BACKGROUND

Persistent high humidity in the built environments favors indoor fungal growth with potential release of fungal particles into the air. Exposure to high levels of fungal aerosols is suggested as hazardous with critical implications for human health.\(^1,2\) A recent review of epidemiological studies concluded that exposure to fungi indoors is associated with the development and/or exacerbations of asthma, particularly in children.\(^3\) However, fungal exposure levels in moldy indoor environments are generally below that of outdoors, and associations with respiratory symptoms are generally not significant.\(^4\) Nonetheless, qualitative parameters such as "visible fungal growth" and "moldy odor" were significantly linked to the development of asthma and/or asthma exacerbations.\(^2,3,5,6\)

While fungal abundance in buildings with dampness problems is regarded as key determinant for symptoms reported by occupants, the causative components from fungi (non-pathogenic) remain unknown. It is likely that the magnitude of fungal exposure and subsequent health outcomes are dependent on multiple factors including fungal species composition, fungal aerosol composition, morphology (particle types, size, and shape), and biochemical constituents. Other not identified agents associated with fungal contamination may also

Abstract

Experimental aerosolization studies revealed that fungal fragments including small fragments in the submicrometer size are released from fungal cultures and have been suggested to represent an important fraction of overall fungal aerosols in indoor environments. However, their prevalence indoors and outdoors remains poorly characterized. Moldy basements were investigated for airborne fungal particles including spores, submicron fragments, and larger fragments. Particles were collected onto poly-L-lysine-coated polycarbonate filters and qualitatively and quantitatively analyzed using immunogold labeling combined with field emission scanning electron microscopy. We found that the total fungal aerosol levels including spores, submicrometer, and larger fragments in the moldy basements (median: \(80 \times 10^3 \text{ m}^{-3}\)) were not different from that estimated in control basements (\(63 \times 10^3 \text{ m}^{-3}\)) and outdoor (\(90 \times 10^3 \text{ m}^{-3}\)). However, mixed effect modeling of the fungal aerosol composition revealed that the fraction of fragments increased significantly in moldy basements, versus the spore fraction that increased significantly in outdoor air. These findings provide new insight on the compositional variation of mixed fungal aerosols in indoor as compared to outdoor air. Our results also suggest that further studies, aiming to investigate the role of fungal aerosols in the fungal exposure-disease relationships, should consider the mixed composition of various types of fungal particles.

KEYWORDS

fungal aerosol composition, fungal fragments, Immunogold labeling-FESEM

Fungal aerosol composition in moldy basements

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be involved. Also, the exposure duration and individual genetic susceptibility of exposed subjects are likely to be of great importance for the resulting adverse health effects.\textsuperscript{1,7}

To date, assessment of human exposure to fungal particles in indoor environments focuses predominantly on the concentrations of airborne or settled spores. Since the discovery that fragments smaller than 1 μm were released in larger numbers than spores in controlled aerosolization studies,\textsuperscript{9–10} detailed characterization of fungal aerosols including these fragments indoors and outdoors has become crucial. So far, related biomarkers and metabolites of fungi have been used in a number of studies aiming to uncover the occurrence and the importance of submicron fungal fragments in indoor environments. For example, β-1,3 glucans were detected in the submicron fraction of size-fractionated air samples or in samples of moldy walls and were assumed to be of fungal origin.\textsuperscript{11–15} Similarly, N-acetylhexosaminidase (NAHA) has also been used as a biomarker for fungal cell biomass.\textsuperscript{16} Although these biomarkers are antigens themselves, they cannot be fully attributed to fungi as they may originate from diverse organisms including bacteria, plants, and insects.\textsuperscript{17,18} To the best of our knowledge, studies reporting fungal aerosols with clear discrimination and enumeration of spores, submicron fragments, and larger fragments, whether in moldy indoor or outdoor air, are still missing. Of note, large hyphal fragments (>1 μm) have been detected and quantified in outdoor\textsuperscript{19–21} and indoor air\textsuperscript{22–24} but not the submicron fragments.

Mixtures of various types of particles and/or diverse microbial species may elicit immune-toxicological reactions that are completely different or similar to that of intrinsic single components.\textsuperscript{25} Dormant fungal spores were reported to induce non-allergic and TH\textsubscript{1}-polarized inflammatory reactions, whereas hyphal fragments elicited allergic and TH\textsubscript{2}-polarized allergenic adaptive responses.\textsuperscript{26} It has also been documented that the in vitro clearance of large fiber-like fungal/hyphal fragments by neutrophils involved NETosis (ie, neutrophil extracellular traps) with consequently release of high levels of reactive oxidative species.\textsuperscript{27} Based on such immunological differences, detailed characterization of the composition of fungal aerosols in moldy indoor versus outdoor may be of great importance in improving our understanding of the link between dampness related health problems and fungal aerosol exposure in indoor environments.

