TÉCNICO LISBOA

Phenotypic characterization of the pathogenic profile of oral *Mucor* species in Eurasian Griffon Vultures

Catarina Caiola Raposo, 97044

Faculdade de Medicina Veterinária da Universidade de Lisboa

Abstract

The Eurasian Griffon Vulture is a specialized scavenger, which inhabits rugged and mountainous areas in southern Europe surrounding the Mediterranean. Due to poisoning and decline in food resources, there has been a rise in the number of vultures needing veterinary care and hospitalization in Wildlife Hospitals and Rehabilitation Centres in the Iberian Peninsula. In captivity, vultures often develop oral and other infectious diseases, which can potentially affect their survival and the probability of reintroduction in the wild. As such, it is important to characterize the pathogenic potential of relevant microbial species present in the oral cavity of these animals. In this work, *Mucor* spp. isolates were obtained from Eurasian Griffon Vulture (*Gyps fulvus*) and Cinereous Vulture (*Aegypius monachus*) oral swabs collected at CERAS, Castelo Branco (n=2) and at RIAS, Olhão (n=5), and their pathogenic potential assessed. The isolates identification was performed through macro and microscopic observation and confirmed by PCR and ITS sequencing, and their phenotypic pathogenic profile determined as well as their inhibitory capacity, using a collection of potentially pathogenic bacteria from the Laboratory of Bacteriology of FMV/ULisboa, as well as two yeasts species isolated from the oral cavity of the vultures, frequently associated with the development of oral disease. Lastly, the oral samples from which an interesting broad inhibitory spectrum was shown, were subjected to a metagenomic analysis to evaluate the presence of pathogenic bacteria belonging to same genera used in the *in vitro* inhibition assays.

It was possible to observe that 85.7% (6/7) of the isolates produced alpha-haemolysis, 71.4% (5/7) expressed DNase, 57.1% (4/7) produce lecithinase and lipase, 28.6% (2/7) expressed gelatinase and, lastly, 28.6% (2/7) revealed to be strong biofilm-producers. Concerning the inhibitory ability, 3 isolates (2, 3 and 4) were suggestive of presenting a broad spectrum of action. Thus, these isolates were subjected to a metagenomic analysis which revealed that the *Mucor* isolates may present that same inhibition *in vivo.*

Keywords: *Mucor* spp., virulence factors, vultures, Eurasian Griffon Vulture, oral diseases, antimicrobial activity

1. Introduction

The Eurasian Griffon Vulture (*Gyps fulvus*) and Cinereous Vulture (*Aegypius monachus*) are extremely important for our ecosystem. The species has undergone dramatic declines leading to extinction in the Alps and the Carpathians. These declines were mainly due to persecution by man and poisoning (Moleón, M. et al, 2014). The remnant populations are isolated and in urgent need of conservation action assisted by international cooperation and provision of expertise. In Portugal, vultures are considered Near-Threatened species and world-wide are considered Least-Concerned, but still their global populations have suffered serious and long-term declines, which is an indicative that scavenger's conservation is imperative, especially in the Iberian Peninsula and in some African and Asian countries (Margalida & Colomer, 2012). However, the number of animals that are entering in the rehabilitation centres is growing, due to food poisoning or decreased food sources. In the rehabilitation centres, the recuperation rate is influenced by several factors including the development of infections such as oral infections (Ferguson-Lees & Christie, 2001). Fungal infections pose a major threat as mycoses are among the most frequent and most serious systemic diseases in birds, the majority of which are caused by ubiquitous microorganisms that birds are continually exposed. The genera *Aspergillus* and *Candida* are the pathogens most frequently associated with diseases (Hubálek, Z., 2004). Stress appears to be a defying factor in the development of fungal infections, thus being associated with captivity, inadequate management, or treatments with antimicrobials for long periods of time. Also, physiological stress in the breeding season for example, may contribute for these infections. the order Mucorales includes several saprophytic fungi associated with underlying diseases (Balseiro A. et al., 2005). The most common infection route is through inhalation of its spores and the infections caused by *Mucor* spp. have been reported as possible aetiological agents of meningoencephalitis in birds (Vellanki et al., 2018). Oral mycosis has also been described in wild birds in captivity and therefore have predisposing factors as underlying immunosuppressive or debilitating diseases, prolonged therapy with broad-spectrum antibiotics, malnutrition, unsanitary conditions, and captivity related stresses (Mukherjee, P. et al., 2017). In the other hand, fungal infections causing clinical lesions are rarer in free-range wild birds.

