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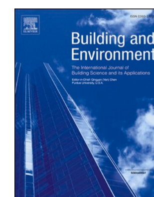

The aerobiome in a hospital environment: Characterization, seasonal tendencies and the effect of window opening ventilation

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ABSTRACT

The urban atmosphere carries biological particles (bioaerosols) that may cause several diseases and allergies. These bioaerosols infiltrate and mix with those present inside the buildings, including hospitals. However, little is known about the behavior of these particles around health facilities. Here, we described the bioaerosols composition of an urban hospital indoor and outdoor at two different periods (winter and summer) using DNA sequencing. We observed that the seasonality and composition of the bioaerosols outdoor was also displayed indoor, and, in some cases, the taxa showed different trends depending on the season. Pathogenic species of bacteria and fungi were found indoors at low levels but also outdoors, being mostly environmental species, which would reject the idea that hospitals may be acting as a source of emission via aerosols. Skin-related bacteria were the most prevalent group related to human microbiome, being more abundant indoors. Air temperature was the principal factor affecting the bioaerosols composition in the samples but, in general, meteorological parameters outdoors were poor descriptors of the bioaerosols indoors. Similarly, the concentrations of the main indoor air pollutants did not correlate with microbial abundances. Globally, natural ventilation through a window opening did not significantly alter the composition of the bioaerosols indoor.

1. Introduction

In our daily routine, we are unavoidably exposed to a wide variety of aerosols of biological origin, both indoors and outdoors, due to their ubiquity in any habitat on the planet [1]. Pollen grains and airborne fungal spores can trigger or worsen symptoms of asthma, allergy, rhinitis and obstructive lung diseases, which affect millions of people worldwide [2–4]. In addition, bacterial infectious diseases such as tuberculosis or legionellosis [5,6], or mycoses as aspergillosis and histoplasmosis [7,8] are airborne transmitted. Moreover, it has been proposed that bioaerosols can exacerbate respiratory diseases and infections associated with air pollutants (mainly particulate matter concentration) in human and animal models [9–11], and also alter the natural microbiota in the respiratory tract or the gut microbiome [12,13]. Thus, inhaled bioaerosols may have potential consequences for other non-respiratory maladies such as obesity or diabetes [14]. On the other hand, the abundance, presence, dispersion and exchange of bioaerosols between indoor and outdoor environments are under the influence of numerous variables such as the proximity of the emission sources, meteorological factors, anthropogenic activities, seasonal changes on environmental and human patterns, etc. [1,15–17]. Therefore, because of its impact on health and the complexity of analyzing the multiple variables that may be involved in its diversity and dynamics, the so-called aerobiome is currently a flourishing field of study.

Since we spend most of the day indoors [18], many studies analyzing the microbial composition of the air inside different types of buildings have been performed, including houses, schools, offices, college and university premises, daycare centers, kindergartens, etc. [19–24]. As a result, a series of parameters determining the bioaerosol composition indoor have been identified, like the composition outdoor, the time of the year (season), type of ventilation, occupancy or human activities in the rooms [15,17,25]. Among all the types of buildings, healthcare centers and hospitals are particularly interesting for their association with pathogenic microbes. Hospitalized patients are especially sensitive to nosocomial infections, affecting ca. 15% of the inpatients [26]. Moreover, some areas are dedicated to infectious diseases, which might act as a point of dissemination. Additionally, many microbes isolated from hospitals are highly resistant to treatment because they carry antibiotic resistance genes (ARGs), which seems to be favored by the selective pressure of this particular environment on the microorganisms

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[27–29]. However, there is a lack of surveys conducted in this type of buildings addressing the study of bioaerosols by molecular methods. Most works are focused on one group of microorganisms (bacteria, fungi or particular species), and/or use culture techniques, neglecting the wide diversity that cannot be recovered by culture and only detectable by DNA-based analyses. Furthermore, samplings are usually carried out in a short period of time when bioaerosols outdoors fluctuate remarkably along the year [30,31].

Consequently, the air composition inside hospitals and the influencing factors are still poorly elucidated. Here, we compared the bioaerosols present in a hospital, indoor and outdoor, at two different seasons, to assess the influence of the urban atmosphere within this particular facility. Airborne bacterial and fungal communities were surveyed by DNA sequencing to identify general and particular tendencies. We also examined the presence of pathogens and human-related microorganisms to determine if they follow similar trends. Moreover, we evaluated whether natural ventilation throughout a window opening, a common strategy in many buildings, is really effective in altering the bioaerosols indoors. Finally, we analyzed the relationships of these biological particles with meteorological parameters and air pollutants to evaluate their use as predictors of the bioaerosols composition.

2. Material and methods

2.1. Site description and air samples collection

The bioaerosols sampling was conducted in "Hospital Clínico San Carlos", a healthcare institution located within the urban environment of Madrid (Spain) but surrounded by several wide urban parks (40.4417392°N 3.7206851°W, 675 m AMSL). A total of 45 samples were collected during two collection campaigns: Summer (23rd June to 10th July 2020) and Winter (10th to 26th February 2021), with the same collection points: one indoor and one outdoor. Additionally, within each campaign, the samples indoor were taken under two situations: window open or closed (see Table S1 for detailed conditions of each sample).

The samples outdoor were taken at 1.5 m above the ground, on the roof of one of the wings of the building, which is at a lower height than the main building (2nd floor and 6 m below the location of the room where the samples indoor were collected). The room used for the sampling (7.2 × 5.7 × 3.0 m, w x l x h) was next to an inpatient wing on the 4th floor of the building, at 12 m AGL. There was a window of 2.1 × 2.4 m (w x h) facing the collection point outdoor, which was used to evaluate the effect of the window opening according to the sampling design (see Fig. S1 for the location of the collection sites). Any human activity in the room was cancelled during the sampling periods and neither heating nor AC was used. No special cleaning treatments were carried out before the sampling campaigns and the regular cleaning was cancelled.

Two identical impactor-type devices (DUO SAS Super 360 (VWR)) were employed to collect the samples. The two heads for Petri dishes of this particular model allowed us to collect replicates. The devices were placed on their respective locations (indoor and outdoor) and the samples were taken simultaneously at an airflow rate of 180 L/min for 2 h (−21.6 m^3 of air per Petri dish). Previously, under aseptic conditions in a biosafety cabinet and using sterile gloves, the Petri dishes were covered with pharmaceutical petroleum jelly (Vaseline, Interapothek, Spain), which acted as adhesive surface to collect the airborne particles. The Petri dishes were kept closed at 4 °C until the sampling time. The heads of the samplers were cleaned and autoclaved each day after sampling. The collections were carried out within the timeframe 9:00–14:00 a.m. throughout the two campaigns. The window in the room was kept closed at all times, the whole day during the campaigns, taking the samples WinC under this condition. In the last five days of each campaign, we evaluated the effect of the natural ventilation by window opening (samples WinO). To do so, the window of the room was open while the collection was conducted (2 h), providing an opening area of 2 m^2 to the air outdoor, and then it was closed again for the remainder of the day.

