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

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"The Road goes ever on and on

Down from the door where it began.

Now far ahead the Road has gone,

And I must follow, if I can,

Pursuing it with eager feet,

Until it joins some larger way

Where many paths and errands meet.

And whither then? I cannot say"

- **J.R.R. Tolkien**

Preface

The work presented in this thesis was performed at Laboratory of Veterinary Bacteriology of Faculty of Veterinary Medicine (Lisbon, Portugal), during the period September 2020 – September 2021, under the supervision of Prof. Doc. Maria Manuela Castilho Monteiro de Oliveira. This thesis was co-supervised at Instituto Superior Técnico by Prof. Doc. Jorge Humberto Gomes Leitão.

Declaration

I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the Lisbon University.

Acknowledgements

Terminando mais uma etapa da minha vida, chegou a altura de mencionar algumas pessoas importantes para tal.

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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. The order Mucorales includes several saprophytic fungi associated with relevant opportunistic diseases. The most common infection route is through spores' inhalation, with *Mucor* spp. previously been reported as possible aetiological agents of meningoencephalitis in birds.

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. Swab samples were cultured in Sabouraud dextrose agar (SDA), incubated at 27ºC for 5 days. Afterwards, isolates identification was performed through macro and microscopic observation and confirmed by PCR and ITS sequencing, and their phenotypic pathogenic profile determined by assessing the production of lipase, lecithinase, gelatinase, DNase, haemolysins and biofilm, after 24, 48 and 72 hours of incubation in specific media. Furthermore, the inhibitory capacity of the *Mucor* isolates was evaluated 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.* 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.

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

Resumo

O Grifo Euroasiático é um necrófago especializado que habita áreas montanhosas no sul da Europa que rodeia o Mediterrâneo. Devido a envenenamentos e decréscimo nos seus recursos alimentares ao longo dos anos, têm aumentado o número de abutres que necessitam de cuidados veterinários e hospitalização no hospitais e centros de reabilitação animal da Península Ibérica. Em cativeiro, os abutres desenvolvem várias doenças infeciosas, como acontece na cavidade oral, que levam a que potencialmente a sua capacidade de sobrevivência e reintrodução na natureza sejam afetadas. A ordem Mucorales inclui vários fungos saprófitas associados com doenças oportunistas relevantes. A via de infeção mais comum, é através de inalação de esporos, sendo que a espécie *Mucor* já veio a ser reportada como possível agente de meningoencefalite em aves.

Neste trabalho, a espécie *Mucor* foi isolada de colheitas orais de Grifos Euroasiáticos (*Gyps fulvus*) e de Abutres Cinzentos (*Aegypius monachus*) recolhidas no CERAS em Castelo Branco (n=2) e no RIAS em Olhão (n=5) e o seu potencial patogénico investigado. As amostras foram cultivadas em Sabouraud dextrose agar (SDA), e incubadas a 27ºC durante 5 dias. Posteriormente, os isolados foram identificados sob observação macro e microscópica e confirmados por PCR e sequenciação ITS. O seu perfil patogénico foi determinado através do estudo da produção de lipase, lecitinase, gelatinase, DNase, hemolisinas e biofilme após 24, 48 e 72 horas de incubação nos meios de crescimento específicos. Posteriormente, a capacidade de inibição dos isolados foi avaliada usando uma coleção de bactérias potencialmente patogénicas e leveduras frequentemente encontradas nas cavidades orais de abutres fornecidas pelo Laboratório da FMV/ULisboa. Após determinação da sua habilidade inibitória, os isolados com o espetro de ação mais interessantes foram submetidos a uma análise metagenómica no sentido de avaliar a presença de bactérias potencialmente patogénicas pertencentes aos géneros utilizados nos testes de inibição *in vitro*.

Foi possível observar que 85.7% (6/7) dos isolados produziam alfa-hemólise, 71.4% (5/7) produziam DNase, 57.1% (4/7) produziam lecitinase e lipase, 28.6% (2/7) gelatinase e por último, 28.6% (2/7) demostraram ser forte produtores de biofilme. Relativamente ao estudo da capacidade inibitória, 3 isolados (2, 3 e 4) foram sugestivos de apresentar um espetro de ação diversificado. Consequentemente, estes isolados foram submetidos a uma análise metagenómica que relevou que possivelmente estes isolados podem demostrar essa inibição *in vivo.* Dado o perfil patogénico demostrado pelos fungos em estudo, e que os abutres são uma espécie ameaçada, mais estudos deveriam ser conduzidos de maneira a caracterizar completamente o seu microbioma oral, e sobretudo o potencial patogénico de espécies microbianas relevantes, contribuindo para o desenvolvimento de programas de recuperação adequados.

Palavras-chave: *Mucor* spp., fatores de virulência, abutres, Grifo Euroasiático, infeções orais, atividade antimicrobiana.

Table of Contents

LIST OF FIGURES .... **x**

LIST OF TABLES .... **xi**

LIST OF GRAPHICS .... **xi**

LIST OF ABREVIATIONS .... **xii**

1. INTRODUCTION **1**

2. LITERATURE REVIEW **2**

2.1.1. The Eurasian Griffon Vulture (*Gyps fulvus*) 2

2.1.2. Distribution and population in Portugal and Iberian Peninsula 3

2.1.3. Conservation and threats 4

2.2. Microbiota **5**

2.2.1. *Mucor* species 6

2.2.1.1. Morphological forms and Morphogenetic transformations 8

2.2.1.2. Environmental factors influencing morphogenesis 9

2.2.1.3. Macroscopic Features 9

2.2.1.4. Microscopic Features 9

2.3. Virulence Factors **10**

2.3.1. Previous studies on virulence factors 14

2.4. The microbiome **15**

2.4.1. Assessment of Microbiota 15

2.4.1.1. Culture based methods 15

2.4.1.2. Molecular based methods 16

2.4.1.3. Non sequencing methods 16

2.4.1.4. Sequence-based methods 16

3. MATERIAL AND METHODS **19**

3.1.1. Sample collection and handling 19

3.1.2. DNA extraction 19

3.1.3. DNA amplification 20

3.1.4. Sanger sequencing 20

3.2. Phenotypical identification 21

3.3. Inhibitory ability 22

3.4. Microbiome Characterization 23

4. RESULTS **25**

4.1. *Mucor* spp. isolation and identification 25

4.2. Molecular identification 25
4.3. Sanger Sequencing 26
4.4. Phenotypical identification 27
4.4.1. Descriptive analysis 31
4.5. Inhibitory ability 32
4.5.1. Descriptive analysis 38
4.6. Microbiome Characterization 40
5. DISCUSSION 41
5.1. Phenotypical identification 42
5.2. Inhibitory ability 43
5.3. Microbiome Characterization 44
6. CONCLUSION 45
7. REFERENCES 46
8. ANNEX I 52

List of Figures

FIGURE 1. Priority areas for vulture conservation **1**

FIGURE 2. Distribution of the Griffon Vulture compared between 1986 and 2002 ..... **3**

FIGURE 3. Alternative morphogenetic fates of sporangiospores from dimorphic *Mucor* spp. **7**

FIGURE 4. *Mucor* spp. colonies isolated from a case of mucormycosis on Sabouraud dextrose agar plates. A- Initially the colonies are white; B- The colonies become greyer when they develop sporangia. **9**