Mucor is composed by filamentous fungi found in soil, plants, decaying fruits and vegetables. It belongs to phylum Zygomycota, order Mucorales and family Mucoraceae. This genus is known for being ubiquitous in nature and a common laboratory contaminant. Though *Mucor* spp. are usually not pathogens, several species are among the various zygomycetes identified as causing a type of opportunistic fungal infection called mucormycosis (Marques, S. et al., 2010). Also, it is responsible for several fungal infections in humans, cattle, swine, and some birds. *Mucor* spp. constitutes a group of microorganisms responsible to produce several enzymes such as amylases, lipases,

pectinases, and proteases. The enzymes are essential proteins for the metabolic system of all living organisms and have an important role in the degradation of organic matter, in host infection and food spoilage. Regarding the metabolic pathways, they act in organized sequences of catabolic and anabolic routes (Ainsworth, G. & Sussman, A., 1965). Also, they may be isolated from animals, plants and microorganism which are considered good sources of industrial enzymes for the great diversity of enzymes that have been found (Alves, M. et al., 2002).

In this study, the aim was to evaluate the pathogenic profile of oral *Mucor* species from Eurasian Griffon Vultures trough phenotypic characterization and access the ability of these isolates to produce inhibitory composts.

2. Material and methods

2.1. Sample collection and handling

The oral samples were collected with AMIES swabs and transported to the Laboratory of Mycology from Faculty of Veterinary Medicine of the University of Lisbon. The samples were cultured in Sabouraud medium and incubated for 4 days at 27ºC. After incubation, through macroscopic and microscopic evaluation it was possible to presumptive identify 7 isolates as *Mucor* spp. Isolates were maintained in Sabouraud agar at room temperature throughout the assays.

Table 1- Overview of the samples, species, collecting place and age of the vultures in study. GF- *Gyps fulvus*; AM- *Aegypius monachus*; D- samples from animals diagnosed with an oral disease; S- healthy

Sample	Animal ID	Vultures	Rehabilitation Center	Age	Sex
D2	1	GF	CERAS	JUVENILE	Unknown
D4	2	GF	RIAS	JUVENILE	Unknown
S6	3	GF	CERAS	JUVENILE	Unknown
S8	4	AM	RIAS	Unknown	Unknown
S9	5	GF	RIAS	JUVENIL	Unknown
S12	6	GF	RIAS	Unknown	Unknown
S13	7	GF	RIAS	Unknown	Unknown

2.2. DNA extraction

The DNA extraction was carried out following the instructions on the kit NucleoSpin Plant II by Marcherey-Nagel. First the mycelium was washed and mixed in ethanol. Then, the ethanol was retrieved, the sample placed in a reaction tube and siliconized glass beads were add along in 200µL of PL1 (lysis buffer). After homogenization, chloroform was added, and the sample centrifuged for 5 minutes at 11000 rpm. The supernatant was kept in a centrifuge tube and incubated at 65ºC for 30 minutes. Using the violet ring (NucleoSpin Filter) it was possible to collect the transparent fluid after centrifugation for 2 minutes. Again, the supernatant was placed in a reaction tube and the filter discarded. After, 450µL of binding buffer (PC) was joined and mixed and the sample was put in a new collecting tube with a green ring (NucleoSpin Plant II). Another centrifugation at 11000 rpm was carried out for 1 minute and the flow-through discarded. After this step, 400µL PW1 (wash buffer) was added to the NucleoSpin column, and the sample was centrifuged again for 1 minute and the flow-through discard. 700µL of PW2 (wash buffer) were included in the column and another centrifugation was performed for 2 minutes to remove the buffer and allow the silica membrane to dry. The column was placed in another centrifuge tube and 50µL of PE (elution buffer),

previously heated to 70ºC, was pipetted on the membrane which was further incubated in 70ºC for 5 minutes. Lastly, the sample was centrifuged for 1 minute to elute the DNA.

