



Fungal signatures of oral disease reflect environmental degradation in a facultative avian scavenger

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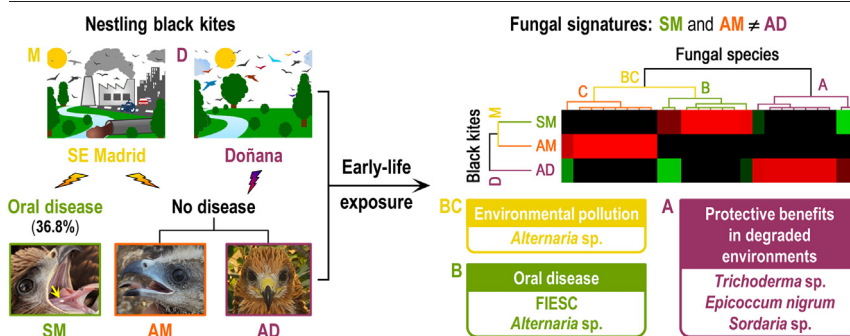
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HIGHLIGHTS

- Exposure to altered environments increased the risk of oral disease in black kites.
- Oral fungal pathogens were more prevalent in nestlings from degraded environments.
- Fungal signatures differed between nestlings from natural and altered habitats.
- *Fusarium incarnatum-equiseti* and *Alternaria* were predictors of oral infection in nestlings.
- Risk and protective factors for exposure to environmental degradation were found.

GRAPHICAL ABSTRACT



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ABSTRACT

Degradation of natural ecosystems increases the risk of infections in wildlife due to microbiota dysbiosis. However, little is known about its influence on the development of fungal communities in predators and facultative avian scavengers. We evaluated the incidence of oral disease in wild nestling black kites (*Milvus migrans*) under contrasting environmental degradation conditions, and explored their oral fungal patterns using molecular methods and multivariate analysis. Oral lesions were found in 36.8% of the 38 nestlings examined in an anthropogenically altered habitat (southeastern Madrid, Spain), but in none of the 105 nestlings examined in a well-conserved natural area (Doñana National Park, Spain). In a subsample of 48 black kites, the composition of the oral fungal community differed among symptomatic nestlings from Madrid (SM) and asymptomatic nestlings from Madrid (AM) and Doñana (AD). Opportunistic fungal pathogens (e.g., *Fusarium incarnatum-equiseti* species complex, *Mucor* spp., *Rhizopus oryzae*) were more prevalent in SM and AM than in AD. Hierarchical clustering and principal component analyses revealed that fungal patterns were distinct between both study areas, and that anthropogenic and natural environmental factors had a greater impact on them than oral disease. Fungal signatures associated with anthropogenic and natural stresses harbored some taxa that could be used to flag oral infection (*F. incarnatum-equiseti* species complex and *Alternaria*), indicate environmental degradation (*Alternaria*) or provide protective benefits in degraded environments (*Trichoderma*, *Epicoccum nigrum* and *Sordaria*). Co-occurrence associations between potentially beneficial and pathogenic fungi were typical of AM and AD, hinting at a possible role in host health. This study shows that early-life exposure to highly

Abbreviations: HCA, hierarchical clustering analysis; PC, principal component; PCA, principal-component analysis; PCR, polymerase chain reaction; PM₁₀, particulate matter of less than 10 µm diameter; OR, odds ratio.

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degraded environments induces a shift towards a higher prevalence of pathogenic species in the oral cavity of black kites, favoring oral disease. Furthermore, our findings suggest potential ecological applications of the monitoring of oral mycobiome as a bioindication of oral disease and environmental degradation.

1. Introduction

The role of degradation of natural ecosystems on wildlife health and diseases are growingly highlighted worldwide. Concerns around these impacts have been especially reported for emerging diseases threatening wildlife just when their effects are patent after dramatic population declines (Aguirre and Tabor, 2008; Smith et al., 2009; Fisher et al., 2012; Tompkins et al., 2015), generally because the lack of widespread surveillance systems (Kelly et al., 2021). Research on disease-causing processes in wildlife increasingly underscores the impact of human disturbances on ecosystems (Acevedo-Whitehouse and Duffus, 2009; Brearley et al., 2013; Hassell et al., 2017). In particular, growing human activities and occupation of natural areas promote frequent and widespread contact between wild animals with food and anthropogenic contaminants (Marcogliese and Pietrock, 2011; Becker et al., 2015; Altizer et al., 2018). Multiple inter-related physiological processes can be affected by contaminants, biotoxins and pathogens acquired through multiple pathways, resulting in nutritional deficiencies, immunosuppression, metabolic disorders, and microbiota alteration (Strandin et al., 2018; Encarnação et al., 2019; Murray et al., 2020; Dujon et al., 2021). Depending on the extent, frequency and intensity of these impacts, populations may be directly and indirectly harmed by environmental and infectious diseases in varying proportion and intensity (Acevedo-Whitehouse and Duffus, 2009; Smith et al., 2009; Gottdenker et al., 2014). The disease triangle framework has often been applied to understand how the interactions between environmental factors, host susceptibility, pathogen virulence and anthropogenic factors such as contaminants lead to different disease stages or impacts (Scholthof, 2007). The incidence of fungal infections has increased over the past few decades in most vertebrate groups, leading to global declines and extinctions of amphibians and bats (Fisher et al., 2012). A wide array of interactions among wild birds, livestock and human populations can contribute to these infections as a result of close contact of birds with high inputs of anthropogenic residues and contaminants (Tompkins et al., 2015; Wiethoelter et al., 2015). However, little is known about the potential of fungal species to cause disease, mortality or population declines in birds (Fisher et al., 2012; Seyedmousavi et al., 2018).

Obligate and facultative avian scavengers can benefit or be harmed by the opportunities and risks associated with living at the edge of livestock-human interfaces, especially through the exploitation of decaying food from anthropogenic subsidies (Cortés-Avizanda et al., 2016). This material can contain variable levels of harmful pathogens and toxins depending on their nature, origin and state (Plaza et al., 2020), that in turn determine the scavenger species able to exploit it without compromising their health (Roggenbuck et al., 2014; Waite and Taylor, 2015; Blumstein et al., 2017). An extreme feeding specialization on carrion by obligate scavengers like vultures has evolved in parallel with diverse adaptive and protective mechanisms to successfully cope with microbial species colonizing and decomposing flesh (Chung et al., 2015; Blumstein et al., 2017; Zepeda-Mendoza et al., 2018). Among them, ubiquitous commensal, saprotroph and specialized decomposer fungi can turn into opportunistic pathogens threatening wildlife populations (Seyedmousavi et al., 2018). This is mainly due to general long environmental persistence and proliferation in places where decaying organic matter accumulates artificially together with refuse and other anthropogenic residues (Becker et al., 2015; Hassell et al., 2017; Plaza et al., 2020). Feeding on this matter, and the regular exposure to host-generalist microorganisms from detritus and contaminated water, can increase the probability of vertebrates to suffer health problems, such as gastrointestinal pathologies because of an imbalance in their normal protective gut microbiota (Trevelline et al., 2019; West et al., 2019). The overgrowth of these microorganisms may also be promoted by food-

borne pharmaceuticals, which lead to an alteration (dysbiosis) of the normal microbiota, as described in domestic animals and humans (Hazen, 1995; MacCallum, 2010; Keeney et al., 2014; Mukherjee et al., 2015).

Pharmaceuticals and other pollutants can promote immunosuppression and other processes that disrupt homeostasis, negatively affecting wildlife health (Arnold et al., 2014; Desforjes et al., 2016; Aulsebrook et al., 2020), and be spatiotemporally associated with pathogens promoted by human activities (e.g. pathogen pollution from farming and urban areas that eventually cause disease) (Brearley et al., 2013; Sánchez et al., 2020; Blanco and de Tuesta, 2021). In contrast, populations inhabiting environments where ecosystem structure, diversity, and functionality remain relatively well conserved are expected to be more resilient to disease (Tompkins et al., 2011; De Vos et al., 2016). These differences can be examined to evaluate whether environmental degradation may exert an overall effect on body condition, infection and disease, and to assess the main factors behind.

Recent studies have reported the occurrence of oral mycoses in vultures and other avian scavengers regularly exposed to antimicrobial agents from livestock carcasses (Blanco et al., 2016, 2017a, 2017b; Pitarch et al., 2017, 2020). The presence of abundant yeast-like fungal species forming part of the oral lesions has been highlighted as a likely consequence of such alterations related to the negative effect of antimicrobials on the host microbiome (Blanco et al., 2017a; Pitarch et al., 2017, 2020). These lesions have been proposed as an early warning of physiological alterations in avian scavengers (Pitarch et al., 2017), especially in nestlings that have not fully developed normal protective microbiota and immune system (López-Rull et al., 2015). Filamentous fungi isolated at high prevalence from the same lesions, but not identified at the species level, have been suggested to act as opportunistic pathogens contributing to disease (Pitarch et al., 2017). The potential impact of these fungi together with the exposure to environmental pollutants may represent a dangerous combination encountered by avian scavengers in places where food subsidies and refuse accumulate (Plaza et al., 2020). Remarkably, early-life exposure to anthropogenic pollution can alter the establishment of host microbial communities, and trigger imbalances in genes related to health protection and pathogenicity within the host microbiome (Trevelline et al., 2019; West et al., 2019; Bernardo-Cravo et al., 2020; Mueller et al., 2020). This altered microbiota during early life can affect immune development and lead to microbial dysbiosis with negative effects on their life-long health (Vallès and Francino, 2018).