FIGURE 5. Sporangia, columella and sporangiospores of *Mucor* spp. **10**

FIGURE 6. Analytical pipeline of the analysis developed by BioISIGenomics for taxonomic classification of 16S sequencing metagenomic data. **24**

FIGURE 7. *Mucor* chlamydospore; B- *Mucor* conidia; C- *Mucor* sporangia; D- *Mucor* sporangia, columella, and conidiophore. 400x. **25**

FIGURE 8. PCR products after separation by 1,5% agarose gel electrophoresis **25**

FIGURE 9. A negative result (isolate 6), a weak positive result (isolate 2) and a positive result (isolate 4) for biofilm production at 72 hours **27**

FIGURE 10. Isolate 4. Positive result for biofilm production at 72 hours. **27**

FIGURE 11. Positive result for lipase production. Isolate 3 at 72 hours **28**

FIGURE 12. Negative result for lipase production. Isolate 7 at 72 hours **28**

FIGURE 13. α-haemolysis of isolate 6 at 48 hours **29**

FIGURE 14. Negative result for haemolytic activity of isolate 4 at 72 hours **29**

FIGURE 15. Positive result for gelatinase production by isolate 6 at 72 hours **29**

FIGURE 16. Negative result for gelatinase production by isolate 3 at 72 hours **29**

FIGURE 17. Positive result for DNase production from isolate 6 at 72 hours **30**

FIGURE 18. Negative result for DNase production from isolate 5 at 24 hours **30**

FIGURE 19. Negative result for lecithinase production from isolate 3 at 48 hours **31**

FIGURE 20. Positive result for lecithinase production from isolate 3 at 72 hours **31**

FIGURE 21. Inhibitory ability of isolate 3 and 4 in *N. zoodegmatis* at 48 hours. Second round of results **33**

FIGURE 22. Inhibitory ability of isolate 7 in *P. aeruginosa* 413/18 at 48 hours. Second round of results **34**

FIGURE 23. Inhibitory ability of isolate 4 in *P. aeruginosa* ATCC at 72 hours. First round of results **34**

FIGURE 24. Inhibitory ability of isolates 1, 2 and 4 in *E. coli* ATCC at 48 hours. Second round of results **35**

FIGURE 25. - Inhibitory ability of isolates 2 and 4 in *E. faecium* CCUG at 48 hours. Second round of results. **36**

FIGURE 26. Inhibitory ability of isolates 6 in *S. aureus* ATCC at 48 hours. First round of results **36**

FIGURE 27. Inhibitory ability of isolates 6 in *S. aureus* Z25.2 at 72 hours. First round of results **37**

List of Tables

TABLE 1. Overview of the samples, species, collecting place and age of the vultures in study . **19**

TABLE 2. Microorganisms used to test the inhibitory potential of *Mucor* isolates **22**

TABLE 3. ID number of the samples, scientific name, query cover, e-value, percentage of identity and sequence with ITS1 and ITS2 primers **26**

TABLE 4. Summary of the results of the phenotypic identification after 24, 48 and 72 hours .. **27**

TABLE 5. Results of the inhibitory ability assays after 24, 48 and 72 hours respectively........... **33**

TABLE 6. Raw reads classification statistics in percentage and in raw read numbers and with sample information **40**

TABLE 7. Table showing the read counts percentages per each Genus (row) on each sample (column) for sample that originate isolate 2........ **40**

TABLE 8. Table showing the read counts percentages per each Genus (row) on each sample (column) for sample that originate isolate 3........ **40**

TABLE 9. Table showing the read counts percentages per each Genus (row) on each sample (column) for sample that originate isolate 4........ **40**

TABLE A1. Inhibitory potential of *Mucor* isolates towards *Neisseria zoodegmatis* CCUG 52598T throughout 72h. 1st round........ **52**

TABLE A2. Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* 413/18 throughout 72h. 1st round........ **52**

TABLE A3. Inhibitory potential of *Mucor* isolates towards *Enterococcus faecium* CCUG 36804 throughout 72h. 1st round........ **52**

TABLE A4. Inhibitory potential of *Mucor* isolates towards *Escherichia coli* ATCC 25922 throughout 72h. 1st round........ **53**

TABLE A5. Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* ATCC 29213 throughout 72h. 1st round........ **53**

TABLE A6. Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* Z25.2 throughout 72h. 1st round........ **53**

TABLE A7. Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* ATCC 27853 throughout 72h. 1st round........ **53**

TABLE A8. Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* Z25.1 throughout 72h. 1st round........ **54**

TABLE A9. Inhibitory potential of *Mucor* isolates towards *Candida* spp. S2-1 throughout 72h. 1st round **54**

TABLE A10. Inhibitory potential of *Mucor* isolates towards *Rhodotorula* spp. S2-2 throughout 72h. 1st round **54**

TABLE A11. Inhibitory potential of *Mucor* isolates towards *Neisseria zoodegmatis* CCUG 52598T throughout 72h. 2nd round **55**

TABLE A12. Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* 413/18 throughout 72h. 2nd round **55**

TABLE A13. Inhibitory potential of *Mucor* isolates towards *Enterococcus faecium* CCUG 36804 throughout 72h. 2nd round **55**

TABLE A14. Inhibitory potential of *Mucor* isolates towards *Escherichia coli* ATCC 25922 throughout 72h. 2nd round **55**

TABLE A15. Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* ATCC 29213 throughout 72h. 2nd round **56**

TABLE A16. Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* Z25.2 throughout 72h. 2nd round **56**

TABLE A17. Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* ATCC 27853 throughout 72h. 2nd round **56**

TABLE A18. Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* Z25.1 throughout 72h. 2nd round **57**

TABLE A19. Inhibitory potential of *Mucor* isolates towards *Candida* spp. S2-1 throughout 72h. 2nd round **57**

TABLE A20. Inhibitory potential of *Mucor* isolates towards *Rhodotorula spp.* S2-2 throughout 72h. 2nd round **57**

TABLE A21. Inhibitory potential of *Mucor* isolates towards *Neisseria zoodegmatis* CCUG 52598T throughout 72h. 2nd round **58**

TABLE A22. Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* 413/18 throughout 72h. 2nd round **58**

TABLE A23. Inhibitory potential of *Mucor* isolates towards *Enterococcus faecium* CCUG 36804 throughout 72h. 2nd round **58**

TABLE A24. Inhibitory potential of *Mucor* isolates towards *Escherichia coli* ATCC 25922 throughout 72h. 2nd round **58**

TABLE A25. Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* ATCC 29213 throughout 72h. 2nd round **59**

TABLE A26. Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* Z25.2 throughout 72h. 2nd round **59**

TABLE A27. Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* ATCC 27853 throughout 72h. 2nd round **59**

TABLE A28. Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* Z25.1 throughout 72h. 2nd round **60**

TABLE A29. Inhibitory potential of *Mucor* isolates towards *Candida* spp. S2-1 throughout 72h. 2nd round **60**

TABLE A30. Inhibitory potential of *Mucor* isolates towards *Rhodotorula* spp. S2-2 throughout 72h. 2nd round **61**

List of Graphics

GRAPHIC 1. Percentage of isolates with virulence capacity. CERAS vs RIAS **31**

GRAPHIC 2. Percentage of isolates with virulence capacity considering the health status of the sampled vulture **32**

GRAPHIC 3. Percentage of *Mucor* isolates with inhibitory ability at 24, 48 and 72 hours **38**

GRAPHIC 4. Inhibitory capacity of the Mucor isolates in the microorganisms in study **39**