2.2. Meteorological data and air pollutants concentrations

Meteorological data of temperature, relative humidity, wind speed and direction were obtained from a portable unit sited on the roof of the main building (8th floor), at 28 m AGL. Atmospheric pressure and solar radiation measurements were compiled from a station installed in "Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas" (CIEMAT), at 1.8 km from the sampling site. Concentrations of air pollutants (nitrogen monoxide (NO), nitrogen oxides (NO_x), nitrogen dioxide (NO_2), particulate matter < 1 μm (PM_1), <2.5 μm ($PM_{2.5}$), <10 μm (PM_{10}), and Total Number of Particles (TNP)) were measured indoor and outdoor, the latter from a mobile air quality station located in the hospital parking lot (see Fig. S1).

2.3. DNA extraction and sequencing

The Petri dishes with the collected material were kept at 4 °C until DNA extraction was performed later in the same day. The petroleum jelly (with the particles adhered) was recovered using a sterilized razor and put into an extraction tube of DNeasy Powersoil Kit (Qiagen). DNA was extracted and purified following steps described in the manufacturer's guideline. Our previous surveys showed that the DNA concentration in this type of samples is low, so the buffers containing the DNA of each replicate were merged and spun through the same purification column of the kit. In such way, we increased the DNA concentration to carry out the following DNA sequencing protocol and, in addition, we obtained a more representative sample because combining two replicates reduces the variability associated with sampling airborne biological particles. DNA samples were submitted to high-throughput amplicon sequencing at the Genomics service of "Parque Científico de Madrid" (Madrid, Spain), using Illumina® Mi-Seq platform (2 × 300 reads). The following universal primers sets were used to generate the amplicon libraries: Bakt_341 (F): 5′-CCTACGGGNGGCWGCAG-3′; Bakt_805 (R): 5′-GACTACHVGGGTATCTAATCC-3′ [32] for the amplification of the regions V3–V4 of the gene 16S rRNA of bacteria; and ITS86 (F): 5′-GTGAATCATCGAATCTTTGAA-3′ [33], ITS-4 (R): 5′-TCCTCCGCTTATTGATATGC-3′ [34], for the region 5.8S – ITS2 of fungi. The negative control (the same sampling practice but with the air sampler turned off, followed by the same DNA extraction procedure) resulted in no DNA amplification using the same protocol. As a part of the quality control of the sequencing process, a sample with DNA from the phage PhiX174 was loaded to discard the possibility of cross-contamination between samples. Additionally, an air sample processed as described here and previously sequenced was also included as a positive control to verify that similar results were obtained.

2.4. Sequence assembly, pre-processing and normalization

DNA sequences were submitted to DADA2 pipeline (v1.17.5) [35] with default parameters in R environment [36] and the identified chimeras were filtered out during the processing. The taxonomy assignment of the Amplicon Sequence Variants (ASVs) was performed using SILVA [37] (release 138) and UNITE [38](version 8.3) databases for bacteria and fungi, respectively. Despite of using primers sets designed to amplify microbial DNA, plant DNA is usually detected due to the presence of pollen grains in the air, which is identified as Order "chloroplast" in the 16S rRNA analysis and as Kingdom "*Viriplantae*" in the ITS analysis. Those were correspondently annotated as "Plants" at Phylum and Genus ranks for figures and analyses purposes. The package "metagenomeSeq" [39] was used to normalized the number of reads caused by the differences in sequencing depth and processing steps.

2.5. Data analyses and statistics

General analyses were conducted in R environment with the packages “phyloseq” [40] (version 1.34.0) and “vegan” (version 2.5–7). Bray-Curtis dissimilarity matrices were used to conduct Principal Coordinates Analyses (PCoA), and the analysis of similarity (ANOSIM) was set to 999 permutations. Statistical differences between the abundances of taxonomical groups were tested by pairwise comparisons using Welch’s test. Alpha-diversity indexes were calculated after rarefying the abundance matrices, setting the threshold to the sample with the lowest number of reads in the matrix. For the redundancy analyses, the respective matrices were transformed using Hellinger standardization, submitted to distance-based redundancy analysis (dbRDA) with log-transformed meteorological and pollutants data, and the explained variances were corrected using adjust R^2. Using the function “ordiR2-step” in the package “vegan”, we selected the variables significantly associated with our biological data (marked in the figures with asterisks, which also indicate the level of significance). We improved the explanatory model by exploring the rest of the variables, selecting those that improved the R^2 value but also restraining the values of variance inflation factors (vif) < 3 in order to discard collinear variables.

The correlations between the abundances of microbial phyla and meteorological parameters or air pollutants concentrations were evaluated using Spearman’s rank correlation test implemented in the R package “psych” (version 2.2.5) and correcting p-values by Benjamini & Hochberg approach to reduce the false discovery rate (FDR).

2.6. Pathogens and human-related bacteria

To study the human pathogens present in our work, the list of microbial pathogens having *Homo sapiens* as a host was obtained from PHI-base (http://www.phi-base.org/) [41], and complemented with the list of biological risks at work from the National Institute for Occupational Safety and Health in Spain (INSST; https://www.insst.es/) (Tables S2 and S3). For the human-related bacteria, recent works providing a list of microorganisms isolated from the most relevant parts of the human body (skin [42], oral (including mouth, nostrils and throat [43]), and gut [44]), were used to create a customized database with their respective localization (Table S4). For those species found in different parts, combined categories were created.

2.7. Total cultivable bacteria

Colony Forming Units (CFU) of airborne bacteria were estimated during the Summer campaign, both indoor and outdoor, employing the air sample device running for 20 min (3.6 m^3 of air), and Petri dishes containing Nutrient Agar (CM0003, *ThermoFischer Scientific*) supplemented with 2.5 μg/mL amphotericin B (171375, *Calbiochem*) to prevent fungal growth. The Petri dishes were incubated for 3 days at 35 °C before the colony counting.