The food-pollution-disease connections can be flagged by particular signatures in microbiomes of endangered wildlife (Trevelline et al., 2019; West et al., 2019), including co-occurrence relationships between microbial species, which can provide insight into patterns associated with the emergence of pathogenic taxa or the protection of beneficial microbial taxa (Bernardo-Cravo et al., 2020; Mueller et al., 2020). Some of these microbial taxa may be used as potential predictive biomarkers of disease and environmental pollution or as potential environmental bioremediators (De et al., 2007; Mancabelli et al., 2017; Gao et al., 2020; Nolorbe-Payahua et al., 2020). Uncovering these relationships within the microbiome of particular hosts may be essential to understand the epidemiology of infectious diseases in wildlife conservation (Freilich et al., 2018; West et al., 2019), and to forecast potential disease outbreaks in order to prevent or mitigate them. To our knowledge, no information is available on biodiversity patterns of fungal communities in nestlings of facultative avian scavengers living under contrasting environmental degradation conditions, and on factors driving microbial interactions within their microbiomes either promoting or mitigating disease.

In this study, we investigated whether early-life exposure to highly degraded environments could act as a predisposing factor that makes

nestlings of a facultative avian scavenger, the black kite (*Milvus migrans*), more susceptible to developing filamentous fungal infections. We first compared the occurrence of disease expressed in oral lesions of nestling black kites from two populations living in contrasting conditions of environmental degradation generated by habitat transformation and pollution. We then identified the filamentous fungal species present in the oral cavity of a selected subgroup of nestling black kites by molecular methods, and assessed whether their fungal occurrence patterns were shared or differed between nestlings with or without oral lesions under these contrasting environmental conditions using multivariate analysis. We further explored whether their oral fungal signatures could conceal potential biomarkers of disease and environmental contamination. Finally, we examined potential ecological interactions between members of their oral fungal communities.

2. Materials and methods

2.1. Ethics statement

Bird-handling procedures and bird marking were carried out in accordance with permits from the Spanish Bird Ringing Centre (Permit Number: 530115), and authorized by the regional governments of Madrid and Andalusia.

2.2. Study areas, study species and black kite populations

The study areas were located in South-Eastern Madrid (central Spain) and in Doñana National Park (southern Spain). These areas can be considered as extreme examples of the degradation continuum used by black kites for breeding in the southern portion of their European range.

Given the frequent association with human settlements, rubbish dumps and anthropogenic food subsidies (Blanco, 1994; Kumar et al., 2014), the black kite represents a good model to evaluate the impact of human activities on wildlife health. This is a medium-sized opportunistic predator and facultative scavenger with a widespread distribution. Like habitat-generalists, black kites exploit a wide gradient of habitat transformation, from well-conserved natural environments to large rubbish dumps and big cities, where they show an eclectic diet including young, debilitated and injured vertebrate prey, invertebrates, carcasses of wild animals and livestock, and remains from urban refuse (Blanco, 1997; Kumar et al., 2019). In anthropogenically altered and urban habitats, they are exposed to a wide array of pollutants of variable origin acquired from different uptake routes (Blanco et al., 2003, 2018), which can potentially cause variable detrimental effects on health (Blanco et al., 2004; Baos et al., 2006). Strikingly, while the black kite is probably the most abundant raptor worldwide, a paucity of information still exists on its microbial communities, pathogens and disease directly or indirectly linked to environmental alteration, foraging conditions and food sources.

South-Eastern Madrid (Madrid hereafter) is a highly transformed region devoted to irrigated intensive agriculture, gravel extraction, and other environmentally-impacting activities such as urbanization. The latter includes legal and illegal settlements, landfills, urban waste incineration, waste-water treatment plants and industrial developments at the peri-urban area of Madrid city. As consequence of these activities, this is as a highly contaminated area with multiple pollutants (Fernández et al., 2000; Jiménez et al., 2000; Valcárcel et al., 2011; Escobar-Arnanz et al., 2018). These pollutants have been recorded at high levels, with detrimental effects on health and reproduction of black kites and other avian species (Merino et al., 2002, 2005; Blanco et al., 2004, 2018; Jiménez et al., 2004; De Sanctis et al., 2013). Specifically, high mean \pm SE levels (ppb wet weight) of cadmium (1.83 ± 2.91 , range = 0.00–18.00), lead (84.13 ± 52.45 , range = 7.21–221.40) and arsenic (41.25 ± 63.49 , range = 0.10–288.0) were found in the blood of nestling black kites ($n = 69$) (Blanco et al., 2003). High mean \pm SD levels of non-ortho polychlorinated biphenyls (PCBs) (432.49 ± 358.85 pg/g in 2001; $n = 13$), ortho PCBs (6621.86 ± 4786.81 ng/g in 2002; $n = 10$), dichlorodiphenyltrichloroethane (DDT) (86.29 ± 25.21 ng/g in 2003; $n = 9$), and

dichlorodiphenyldichloroethylene (DDE) (202.92 ± 181.72 ng/g in 2003; $n = 9$) were reported in failed eggs (Jiménez et al., 2004). The levels of polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) found in eggs ($n = 15$) in 2001 ranged from 8.9 to 85.9 pg/g on a wet weight basis (Merino et al., 2002). The mean \pm SD levels of flame retardants found in eggs ($n = 28$) between 2007 and 2016 were also high, including polybrominated diphenyl ethers (PBDEs) (270.12 ± 289.71 ng/g lw) and dechloranes (Dec) (362.87 ± 513.29 ng/g lw) (Blanco et al., 2018). In this area, black kites nest in highly fragmented and degraded riparian forests along the highly contaminated Manzanares and Jarama Rivers (see nest distribution in Hernández-Brito et al., 2020). Breeding individuals feed on a variety of medium-sized vertebrates, primarily European rabbits (*Oryctolagus cuniculus*), as well as on small remains of livestock and sea fish carcasses from domestic and market waste discarded in large landfills (Blanco, 1997). Large numbers of black kites and other avian scavengers forage on these landfills, especially during their migratory passages (Blanco, 1994).

Doñana National Park (Doñana hereafter) is a large protected area with one of the largest and most biodiversity-rich wetlands of Europe, classified as a UNESCO Biosphere Reserve, a World Heritage Site and a Ramsar wetland (García-Novo and Marín-Cabrera, 2006), and therefore it was selected as a comparatively well-conserved area. In Doñana, black kites breed at high density in the proximity of the extensive Guadalquivir marshes (Sergio et al., 2005), and forage on a large variety of resident and migratory prey from aquatic and terrestrial environments, with a diet ultimately dominated by rabbits, waterbirds and fishes (Delibes, 1975; Veiga and Hiraldo, 1990).

2.3. Fieldwork

During the breeding season of 2018, black kite nests were accessed to band the nestlings when they were approximately 30 days old, i.e., when they were feathered but not at risk of abandoning the nests (details in Blanco et al., 2004, 2006). We recorded the occurrence of disease represented by gross lesions in the oral cavity of nestlings by conducting a visual inspection as described previously for several vulture species (Pitarch et al., 2017).

Samples of lesions were collected from the oral cavity (tongue, pharynx and palate) of affected nestling ($n = 13$ from 9 nests) from Madrid by gently scraping the surface of the lesion with sterile swabs. These were subsequently stored and labelled in Eppendorf tubes. In apparently asymptomatic nestling black kites ($n = 22$ from 15 nests in Madrid and $n = 13$ from 9 nests in Doñana), scraping was also carried out on the surface of the tongue, pharynx and palate, in the same sites where lesions appeared in symptomatic nestlings. The samples were maintained at 4 °C, labelled with a code, and processed in the facilities of the Real Jardín Botánico-CSIC (Madrid, Spain) within 14 h after collection.

2.4. Fungal isolation

Once in the laboratory, the swabs were used to inoculate and streak peptone-glucose agar (PGA) plates supplemented with penicillin (100 mg/L). Plates were incubated at 20 °C for 48 h. After detecting the presence of filamentous fungi, each colony with different macroscopic or microscopic features was then subcultured using a glass-ring technique as described in Sarmiento-Ramírez et al. (2014) and grown at 20 °C to avoid the bacterial growth (Fig. 1). The resulting cultures free of bacteria were maintained on PGA media at 4 °C in the culture collection of the Real Jardín Botánico, CSIC, Madrid, Spain.

2.5. Molecular identification of isolated fungi

The molecular characterization of the fungal isolates was performed according to Sarmiento-Ramírez et al. (2014) with some modifications (Fig. 1). Briefly, the cultures were transferred to 2 mL-tubes, frozen at -80 °C, and later lyophilized (VirTis Benchtop K Series Freeze Dryers) for 24 h (≤ 80 °C; ≤ 20 mTorr). The samples were mechanically broken using glass beads in a TissueLyser (Qiagen Iberia, Madrid, Spain). Genomic

