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A Walk in the Park: Influence of Natural Co-Exposure to Grass Pollen and Fungal Spores on Nasal Mycobiome and Cytokine Responses

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ABSTRACT

Background: During the grass flowering season, fungal spores are abundant in outdoor air. We tested for co-sensitisations to grass pollen and fungal spores, assessed the degree of co-exposure, and studied its impact on the nasal mycobiome and immune responses.

Methods: Fungi-specific IgE-levels were studied in 277 individuals with and without grass pollen sensitisation. In a small cohort ($n = 7$), exposure to grass pollen and fungal spores was monitored during 5 consecutive indoor and outdoor stays in a flowering meadow and correlated with changes in the nasal mycobiome. Cytokines of nasal epithelial cells were studied under stimulation with recombinant grass pollen allergens, with and without fungal spores derived from outdoor isolates.

Results: IgE-sensitisation against the studied fungi was significantly more frequent among individuals with grass pollen sensitisation than among those without grass pollen sensitisation. Outdoor exposure resulted in changes in the nasal mycobiome, with a transitory enrichment of environmental fungi, for example, *Cladosporium* species. Most of the fungi cultivated from outdoor air samples belonged to the genera *Fusarium*, *Cladosporium* and *Penicillium*. Apical co-stimulation of nasal epithelial cells with grass pollen allergens and *Fusarium*, *Cladosporium* or *Penicillium* spores led to an increased loss of transepithelial electrical resistance and induction of pro-inflammatory cytokine release compared to mono-stimulation.

Conclusion: Frequent co-exposure to fungal spores and grass pollen may increase the chance of acquiring a co-sensitisation to both allergens. Environmental fungi interact with and transitorily change the local mycobiome. Under co-exposure, fungal spores induce nasal inflammation and foster immune responses to otherwise poorly immunogenic pollen allergens.

Abbreviations: ALI, air-liquid interface; DAMP, danger-associated molecular pattern; EU/µg, European units per microgram; GPE, grass pollen extract; HNEC, human nasal epithelial cell; IMNGS, integrated microbial next generation sequencing; kDa, kilo dalton; PAMP, pathogen-associated molecular pattern; PDA, potato dextrose agar; Phl p, *Phleum pratense*; rpm, rotations per minute; RT, room temperature; TEER, transepithelial electrical resistance.

Annika Eggestein and Denise Rauer equally contributed to this work. Athanasios Damialis and Stefanie Gilles jointly led the project.

Key Messages

- Co-sensitisation to a variety of fungi is more frequent in grass pollen-sensitised individuals than in individuals not sensitised to grass pollen.
- Airborne fungi transiently modify the nasal mycobiome of study participants after 1 h exposure in a flowering meadow.
- Fungal spores that were most abundant in the air during the study period have the potential to alter the physical and immunological barrier of nasal epithelial cells.

1 | Introduction

Grass pollen causes allergic symptoms in sensitised individuals and is the main cause of seasonal allergic rhinitis and asthma in the summer months [1, 2]. The major allergens of all sweet-grass pollen are Phl p 1 and Phl p 5 [3]. Although the major part of the IgE response is directed towards these allergens, it is presently unknown how they trigger allergic sensitisation. For some plant allergens, for example, birch and ragweed pollen, it has been shown that the main allergens are not immunogenic enough to explain the resulting Th2-mediated, IgE-driven immune response [4, 5]. Therefore, co-factors like PAMPs (pathogen-associated molecular patterns) and DAMPs (damage-associated molecular pattern) must play a role in activating the immune system. These substances lead to the recruitment of immune cells, especially eosinophils and neutrophils, and activate the innate immune system, which may facilitate the priming of Th2 responses [6, 7]. PAMPs and DAMPs can originate from the pollen matrix itself [6, 8–10] or from microorganisms to which the pollen is exposed [11, 12].

Airborne microorganisms that could easily come into contact with grass pollen are fungal spores, which play a role as allergens themselves and as such can cause allergic rhinitis or asthma [13]. *Penicillium* and *Aspergillus* spores in particular are recognised as indoor allergens [14, 15]. Most other fungal taxa are mainly found in the atmosphere during late summer after the pollination period of allergenic trees and are considered less relevant for the allergy symptom burden.

We have previously shown that fungi can be found on the surface of pollen [11]. It is also evident from the literature that there is no general fungal spore season, but that each individual taxon has its own season, frequently much longer compared to pollen, with no distinct seasonality for the majority of taxa, the spore peak by rule taking place in the middle of summer [16]. Thus, co-exposure to some fungal spores and grass pollen can occur. Indeed, *Cladosporium* spores were found to co-occur in the air together with grass pollen on > 50% (28/50) of days during the grass pollen season, and *Alternaria* spores on 14/50 days [17, 18]. Fungal co-exposure may even be relevant beyond the main grass pollen season as grass pollen allergens have been detected in air samples up to the autumn [19].

In this paper, the questions were asked whether significant co-sensitisations to grass pollen and fungal spores exist, which fungal spores co-occur with airborne grass pollen, whether those fungi can be detected in and modify the composition of the nasal mycobiome of humans, and whether they trigger a pro-inflammatory immune response of nasal epithelial cells to isolated grass pollen allergens. To answer these questions, we performed a data analysis of IgE profiles measured in participants ($n = 277$) of various studies in the outpatient clinic of Environmental Medicine in Augsburg during the years of 2015–2023. In addition, we performed a prospective study with a small number of volunteers with repetitive environmental and nasal sampling on 5 consecutive days, before and after a 1 h stay in a flowering meadow. Finally, we isolated and sequenced cultivable fungi from air samples, stimulated fully differentiated nasal epithelial cell cultures with their spores, either alone or in combination with recombinant grass pollen allergens (Phl p 1 and Phl p 5), and analysed the physical and immunological barrier response of the cells.

2 | Methods

2.1 | Study Centre Cohort ('CoSens' Study)

Comprehensive IgE profiles (ImmunoCAP, Phadia, Sweden) had been obtained during routine screening from a total of > 1000 former study participants in the outpatient clinic of Environmental Medicine at the University hospital of Augsburg during the years of 2015–2023. In the frame of the 'CoSens' study, informed consent to re-analyse the IgE data stored in the study centre database was obtained via a web-based survey (Qualtrics). IgE profiles of 277 individuals (mean age 39.7 years, range: 18–82 years; 30/70% m/f) were included and subjected to retrospective data analysis to test for co-sensitisations to grass pollen and various fungal spores. The local ethics committee approved the study (code: 2022-577-S-KH).

2.2 | Exposure Cohort ('Picnic' Study)

Seven adult volunteers with and without sensitisation to aeroallergens (Table 1) were enrolled after written informed consent. The study was approved by the local ethics committee (code: 54/17 S) and conformed to the guidelines of Helsinki. Serum was tested for specific IgE against aeroallergens, including tree-, grass-, and weed pollen, house dust mite, and fungal spores (ImmunoCAP, Phadia, Sweden).