2.3. DNA amplification

PCR was carried in a 25μL volume consisting in 0,4μL (0,8uM) of each primer ITS1 (5-TCC GTA GGT GAA CCT GCG G) and ITS2 (5-GCT GCG TTC TTC ATC GAT GC), 10μL DNA, 10μL MasterMix (NZYtaq 2x Green, NZYtech®) consisting in 1x reaction buffer (50 mM Tris – HCl, pH 9.0, 50 mM NaCl, 2.5 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP, dTTP) and 4,2μL of PCR water according to the protocol used by Lau et al, 2007. Amplification was performed on a Doppio thermocycler (VWR®). The conditions were 95ºC for 10 minutes, followed by 60 cycles of 94ºC for 15 seconds, 55ºC for 30 seconds and 72ºC for 30 seconds, and a final extension at 72ºC for 5 minutes. After the amplification, the PCR products were separated by 1,5% agarose gel electrophoresis stained with Green Safe (NZYtech®). Results were visualized by transillumination (ChemiDoc XRS+, Bio-rad®).

2.4. Sanger sequencing

After the results from amplification, the PCR products were sent to sequencing to know if they were in fact *Mucor* spp. samples.

The samples were prepared so that the DNA had a concentration of 20mg/μL and the primers a concentration of 10 pmol/mL. The tubes sent contained 10 μL of DNA and 3 μL of primer being 1 tube for each combination of sample/primer. The primers as referred above were ITS1 (5-TCC GTA GGT GAA CCT GCG G) and ITS2 (5-GCT GCG TTC TTC ATC GAT GC). Finally, the samples were evaluated in Nanodrop to understand if they were pure. For the samples to be pure the ratio between the absorbance at 260nm and 280nm must be ~1.8.

2.5. Phenotypical identification

In order to test the virulence potential of the *Mucor* isolates under analysis, several tests were performed namely to evaluate the production of enzymes such as lipase, lecithinase, gelatinase, DNase and haemolysins. Biofilm production by the isolates was also tested. All assays were monitored at 24, 48 and 72 hours. **Lipase**: The lipase medium contained 1% of peptone, 5% of sodium chloride, 0,01% of calcium chloride and 2% of agar. The medium was autoclaved and 1% tween 80 was added to the molten medium at approximately 50ºC. Afterwards, isolates were inoculated in the medium and further incubated at 37ºC. **Lecithinase**: The medium used for testing the production of lecithinase by the *Mucor* isolates contained 1% of peptone, 2% of dextrose, tryptic soy agar (35g/L) and egg yolk. For 1L of medium, 900mL of water were added to TSA. After autoclave, 100ml of egg yolk was added. After the isolates were inoculated and incubated. **Gelatinase**: Nutrient gelatin stab method was performed for the detection of gelatin hydrolysis of the *Mucor* isolates. The nutrient gelatin medium contained peptone special (5 g/L), beef extract (3 g/L) and gelatin (120 g/L) being sterilized by autoclave. A total volume of 5 ml was added in each test tube and allowed to cool in an upright position.

The isolates were stab inoculated into gelatin tubes and incubated at 37°C and reviewed for gelatin liquefaction. **DNase**: The DNase medium was prepared and sterilized by autoclave. Afterwards, the toluidine blue reagent (0,1g/L) was added. Inoculation and incubation at 37ºC. **Haemolysins**: To test this virulence potential, Columbia agar plates supplemented with sheep blood (COS) from Biomérieux® were used. The isolates were inoculated and incubated at 37ºC. **Biofilm**: Firstly, the saccharose (50g/L) was filtrated. Afterwards, brain heart infusion (37g/L) was added to agar (20g/L) and to saccharose and the medium was autoclaved. The congo red (8g/L) was diluted apart from the mix and added after autoclavation. Lastly, the isolates were inoculated and incubated at 37ºC.