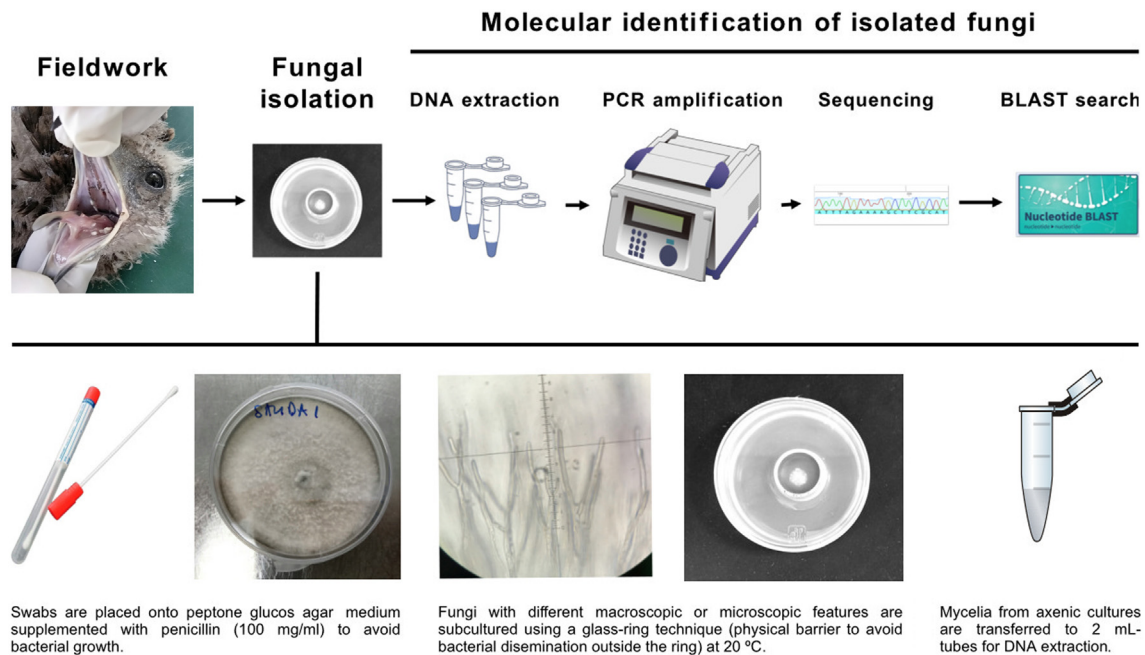


Fig. 1. Overview of the procedure for isolation and molecular identification of filamentous fungi in the oral cavity of nestling black kites. Samples were obtained by gently scraping the surface of the oral lesion (or the same sites where lesions appeared in symptomatic nestlings if the individuals were apparently asymptomatic) with sterile swabs. Each swab was placed within a glass ring previously placed onto a peptone-glucose agar (PGA) medium. Hyphae reaching the outside area of the ring were picked and used for DNA extraction. Isolated fungi were molecularly identified after DNA extraction, PCR amplification, sequencing and BLAST search.

DNA was extracted using the E.Z.N.A. SP Plant-DNA kit (Omega Bio-Tek, Norcross, Georgia, USA). The primers used for the polymerase chain reaction (PCR) were the universal primer pair ITS5 and ITS4 (5'-GGAAGT AAAAGTCGTAACAAGG-3' and 5'-TCCTCCGCTTATTGATATATGC-3'), corresponding to the transcribed internal spaced region of the nuclear ribosomal DNA (ITS nrDNA). The PCR program started with an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 25 s, and elongation at 72 °C for 45 s. The reaction was completed with a final elongation at 72 °C for 10 min. The amplified products were visualized by electrophoresis in a 1% agarose gel stained with SYBR Safe DNA (Invitrogen, Madrid, Spain). Positive amplified products were purified using QIAquick PCR Purification Kit (Qiagen, Germany). Double strand PCR positive products were sequenced using an automated sequencer (Applied Biosystems 3730xl DNA, Macrogen, Inc. Madrid, Spain). Both sequence strands were assembled and the chromatograms were revised for double-peaks using the program Geneious v10.0.3 (<http://www.geneious.com>) (Kearse et al., 2012). We ran a BLAST search to check the nature of each of the consensus generated sequences on the nucleotide sequence databases of the National Center for Biotechnology Information (NCBI) (<http://blast.ncbi.nlm.nih.gov>).

2.6. Statistical analyses

Population-prevalence estimates and 95% confidence intervals (CI) were calculated with EpiTools (Sergeant, 2018). Kolmogorov-Smirnov and Shapiro-Wilk tests were used to assess whether data followed a normal distribution. Differences in the number of fungal species isolated per individual among the black kite groups were assessed with the Kruskal-Wallis *H* test. Venn diagrams were performed to reveal the number of fungal (phylum, order or species) taxa that were shared by the three study groups or by two out of the three study groups or that were specific to each study group (Shade and Handelsman, 2012; Cardoso et al., 2017).

Two-way hierarchical clustering analysis (HCA) was applied to group nestling black kites and identified fungal (order or species) taxa according to the similarities in the fungal order or species occurrence profiles (i.e., patterns of relative occurrence rate of fungal orders or species, respectively) of each black kite group and the occurrence patterns of each

identified fungal order or species across all black kite groups, respectively. Relative fungal taxonomic occurrence rates (i.e., the proportion of samples positive for each fungal species or order) were normalized by median-centering oral fungal occurrence profiles for each nestling group and then by median-centering each pattern of each identified fungal taxon across all nestling groups. Principal component analysis (PCA) was used to extract the main factors that capture most of the information from the dataset (relative fungal taxonomic occurrence rates) through principal components (PCs), which are orthogonal or linearly independent to each other (Broa and Smildeab, 2014; Jolliffe and Cadima, 2016). PCA was performed on the global fungal order or species occurrence patterns of each black kite group to cluster nestling individuals. PCA was further carried out on the occurrence profiles of each identified fungal order or species across all black kite groups to cluster the identified fungal taxa. The degree of homology or relative similarity of these profiles between the groups was evaluated with the *t*-test or the Mann-Whitney test, as appropriate.

The ability of the fungal signatures to discriminate between symptomatic and asymptomatic nestling individuals or between nestling black kites from natural and altered environments was evaluated by receiver-operating-characteristic (ROC) curve analysis. It is a useful tool for assessing the discrimination accuracy of a prediction model or diagnostic test (Obuchowski and Bullen, 2018). Odds ratios (ORs), which indicate the probability that an event will occur relative to the probability that it will not occur, were calculated to examine the association of the occurrence of fungal species identified in the oral cavity with the presence of oral lesions or exposure to highly altered environments (Pitarch et al., 2011; Miller and Looney, 2012). Multivariate logistic regression models were built to define independent predictors of infection and environmental degradation. In this analysis based on the Wald statistic (Pitarch et al., 2007; Ascha et al., 2017), the response variables were oral disease (presence or absence of oral lesions) and environmental pollution (Madrid or Doñana), respectively, while the explanatory variables were the fungal taxa identified in their corresponding HCA-defined signatures. The Hosmer-Lemeshow test based on the deciles of probability was used to assess the goodness of fit of the models (Paul et al., 2013).

Pairwise Spearman rank-based correlation analyses were carried out to evaluate potential co-occurrence, co-exclusion or random associations

(positive, negative or random pairwise correlations, respectively) between the identified fungal species within each of the black kite groups. Statistical analyses were performed with the IBM SPSS Statistics programs, as well as with the Python SciPy and Seaborn packages. Statistical significance was set at $P < 0.05$ (two-sided).

3. Results

3.1. Appearance, prevalence and number of oral lesions

Oral lesions often appeared as strongly attached discrete nodules of variable size, elliptical or irregular form, and white-beige color, mostly located on the lower part of the tongue and on the palate (Fig. 2A). Less frequently, the lesions extended throughout the oral cavity, including pharynx, choana and cavity walls, combining discrete nodules, irritated areas, ulcerated plaque-like areas and less compact material with a cotton-like aspect (see examples in Fig. 2B and C).

In Madrid, we examined 38 nestlings from 18 broods (median number of nestling sampled per brood = 2, range = 1–3), and found lesions in 14 nestlings from 9 nests (prevalence, 36.8%, 95% CI: 23.4–52.7). The number of oral lesions recorded in individual nestlings ranged between 1 and 29 (median, 2). In Doñana, we examined 105 nestlings from 75 nests, and we did not find any nestling with lesions.

3.2. Isolated fungal species in the oral lesions and cavity

Fungal species were isolated from the oral lesions of all symptomatic nestling black kites from Madrid ($n = 13$) as well as the oral cavity of all asymptomatic nestling black kites from Madrid ($n = 22$) and Doñana ($n = 13$) sampled. A total of 56 fungal isolates were obtained from this study subpopulation, of which 16 were identified in symptomatic nestlings from Madrid, 24 in asymptomatic nestlings from Madrid, and 16 in

nestlings from Doñana. No significant differences in the number of isolated fungal species per individual were found among the black kite groups (median = 1 for all groups; range = 1–3 for nestlings from Madrid with oral lesions and from Doñana, and 1–2 for nestlings from Madrid without oral lesions; Kruskal-Wallis test, $H = 0.52$; $df = 2$, $P = 0.08$; Table 1).

Further analysis of the fungal community composition in the oral cavity revealed that Ascomycota and Mucoromycota were the dominant phyla (Fig. 3A). However, their relative proportion differed among nestling individuals of the three black kite groups. Ascomycota was more prevalent in nestlings from Doñana than in those from Madrid, while Mucoromycota was more frequently isolated in individuals from Madrid than in those from Doñana. The relative occurrence of Ascomycota in nestlings from Doñana (14 out of 16, 87.5%) and symptomatic nestlings from Madrid (10 out of 16; 62.5%) was higher than Mucoromycota, whereas both fungal phyla showed the same occurrence in asymptomatic nestlings from Madrid (12 out of 24; 50%).