A recently developed method combining immunogold labeling and field emission scanning electron microscopy (IGL-FESEM) enabled us to identify and enumerate submicrometer fungal fragments as well as large fragments and spores in complex environmental air sample matrix. When this method was applied to personal samples collected in sawmills, we found a substantial proportion of fragments including submicrometer (9%) and larger fragments (62%) in addition to spores in the fungal aerosols.\textsuperscript{28} We therefore aimed at a detailed characterization of the fungal aerosols, including spores and fragments in the submicrometer and larger size ranges, in moldy basements of townhouses and simultaneously in outdoor air. Because basements often used for storage in Norway generally have poor ventilation, dampness with mold growth on building and/or stored materials is more common than in other rooms or apartments in houses.\textsuperscript{29,30} We selected therefore the basements to study the prevalence and levels of submicron fragments and larger fragments as well as spores. Moldy basements with visible mold were regarded as "worse case situation" of moldy indoor conditions. We focused particularly on the compositional variation of the fungal aerosols between indoor and outdoor as well as the influence of seasons (summer and autumn).

2 | MATERIALS AND METHODS

2.1 | Sampling of airborne particles

Air samples were collected in moldy basements of townhouse buildings (n = 7), control basements (n = 3), and simultaneously in outdoor air of the buildings backyards (n = 10). Sampling sites were located in the Oslo region, and one site located in Østfold County, all with a coastal climate. We used visible mold on walls or storage materials as the criterion in classifying problem and control basements. For details, photographs of the sampling sites are shown in Figure 1. Stationary sampling was adopted following the procedure described previously\textsuperscript{31} but with minor adjustments. Briefly, airborne particles were sampled onto poly-L-lysine-coated polycarbonate filters mounted in open 37-mm-diameter standard aerosol SKC cassettes (SKC Inc). During sampling, the cassettes were mounted on speaker stands at ~1.5 m aboveground level and were connected to a vacuum pump (Leybold Trivac DBA vacuum pump W/AEG MOTOR AMEB 90S4R3). The airflow was monitored with a mass flow meter (822 Top-Track; Sierra Instruments) mounted between the cassette and the pump. The average airflow rate was 14.7 L min\textsuperscript{−1} (SD: 2.24) at the start and 14.2 L min\textsuperscript{−1} (SD: 2.22) at the end of sampling. Sampling period was 3 hours. Filter samples were stored dry (RH ~ 30 ± 5%) at room temperature until preparation for IGL-FESEM. Meteorological conditions including temperature and relative humidity indoors were measured while outdoor parameters were obtained from www.yr.no (https://www.yr.no/place/Norway/Oslo/Oslo_Oslo_Tinghus/almanakk.html?dato=2017-03-16) (Table 1).

Practical Implications

- The present study provides new insight on the compositional variation of fungal particles including spores and fragments in indoor as compared to outdoor air.
- Fungal exposure in moldy indoor environments seems to be dominated by fungal fragments as compared to outdoor environments (commonly judged as normal) that contained mostly fungal spores.
- Our results suggest that further studies, aiming to investigate the role of fungal aerosols in the fungal exposure-disease relationships, should consider the mixed composition of various types of fungal particles.
2.2 Immunogold labeling of fungal fragments

Fragments were labeled on ¼-sector cutout of the sample filter. Prior to labeling, particles were first vapor-fixed with 25% glutaraldehyde overnight. The fixation step aimed to fix soluble antigens on the particle surface and simultaneously link permanently the particles onto the filter membrane through poly-lysine bonds. The filter specimens were then mounted onto a 25-mm carbon tab attached to a circular supporting metal grid and placed in a 6-well cell culture plate for downstream treatments. Immunogold labeling of fragments was performed following the procedure previously described.31 Briefly, free aldehyde binding sites were first quenched with 0.02 M glycine (MERCK) before being blocked with 5% skimmed milk freshly dissolved in Tris-buffered saline (pH 8, MERCK) and containing 0.05% Tween 20 (TBSTSM). Samples were then incubated with primary anti-fungi IgY antibodies (NABAS, Kroer Norway), washed with TBSTSM, and subsequently labeled with gold probes (25 nm) conjugated goat anti-IgY secondary antibodies (Aurion). After another washing step, the filter membranes were rinsed in water before being subjected to silver enhancement treatment for 15 min. using the Aurion kit (Aurion R-gent SE-LM). After a washing step that removes unreacted silver reagents, the samples were air dried under aseptical conditions. Dry membranes were, by mean of carbon adhesives, mounted onto aluminum specimen stubs (25 mm) and coated with 5-6 nm platinum in a Cressington 208HR Sputter Coater (Cressington Scientific Instruments Ltd.), before being subjected to FESEM analysis. All treatments were performed at room temperature.