The 'Picnic' study (Figure 1) consisted of 5 weekly visits during the main grass pollen season in Augsburg (June–July 2018). At each visit, the participants spent ≥ 3 h indoors and subsequently 1 h outdoors in a flowering meadow. Cotton swabs of four participants were taken immediately before and after the outdoor exposure for ITS sequencing of the nasal mycobiome. Three participants wore nasal filters during the outdoor stay. Portable Burkard pollen traps were set up indoors and outdoors to measure the on-site bioaerosol concentration. Additionally,

TABLE 1 | Overview over IgE profiles of the participants of the 'Picnic' study.

ID	Total IgE (kU/L)	Sex	Age (years)	Grasses (kU/L)	Birch (kU/L)	Mugwort (kU/L)	Fungi (kU/L)	HDM (kU/L)
G1	570.0	F	27	28.0	1.4	0.3	0.0	0.2
G2	22.0	F	28	5.1	0.0	0.0	0.5	0.3
G3	42.0	F	47	3.9	0.0	0.0	0.0	0.0
N1	19.0	F	29	0.0	0.0	0.0	0.0	0.0
N2	5.1	M	33	0.0	0.0	0.0	0.0	0.0
N3	6.3	M	22	0.0	0.0	0.0	0.0	0.0
N4	9.7	F	39	0.0	0.0	0.0	0.0	0.0

Note: Given are specific IgE levels (kilo units per litre) as measured by ImmunoCAP (Phadia) in serum.
Abbreviations: F, female; G, grass pollen allergic; HDM, house dust mite; M, male; N, non-allergic.

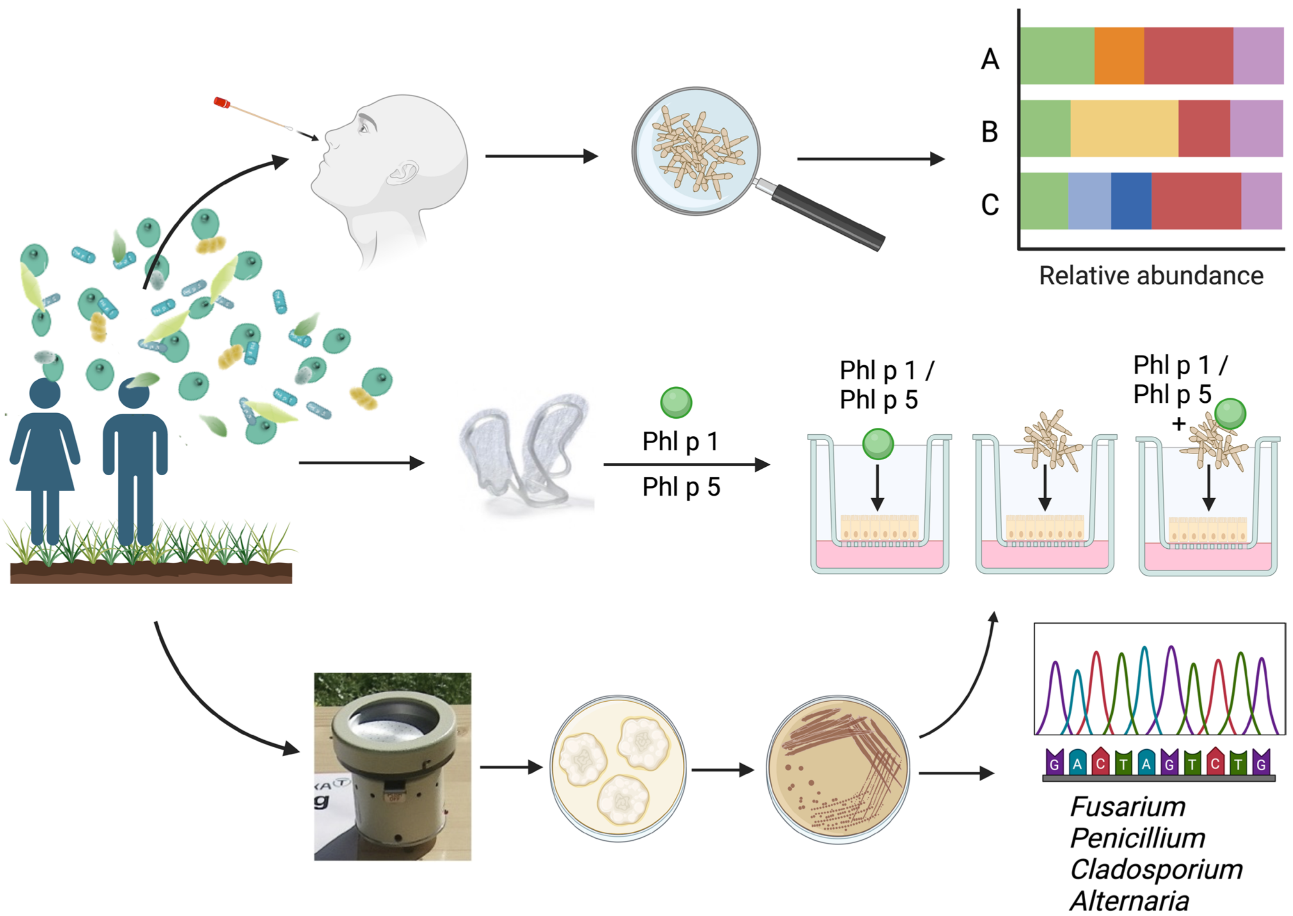

FIGURE 1 | Experimental setup of the 'Picnic' study. On five successive days during the peak grass pollen season in June 2018, seven volunteers (four non-allergic, three grass pollen allergic) stayed 3 h indoors, followed by a 1-h stay in a flowering meadow. Nasal swabs of four participants, taken before and after the outdoor stay, were subjected to mycobiome analysis. Nasal filters worn by three participants during the outdoor stay were eluted and analysed for aeroallergen content by MARIA assay. Fungal spores were quantified in air samples with Burkard traps, and fungal isolates were collected and sequenced. Fungal spores from air samples were used for stimulation of nasal epithelial cells.

Hirst-type traps (Burkard Co. Manufacturing, UK) were set up with potato-dextrose agar (PDA) plates to collect and sequence cultivable fungal species. The traps were placed in a common room for 1 h indoors and next to the study participants during 1 h outdoor exposure.

2.3 | Airborne Pollen and Fungal Spore Measurements

Pollen grains and fungal spores were identified microscopically in Hist-type trap samples by a trained technician and particle concentrations were estimated per m^3 of air for each sampling interval as described previously [20].

2.4 | Cultivation of Fungal Species From Air Samples and Isolation of Fungal Spores

Exposed PDA plates were incubated at room temperature (RT) for 5 days. Resulting colonies were picked with a single-use inoculation loop and streaked on fresh agar plates. After 4–5 days, single colonies were picked for DNA extraction. Subcultures of pure strain fungal cultures were obtained by cutting 1 cm^2 pieces of the original culture and placing them on a fresh PDA plate. The plate was incubated at RT (*Fusarium* sp.) or 25°C (*Cladosporium cladosporioides*, *Penicillium manginii*) for 1–3 weeks until spores were produced. A swab was used to scrape the spores off the culture dish and placed into 1 mL synthetic nasal fluid medium [21]. The spores were counted and diluted for cell culture experiments.