In contrast, common and unique fungal orders, genera and species were identified in nestling black kites from the three studied groups (Fig. 3B and C, and Table 1). At the order taxonomic level, the most abundant fungal orders in nestlings from Madrid with and without oral lesions were Mucorales (31.3% and 50.0%, respectively) and Pleosporales (31.3% and 29.2%, respectively), followed by Hypocreales (18.8% and 12.5%, respectively), while those in nestlings from Doñana were Pleosporales (43.8%), followed by Sordariales (18.8%), Mucorales (12.5%) and Hypocreales (12.5%) (Fig. 3B). These represented 81.3% of the total identified fungal orders in symptomatic nestlings from Madrid, 91.7% in asymptomatic nestlings from Madrid, and 87.5% in nestlings from Doñana. Pleosporales, Mucorales and Hypocreales were common in all groups, whereas Eurotiales was identified in nestlings from Madrid with oral lesions and from Doñana, and Sordariales was shared by asymptomatic nestlings from Madrid and Doñana. Saccharomycetales and Mortierellales were exclusively isolated in symptomatic nestlings from Madrid, and Helotiales and Xylariales were

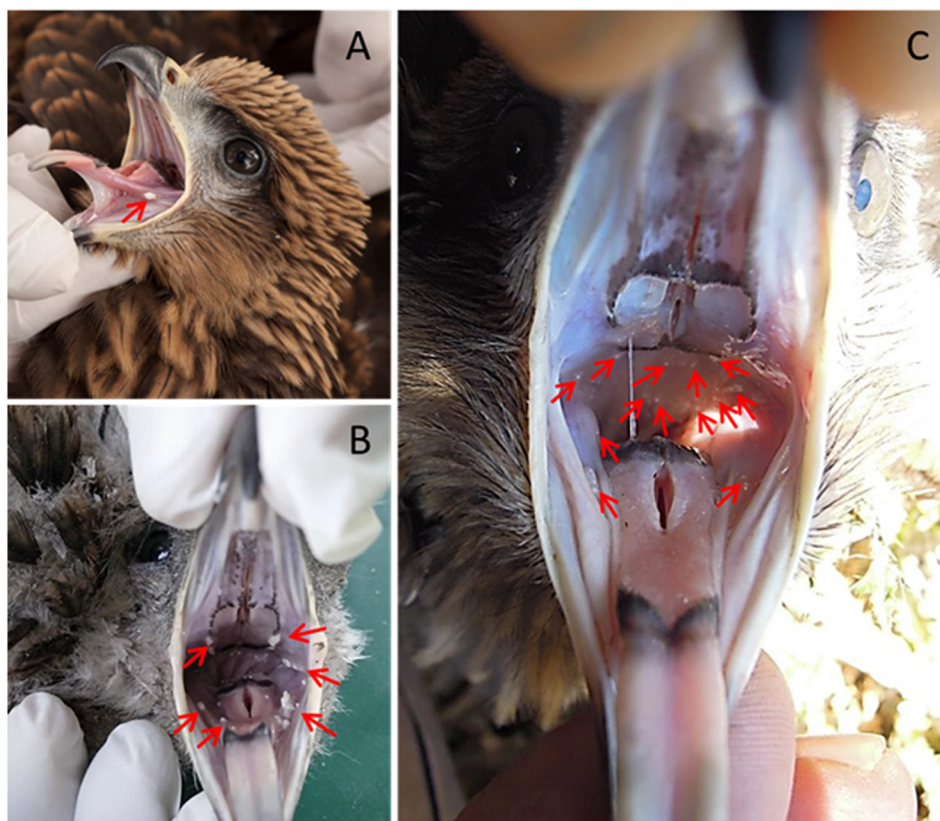


Fig. 2. Examples of mucosal lesions in the oral cavity of nestling black kites examined in Madrid, central Spain, showing (A) discrete nodules, (B) multiple lesions with a cotton-like aspect, and (C) ulcerated plaque-like areas. Arrows indicate the location of lesions.

Table 1

Distribution of the different fungal species isolated and identified from the oral cavity of nestling black kites with and without lesions from Madrid and Doñana.

Fungal species	Black kites ¹				All fungal isolates (n = 56)
	SM (n = 13)	AM (n = 22)	AD (n = 13)	All (n = 48)	
Number (%) of fungal isolates					
Phylum Ascomycota					
Order Eurotiales					
<i>Penicillium brevicompactum</i>	1 (7.7)	0 (0)	0 (0)	1 (2.1)	1 (1.8)
<i>Penicillium/Talaromyces</i> sp. ²	0 (0)	0 (0)	1 (7.7)	1 (2.1)	1 (1.8)
Order Helotiales					
<i>Botrytis</i> sp.	0 (0)	1 (4.5)	0 (0)	1 (2.1)	1 (1.8)
Order Hypocreales					
<i>Fusarium incarnatum-equiseti</i> species complex	3 (23.1)	1 (4.5)	0 (0)	4 (8.3)	4 (7.1)
<i>Fusarium</i> sp.	0 (0)	1 (4.5)	0 (0)	1 (2.1)	1 (1.8)
<i>Trichoderma gamsii</i>	0 (0)	0 (0)	1 (7.7)	1 (2.1)	1 (1.8)
<i>Trichoderma</i> sp.	0 (0)	1 (4.5)	1 (7.7)	2 (4.2)	4 (3.6)
Order Pleosporales					
<i>Alternaria didymospora</i> (formerly <i>Embellisia didymospora</i>)	0 (0)	0 (0)	1 (7.7)	1 (2.1)	1 (1.8)
<i>Alternaria</i> sect. <i>Ulocladium</i> (formerly <i>Ulocladium</i>)	0 (0)	1 (4.5)	0 (0)	1 (2.1)	1 (1.8)
<i>Alternaria</i> sp.	5 (38.5)	5 (22.7)	3 (23.1)	13 (27.1)	13 (23.2)
<i>Drechslera/Pyrenophora</i> sp. ²	0 (0)	0 (0)	2 (15.4)	2 (4.2)	2 (3.6)
<i>Epicoccum nigrum</i>	0 (0)	1 (4.5)	1 (7.7)	2 (4.2)	2 (3.6)
Order Saccharomycetales					
<i>Galactomyces</i> sp.	1 (7.7)	0 (0)	0 (0)	1 (2.1)	1 (1.8)
Order Sordariales					
<i>Sordaria</i> sp.	0 (0)	1 (4.5)	3 (23.1)	4 (8.3)	4 (7.1)
Order Xylariales					
<i>Truncatella</i> sp.	0 (0)	0 (0)	1 (7.7)	1 (2.1)	1 (1.8)
Phylum Mucoromycota					
Order Mortierellales					
<i>Mortierella reticulata</i>	1 (7.7)	0 (0)	0 (0)	1 (2.1)	1 (1.8)
Order Mucorales					
<i>Mucor circinelloides</i>	1 (7.7)	3 (13.6)	1 (7.7)	5 (10.4)	5 (8.9)
<i>Mucor fragilis</i>	0 (0)	0 (0)	1 (7.7)	1 (2.1)	1 (1.8)
<i>Mucor hiemalis</i>	1 (7.7)	1 (4.5)	0 (0)	2 (4.2)	2 (3.6)
<i>Mucor irregularis</i> (formerly <i>Rhizomucor variabilis</i>)	1 (7.7)	0 (0)	0 (0)	1 (2.1)	1 (1.8)
<i>Mucor mucedo</i>	0 (0)	1 (4.5)	0 (0)	1 (2.1)	1 (1.8)
<i>Mucor racemosus</i>	0 (0)	1 (4.5)	0 (0)	1 (2.1)	1 (1.8)
<i>Mucor racemosus/Mucor plumbeus</i> ³	1 (7.7)	4 (18.2)	0 (0)	5 (10.4)	5 (8.9)
<i>Mucor saturninus</i>	0 (0)	1 (4.5)	0 (0)	1 (2.1)	1 (1.8)
<i>Rhizopus oryzae</i>	1 (7.7)	1 (4.5)	0 (0)	2 (4.2)	2 (3.6)
Fungal species/black kite ratio (total numbers)	1.2 (16/13)	1.1 (24/22)	1.2 (16/13)	1.2 (56/48)	
Median number (range) of fungal species per individual	1 (1–3)	1 (1–2)	1 (1–3)	1 (1–3)	

¹ SM, symptomatic nestling black kites from southeastern Madrid; AM, asymptomatic nestling black kites from southeastern Madrid; and AD, asymptomatic nestling black kites from Doñana.

² Molecular methods used for fungal identification could not discriminate between both genera from the same order.

³ Molecular methods used for fungal identification could not differentiate between both species.

only detected in asymptomatic nestlings from Madrid and Doñana, respectively.

Alternaria was one of the most dominant fungal genera in all groups (38.5% in symptomatic nestlings from Madrid, 27.3% in asymptomatic nestlings from Madrid, and 30.8% in nestlings from Doñana) (Table 1). The other most prevalent fungal genera were *Mucor* (30.8%), followed by *Fusarium* (23.1%) in symptomatic nestlings from Madrid, *Mucor* (50.0%) in asymptomatic nestlings from Madrid, and *Sordaria* (23.1%) in nestlings from Doñana. *Alternaria* sp. and *M. circinelloides* were isolated in all groups, while four fungal taxa were uniquely detected in symptomatic nestlings from Madrid, six in asymptomatic nestlings from Madrid, and six in nestlings from Doñana (Fig. 3C and Table 1). Although *F. incarnatum-equiseti* species complex, *Mucor hiemalis*, *M. racemosus*/*M. plumbeus* and *Rhizopus oryzae* were common in nestlings from Madrid with and without oral lesions, no fungal genera or species other than those isolated in all groups were shared by nestlings from Madrid with oral lesions and from Doñana. *Trichoderma* sp., *Epicoccum nigrum* and *Sordaria* sp. were identified in asymptomatic nestlings from Madrid and Doñana, but not in symptomatic nestlings from Madrid.

3.3. Fungal species patterns in nestlings of the same brood

Of the 35 nestlings sampled in Madrid for fungal isolation and identification, there were six nests with a single nestling (two nests with a single

symptomatic nestling and four nests with a single asymptomatic nestling), and thirteen nests with two or three siblings (two nests with oral lesions present in all siblings, five nests with oral lesions present in some siblings but not in others, and six nests with no lesions in all siblings of the same brood) (Figs. 4A–C). Of the 13 nestlings sampled in Doñana, there were six nests with a single nestling and three nests with two or three siblings (Fig. 4D).

Overall, oral fungal occurrence patterns differed among siblings of the same brood in any of the study areas (Fig. 4). No common fungal species or genera were identified in nests with oral lesions in all siblings (in Madrid; Fig. 4A). The proportion of nests with some or all siblings sharing a common fungal species or genus in their oral cavity was higher when oral lesions were present in some siblings but not in others of the same brood (in Madrid; Fig. 4B) than when all siblings had no oral lesions (in Madrid and Doñana; Fig. 4C and D).

