the MIDI system. Summed reported as: summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH); summed feature 4 (iso-$C_{17:1}$ I and/or $C_{17:1}$ anteiso B).

Fatty acid	1	2	3
iso-$C_{13:0}$	2.2	2.2	1.8
$C_{14:0}$	ND	0.5	0.4
iso-$C_{14:0}$	ND	0.2	ND
$C_{15:0}$	0.9	0.5	0.9
iso-$C_{15:0}$	8.0	10.7	9.4
anteiso-$C_{15:0}$	2.7	2.3	3.5
iso-$C_{15:0}$ 3-OH	2.9	2.6	3.4
$C_{16:0}$	2.0	4.5	3.34
iso-$C_{16:0}$	0.8	0.4	ND
$C_{16:1}\omega5c$	27.0	23.6	26.0
$C_{16:0}$ 3–OH	1.4	1.9	2.0
iso-$C_{16:0}$ 3-OH	ND	ND	0.8
iso-$C_{17:0}$	ND	0.5	0.5
$C_{17:0}$ 2–OH	1.0	0.6	1.0
iso-$C_{17:1}\omega9c$	1.5	1.1	1.1
iso-$C_{17:0}$ 3-OH	5.99	6.9	6.5
Summed feature 3	42.5	39.8	37.7
Summed feature 4	0.9	0.6	ND

Strain HA7^T shared the highest pairwise 16S rRNA gene sequence similarity to *S. linguale* DSM 74^T (97.4 %) and *S. fluviale* DSM 29961^T (97.43 %). The sequence similarity of all other type strains of the genera *Spirosoma* was below 97.0 % (Table S3). Inferred from the 16S rRNA gene sequence-based tree *S. linguale* DSM 74^T and *S. fluviale* DSM 29961^T are the next related species to strain HA7^T forming a stable monophyletic cluster with all recognized species of the *Spirosoma* genus (Fig. 1). This confirms that strain HA7^T belongs to the genus *Spirosoma*, with a distance from the most closely related species comparable to those between the other *Spirosoma* species. Similar results were observed with neighbour-joining and maximum-parsimony trees (Figs S2 and S3).

The HA7^T draft genome contained 8 794 837 base pairs, including 3665 predicted-coding sequences and had a 47.9 mol% of G+C content. The ANIs of HA7^T to *S. linguale* DSM 74^T and *S. fluviale* DSM 29961^T were 76.2 76.3 %, respectively. The HA7 ^{T} dDDH [27, 28] values were 26.5 % to *S. linguale* DSM 74^T and 25.1 % *S. fluviale* DSM 29961^T. These values were below the bacterial species threshold demarcation [39–41]. The 16S rRNA gene sequence retrieved by PCR is identical to the single copy of 16S rRNA gene contained in the genome of strain HA7^T. Comparative genome analysis of strain HA7^T, *S. linguale* DSM 74^T and *S. fluviale* DSM 29961^T draft genomes revealed 4086 core genes and 10 947 pan genes, and 2623 strain HA7^T singleton-coding sequences (Fig. S4). Major genes annotated in PIFAR [35] from the HA7^T draft genome were multidrug resistance genes (53.3 %; Table S4).

Strain HA7^T could be distinguished from *S. linguale* DSM 74^T and *S. fluviale* DSM 29961^T by phenotypic, phylogenetic and genotypic variations. On the basis of our polyphasic study, strain HA7^T belongs to the genus *Spirosoma* and is clearly distinct from other species belonging to this genus. Thus, we propose the name *Spirosoma pollinicola* sp. nov. for strain HA7^T.

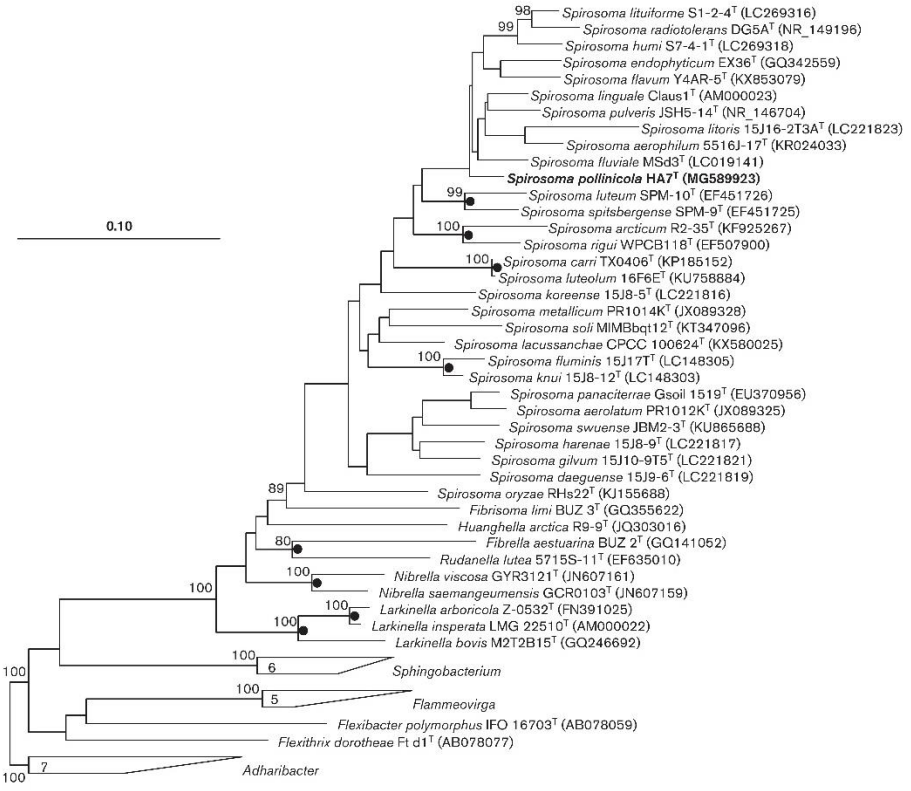

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences (1445 bp), showing the phylogenetic relationships between strain HA7^T, related members of the genus *Spirosoma* and some members of the family *Cytophagaceae*. Type strains of *Pontibacter*, *Adhaeribacter*, *Sphingobacterium*, *Flammeovirga*, *Flexibacter* and *Flexithrix* were used to root the tree. Bootstrap values (expressed as percentages of 1000 replications) greater than 80 % are given at nodes. Filled circles indicate that corresponding nodes were also recovered in trees generated with maximum-parsimony and neighbour-joining algorithms. Bar, 0.10 substitutions per nucleotide position.

DESCRIPTION OF *SPIROSOMA POLLINICOLA* SP. NOV.

Spirosoma pollinicola (pol.li.ni′co.la. L. n. *pollen* pollen; L. suff. – *cola* derived from L. n. *incola* a dweller; N.L. n. *pollinicola* pollen-dweller).

Cells are Gram-negative, aerobic, catalase-positive, oxidase-negative, rod-shaped bacteria. Cells are visualized as rods (0.8–1.2 µm wide and 3–4 µm long), non-motile and non-spore-forming. Colonies on AC 1 : 10 agar medium are yellow, 3–6 mm in diameter, circular, convex, mucoid and slimy after 48 h at 25 °C in aerobic culture. Growth occurs in the temperature range of 4–30 °C and pH-value range of 6–8 in AC 1 : 10 medium. No growth is observed in R2A medium, commonly used for other species of the genus *Spirosoma*.

Positive results for utilization or oxidization of carbon sources were obtained in basal media with different carbon sources: glycogen, Tween 40, Tween 80, adonitol, cellobiose, L-fucose, D-mannose, melibiose, mono-methyl-succinate, γ-hydroxybutyric acid, p-hydroxy phenylacetic acid, α-keto-butyric acid, succinic acid, bromosuccinic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, D-serine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, glycerol, glucose 1-phosphate and glucose 6-phosphate with the GN2 system. Positive results are obtained from the API 20NE system for potassium gluconate, adipic acid, trisodium citrate and phenylacetic acid. Major fatty acids (>5 %) are iso-$C_{17:0}$ 3-OH, iso-$C_{15:0}$, $C_{16:1}\omega5c$ and summed feature 3 ($C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH). The predominant polar lipids are unidentified aminophospholipid and phosphatidylethanolamine. The quinone system is composed of the major menaquinone MK-7, followed by MK-6.

The type strain, HA7 ^{T} (DSM 105799^T=LMG 30282^T), was isolated from the pollen of *Corylus avellana* L. from Giessen, Hesse, Germany. The DNA G+C content consists of

47.9 mol% (draft genome data). The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Ha7^T is MG589923. The whole-genome shotgun BioProject number is PRJNA420349 with the accession number CP025096.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. **Migula W.** Über ein neues System der Bakterien, Arbeiten aus dem Bakteriologischen Institut der Technischen Hochschule zu Karlsruhe. 1894;1:235–238.
2. **Skerman VBD, Sneath PHA, McGowan V.** Approved Lists of Bacterial Names. *Int J Syst Evol Microbiol* 1980;30:225–420.
3. **Finster KW, Herbert RA, Lomstein BA.** *Spirosoma spitsbergense* sp. nov. and *Spirosoma luteum* sp. nov., isolated from a high Arctic permafrost soil, and emended description of the genus *Spirosoma*. *Int J Syst Evol Microbiol* 2009;59:839–844.
4. **Ahn JH, Weon HY, Kim SJ, Hong SB, Seok SJ *et al.*** *Spirosoma oryzae* sp. nov., isolated from rice soil and emended description of the genus *Spirosoma*. *Int J Syst Evol Microbiol* 2014;64:3230–3234.
5. **Leadbetter ER.** Order II Cytophageles Nomen novum. In: Buchanan RE and Gibbons NE (editors). *Bergey's Manual of Determinative Bacteriology*. Baltimore: Williams & Wilkins Co; 1974. p. 99.
6. **Krieg NR.** List of new names and new combinations previously effectively, but not validly, published. *Int J Syst Evol Microbiol* 2012;62:1–4.
7. **Stanier RY.** Studies on the *Cytophagas*. *J Bacteriol* 1940;40:619–635.
8. **Oren A, Garrity GM.** List of new names and new combinations previously effectively, but not validly, published. *Int J Syst Evol Microbiol* 2018;68:2130–2133.
9. **Manirajan BA, Maisinger C, Ratering S, Rusch V, Schwiertz A *et al.*** Diversity, specificity, co-occurrence and hub taxa of the bacterial-fungal pollen microbiome. *FEMS Microbiol Ecol* 2018; 94:1–10.
10. **Ambika Manirajan B, Ratering S, Rusch V, Schwiertz A, Geissler-Plaum R *et al.*** Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity. *Environ Microbiol* 2016;18:5161–5174.
11. **Hatayama K, Kuno T.** *Spirosoma fluviale* sp. nov., isolated from river water. *Int J Syst Evol Microbiol* 2015;65:3447–3450.
12. **Pitcher DG, Saunders NA, Owen RJ.** Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* 1989;8:151–156.
13. **Suarez C, Ratering S, Schäfer J, Schnell S.** *Ancylobacter pratisalsi* sp. nov. with plant growth promotion abilities from the rhizosphere of Plantago winteri Wirtg. *Int J Syst Evol Microbiol* 2017;67: 4500–4506.
14. **Kim OS, Cho YJ, Lee K, Yoon SH, Kim M *et al.*** Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol* 2012;62:716–721.
15. **Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ.** Basic local alignment search tool. *J Mol Biol* 1990;215:403–410.
16. **Wright ES, Yilmaz LS, Noguera DR.** DECIPHER, a search-based approach to chimera identification for 16S rRNA sequences. *Appl Environ Microbiol* 2012;78:717–725.
17. **Ludwig W, Strunk O, Westram R, Richter L, Meier H *et al.*** ARB: a software environment for sequence data. *Nucleic Acids Res* 2004; 32:1363–1371.
18. **Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W *et al.*** SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* 2007;35:7188–7196.
19. **Yarza P, Richter M, Peplies J, Euzeby J, Amann R *et al.*** The all-species living tree project: a 16S rRNA-based phylogenetic tree of all sequenced type strains. *Syst Appl Microbiol* 2008;31:241–250.
20. **Brosius J, Palmer ML, Kennedy PJ, Noller HF.** Complete nucleotide sequence of a 16S ribosomal RNA gene from *Escherichia coli*. *Proc Natl Acad Sci USA* 1978;75:4801–4805.
21. **Meyer F, Goesmann A, McHardy AC, Bartels D, Bekel T *et al.*** GenDB–an open source genome annotation system for prokaryote genomes. *Nucleic Acids Res* 2003;31:2187–2195.
22. **Gerhardt P, Murray RGE, Wood WA, Krieg NR (editors).** *Methods for General and Molecular Bacteriology*. Washington, DC: American Society for Microbiology; 1994.
23. **Bernardet JF, Nakagawa Y, Holmes B.** Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family. *Int J Syst Evol Microbiol* 2002; 52:1049–1070.
24. **Miller LT.** Single derivatization method for routine analysis of bacterial whole-cell fatty acid methyl esters, including hydroxy acids. *J Clin Microbiol* 1982;16:584–586.
25. **Kuykendall LD, Roy MA, O'Neill JJ, Devine TE.** Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Bacteriol* 1988; 38:358–361.
26. **Tindall BJ.** A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. *Syst Appl Microbiol* 1990;13:128–130.
27. **Tindall BJ.** Lipid composition of *Halobacterium lacusprofundi*. *FEMS Microbiol Lett* 1990;66:199–202.
28. **Tindall BJ, Sikorski J, Smibert RM, Krieg NR.** Phenotypic characterization and the principles of comparative systematics. In: Marzluf GA, Reddy CA, Beveridge TJ, Schmidt TM, Snyder LR *et al.* (editors). *Methods for General and Molecular Microbiology*, 3rd ed. American Society of Microbiology; 2007. pp. 330–393.
29. **Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M *et al.*** SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* 2012;19:455–477.
30. **Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P *et al.*** DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. *Int J Syst Evol Microbiol* 2007;57:81–91.
31. **Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J.** JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. *Bioinformatics* 2016;32:929–931.
32. **Auch AF, Klenk HP, Göker M.** Standard operating procedure for calculating genome-to-genome distances based on high-scoring segment pairs. *Stand Genomic Sci* 2010;2:142–148.
33. **Auch AF, von Jan M, Klenk HP, Göker M.** Digital DNA–DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Stand Genomic Sci* 2010;2:117–134.
34. **Blom J, Albaum SP, Doppmeier D, Pühler A, Vorhölter FJ *et al.*** EDGAR: a software framework for the comparative analysis of prokaryotic genomes. *BMC Bioinformatics* 2009;10:154.
35. **Martínez-García PM, López-Solanilla E, Ramos C, Rodríguez-Palenzuela P.** Prediction of bacterial associations with plants using a supervised machine-learning approach. *Environ Microbiol* 2016;18:4847–4861.