2.5 | Mycobiome Analysis

Microbial material was sampled bilaterally from the middle nasal meatus with cotton swabs. Microbial DNA was extracted following the instructions of the QIAamp UCP Pathogen Mini Kit with the following modifications: 650 μL of ATL buffer containing 4.3 μL D× buffer were added to swabs in tubes containing 500 mg zirconia beads and 500 μL Stool stabilising solution. Mechanical pre-lysis was performed by shaking at 5600 rpm, 2×90 s with a 15 s break in between on a Precellys Evolution bead beater. The volumes of the reagents were adjusted to the supernatant obtained from step 7 of the pre-lysis protocol. Finally, the DNA was eluted twice with 2×50 μL elution buffer and stored at −20°C.

Pure fungal colonies grown 4–5 days were picked, suspended in 200 μL fungal lysis buffer and vortexed vigorously. The samples were incubated for 10 min at 95°C and shaking at 600 rpm. After centrifugation for 2 min at 10,000×g, the supernatants were collected, and species were identified by Illumina Sequencing (ITS primers: fw: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTA AAA GTC GTA ACA AGG TTT C-3′; rev: 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGT GTT CAA AGA YTC GAT GAT TCA C-3′) [22]. Barcoding and sequencing was carried out at the TUM Core Facility Microbiome at the ZIEL Institute for Food and Health in Freising, Germany using an Illumina MiSeq platform according to the recommendations of the manufacturer.

2.6 | Quantification of Aeroallergens in Nasal Filters

Nasal filters were collected in 5 mL D-PBS + 0.05% Tween20 and washed for 2 h at RT on an Eppendorf shaker (300 rpm). Aliquots of the extracts were stored at −80°C. For analysis, the aliquots were thawed and diluted 1:10 in D-PBS to a final concentration of 0.05 mM Tween-20. The extracts were then re-concentrated using 3 kDa cutoff ultrafilters (Amicon Ultra, Millipore). Phl p 5 content in nasal filter extracts was analyzed using a single-plex array for allergens (MARIA, InBio, Charlottesville, VA, USA) according to the manufacturers' instructions and measured on a Bio-Plex 200.

2.7 | Human Primary Nasal Epithelial Cell Models

Human nasal epithelial cells (HNECs) were isolated from turbinoplastic surgery specimens of non-allergic donors as previously described [23]. For air-liquid interface (ALI) cultures, second passage HNECs were transferred to collagen-coated transwells, grown in submerged culture in Airway Epithelial Cell growth medium (PromoCell, Heidelberg, Germany) until confluent and air-lifted after 5–7 days. The cultures were maintained for 21 days in basolateral ALI medium (PromoCell), with TEER measurement and basolateral medium change every 2 days. After the cultures had reached a TEER of ≥ 1000 Ω/cm^2, they were stimulated apically (30 μL) with synthetic nasal fluid medium (ctrl.), aqueous grass pollen extract (GPE; concentration corresponding to 10 μg/mL total protein), recombinant Phl p 1 or Phl p 5 (1 μg/mL), fungal spores (1×10^4 spores) or combinations of fungal spores and grass pollen allergens. TEER measurements were performed at baseline, 1 h, 6 h, 24 h and 48 h after stimulation. Basolateral supernatants and apical washes were taken after 24 h and stored at −80°C. IL-1β, IL-8 and MCP-1 were measured in the samples by ELISA (BD OptEIA).

2.8 | Statistical Data Analysis

Fisher exact X^2 test was performed to test for differences in the frequency of fungal sensitisations between individuals with and without grass pollen sensitisation. One-way ANOVA with post hoc Dunn's test was performed to test for differences in cytokine secretion of stimulated HNEC cultures. To compare the results of TEER measurements, mixed effects model ANOVA with post hoc Dunnett's test was used. Plots were generated and statistical analyses carried out with Graph Pad Prism 10. Mycobiome: Demultiplexing, sequence merging, filtering and removal of chimera artefacts of sequencing data of the mycobiome was performed using the Integrated Microbial Next Generation Sequencing (IMNGS) platform [24]. Quality filtering to remove contaminants was done using microbIEM v0.41 [25]. Filtered data were further analysed using MicrobiomeAnalyst v4.0 [26]. To compare the groups of interest, paired and unpaired *t*-test or one-way ANOVA were performed, and statistical significance was determined as $p < 0.05$. Statistical analysis of microbiome data was performed in R 3.6.2 using the 'ggpubr' package; graphs were prepared in GraphPad Prism version 8.4.3.

3 | Results

3.1 | Co-Sensitisation Against Fungal Spores in Individuals With or Without Specific IgE Against Grass Pollen

In order to investigate the frequency of co-sensitisations to grass pollen and fungi, IgE profiles of 277 subjects were analysed. Individuals sensitised and non-sensitised to grass pollen were compared for their IgE levels against *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium* and *Penicillium* spores.

Individuals with specific IgE (≥ 0.35 kU/L) against grass pollen (GP+) had significantly higher ($p < 0.0001$, Mann–Whitney test) IgE levels against all tested fungal spores than individuals who were not grass pollen-sensitised (GP−) (Figure 2A). Concurrently, multiple fungal sensitisations were observed mainly among participants with a strong grass pollen sensitisation (Figure 2B). As shown in the contingency plot (Figure 2C), the frequency of sensitisation to any fungus was significantly higher among grass pollen-sensitised than among grass pollen-non-sensitised individuals ($p < 0.0001$, Fisher's exact X^2 test).

3.2 | Nasal Levels of Grass Pollen Major Allergen Is Mirrored by the Levels of Fungal Spores and Grass Pollen Particles in the Air

To draw a possible connection between the occurrence of grass pollen and fungal spores, their concentrations in the air were measured during the study period. Airborne pollen and fungal spore concentrations were compared with the levels of allergens eluted from the nasal filters that a subset (3/7) of the volunteers wore during the visits.

The total concentration of pollen and fungal spores was significantly lower indoors than outdoors (Figure 3A). Airborne grass pollen concentrations outdoors peaked on the visits 1, 2 and 5 (Figure 3B). Of the fungal and pollen allergens tested by immunoassay in nasal filter extracts, only Phl p 5 was measurable, and its peaks coincided with the peaks of airborne grass pollen (Figure 3C).