3. Results

3.1. Overview

Two campaigns (Summer and Winter) were carried out to collect samples of bioaerosols indoor (“Indoor”) and outdoor (“Outdoor”) within the hospital premises, compiling a total of 45 air samples (25 in Summer and 20 in Winter; see Table S1). For each one, some samples were taken with the window closed (“Indoor WinC”), so the air exchange with outdoor could only occur by infiltration (unintentionally through holes, cracks, window and door frames, etc.). In these samples, we evaluated the existence of differences of bioaerosols between indoor and outdoor because of the characteristics of the building (structural and activity-related). Additionally, a set of samples was collected with the window open (“Indoor WinO”; see Materials and methods for details) to analyze the effect of natural ventilation on the bioaerosols in the room using this common strategy to airing rooms.

The bioaerosols were characterized using high-throughput sequencing, targeting bacterial and fungal DNA, obtaining a total of 19,267 and 9,247 ASVs, respectively.

An overview of the samples by Principal Coordinates Analysis (PCoA) revealed a striking variability associated with the period of the year in which the samples were taken (Campaign). This suggests a marked seasonality in the bioaerosols present outdoor but also indoor, both for bacteria and fungi (ANOSIM test R = 0.685 and 0.833, respectively; Fig. 1).

These seasonal patterns were also observed for bacterial richness and diversity indices (Table S5), where the species richness was higher in Summer, as were the Shannon and Simpson diversity indices. These tendencies were similar for both indoor and outdoor environments. On the contrary, Chao1 and Observed species indices were not remarkably different for fungi between seasons. This contrasts with the marked seasonality distribution obtained in Fig. 1, suggesting that some species are replaced by others in an equivalent number.

3.2. Microbial communities in the air indoor

Previous works have reported that airborne microbial communities tend to develop a core of species or characteristic taxa composition [30, 45,46]. Therefore, we analyzed the bioaerosols indoor (samples WinC) for both periods (Summer and Winter) to evaluate the existence of a microbial core in this environment. As shown in Fig. 2a, the most abundant taxa were sensitive to seasonal changes. For instance, the phyla *Proteobacteria*, *Bacteroidetes* (bacteria) and *Ascomycota* (fungi) were more abundant in Summer than in Winter (Welch’s test, $P < 0.05$), suggesting changes in the communities. Among the most abundant bacterial genera we found *Sphingomonas*, *Streptomyces*, *Massilia*, *Hymenobacter* or *Methylobacterium-Methylorubrum* (Fig. S2a); while for fungi, the genera *Cladosporium*, *Alternaria*, *Filobasidium* or *Penicillium* were predominant indoors (Fig. S2b).

However, within each campaign, numerous ASVs were highly prevalent and found in all the samples (Fig. 2b). 107 bacterial ASVs (29 defined species) were found only in Summer, which contributed 33.9–54.9% of the relative abundance in each sample. In contrast, only 52 bacterial ASVs were present in all the samples of the Winter campaign, and their contribution was lower in each sample (22.3–32.0%), compensated by higher levels of plant DNA. Merging these records, 38 ASVs (13 defined species; top ellipse in Fig. 2b) were present in all the samples throughout both campaigns, which would constitute a bacterial core for the air indoor. This core agglomerated 19.7–35.8% of the samples indoor WinC, being *Streptomyces* sp. the most abundant genus during Winter, and *Sphingomonas* (*S. faeni* and unidentified species) during Summer, although the latter was always one of the most abundant in both periods (Table S6). Unexpectedly, this core was not composed only by bacteria typically associated with humans, as it would be expected inside a building. Most of them were related to an environmental origin, suggesting a strong influence of the biological components of the air outdoor inside the building. In fact, when the ASVs indoors and outdoors are compared, 82% were found in both environments. Moreover, these shared taxa represented 96.0–99.1% of the relative abundance of each sample taken indoor, indicating the high influence of the external income to the air in the room despite the fact that the window was closed all day during the collection of these samples.

In regard to fungi, more prevalent ASVs were found in Winter (108; 67 defined species) compared to Summer (45; 29). Their contributions ranged 54.1–72.0% and 60.1–86.1%, respectively. The fungal core was formed by 20 ASVs (12 defined species), agglomerating 13.0–77.8% of the relative abundance for each sample indoor WinC (excluding Plants). The aeroallergen *Cladosporium* was the most abundant genus in both periods, followed by the genera *Alternaria* in Summer and *Penicillium* in

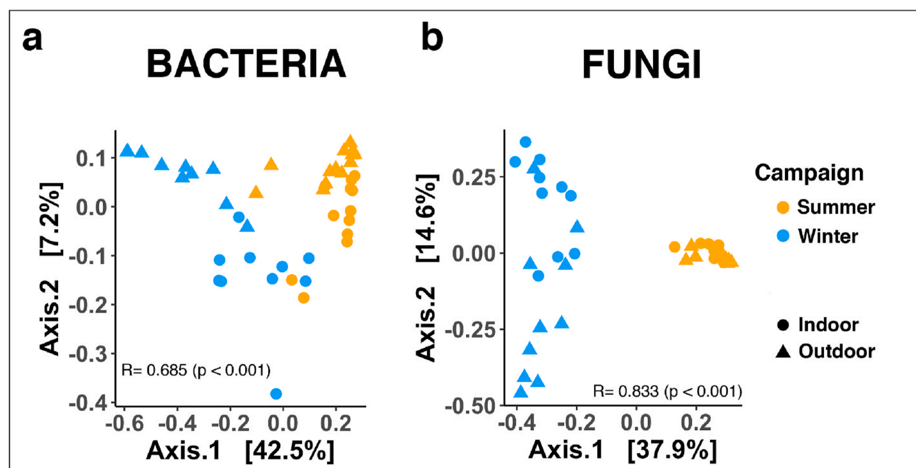

Fig. 1. Bioaerosols indoor and outdoor are subjected to seasonality. Principal Coordinates Analysis (PCoA) for bacteria (a) and fungi (b) based on Bray-Curtis dissimilarity matrices. The color indicates the Campaign (Summer or Winter) while the shape is related to the sampling site (indoor or outdoor). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