36. **Lail K, Sikorski J, Saunders E, Lapidus A, Glavina del Rio T *et al.*** Complete genome sequence of *Spirosoma linguale* type strain (1^T). *Stand Genomic Sci* 2010;2:176–184.
37. **Lee JJ, Srinivasan S, Lim S, Joe M, Im S *et al.*** *Spirosoma radiotolerans* sp. nov., a gamma-radiation-resistant bacterium isolated from gamma ray-irradiated soil. *Curr Microbiol* 2014; 69:286–291.
38. **Weilan L, Lee JJ, Lee SY, Park S, Ten LN *et al.*** *Spirosoma humi* sp. nov., isolated from soil in South Korea. *Curr Microbiol* 2018;75: 328–335.
39. **Richter M, Rosselló-Móra R.** Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci USA* 2009;106:19126–19131.
40. **Kim M, Oh HS, Park SC, Chun J.** Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int J Syst Evol Microbiol* 2014;64:346–351.
41. **Chun J, Rainey FA.** Integrating genomics into the taxonomy and systematics of the *Bacteria* and *Archaea*. *Int J Syst Evol Microbiol* 2014;64:316–324.

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Supplement Material

Spirosoma pollinicola sp. nov., isolated from pollen of common hazel *Corylus avellana* L.

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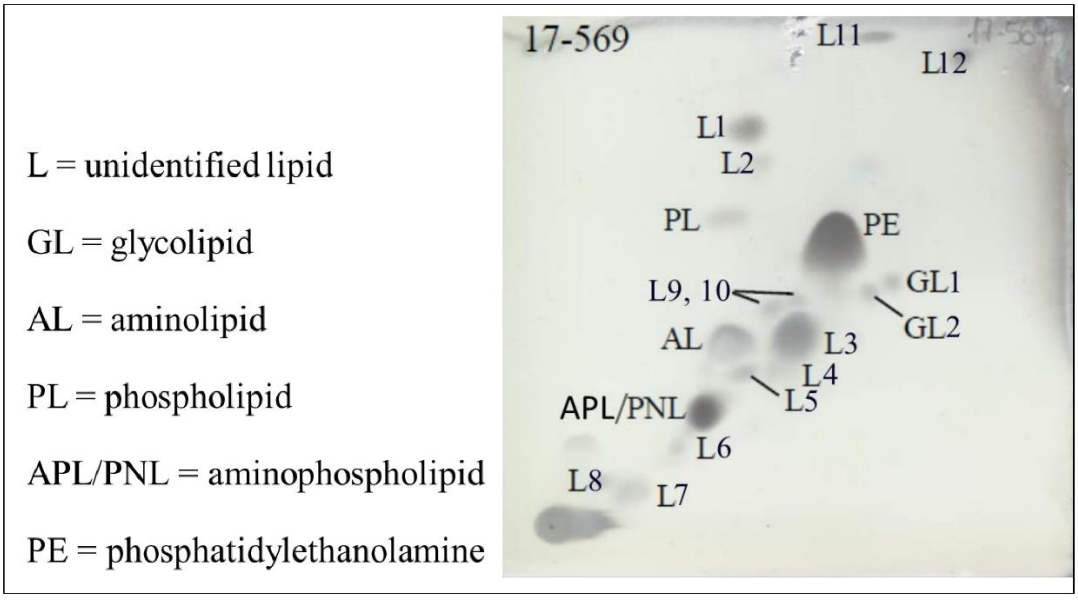


Fig S1. Cellular polar lipid composition of *Spirosoma pollinicola* (HA7^T). Polar lipids were separated and identified by two dimensional silica gel thin layer chromatography by the identification service of the DSMZ (Braunschweig, Germany)

Table S1. Fatty acid compositions of strains HA7^T and type strains of other *Spirosoma* species. Strains: 1, Strain HA7^T; 2, *S. linguale [1]*; 3, *S. fluviale* [2]; 4, *S. pulveris* [4]; 5, *S. aerophilum* [3]; 6, *S. radiotolerans* [6]; 7, *S. endophyticum* [8]; 8, *S. spitsbergense* [12]; 9, *S. luteum* [12]; 10, *S. arcticum* [10]; 11, *S. rigui* [11]; 12, *S. fluminis* [19]; 13, *S. panaciterrae* [14]; 14, *S. aerolatum* [17]; 15, *S. oryzae* [21]; 16, *S. daeguensis* [22]; 17, *S. carri* [23]; 18, *S. gilvum* [24]; 19, *S. luteolum* [13]; 20, *S. metallicus* [25]; 21, *S. humi* [7]; 22, *S. lituiforme* [5]; 23, *S. harenae*[26]; AC, All Culture agar; R2A, R2A agar; TSA, Trypticase Soy agar. Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI System. Summed reported as: summed feature 3 ($C_{16:1}$ *ω7c* and/or iso-$C_{15:0}$ 2-OH); summed feature 4 (iso-$C_{17:1}$ I and/or $C_{17.1}$ anteiso B); summed feature 9 (iso-$C_{17:0}$ *ω9*c and/or $C_{16:0}$ 10-methyl). Results from the AC 1:10 medium are from this study.

	1	2			3		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Medium / Fatty acid	AC 1:10	AC 1:10	DSMZ medium	R2A	AC 1:10	R2A	R2A	R2A	R2A	TSA 10 %	R2A	R2A	R2A	TSA 10 %	R2A	R2A	R2A	R2A	R2A	R2A	R2A	R2A	R2A	R2A	R2A	R2A
iso-$C_{13:0}$	2.2	2.2	2.2	1.9	1.8	1.2	2.2	–	3.1	2.3	–	–	–	–	–	–	–	–	–	2.6	–	2.2	–	1.9	1.9	2.4
iso-$C_{13:0}$ 3-OH	–	–	–	–	–	–	–	–	–	–	–	–	3	–	–	–	–	–	–	–	–	–	–	–	–	–
$C_{14:0}$	–	0.5	–	0.7	0.4	0.5	–	1.5	1	–	0.5	0.3	–	1.3	2.36	1.6	–	–	1.6	1	1	–	–	–	1.5	1.3
iso-$C_{14:0}$	–	0.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
$C_{15:0}$	0.9	0.5	–	–	0.9	–	–	–	–	–	0.3	0.3	–	–	–	2.7	–	–	–	–	–	–	–	–	–	–
iso-$C_{15:0}$	8	10.7	9.3	7.2	9.4	7.5	14.4	8.4	14.9	5.4	5.7	2.6	4.8	9.5	6.67	15.1	9.5	9.9	13.3	4.2	11.6	3.3	17.3	9.3	5.2	13.5
anteiso-$C_{15:0}$	2.7	2.3	2.6	1.5	3.5	2.1	4.6	3.8	2.8	–	–	–	3.5	1.3	1.9	1.9	1.2	2.1	2.9	1	2.5	–	1.2	1.8	1.6	2.9
anteiso-$C_{15:0}$ 3-OH	–	–	–	–	–	–	–	–	–	–	1.1	0.9	–	2.6	–	–	–	–	–	–	–	–	–	–	–	–
iso-$C_{15:0}$ 3-OH	2.9	2.6	3.4	2.7	3.4	3.4	3.3	2.8	12	3.2	2.3	2.5	3.2	–	1.9	–	2.9	2.8	1.8	3.3	3.3	–	4.2	1.6	1.9	2.1
$C_{16:0}$	2	4.5	3.6	6.7	3.34	8.8	–	7.1	4.9	3.9	7	7.3	10.7	8.8	13.14	5.2	6.3	4.8	8.4	10.6	3.6	9.3	18.8	3	12.5	11.5
iso-$C_{16:0}$	0.8	0.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
$C_{16:1\ \omega 5c}$	27	23.6	22.2	17.3	26	19	24.9	18.4	29.6	23.8	21.3	19.1	16.5	18.5	22.3	33.4	24	30	23.1	21.4	27.7	21.9	12.7	24.9	20.4	23.4
$C_{16:0}$ 3–OH	1.4	1.9	2.2	3.3	2	2.9	–	1.7	0.7	2.4	4.5	3.5	1.9	2.8	1.5	–	2.7	2	1.5	3.6	3.2	–	4.2	1.2	1.2	1.5
iso-$C_{16:0}$ 3-OH	–	–	–	–	0.8	1	–	–	–	–	0.7	–	–	–	–	–	–	–	–	–	–	–	1.2	–	–	–
iso-$C_{17:0}$	–	0.5	–	–	0.5	0.8	–	–	0.3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
anteiso-$C_{17:0}$	–	–	–	–	–	–	1.3	–	–	–	–	–	0.9	–	–	–	–	–	–	–	–	–	–	–	–	–
$C_{17:0}$ 2–OH	1	0.6	1	–	1	0.9	–	1	–	–	–	–	1	–	–	–	–	–	–	–	–	–	–	–	–	–
iso-$C_{17:1\ \omega 9c}$	1.5	1.1	1.2	–	1.1	–	–		0.4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
iso-$C_{17:0}$ 3-OH	5.99	6.9	8.6	7.1	6.5	8.2	–	8.6	1.3	6.2	6.1	4.7	7.4	3.5	8.1	3.2	4.9	8.2	6.1	4.9	6.6	–	8.5	4.4	4.5	7.2
$C_{18:0}$	–	–	–	–	–	–	–	3.1	–	–	–	–	–	1.2	–	–	3.6	–	4.8	–	–	1.8	–	2	6.9	4.3
$C_{18:1\ \omega 9c}$	–	–	–	–	–	–	–	2	–	–	–	–	2.8	1.5	–	–	–	–	–	–	–	–	–	–	1.1	–
summed feature 3	42.5	39.8	42.4	51.8	37.7	43.4	28.7	33.9	36.9	49.3	42.9	45	24.6	45.6	30.8	–	33.3	38.2	30.1	42.1	36.4	33	28.4	41	39.1	28
summed feature 4	0.9	0.6	–	–	–	–	1.2	1.1	–	–	–	–	1.7	–	–	33.5	9.3	–	–	–	2.7	–	–	–	–	–
summed feature 9	–	–	–	–	–	0.5	2.5	1.4	–	–	–	–	1.9	–	–	–	–	–	–	–	1.9	–	–	–	–	–

Table S2. Comparison between strain HA7^T polar lipid profiles with nearest *Spirosoma* species. (Abbreviations: phosphatidylethanolamine (PE); glycolipid (GL); phospholipid (PL); aminolipid (AL); unknown aminophospholipids (PNL/APL) and unidentified lipid (L)). #, data from present study.