3.3 | Transient Changes in the Nasal Microbiome Evident Between Indoor and After Outdoor Exposure

A total of 48 fungal families were identified overall in nasal swabs, and 39/48 families were found in samples from indoor and outdoor exposure. Fungal families that showed significantly higher abundance after indoor exposure were Malasseziaceae ($p < 0.0001$), Globuleviaceae ($p < 0.05$) and Sporidiobolaceae ($p < 0.05$); Cladosporiaceae ($p < 0.01$), Erysiphaceae ($p < 0.01$), Didymellaceae ($p < 0.05$), Phaeosphaeriaceae ($p < 0.05$), Dotioraceae ($p < 0.05$) and Russulaceae ($p < 0.05$) (Figure 4A). On the species level, *Malassezia restricta* was the most abundant fungal species after indoor exposure, and *Cladosporium angustiherbarum*, *C. cladosporoides* and *Neoerysiphe hiratae* were the most abundant species after outdoor exposure (Figure 4B). Transient shifts observed in the relative abundance of the top 10 families, genera and species are depicted in Figure 4C–E.

We further compared the alpha-diversity of the nasal mycobiome in pre- and post-exposure samples. The richness of the nasal mycobiome did not differ significantly between before and after outdoor

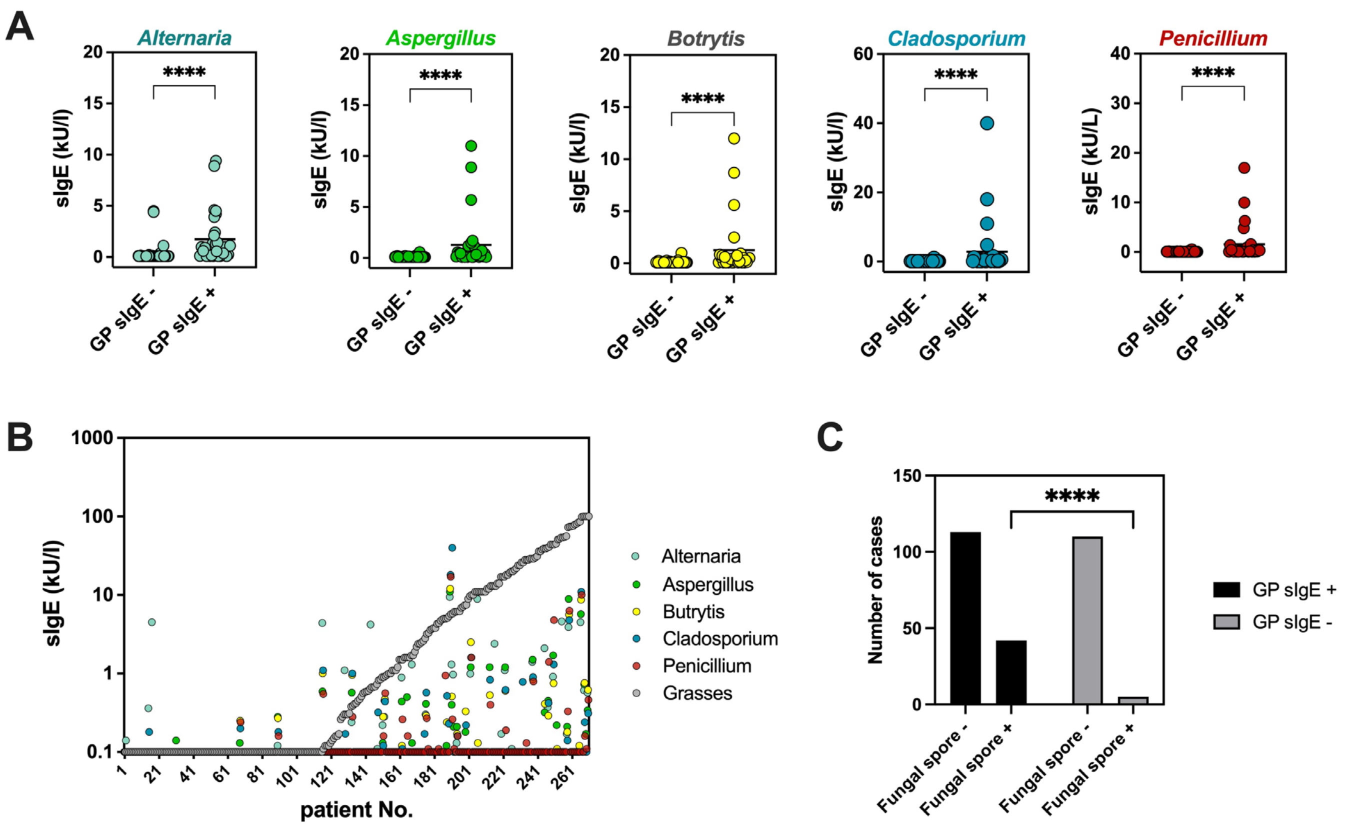

FIGURE 2 | Co-sensitisation against fungal spores in individuals with or without specific IgE against grass pollen. $N = 277$ subjects from a clinical study centre database in Augsburg, Germany, were tested for specific IgE against a panel of aeroallergens by ImmunoCAP (Phadia). Specific IgE levels against common allergenic fungi were compared between grass pollen (GP) sensitised and grass pollen non-sensitised patients. (A) Specific IgE levels against fungi in study participants with and without grass pollen sensitisation. (B) Plot of fungal sIgE levels against the sIgE levels to grass pollen in the same patients. (C) Contingency analysis plot. ****$p < 0.0001$, X^2 Fisher exact test. sIgE, specific IgE.

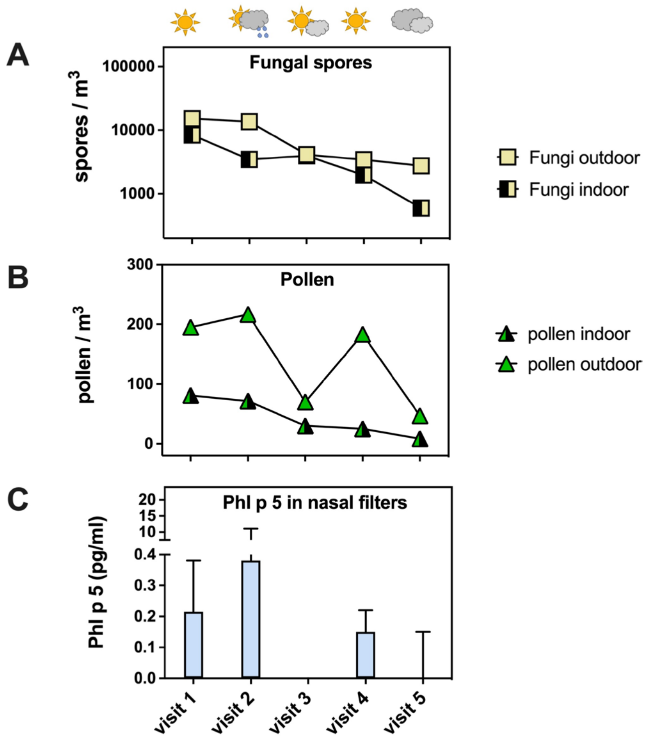

FIGURE 3 | Grass pollen allergen in nasal filters in relation to airborne pollen and fungal spores. During the peak grass pollen season in Augsburg, human volunteers ($n=6$) underwent repeated nasal sampling after an indoor- and a subsequent 1-h outdoor stay in a flowering meadow. (A) Abundance of airborne fungal spores; (B) Abundance of airborne pollen as measured with a portable Burkard trap outdoors and indoors. (C) Concentration of Phl p 5 (median + 5%–95% range, triplicate measurements) as measured in nasal filter extracts.

exposure (Figure 5A). In contrast, evenness was significantly ($p<0.001$) increased overall after the outdoor stay compared to indoors. The differences in alpha-diversity were observed throughout the visits but were not statistically significant per day (Figure 5B).