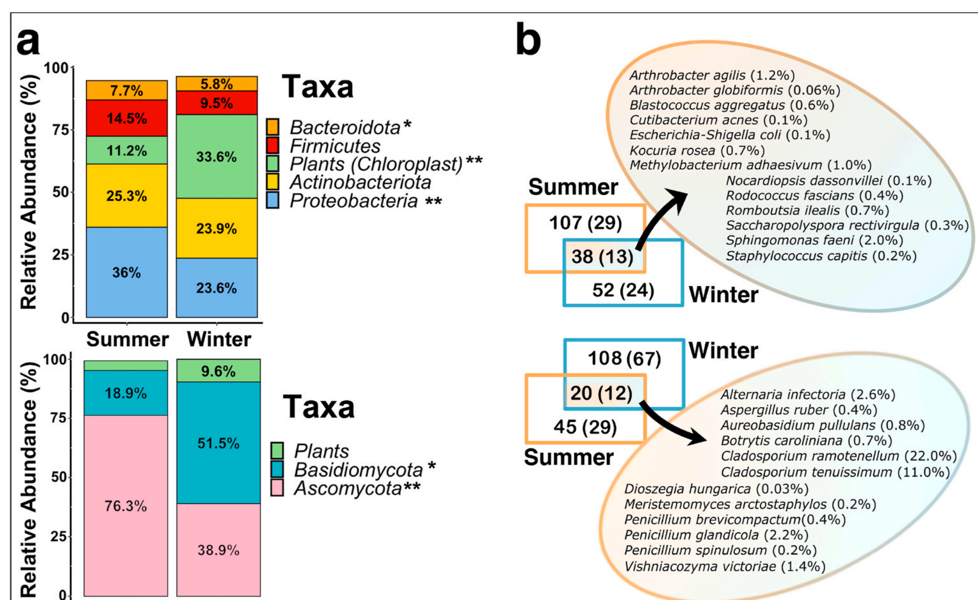

Fig. 2. Bioaerosols composition of the air indoor and microbial cores. (a) Accumulative abundance of the most abundant bacterial and fungal taxa (top and bottom, respectively) and their contribution for the different campaigns (Winter and Summer). Percentages <2% are omitted in the plot for visualization purposes. The asterisks next to the taxa indicate statistically significant differences of the abundances between the two campaigns (Welch's test, *: $0.05 \leq P < 0.01$; **: $0.01 \leq P < 0.001$). (b) The number of ASVs composing the bacterial (top) or fungal (bottom) cores of each campaign is indicated inside the boxes with the number of taxa defined at species level within parentheses. The number of ASVs shared between the cores of both campaigns is indicated in the intersection of the boxes and the shared species are shown inside the ellipses, with the average abundance across the samples within parentheses.

Winter (Table S7). Similar to bacteria, a high percentage of the ASVs indoor were present outdoor as well (80%), accumulating 96.5–99.6% of the relative abundance in each sample indoors.

Given the high similarity of the bioaerosols with the outdoors air, we also evaluated the different profiles indoor-outdoor in order to find an interpretation (Fig. S3). As a result, the bioaerosols found indoor showed a different response depending on the type of bioaerosol (Fig. 3). Firstly, we observed that some level of plant DNA was always detected indoors. Since this occurs even when the window was closed (WinC), this would support the idea of high levels of penetration via infiltration. When the levels outdoor were low (Summer; Fig. S3, left panels), the abundances indoors were similar (Indoor WinC and Outdoor WinC showed no significant differences; Welch's test, $P > 0.05$). However, when the levels of pollen outdoor increased (Winter; Fig. S3, right panels), the effect of infiltration seems limited, creating a significant difference with the levels indoors and keeping the latter lower than outside (samples Outdoor WinC vs. Indoor WinC, $P < 0.05$).

Among the main bacterial phyla (Fig. S3a), only the abundance of *Firmicutes* indoors showed significant differences with the air outdoor in both campaigns (Welch's test, $P < 0.05$), while other phyla (*Bacteroidota* and *Actinobacteriota*) displayed some divergences between indoor and outdoor exclusively in the Winter campaign. Similarly, the fungal composition showed some differences exclusively during the Winter campaign, when the levels of *Basidiomycota* were higher indoor compared to outdoor (Fig. S3b). Globally, the high resemblance in the composition of the bioaerosols indoor and outdoor, even by taxonomical groups, would indicate that the atmosphere inside the building is strongly affected by infiltration.

3.3. Effect of the window opening

Next, we analyzed how the biological communities indoor were influenced by opening a window (natural ventilation). To do so, a group of samples in each campaign (see Table S1 for details) was taken keeping the window open (samples WinO). The samples indoor were collected during 2 h under this condition, and after this time the window was closed until the next day collection. To examine the effect of this strategy of ventilation, pairwise comparisons of the abundances of bioaerosols

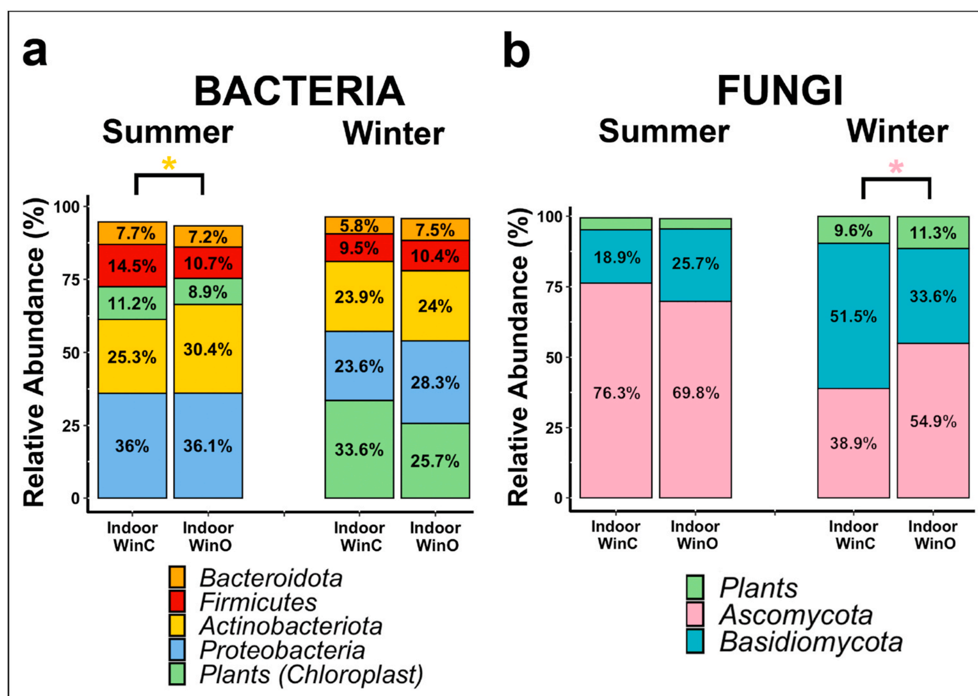

Fig. 3. - Comparison of the relative abundances of the bioaerosols indoor. The mean values for each type of sample and campaign are shown for the bacterial (a) and fungal (b) analyses. Percentages <2% are omitted in the plot for visualization purposes. The asterisks indicate statistically significant differences in the abundances between the two samples compared (Welch's test, *: 0.05 ≤ P < 0.01), while the color of the asterisks correlates with the taxa. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

between the samples WinC and WinO were performed (Fig. 3). Additionally, comparisons between the samples outdoor WinC and WinO were also tested to detect potential changes in the aerobiome outdoor that could bias the analyses, but no significant differences were found (Welch's test, P > 0.05; Fig. S3).