2.6. Inhibitory ability

The ability of the isolates being able to produce inhibitory compounds was evaluated, by using a collection of potentially pathogenic bacteria from the Bacteriology Lab of FMV/ULisboa, as well as two yeasts species isolated from the oral cavity of the vultures (Table 2), frequently associated to the development of oral disease.

The first step was to grow 3 sets of the seven *Mucor* isolates in liquid Sabouraud medium during 24h, 48h and 72h respectively. Apart from that, the bacteria and the yeasts mentioned in Table 2, were inoculated in brain heart infusion (BHI) plates regarding the bacteria and the yeasts were grown in Sabouraud medium. All plates were incubated at 37ºC for 24 hours. After the 24 hours of incubation, the broth culture of the first set of seven *Mucor* isolates was filtrated. In order to prepare the plates of each bacteria and yeast, these microorganisms were diluted in sodium chloride (NaCl) until a density of 0.5 McFarland turbidity was reached. Once this density was established, the inoculation was performed and 10µL of each *Mucor* isolate was applied over each microorganism mentioned above (Table 2). These plates were incubated for 24 hours at 37ºC. Afterwards, the inhibitory ability was observed by measuring the halo formation around the spot where the *Mucor* isolates were applied. Furthermore, the process was repeated for the second set of *Mucor* isolates that had been growing for 48 hours. The filtrated isolates were applied in new plates with the same bacteria and yeasts. Again, after 24 hours of incubation at 37ºC, the inhibitory ability was noted. Lastly, the last set of *Mucor* isolates (grown for 72

Table 2- Microorganisms used to test the inhibitory potential of *Mucor* isolates.

Bacteria/Yeast	
***Escherichia coli* ATCC 25922**	Reference isolate
***Pseudomonas aeruginosa* ATCC 27853**	Reference isolate
***Pseudomonas aeruginosa* 413/18**	Isolated from an otitis from a dog
***Pseudomonas aeruginosa* Z25.1**	Isolated from a patient diagnosed with diabetic foot
***Staphylococcus aureus* ATCC 29213**	Reference strain
***Staphylococcus aureus* Z25.2**	Isolated from a patient diagnosed with diabetic foot
***Enterococcus faecium* CCUG 36804**	Reference Van A positive
***Neisseria zoodegmatis* CCUG 52598T**	Reference type
***Candida* spp. S2-1**	Isolated from the oral cavity of the vultures under study
***Rhodotorula* spp. S2-2**	Isolated from the oral cavity of the vultures under study

hours) was filtrated and administrated in new plates. After 24 hours of incubation, the inhibitory ability of this last set of isolates was evaluated. This process was performed in triplicates.