HA7^T and nearest species	**PE**	**GL**	**PL**	**AL**	**PNL/ APL**	**L**	**Reference**
HA7^T	+	+	+	+	+	+	#
S. linguale	+	-	-	+	-	+	[1]
S. fluviale	+	-	-	-	+	+	[2]
S. aerophilum	+	-	-	+	+	+	[3]
S. pulveris	+	-	+	+	+	+	[4]
S. lituiforme	+	+	+	+	+	+	[5]
S. radiotolerans	+	-	+	+	+	+	[6]
S. humi	+	-	+	+	+	+	[7]
S. endophyticum	+	+	-	+	+	+	[8]
S. flavum	+	-	+	+	+	+	[9]
S. arcticum	+	-	-	+	-	+	[10]
S. rigui	+	-	-	+	-	+	[11]

Table S3. 16S rRNA gene similarities (EzBioCloud) of HA7^{T}with other *Spirosoma* species (March 2018)

Available species in *Spirosoma* genera	**Accession number**	**similarity (%) with HA7^T (EzTaxon)**	**Reference**
S. linguale	CP001769	97.4	[1]
S. fluviale	LC019141	97.4	[2]
S. pulveris	KP974819	96.9	[4]
S. endophyticum	GQ342559	96.4	[8]
S. aerophilum	KR024033	96.1	[3]
S. radiotolerans	CP010429	95.8	[6]
S. rigui	CP020105	94.4	[11]
S. spitsbergense	ARFD01000223	93.4	[12]
S. luteum	ARFC01000063	93.3	[12]
S. luteolum	KU758884	92.8	[13]
S. panaciterrae	ARFA01000077	92.1	[14]
S. swuense	KU865688	92.1	[15]
S. soli	KT347096	91.9	[16]
S. aerolatum	CP020104	91.8	[17]
S. lacussanchae	KX580025	91.8	[18]
S. arcticum	KF999686	91.4	[10]
S. fluminis	LC148305	90.7	[19]
S. knui	LC148303	90.4	[20]
S. oryzae	KJ155688	90.2	[21]

Table S4. PIFAR- annotated genes against HA7^T draft genome.

Gene annotated	% observed
Multidrug resistance genes	53.3
Metabolism of compounds involved in the proliferation of bacteria inside the plant	11.1
Plant cell wall-degrading enzyme	8.9
Detoxification compounds	5.6
Antibiotics	4.4
Phytotoxic compounds	3.3
Phytohormones	2.2
Exopolysaccharides	2.2
Volatile compounds	2.2
Microbe-associated molecular patterns	2.2
Proteases	1.1
Bacterial lipopolysaccharides	1.1
Factors involved in biofilm formation	1.1
Type III effector proteins	1.1

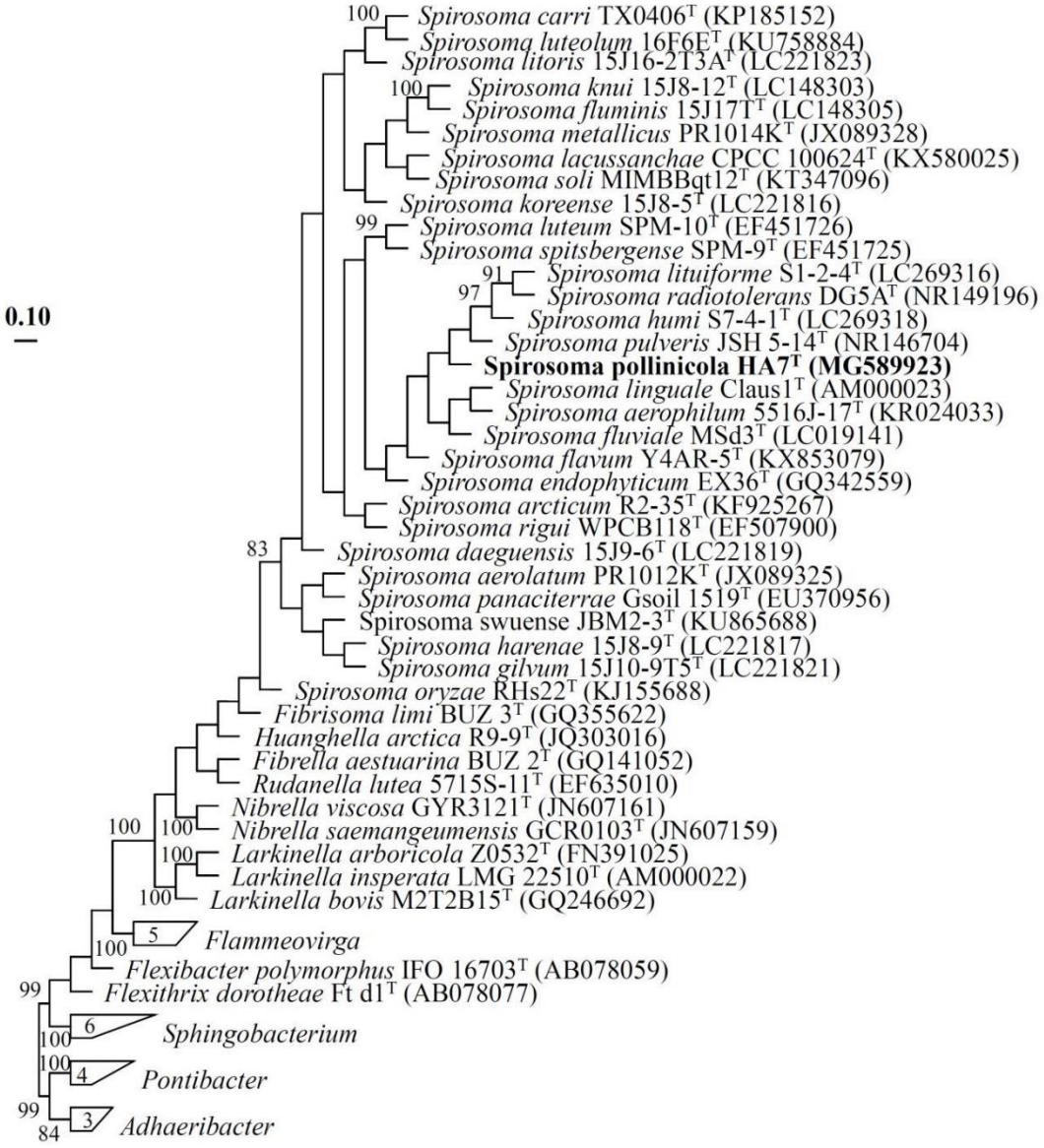


Fig S2. Maximum-parsimony phylogenetic tree based on 16S rRNA gene sequences for the strain HA7^T, members of the recognized genus *Spirosoma* and some members of the family *Cytophagaceae*. Type strains *of Pontibacter, Adhaeribacter, Sphingobacterium, Flammeovirga, Flexibacter and Flexithrix* were used to root the tree. Bootstrap percentages (based on 1000 replicates) are showed at nodes. Bar, 0.10 substitutions per nucleotide position. Only bootstrap percentages greater than 80 are shown.

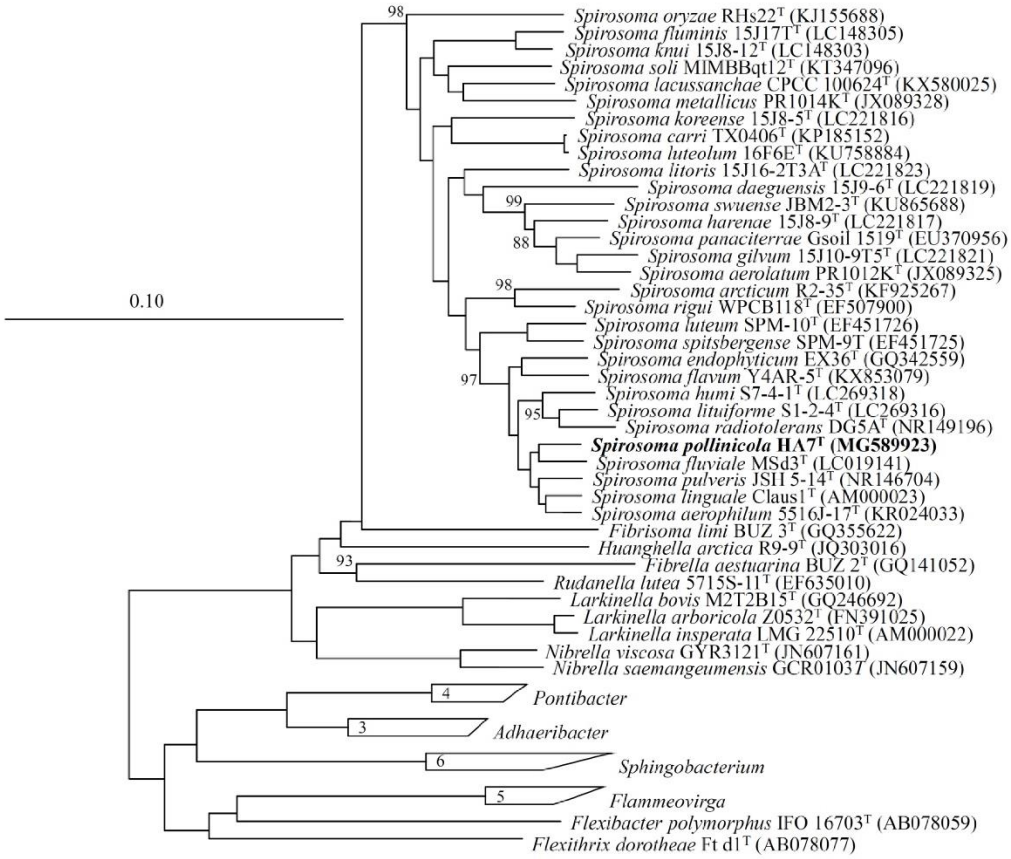


Fig S3. Neighbour-joining tree based on 16S rRNA gene sequences for the strain HA7^T, members of the recognized genus *Spirosoma* and some members of the family *Cytophagaceae*. Type strains *of Pontibacter, Adhaeribacter, Sphingobacterium, Flammeovirga, Flexibacter and Flexithrix* were used to root the tree. A termini filter between positions 71 and 1354 (*Escherichia coli* numbering) of the 16S rRNA gene sequences was used. Bootstrap percentages (based on 1000 replicates) are showed at nodes. Bar, 0.10 substitutions per nucleotide position. Only bootstrap percentages greater than 80 are shown.

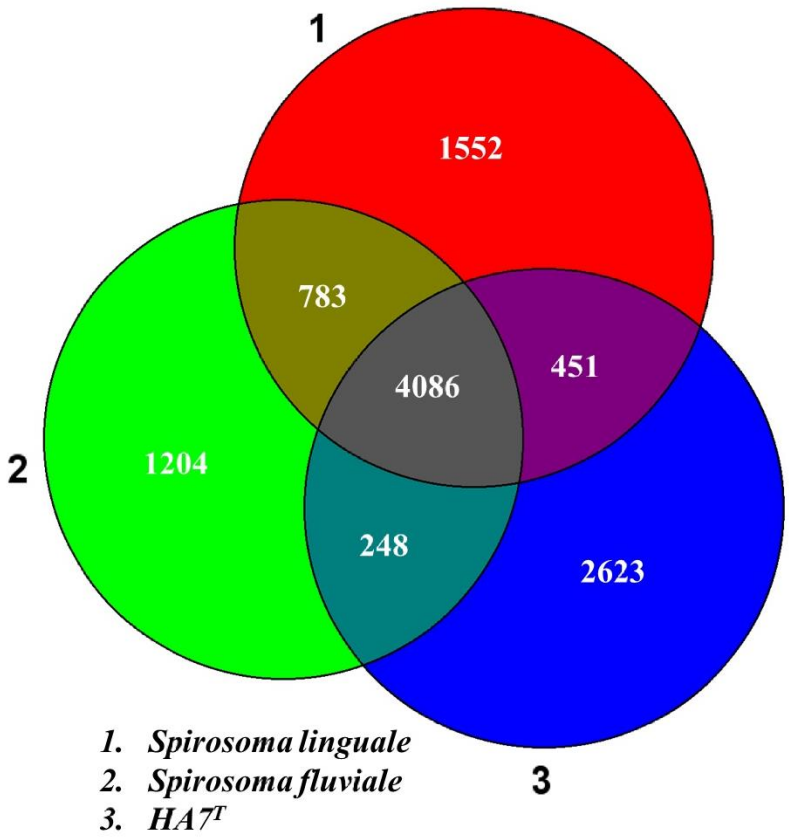


Fig S4. Venn diagram showing the coding sequences of the draft genome shared between HA7^T, *S. linguale* Claus1^T and *S. fluviale* MSd3^T.

References

[1] **Lail K, Sikorski J, Saunders E, Lapidus A, Glavina Del Rio T *et al.*** Complete genome sequence of *Spirosoma linguale* type strain (1), *Standards in Genomic Sciences* 2010;2:176–185.

[2] **Hatayama K, Kuno T.** *Spirosoma fluviale* sp. nov., isolated from river water, *Int J Syst Evol Microbiol* 2015;65:3447–3450.

[3] **Kim S-J, Ahn J-H, Weon H-Y, Hong S-B, Seok S-J *et al.*** *Spirosoma aerophilum* sp. nov., isolated from an air sample, *Int J Syst Evol Microbiol* 2016;66:2342–2346.

[4] **Joo ES, Lee J-J, Cha S, Jheong W, Seo T *et al.*** *Spirosoma pulveris* sp. nov., a bacterium isolated from a dust sample collected at Chungnam province, South Korea, *J Microbiol* 2015;53:750–755.