3.4. Oral fungal signatures

Two-way HCA on the fungal occurrence rates of each black kite group showed two major clusters of highly correlated fungal occurrence profiles at the order and species levels (clusters BC and A; geographical area-associated fungal signatures) that separated nestling black kites from Madrid and Doñana into two discrete groups (clusters M and D, respectively; Fig. 5). In turn, cluster BC (Madrid-associated fungal signature)

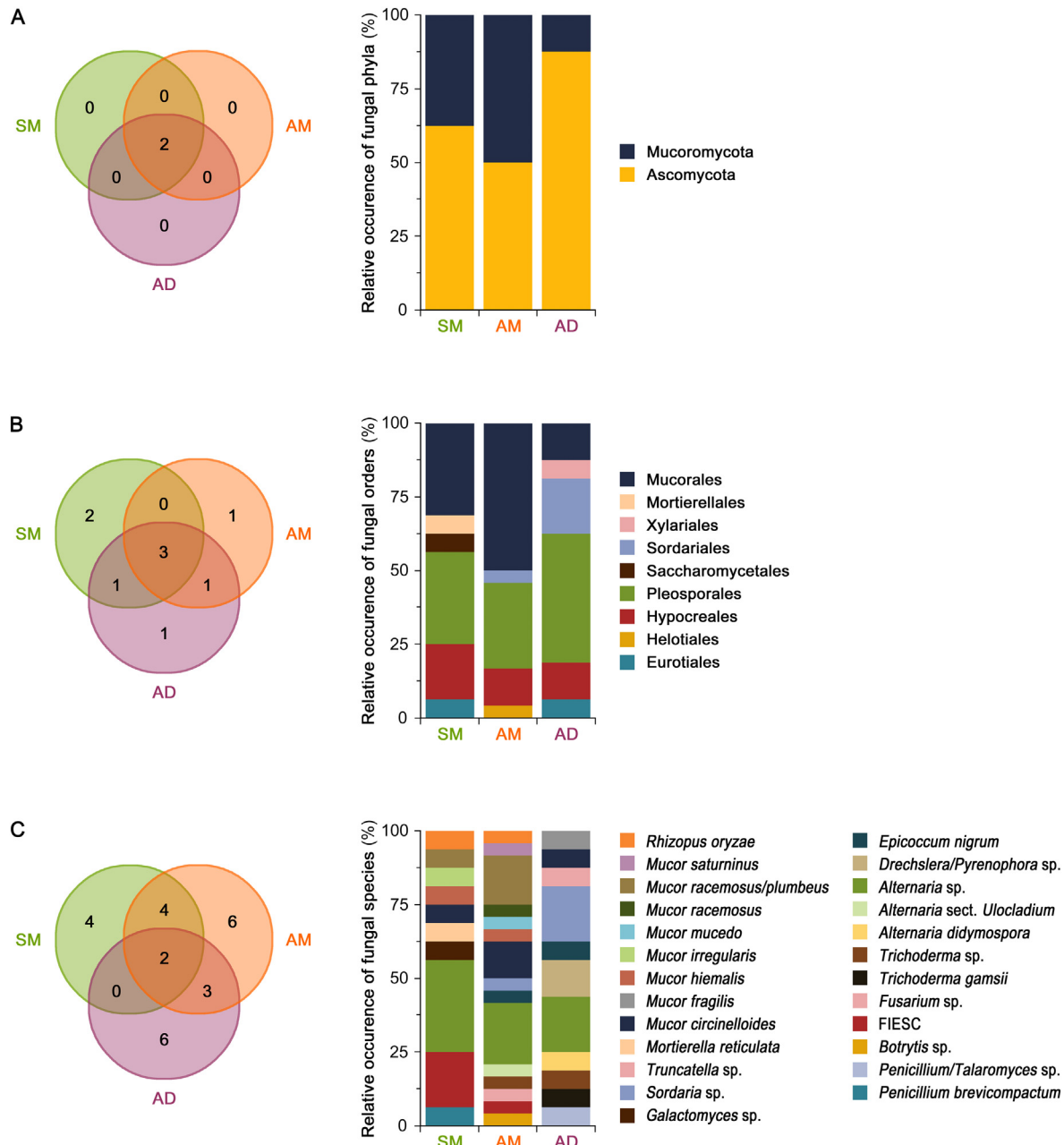


Fig. 3. Oral fungal community composition of nestling black kites with and without lesions from Madrid and Doñana at the (A) phylum, (B) order and (C) species taxonomic level. In the *left panels*, Venn diagrams indicate the common and unique fungal taxa at the phylum, order and species level identified in the three study groups. In the *right panels*, bar charts show the distribution of relative occurrence rates of fungal taxa at the phylum, order and species level isolated in each black kite group. SM, symptomatic nestling black kites from southeastern Madrid; AM, asymptomatic nestling black kites from southeastern Madrid; AD, asymptomatic nestling black kites from Doñana; and FIESC, *Fusarium incarnatum-equiseti* species complex.

comprised two subclusters of more strongly correlated fungal order or species occurrence profiles that segregated symptomatic from asymptomatic nestlings from Madrid (subclusters B and C, respectively).

PCA on the global fungal occurrence patterns of each black kite group at the order and species taxonomic levels confirmed that these profiles were distinct between Madrid and Doñana (clusters M and D; Fig. 6A). PCA further revealed that 96.7% (79.2% and 17.5% for PC1 and PC2, respectively) and 87.0% (58.6% and 28.4% for PC1 and PC2, respectively) of the total variance of the fungal order and species occurrence profiles, respectively, were caused by differences between both geographical locations under contrasting environmental conditions (primary contributor to PC1 and PC2; M vs D; Fig. 6A), whereas the remaining dataset variance (3.3% and 13% for PC3 in

the order and species taxonomic level, respectively) was related to presence and absence of oral lesions (primary contributor to PC3; symptomatic nestlings from Madrid vs asymptomatic nestlings from Madrid and Doñana; Fig. 6B).

The different HCA-defined fungal signatures ((sub)clusters A, BC, B and C) were also discerned by the two first PCs of PCA on the occurrence profiles of each identified fungal order or species across all nestling groups (Fig. 6C). PC1 and PC2 explained 100% of the total variations in the dataset: 54.2% and 57.5% for PC1 in the order and species taxonomic level, respectively, and 45.8% and 42.5% for PC2 in the order and species taxonomic level, respectively. The fungal signatures found for both order and species taxonomic levels and related to distinct geographical areas

SE Madrid

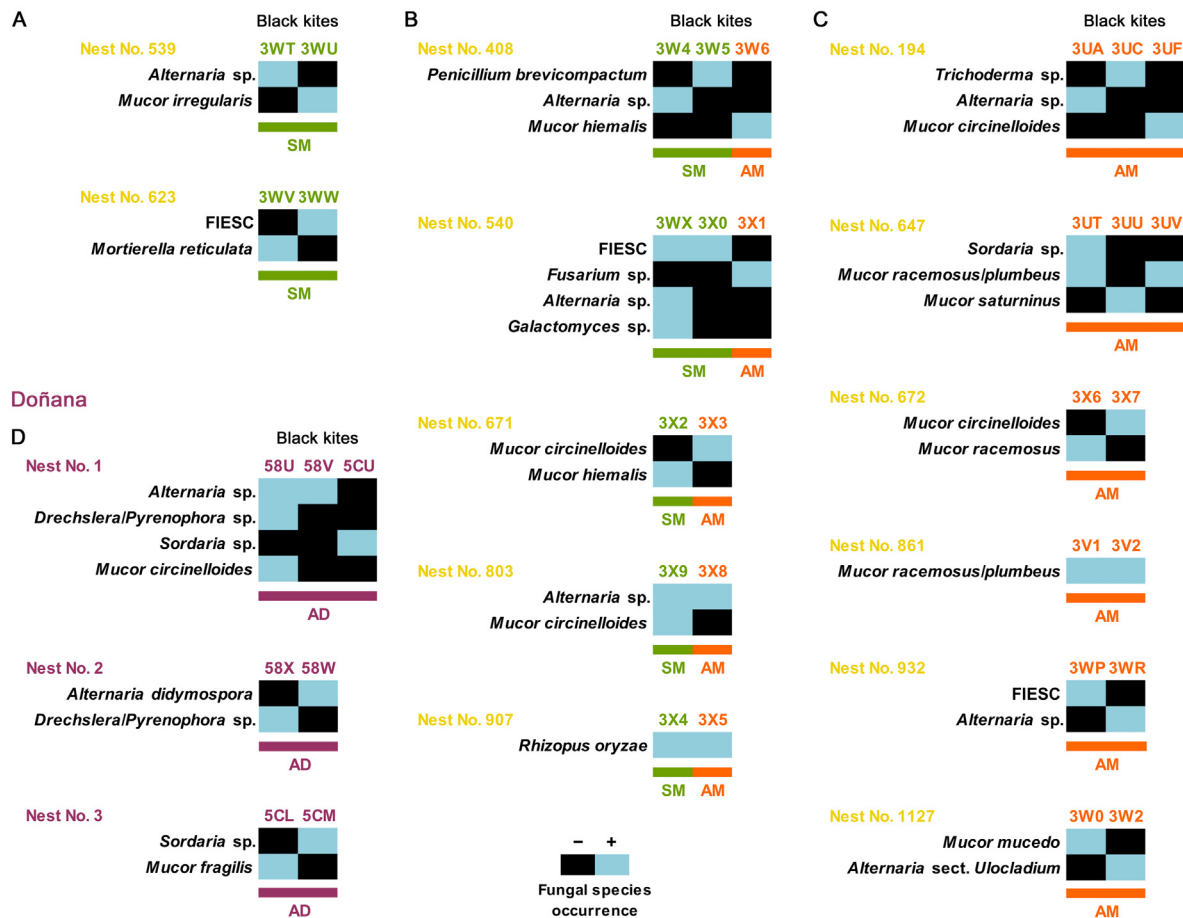


Fig. 4. Fungal species profiles in black kite siblings of the same brood in the two study areas. A. Nests with oral lesions present in all siblings in Madrid. B. Nests with oral lesions present in some siblings but not in others in Madrid. C. Nests with no lesions in all siblings in Madrid. D. Nests with no lesions in all siblings in Doñana. The occurrence of fungal species (*oblongs*) are indicated by color in the legend (absence in black and presence in blue). Black kite siblings of the same brood are shown in columns, and fungal species in rows. The horizontal colored bars below the heat maps represent black kite groups (SM, AM and AD). Ring codes of siblings in Madrid (SM in green and AM in orange) and Doñana (AD in violet) and their corresponding nest codes (Madrid in yellow and Doñana in violet) are shown. SM, symptomatic nestling black kites from southeastern Madrid; AM, asymptomatic nestling black kites from southeastern Madrid; AD, asymptomatic nestling black kites from Doñana; and FIESC, *Fusarium incarnatum-equiseti* species complex.