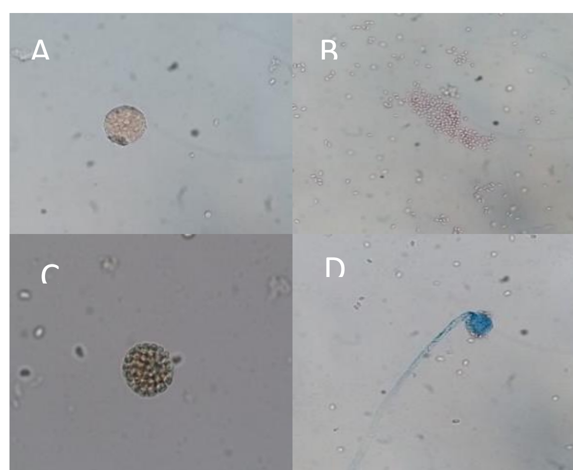
2.7. Microbiome characterization

Finally, the vulture samples from which the *Mucor* isolates showed an interesting broad spectrum (2, 3, 4 and 6) were subjected to a metagenomic analysis to evaluate the presence of pathogenic bacteria belonging to same genera used in the inhibition assays in vitro. The samples used in this study were analysed by a customized analytical pipeline developed by BioISIGenomics® for long-read targeted nanopore sequencing in order to obtain high-accuracy taxonomical classification. Sequencing data was obtained from 16S amplicons, low quality reads were removed, and the remaining reads were size selected (keeping reads with lengths higher than 300 bps) using prinseq-lite (Schmieder and Edwards, 2011). Taxonomic classification was performed using a Lowest Common Ancestor approach: indexing based on Burrows-Wheeler transform (BWT) and the Ferragina-Manzini (FM) index through Karkkainen's blockwise algorithm using the NCBI Refseq Archaea and Bacteria genome database (Kim et al., 2016). Following classification, data were subjected to: linear discriminant analysis (LDA) Direto with effect size measurements (LEfSe) for biomarker determination (Segata et al., 2011); sample-to-sample analysis - represented on a heatmap for sample-to-sample comparison based on the Bray-Curtis dissimilarity method and with hierarchical reconstruction performed using Ward's method (McMurdie and Holmes, 2013); Alpha diversity group siginificance analysis (Bolyen et al., 2018); sample dissimilarity analysis – Principal Coordinates Analysis (PCoA) for beta diversity analysis based on the Jaccard similarity index and determination of taxa abundance (after normalization into counts per million, and with a genera prevalence cutoff of ≥ 0.01) (Bolyen et al., 2018); phylogenetic reconstruction – construction of phylogenetic trees (Sankey Network Graph using raw OTU counts) and Krona Plots (by hierarchical order of OTU frequency) (Allaire et al., 2017; Ondov et al., 2011).

3. Results

In the first part of the experimental work, it was possible to isolate and identify seven *Mucor* spp. isolates through their macro and microscopic features. The colonies of *Mucor* are typically white or beige and grow at a very fast rhythm, becoming grey or brownish due to the development of its spores. *Mucor* spores or sporangiospores can be simple or branched and form apical, globular sporangia that are supported and elevated by a column-shaped columella as can be observed in Figures 1. C and D. Some *Mucor* species produce chlamydospores, Figure 1. A.

Figure 1- A- Chlamydospore of *Mucor*; B- Conidia of *Mucor*. C- Sporangia of *Mucor*; D- Sporangia, columella, and conidiophore of *Mucor*. 400x. Original.



3.1. Phenotypic identification

Regarding biofilm production, it was possible to observe that only isolates 2 and 4 (28.6%) were able to produce biofilm. Isolate 4 showed a strong positive result throughout the 72 hours of incubation whereas isolate 2 showed a weaker positive result only after 72 hours of incubation. Regarding lipase production isolates 2, 4 and 6 (42,9%) showed a positive result after 24h, 48h and 72 hours of incubation. The other isolates showed a negative result throughout the whole experience. Mentioning haemolysins, it was the strongest virulence factor observed as 6 of the 7 isolates (85,7%) were able to produce α-haemolysis throughout the 72 hours of experience. When testing the production of gelatinase, it was perceived that none of the isolates had a positive result for gelatinase production after 48 hours of incubation. Nevertheless, at 72 hours of incubation two of the isolates, isolate 1 and 6 (28,6%) were found to be positive for gelatinase production. Evaluating DNase production, at 24 hours of incubation all isolates were negative for DNase production. Moreover, at 48 hours isolates 3 and 6 revealed to be weak DNase producers, while at 72 hours, 5 isolates (2, 3, 5, 6 and 7), 71,4% were found to have the ability to produce DNase. It was the strongest virulence factor after the haemolysis as 5 out of the 7 isolates showed a positive result for DNase production by the end of the experience. Regarding lecithinase production, all isolates presented a negative result at 24 hours. At 48 hours, isolates 1, 2 and 5 showed a positive result. Finally, at 72 hours of incubation, one more isolate showed a positive result for lecithinase production. Thus, at end of the experience isolates 1, 2, 3 and 5 (57,14%) were showing signs of lecithinase activity.