[5] **Li W, Lee S-Y, Park S, Kim B-O, Ten LN *et al.*** *Spirosoma lituiforme* sp. nov., isolated from soil, *J Microbiol* 2017;55:856–861.

[6] **Lee J-J, Srinivasan S, Lim S, Joe M, Im S *et al.*** *Spirosoma radiotolerans* sp. nov., a gamma-radiation-resistant bacterium isolated from gamma ray-irradiated soil, *Curr Microbiol* 2014;69:286–291.

[7] **Weilan L, Lee J-J, Lee S-Y, Park S, Ten LN *et al.*** *Spirosoma humi* sp. nov., Isolated from Soil in South Korea, *Curr Microbiol* 2017.

[8] **Fries J, Pfeiffer S, Kuffner M, Sessitsch A.** *Spirosoma endophyticum* sp. nov., isolated from Zn- and Cd-accumulating Salix caprea, *Int J Syst Evol Microbiol* 2013;63:4586–4590.

[9] **Zou R, Zhang Y, Zhou X, Wang Y, Peng F.** *Spirosoma flavum* sp nov., isolated from Arctic tundra soil, *Int J Syst Evol Microbiol* 2017;67:4911–4916.

[10] **Chang X, Jiang F, Wang T, Kan W, Qu Z *et al.*** *Spirosoma arcticum* sp. nov., isolated from high Arctic glacial till, *Int J Syst Evol Microbiol* 2014;64:2233–2237.

[11] **Baik KS, Kim MS, Park SC, Lee DW, Lee SD *et al.*** *Spirosoma rigui* sp. nov., isolated from fresh water, *Int J Syst Evol Microbiol* 2007;57:2870–2873.

[12] **Finster KW, Herbert RA, Lomstein BA.** *Spirosoma spitsbergense* sp. nov. and *Spirosoma luteum* sp. nov., isolated from a high Arctic permafrost soil, and emended description of the genus Spirosoma, *Int J Syst Evol Microbiol* 2009;59:839–844.

[13] **Lee J-J, Park S-J, Lee Y-H, Lee S-Y, Park S *et al.*** *Spirosoma luteolum* sp. nov. isolated from water, *J Microbiol* 2017;55:247–252.

[14] **Ten LN, Xu J-L, Jin F-X, Im W-T, Oh H-M *et al.*** Spirosoma panaciterrae sp. nov., isolated from soil, *Int J Syst Evol Microbiol* 2009;59:331–335.

[15] **Joo ES, Kim EB, Jeon SH, Srinivasan S, Kim MK.** *Spirosoma swuense* sp nov., isolated from wet soil, *Int J Syst Evol Microbiol* 2017;67:532–536.

[16] **Yang SS, Tang K, Zhang X, Wang J, Wang X *et al.*** *Spirosoma soli* sp nova, isolated from biological soil crusts, *Int J Syst Evol Microbiol* 2016;66:5568–5574.

[17] **Kim D-U, Lee H, Kim S-G, Ahn J-H, Yoon Park S *et al.*** *Spirosoma aerolatum* sp. nov., isolated from a motor car air conditioning system, *Int J Syst Evol Microbiol* 2015;65:4003–4007.

[18] **Li Y, Ai M-J, Sun Y, Zhang Y-Q, Zhang J-Q.** *Spirosoma lacussanchae* sp nov., a phosphate-solubilizing bacterium isolated from a freshwater reservoir, *Int J Syst Evol Microbiol* 2017;67:3144–3149.

[19] **Lee J-J, Lee YH, Park SJ, Lim S, Jeong S-W *et al.*** *Spirosoma fluminis* sp. nov., a Gamma-Radiation Resistant Bacterium Isolated from Sediment of the Han River in South Korea, *Curr Microbiol* 2016;73:689–695.

[20] **Lee J-J, Lee Y-H, Park S-J, Lee S-Y, Kim B-O *et al.*** *Spirosoma knui* sp. nov., a radiation-resistant bacterium isolated from the Han River, *Int J Syst Evol Microbiol* 2017;67:1359–1365.

[21] **Ahn J-H, Weon H-Y, Kim S-J, Hong S-B, Seok S-J *et al.*** Spirosoma oryzae sp. nov., isolated from rice soil and emended description of the genus Spirosoma, *Int J Syst Evol Microbiol* 2014;64:3230–3234.

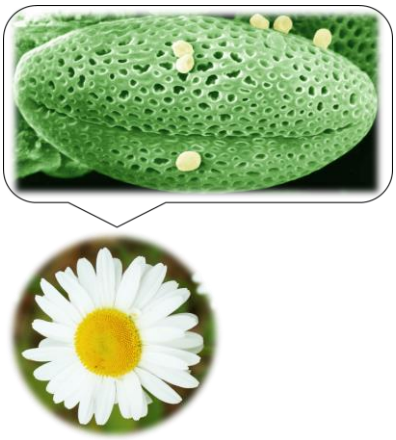
[22] **Elderiny N, Ten LN, Lee J-J, Lee S-Y, Park S *et al.*** *Spirosoma daeguensis* sp. nov., isolated from beach soil, *J Microbiol* 2017;55:678–683.

[23] **Kim D-U, Lee H, Lee S, Park S, Yoon J-H *et al.*** *Spirosoma carri* sp. nov., isolated from an automobile air conditioning system, *Int J Syst Evol Microbiol* 2017;67:4195–4199.

[24] **Lee J-J, Elderiny N, Lee S-Y, Sung Lee D, Kyum Kim M *et al.*** *Spirosoma gilvum* sp. nov., Isolated from Beach Soil, *Curr. Microbiol.* 2017.

[25] **Lee H, Kim D-U, Lee S, Park S, Yoon J-H *et al.*** *Spirosoma metallicus* sp. nov., isolated from an automobile air conditioning system, *J Microbiol* 2017;55:673–677.

[26] **Ten LN, Elderiny N, Lee J-J, Lee S-Y, Park S *et al.*** *Spirosoma harenae* sp. nov., a Bacterium Isolated from a Sandy Beach, *Curr Microbiol* 2017.



Chapter 6

[Manuscript prepared]

Ambika Manirajan, B., Ratering, S., Rusch, V., Schwiertz, A., Geissler-Plaum, R., Cardinale, M., Schnell, S. Bacterial species associated with highly allergenic pollen habitat yield a high level of endotoxin.

Bacterial species associated with highly allergenic pollen habitat yield a high level of endotoxin

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Abstract

The prevalence of the sensitization to pollen allergens has been seen increased in Europe every year. Most studies in this field are related to climate change, phenology, allergens associated with different pollen and allergic disorders. As a plant microhabitat, pollen is also colonized by diverse microorganisms including bacteria. Hence, our hypothesis was that some bacterial species inhabiting in the pollen microhabitat might be producing endotoxins and thereby playing a major role in pollinosis. Here we compared morphologically different bacterial isolates from nine different pollen species (four wind–pollinated high-allergic, four insect–pollinated low-allergic and one wind–pollinated low-allergic). The concertation of endotoxins (lipoteichoic acids and lipopolysaccharides) was measured directly from the pollen samples as well as from the bacterial isolates, and was analysed according to the high allergic and low allergic groups. High-allergenic pollen showed a significantly higher level of bacterial endotoxins; interestingly, also in the bacterial isolates from high-allergenic pollen the endotoxins level was significantly higher compared to bacteria from low-allergenic pollen. Moreover, the bacterial lipopolysaccharide concentration across the nine different pollen species positively correlated with the lipopolysaccharide concentration across their corresponding bacterial isolates. This study suggests a role of bacteria and bacterial endotoxins in the pollen allergy.

Keywords:

Pollen bacteria, endotoxin, lipopolysaccharide, lipoteichoic acid, ELISA**.**

Introduction

Pollen allergy is a major public health problem which has been found regularly increasing (Platts-Mills *et al.*, 2015). In Europe, the extent of pollen allergy is estimated in around forty percent of the population (D'Amato *et al.*, 2007), while the economic impact of allergic diseases is between 55 and 151 billion euros per year (Zuberbier *et al.*, 2014). Pollen grains from many plants like grasses, weeds and trees are recognized as liable for pollinosis (Emberlin 1997; D'Amato *et al.*, 2007). Increased number of pollen count in the environment, changes in the weather and pollution are the possible reasons for increasing pollinosis incidence (Javier *et al.* 2015). Plant pollen grains carry a diverse bacterial population and this includes a number of Gram-positive and -negative species (Ambika Manirajan *et al.*, 2016). Colldahl and Nilsson (1973) initially reported the possible influence of the co-existent microorganisms in pollen allergy. Moreover, bacterial endotoxins associated with pollen grains were shown to play a major role in pollinosis (Spiewak *et al.*, 1996).

Endotoxins have been associated in the pathogenesis of a variety of different clinical conditions. The study of endotoxins started since the 19th century by Richard Pfeiffer and the term 'endotoxin' was given for the heat stable toxin found from heat inactivated *Vibrio cholera* (Bayston and Cochen, 1990). Lipoteichoic acid (LTA) and lipopolysaccharide (LPS) are the major endotoxins produced by Gram-positive and Gram-negative bacterial species, respectively, which trigger host immune responses (Dai *et al.*, 2014; Parolia *et al.*, 2014; Rosenfeld *et al.*, 2006). Endotoxin of Gram-negative bacteria (LPS) consists of a hydrophilic heteropolysaccharide part and a covalently linked hydrophobic lipid portion anchored in the outer membrane. The LPS molecule commonly consists of three structural components: lipid A, a non-repeating core oligosaccharide, and the polysaccharide O-antigen (Raetz and Whitfield, 2002; Bayston and Cochen, 1990; Rietschel *et al*., 1982). The hydrophobic region

consists of lipid A portion which is responsible for the toxic biological effect (Steimle *et al.*, 2016; Watson and Kim, 1963), while the O-antigen is responsible for the immunological response (Mäkelä *et al*., 1973). Lipoteichoic acids are commonly composed of hydrophilic repetitive glycerophosphate units and D-alanine or hexose substituents as well as a lipophilic glycolipid anchor (Schneewind and Missiakas, 2014; Morath *et al.*, 2005). The glycolipid anchors of LTA are the crucial molecules that trigger the immunity (Jang *et al.*, 2011).

Mostly the sources of allergic pollen are the wind-pollinated plants (ECARF, 2016). The morphological characters of wind-pollinated pollen and its exine are different from other types of pollen grains (Asam *et al*., 2015; Schwendemann *et al*., 2007). Our previous studies reported that the bacterial community structure and diversity associated with pollen microhabitat were clearly affected by pollination type, as analysed by both cultivation-dependent and –independent methods (Ambika Manirajan *et al.* 2016; 2018). We also observed the presence of bacterial hub species and the microbiome interactions in the pollen habitat.

In this study, we aimed to investigate the possible relationships of bacterial endotoxins associated with both the pollen and the respective bacterial isolates in pollen allergy. The objectives were, (i) to compare endotoxin level in pollen grains between four allergic and five non-allergic pollen species; (ii) to compare the endotoxin level of bacterial isolates from allergic and non-allergic pollen species; and (iii) to correlate the endotoxin levels found in different allergic and non-allergic pollen species with that of the bacterial isolates from these pollen species.

Materials and methods

Nine different plants, including four allergic [birch (*Betula pendula* Roth.), winter rye (*Secale cereale* L.), common hazel (*Corylus avellana* L.) and common mugwort (*Artemisia vulgaris*

L.)] and five non-allergic [autumn crocus (*Colchicum autumnale* L.), winter rapeseed (*Brassica napus* L.), blackthorn (*Prunus spinosa* L.), cherry plum (*Prunus cerasifera* Ehrh.) and Hemp (*Cannabis sativa* L.)] species, were selected for pollen sampling. Flowers were collected from the Giessen district (Hessen, Germany) (for details, see Ambika Manirajan *et al*., 2018). The pollen grains were then extracted by shaking, as described in Ambika Manirajan *et al.* (2016, 2018).

The pollen samples were shaked each in 5 ml of shaking solution (0.05% Tween 80 and 0.18% $Na_4P_2O_7$; Musovic *et al*., 2006) for 30 min and then serially diluted using 0.02% Tween 80 + 0.085% NaCl to a dilution of 10^{-5}. This was followed by plating of 100 µl of each dilution were plated onto 1:10 diluted AC agar medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), as well as in pollen enriched minimal salt medium (Ambika Manirajan *et al.*, 2016). These plates were incubated for five days at 25ºC in aerobic culture. Total colony forming units (CFUs) per gram of each species were calculated and compared between allergic and non-allergic pollen species using Student's t-test (Gosset, 1908). Morphologically different colonies were sub-cultured from single colonies and pure cultures were prepared. Genomic DNA of these pure bacterial cultures were isolated using the NucleoSpin DNA isolation kit (MACHEREY NAGEL GmbH & Co. KG, Düren, Germany). Primers EUB9F (5'-GAGTTTGATCMTGGCTCAG-3') and EUB1492R (5'-ACGGYTACCTTGTTACGACTT-3') (Lane, 1991) were used to amplify 16S rRNA gene. The PCR products were further purified using the QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany) and sequenced by LGC genomics (Berlin, Germany). The high quality region of the 16S rRNA gene sequences was used for comparing with the reference sequences by BLAST (Zhang *et al*., 2000) and Ez Taxon (Kim *et al*., 2012) alignment. Bacterial isolates from birch, winter rye, autumn crocus and winter rapeseed were already reported in our previous publication (Ambika Manirajan *et al.*, 2016).