(Madrid and Doñana) were distinguished along PC1 (clusters BC vs A), while those associated with the presence or absence of oral lesions were differentiated along PC2 ((sub)clusters B vs C and A).

Oral infection-associated fungal species signature (subcluster B; Fig. 5, right) was able to discriminate diseased from healthy nestling black kite individuals (ROC areas, 0.85 and 0.92; 95% CI, 0.72–0.99 and 0.80–1.00; $P = 0.001$ and <0.001 , respectively). Doñana- or natural environment-associated fungal species signature (cluster A; Fig. 5, right) accurately identified the oral fungal patterns of nestlings from Doñana from those of nestlings from Madrid (ROC areas, 0.96 and 0.94; 95% CI, 0.87–1.00 and 0.84–1.00; $P < 0.001$, respectively). In the same way, Madrid- or highly altered environment-associated fungal species signature (cluster BC; Fig. 5, right) accurately differentiated between nestling black kites from contrasting environmental areas (nestlings from Madrid vs Doñana) (ROC area, 0.93; 95% CI, 0.85–1.00; $P < 0.001$).

3.5. Risk and protective factors for oral disease and environmental degradation

We next investigated whether subcluster B (Fig. 5, right) could conceal predictors of oral infection. A positive association was observed between the risk of oral disease and the occurrence of *F. incarnatum-equiseti* species

complex (OR, 40.0; 95% CI, 2.1–1092.1; $P = 0.01$) and *Alternaria* sp. (OR, 12.0; 95% CI, 1.2–123.7; $P = 0.04$) in nestlings. These analyses were then conducted on clusters BC and A (Fig. 5, right). A positive association was found between exposure to highly altered environments and the occurrence of *Alternaria* sp. on the oral cavity of nestling black kites (OR, 6.7; 95% CI, 1.1–42.5; $P = 0.04$). On the contrary, there was a negative relationship between this exposure and the presence of *Sordaria* sp. (OR, 0.01; 95% CI, 0.001–0.2; $P = 0.003$), *E. nigrum* (OR, 0.03; 95% CI, 0.001–0.9; $P = 0.047$) and *Trichoderma* sp. (OR, 0.03; 95% CI, 0.001–0.9; $P = 0.047$) in their oral cavity.

3.6. Oral fungal species co-occurrence interactions

No significant co-occurrence interactions between the identified fungal species across oral samples were observed in symptomatic nestlings from Madrid. In contrast, fungal species belonging to distinct phyla (Ascomycota and Mucoromycota) co-occurred in the microbial communities of nestling black kites without oral lesions. In particular, co-occurrence associations were detected between *Sordaria* sp. and *M. racemosus*/*M. plumbeus* in asymptomatic nestlings from Madrid, as well as between *Drechslera*/*Pyrenophora* sp. and *M. circinelloides* in nestlings from Doñana. No fungal co-exclusion relationships were found in any black kite group.

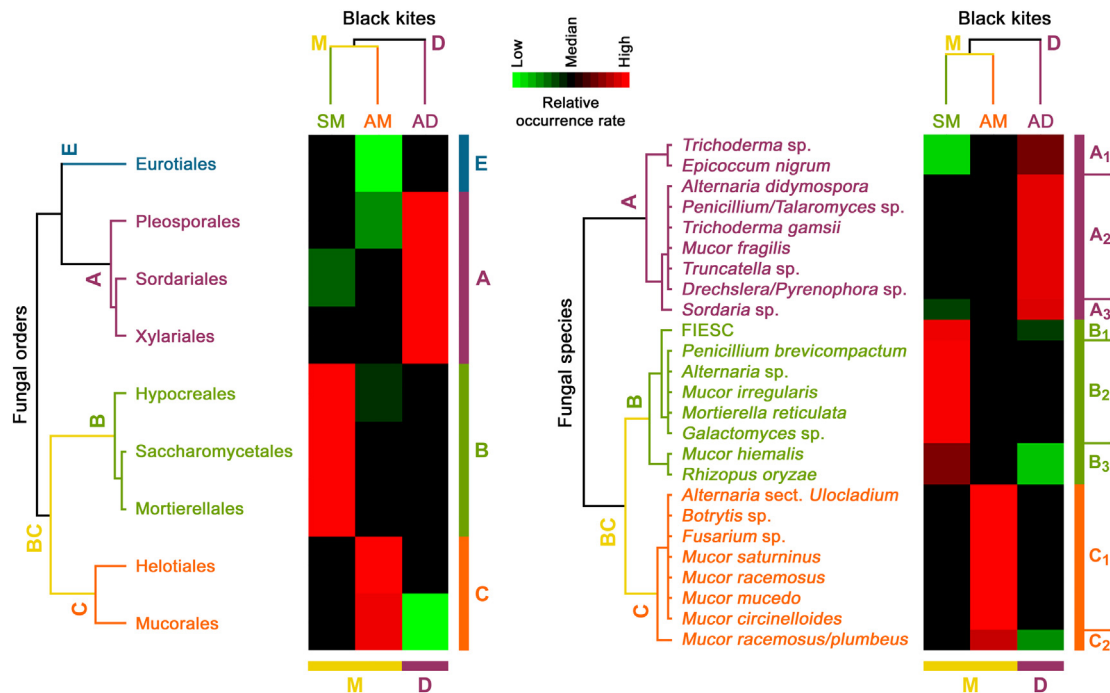


Fig. 5. Two-way HCA on the occurrence rates of oral fungal communities of nestling black kites with and without lesions from Madrid and Doñana at the order (left panel) and species (right panel) taxonomic level. Dendrograms and heat maps show the clustering of black kite groups (columns) and fungal taxa (rows) based on overall similarities of the occurrence patterns of fungal taxa in each black kite group and the occurrence profiles of each fungal taxon identified in all nestling groups, respectively. Relative occurrence rates of fungal taxa (oblongs) are indicated by color in the legend (low occurrence in green, medium occurrence in black and high occurrence in red). Nestling black kites from Madrid (SM and AM) and Doñana (AD) were separated into two clusters (M and D, respectively). The vertical colored bars to the right of the heat map represent fungal taxonomic signatures (clusters and subclusters of fungal taxa whose occurrence rates were highly correlated across all black kite groups). SM, symptomatic nestling black kites from southeastern Madrid; AM, asymptomatic nestling black kites from southeastern Madrid; AD, asymptomatic nestling black kites from Doñana; and FIESC, *Fusarium incarnatum-equiseti* species complex.

4. Discussion

4.1. Early-life exposure to environmental degradation induces a shift towards a higher prevalence of oral opportunistic pathogenic species in nestlings, promoting oral disease

This study provides compelling evidence that early-life exposure to highly degraded environments favors oral opportunistic infections in nestling black kites, as reflected in gross lesions in their oral cavity. In accordance with the hypothesis that environmental degradation increases the impact of disease in wildlife, nestlings affected by oral lesions were found at a relatively high frequency in Madrid. The more degraded and contaminated breeding habitats are often linked to disrupted natural food-web conditions, specifically depending on the exploitation of abundant, concentrated and predictable but risky contaminated food obtained in large dumps of refuse from urban areas (Batley et al., 2013; Lehel and Murphy, 2021). On the contrary, we observed no nestling with oral lesions in Doñana, where black kites nest and forage in a comparatively well-conserved environment and mostly feed on wild prey. The appearance of the lesions was similar to that reported in several species of nestling vultures, which suffered from oral dysbiosis induced by early-life exposure to antibiotics and other pharmaceuticals when feeding on medicated livestock carrion (Blanco et al., 2016, 2017a, 2020; Pitarch et al., 2017, 2020; Gómez-Ramírez et al., 2020). However, the prevalence of lesions was lower in nestling black kites than in nestling vultures (Pitarch et al., 2017).

We found that the oral fungal occurrence patterns in nestling black kites were distinct between the two study areas. Our results revealed a higher occurrence of Mucoromycota in the oral cavity of nestling individuals that inhabited in a highly degraded environment (Madrid) than in a natural environment (Doñana). The most prevalent fungal species identified in this phylum (*Mucor* spp. and *Rhizopus* spp.) were the most common etiologic

agents of mucormycosis, a serious fungal infection associated with fatal clinical outcomes in humans and animals with compromised immunity (Mendoza et al., 2014; Seyedmousavi et al., 2018; Reid et al., 2020; Steinbrink and Miceli, 2021). This shift towards a higher occurrence of opportunistic pathogenic fungi in the oral cavity of nestlings from natural to highly-altered environments could contribute to the increased risk of oral lesions observed in individuals from Madrid. This finding is consistent with recent studies showing an association between environmental contamination and microbiome dysbiosis with subsequent health effects (Vallès and Francino, 2018; Nolorbe-Payahua et al., 2020; Vignal et al., 2021).