3.4 | *Cladosporium*, *Fusarium* and *Penicillium* Were the Most Frequently Identified Cultivable Fungi in the Air Samples

To determine the taxonomic spectrum of cultivatable fungi in the air samples, single colonies were isolated from indoor and outdoor air samples and sequenced. We found that most of the cultivable fungi identified in the air samples belonged to only three taxa: *Cladosporium*, *Fusarium* and *Penicillium*. An overview of the abundances of cultivable fungi in the air samples is provided in Table 2.

3.5 | Co-Exposure of Nasal Epithelial Cells to Grass Pollen Allergens and Fungal Spores Results in Changed Physical Barrier Integrity

In a set of in vitro experiments, we aimed to determine whether mono- and co-exposure to grass pollen allergens and fungal spores would result in differences in the barrier integrity of nasal epithelial cells. Therefore, HNEC ALI cultures were mono-exposed to synthetic nasal fluid medium (ctrl.) recombinant grass pollen allergens (Phl p 1, Phl p 5), aqueous grass pollen extract (GPE), spores of fungal isolates (*C. cladosporioides*, *Fusarium equiseti*, *Alternaria alternata*), or co-exposed to combinations of grass pollen allergens and fungal spores.

Mono-exposure with Phl p 1 led to a significant ($p<0.05$) reduction in TEER after 6 h as compared to the initial value (Figure 6A). Mono-exposure with Phl p 5 led to a significant decrease in TEER after 6 h and 24 h ($p<0.05$) (Figure 6B). Mono-exposure to GPE did not lead to differences in TEER compared to its initial value or the control (Figure 6C).

Under Phl p 1 stimulation (Figure 6A), TEER was significantly reduced after 6 h co-exposure to *Fusarium* ($p<0.05$), after 24 h co-exposure to *Cladosporium* ($p<0.01$) and *Fusarium* ($p<0.01$), and after 48 h co-exposure with *Fusarium* ($p<0.01$) and *Cladosporium* ($p<0.001$) spores.

Under Phl p 5 stimulation (Figure 6B), TEER was reduced after 1 h co-exposure with *Cladosporium* ($p<0.05$), after 6 h co-exposure with *Cladosporium* ($p<0.001$), *Fusarium* ($p<0.05$) and *Alternaria* ($p<0.05$), after 24 h co-exposure to *Cladosporium* ($p<0.01$) and *Fusarium* ($p<0.01$), and after 48 h co-exposure to *Cladosporium* ($p<0.01$) and *Fusarium* spores ($p<0.05$).

In GPE-treated cells (Figure 6C), the TEER was significantly increased after 6 h co-exposure to *Cladosporium* ($p<0.01$) and decreased after 6 h co-exposure with *Alternaria* spores ($p<0.01$).

3.6 | Fungal Spore Co-Exposure Leads to an Increased Pro-Inflammatory Response of Nasal Epithelial Cells to Grass Pollen Allergens

Lastly, we stimulated HNECs with nasal fluid medium (ctrl.), Phl p 1, Phl p 5 and GPE, either alone or in combination with fungal spore isolates (*C. cladosporioides*, *Fusarium equiseti*, *Penicillium magnum*, *Alternaria alternata*), and assessed the cytokine response of the cells in supernatants. The secretion of MCP-1 was not significantly regulated by any of the tested stimuli (Figure 7A). IL-8 secretion was significantly increased after mono-exposure to *Penicillium* spores ($p<0.05$) and after co-exposure to Phl p 5 and *Cladosporium* spores ($p<0.05$) (Figure 7B). IL-1β secretion was increased in cells mono-exposed to *Fusarium* spores ($p<0.05$) and co-exposed to Phl p 5 and *Cladosporium* spores ($p<0.01$) (Figure 7C). The co-exposure of cells with Phl p 1 or Phl p 5 with *Alternaria* spores ($n=3$) resulted in a higher IL-1β release compared to the respective mono-exposures (Figure 7C); however, due to low cell donor numbers, this effect did not reach statistical significance.

4 | Discussion

The increasing number of patients suffering from pollen allergies is a great challenge in the face of climate change [27, 28]. It