Globally, no significant effect of the window opening was observed in the composition of the samples indoor (WinC vs. WinO). For those bioaerosols with similar levels indoor and outdoor, no changes would be thought to occur, but for those with higher abundances in one of the sides, a variation when the window was kept open during the collection would be expected. For example, the levels of pollen (Plants) in Winter were higher outdoor, but maintaining the window open for 2 h did not vary the abundances indoor significantly (WinC vs. WinO; Welch's test, P > 0.05), and these results were found consistently across both analyses for bacterial and fungal DNA (Fig. 3).

On the contrary, the abundances of *Firmicutes* were higher indoors, but the window opening caused no significant alteration for this phylum, and only a statistically significant change was observed for *Actinobacteriota* in the Summer campaign (P < 0.05) (Fig. 3a). Regarding the fungal composition, only the levels of *Ascomycota* seemed to change when using the window for ventilation of the room during the Winter campaign (WinO vs. WinC, P < 0.05), while no other effect was observed for any other taxa in any campaign (Welch's test P > 0.05 when the abundances in the samples WinC vs. WinO were compared).

In order to have an estimate of the concentration of airborne bacteria present in the hospital environment, we also collected culturable bacteria during the Summer campaign (Fig. S4). The concentration of bacteria indoors tended to be lower (6 ± 3 CFU/m^3 vs. 11 ± 7 CFU/m^3 outdoors) although no statistical differences were found compared to outdoors (Welch's test, P > 0.05). In agreement with the other

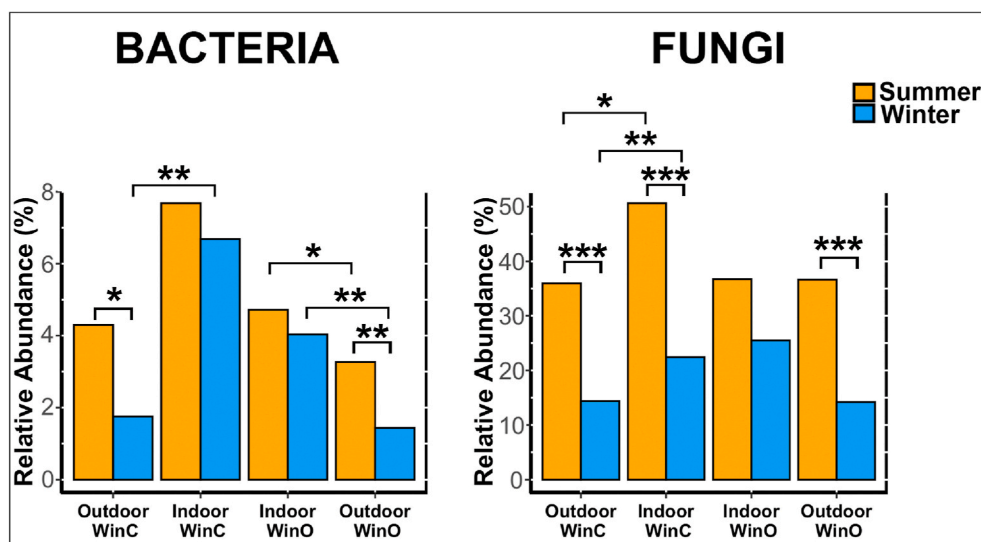

Fig. 4. Relative abundance compiled by pathogenic genera. The asterisks indicate statistically significant differences of the abundances between samples (Welch's test, *: 0.05 ≤ P < 0.01; **: 0.01 ≤ P < 0.001; ***: P < 0.001).

observations, opening the window did not show a significant effect on the concentration of bacteria indoors (Welch's test, $P > 0.05$).

3.4. Pathogens and human-related bacteria

One advantage that DNA analyses can offer over a culture approach is to identify the presence of a variety of pathogens. As shown in Fig. 4, the relative abundances of potentially harmful genera (see Tables S2 and S3 for the lists of the species included) tended to be higher indoors (Outdoor WinC vs. Indoor WinC), with significant differences in Winter (Welch's test, $P < 0.01$ for both bacteria and fungi), and also in Summer for fungi ($P < 0.05$). Regarding indoor air (indoor WinC), the levels of pathogenic bacterial genera were similar in both campaigns ($P > 0.05$ for Summer vs. Winter samples). In the case of fungal taxa, the seasonality was evident in both indoor and outdoor environments (samples WinC vs. WinO, $P < 0.001$). No significant changes in the abundances of bacterial or fungal pathogens indoor were observed when the window was kept open for 2 h ($P > 0.05$ for WinC vs. WinO in Summer or Winter campaigns).

When the analyses are focused at species rank (Fig. S5), only 25 microbial species were detected in our survey (14 bacteria, 11 fungi). The mean abundance of these particular taxa in each sample was very low (<0.14% for bacteria, with *Escherichia-Shigella coli* as the most abundant species; and <0.6% for fungal species, being *Aspergillus fumigatus* the most abundant). Only *Escherichia-Shigella coli* was part of the microbial core defined above, suggesting that the presence of most of these species was infrequent.

Compared to outdoors, the number of pathogenic species was similar (14 bacteria, 12 fungi). Most of them were also present indoors, suggesting that the air outdoor could be the true source for these pathogens. *Coxiella burnettii* and *Enterococcus faecalis* were only detected indoors, but their low abundances and occasional appearances would make it difficult to confirm a source inside the building. For fungal pathogens indoors, they were all found in the air outdoors, indicating an environmental source for those found inside the building.

We also analyzed the contribution of the bacteria related to the human microbiome present in our samples (Fig. 5). The highest abundances were found in the samples indoor, especially when the window was closed (samples WinC), with a maximum contribution of 16.6%. Opening the window only had a significant effect in Summer, tending towards reduction (Welch's test, $P < 0.05$). In regard to the taxa, bacteria related to human skin were notably higher (Fig. 5), with *Kocuria rosea* (phylum *Actinobacteriota*) as the most abundant species, and *Sphingomonas echinoides* (*Proteobacteria*) and *Prevotella copri* (*Bacteroidota*) as the most significant representatives for oral and gut-related microbiota, respectively. Interestingly, these taxa were the most abundant both inside and outside the building sharing >70% of the species (273 out of 385), which may suggest an environmental origin and penetration in the building via infiltration. The number of ASV identified only in one environment (indoor or outdoor) was similar (350 and 308), the abundance compiled indoors was higher (1.6% and 0.3%, respectively), with a predominance of the taxa associated with the phylum *Firmicutes*.