GRAPHIC 5. Genera composition plot of samples corresponding to isolates 2, 3 and 4, respectively **41**

List of Abbreviations

BHI – brain heart infusion

Bps – Plural of Bp, base pair

BWT- Burrows-Wheeler transform

CERAS – Centro de Recuperação de Animais Silvestres – Castelo Branco

COS – columbia blood agar

DNA – Deoxyribonucleic acid

E. coli- Escherichia coli

E. faecium- Enterococcus faecium

e.g- for example

EU- European Union

FM- Ferragina-Manzini

FMV/ULisboa- Faculdade de Medicina Veterinária da Universidade de Lisboa

G. f. fulvus- Gyps fulvus fulvus

ID- Identification

ITS – Internal transcribed spacer

L. corymbifera- Lichtheimia corymbifera

LEfSe – Linear discriminant analysis Effect Size

M. bacilliformis- Mucor bacilliformis

M. circinelloides- Mucor circinelloides

M. corticolus- Mucor corticolus

M. miehei- Mucor miehei

M. racemosus- Mucor racemosus

N. zoodegmatis- Neisseria zoodegmatis

NaCl- sodium chloride

NC – negative control

NCBI- National Centre for Biotechnology Information

NGS- Next Generation Sequencing

P. aeruginosa- Pseudomonas aeruginosa

PC – positive control

PCR – Polymerase chain reaction

R. arrhizus- Rhizopus arrhizus

R. oryzae- Rhizopus oryzae

R. pusillus- Rhizomucor pusillus

R. rfrcinurns- Rhizopus rfrcinurns

RIAS- Centro de Recuperação e Investigação de Animais Selvagens- Olhão

rRNA – Ribosomal ribonucleic acid

S. aureus- Staphylococcus aureus

spp.- species

TSA – tryptic soy agar

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. The remnant populations are isolated and in urgent need of conservation action assisted by international cooperation and provision of expertise. Furthermore, *Gyps fulvus* and other Vulture species, regulate the spreading of pathogenic agents and diseases and contribute to nutrients recycling. Is estimated that the species can remove more than 8000 tons of livestock carcasses from the field, per year (Margalida & Colomer, 2012) leading to a reduction of greenhouse gases emission (Moleón, M. et al., 2014).

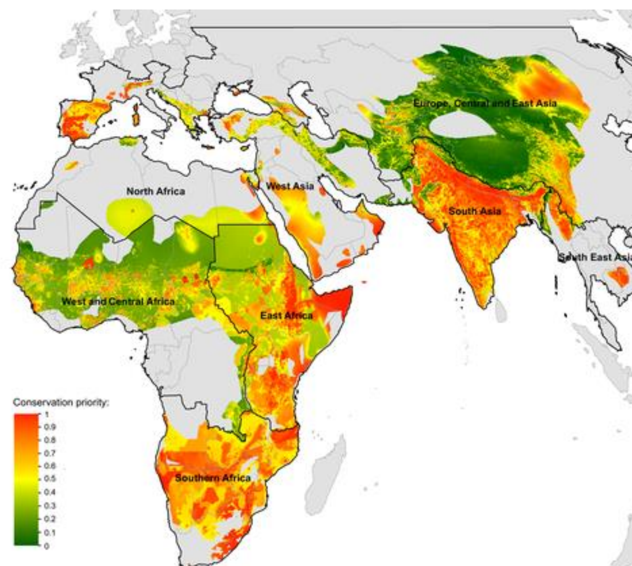


Figure 1- Priority areas for vulture conservation. Retrieved from: Santangeli, A. et al., 2019.

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 as may be observed in Figure 1 above (Ferguson-Lees & Christie, 2001). 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. Thus, is imperative that new studies are performed focusing on pathogenic species present in the oral cavity of these animals that may be responsible infections development. 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 (Garcia, M. et al., 2007).

2. Literature Review

2.1.1. The Eurasian Griffon Vulture (*Gyps fulvus*)

The Eurasian Griffon Vulture is a specialized scavenger, which inhabits rugged and mountainous areas in southern Europe surrounding the Mediterranean. Belongs to the species *Gyps fulvus*, Accipitriformes Order, *Accipitridae* Family that includes the Old-World Vultures. There are two subspecies of *G. fulvus* recognized, namely *Gyps fulvus fulvus* (Hablizl, 1783) and *Gyps fulvus fulvescens* (Hume, 1869) (Slotta-Bachmayr, L & Camiña, A., 2004). *Gyps fulvus* is usually a colonial breeder with colonies, comprising from one to more than 100 pairs. Is a slow-reproducing raptor which makes the species highly sensitive to a decrease in adult survival, whereas low juvenile survival or a temporary decrease in productivity will have much lower effect on population dynamics. Breeding is mainly restricted to cliffs reaching from just above sea-level to heights of up to 2,500m. In Spain and Portugal, nests have also been located on trees where Griffons occupy Cinereous Vulture (*Aegypius monachus*) nests (Traverso, 2001).

The Cinereous Vulture, also known as the Eurasian Black Vulture, is the largest European vulture, and one of the heaviest and largest raptors in the world, with its wingspan almost reaching 3 metres. The Cinereous Vulture faced a dramatic decline in Europe, but thanks to the increase of the Spanish population, the species is making a comeback in other parts of Europe, being now present from Portugal to France and Bulgaria (Vulture Conservation Foundation, 2021).

Vultures are the only vertebrates that are obligate scavengers. The Griffon Vulture feeds on carrion and their diet is mainly composed by livestock species as sheep, goats, and horses or by wild ungulate species in certain areas (Pyrenees, Alps) where these species are abundant, and carcasses are accessible. Since carrion is an unpredictable food resource, in regards of its availability and geographic distribution (Margalida & Ogada 2018), vultures have developed morphological and behavioural features to guarantee their own survival even in harsh conditions (Moreno-Opo et al., 2016). The adult vultures are sedentary, with partial migration occurring by juvenile and immature birds as they leave their breeding areas at the end of the breeding season thus avoiding competition with adults for food which is relevant since juveniles are less efficient both time and energy wise.

Regarding reproduction, *Gyps fulvus* individuals are usually monogamous, with a breeding pair composed by sexually mature individuals, raising a single nesting per year around their 4th or 5th year of age. The peak of nesting season is in the coldest months thus usually not starting later than January. Although it varies with the regions, breeding may begin from December to April leading to females laying the eggs in hotter areas and clutches peaking in February, which is probably associated with food resources availability and with weather conditions (Slotta-Bachmayr, L. & Camiña, A., 2004).

Regarding the Cinereous Vulture, they nest in trees and sometimes on cliffs. Like most other vultures, cinereous vultures are monogamous. Couples build huge nests that are reused each year. Eggs hatch in 50 to 55 days. Cinereous vultures have a specialised type of

haemoglobin in their blood, allowing them to effectively absorb oxygen even at great heights. These animals use thermal winds they 'float' to reach great heights in search of carcasses (Vulture Conservation Foundation, 2021).