The pollen samples were suspended in sterile pyrogen free water in the proportion of 1 mg ml^{-1}. This was centrifuged and concentrated to OD 50 and used for LPS and LTA quantification analysis. The bacteria isolates were cultivated in liquid AC 1:10 medium. The bacterial liquid cultures were centrifuged, the pellet was collected (OD of each culture were adjusted to 50). It was suspended in 1 ml sterile pyrogen free water and four time dilution was made for avoiding to cross upper standard value in standard curve. Gram-negative and Gram-positive bacterial suspensions (OD of 12.5) were used for LPS and LTA - ELISA test, respectively. Quantification of endotoxin in the pollen sample, as well as in bacterial isolates was done using Lipopolysaccharides (LPS) ELISA kit (Cusabio Biotech, China) and the Lipoteichoic acid (LTA) with ELISA kit (MyBioSource, USA), respectively, following the manufacturer's instructions. A standard solution of LPS (concentrations of 0 to 400 ng mg^{-1}) and LTA (concentration of 0 to 20 ng mg^{-1}) was used to quantify the amounts of each pollen and each bacterial strains in ng ml^{-1}. The LPS - ELISA was performed by adding 100 µl of pollen or Gram-negative bacterial suspension to each well of the microtiter plate togrther with standard and blank, and this was incubated for 2 hours at 37ºC. Liquid of each well were carefully removed by using micropipette after incubation. This was followed by 100 µl of Biotin-antibody added to each well and incubated for 1 hour at 37ºC. Aspirated and washed the wells three times with wash buffer (200 µl each wash) after incubation. Later added 100 µl of HRP-avidin to each well and incubated for 1 hour at 37ºC. Repeated the washing step again five times, added 90 µl of TMB substrate to each well after the wash, and incubated 15-30 minutes at 37ºC in dark. After incubation added 50 µl of stop solution to each well with gentle taping the plate and determined the OD using 450 nm. The LTA-ELISA was performed by adding 50 µl of standard, samples (pollen and Gram-positive isolates suspension) and blank in respective wells and immediately added 50 µl of Detection A working solution to each well. This was incubated for 1 hour at 37ºC. This was followed by

aspirated and washed the wells three times with wash buffer (400 µl each wash). Added 100 µl of Detection B working solution in each washed wells and incubated for for 45 minutes at 37ºC. Repeated the washing step five times, added 90 µl of substrate in each well after washing and incubated for for 15-30 minutes at 37ºC in dark. After incubation added 50 µl of stop solution to each well, gently taped the plate and determined the OD using 450 nm. A standard curve with four parameter logistic curve-fit was created to calculate the concentration of LPS and LTA. The mean LPS concentration of pollen as well as Gram-negative and LTA concentration of pollen as well as Gram-positive bacterial strins were compared between high allergenic and low allergenic plant pollen species. The Spearman's correlation and Pearson correlation (*r*-value) between endotoxin concentrations of nine different pollen and the mean endotoxin concentrations of all isolates from nine different pollen species respectively were calculated using the software SPSS version 22 (SPSS Inc., Chicago, IL).

Results

Isolation and characterization of bacteria from pollen

A total of 157 morphologically different bacteria were isolated from nine different plant pollen species [bacterial isolates: 18 from winter rye, 16 from birch, 15 from winter rapeseed and 12 from autumn crocus used in work were from Ambika Manirajan *et al*. (2016)] including 62 Gram-positive and 95 Gram-negative isolates (Table S1). The total count of pollen bacterial populations ranged from 3.8×10^5 CFU g^{-1} (in hazel) to 8.5×10^8 CFU g^{-1} (in blackthorn). The trend of CFUs showed that the high-allergenic pollen (birch, winter rye, mugwort and hazel) have lower CFU numbers than low-allergenic plants (winter rapeseed, autumn crocus, cherry plum, blackthorn and hemp). The numbers of CFUs in different plant pollen species were found significantly different from each other (Kruskal-Wallis test,

p<0.003) (Fig. 1) Moreover, the bacterial CFU values between high allergenic and low allergenic plant pollen species also show significant differences (Student's t-test, *p*=0.02).

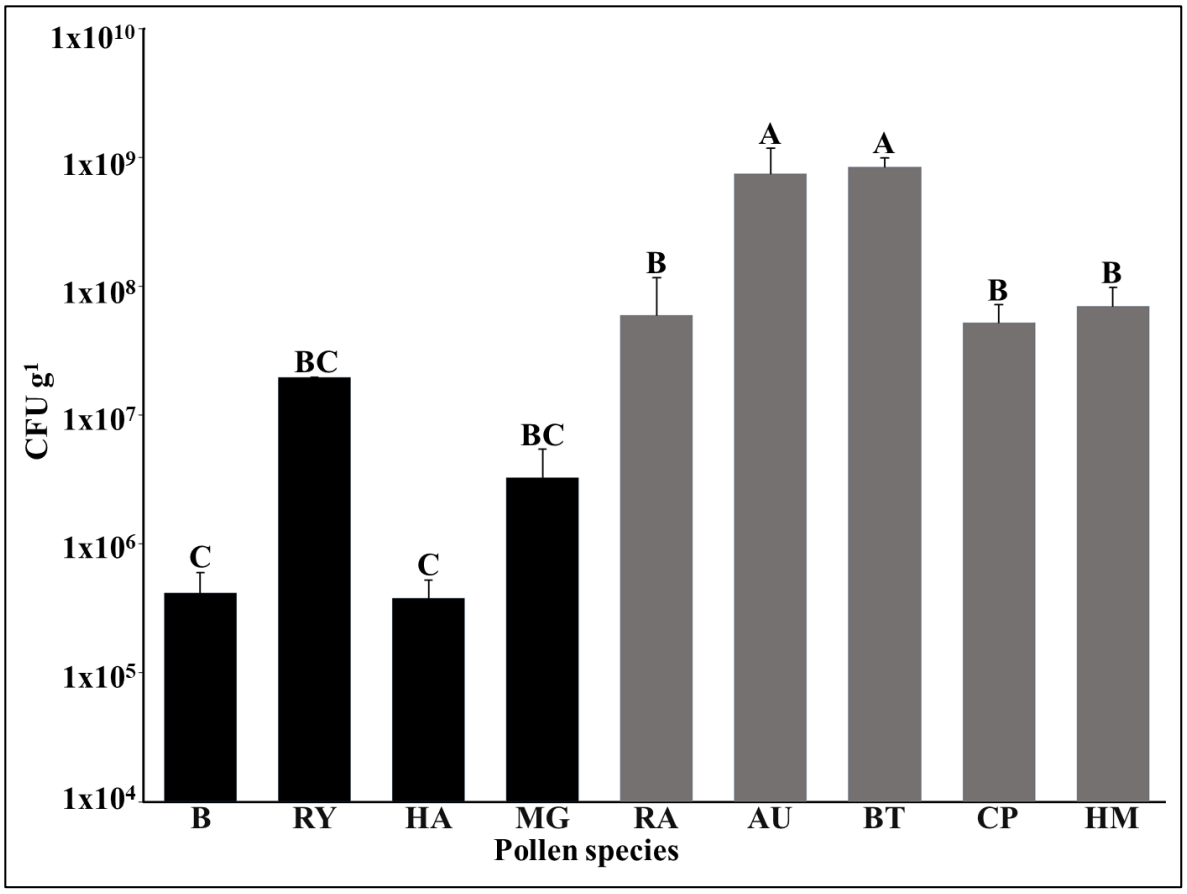

Fig 1. Numbers of cultivable bacteria on flower pollen (CFUs per gram of dry pollen weight). The values are means of three samples per pollen species. Kruskal-Wallis test, *p*=0.003 for the factor 'species'; Student's t-test, *p*=0.02 for the factor 'allergenic potential'. Different letters indicate significantly different means (Tukey test, *p*< 0.05). B=birch; RY=winter rye; HA=hazel; MG=mugwort; RA=winter rapeseed; AU=autumn crocus; BT=blackthorn; CP=cherry plum; HM=hemp. High-allergenic pollen species=black; low-allergenic pollen species=grey. Data of B, RY, HA and MG from Ambika Manirajan *et al*. (2017)

Endotoxin quantitative determination assay

The results of LPS-ELISA quantitative determination assay of high allergenic and low allergenic pollen samples showed significantly higher LPS concentration in high allergenic pollen samples (*t*-test, p<0.0001). Mugwort pollen samples exhibited the highest LPS quantities while the autumn crocus had the least. Moreover, hemp pollen samples had a medium level of LPS concentration (Fig 2A). The result of LPS-ELISA quantitative

determination assay between the different bacteria isolated from high allergenic and low allergenic pollen samples, revealed significantly high LPS concentrations in bacterial isolates from high allergenic pollen samples (*t*-test, $p<0.0001$) (Fig 2B).

The results of LTA-ELISA quantitative determination assay between high allergenic and low allergenic pollen samples showed significantly high LTA concentration in high allergenic pollen samples (*t*-test, $p<0.0001$). Birch pollen samples accounted for the highest LTA quantity and the cherry plum pollen samples with the least LTA quantity. Moreover, hemp pollen showed a level of LTA concentration slightly higher than the other low-allergic pollen species (Fig 2C). The result of LTA-ELISA quantitative determination assay between the different bacterial species isolated from high allergenic and low allergenic pollen samples showed significantly higher LTA concentrations in bacterial species, isolated from high allergenic pollen samples (*t*-test, $p=0.06$) (Fig 2D). Even though the five isolates (*Clavibacter michiganensis, Brevibacterium frigoritolerans, Agreia pratensis, Amnibacterium soli* and *Frondihabitans sucicola*) from low-allergenic pollen samples showed higher value in LTA-ELISA, very little reads were found in high-throughput sequencing in the specific pollen species corresponding to the genus of these isolates (Ambika Manirajan et al. 2018). Moreover, out of the ten isolates from high allergic pollen samples, eight isolates [*Arthrobacter oryzae, Bacillus simplex, Clavibacter michiganensis, Rathayibacter festucae, Amnibacterium kyonggiense, Bacillus aerophilus* and *Bacillus safensis* (two isolates)] showed high LTA results and were found to have comparatively high number of reads in high-throughput sequencing in the specific pollen species corresponding to the genus of these isolates.