3.5. Correlation with meteorological parameters and air pollutants

Previous works have established that the composition of the microbial communities in the air outdoors is highly influenced by meteorological factors and correlated with some air pollutants [31,47,48]. Accordingly, the air temperature (Temp) outdoors was the most important variable influencing the composition of the bioaerosols (Fig. 6, left panels), being a key factor in differentiating the samples collected in Summer (higher temperatures) from those taken in Winter (variation explained 32% and 39% for bacteria and fungi analyses, respectively). Atmospheric pressure was also a significant element for fungi outdoors, while other meteorological factors such as wind speed (WS) and direction (WD) contributed to a lesser extend to explain the communities' composition. On the other hand, the high concentration of several air pollutants (NO, NO_2 and TPN but not PMs) contributed to the explanatory model for the samples in Winter but only for the prokaryotic components.

In regard to the bioaerosols indoor (samples WinC; Fig. 6, right panels), we evaluated the use of the same external data as predictors for indoors because they are easily available from public sources and also because air pollutants are not usually measured inside the buildings. Moreover, given the high influence of the bioaerosols outdoor, which

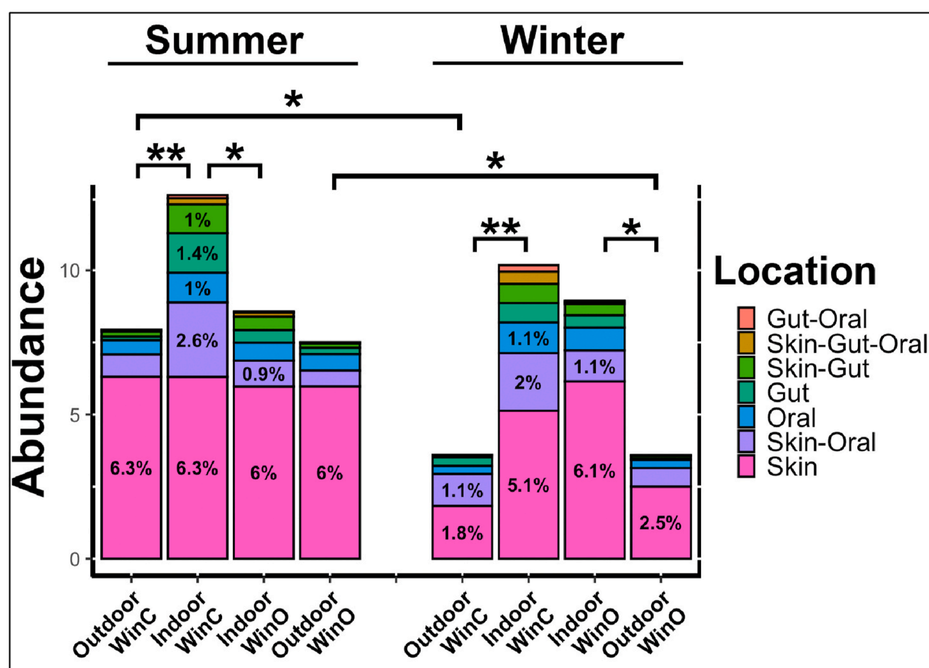

Fig. 5. Distribution of the relative abundances of human-related bacteria and their location. The asterisks indicate statistically significant differences of the abundances by pairwise comparison using Welch's test (*: $0.05 \leq P < 0.01$; **: $0.01 \leq P < 0.001$; ***: $P < 0.001$).

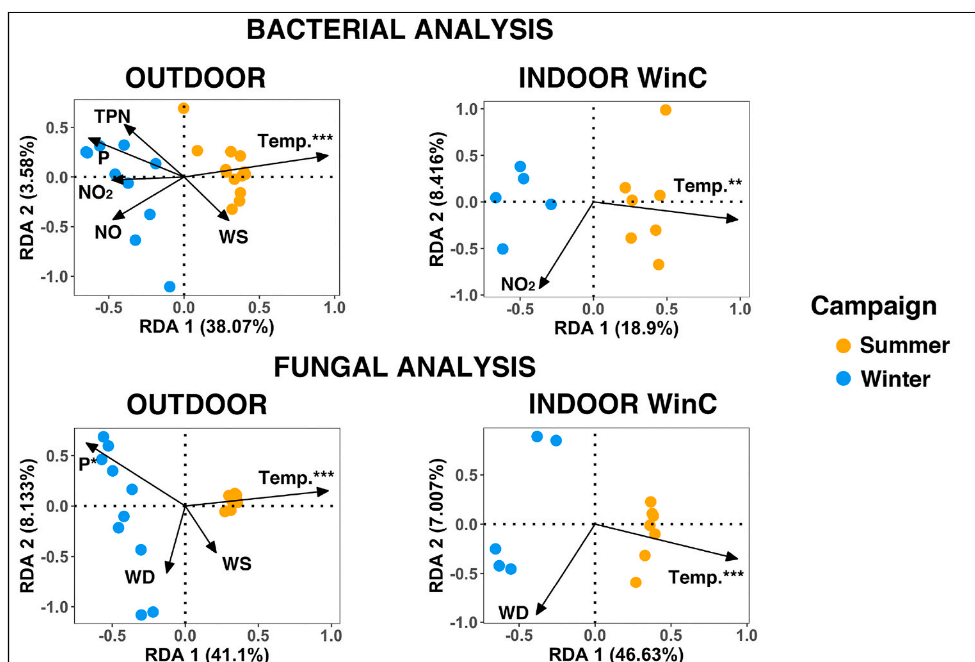

Fig. 6. Redundancy Analysis (RDA) of the bioaerosols communities and meteorological factors and air pollutants. NO (concentration of nitrogen monoxide), NO_2 (concentration of nitrogen dioxide), P (atmospheric pressure), Temp (air temperature), TPN (total particles number), WD (wind direction) and WS (wind speed). The asterisks indicate statistical significance under permutation (**: $0.01 \leq P < 0.001$; ***: $P < 0.001$).

are subjected to these parameters, we decided to test these records as explanatory variables. As a result, the bioaerosols indoor were also highly influenced by the air temperature outdoor for both bacterial and fungal analyses, with a minor influence from other factors such as NO_2 and WD, respectively, and not significantly related with the concentrations of PMs. Additionally, we employed the values of air pollutants indoors for the analyses, but similar results were obtained (Fig. S6).