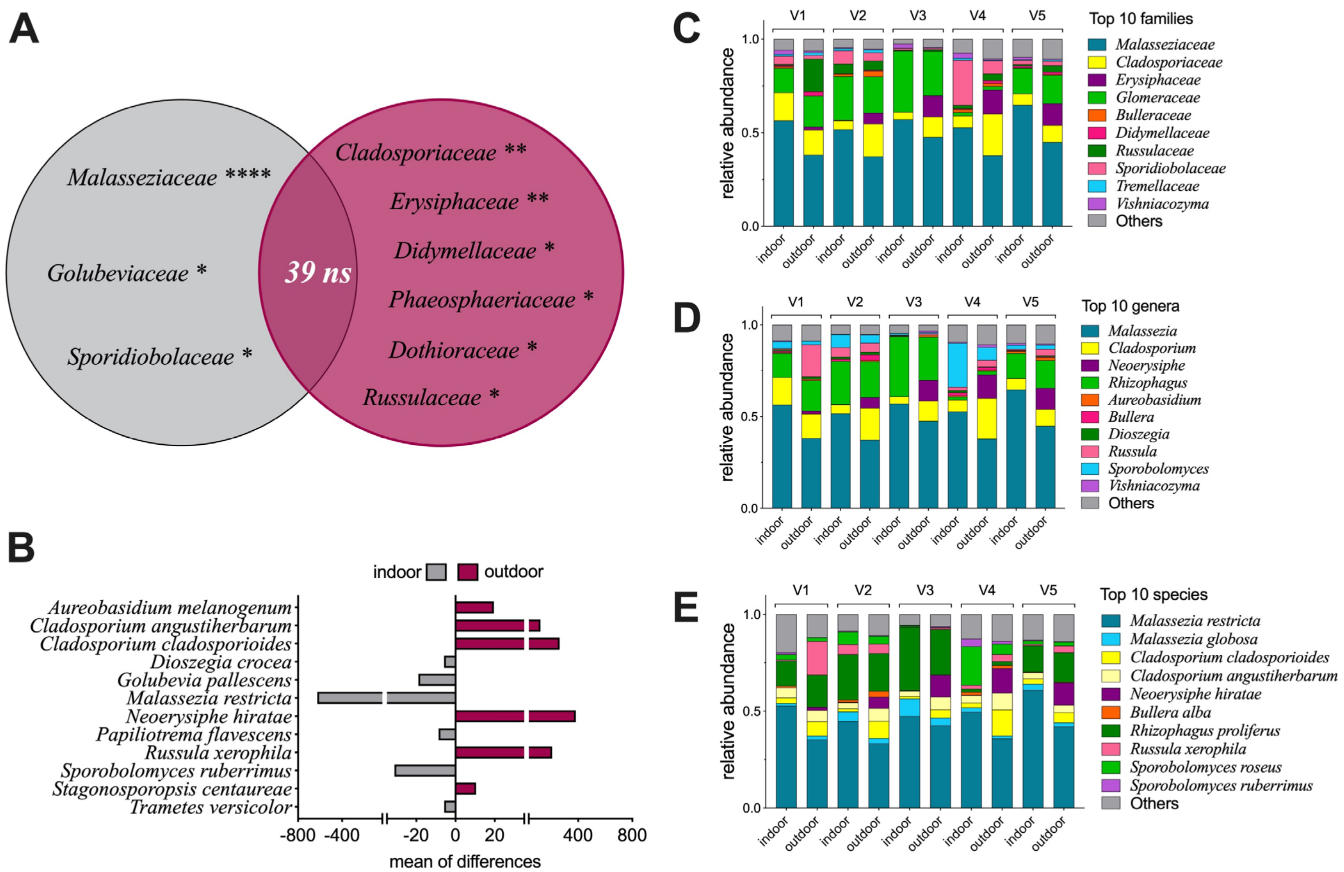

FIGURE 4 | Changes in the nasal mycobiome after indoor and outdoor stays. (A) Venn diagram of the top fungal families enriched in the nasal swabs of 'Picnic' panel study participants after the indoor (grey) and outdoor stays (purple). (B) Fungal species enriched after indoor and outdoor stays. (C–E) Relative abundance of the 10 most abundant fungi in nasal swabs of the participants after indoor and outdoor stays, depicted for all five visits on family (C), genus (D) and species level (E).

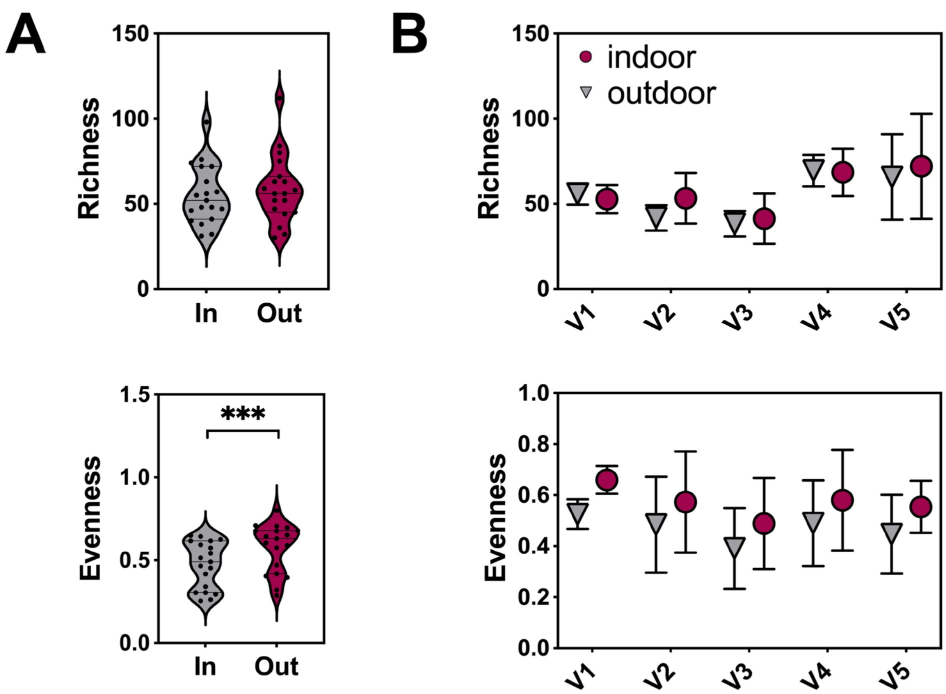

FIGURE 5 | Alpha diversity of the nasal mycobiome after indoor and outdoor stays. (A) Overall alpha-diversity in nasal swabs during the entire study period, pre- (grey violin plots) versus post-exposure (purple violin plots) in the flowering meadow. (B) Alpha diversity in the samples from each of the five visits, pre- (grey dots) versus post exposure (purple dots). ***$p < 0.001$, Wilcoxon test.

TABLE 2 | Fungal taxa cultivated from outdoor air samples during the 'Picnic' panel study.

Lab-ID	Visit #	Species	Indoor	Outdoor
F1	1	*Cladosporium tenuissimum*	−	+
F2	1	*Epicoccum nigrum*	−	+
F3	2	*Penicillium commune*	−	+
F4	2	*Cladosporium cladosporioides*	−	+
F5	3	*Penicillium chrysogenum*	+	−
F6	3	*Gibberella intricans*	+	−
F7	3	*C. cladosporioides*	−	+
F8	3	*G. intricans*	−	+
F9	4	*C. cladosporioides*	+	−
F10	4	*C. cladosporioides*	−	+
F11	5	*C. tenuissimum*	+	−
F12	5	*C. tenuissimum*	+	−
F13	5	*Penicillium magnii*	+	−
F14	5	*Trichoderma koningiopsis*	−	+
F15	5	*T. koningiopsis*	−	+
F16	5	*E. nigrum*	−	+
F17	5	*C. tenuissimum*	−	+
F18	5	*C. tenuissimum*	−	+
F19	5	*T. koningiopsis*	−	+
F20	5	*T. koningiopsis*	−	+
F21	5	*T. koningiopsis*	−	+

Note: Single colonies from the air samples were sequenced by NGS (ITS genes) and annotated with microbIEM.

is therefore important to shed light on possible co-factors that may foster pollen sensitisation or aggravate symptoms in those already sensitised. Here, we focused on fungal spores that could be of relevance during the grass pollen season.

The analysis of IgE profiles showed that individuals with elevated IgE to grass pollen also had more frequently a co-sensitisation to various fungi. Interestingly, many study participants had simultaneous sensitisation to several fungal species. This finding might either reflect true co-sensitisations or IgE cross-reactivity between certain fungal proteins, and unfortunately our extract-based immunoassay was not able to distinguish between these possibilities. We tested for IgE against *Aspergillus*, *Alternaria*, *Botrytis*, *Cladosporium* and *Penicillium* because many of these fungi are known allergens [29–31] and the tests were available in our hospital lab. *Cladosporium* was the most frequent fungal isolate in the air samples collected in a flowering meadow during the 'Picnic' study, followed by *Fusarium* and *Penicillium*. Even though *Fusarium*-specific IgE could not be measured in the clinical cohort, our comprehensive IgE data, in combination with the data from the air samples, suggest that co-exposure and co-sensitisation might be causally linked, which could indicate a common sensitisation mechanism. Like pollen grains, fungal spores enter the human body through inhalation, and pollens can have fungal cells attached to their surface [11]; thus, inhalation of microbe-laden pollen grains could result in a mixed exposure. Alternatively, fungal spores may simply be inhaled along with pollen grains at times when both particle-types are present at high enough concentrations in the same volume of air. There are studies showing a temporal overlap with the grass pollen season and the seasons of various fungal spores, such as *Cladosporium* [32] and *Alternaria* [33].