4.2. Siblings of the same brood have different oral fungal patterns but may share a fungal taxon

Our data showed that the presence or absence of nestlings with oral lesions in Madrid does not appear to follow a clear pattern within each nest, supporting the view that there should be no pseudoreplication in terms of the oral fungal species present in siblings with or without lesions. This also suggests particular phenotypic, genetic and epigenetic variance among individuals in shaping the organism's competence to cope with environmental stress that is reflected in condition-mediated disease (Blanco et al., 2022).

In most nests with more than one nestling, oral fungal patterns were markedly different among siblings of the same brood in any of the study areas. These data, together with the observation that no common fungal species was isolated from nests in which all siblings had lesions, prompt the proposal that oral lesions in nestling black kites are caused by a non-contagious fungal disease. Despite this dissimilarity in fungal patterns among siblings, a higher proportion of nests with some or all siblings sharing a common fungal species or genus were found in broods where there were siblings with or without lesions than in broods where all siblings

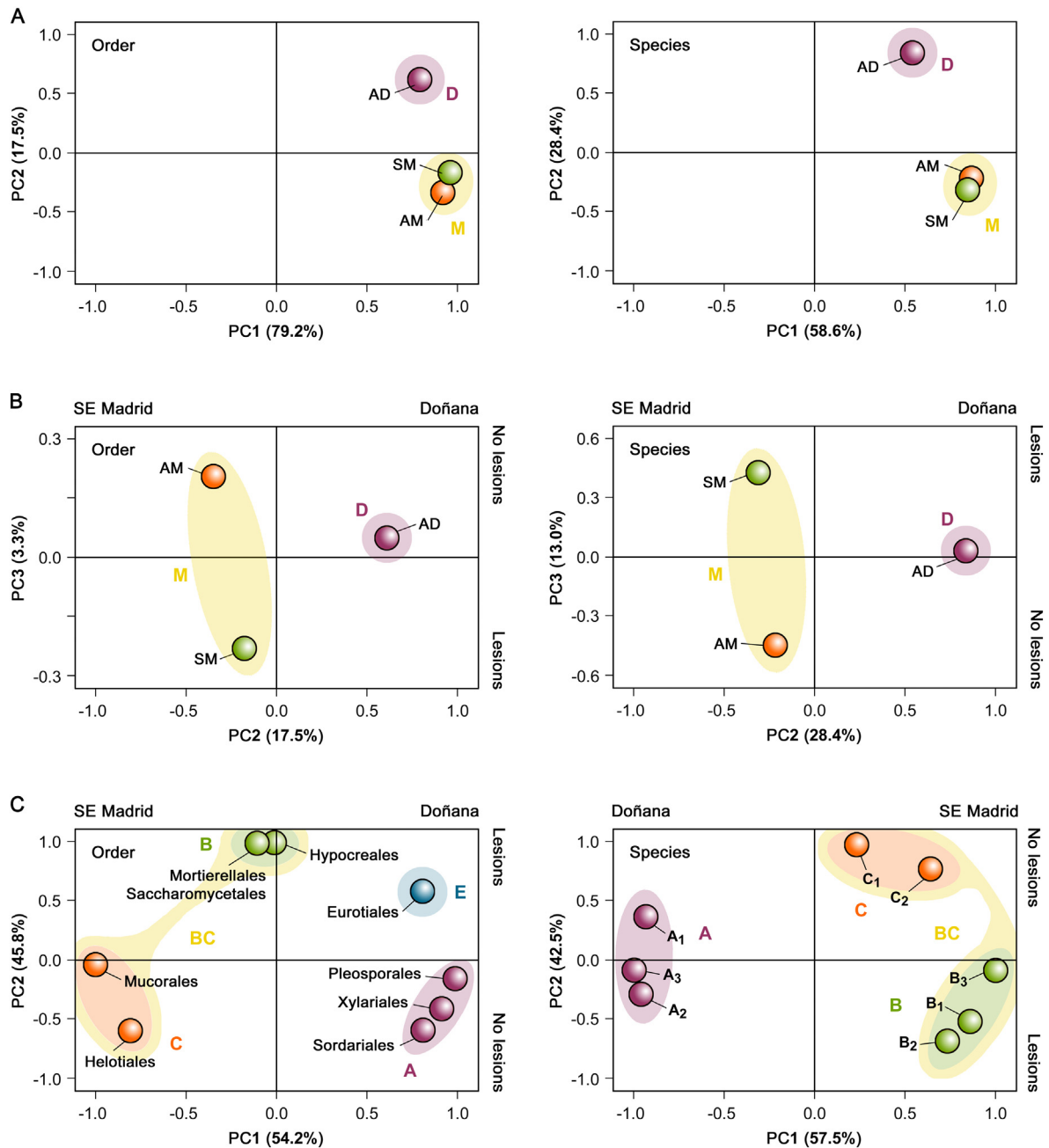


Fig. 6. PCA on the occurrence rates of the oral fungal communities of nestling black kites with and without lesions from Madrid and Doñana at the order (left panels) and species (right panels) taxonomic level. A and B. PCA of the overall patterns of fungal taxon occurrence of each black kite group within a two-dimension vector space showing (A) PC1 vs PC2 and (B) PC2 vs PC3. Each circle denotes the occurrence pattern of a single black kite group. Areas shaded in color represent clustering of nestling groups. SM, symptomatic nestling black kites from southeastern Madrid; AM, asymptomatic nestling black kites from southeastern Madrid; and AD, asymptomatic nestling black kites from Doñana. C. PCA of the occurrence patterns of each fungal taxon identified in all black kite groups within a two-dimension vector space. Each circle shows the occurrence pattern of a single fungal taxon or subgroup of fungal taxa (labelled as in Fig. 5) in the study population. Areas shaded in color depict clustering of fungal taxa. In A–C, the percentages of the variance of the data set explained by the principal components (PC1, PC2 or PC3) are indicated on their corresponding axes. Note that color codes are the same as in Fig. 5.

had no oral lesions (both in Madrid and Doñana). This result is in line with an earlier report showing that microbial patterns are more similar between diseased individuals and their healthy siblings than among all-healthy siblings because of the lower diversity of the core microbiota among the former than among the latter (Hedin et al., 2017). These findings should facilitate further longitudinal studies that include data on nestling feathers or blood to elucidate whether siblings of symptomatic nestlings might have lower levels of exposure to certain anthropogenic pollutants or some immunological advantage that could explain their asymptomatic state, as well as

to assess the impact of highly degraded environments on broods composed of symptomatic and asymptomatic siblings.

4.3. Anthropogenic and natural environmental factors have a greater impact on fungal patterns of nestlings than oral disease

Previous studies have highlighted that exposure to environmental pollutants (such as heavy metals, pesticides, fertilizers, microplastics, nanoparticles, urban and industrial wastes, sewage, antibiotics and other

pharmaceutical chemicals), dietary changes related to natural and anthropogenic stresses, and spatial and climatic signals, among others, can exert a great impact on the host microbiota composition (Wang et al., 2015; Jin et al., 2017; Leeming et al., 2019; Zuza-Alves et al., 2019; Nolorbe-Payahua et al., 2020; Pitarch et al., 2020; Teyssier et al., 2020). The dissimilarities in oral fungal patterns between nestling black kites from Madrid and Doñana could thus be attributed to differences of anthropogenic and natural origin between the two study areas, in line with earlier works (Wang et al., 2015; Zuza-Alves et al., 2019; Nolorbe-Payahua et al., 2020; Pitarch et al., 2020). Our PCA data support the notion that anthropogenic environmental factors (i.e., environmental pollution due to anthropogenic activities, such as those related to solid waste incinerators, fire places, furnaces, pesticide and fertilizer pollution from the agricultural area, industry and vehicular traffic, which are characteristic of the Madrid study area (Jiménez et al., 2000; Blanco et al., 2003, 2018; Escobar-Arnanz et al., 2018)) and natural environmental factors (e.g., climate, geographic distance, habitat, ecology, food resource, and long-term diet, among others) (primary contributors to PC1 and PC2) had a more profound impact on the oral fungal patterns of nestlings than their health or disease states, as estimated by visible oral lesions (primary contributor to PC3; Fig. 6A and B). Therefore, the presence of certain fungal species in the oral cavity of nesting black kites could be the result, at least to a greater extent, of repeated or continuous exposure to sources in their immediate environment, which differ between the two study areas. These findings suggest that early exposure to anthropogenic environmental stress critically affects the behavior of the oral fungal patterns of nestlings, which is already visible in early life. In agreement with previous studies (Vallés and Francino, 2018; Evariste et al., 2019), these alterations can have short-term consequences on health and survival, and potential long-term effects on fitness. Future studies are needed to evaluate the impact of oral disease on nestling health, body condition and survival during the nestling stage and its long-term fitness consequences, as well as to assess the influence of natural environmental factors (such as spatial and climatic cues, among others) on oral fungal diversity patterns and disease.

4.4. Anthropogenic pressure-associated fungal signatures harbor opportunistic pathogenic species that could be used as bioindicators of oral disease and environmental pollution

We identified distinct fungal signatures within the oral cavity of nestling black kites that were associated with oral disease or different environmental pressures. In particular, the oral disease-associated fungal signature (subcluster B) encompassed opportunistic pathogens of plants and, more rarely, of vertebrates (including humans), such as *F. incarnatum-equiseti* species complex (Khan et al., 2021), *Penicillium brevicompactum* (Caro-Vadillo et al., 2007), *Alternaria* sp. (Williams et al., 2008), *Mucor irregularis* (Tang et al., 2021), *M. hiemalis* (Desai et al., 2013) and *R. oryzae* (Doub et al., 2020). *F. incarnatum-equiseti* species complex and *Alternaria* sp. were identified as potential microbial predictors of oral disease. These are mycotoxigenic pathogens that can release diverse toxic metabolites and mycotoxins with a deleterious impact on human and animal health (Munkvold, 2017; Pinto and Patriarca, 2017). Their emergence in the oral cavity of nestling black kites could therefore play an important role in disease pathogenesis and be involved in the development of the observed oral lesions in symptomatic nestlings. This is consistent with earlier studies reporting the presence of oral lesions in broiler chickens and laying hens exposed to *Fusarium* mycotoxins in early life (Ademoyero and Hamilton, 1991; Brake et al., 2000), as well as alterations of esophageal mucosa of mice exposed to *Alternaria* mycotoxins (Yekeler et al., 2001). Accordingly, further research should aim to determine whether these microbial predictors and their released mycotoxins could be a cause or a consequence of oral disease in developing nestlings, in order to establish future medical and health approaches to counteract early imbalances in oral microbial communities of this facultative avian scavenger.