When specific taxonomic groups were analyzed (Fig. S7), the influence of external temperature was positively correlated with the abundances of *Actinobacteriota*, *Bacteroidota*, *Proteobacteria* and *Ascomycota* outdoors. The same was observed for solar radiation because of the collinearity of both variables. The abundances of plant DNA followed the opposite trend, with the highest abundances peaking in Winter, and positively correlated with relative humidity. The abundances of these groups did not show any significant correlation with the air pollutants analyzed (only *Firmicutes* with nitrogen monoxide concentration, NO). Most of these parameters measured outdoors were not able to predict the abundances of bacteria indoors and only a few showed significant correlations with fungi: *Ascomycota* positively with temperature and solar radiation, and the opposite trend for *Basidiomycota*. Among the air pollutants, only NO concentrations outdoor were positively correlated with *Basidiomycota* abundances, while no significant correlations were found when the data of air pollutants indoor were used (Fig. S8).

4. Discussion

4.1. Indoor and outdoor bioaerosols similarities and divergences

Hospitals are expected to be safe buildings within the urban environments in regard to human health. However, excepting the operating rooms where the protocols impose to be sterile areas, the rest of the premises are not free of biological particles, including bacteria and fungi. Our survey showed that the composition of bioaerosols inside was highly influenced by the air outdoor. Over 80% of the ASVs were present indoor and outdoor, representing >96% of the relative abundances of each sample indoor. Moreover, the bioaerosols of the samples indoor and outdoor collected in the same season exhibited a high similarity between them, more significant than the resemblance between the samples indoor or outdoor from different seasons (Indoor-Summer vs. Indoor-Winter, or Outdoor-Summer vs. Outdoor-Winter) (Fig. 1 and Table S5). Since the similarities in the bioaerosols composition were present even when the window was closed, they could be attributed to a high influence of infiltration (building faults, window frames, leaks, etc.). In fact, no significant differences were observed in the concentration of particles (PMs and TPN, Table S1) between indoor and outdoor, regardless of the state of the window (Welch's test, $P > 0.05$). The building dates from the 30s and the wear of the structures over time would decrease the isolation of the room and facilitate the entry of air from outside. Therefore, the tendencies inside the hospital resembled those outdoor, in agreement with previous observations in other urban buildings [19–21,49], especially when no mechanical ventilation is used. Furthermore, the high concentration of plant DNA indoors must be associated with infiltration since the natural source is outdoors, supporting that infiltration plays a major role in this room. Also, the lack of human activities in the room may have contributed to keep the resemblance with the composition outdoor.

However, two remarked disparities were found: i) seasonal differences between the abundances indoor/outdoor of some bioaerosols; and ii) divergences in the *Firmicutes* group, with higher abundances indoor. First, the abundances of pollen grains were significantly higher outdoors than inside the building in Winter (Fig. S3), suggesting a limiting infiltration for these bioaerosols, likely because of their large size (>20 μm). In fact, different pollen grains can have different penetration rates inside the buildings based on their morphologies and buoyancy properties, even through open windows [50,51]. Other bacterial phyla like *Actinobacteria*, *Bacteroidota* or the fungi *Basidiomycota* also showed differential contributions between indoor and outdoor only during Winter, which could be the result of ecological alterations in the abundances of the bioaerosols outdoor (seasonality) combined with changes in environmental factors (e.g., wind direction and speed) affecting locally. Second, the differential distribution of *Firmicutes* was consistently found between campaigns. Although the number of different species of this phylum was higher outdoor (464 vs. 413 indoor), the average abundance in the samples was notably lower (3.6% vs. 12.4% indoor).

Moreover, the window opening did not vary significantly these values, which suggests the existence of a source or an accumulation favored by the conditions inside the room. The proportion of the taxa belonging to *Firmicutes* related to human microbiome was similar in both indoor and outdoor (17% and 19%, respectively), and the window opening tended to reduce this particular group of *Firmicutes* indoor. Since a source of these bacteria associated with human activities is negligible in our case (excepting the brief entries and exits in the room for sampling procedures), these observations aim to prove the second hypothesis, that the prevalence and growth of environmental *Firmicutes* in the room is favored.

4.2. Microorganisms and microbial cores indoor

Proteobacteria, Actinobacteriota, Firmicutes and Bacteroidota were the predominant bacterial phyla indoors, coinciding with similar studies [52,53]. The most abundant genera (*Sphingomonas*, *Streptomyces*, *Massilia*, *Methylobacterium*) showed a high resemblance to those described by Gao et al. [54], who sampled bioaerosols in different hospitals, suggesting that healthcare environments promotes the growth of these particular taxa since they were also found outdoor but with less abundance. We identified a microbial core with those ASVs present in all the samples of both campaigns, mainly formed by environmental bacteria and fungi, as expected in a room with no occupancy and high infiltration, and based on the fact that all the taxa of this core were found outdoors too. Many of them were not specially abundant and differed from those described by Chen et al. [55], who found a bacterial core formed mostly by human-related microbes (*Streptococcus*, *Staphylococcus*, *Propioinibacterium*, *Corynebacterium*, *Acinetobacter*, etc.). However, their survey was conducted in areas with high human activities and also, they sampled around highly touched items in rooms and workplaces instead of air, which, partly, could explain the differences in results. This variability associated with the items analyzed suggests a very interesting factor to take into account when conducting this type of studies because the composition of the deposited particles may be different from the airborne ones.

The fungal core has been less explored in hospitals. *Cladosporium* spp. was the most abundant fungus in our study, in agreement with the levels outdoors. However, different species of *Aspergillus* have been usually found in previous works [53,56–58], which is of special interest due to its infectious potential. 24 different species of *Aspergillus* were detected indoors (with accumulated abundances that ranged 0.6–3.7%), including *A. fumigatus*, *A. flavus*, *A. terreus* or *A. niger*. More species were found outdoors (28), but their relative abundances were lower (0.1–1.9%).

4.3. Effect of natural ventilation using a window

Our results indicate that keeping the window open for 2 h (a longer time than recommended for health reasons) showed minor variations in the bioaerosols indoors. Significant changes were observed in some cases comparing WinC and WinO, like *Actinobacteriota* in Summer or *Ascomycota* in Winter. However, the levels indoors with the window closed kept the same relation with those observed outdoors, suggesting little relevance for these observations. On the other hand, the richness and diversity of both bacteria and fungi increased when the window was open (Table S5), indicating that there is a perceptible exchange with the air outside to some extent. Similar results were observed when the same experience was assessed in a smaller room without additional ventilation systems [59], so the low effect on bioaerosols does not seem to be linked to the size of the room. The exchange of air between indoor and outdoor in these cases is promoted by the gradient of temperature and pressure. In a single-sided natural ventilation scenario as the one surveyed (one window open, door closed and without temperature control by AC or HVAC), the pressure caused by the wind on the building surface is the most significant force to promote the exchange of air inside the room [60]. Most of the days with the window open, the wind direction was oriented towards the window (NE), although the speed was not particularly high (usually <2 m/s). Also, the estimations of the ventilation flow rate through the window in the room and air changes per hour (ACH; Table S1) were low, <3 ACH in most days, when generally 4 or higher ACH are recommended. This would explain the scarce changes when using only one window with the aim of inducing a change on the bioaerosols indoor. Thus, it would be interesting to address more intensive campaigns where other strategies are developed such as the combination of opening doors and windows to increase the air movements and facilitate the dispersion of bioaerosols.