2.1.2. Distribution and population in Portugal and Iberian Peninsula

The *Gyps* species feed through large areas of Africa, Asia, and Europe, with the juveniles experiencing a wider distribution. The populations are distributed over the western Palearctic from India, Pamirs, and Altai in the east, to Portugal and Spain in the west (Slotta-Bachmayr, L & Camiña, A., 2004). The total population comprises around 20.000-30.000 breeding pairs. As may be observed in Figure 2 below, in 1986, the distribution of the Griffon Vulture was clearly distributed into two regions. In the western region, it was mainly found on the Iberian Peninsula and northern Morocco, whereas in the east, the Griffon Vulture occurred in Greece, Turkey, the Ural Mountains and certain regions of Israel and Jordan. In between, there are only a few small populations mainly on the Balkans (Xirouchakis, S. & Mylonas, M., 2004). The European population of the Eurasian Griffon Vulture consists only of the subspecies *G. f. fulvus* with an estimation of 64800 to 68800 mature individuals, considered to be at least 10% of the Global population of *G. fulvus.*

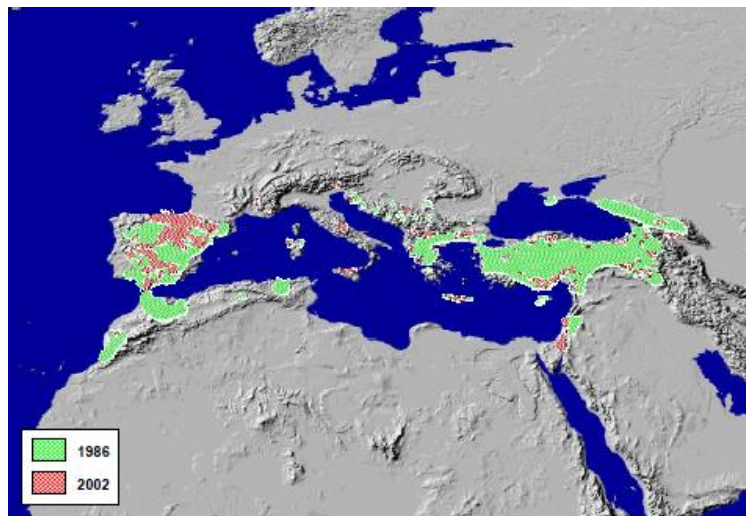


Figure 2 - Distribution of the Griffon Vulture compared between 1986 and 2002. Retrieved from Slotta-Bachmayr, L. et al., 2004.

Once vultures spread across the continent, they faced dramatic species declines leading to extinction in the Alps and the Carpathians. This happened mainly due to persecution by man and poisoning. The remnant populations are isolated and in urgent need of conservation action assisted by international cooperation and provision of expertise. In 2002, distribution became more irregular but with similarities comparing to 1986. Regarding Iberian Peninsula, the Griffon Vulture is well established, doubling its population size every 8 years. (Slotta-Bachmayr, L & Camiña, A., 2004).

If we exclude the Iberian Peninsula, the population of vultures in Europe is stable with about 2000 pairs for the last 16 years (Camiña A., 2004). According to BirdLife census in 2004, is important to refer the relevant role of the Iberian Peninsula countries on the conservation of the Eurasian Griffon as Spain holds the 86.19-91.05% of the all the population (Papazoglou, C. et al., 2004). Portugal, however, is only partly occupied by Griffon vultures since the Atlantic corridor is not suitable as griffon's habitat. In Portugal, there are approximately 267 to 272 breeding pairs, distributed basically on the large river's basins: Douro (43.1% of griffon population), Tejo (56.5%) and Guadiana (0.4%). The Guadiana basin was re-colonised in 1999 after more than twenty years without vultures (Slotta-Bachmayr, L & Camiña, A., 2004). The success of griffon vulture in Portugal is due to the traditional farming practices of leaving carcasses in the field thus providing food supply for vultures. However, there is a massive difference between the vulture population in Spain and in Portugal as in 2001 an EU directive aiming the reduction of the mad cow disease, mandated the immediate burial or incineration of cattle found dead in the fields. Spain abandoned the measure a few years later, but it is still performed in Portugal (BigThink, 2018).

2.1.3. Conservation and threats

The major threat for vultures is food poisoning and habitat loss. Usually, the poison is meant to affect the populations of mammalian predators as wolves, bears, foxes, and jackals, but end up affecting the vultures since they are obligate scavengers, being particularly vulnerable to all sorts of poisons and chemicals present on the terrestrial surface of the planet, through baits, pesticides, and lead poisoning, among other contaminants. In fact, large numbers of individuals can be poisoned by a single carcass (Margalida, A. & Ogada, D., 2018).

Regarding habitat loss, it is a problem that refers not only to food availability but also to breeding sites. In Europe, extensive farming changes along with socio-economic developments may lead to a massive decrease in the food supply. In 2001, the "mad cow" disease promoted that all carcasses of domestic animals had to be collected from farms and transformed or destroyed at authorized facilities, which resulted in the closing of many vulture feeding stations. In Spain, it is estimated that 80% of all sheep and goat carcasses and 100% of all cow carcasses were being disposed (Donázar, J. et al., 2009). Between 2005 and 2008, in the new Navarre and Aragon regions, it is believed that 80% of all feeding stations were forced to close.

The new regulations were responsible for an annual decrease in breeding success, reduced population growth, increased mortality of young age classes, changes in the diet and behaviour of Eurasian griffon vultures and a reduction on egg quality (Margalida, A. et al., 2017; Hernández, M. et al., 2018). Fortunately, recommendations made by scientists and conservationists recently allowed the creation of new EU guidelines allowing farmers to abandon dead animals in the field or at feeding stations (Margalida, A. et al., 2012).

The cinereous vulture greatly decreased in numbers in most distribution areas, especially by poisoning, habitat destruction and reduction of the food supply. In many European countries

(Portugal, France, Italy, Austria, Poland, Slovakia and Romania), the species became extinct. Thanks to strong efforts in many different projects, the numbers are now slowly increasing, especially in Spain, where the population is now of more than 2,000 pairs. Furthermore, in France a successful reintroduction project has resulted in the re-establishment of the species. The total European breeding population (including Turkey and Russia) is approximately 1800 pairs (Vulture Conservation Foundation, 2021).

2.2. Microbiota

The exposure to antimicrobial pharmaceuticals as environmental contaminants can exert direct and indirect effects on wildlife health. Fungal infections pose a major threat as mycosis are among the most frequent and most serious diseases in birds, the majority of which are caused by ubiquitous microorganisms (Garcia, M. et al., 2007).

The genera *Aspergillus* and *Candida* are the pathogens most frequently associated with diseases (Hubálek, Z., 2004). Stress appears to be an important factor in the development of fungal infections, as it is 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.

In birds, opportunistic fungal infections have been reported in stressed, immunocompromised, and medicated poultry and pets (Pitarch, A. et al., 2017). These animals have a predisposition to systemic mycosis as the absence of the epiglottis makes it impossible to prevent matter from entering the lower respiratory tract (Balseiro A. et al., 2005). Moreover, they lack the diaphragm, resulting in the inability to produce a strong cough reflex. Airborne fungal spores such as these from *Aspergillus* enter the upper and lower respiratory tract by inhalation but, usually, they are not pathogenic to healthy individuals (Tell, L., 2005). For this reason, the vultures may be carriers of the fungi but do not develop diseases unless stimulated by a decreased resistance in consequence of stress or malnutrition.

Also, the order Mucorales includes several saprophytic fungi associated with underlying diseases. 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; Neville, B. et al., 2015). In the other hand, fungal infections causing clinical lesions are rarer in free-range wild birds.