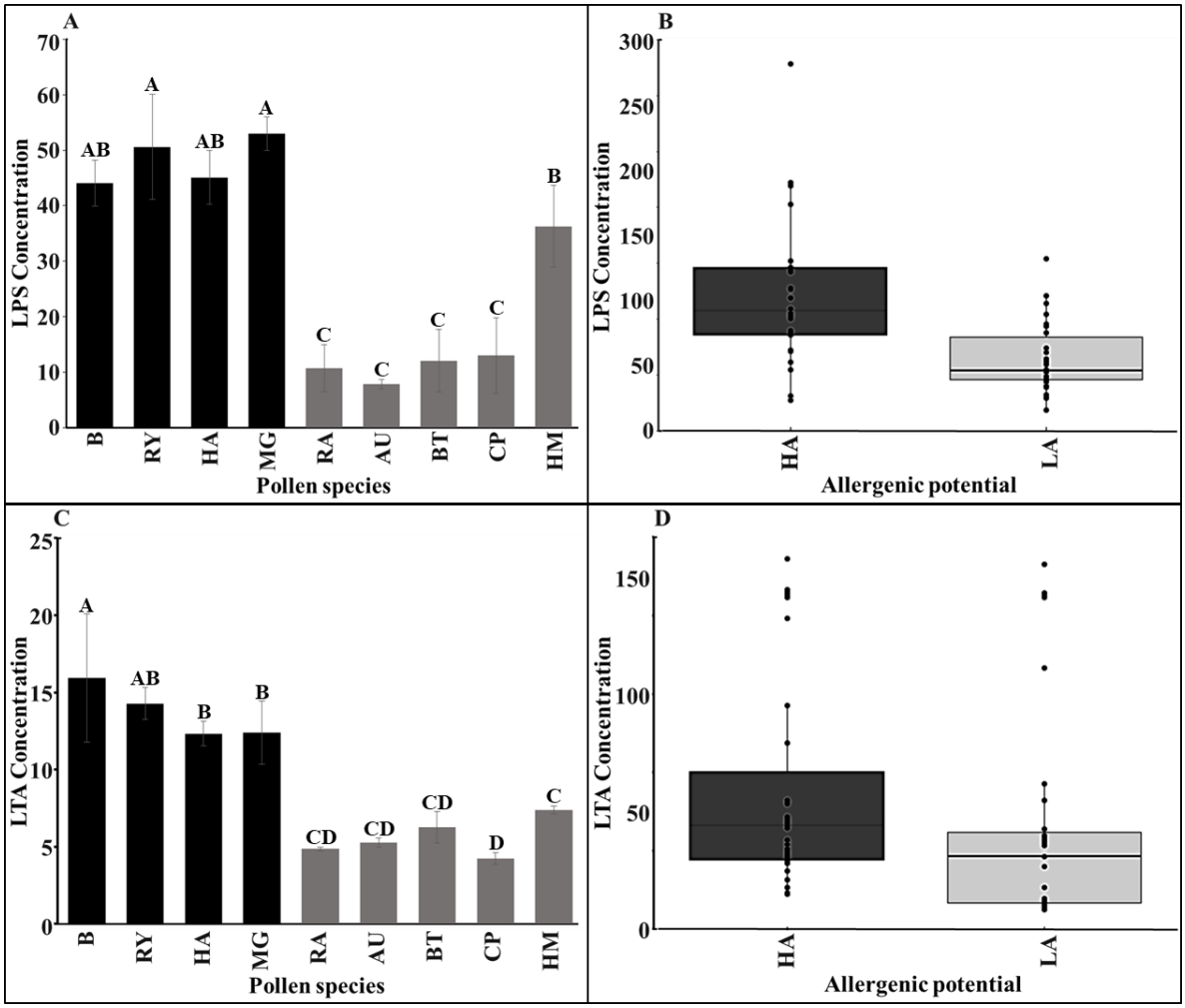

Fig 2. (A) LPS-ELISA quantitative determination assay (ng ml^{-1}) of different pollen species (between allergenic potential: *t*-test, p<0.0001). Different letters indicate significantly different means (Tukey test, $p< 0.05$). (B) Mean of LPS quantity observed in bacterial strains from high allergenic (HA) and bacterial strains from low allergenic (LA) pollen species (*t*-test, p<0.0001). (C) LTA-ELISA quantitative determination assay (ng ml^{-1}) of different pollen species (between allergenic potential: *t*-test, p<0.0001). (D) Mean of LTA quantity observed in bacterial strains from high allergenic (HA) and bacterial strains from low allergenic (LA) pollen species (*t*-test, p<0.06). B=birch; RY=winter rye; HA=hazel; MG=mugwort; RA=winter rapeseed; AU=autumn crocus; BT=blackthorn; CP=cherry plum; HM=hemp. HA= high allergenic pollen; LA= low allergenic pollen; high-allergenic plant pollen species=black; low-allergenic plant pollen species=grey.

Correlation of endotoxin concentration between pollen and isolates

The LPS concentration of nine different pollen species were positively correlated with the mean LPS concentration of all Gram-negative bacterial isolates from this respective pollen species (Spearman R=0.83, *p*=0.005; Pearson R=0.85, *p*=0.004) (Fig 3). No correlation was

found between LTA concentrations from nine different pollen species and the corresponding bacterial isolates analyzed.

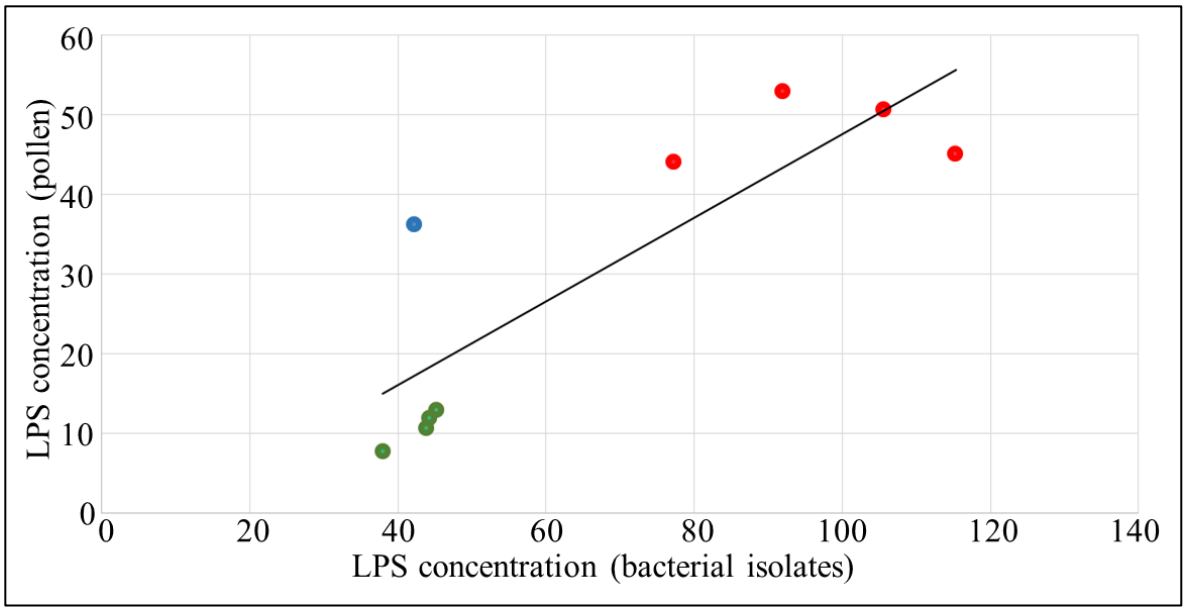

Fig 3. Correlation between LPS concentrations from pollen species and the mean LPS of bacterial isolates from this pollen (Spearman R=0.83, *p*=0.005; Pearson R=0.85, *p*=0.004). Anemophilus high-allergenic–red; entomophilus low-allergenic–green; anemophilus low-allergenic–blue.

Discussion

In regards to the increasing number of pollen related allergenic incidence all over the world, subsequent studies about pollen associated bacteria are of high importance. In this study, we tried to compare the clinically important molecules such as LTA and LPS associated with Gram-positive and negative bacteria from high allergenic as well as low allergenic pollen habitat.

A mixed bacterial flora, consisting of Gram-negative and Gram-positive bacteria, contribute to the endotoxin level of different plant pollen species. Quantification of endotoxin from the pollen samples and the bacterial isolates from pollen provide basic knowledge about the potential role of bacterial species living on pollen in pollen allergy. Endotoxins are the major factors for the pathogenesis of bacterial infection and are known as airborne immunotoxicant

for human (Michel *et al.*, 1991; Michel *et al.*, 1989). Inhalation of bacterial endotoxin causes blood and lung inflammatory reaction, fever and shaking chills (Michel *et al.*, 1997; Sandström *et al.*, 1992; Rylander *et al.*, 1989). So far, very little attention has been given towards the role of pollen bacterial inhabitance in pollen allergy.

A significant variation of CFU numbers of nine different pollen species indicates that the species specific structure and allergenic potential of pollen could affect the size of the bacteria inhabiting pollen. The bacterial cultivable fraction (CFU counts) in high allergenic pollen species was significantly less compared to low allergenic plants. This might be due to morphological difference between high allergenic and low allergenic pollen exine. Morphology of pollen and its exine is mainly correlated with the pollination type (Tanaka *et al.*, 2004). Wind-pollinated pollen grains are usually dry, smooth walled, small in diameter, non-sticky and with limited pollen coat (Schwendemann *et al*., 2007; Shukla *et al*., 1998; Heslop-Harrison, 1979). In contrast, insect pollinated pollen grains are moist, sticky and rough, with abundant pollen coat (Dickinson *et al*., 2000; Shukla *et al*., 1998; Pacini and Franchi, 1996). Watson *et al.* (1977) initially reported the positive relationship between endotoxin concentration and total bacterial count: here we did not observe the same trend.

The LPS-ELISA demonstrated the presence of high level of LPS in high allergenic pollen species, as expected (Spiewak *et al*., 1996). Interestingly, also the Gram-negative bacterial species isolated from high allergenic pollen had a higher amount of LPS quantity compared to the isolates from low allergenic pollen species. Colldahl and Carlsson (1968) first reported that the extracts of bacterial isolates from allergenic pollen induce clinical symptoms (skin reaction test, eye or nasal provocation test) in pollen sensitive patients. Later Spiewak *et al*. (1996) showed that the allergenic plant pollen, as well as Gram-negative bacterial isolated from this pollen, contain high LPS level. The LPS exposure triggers immune responses in bronchial epithelial cells and thus might influence the development of allergic asthma

(Eisenbarth *et al.*, 2002; Braun-Fahrlander *et al.*, 2002). Oteros *et al.* (2018) reported that the *Artemisia* (mugwort) pollen act as vector for airborne bacterial LPS.

Similar to Gram-negative bacteria, Gram-positive bacteria can also trigger the immune response (Georgiev, 2009). Gram-positive bacteria associated and colonized with the grass pollen influences the allergic immune responses during skin prick test in human as well as in cell culture (Heydenreich *et al*., 2012). The LTA-ELISA results of pollen samples revealed the presence of high level of LTA concentration in high-allergenic pollen samples compared to low-allergenic pollen samples. Even though five isolates from low-allergenic pollen exhibited higher LTA concentration, the numbers of reads of these bacterial genera (used as a proxy for their actual abundance) were found very low in high-throughput sequencing from the respective pollen samples (data from Ambika Manirajan et al, 2018). The origin of these five isolates could be contamination with other pollen through wind, insect, pollution, presence of other plant materials, etc. (Hani *et al*., 2012).

Moreover, the LPS quantities between nine different plant pollen species were positively correlated with the average LPS concentration of the bacterial species isolated from respective pollen species. This result indicates that a few bacterial inhabitants in the pollen habitat contribute to the endotoxin quantity level and are influenced by the factor allergenic potential. The level of endotoxin concentration had a significant correlation with the viable number of Gram-negative bacteria present in water-soluble metalworking fluids (Cyprowski *et al.*, 2007), dust of livestock barns and poultry houses (Bakutis *et al.*, 2004), air of wastewater treatment plants (Laitinen *et al.* 1992) and in fresh water (Dawson *et al.*, 1988). Biologically active lipopolysaccharides associated with dust can induce bronchial inflammation and asthma (Park *et al.*, 2001; Rizzo *et al.*, 1997). Jagielo *et al.* (1996) reported that the concentration of endotoxin in the corn dust strongly influences the physiologic and biological response in grain dust causing acute airway injury.

These results determine the presence of high concentration of endotoxins in the pollen of high- allergenic plant pollen species. Moreover, bacterial endotoxins from organic and cotton dust are the major causative agents for the development of immune modulatory reactions like bronchial reactivity including fever, asthma and wheezing etc (Williams *et al.*, 2005; Rylander *et al.*, 1989). Bacterial endotoxin associated with pollen causes airborne respiratory inflammatory effect (Oteros *et al*., 2018). Bacterial compounds together with allergens contribute a major role in allergic immune response (Bublin *et al* 2014; Traidl-Hoffmann *et al.*, 2009).

Here we showed the prevalent occurrence of LPS in different allergenic pollen species, which suggest that the LPS from Gram-negative bacteria might be playing a major role in pollinosis. Further clinical studies are necessary to conclude the activity of LPS and LTA associated with pollen in pollinosis.

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References

Ambika Manirajan, B., Maisinger, C., Ratering, S., Rusch, V., Schwiertz, A., Giessler-Paum, R., Cardinale, M., & Schnell, S. (2018). Diversity, specificity, co-occurrence and hub taxa of the bacterial–fungal pollen microbiome. *FEMS Microbiol Ecol*, *94*. DOI*:10.1093*/femsec/fiy112.

Ambika Manirajan, B., Ratering, S., Rusch, V., Schwiertz, A., Giessler-Paum, R., Cardinale, M., & Schnell, S. (2016). Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity. *Environ Microbiol*, *18*, 5161–5174.

Asam, C., Hofer, H., Wolf, M., Aglas, L., & Wallner, M. (2015). Tree pollen allergens—an update from a molecular perspective. *Allergy*, *70*, 1201–1211.

Bakutis, B., Monstviliene, E., & Januskeviciene, G. (2004). Analyses of airborne contamination with bacteria, endotoxins and dust in livestock barns and poultry houses. *Acta vet Brno*, *73*, 283–289.

Bayston, K.F., & Cohen, J. (1990). Bacterial endotoxin and current concepts in the diagnosis and treatment of endotoxaemia. *J Med Microbiol*, *31*, 73–83.

Bayston, K.F., & Cohen, J. (1990). Bacterial endotoxin and current concepts in the diagnosis and treatment of endotoxaemia. *J Med Microbiol*, *31*, 73–83.

Braun-Fahrlander, C., Riedler, J., Herz, U., Eder, W., Waser, M., Grize, L., Maisch, S., Carr, D., Gerlach, G., Bufe, A., Lauener, R.P., Schierl, R., Renz, H., Nowak, D., & von Mutius, E. (2002). Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med*, *347*, 869–877.

Bublin, M., Eiwegger, T., & Breiteneder, H. (2014). Do lipids influence the allergic sensitization process? *J Allergy Clin Immunol*, *134*, 521–529.

Colldahl, H., & Nilsson, L. (1973). Possible relationship between some allergens (pollen, mites) and certain microorganisms (bacteria and fungi). *Acta Allergol*, *28*, 283–295.