Cladosporium was not only abundantly present in the environmental air, but it was also transiently detected in the human nose after exposure to outdoor air. Temporal changes in the composition of nasal microbiota of adults were demonstrated before [34] but were never studied in the context of environmental exposure. No report on temporal fluctuations in the nasal mycobiome existed prior to our study. How long the environmental fungi can last in the microbial ecosystem of the human nose needs to be investigated in more detail in further studies. Interestingly, *Fusarium* and *Penicillium*, which were also abundant among cultivable fungi in outdoor samples, were not detected in the nose, whereas in a previous study, conidia and hyphal fragments of *Fusarium*, *Cladosporium*, *Epicoccum* and *Penicillium* (among others) could be detected in the human nasal cavity [35, 36]. Possibly, only *Cladosporium* spores were abundant enough in the air to cause measurable changes in the mycobiome in our study. In accordance with previous reports [37–39], *Cladosporium* was by far the most abundant spore type counted in air samples, whereas the concentrations of other fungal spores were approximately 100 times lower, even though some of them were readily cultivated (data not shown).

Cladosporium spores, among others, are themselves a common cause of allergy [29]. One of the major allergens of *Cladosporium cladosporides*, Cla c 9, is a vacuolar serine protease. In our in vitro experiment, *Cladosporium*, *Fusarium* and *Alternaria* spores significantly decreased the TEER in nasal epithelial cell cultures in the presence of recombinant Phl p 1 and Phl p 5, and the effect lasted up to 48 h, at least for *Cladosporium* and *Fusarium* co-exposure. Whether tight junction degradation is the reason and whether this might be mediated by fungal allergens directly or by other fungal proteases remains to be assessed. Interestingly, 6 h co-exposure to BPE and *Cladosporium* led to an increased TEER, whereas 6 h co-exposure to BPE and *Alternaria* resulted in a significant TEER decrease. The differences in the physical barrier responses of the cells to recombinant grass pollen allergens and GPE highlight the importance of pollen matrix constituents in eliciting cellular responses [4]. As previously reported,

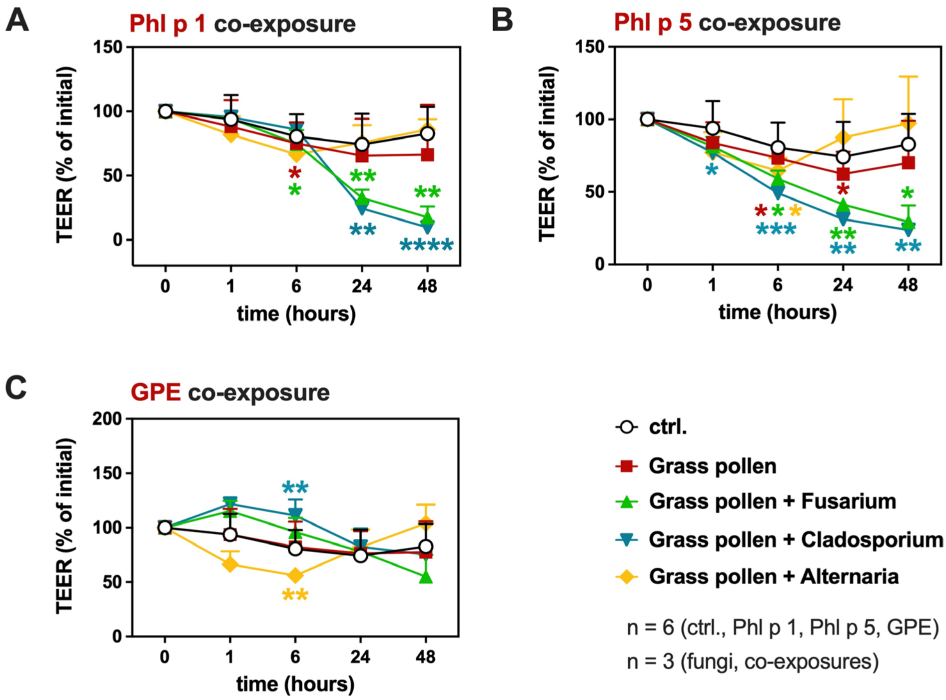

FIGURE 6 | Transepithelial electrical resistance of nasal epithelial cells after exposure to grass pollen allergens and fungal spores. Cells were apically exposed recombinant grass pollen allergens (1 µg/mL) Phl p 1 (A), Phl p 5 (B), or grass pollen extract (GPE; 100 µg/mL) (C), either alone or in combination with spores (10^4) of *Fusarium equiseti*, *Cladosporium cladosporioides*, or *Alternaria alternata* isolates. TEER was measured at different time points after the stimulation. Shown are the results of 3 (fungi, co-exposures) and 6 (ctrl., Phl p 1, Phl p 5, GPE) independent experiments performed with cells of different, non-allergic donors (mean ± SD). *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$, mixed effects model ANOVA with post hoc Dunnett's test (comparison vs. 0 h for each stimulation).

GPE by itself did not lead to a decrease in TEER [23, 40] which could reflect the presence of barrier-stabilising compounds in the complex pollen matrix.

In our experiments, nasal epithelial cells co-stimulated with grass pollen allergens and fungal spores released above-baseline levels of pro-inflammatory cytokines. This matches a previous publication [41, 42], which had reported an activation of the immune system by *C. cladosporioides* in a mouse model. Previously, a pulmonary epithelial cell line stimulated with Phl p 1 was shown to secrete pro-inflammatory cytokines and chemokines, among them IL-6 [43]. The discrepancy with our study could be explained by the fact that the mentioned study had used natural Phl p 1 purified from grass pollen, which could contain residual pollen-derived adjuvants. The endotoxin levels in the recombinant grass pollen proteins used in our study were very low (Phl p 1: < 0.01 EU/µg; Phl p 5: 0.003 EU/µg, as specified by the manufacturer), which could explain why they elicited no above-baseline cytokine response in our hands. Another explanation could be that different respiratory epithelial cell types might have different pattern recognition receptor profiles, explaining their different responses to the same protein [44, 45]. Several highly purified or recombinant pollen allergens, such as Bet v 1 (birch) and Amb a 1 (ragweed), were proven to be rather poor immunogens in the absence of an extrinsic (e.g., Alum) or pollen matrix-intrinsic adjuvant [4, 5]. Our results indicate that the recombinant grass pollen allergens alone are not enough to trigger a sustained barrier disruption and immune response in nasal epithelial cells. Fungi, including their spores, contain a plethora of PAMPs, such as glucans and chitins, which could provide innate signals that promote sensitisation to poorly immunogenic grass pollen proteins. This could even occur in the absence of any specific sensitisation to fungal proteins. Co-sensitization might also occur as a result of allergens triggering the formation of pores in epithelial cells [46]. Alternatively, co-sensitisation might simply be a stochastic outcome of frequent and intense co-exposure, without a direct mechanistic link. A general limitation of our study is the lack of molecular characterisation of the fungal spore preparations used to stimulate the cells. While we provide evidence that co-exposure to grass pollen and certain fungal spores exists and might be clinically relevant, the molecular mechanism of action of fungal spore- and grass pollen-derived substances possibly facilitating a co-sensitisation needs to be further investigated in targeted molecular biological experiments in vitro or in a mouse model. Specifically, it should be investigated if possible direct molecular interactions between fungal and grass pollen allergens, e.g., Alt a 1 and Phl p 1, contribute to the observed effects [47].