Since yeast and fungi are cosmopolitan and ubiquitous saprophytes, certain contaminated environments, wastewater, or litter and excreta from livestock can contribute to their proliferation and dissemination (Redig, P., 2003). These unsanitary conditions, including the

accumulation of decomposing carcasses and faecal material, often concur in feeding stations used as management and conservation tools for vultures and other avian scavengers.

Specially in Europe, vultures have increased their dependence on livestock carcasses deposited in feeding stations over carcasses of free-ranging livestock, which may represent health risks due to ingestion of veterinary pharmaceuticals, exposure to parasites and pathogens. The risk is high since carrion used for this purpose is generally from poultry and swine, that are often sick, medicated and disposed of in feeding stations a short time after slaughter, without a withdrawal period or residue control, thus potentially leading to negative effects on the health of scavengers (Garcia, M. et al., 2007). The improper contact or consumption of antibiotic residues can stimulate the growth of opportunistic fungi, as *Mucor* spp. or more frequently *Candida* spp., that may lead to invasive mycosis by eliminating or disturbing competing bacteria of the host microbiome. Several diseases represented by lesions compatible with fungal infections have been recorded in the oral cavity of nestling and adult wild vultures exposed to livestock antibiotics (Pitarch, A. et al., 2017).

2.2.1. *Mucor* species

The first illustrations clearly recognizable as *Mucor* representatives appeared in 1665. Although, by the end of 1838 it was still mistaken with a filamentous form of the organism originated from *Saccharomyces* spp. by species transmutation. In 1876, Pasteur implicated *Mucor* spp. as an important fungal contaminant of diseased beer as he noted its fermentative capacity and described the dimorphic habit of *Mucor racemosus*. Also, Pasteur related morphological conversions to the oxygen tension of the growth medium (Ainsworth, G. & Sussman, A., 1965). To this day, the genus *Mucor* continues to be the focus of study by a considerable number of microbiologists and mycologists with a broad spectrum of interests in both the basic and applied areas of biological research.

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. Also, it is responsible for several fungal infections in humans, cattle, swine, and some birds. The genus *Mucor* contains several species, being the most common ones *Mucor amphibiorum*, *Mucor circinelloides*, *Mucor hiemalis*, *Mucor indicus*, *Mucor racemosus*, and *Mucor ramosissimus* (Dr. Fungus, 2021).

Mucor spp. cause infections as mucormycosis. This group of diseases includes mucocutaneous and rhino cerebral infections, as well as septic arthritis, dialysis-associated peritonitis, renal infections, gastritis, and pulmonary infections. It appears more frequent in patients with diabetic ketoacidosis and immunosuppression. Also, desferoxamine treatment, renal

failure, extensive burns, and intravenous drug use may also predispose to development of mucormycosis. These infections cause necrosis of the infected tissue, and perineural invasion (Marques, S. et al., 2010).

Mucor spp. display properties typical of other zygomycetes. Although there is one significant difference that distinguish *Mucor* spp. from other zygomycetes namely the property of dimorphism. Thus, all species of *Mucor* have a variety of several hyphal morphologies, usually associated with the production of arthrospores, sporangiospores, or zygospores, only those that can grow in the form of spherical multipolar budding yeasts are referred to as dimorphic (Orlowski, M., 1991). Dimorphic *Mucor* species can grow as aseptate filamentous mycelia or as multipolar budding yeasts, depending on the environment. In aerobic conditions, the hyphal growth is stimulated, whereas strict anaerobiosis is required for the development and maintenance of the yeast form (Figure 3). Also, carbon source is an important factor as the yeast form can only grow using fermentable hexoses while the hyphae is proved to grow using various substrates including complex carbon sources (Arroyo et al., 2016).

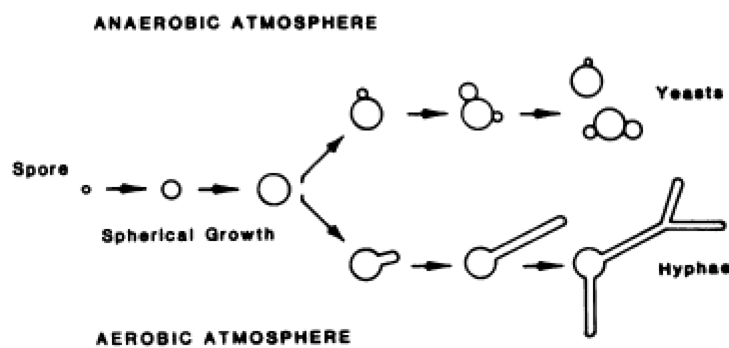


Figure 3 – Alternative morphogenetic fates of sporangiospores from dimorphic *Mucor* spp. Retrieved from Orlowski, M., 1991.

Mucor circinelloides is a common and variable species that grows as mould in the environment and is a biodiesel producer. Its predominant form is filamentous, although it can grow as a yeast under certain conditions (Vellanki et al., 2018). In the presence of eggs or ascarids, its spores develop a mycelium which adheres to the eggshell, penetrate, and eliminates the embryo.

2.2.1.1. Morphological forms and morphogenetic transformations

Fungal morphogenesis is a cyclical process being usually convenient to consider the spore as the point of reference. *Mucor* spp. can construct three different types of spores (Orlowski, M., 1991). The zygospore effects genetic recombination, the sporangiospore serves as a dispersal mechanism and the arthrospore ensures survival under environmental duress. Though it derives exclusively from the mycelial habit of *Mucor* spp., the sporangiospore can develop into either the yeast or hyphal form upon germination, the precise morphological direction taken being

dependent only on the nutritional and gaseous environments. Whether the sporangiospore ultimately develops into a budding yeast cell or a hyphal germ tube, it first undergoes a common period of spherical growth where the new vegetative wall is synthesized underneath the existing spore wall (Genthner, F. & Borgia, P., 1978; Li, C. et al., 2011). The composition of the vegetative wall is different from that of the spore wall and is specific to the incipient cell morphology. The spore wall stretches and cracks above the expanding germ sphere, its fragments adhering to the new surface but having no molecular continuity with it. Upon such emergence, new-wall deposition is uniformly dispersed in yeasts and apically localized in hyphae. The vegetative wall deposited is much thicker in spores developing as yeasts and differs in microfibrillar structure from spores converting to hyphae (Storck, R. & Morril, R., 1977; Lübbehüsen, T. et al., 2003).

The arthrospore is the least studied, most poorly understood type of cell made by *Mucor* spp. It is associated to a survival mechanism because it is formed after the cessation of logarithmic growth or under unfavourable nutritional or other environmental conditions. These spores are formed in submerged cultures trough septation of normally coenocytic hyphae and the deposition of a new three-layered wall beneath and distinct from the original hyphal wall (Bartnicki-Garcia, S. & Nickerson, W., 1962).

Mucor hyphae can develop from any of the spore types mentioned above and from *Mucor* yeasts. A typical hypha initially emerges from the spherical cell in the form of a short projection called a germ tube, through factors which are not yet understood. The wall and plasma membrane of yeasts and sporangiospore-derived germ spheres remain ultra-structurally undifferentiated until germ tube formation initiates (Bartnicki-Garcia, S. et al., 1968; Li, C. et al., 2011). *Mucor* yeasts may be developed from sporangiospores, arthrospores, and hyphae of dimorphic species. They are large, spherical, multinucleate cells that grow and propagate by the production of multiple buds that appear to be initiated at random locations over the surface of the mother cell. The cells walls are much thicker, diffuse, and fibrous than the walls of the hyphae. Also, it is differentiated into two discrete layers, whereas the hyphal wall is differentiated is structurally uniform. The formation of yeasts from hyphae involves spherical bud production on the sides and tips of the filaments, which themselves remain morphologically unchanged. Hyphal fragments persist as a significant portion of the cell population for a considerable time after the initiation of hypha-to-yeast morphogenesis. The resulting mixed-cell populations have dissuaded researchers from studying morphological conversions in this direction (Bartnicki-Garcia, S., 1963; Arroyo, M. et al., 2016).