Within highly degraded environment-associated fungal signature (cluster BC), *Alternaria* sp. was also identified as a potential microbial biomarker

of exposure to environmental contaminants derived from anthropogenic activities. *Alternaria* spores are associated with biological air contamination (Grinn-Gofroń et al., 2011; Kasprzyk et al., 2015), and can coexist simultaneously with anthropogenic air pollutants during long-distance transport of ragweed pollen (Grewling et al., 2019). Earlier studies have reported that the concentration of airborne *Alternaria* spores correlates with the levels of atmospheric particulate matter of less than 10 µm diameter (PM₁₀) (Adhikari et al., 2006; Sousa et al., 2008). PM₁₀ is linked to air pollution due to anthropogenic activities, such as those related to solid waste incinerators, fire places, furnaces, industry, and vehicular traffic (Fischer et al., 2020; Ziegler et al., 2021), which are characteristic of the Madrid study area (Jiménez et al., 2000; Blanco et al., 2003, 2018; Escobar-Arnanz et al., 2018). Long-term exposure to particulate (PM₁₀) air pollution can cause detrimental effects on health and environment (Fischer et al., 2020). Further studies are now warranted to validate the usefulness of the occurrence of this fungus as a bioindicator of environmental pollution of anthropogenic origin as well as for environmental biomonitoring in highly contaminated and industrialized urban areas.

This fungal signature that differentiated between nestlings from natural and altered environments (cluster BC) also integrated several fungal taxa that are frequently isolated in anthropogenically polluted soil, water or air, such as *Fusarium*, *Penicillium* (*P. brevicompactum*), *Alternaria*, *Alternaria* section *Ulocladium*, *Mucor* (*M. hiemalis*, *M. plumbeus* and *M. circinelloides*), and *Rhizopus* species (Adhikari et al., 2006; Evdokimova et al., 2013; Novak-Babic et al., 2017). Many of these filamentous fungi are opportunistic pathogens to humans, animals or plants, and producers of secondary metabolites, such as mycotoxins (*Fusarium*, *Penicillium* and *Alternaria* species), allergenic compounds (*Fusarium*, *Penicillium*, *Alternaria*, *Mucor* and *Rhizopus* species) and microbial volatile organic compounds (*Penicillium* spp.) (Novak-Babic et al., 2017; Seyedmousavi et al., 2018), which can cause adverse effects on host health (Novak-Babic et al., 2017). These findings support the view that early-life exposure to widespread degraded environments related to anthropogenic activities and urban waste may promote the proliferation of opportunistic pathogens that will determine the health or disease status of nestling black kites.

Some of these opportunistic filamentous fungi present in the oral cavity of nestling black kites have been found in nests of birds (including raptors), such as *Fusarium* sp., *Penicillium* sp., *Alternaria* sp., *M. hiemalis*, and *M. circinelloides* (Kornilowicz-Kowalska et al., 2011; Kornilowicz-Kowalska and Kitowski, 2018; Ogórek et al., 2022). In addition to growing on and colonizing plant fragments of the bird nests (Ogórek et al., 2022), some of the potentially phytopathogenic and zoopathogenic fungi identified in nestling black kites have also been detected in bird droppings, such as *Penicillium* sp., *Mucor* sp. and *Rhizopus* sp. (Mendes et al., 2014; Ghaderi et al., 2019). These observations raise the possibility that bird nests and droppings could represent important sources of dissemination of these saprobic and coprophilous fungi in nestling black kites, and play an active role in the establishment and proliferation of oral infections during their early stages of life.

4.5. Natural environment-associated fungal signature reveals microbial taxa that may provide protective benefits in highly degraded environments

The natural environment-associated fungal signature (cluster A) mostly comprised several fungal species that may act as phytopathogen antagonists or biocontrol agents against plant fungal pathogens as well as environmental bioremediators, such as *Trichoderma* (Tripathi et al., 2013; Chen et al., 2016), *E. nigrum* (Ogórek and Plaskowska, 2011; Elsayad et al., 2020), *Penicillium/Talaromyces* (Dusengemungu et al., 2020), *Truncatella* (Busby et al., 2016), *Drechslera/Pyrenophora* (Solai et al., 2014; d'Errico et al., 2020) and *Sordaria* (Abdallah et al., 2018) species. These fungal biocontrol agents have evolved diverse mechanisms to protect plants against fungal pathogens (Köhl et al., 2019), and may be used for bioremediation of inorganic and organic pollutants, agrochemicals, and other environmental contaminants (Tripathi et al., 2013). These fungal taxa could therefore

be considered beneficial to the oral health of nestling black kites that are exposed to the adverse effects of environmental contaminants.

Trichoderma sp., *E. nigrum* and *Sordaria* sp. were identified as protective factors for exposure to environmental pollution. As these were identified exclusively in asymptomatic nestlings, their presence in the oral cavity could be playing a crucial role in host health. This possibility offers an explanation why asymptomatic nestlings from Madrid did not develop oral lesions despite sharing repeated or permanent exposure to the same anthropogenic stresses as symptomatic nestlings from Madrid. These fungi could help asymptomatic nestlings from Madrid to tolerate, detoxify and resist diverse anthropogenic environmental pollutants (Katayama and Matsumura, 1993; Bordjiba et al., 2001; Srivastava et al., 2011; Tripathi et al., 2013; Ahumada-Rudolph et al., 2021). Furthermore, the potential of these fungi as antagonists and biocontrol agents against several fungal pathogens (including *Fusarium* spp., *Alternaria* spp., *Mucor* spp. and *R. oryzae*) in plants (Watanabe, 1991; Dewan et al., 1994; Howell, 2002; Restuccia et al., 2006; Abdallah et al., 2018; Ramírez-Cariño et al., 2020; Pellán et al., 2021) could also be contributing to control possible infections caused by fungal pathogens (*Fusarium* spp., *Alternaria* spp., *Mucor* spp. and *R. oryzae*) present in the oral cavity of asymptomatic nestlings from Madrid and Doñana, and support the absence of oral lesions in these nestlings exposed to anthropogenic and natural pressures, respectively. These findings may open new research avenues on the prophylactic use of these fungi as potential probiotics against oral infections in nestling black kites.

4.6. Co-occurrence associations between potentially beneficial and pathogenic fungi may play a key role in health of nestlings

We detected co-occurrence associations between potentially beneficial fungi (fungal biocontrol agents or pathogen antagonists; such as *Sordaria* sp. or *Drechslera/Pyrenophora* sp.) and pathogenic fungi (*M. racemosus/M. plumbeus* or *M. circinelloides*) in asymptomatic individuals from Madrid and Doñana but not in symptomatic individuals from Madrid. These microbial co-occurrence interactions could provide health benefits to black kites, and represent a defense mechanism to cope with opportunistic fungal pathogens present in their oral cavity. In line with previous studies in plants (Watanabe, 1991; Dewan et al., 1994; Restuccia et al., 2006; Abdallah et al., 2018; Kornilowicz-Kowalska and Kitowski, 2018; d'Errico et al., 2020), these beneficial biocontrol agents or pathogen antagonists could reduce the pathogenicity of these opportunistic fungi and minimize the subsequent risk of oral infection. A better understanding of these relationships between potential beneficial and pathogenic fungal species within the oral microbial communities of nestling black kites could help policy-makers and conservation managers design new conservation initiatives aimed at ensuring the development of a health-promoting microbiota in the future (Bahrdorff et al., 2016; Vallès and Francino, 2018).

5. Conclusions

This work reveals a shift towards a higher prevalence of fungal species with pathogenic potential in the oral cavity of nestling black kites exposed to highly degraded environments in early life, increasing the risk of developing oral lesions. As a result, nestling individuals in natural and anthropogenically altered habitats harbor distinct fungal signatures within their oral microbial communities. Some of their microbial taxa could be used as potential predictors of oral disease (*F. incarnatum-equiseti* species complex and *Alternaria* sp.), bioindicators of anthropogenic environmental pollution (*Alternaria* sp.), and protective factors for exposure to environments altered by anthropogenic activities (*Trichoderma* sp., *E. nigrum* and *Sordaria* sp.). These protective factors as well as co-occurrence associations between potential beneficial fungi (fungal biocontrol agents or antagonists against fungal pathogens) and fungal pathogens are distinctive hallmarks of asymptomatic nestling black kites. These could be helping asymptomatic nestlings to tolerate, detoxify or resist some natural and anthropogenic environmental stresses and pollutants, and thus play a key role in their health. Further studies should aim to validate the potential prophylactic role of

these beneficial fungi for nestling black kites, as well as the use of the identified microbial biomarkers for oral disease and environmental biomonitoring in anthropogenically contaminated habitats. This could give rise to the potential future development of specific conservation measures and management recommendations to counteract microbiota imbalances and promote the health of black kites and other raptors affected by anthropogenic environmental degradation in early life stages.

CRedit authorship contribution statement

Aida Pitarch: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Javier Diéguez-Urbeondo:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – review & editing, Funding acquisition. **Laura Martín-Torrijos:** Methodology, Investigation, Formal analysis, Visualization, Writing – review & editing. **Fabrizio Sergio:** Conceptualization, Methodology, Investigation, Visualization, Writing – review & editing, Funding acquisition. **Guillermo Blanco:** Conceptualization, Methodology, Investigation, Visualization, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition.

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.

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