2.3 FESEM analysis

The procedure used here was described elsewhere.28 Briefly, the composition of fungal aerosols including spores (S), submicron fragments (SF: ≤1 µm), and larger fragments (LF: >1 µm) in the samples was assessed using a FESEM (SU 6600 HITACHI) operated at low vacuum (25-30 Pa) and in the back-scattered electron (BSE) imaging mode. We imaged samples at 6-7 mm working distance and with an acceleration voltage of 15 kV. While spores were recognized by their morphology, fragments were identified as of fungal origin when labeled with gold particles. Significantly, labeled submicron fragments and larger fragments required at least two and four visible gold particles, respectively, so to distinguish fragments of fungal origin from background staining.31 The two types of particles were counted in 100 randomly selected fields imaged at 3000× magnification. The lowest detectable number of fungal particles at this magnification amounted to 3.0 × 10³ m⁻³ for an average of 3-hour air sampling (arithmetic mean of air volume: 2.7 m³). The levels of airborne fungal particle were estimated as the sum of the number of spores, submicron fragments, and larger fragments and reported as numbers of fungal particles per m³ air. Two unexposed filters were used as blank and revealed no background contamination by fungal particles.

2.4 Statistical analysis

To enable ratio and log calculations, all zero counts were arbitrary substituted by 0.1 before performing statistical analysis. We estimated the levels and the fractions of spores (S), submicron fragments (SF: ≤1 µm), and larger fragments (LF: >1 µm) by the arithmetic mean (AM) with standard deviation (SD) and the median with minimum-maximum concentrations. The levels and fractions of each fungal particle type from indoor (moldy and controls) air were compared with outdoor air and likewise for the seasons (summer versus autumn). For this purpose, we used the non-parametric Kruskal-Wallis (K-W) test as our data were not normally distributed (Shapiro test: P < 0.05) and of small size. Following a significant K-W-test, post hoc Wilcoxon rank-sum tests (or Mann-Whitney test) were used for multiple category
comparisons and Bonferroni adjusted the P-values (significant P-values < 0.017) were applied. Correlation between different types of fungal particles was also estimated using Spearman rank-sum correlation. Further, we applied linear mixed effect regression analysis with interaction terms to depict changes in the fungal aerosol composition in indoor versus outdoor (I/O) and between summer and autumn (seasons). To achieve this, we used centered log ratio (clr) transformed percentages of the particle types as reported previously.28 Schematically, the models were as follows:

\[
\text{For IO: } \text{clr} = \text{Intercept} + \text{Types} + \text{Types} \times \text{I/O} + \text{Repeated samples (random)} + \text{residuals}
\]

\[
\text{For Season: } \text{clr} = \text{Intercept} + \text{Types} + \text{Types} \times \text{Season} + \text{Repeated samples (random)} + \text{residuals}
\]

For all comparison tests in this study, a two-sided P-value of 0.05 was regarded as statistically significant. Benjamini and Hochberg's false discovery rate (5%) was used for multiple comparison adjustment in the clr models. All statistical analysis was performed with STATA 15 (Stata Corps).

### RESULTS

#### 3.1 Fungal spore and fragment concentrations

Spores and larger fragments as shown in Figure 2A and C, respectively, were detected in both outdoor and indoor air samples. Spores were found in all samples, whereas submicron fragments were only detected in two indoor samples. Larger fragments were detected in 17 out of 20 samples. The concentrations of total fungal aerosol particles, regardless of types, were not different in outdoor air samples (median: 90 × 10^3 m^{-3}) as compared to air samples collected in moldy (median: 80 × 10^3 m^{-3}) and control basements (median: 63 × 10^3 m^{-3}). With the IGL-FESEM method, the overall levels of submicron fragments and larger fragments were (median/AM ± SD) 1.3/2.0 ± 3.0 × 10^3 m^{-3} and 19/30 ± 20 × 10^3 m^{-3}, respectively, whereas the concentrations of spores were 41/70 ± 90 × 10^3 m^{-3}. Overall, the levels of spores were significantly higher than that of LF (P = 0.01) and SF (P < 0.001). Spearman correlation revealed that the spore concentrations were weakly correlated (r = -0.14, P = 0.5) and larger fragments were also weakly correlated (r = 0.15, P = 0.3). Similarly, submicron fragments and larger fragments were also weakly correlated (r = 0.10, P = 0.4).