2.2.1.2. Environmental factors influencing morphogenesis

Dimorphic species of *Mucor* react morphologically different to changes in the environment. Usually, there are two generalities that apply, being one that a fermentable hexose is always required for growth in the yeast form and another that anaerobiosis generally favours growth in the yeast form, whereas an aerobic atmosphere normally stimulates the hyphal growth. A given set of environmental conditions will usually evoke the same morphological response from sporangiospores, arthrospores, and vegetative cells (Orlowski, M., 1991).

2.2.1.3. Macroscopic features

In the laboratory, colonies of *Mucor* grow rapidly at 25-30ºC and quickly cover the surface of the agar. Its fluffy appearance with a height of several cm resembles cotton candy. The colour is initially white, becoming more greyish brown along the incubation period. *Mucor indicus* is an aromatic species and may grow at temperatures as high as 40°C. On the other hand, *Mucor racemosus* and *Mucor ramosissimus*, grow poorly or do not grow at all at 37°C (Dr. Fungus, 2021).

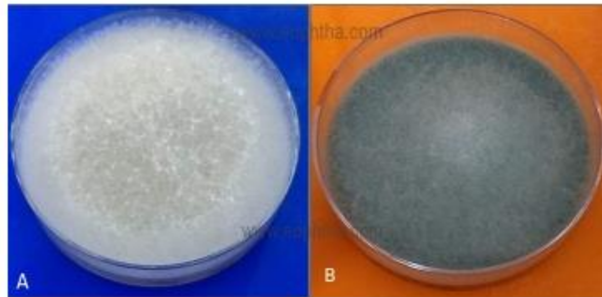


Figure 4 – *Mucor* spp. colonies isolated from a case of mucormycosis on Sabourad dextrose agar plates. A- Initially the colonies are white; B- The colonies become greyer when they develop sporangia. Retrieved from eOphtha, 2021.

2.2.1.4. Microscopic features

Regarding *Mucor* spp. microscopic characteristics, the microorganism is nonseptate or sparsely septate, and present broad (6-15 µm) hyphae, sporangiophores, sporangia, and spores. Intercalary or terminal arthrospores (oidia) located through or at the end of the hyphae and few chlamydospores may also be produced by some species. Sporangiophores are short, erect, taper towards their apices and may form short sympodial branches. Columellae are hyaline or dematiaceous and are hardly visible if the sporangium has not been ruptured. Smaller sporangia may lack columella. Sporangia are round, grey to black in colour, and are filled with sporangiospores. Following the rupture of the sporangia, sporangiospores are freely spread. A collarette may sometimes be left at the base of the sporangium following its rupture. The sporangiospores are round or slightly elongated. Zygospores, if present, arise from the mycelium (Mycology Online, 2016).

The branching of sporangiophores (branched or unbranched), the shape of the sporangiospores (round or elongated), maximum temperature of growth, presence of chlamydospores, assimilation of ethanol, and molecular analysis aid in the differentiation of *Mucor* species (Dr. Fungus, 2021).

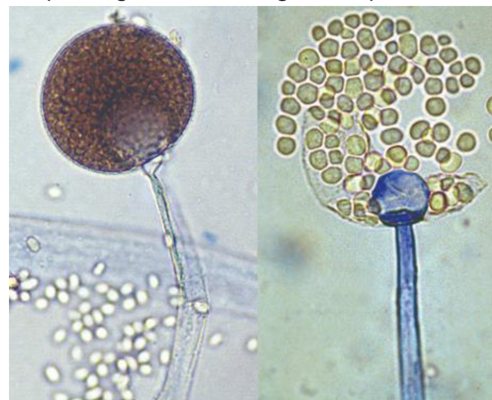


Figure 5 –Sporangia, columella and sporangiospores of *Mucor* spp. Retrieved from Mycology Online, 2016.

2.3. Virulence factors

Despite the order Mucorales being significantly neglected comparing to others, several studies are being conducted to investigate the virulence of *M. circinelloides* as its ability to cause disease is alarming (Lax, C. et al., 2020). Fungi are well known by their ability to excrete enzymes into the environment. Among the species belonging to this genus, representatives of *Mucor* have important biotechnological potential and some of them produce industrial enzymes.

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. Enzymes may also act in the control of biochemical processes in the living cells. They may be isolated from animals, plants, and microorganisms (Baldin, C. & Ibrahim, A., 2017).

Extracellular enzymes may be produced in liquid or solid media. The use of solid media permits a fast screening of large populations of fungi, allowing the detection of specific enzymes and helping in the chemotaxonomically differentiation of many microorganisms. The production of enzymes by microorganisms assures a potential and unlimited supply and makes it possible the genesis of new enzymatic systems that cannot be obtained from plant or animal sources (Hassan, M. & Voigt, K., 2019). In the metabolic pathways, they act in organized sequences of catabolic and anabolic routes. Enzymes may also act in the control of biochemical processes in the living cells. The production of enzymes by microorganisms assures a potential and unlimited supply and makes it possible the genesis of new enzymatic systems that cannot be obtained from plant or animal sources (Alves, M. et al., 2002).

Mucor spp. constitutes a group of microorganisms responsible to produce several enzymes such as amylases, lipases, pectinases, and proteases. *Mucor hiemalis*, *M. racemosus*, *M. bacilliformis* and *M. miehei* present protease activity of commercial value and *M. miehei* is the most studied specie concerning the production of lipase. 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. 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).

Lipase production

Lipases catalyse both the hydrolysis and synthesis of triacylglycerols. Many of these enzymes are characterized by stability at high temperatures and in organic solvents, high enantioselectivity, and resistance to proteolysis, which make them ideal candidates for diverse commercial applications. The putative roles of microbial extracellular lipases include digestion of lipids for nutrient acquisition, adhesion to host cells and host tissues, synergistic interactions with other enzymes, nonspecific hydrolysis due to additional phospholipolytic activities, initiation of

inflammatory processes by affecting immune cells, and self-defence mediated by lysing competing microflora (Gácser, A. et al., 2007).

Lipases are widely distributed in nature being found in animals, in plants (mainly in oily seeds); in bacteria, mainly in species of the genera *Chromobacterium*, *Pseudomonas* and *Staphylococcus*; in yeasts, mainly in species of the genera *Candida* and *Yarrowia lipolytica* and in fungi especially in species of the genera *Aspergillus, Geotrichum, Rhizopus, Mucor, Penicillium* (Pascoal, A. & et al., 2018)

Lecithinase production

Lecithinase is a type of phospholipase that acts upon lecithin. Lecithinases or phospholipases are enzymes released by bacteria that can destroy animal tissues and play a role in pathogenicity. Lecithinase, which is also called phospholipase C, is such an enzyme that splits the phospholipid lecithin. Phospholipid complexes are usually emulsifying agents occurring in tissues, serum, and egg yolk. Lecithin is a normal component of the egg yolk (MicrobeNotes, 2018).