Also, it was evaluated the prevalence of *Mucor* isolates with the ability to produce virulence factors in the two vulture recovery centres in which the samples were collected and if the presence of an oral disease had any influence on the virulence of the samples. It was possible to understand that the isolates retrieved from CERAS (1 and 3) presented a higher virulence ability regarding haemolysis and lecithinase production. The isolates from RIAS, were stronger biofilm producers. Regarding the presence of an oral disease, these isolates seem to have a higher ability to produce virulence determinants except regarding DNase and lipase production. The most predominant virulence factor in these isolates were haemolysis and lecithinase production, as both isolates were strong producers.

3.2. Inhibitory ability

The results obtained revealed that *E. coli* ATCC 25922 was the bacteria more susceptible to *Mucor* isolates extracts followed by *N. zoodegmatis* CCUG 52598T, *E. faecium* CCUG 36804 and *S. aureus* Z25.2. On the opposite, *P. aeruginosa* Z25.1 as well as both yeasts (*Candida* spp. S2-1 and *Rhodotorula* spp. S2-2) were not affected by none of the *Mucor* isolates extracts. Also, the biggest percentage of isolates with inhibitory properties was seen in *E. coli* ATCC 25922 at 48h with 8 isolates between the three rounds of results. Regarding the incubation period, it was observed that at 48 hours of incubation was

when more isolates showed a broader spectrum of action.

Isolate 4 produced the extract with a broader spectrum of action as it demonstrated activity towards *N. zoodegmatis* CCUG 52598T*, P. aeruginosa* 413/18, *E. coli* ATCC 25922*, E. faecium* CCUG 36804, *S. aureus* ATCC 29213 and *S. aureus* Z25.2 throughout the 3 rounds of 72 hours of experience. Thus, it demonstrated a bigger activity in *N. zoodegmatis* CCUG 52598T as 3 out of the 9 extracts (33,33%) (3 at 24h, 3 at 48h and 3 at 72h) throughout the 3 rounds of 72 hours, showed inhibitory capacity. Moreover, isolate 2 also showed an interesting inhibitory potential as it inhibited the growth of *N. zoodegmatis* CCUG 52598T*, E. coli* ATCC 25922*, E. faecium* CCUG 36804 and *S. aureus* Z25.2. Also, this isolate represented the biggest percentage of inhibitory capacity as 7 extracts in 9 (77,77%) demonstrated inhibitory properties in *E. coli* ATCC 25922. Isolate 3, also exhibited a good result as its extract presents inhibitory towards *N. zoodegmatis* CCUG 52598T, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213.

Furthermore, isolate 1 was the isolate that demonstrated less inhibitory capacity as it only one extract revealed activity towards *E. coli* ATCC 25922.

3.3. Microbiome characterization

After the analysis on inhibitory proprieties of *Mucor* isolates, the samples from which the extracts with a broader spectrum of action (isolate 2, 3 and 4) were subjected to a metagenomic analysis to understand if the pathogenic bacteria previously studied in vitro were part of the oral microbiome of the vultures by characterization of 16S rRNA region of the isolates mentioned above. Regarding sample 2 is possible to understand that 57.79% of its reads correspond to the genus *Clostridium*, 31.84% of the reads correspond to *Paeniclostridium* genus, 1.084% to *Dysgonomonas* genus and 1.018% to *Yersinia* genus. Referring to sample 3, 39.41% of the reads also belong to genus *Clostridium*, 31.63% to *Paeniclostridium* genus, 8.726% of the reads belong to *Enterococcus* genus and 3.278% to *Yersinia* genus. Lastly, evaluating sample 4, 25.43% of its reads correspond to *Paeniclostridium* genus, 19.85% to *Clostridium* genus, 18.67% to *Peptoniphilus* genus and 7.14% to *Gudongella* genus.