### Table 1: Sampling sites and meteorological conditions

| Sample sites | Sampling date | Season | Outdoor temperature, °C (min-max)a | Outdoor precipitation, mm AM (min-max)b | Outdoor RH% AM (min-max)a | Outdoor wind speed, m/s AM (min-max)a | Indoor temperature, °C (min-max) | Indoor RH% (min-max) |
|--------------|---------------|--------|-----------------------------------|-----------------------------------------|--------------------------|----------------------------------------|-------------------------------|---------------------|
| A            | 23.09.2015    | Autumn | 13.3 (13-15)                      | 0.02 (0-0.1)                            | 89 (86-92)               | 2.8 (2.2-4.1)                         | 13-15                         | 62-65               |
| B            | 01.10.2015    | Autumn | 13.8 (11-14)                      | 0                                       | 70 (62-83)               | 7.6 (3.4-11)                         | 12-14                         | 74-76               |
| C            | 14.10.2015    | Autumn | 7.2 (5-12)                        | 0                                       | 70 (60-80)               | 2.2 (1.8-2.8)                        | 15-17                         | 81-83               |
| D            | 15.10.2015    | Autumn | 7.2 (3-11)                        | 0                                       | 77 (60-96)               | 2.0 (0.9-3.7)                        | 15-16                         | 47-49               |
| E            | 26.11.2015    | Autumn | -1.7 (3-0.2)                      | 0                                       | 94 (92-96)               | 2.1 (1.7-2.5)                        | 8-9                           | 49-53               |
| F            | 14.06.2016    | Summer | 21.2 (19-22)                      | 0                                       | 32 (26-43)               | 5.1 (2.6-6.7)                        | 15-17                         | 43-52               |
| G            | 15.06.2016    | Summer | 13.2 (13-14)                      | 0.46 (0.3-0.7)                          | 81 (73-86)               | 7.7 (5.3-9.2)                        | nd                            | nd                  |
| H            | 23.06.2016    | Summer | 18.6 (16-21)                      | 0                                       | 69 (61-82)               | 5.6 (4.5-7.5)                        | nd                            | nd                  |
| I            | 16.03.2017    | Summer | 6 (5-7)                           | 0                                       | 62 (57-67)               | 4.4 (2.0-6.6)                        | nd                            | nd                  |
| J            | 05.07.2017    | Summer | 17.4 (15-19)                      | 0                                       | 43 (41-53)               | 5.5 (4.2-6.7)                        | 17-19                         | 45-49               |
|              | Autumn        | 8 (3-15)                         | 0.004 (0-0.07)                         | 80 (50-96)               | 3.4 (0.9-11)                       | 0.9-16                         | 47-83             |
|              | Summer        | 15.3 (5-22)                       | 0.09 (0-0.7)                           | 58 (26-86)               | 5.7 (2.0-9.2)                      | 15-19                         | 43-52               |

Abbreviations: AM, arithmetic mean; m/s, meter per second; nd, not done.

aData during the sampling period gathered from yr.no.
As summarized in the Table 2, the outdoor concentrations of spores were significantly higher than the levels measured in the moldy and control basements \((P = 0.04)\), but the total fungal particle levels were not different between the three environments \((P = 0.3)\). Further, the level of spores was significantly influenced by season \((P = 0.03)\) with about 2-fold higher average concentration of spores in autumn than that in the summer season while the levels of submicronic and larger fragments were not affected by season \((P = 0.7 \text{ and } 0.3, \text{ respectively})\).

### Table 2 Overall concentrations of fungal particles (number of particles \(\times 10^3\) per m\(^3\)) and stratified by site and season

|          | Spores                          | Submicronic fragments          |
|----------|---------------------------------|--------------------------------|
|          | N  | AM  | SD  | Median | min  | max  | P-value | AM  | SD  | Median | min  | max  | P-value |
| Overall  | 20 | 72  | 88  | 41     | 8    | 332  |         | 2   | 2.9 | 1.3    | 0.7  | 13  |         |
| Sites    |     |     |     |        |      |      |         |     |     |        |      |      |         |
| Indoor   | 7  | 33  | 19  | 38\(a\) | 8    | 65   | 0.04    | 2   | 2.4 | 1.3    | 0.8  | 7.6 | 0.7     |
| Outdoor  | 10 | 112 | 110 | 69\(b\) | 16   | 330  |         | 1.3 | 0.2 | 13     | 0.7  | 1.6 |         |
| Control  | 3  | 30  | 19  | 27\(b\) | 13   | 50   |         | 5   | 7   | 1.3    | 1.2  | 13  |         |
| Seasons  |     |     |     |        |      |      |         |     |     |        |      |      |         |
| Summer   | 10 | 40  | 39  | 24     | 8.3  | 120  | 0.03    | 3   | 4   | 1      | 0.7  | 13  | 0.9     |
| Autumn   | 10 | 103 | 111 | 52     | 38   | 331  |         | 1.3 | 0.3 | 1.3    | 0.05 | 1.4 |         |

Note: \(P\)-values based on Kruskal-Wallis test for sites or Mann-Whitney test for seasons. Significant \(P\)-values in bold. Post hoc test using Mann-Whitney for the difference in spore concentrations between outdoor and moldy basement \((a: P = 0.02)\) and outdoor and control basement \((b: P = 0.06)\).

Abbreviations: AM, arithmetic mean; N, number of samples; SD, standard deviation.
spores, submicron fragments and larger fragments, and total fungal aerosols, respectively. The average I/O ratio of total fungal particles in the moldy basements was slightly higher than that in the controls but the difference was not significant. The average I/O ratios of spore and total fungal aerosols were below 1 and were significantly lower than that of fragments (P < 0.05). We found no significant difference between particle types related I/O ratios in the moldy basements as compared to the control basements. Further, the overall F/S ratios (ie, submicron and larger fragment/ spores) were AM/ median (range): 1.7/1.0 (0.2-5.5), 1.6/0.55 (0.3-4.0), and 0.32/0.22 (0.01-1.03) in moldy basements, in control basements, and in outdoor, respectively. The F/S ratio in moldy basements was significantly higher than that from outdoor (P = 0.005) but not different from the controls.