5 | Conclusion

Co-sensitisation to a variety of fungi is more frequent in grass pollen-sensitised individuals. Airborne fungi transiently modify the nasal mycobiome, and spores of fungi that were most abundant in the air during the study period have the potential to alter the physical and immunological barrier of nasal epithelial cells.

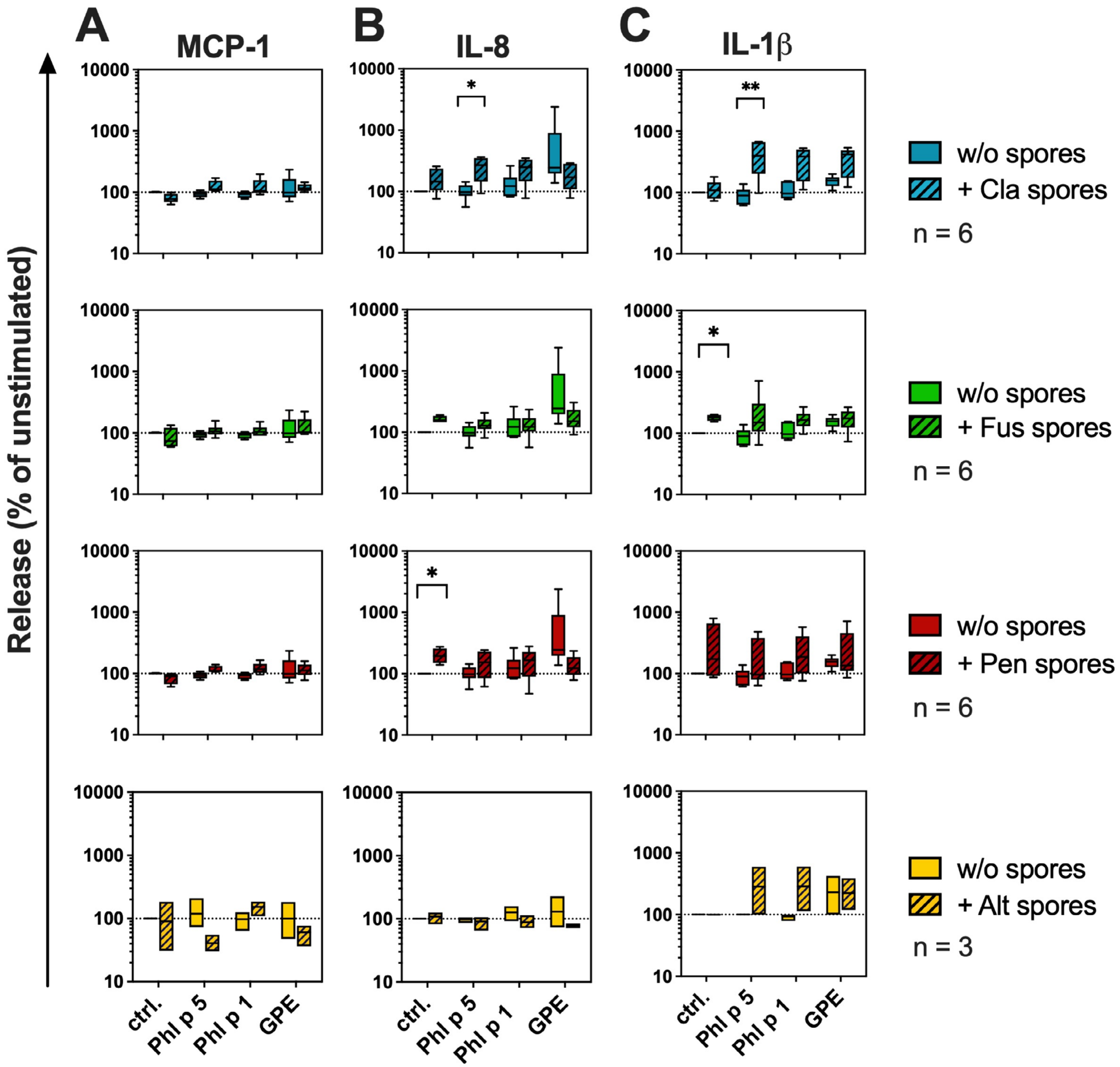

FIGURE 7 | Cytokine profiles in differentiated human nasal epithelial cells after exposure to grass pollen and fungal spores. Fungal spores were obtained from *Cladosporium cladosporioides* (blue), *Fusarium equiseti* (green), *Penicillium magnum* (red) and *Alternaria alternata* (yellow) isolated from air samples during the visits. The spores (10^4/well) were added to the apical side of the cells. Shown are normalised levels of MCP-1 (A), IL-8 (B) and IL-1β (C) measured in supernatants of HNECs of 6 (Cladosporium, Fusarium and Penicillium) or 3 (Alternaria) donors after 24 h of stimulation, as indicated on the *x*-axis. Ctrl, unstimulated control; GPE, aqueous grass pollen extract. *$p < 0.05$; **$p < 0.01$, one-way ANOVA with Dunn's post hoc multiple comparison test (fungal spores vs. w/o fungal spores).

Author Contributions

D.R. performed clinical study, DNA isolation from nasal swabs, allergen measurements in extracts of nasal filters, and analysis of the fungal microbiome. A.E. performed ALI culture experiments and drafted the manuscript. S.M.H. isolated and cultured fungi from air samples and stimulated nasal epithelial cells during her bachelor's thesis. F.K. and V.L.-W. classified airborne pollen and fungal spores, respectively. C.H. and M.B. helped with the annotation of fungal taxa and with microbiome analysis. B.F. and M.S. provided the PCR protocol for nasal mycobiome analysis. V.S. provided support in the clinical part of the study. C.T.-H. and M.R. provided discussion and critically reviewed the manuscript. A.D. designed the environmental sampling setup and supervised all fungal spore and pollen-related work. S.G. planned and supervised the clinical and immunological part of the project, prepared figures and co-drafted the manuscript. A.D. and S.G. shared the original idea for the study.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Clinical study data will be made available on request.

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