4. Discussion

Lipase was expected to have a higher production as most of the isolates investigated in previous studies showed a capacity to produce lipase production. Regarding the production of DNase, the results showed by Thompson are compatible to the ones presented in this study as 71.4% of isolates were found to have the ability to produce DNase being one the virulence factors most exhibited by *Mucor* isolates just like it was verified in the previous study (Thompson, D. et al., 1984).

As it concerns the biofilm-forming capacity, the results of the present studied revealed that two of the *Mucor* isolates also had the ability to form biofilm, both were identified as *M. circinelloides.* Referring to the results of the gelatinase production the present study did not show a high virulence capacity regarding the production of gelatinase. The

production of lecithinase was shown 4 out of 7 isolates, being one of the most predominant virulence factors recorded. These results are like studies developed in several filamentous fungi, which detected the production of lecithinase as one of the most predominant enzymatic activities. Investigating the role of hemolysins during the early stages of growth, especially in filamentous fungi could provide valuable information regarding the functional role of these proteins. It does not appear that these proteins are critical for fungal growth but based on these observations, it is probable that these proteins have a role in regulating fungal growth (Ghannoum, M., 2000). In this study, haemolysis activity was the most recorded virulence factor as from the 7 isolates, only one did not show signs of haemolysis.

Previously *Mucor* spp. were proved to have antibacterial activity towards Gram-negative bacteria namely *Klebsiella pneumoniae, Pseudomonas brassicacearu, Aeromonas hydrophila, Escherichia coli* and two Gram-positive bacteria, namely *Bacillus cereus* and *Staphylococcus aureus.* Also, *Mucor* spp. have proved to have antifungal activity (Aziz, N. et al., 2016). In this study, inhibitory ability of the *Mucor* extracts was detected towards *towards N. zoodegmatis CCUG 52598T, P. aeruginosa 413/18, E. coli ATCC 25922, E. faecium CCUG 36804, S. aureus ATCC 29213 and S. aureus Z25.2.*

The most abundant genus identified in the Eurasian Griffon Vulture samples analysed included *Clostridium*, *Paeniclostridium, Enterococcus* and *Peptoniphilus.* The genera *Yersinia*, *Dysgonomonas* and *Gudongella* were found in considerably lower percentages. These results match the ones obtained in previous studies regarding other scavengers' species, as according to Vela et al. (2015) the most common genera are *Escherichia, Enterococcus, Staphylococcus, Clostridium* and *Lactococcus*. *Escherichia coli* and *Enterococcus faecalis* the most common species in cloacal and pharyngeal samples of Eurasian Griffon Vultures. Thus, it was expected that genera as *Escherichia* or *Staphylococcus* were present in the metagenomic analysis performed in this study as they are one of the most present genera commonly found in vultures. However, those genera were not found meaning that perhaps the fact that *Mucor* spp. is present in the oral cavity of vultures may contribute to the decrease of the presence of bacteria, as it was demonstrated in this study that the *Mucor* extracts have an inhibitory capacity towards *E. coli* and *S. aureus.* Moreover, the isolates also showed an inhibitory ability towards *E. faecium.* Although, the genera Enterococcus was reported as part of the bacterial reads of the metagenomic analysis of one of the samples in study (sample 3), but it was only registered in one of the samples analysed and in a smaller percentage. Further studies must be performed to confirm this hypothesis.

5. Conclusion

The results obtained, indicated that these fungi have a high virulence capacity as all isolates showed a positive result for at least 2 of the virulence factors tested. Also, the *Mucor* extracts presented inhibitory capacity towards *N. zoodegmatis* CCUG5 2598T, *P.*

aeruginosa 413/18, *E. coli* ATCC 25922, *E. faecium* CCUG 36804, *S. aureus* ATCC 29213 and *S. aureus* Z25.2. Furthermore, metagenomic analysis of the samples that exhibited a broader spectrum of action pointed out the possibility of that inhibition activity detected *in vitro* actually being verified *in vivo*. Although, this study does not provide enough data to confirm this hypothesis.