Colldahl, H., & Nilsson, L. (1973). Possible relationship between some allergens (pollens, mites) and certain microorganisms (bacteria and fungi). *Acta Allergologica*, *28*, 283–295.

Colldahl, H., Carlsson, G. (1968). Allergens in pollen. *Acta Allergologia*, *XXIII*, 387–397.

Cyprowski, M., Piotrowska, M., Zakowska, Z., & Szadkowska-Stanczyk, I. (2007). Microbial and endotoxin contamination of water-soluble metalworking fluids. *Int J Occup Med Environ* Health, *20*, 365–371.

D'Amato, G., Cecchi, L., Bonini, S., Nunes, C., Annesi-Maesano, I., Behrendt, H., Liccardi, G., Popov, T., & Van Cauwenberge, P. (2007). Allergenic pollen and pollen allergy in Europe. *Allergy*, *62*, 976–990.

Dai, L., DeFee, M.R., Cao, Y., Wen, J., Wen, X., Noverr, M.C., & Qin, Z. (2014). Lipoteichoic acid (LTA) and lipopolysaccharides (LPS) from periodontal pathogenic bacteria facilitate oncogenic herpesvirus infection within primary oral cells. *PLoS One*, *9*, e101326.

Dawson, M.E., Novitsky, T.J., & Gould, M.J. (1988). Microbes, endotoxins and water. *Pharm Engineering*, *8*, 9–12.

Dickinson, H.G., Elleman, C.J., & Doughty, J. (2000). Pollen coatings: Chimaeric genetics and new functions. *Sex Plant Reprod*, *12*, 302–309.

Eisenbarth, S.C., Piggot, D.A., Huleatt, J.W., Visintin, I., Herrick, C.A., & Bottomly, K. (2002). Lipopolysaccharide-enhanced, Toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med*, *196*, 1645–1651.

Emberlin, J. (1997). Grass, Tree, and Weed Pollen, in: *Allergy and allergic diseases*, Vol. 2, eds. Kay, A.B., Kaplan, A.P., Bousquet, J., & Holt, P.G. Blackwell Science Ltd, Oxford. 942–962.

Emberlin, J. (1998). The effect of air pollution on allergic pollen. *J Europian Resir Rev*, *53*, 164–167.

Georgiev V.S. (2009). *Mammalian Host Defenses: Innate and Adaptive Immunity*. In: Georgiev V.S. (eds) National Institute of Allergy and Infectious Diseases, NIH. Infectious Disease. Humana Press. pp 577–626.

Gosset, W.S (Student). (1908). The probable error of a mean. *Biometrika*, *6*, 1–25.

Hani, B., Dalila, B., Saliha, D., Daoud, H., Mouloud, G., & Seddik, K. (2012). Microbiological sanitary aspects of pollen. *Adv Environ Biol*, *6*,1415–1420.

Heslop-Harrison, J. (1979). Aspects of the structure, cytochemistry and germination of the pollen of rye. *Ann Bot*, *44*, 1–47.

Heydenreich, B., Bellinghausen, I., König, B., Becker, W.-M., Grabbe, S., Petersen, A., & Saloga, J. (2012). Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses. *Clin Exp Allergy*, *42*, 76–84.

Jagielo, P.J., Thorne ,P.S., Watt, J.L., Frees, K.L., Quinn, T.J., & Schwartz, D.A. (1996). Grain dust and endotoxin inhalation challenges produce similar inflammatory responses in normal subjects. Chest, *110*, 263–270.

Jang, K.S., Baik, J.E., Han, S.H., Chung, D.K., & Kim, B. G. (2011). Multi-spectrometric analyses of lipoteichoic acids isolated from *Lactobacillus plantarum*. *Biochem Biophys Res Commun*, 407, 823–830.

Javier, F., Emilio, F., Montserrat, V., Victor, S., & Purificación, G. (2015). Evolution of the incidence of pollen grains and sensitivity to pollen in the city of Elche (Spain). *Asian Pac J Allergy Immunol*, *33*, 196–202.

Kim, O.S., Cho, Y.J., Lee, K., Yoon, S.H., Kim, M., Na, H., Park, S.C., Jeon, Y.S., Lee, J.H., Yi, H., Won, S., & Chun, J. (2012). Introducing EzTaxon-e: A prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. *Int J Syst Evol Microbiol*, *62*, 716–721.

Laitinen, S., Nevalainen, A., Kotimaa, M., Liesivuori, J., Martikainen, P.J. (1992). Relationship between bacterial counts and endotoxin concentrations in the air of wastewater treatment plants. *Appl Environ Microbiol*, *58*, 3774–3776.

Lane, D.J. (1991). 16S/23S rRNA sequencing. *In Nucleic Acid Techniques in Bacterial Systematics*. Stackebrandt, E., and Goodfellow, M. (eds). New York: John Wiley and Sons Inc, pp. 115–175.

Mäkelä, P.H., Valtonen,V.V., & Valtonen, M. (1973). Role of O-antigen (Lipopolysaccharide) factors in the virulence of *Salmonella*. *J Infect Dis*, *128*, 81–85.

Michel, O., Duchateau, J., & Sergysels, R. (1989). Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. *J Appl Physiol*, *66*, 1059–1064.

Michel, O., Ginanni, R., Le Bon, B., & Duchateau, J. (1991). Effect of endotoxin contamination on the antigenic skin test response. *Ann Allergy*, *66*, 39–42.

Michel, O., Nagy, A-M., Schroeven, M., Duchateau, J., Neve, J., Fondu, P., & Sergysel, R. (1997). Dose-response relationship to inhaled endotoxin in normal subjects. *Am J Respir Crit Care Med*, *156*, 1157–1164.

Morath, S., von Aulock, S., & Hartung, T. (2005). Structure/function relationships of lipoteichoic acids. *J Endotoxin Res*, 11, 348–356.

Musovic, S., Oregaard, G., Kroer, N., & Sorensen, S.J. (2006). Cultivation-independent examination of horizontal transfer and host range of an IncP-1 plasmid among Gram-positive and gram-negative bacteria indigenous to the barley rhizosphere. *Appl Environ Microbiol*, *72*, 6687–6692.

Obersteiner, A., Gilles, S., Frank, U., Beck, I., H€aring, F., Erns, D., Rothballer, M., Hartmann, A., Traidl-Hoffmann, C., & Schmid, M. (2016). Pollen-associated microbiome correlates with pollution parameters and the allergenicity of pollen. *Plos One*, *11*, e0149545.

Oteros, J., Bartusel, E., Alessandrini, F., Nünez, A., Moreno, D.A., Bahrendt, H., Schmidt-Weber, C., Traidl-Hoffmann, C., & Buters, J. (2018). *Artemisia* pollen is the mein vector for airborne endotoxin. *J Allergy Clin Immunol*, pii: S0091-6749(18)30999-0.

Pacini, E., & Franchi, G.G. (1996). Some cytological, ecological and evolutionary aspects of pollination. *Acta Soc Bot Pol*, *65*, 11–16.

Park, J.H., Gold, D.R., Spiegelman, D.L., Burge, H.A., & Milton, D.K. (2001). House dust endotoxin and repeated wheeze in the first year of life. *Am J Respir Crit Care Med*, *163*, 322–328.

Parolia, A., Gee, L.S., de Moraes Porto, ICC, & Mohan, M. (2014). Role of cytokines, endotoxins (LPS), and lipoteichoic acid (LTA) in endodontic infection. *J Dent Oral Disord Ther*, *2*, 1–5.

Platts-Mills, A.E., & Thomas, A.E. (2015). The Allergy Epidemics: 1870–2010. *J Allergy Clin* Immunol, *136*, 3–13.

Raetz,C.R., & Whitfield, C. (2002). Lipopolysaccharide endotoxins. *Annu Rev Biochem*, *71*, 635–700.

Rietschel, E.T., Schade, U., Jensen, M., Wollenweber, H-W., Luderitz, O., & Greisman, S.G. (1982). Bacterial endotoxins: chemical structure, biological activity and role in septicaemia. *Scand J Infect Dis*, *31*, 8–21.

Rizzo, M.C., Naspitz, C.K., Fernandez-Caldas, E., Lockey, R.F., Mimica, I., & Sole, D. (1997). Endotoxin exposure and symptoms in asthmatic children. *Pediatr Allergy Immunol, 8*, 121–126.

Rosenfeld, Y., & Shai, Y. (2006) Lipopolysaccharide (endotoxin)-host defense antibacterial peptides interactions: role in bacterial resistance and prevention of sepsis. *Biochim Biophys Acta, 1758*, 1513–1522.

Rylander, R., Bake, B., Fisher, J.J., & Helander, I.M. (1989). Pulmonary function and symptoms after inhalation of endotoxin. *Am Rev Respir Dis*, *140*, 981–986.

Sandström, T., Bjermer, L., & Rylander, R. (1992). Lipopolysaccharide (LPS) inhalation in healthy subjects increases neutrophils, lymphocytes and fibronectin levels in bronchoalveolar lavage fluid. *Eur Respir J*, *5*, 992–996.

Schneewind, O., & Missiakas, D. (2014). Lipoteichoic acids, phosphate-containing polymers in the envelope of Gram-positive bacteria. *J Bacteriol*, *196*, 1133–1142.

Schwendemann, A.B., Wang, G., Mertz, M.L., McWilliams, R.T., Thatcher, S.L., & Osborn, J.M. (2007). Aerodynamics of saccate pollen and its implications for wind pollination. *Am J Bot*, *94*, 1371-1381.

Shukla, A.K., Vijayaraghavan, M.R.., & Chaudhry, B. (1998). "Abiotic pollination". Biology of pollen. *APH Publishing*, New Delhi. pp. 67–69.

Spiewak, R., Skorska, C., Prazmo, Z., & Dutkiewicz, J. (1996). Bacterial endotoxin associated with pollen as a potential factor aggravating pollinosis. *Ann Agric Environ Med*, *3*, 63–66.

Steimle, A., Autenrieth, I.B., & Frick, J-S. (2016). Structure and function: Lipid A modifications in commensals and pathogens. *Int J Med Microbiol, 306*, 290–301.

Tanaka, N., Uehara, K., & Murata, J. (2004). Correlation between pollen morphology and pollination mechanisms in the *Hydrocharitaceae. J Plant Res, 117*, 165–276.

Tanaka, N., Uehara, K., Murata, J. (2004) Correlation between pollen morphology and pollination mechanisms in *Hydrochariataceae*. *J Plant Res, 117*, 265–276.

Traidl-Hoffmann, C., Jakob, T., & Behrendt, H. (2009). Determinants of allergenicity. *J Allergy Clin. Immunol*, *123*, 558–566.

Watson, D.W., & Kim, Y.B. (1963). Modification of host responses to bacterial endotoxins. I. Specificity of pyrogenic tolerance and the role of hypersensitivity in pyrogenicity, lethality, and skin reactivity. *J Exp Med*, *118*, 425–446.

Watson, S.W., Novitsky, T.J., Quinby, H.L., & Valois. F.W. (1977). Determination of bacterial number and biomass in the marine environment. *Appl Environ Microbiol*, *33*, 940–946.

Williams, L.K., Ownby, D.R., Maliarik, M.J., & Johnson, C.C. (2005). The role of endotoxin and its receptors in allergic disease. *Ann Allergy Asthma Immunol*, *94*, 323–332.

Zhang, Z., Schwartz, S., Wagner, L., & Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *J Comput Biol*, *7*, 203–214.

Zuberbier, T (2016). Pollen allergy. *ECARF* websit. www.ecarf.org/en/information-portal/allergies-overview/pollen-allergy/.

Zuberbier, T., Lötvall, J., Simoens, S., Subramanian ,S.V., & Church, M.K. (2014). Economic burden of inadequate management of allergic diseases in the European Union: a GA2LEN review. *Allergy*, *69*, 1275–1279.

Supplimentary material:-

Bacterial species associated with highly allergenic pollen habitat yield a high level of endotoxin

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Table S1. Cultured bacteria associated with nine different flower pollen species, identified using the EzTaxon server on basis of 16S rRNA gene sequence data.