4.4. Potentially harmful species in the hospital environment

Nosocomial infections due to antimicrobial-resistant microorganisms are a major risk for long-stay immunocompromised patients and users in intensive care units, who usually require long-time catheterization [61]. Accordingly, recent works have proposed hospitals as emission hotspots of antibiotic-resistant bacteria in urban environments [62–64]. In contrast, our study suggests that their role as emission points through aerosols of pathogenic microbes is not relevant compared with the environmental sources. On the one hand, the representation of harmful species in the hospital was very low (mean < 0.8% or 2.4% for bacteria or fungi, respectively; Fig. S5). On the other hand, most of bacteria (pathogenic or not) were not associated with the human microbiome and have an environmental origin, where they can acquire ARGs without being exposed to a nosocomial habitat [65–67]. In fact, most species have been detected in previous studies that analyzed the air outdoor in different urban areas throughout the province without a nearby hospital [30,68]. Furthermore, although some species were only found indoors (*Staphylococcus lugdunensis*, *Coxiella burnetii*, *Enterococcus faecalis, Cladosporium cladosporoides*), their low abundances and sporadic appearance make it difficult to conclude that the hospital is their true origin. Nonetheless, it should be noted that the samples indoor were taken in an isolated room, so it cannot be ruled out that other areas within the hospital may show different levels of pathogens. In addition, DNA sequencing does not provide information on viable bacteria, so the estimate of pathogenic bacteria could be misjudged. The concentration of total bacteria indoor and outdoor (6 and 11 CFU/m^3, respectively) was considerably below the values described in other works conducted in similar environments [69–71]. Thus, based on our results, hospital premises present a low risk in terms of the emissions of pathogenic species.

4.5. Environmental factors and air pollutants as predictor of the bioaerosol composition

Monitoring the biological components in the air is still an expensive and laborious task because of their high diversity and the lack of technologies capable of providing results in real-time. Until the technology can address such complexity, using environmental predictors such as meteorological factors and air pollutants may provide an overview of the biological components present in the urban atmosphere. In our survey, despite the high influence of the air outdoor on the composition of bioaerosols indoors, the external values were not reliable predictors of the abundances of microorganisms inside the building. As previous works have established (reviewed in Ruiz-Gil et al. [72]), we found that air temperature is a major driver of the airborne communities (Fig. 6). Guo et al. [20] also described a positive correlation with the temperature indoor and the concentration of bacteria. However, although the temperature outdoor was useful to differ the composition of bacteria indoor between seasons, we did not find any significant correlation with this or any other environmental parameter and the abundance of specific groups of bacteria (phyla) (Fig. S7). This indicates the limited scope of these measurements to infer the bacterial composition indoors. Only the abundances of the fungal phyla were significantly correlated with

temperature and relative humidity. Nonetheless, we cannot rule out that precise measurements of these parameters indoor and more exhaustive campaigns with a larger number of samples would hint to discover any relationships with the variables indoors.

Similarly, the concentration of air pollutants did not allow us to infer the composition of bioaerosols neither outdoor or indoor. Several works have described relationships between bioaerosols and atmospheric pollutants [73–75]. However, the associations remain imprecise, changing between studies, locations and even different relationships were described across the seasons [76]. These discrepancies could be explained by the fact that these studies are usually based on detecting the DNA and not the viability of the microorganisms. Most air pollutants in urban environments have an anthropogenic origin and their concentrations are expected to affect the viability of the microorganisms rather than the concentration and composition of the bioaerosols because their habitats (soil, water, plant surface, etc.) would tend to persist unaffected. Thus, the relationships between air pollutants and bioaerosols could be masked in some cases and more surveys are needed to reach reliable conclusions. Nonetheless, the idea that environmental variables have a stronger influence on the biological particles present in the atmosphere than the air pollutants is very consistent between studies.

5. Limitations of the study and perspectives for future works

Some points must be taken into account for the extrapolation of our results, starting with the number of samples, which could hamper the detection of particular trends, especially across seasons, and the identification of some environmental parameters as predictors of the composition of bioaerosols in the air. Moreover, surveying other hospitals in the region would provide more representative data. Human activities create airflows that can provide an additional income of microorganisms. In our survey, the lack of these activities in the room may contribute to keep the air composition more stable. Thus, future works should cover additional areas in the hospital, including those with diverse human activities and mechanically ventilated rooms, which modify artificially the atmosphere and would show certain dissimilarities. It is also worth noting that DNA sequencing data cannot estimate *per se* the concentration of microorganisms. Although we carried out a limited survey of the bacterial concentration, molecular and culture-based data are complementary and further research should consider a wider spectrum of microbial culture with different media. Molecular methods such as specific DNA probes to detect ARGs could also be interesting to detect potential pathogens. Furthermore, we analyzed airborne particles because inhalation is the easiest way for microorganisms to come into close contact with our body, while other works have used dust or particles deposited on surfaces. A combination of both sampling methodologies could clarify some of the divergences found between studies.

6. Conclusions

Our work shows that the composition of bioaerosols inside a hospital facility is highly influenced by those found outdoors in areas without mechanical ventilation. The main bioaerosols (pollen, bacteria, fungi) exhibited different trends throughout the year, which created differences in composition between indoor and outdoor, a fact to be taken into account when monitoring these particles. The abundance of the bacterial phylum *Firmicutes* was higher indoors compared to outdoors, likely because the conditions indoors are favorable for this group rather than the contribution from the human presence. The abundances of human-related bacteria tended, in general, to be higher indoors. The number of pathogenic microbes was low, their presence was rare in most cases and the majority was also found outdoors, discarding hospitals as a controversial source of airborne pathogens. Based on our results, opening a window for 2 h has little effect on the composition of bioaerosols in the room at any season. Finally, meteorological parameters outdoors and air pollutants concentrations indoors or outdoors were poor predictors for the specific composition of bioaerosols inside the building.

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CRediT authorship contribution statement

Andrés Núñez: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ana M. García:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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