3.3 | Fungal aerosol composition

Considering the fungal aerosols as a mixture of spores and fragments, we analyzed the variations in the fungal composition in moldy indoor versus outdoor air and compared summer to autumn. We found that the proportions of spores dominated the fungal aerosol in outdoor air samples, whereas the fraction of larger fragments was dominant in the samples from moldy basements (Figure 3). We found significant differences between moldy indoor basements, control basements, and outdoor for the fractions of spores (P = 0.01) and larger fragments (P = 0.02). Further, the proportions of spores and larger fragments differed significantly between outdoor and moldy indoor air (both P < 0.01, Table 3), whereas these fractions were not significantly different in outdoor as compared to control basements (P = 0.1). Moreover, the proportion of fragments in control basements was more similar to that of the moldy basements. We also found that the proportions of the three types of fungal particles in autumn were not significantly different in summer (P > 0.05). However, a mixed effect linear regression modeling of the centered log ratio (clr), including the proportions of the three types of particles, showed significant decrease of spore fractions indoors versus outdoors (P = 0.001), whereas larger fragments increased significantly indoors versus outdoor (P = 0.003). This trend was similar for spores in moldy basements and outdoor (P = 0.008) but not for larger fragment proportions that did not change significantly between control basements and outdoors (Table 4). With seasons, this approach revealed that the fraction of spores increased significantly in autumn as compared to summer (P = 0.012) while the fragment fractions remained unchanged (Table 4).

4 | DISCUSSION

With the IGL-FESEM method, we were able to visualize and quantify submicron fragments, larger fragments, and spores simultaneously in both indoor and outdoor air samples. The detection of the three

FIGURE 3 Bar chart of the arithmetic mean percentages of spores, submicron fragments and larger fragments in moldy indoor (I), outdoor (O), and control basements (C). Error bars represent standard deviation of each fungal particle proportion. Bars with similar letter are significantly different according to post hoc test using Mann-Whitney, (ie, P < 0.017) for a, P = 0.005 and for b, P = 0.007
types of fungal particles at the investigated sites confirmed the mixed composition of fungal aerosols in moldy basements as suggested from laboratory aerosolization studies.8–10 The mixed effect regression modeling of the proportions of fungal particles revealed that there is a significant change in the composition of the fungal aerosol, as there is a significant shift of the dominating particle type fraction in indoor as compared to outdoor, as well as in the summer season versus autumn. To the best of our knowledge, no study has investigated such compositional changes of complex fungal aerosols in indoor environments versus outdoor or between seasons. The characterization of the mixed fungal aerosols as described in the present study also provides a broader and holistic analytical characterization that revealed how the fungal aerosol composition including spore and fragment fractions varied in moldy basements versus outdoor air.

Based on the properties of outdoor mixed particulate matters that can differentially influence the innate and adaptive immune responses because of the diverse origins and chemical compositions of constituent particles, we can speculate that changes in the composition of fungal aerosols, as revealed in this study, may likewise influence the adverse health effects related to fungal exposure. Dormant spores were reported to elicit non-allergic inflammation, whereas hyphal fragments and germinating spores induced allergic inflammatory responses.26,33 As such, adverse health effects induced by fungal exposure indoors may, not only, be depended on the levels of fungal aerosols (that are commonly low in such environments), but also on the prevalence of various types of fungal particles in the mixed fungal aerosols.

Although the enumeration of submicron fungal fragments has never been reported in moldy basements and outdoor air samples, several studies have indirectly investigated their occurrence by detection of fungal related constituents in suspected environments, namely beta-glucan and N-acetylhexosaminidase (NAHA). Measured (1, 3)-beta-glucans and NAHA levels in the submicrometer size fraction of air samples from indoor environments were assumed to originate from the submicron fungal fragments.11,16,34 But, as opposed to our study where SF were convincingly identified and visualized, the fungal origin of these biomarkers could not be guaranteed since they may originate from bacteria, plants, and insects.17,18 Additionally, the size fractionation of dust is not fully efficient as larger fragments and spores have been found in the assumed submicrometer fraction.12,14,35 Projections based on (1, 3)-beta-glucans and fragment/spore ratios suggested about 10^3 to 10^6 times the levels of SF as compared to spores.11 In our study, SF were found only in two indoor samples at average concentrations (0.02-0.05 × 10^3 m^-3) and were significantly below the spore concentrations. These data are consistent with previous findings in sawmills,8 but departs largely from levels measured in aerosolization studies and (1, 3)-beta-glucans-based projections, that is, that the spore concentrations are significantly higher than that of submicron fragments. The low levels of SF in present study may be due to the differences in the aerosolization mechanisms governing the release of these particles from fungal biomass in the indoor