Gelatinase production

Nutrient gelatine is a differential medium that tests the ability of an organism to produce an exoenzyme, called gelatinase, that hydrolyses gelatine. Gelatin is a protein derived from connective tissue. When gelatin is at a temperature below 32°C (or within a few degrees thereof), it is a semisolid material. At temperatures above 32°C, it is a viscous liquid (dela Cruz, T. & Torres, M., 2012).

Gelatinase allows the organisms that produce it to break down gelatine into smaller polypeptides, peptides, and amino acids that can cross the cell membrane and be utilized by the organism. When gelatine is broken down, it can no longer solidify. If an organism can break down gelatine, the areas where the organism has grown will remain liquid even if the gelatine is refrigerated (Loperena, L. et al., 2012).

In recent years, there have been attempts to produce different types of proteases, using several different types of substrates. A great number of fungal strains have been used to produce proteases belonging to the genera *Aspergillus, Penicillium, Rhizopus, Mucor, Humicola, Thermoascus, Thermomyces*, among others (Souza, P. & et al., 2015).

DNase production

Deoxyribonucleic acid (DNA) is a large molecular-sized polymer composed of multiple nucleotides monomer that is large and thus, cannot enter the bacterial cell membrane.

Microorganisms produce the deoxyribonuclease enzyme to breakdown the DNA into smaller monomers which can then be taken into the cell easily. Furthermore, the nucleotides are used to make nucleic acid for the bacteria as well as a source of nitrogen, phosphorus, and carbon (Haas, B. et al., 2014).

Also, some microorganisms can even produce extracellular DNase that breaks down larger DNA into smaller monomeric units so that they can be taken into the cell via the transport proteins present on the cell membrane. Moreover, the degradation of DNA is also considered a virulence factor as it causes the degradation of the host’s DNA (Park, H. et al., 2019).

Haemolytic activity

Hemolysins are a class of proteins defined by their ability to lyse red cells but have been described to exhibit pleiotropic functions. These proteins have been extensively studied in bacteria and more recently in fungi. Within the last decade, several studies have characterized fungal hemolysins and revealed a fascinating yet diverse group of proteins.

Hemolysins were first reported in higher fungi in 1907 and 1911 by W. W. Ford while studying various basidiomycete genera including *Amanita, Entoloma, Lactarius,* and *Inocybe.* The authors reported that extracts collected from the mycelium of the fungus grown for 2 – 4 days were heat sensitive, haemolytic, and produced necrosis and enema when introduced in experimental animals (Nayak, A. et al., 2013).

Alpha haemolysis is the partial destruction of red blood cells in the blood and can turn the agar under the colony dark and greenish. Beta haemolysis is the complete destruction of red blood cells in the blood, while gamma haemolysis does not involve any breakdown of red blood cells (Buxton R., 2005).

Biofilm production

Biofilms are dense, highly hydrated cell clusters that are irreversibly attached to a substratum, to an interface or to each other, and are embedded in a self-produced gelatinous matrix composed of extracellular polymeric substances. The micro-organisms in these biofilms exhibit an altered phenotype with respect to growth rate, gene transcription, and resistance to physical, chemical, and biological stresses (Singh, R. et al., 2011).

These biofilms are commonly found during infection caused by a variety of fungal pathogens. Clinically, biofilm infections can be extremely difficult to eradicate due to their resistance to antifungals and host defences. Biofilm formation can protect fungal pathogens from many aspects of the innate immune system, including killing by neutrophils and monocytes. Altered immune recognition during this phase of growth is also evident by changes in the cytokine profiles of monocytes and macrophages exposed to biofilm (Kernien, J. et al., 2018).

One defining trait of biofilm formation is the production of a microbial-produced extracellular matrix, or the "glue" necessary for adhesion, which also serves as a shield that creates protected reservoirs of infection. As investigations reveal the complex composition of the extracellular matrix for several fungal pathogens, it has become increasing clear that this material is distinct from the cell wall. Therefore, biofilm growth provides a means to present unique moieties and conceal cell wall ligands (Kernien, J. et al., 2018).

Most of the work describing biofilm genesis, architecture, chemical composition, genetic regulation, and antimicrobial drug resistance has focused on bacteria and yeasts with some filamentous fungi reported to form biofilms. Although filamentous fungi often penetrate the substrates that they grow on, and this invasive growth may not accurately represent the term biofilm. Filamentation in fungi may therefore be a requisite for robust biofilm development and virulence and fungal biofilms perhaps represent much more than a mere biological coating (Singh, R. et al., 2011).

2.3.1. Previous studies on virulence factors

Alves conducted a study on the production of amylase, lipase, polygalacturonate and protease by 56 isolates of *Mucor* belonging to 11 different taxa, selected from herbivores dung using solid media. The results were alarming as most of the isolates presented several enzymatic activities with the predominance of polygalacturonate (96%), followed by amylase (84%), protease (82%) and lipase (66%). Also, the results from this study show that all *Mucor* isolates possessed a high potential for enzyme production, especially lipase, which was present in most of the taxa studied. It was observed that enzymatic activity does not establish true standards for separation of the taxa at a specific level since it varied in different isolates belonging to the same taxon (Alves, M. et al., 2002).

Another study carried on by Thompson, evaluated the use of solid media for the detection of enzyme production by *Rhizopus* and *Mucor* were the species used in this study. Proteolytic activity was detected in all the fungi isolates under study. RNase and DNase production were also detected in most of the isolates. Regarding lipolytic activity only two isolates showed potential to produce lipase. Urease activity was detected in all fungi, except *R. arrhizus and R. rfrcinurns* (Thompson D. et al., 1984).

In 2014, cellulolytic, lipolytic and proteolytic enzyme production of zygomycetes *Mucor corticolus, Rhizomucor miehei, Gilbertella persicaria* and *Rhizopus niveus* were investigated using agro-industrial wastes as substrates. Isolates selected for this study proved to be good extracellular β-glucosidase and/or lipase producers. Among the tested fungi, *M. corticolus* seemed to be the most promising fungus for cellulase and lipase secretion.

Finally, Singh conducted a study about the biofilm formation by zygomycetes. The author claimed that most studies on fungal biofilms have focused on *Candida* in yeasts and *Aspergillus*

in mycelial fungi. To the authors knowledge their study was the first regarding biofilm formation by zygomycetes. They focused on the biofilm-forming capacity of *Rhizopus oryzae*, *Lichtheimia corymbifera*, *Rhizomucor pusillus* and *Apophysomyces elegans.* They concluded that *R. oryzae*, *L. corymbifera* and *R. pusillus* produced highly intertwined, adherent structures on flat-bottomed polystyrene microtiter plates after 24h at 37ºC. This study was very important to highlight the potential for biofilm production of these three important species of zygomycetes (Singh, R. et al., 2011).

2.4. The microbiome

Vultures are scavengers that fill a key ecosystem niche as they have evolved a remarkable tolerance to bacterial toxins in decaying meat. In the case of vertebrates, their microbiota begins to decompose rapidly after death. During the subsequent breakdown of tissue, these microorganisms excrete toxic metabolites turning the carcass a hazardous food source for most carnivorous and omnivorous animals (Roggenbuck, M. et al., 2014). Vultures have evolved adaptive mechanisms to prevent infections associated with their scavenging lifestyle as showed by recent studies at metagenomic and transcriptomic level (Roggenbuck, M. et al., 2014). Although most of the ingested bacteria will not survive the acidic gastric passage before hindgut colonization, some toxins as are able to survive compromising the health of the consumer. Also, food-borne exposure to antimicrobial pharmaceuticals can promote opportunistic infections with adverse outcomes (Deo, P. & Deshmukh, R., 2019).