Name of the isolate	**Accession number of the isolate**	**Winter rye**	**Birch**	**Hazel**	**Mugwort**	**Blackthorn**	**Cherry plum**	**Autumn crocus**	**Winter rarapeseed**	**Hemp**	**Taxonomical affiliation (Accession number) [% of pairwise sequence similarity]**
RYC17	KX450460	✓									*Pantoea agglomerans* (AJ233423) [99.7]
RYA3, CP19	KX450447, MH813400	✓					✓				*Pseudomonas poae*(AJ492829), *Pseudomonas trivialis* (JYLK01000002) [100, 99.9]
RYC16	KX450459	✓									*Stenotrophomonas rhizophila* (CP007597) [100]
RYA10	KX450454	✓									*Bacillus simplex* (AB363738) [100]
RYA7	KX450451	✓									*Exiguobacterium sibiricum* (CP001022) [100]
RYB14	KX450457	✓									*Pseudarthrobacter defluvil* (AM409361), *Pseudarthrobacter niigatensis* (AB248526) [99.7]
RYA9	KX450453	✓									*Arthrobacter ginsengisoli* (KF212463), *Arthrobacter humicola* (AB279890), *Arthrobacter oryzae* (AB279889) [99.9]
RYB15	KX450458	✓									*Nocardioides cavernae* (KX815990) [99.2]
RYA2, MG4, CP9	KX450446, MH813363, MH813390	✓			✓		✓				*Rathayibacter festucae* (AM410683) [99.7-99.9]
RYC18	KX450461	✓									*Rhodococcus corynebacterioides* (AF430066) [99.7]
RYA5, RYC19	KX450449, KX450462	✓									*Rhodococcus fascians* (JMEN01000010) [100]

RYA1	KX450445	✓									*Streptomyces pratensis* (JQ806215), *Streptomyces anulatus* (DQ026637), *Streptomyces setonii* (MUNB01000146) [100]
RYA4, RYA6, Rb-Pol-2, MG8	KX450448, KX450450, KX450443, MH813366	✓			✓				✓		*Clavibacter michiganensis* (KF663872, HE608962) [100]
RYA11	KX450455	✓									*Clavibacter tessellarius* (99.8) [MZMQ01000001]
RYA8, Ra-AC-3, HA3, HA4, HA15, HA17, BT9A, BT9B	KX450452, KX450432, MH813343, MH813344, MH813352, MH813353, MH813409, MH813410	✓		✓		✓			✓		*Frigoribacterium faeni* (Y18807) [99.0-99.9]
RYB12, Rb-AC-1	KX450456, KX450437	✓							✓		*Exiguobacterium artemiae* (AM072763) [100]
Rb-AC-2, Rb-Pol-1	KX450438, KX450442								✓		*Pantoea vagans* (EF688012) [100]
Ra-Pol-1	KX450434								✓		*Pseudomonas endophytica* (LLWHO1000112) [100]
Ra-Pol-3, Rb-AC-5, Rb-Pol-3	KX450436, KX450441, KX450444								✓		*Rahnella woolbedingensis* (KF308409) [99.9]
Rb-AC-3	KX450439								✓		*Enterococcus haemoperoxidus* (KB946316), *Enterococcus quebecensis* (GU457262), *Enterococcus silesiacus* (CP013614) [99.9]
Ra-AC-4, Rb-AC-4	KX450433, KX450440								✓		*Lactococcus garvieae* (AP009332) [100]
Ra-Pol-2, Ra-AC-2	KX450435, KX450431								✓		*Staphylococcus xylosus* (MRZ001000018) [100]
Ra-AC-1, Bb-AC-3	KX450430, KX450416,		✓						✓		*Rosenbergiella nectarea* (jgi.1084674) [100]
AU2, AU5, AU11	KX450463, KX450464, KX450466							✓			*Rosenbergiella epipactidis* (KF876184) [99.8-100]
Bb-Pol-6	KX450422		✓								*Burkholderia andropogonis* (LAQU01000081) [97.9]
Bd-AC-3, CAN12, CAN19	KX450427, MH813427, MH813434		✓							✓	*Erwinia billingiae* (JN175337) [99.7-100]
Bd-AC-4	KX450428		✓								*Erwinia tasmaniensis* (CU468135) [99.1]
Bd-AC-2	KX450426		✓								Pseudomonas cerasi (LT222319), *Pseudomonas syringae* (KI657453), *Pseudomonas congelans* (AJ492828), *Pseudomonas ficuserectae* (AB021378) [100]
Bd-Pol-1	KX450429		✓								*Rahnella aquatilis* (CP003244) [100]

Bb-Pol-4	KX450420		✓								*Rhizobium soli* (EF363715) [99.7]
Bb-AC-4, Bb-Pol-5	KX450417, KX450421		✓								*Sodalis praecaptivus* (CP006569) [97.7, 97.8]
Bb-Pol-8	KX450424		✓								*Staphylococcus cohnii* (D83361) [100]
Bd-AC-1	KX450425		✓								*Chryseobacterium indoltheticum* (AY468448) [100]
Ba-AC-1	KX450414		✓								*Hymenobacter roseus* (HG965772) [98.3]
Bb-AC-2	KX450415		✓								*Microbacterium phyllosphaerae* (AJ277840) [100]
Bb-Pol-2	KX450419		✓								*Micrococcus yunnanensis* (FJ214355), *Micrococcus luteus* (CP001628) [100]
Bb-Pol-1, MG24	KX450418, MH813381		✓		✓						*Streptomyces albidoflavus* (Z76676), *Streptomyces violascens* (AY999737), *Streptomyces hydrogenans* (AB184868), *Streptomyces daghestanicus* (DQ442497) [100]
AU19, CAN3, AU8, AU14	KX450474, MH813419, KX450465, KX450469							✓		✓	*Erwinia persicina* (BCTN01000053) [97.9-99.5]
AU17	KX450472							✓			*Lonsdalea iberica* (LUTP01000101) [100]
AU15	KX450470							✓			*Micrococcus yunnanensis* (FJ214355) [99.7]
AU12	KX450467							✓			*Erwinia piriflorinigrans* (GQ405202) [98.5]
AU16	KX450471							✓			*Rahnella Victoriana* (KF308403), *Rahnella woolbedingensis* (KF308409 [99.9]
AU13	KX450468							✓			*Brevibacterium frigoritolerans* (AM747813) [100]
AU18, HA8, HA11, HA23, CAN1, CAN2, CAN7, CAN8, CAN11, CAN15, CAN20, CAN21	KX450473, MH813347, MH813350, MH813359, MH813417, MH813418, MH813422, MH813423, MH813426, MH813430, MH813435, MH813436			✓				✓		✓	*Curtobacterium flaccumfaciens* (AJ312209) [98.6-100]
HA1, CP7	MH813341, MH813388			✓			✓				*Aureimonas glaciei* (KU253627) [98.8, 99.2]
HA2	MH813342			✓							*Sphingomonas faeni* (AJ429239) [99.7]
Bb-Pol-7, HA5, MG9	KX450423, MH813345, MH813367		✓	✓	✓						*Methylobacterium pseudosasicola* (jgi.1071178) [99.3-99.9]
HA7	MH813346			✓							*Spirosoma pollinicola* (MG589923) [100]
HA9A, HA9B	MH813348, MH813349			✓							*Amnibacterium kyonggiense* (FJ527819) [98.3, 98.5]

HA13	MH813351			✓							*Methylobacterium marchantiae* (FJ157976) [98.9]
HA18	MH813354			✓							*Pseudomonas agarici* (AKBQ01000002) [98.6]
HA19, BT6, BT7	MH813355, MH813406, MH813407			✓		✓					*Frondihabitans peucedani* (FM998017) [98.5, 100]
HA21A, HA21B, HA22, MG5, MG18, MG19	MH813356, MH813357, MH813358, MH813364, MH813375, MH813376			✓	✓						*Bacillus altitudinis* (ASJC01000029) [99.7-100]
HA24	MH813360			✓							*Paenibacillus kyungheensis* (KF793934) [98.6]
MG1	MH813361				✓						*Bacillus subtilis* (AMXN01000021), *Bacillus tequilensis* (AYTO01000043) [99.9]
MG2, MG10, MG12, MG16	MH813362, MH813368, MH813370, MH813373				✓						*Burkholderia multivorans* (ALIW01000278) [99.9-100]
MG6, MG20	MH813365, MH813377				✓						*Rhodococcus cerastii* (FR714842) [99.9, 98.5]
CP12	MH813393						✓				*Rhodococcus sovatensis* (KU189221), *Rhodococcus cerastii* (FR714842) [98.5]
MG11, MG17	MH813369, MH813374				✓						*Bacillus safensis* (ASJD01000027), *Bacillus zhangzhouensis* (JOTP01000061) [99.9, 100]
MG14	MH813371				✓						*Streptomyces mexicanus* (AF441168) [99.9]
MG15, CP5	MH813372, MH813386				✓		✓				*Agreia pratensis* (AJ310412) [99.9, 99.7]
MG21	MH813378				✓						*Arthrobacter agilis* (X80748) [99.8]
MG22	MH813379				✓						*Microvirga soli* (KX247636) [99.8]
MG23	MH813380				✓						*Sphingomonas aerolata* (AJ429240) [99.5]
BT1, BT16	MH813402, MH813416					✓					*Pseudomonas canadensis* (AYTD01000015) [99.8]
BT2, BT12	MH813403, MH813413					✓					*Glutamicibacter bergerei* (AJ609630) [99.8]
BT3	MH813404					✓					*Microbacterium oxydans* (Y17227), *Microbacterium maritypicum* (AJ853910) [100]
BT5, BT15	MH813405, MH813415					✓					*Curtobacterium oceanosedimentum* (EF592577) [99.7]
BT8, BT10, BT14	MH813408, MH813411, MH813414					✓					*Curtobacterium herbarum* (AJ310413), *Curtobacterium oceanosedimentum* (EF592577) [98.6]

BT11	MH813412					✓					*Brevibacterium aurantiacum* (X76566) [98.7]
CP1	MH813382						✓				*Methylobacterium bullatum* (GU983169) [99.5]
CP2, CP18	MH813383, MH813399						✓				*Methylobacterium cerastii* (FR733885) [100, 99.8]
CP3	MH813384						✓				*Subtercola frigoramans* (AF224723) [97.9]
CP4	MH813385						✓				*Subtercola boreus* (AF224722) [98.4]
CP6, CP13	MH813387, MH813394						✓				*Friedmanniella antarctica* (Z78206) [100, 99.8]
CP8	MH813389						✓				*Amnibacterium soli* (EU432172) [99.8]
CP10, CP11	MH813391, MH813392						✓				*Williamsia limnetica* (HQ157192) [99.2, 99.1]
CP14	MH813395						✓				*Kineococcus aurantiacus* (X77958) [99]
CP15	MH813396						✓				*Nakamurella silvestris* (KP899234) [98.7]
CP16, CP17	MH813397, MH813398						✓				*Frondihabitans sucicola* (JX876867) [98.5, 98.6]
CP20	MH813401						✓				*Caballeronia sordidicola* (FCOC01000044) [98.5]
CAN4, CAN9, CAN10, CAN18	MH813420, MH813424, MH813425, MH813433									✓	*Morganella psychrotolerans* (DQ358135) [98.7]
CAN5	MH813421									✓	Microbacterium testaceum (X77445) [99.8]
CAN13	MH813428									✓	*Pseudomonas coleopterorum* (KM888184), *Pseudomonas rhizosphaerae* (CP009533) [99.8]
CAN14	MH813429									✓	*Bacillus zhangzhouensis* (JOTP01000061), *Bacillus safensis* (ASJD01000027) [99.8]
CAN16	MH813431									✓	*Bacillus tequilensis* (AYTO01000043), *Bacillus subtilis* (AMXN01000021) [99.7]
CAN17	MH813432									✓	*Bacillus aryabhattai* (EF114313) [99.8]

Appendix: 1

International peer reviewed scientific publications:

1. Ambika Manirajan, B., Ratering, S., Rusch, V., Schwiertz, A., Geissler-Plaum, R., Cardinale, M., Schnell, S. (2016) Bacterial microbiota associated with flower pollen is influenced by pollination type, and shows a high degree of diversity and species-specificity. *Environ Microbiol* **18:** 5161–5174. DOI:10.1111/1462-2920.13524.

2. Ambika Manirajan, B., Maisinger, C., Ratering, S., Rusch, V., Schwiertz, A., Cardinale, M., Schnell, S. (2018) Diversity, specificity, co-occurrence and hub taxa of the bacterial-fungal pollen microbiome. *FEMS Microbiol Ecol* **94:** fiy112. DOI:10.1093/femsec/fiy112.

3. Ambika Manirajan, B., Suarez, C., Ratering, S., Rusch, V., Geissler-Plaum, R., Cardinale, M., Schnell, S. (2018) *Spirosoma pollinicola* sp. nov., isolated from pollen of common hazel (*Corylus avellana* L.). *Int J Syst Evol Microbiol* **68:** 3248-3254 DOI:*10.1099*/ijsem.0.002973.

Publication to be submitted:

1. Ambika Manirajan, B., Ratering, S., Rusch, V., Schwiertz, A., Geissler-Plaum, R., Cardinale, M., Schnell, S. (2018) Bacterial species associated with highly allergenic pollen habitat yield a high level of endotoxin (manuscript prepared for submission).

Poster presentation:

1. Ambika Manirajan, B., Cardinale, M., Ratering, S., Schnell, S. (2016) Diversity of bacterial microbiota associated with flower pollen. Annual Conference of the Association for General and Applied Microbiology (VAAM), Jena, Germany.

Oral presentation:

1. Ambika Manirajan, B., Maisinger, C., Ratering, S., Rusch, V., Schwiertz, A., Schnell, S., Cardinale, M. (2018) Diversity, specificity, co-occurrence and hub taxa of the bacterial–fungal pollen microbiome. Annual Conference of the Association for General and Applied Microbiology (VAAM), Wolfsburg, Germany.

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