### TABLE 3

|                | Overall proportion of fungal aerosols (%) | Spores | Submicronic fragments | Large fragments |
|----------------|-----------------------------------------|--------|----------------------|-----------------|
|                | N AM SD Median min max P-value | AM SD Median min max P-value | AM SD Median min max P-value |
| Sites          |                                        |        |                      |                 |
| Indoor         | 20 64 24 66 15.4 99.3 3.3 4.5 1.8 0.4 20 | 7 47 21 3 2 1.4 7.7 49 99 2 23 15 3 2 0.4 8 1.5 60 8 2 0.4 99 16 3 2 0 | 3 2 2 1.4 7.7 (0.07) 50.2 19 49 99 2 23 15 3 2 0.4 8 1.5 15.3 19 49 99 2 23 15 3 2 0.4 8 1.5 0.02 |
| Outdoor        | 10 79 15 9 49 99 2 23 15 3 2 0.4 8 1.5 60 8 2 0.4 99 16 3 2 0 | 10 54 30.5 64.5 20 78 20 78 10 54 30.5 64.5 20 78 20 78 10 54 30.5 64.5 20 78 20 78 | 10 54 30.5 64.5 20 78 20 78 10 54 30.5 64.5 20 78 20 78 10 54 30.5 64.5 20 78 20 78 |
| Control        | 3 54 30.5 64.5 20 78 20 78 10 54 30.5 64.5 20 78 20 78 10 54 30.5 64.5 20 78 20 78 | 3 54 30.5 64.5 20 78 10 54 30.5 64.5 20 78 10 54 30.5 64.5 20 78 | 3 54 30.5 64.5 20 78 10 54 30.5 64.5 20 78 |
| Seasons        |                                        |        |                      |                 |
| Summer         | 10 68 20 75 40 96 7 3 8 2 10 25 20 31 17 77 0.1 | 10 68 20 75 40 96 7 3 8 2 10 25 20 31 17 77 0.1 | 10 68 20 75 40 96 7 3 8 2 10 25 20 31 17 77 0.1 |
| Autumn         | 10 49 22 54 15 78 (0.08) 12 7 10 3 8 2 2 10 18 17 2 53 | 10 49 22 54 15 78 (0.08) 12 7 10 3 8 2 2 10 18 17 2 53 | 10 49 22 54 15 78 (0.08) 12 7 10 3 8 2 2 10 18 17 2 53 |

Note: Kruskal Wallis test for sites and Mann-Whitney test for seasons. Significant P-values in bold. Post hoc test using Mann-Whitney (data with similar letter are significantly different, i.e., P < 0.017) for

Abbreviations: AM, arithmetic mean; N, number of samples; SD, standard deviation.
settings as compared to laboratory experiments. As the release of SF in aerosolization experiments is mainly caused by shearing forces from high velocity air currents blowing over fungal cultures, the much lower velocity of air currents blowing over building material covered with fungal biomass in basements may explain the lower levels of SF in the present study. Also, the large spatial and temporal variation of abiotic and biotic factors may greatly influence the aerosolization of particles indoors. Nevertheless, the documentation of submicron fragments and larger fragments in indoor and outdoor samples provides additional dimensions of the fungal exposure and need to be included in fungal exposure-disease relationships.

Larger fragments were detected in 70% of outdoor samples (concentration range 0.12-48 × 10^3 m\(^{-3}\): AM: 20 × 10^3 m\(^{-3}\)) vs 100% of indoor samples (concentration range 1.3-76 × 10^3 m\(^{-3}\): AM: 35 × 10^3 m\(^{-3}\)). Our AM of outdoor levels were close to outdoor levels reported in Danish strawberry fields: 41 × 10^3 m\(^{-3}\), but were substantially higher than that of hyphal fragments discernible by light microscopy reported in United Kingdom by Pady and Kramer: 1.4 × 10^3 m\(^{-3}\) in Canada by Li and Kendrick: 0.11 × 10^3 m\(^{-3}\) and in Southern California (USA) by Delfino and co-workers: 0.20 × 10^3 m\(^{-3}\). On the other hand, these levels were far lower than those recorded in occupational settings like during renovation of moldy walls: 600 × 10^3 m\(^{-3}\) work in grain farming: 3000 × 10^3 m\(^{-3}\) and both studies from Norway and during seed processing work in Denmark: 360-870 × 10^3 m\(^{-3}\). Regarding the overall indoor concentrations of fragments, they were many folds higher than those reported by Li and Kendrick: 0.15 × 10^3 m\(^{-3}\). These differences may be attributed to aerosolization determinants that influence their release from fungal colonies, but in addition, methodological efficiency may be of great significance here as the microscopic resolution is highly improved with FESEM as compared to light microscopy. Also, the immune-labeling technique helped visualizing the fragments that are difficult to identify by morphology.