Given the pathogenic profile of the tested fungi and that these vultures are endangered species, more studies should be developed to fully characterize the oral microbiome of these animals, and most importantly the pathogenic potential of relevant microbial species, which would ultimately contribute for the development of adequate management programmes.

References

Ainsworth, G. C., & Sussman, A. S. (1965). The Fungi. An advanced treatise. Vol. I. The fungal cell. *The Fungi. An Advanced Treatise. Vol. I. The fungal cell.*

Alves, M. H., Campos-Takaki, G. M., Porto, A. L. F., & Milanez, A. I. (2002). Screening of *Mucor* spp. for the production of amylase, lipase, polygalacturonase and protease. *Brazilian Journal of Microbiology*, *33*, 325-330.

Balseiro, A., Espi, A., Marquez, I., Perez, V., Ferreras, M. C., Marin, J. G., & Prieto, J. M. (2005). Pathological features in marine birds affected by the Prestige's oil spill in the north of Spain. *Journal of Wildlife Diseases*, *41*(2), 371-378.

Camina, A. (2004). Griffon Vulture *Gyps fulvus* monitoring in Spain: current research and conservation projects. In Raptor worldwide. *Proceedings of the 6th world conference on birds of prey and owls*. WGBP/MME, Budapest (pp. 45-66).

Coca Armas, J., Dustet Mendoza, J. C., & Martínez Hernández, J. L. (2008). *Mucor griseocyanus* lipase: Production, characterization, and study of some catalytic properties of the immobilised enzyme. *Food Technology and Biotechnology*, 46(2), 195-201.

Ferguson-Lees, J., & Christie, D. A. (2001). *Raptors of the world*.

Ghannoum, M. A. (2000). Potential role of phospholipases in virulence and fungal pathogenesis. *Clinical microbiology reviews*, 13(1), 122-143.

Hubálek, Z. (2004). An annotated checklist of pathogenic microorganisms associated with migratory birds. *Journal of Wildlife Diseases*, *40*(4), 639-659.

Lau, A., Chen, S., Sorrell, T., Carter, D., Malik, R., Martin, P., & Halliday, C. (2007). Development and clinical application of a panfungal PCR assay to detect and identify fungal DNA in tissue specimens. *Journal of Clinical Microbiology*, 45(2), 380-385.

Margalida, A., & Colomer, M. À. (2012). Modelling the effects of sanitary policies on European vulture conservation. *Scientific Reports*, *2*(1), 1-7.

MarquesI, S. A., de CamargoII, R. M. P., AbbadeIII, L. P. F., & MarquesIV, M. E. A. (2010). Mucormicose: infecção oportunística grave em paciente imunossuprimido. Relato de caso.

Moleón M, Sánchez-Zapata J, Margalida A, Carrete M, Donázar J, Owen-Smith N. 2014. Hominids and scavengers: the evolution of interactions and ecosystem services. *Bioscience*. 64:394–403.

Mukherjee, P. K., Wang, H., Retuerto, M., Zhang, H., Burkey, B., Ghannoum, M. A., & Eng, C. (2017). Bacteriome and mycobiome associations in oral tongue cancer. *Oncotarget*, *8*(57), 97273.

Thompson, D. P., & Eribo, B. E. (1984). Extracellular enzyme production by *Rhizopus* and *Mucor* species on solid media. *Canadian Journal of Microbiology*, *30*(1), 126-128.

Vela, A. I., Casas-Díaz, E., Fernández-Garayzábal, J. F., Serrano, E., Agustí, S., Porrero, M. C. & Domínguez, L. (2015). Estimation of cultivable bacterial diversity in the cloacae and pharynx in Eurasian griffon vultures (Gyps fulvus). *Microbial Ecology*, *69*(3), 597-607.

Vellanki, S., Navarro-Mendoza, M. I., Garcia, A., Murcia, L., Perez-Arques, C., Garre, V. & Lee, S. C. (2018). *Mucor circinelloides*: growth, maintenance, and genetic manipulation. *Current Protocols in Microbiology*, 49(1), e53.