Compared with spore concentrations, the levels of total fungal particles in moldy (median: 80 × 10^3 m\(^{-3}\) vs 38 × 10^3 m\(^{-3}\)) and control basements (median: 63 × 10^3 m\(^{-3}\) vs 27 × 10^3 m\(^{-3}\)) were about 2-fold higher, indicating that fungal aerosol exposure indoors is almost equally distributed between spores and fragments. The concentrations of spores measured in the moldy basements (range: 8-65 × 10^3 spores m\(^{-3}\) were relatively higher than that reported in normal dwelling places with visible fungal growth on walls (range 0.01-2.3 × 10^3 CFU m\(^{-3}\)) assuming that 1 CFU m\(^{-3}\) is equivalent to 10 spores m\(^{-3}\) so to enable comparison with studies that measured CFU m\(^{-3}\). Of note, this equivalence is a crude simplification as the culture counts vary from 0.1% to 100% of the microscopic counts. We suspect that limited ventilation played key role in the high levels of spores in the moldy basements as compared to other

| TABLE 4  | Clr mixed effect regression models stratified by sites (moldy basements (I), control basements (C) and outdoor (O)) and seasons (summer and autumn) with the types of fungal aerosol as fixed effects and the repeated sample ID as random effect |
|----------|--------------------------------------------------------------------------------------------------|
| **Sites**| **B** | **SE** | **P-values** | **Seasons**| **B** | **SE** | **P-values** |
| Intercept | 2.02 | 0.23 | 0.000 | Intercept | 1.88 | 0.23 | 0.000 |
| Particles | | | | | | | |
| S | Reference | | | | | | |
| LF | -1.97 | 0.32 | 0.000 | LF | -1.62 | 0.39 | 0.000 |
| SF | -4.08 | 0.32 | 0.000 | SF | -4.0 | 0.33 | 0.000 |
| Interaction Type × Site | Interaction type × Season | | | | | | |
| LF × O | Reference | | | LF × Autumn | Reference |
| LF × I | 0.97 | 0.36 | 0.003 | LF × Summer | 0.41 | 0.33 | 0.2 |
| LF × C | 0.48 | 0.48 | 0.3 | LF × Autumn | Reference |
| SF × O | Reference | | | SF × Autumn | Reference |
| SF × I | 0.12 | 0.36 | 0.7 | SF × Summer | 0.42 | 0.33 | 0.2 |
| SF × C | 0.69 | 0.48 | 0.1 | S × O | Reference |
| S × O | -1.09 | 0.36 | 0.001 | S × Autumn | Reference |
| S × C | -1.16 | 0.48 | 0.008 | S × Summer | -0.83 | 0.33 | 0.01 |
| LR test | | | | | | | |
| df | 11 | | | df | 8 | | |
| \(\chi^2\) | 0.0003 | | | \(\chi^2\) | 0.012 | | |

Note: P-values: significant values in bold. Abbreviations: B, regression coefficient; df, degree of freedom; LF, larger fragments (>1 µm); LR test, likelihood ratio test; S, Spores; SE, standard error; SF, Submicron fragments (0.2-1 µm).
moldy indoor environments with better ventilation. Limited air exchange or ventilation also favors high humidity that in turn promotes fungal growth.2

The spore levels outdoors were relatively higher than the concentrations measured indoors although not significantly different. This trend was also reported by Shelton and colleagues in one of the largest study on the assessment of cultivable fungi in indoor and outdoor environments in the United States. Significantly higher median levels of fungi (540 CFU m⁻³) were recovered in outdoor samples versus indoors (82 CFU m⁻³).47 Our median levels were 46 times (indoors) and 13 times (outdoors) higher than that reported by Shelton et al. Such discrepancy may be due to methodological efficacy of the microscopic method that counts both dead and viable particles versus cultivation method that considers only particles capable of germinating on artificial media.

It is also possible to link our spore data from outdoor air to many other studies that used microscopic enumeration. For example, our median spore levels (outdoor: 69 × 10³ m⁻³) were many folds higher than that published by Li and Kendrick in samples from outside residences in Kitchener-Waterloo (Ontario, Canada): 2.9 × 10³ m⁻³.21 Codina and colleagues in outdoor samples from Tampa (Florida, USA): 2.4 × 10³ m⁻³.48 Fernandez-Rodriguez et al in samples from Badajoz (Spain): 0.09-10 × 10³ m⁻³49 and Jara et al in samples from outdoor in North American Midwest: 9 × 10³m⁻³.50 However, our data are comparable with the ranges reported by Gots et al when reviewing studies that covered various regions in United States: 0.4-80 × 10³ m⁻³51 and Reponen and co-workers in New Orleans and Ohio (1.1-8.4 × 10³ m⁻³).11 Based on seasons, the moldy indoor levels of spores were highest during autumn (median: 52 × 10³ m⁻³) as compared to summer (median: 24 × 10³ m⁻³) (P = 0.03) and this corroborates well with some earlier studies.47,52 However, in study from southwest United States and Leicestershire in England, higher indoor levels were found in summer and not in autumn in residential buildings.47,53 This difference may be attributed to the large variability in the absolute levels of airborne spores by site and regions. Further comparisons based on data stratified by indoor/outdoor air revealed that only the moldy indoor basement levels of spores were influenced by the season (data not shown). This indicates that despite the limited air exchange rate in townhouse basements, the basement environments are likely affected by high relative humidity outdoors (Table 1), probably by capillary infiltration of water/humidity through basement walls or cracks. As such, the higher levels of spores in autumn may be linked to increased relative air humidity indoors that favored microbial growth with consequent release of high levels of spores. The outdoor levels of spores did not vary similarly contradicting therefore previous reported studies.47,49,54 This discrepancy may be explained by the Scandinavian autumn characterized by high pluviometry that favors an increased sedimentation of outdoor particles.

We estimated also the I/O ratios in the moldy and control basements. Indoor environments with I/O ratio of fungal spores above 16% were suggested critical for IAQ (Indoor Air Quality) assessment although this criterion is not associated with health effects.47,53 Of note, there is no global health-related limit value of I/O ratios for evaluating fungal spore exposure indoors.55 In the present study, the median spore-based I/O ratio in the moldy basements (48%) was higher than that of the controls (28%). This is similar to fragments and total fungal aerosols. The median I/O ratios based on spores of the moldy and control basements were higher than 16% indicating that the moldy basements presented critical IAQ. Moreover, the average I/O ratio based on larger fragments was about twice as high in moldy basements versus the control basements although the difference was not significant. Hence, an evaluation based on I/O ratios of larger fungal fragments should be included in fungi-related IAQ assessment.

Beta-glucans measured from fragments in size separated sample fraction versus spore in the same size fraction (F/S ratio) has previously been used as an indirect approach to estimate the relative amount of fungal fragments in size-fractionated environmental and laboratory samples.11,12 In fact, environments with F/S ratio >1 were suggested of having higher fungal fragments exposure as compared to spores. Interestingly, our results revealed median F/S ratio above the unit for moldy basements while in control basements and outdoor medians were about 2- and 3-fold lower, respectively. Comparison between our findings and previously reported studies using beta-glucans mass-based F/S ratio11,12 is difficult as those studies limited estimated fragment mass to the submicrometer size fraction while larger fragments seem to be the main contributor to the fragment mass. We also found that the F/S ratio in moldy basements (AM/median: 1.71/1.05) was significantly higher than that outdoors (AM/median: 0.32/0.22): P = 0.005, but not different from that the control basements (AM/median: 1.61/0.55) P = 0.6. This is an indication that fragments are more prevalent in basements, probably because of the poor ventilation and low exchange air rate. We can also speculate on the sedimentation velocity of spores that may be higher than that of fragments in such environments due more compact and genus-/species-specific isometric shapes of spores versus the polymorphic shapes of fragments.

We do not know yet the clinical implications of exposure to fungal fragments and mixture of fragments and spores. However, measuring the mixed fungal aerosols during exposure assessment will be of great importance in future studies on the role of fungal exposure indoors and airways diseases for two main reasons: Firstly, the nature of immune-toxicological responses induced by fungal exposure is likely a multifactorial mechanism governed by particle types, morphology, and antigenic composition as well as the host genetic disposition.7 Morphological diversity of fungal particles (spherical, oblong, or fiber-like in shape) may influence the particles aerodynamic deposition patterns in the human respiratory tract upon inhalation,10,56 and the type of immune reactions induced through cytokines, chemokines from involved immune cells dependent on the deposition sites in the airways.7 Species diversity is also of great importance as some common indoor fungi induce particularly allergenic responses versus others that induce non-allergic inflammatory reactions.57 Therefore, we may get new insights in the role played by fungi in respiratory morbidity when we base our characterization on a holistic approach. Secondly, the paradigm of
spores as the major fungal exposure particles bearing allergens and fungal metabolites. Submicron fragments have been suggested as the most prevalent fungal pathogens in indoor exposure. Although our data did not support the idea that spores are more prevalent in indoor environments, the importance of considering the heterogeneous composition of fungal aerosols in both indoor and outdoor settings is highlighted.

The major weakness of the present study is the small size of our data. The small number of sites was due to difficulties of getting approval from home owners for mold sampling. It is therefore important to consider the observed statistical differences with some caution. With increased sample size, future studies linking compositional exposure data to health outcomes will be of great significance in confirming our findings and to further investigate how compositional and concentration based analyses influence the fungal exposure-disease relationship.

5 | CONCLUSIONS

The IGL-FESEM detection approach used in the present study is relatively tedious but allows specific quantification and classification of fungal particles by type and size. Despite the small size of our data, the airborne fungal particles in indoor environments seem to be dominated by large fungal fragments while there are more spores in outdoor air. The fraction of submicron fragments in the fungal aerosol was relatively small, even absent in all outdoor samples, whereas the larger fragments represented the largest fungal fraction in the indoor samples. The present study provides therefore very useful information on the heterogeneous composition of the fungal aerosols in both townhouse basements and outdoor. Further studies are needed to elucidate not only the role of fungal fragments, but also of the mixed fungal aerosols in health effects from indoor and outdoor exposure to fungal aerosols.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

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