2.4.1. Assessment of microbiome

Oral microbiome, oral microbiota or oral microflora refers to the group of microorganisms found in the oral cavity. Although, microbiome studies usually are based on stool analysis, the oral cavity has the second largest and diverse microbiota after the gut harbouring over 700 species of bacteria. Other microorganisms present include fungi, viruses, and protozoa. The mouth is a very complex habitat as microbes colonize the hard surfaces and the soft tissues of the oral mucosa (Killian, M. et al., 2016). Also, the mouth is the initiation point of digestion and is crucial in maintaining oral and systemic health. The oral cavity and the nasopharyngeal regions provide an ideal environment for the growth of microorganisms as the normal temperature is without significant changes providing a stable environment for the survival of microbiota. Saliva also has a stable pH, which favours the growth of bacteria. Moreover, it keeps the bacteria hydrated and serves as a medium for the transportation of nutrients to microorganisms. The oral microbiome as become the most well-studied microbiome thus far due to the ease of sample collection and processing (Deo, P. & Deshmukh, R., 2019).

Previously, the microbiome was studied using culture methods, but the abundant microbiota present in the oral cavity could not be fully cultured (Killian, M. et al., 2016). Thus, the

emergence of new genomic technologies including next generation sequencing and bioinformatics has revealed the complexity of the oral microbiome.

2.4.1.1. Culture based methods

The culture-based methods have been improving, allowing the identification of a broader set of organisms present in different microbiomes. Some previous studies have used bioreactors, to simulate the microbiome to understand more about interactions between different organisms and their hosts, production of metabolites, chemical effects of various antibiotics or polycyclic aromatic hydrocarbons on the community, biofilm dynamics and fermentation processes (McDonald et al., 2015).

The biggest challenge when applying this method is that each organism is different and so are their optimal conditions, being more difficult to mimic that in the laboratory. Culture outcomes rely on selecting the correct pH, oxidation-reduction potential, temperature, and nutrient supplementation. Another challenge is that the relations between the microorganisms and their host *in vitro* are very difficult to replicate as the fact that these enrichment and isolation techniques usually are lower throughput in comparison with molecular techniques (Dickson et al. 2017).

2.4.1.2. Molecular based methods

Although being a more accurate technique, most molecular techniques do not allow the differentiation between living and dead microorganisms, which culture-based methods do.

2.4.1.3. Non sequencing methods

In these techniques, bacterial nucleic acid is extracted from the specimen to be analysed, followed by amplification either of the entire length of the 16S rRNA gene or a segment of this gene that includes one or more selected hypervariable regions. This can be performed using PCR with universal primers corresponding to conserved regions in the bacterial genome flaking the entire gene or the selected hypervariable region(s). The resultant amplified mixture of 16S rRNA genes from all the bacteria contained in the specimen can then be resolved using one of several techniques as electrophoresis-based separation based on fragment length or those based on the presence of specific nucleotide sequences (Dickson et al. 2017).

The main disadvantage of these methods is a limited resolution of bacterial groups. Hence, the bacterial groups present in low abundance are missed, explaining the reason that these methods have been replaced by newer generation sequencing techniques (Sarangi, A. et al., 2019).

2.4.1.4. Sequence-based methods

The traditional Sanger technique for nucleic acid sequencing needs relatively pure DNA as starting material and provides only one sequence per experiment. Thus, it was not possible to sequence a specimen containing a mixture of related nucleic acids using this technique, except by cloning each of these nucleic acid molecules into separate vectors and sequencing each clone, a very tedious and costly undertaking (Ortiz-Estrada et al., 2019). Since microbiota contains a mixture of bacteria with somewhat diverse genomic material, it cannot be established using this technique.

Many methods developed over the years have allowed massively parallel sequencing of each molecule contained in a DNA mixture, such an isolated from a microbiota specimen. However, these techniques pose two challenges as they have for one a large amount of data, posing a nightmare for analysis and second, they provide much shorter read-lengths than were possible from Sanger sequencing. Several different technologies were developed and commercialized for multi-parallel sequencing. However, most of these have fallen by the wayside, and most of the current studies on microbiota use of one of two equipment's from one manufacturer (Illumina). In view of their limited read-lengths, these techniques allow sequencing of only one or two adjacent hypervariable regions of the 16S rRNA gene. This information allows one to determine the types of bacteria present as also their relative frequencies (abundance) in a mixed specimen (Sarangi, A. et al., 2019).

The main advantages of Next Generation Sequencing techniques like Illumina are that is much cheaper and higher-throughput alternative to sequencing DNA than traditional Sanger sequencing because the use of an array instead of capillary sequencing significantly reduces the cost of DNA reaction of sequencing. Also, in this technique the bottlenecks existing in Sanger technology are reduce as colony picking and DNA extraction. In this sequencing method millions of arrays per read are generated. On the other hand, the NGS technologies have some limitations as the generation of millions of reads means that they have short-sequencing lengths (200-500 nucleotides) that lead to sequence errors and assembly problems. Furthermore, in some NGS techniques there is an inaccurate sequencing of homopolymer regions (spans of repeating nucleotides).

Aims of the study

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. The order Mucorales includes several saprophytic fungi associated with relevant opportunistic diseases in bird sheaths.

In this study, the aim was to evaluate the pathogenic profile of oral *Mucor* species from Eurasian Griffon Vultures trough phenotypic characterization. For that, 7 oral swabs were collected from Eurasian Griffon Vulture (*Gyps fulvus*) and Cinereous Vulture (*Aegypius monachus*) and cultured in Sabouraud dextrose agar (SDA), with further incubation. Furthermore, the isolates were identified through macro and microscopic observation and identification was confirmed by PCR and ITS sequencing. Afterwards, their phenotypic pathogenic profile was determined by assessing the production of lipase, lecithinase, gelatinase, DNase, haemolysins and biofilm, after 24, 48 and 72 hours of incubation in specific media.

Another goal of this study was to access the ability of these isolates to produce inhibitory composts. This part was evaluated 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.

This study hopes to bring awareness for this endangered animal species and to help to understand the pathogenic potential of a relevant microbial genus, *Mucor* spp., which would ultimately contribute for the development of adequate management programmes for protection of the vultures.

3. Material and Methods

3.1.1. Sample collection and handling

The group of *Gyps fulvus* and *Aegypius monachus* included in this project was recovering in the rehabilitation centres Centro de Estudos e Recuperação de Animais Selvagens, CERAS (n=2) and Centro de Recuperação e Investigação de Animais Selvagens, RIAS (n=5). These vultures were recovering from bad nutrition and debility. Some of the samples collected were from animals diagnosed with an oral disease.

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

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.

3.1.2. 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.

3.1.3. 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 MgCl2, 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®). The electrophoresis run at 90V for 1 hour. The gel was made joining 1,5g of agarose to 100mL of TBE medium. After homogenization, 3µL of Green Safe were added to the medium. A molecular weight marker, NZYDNA ladder VI (Nzytech®) was also included. Results were visualized by transillumination (ChemiDoc XRS+, Bio-rad®).

3.1.4. Sanger sequencing

After the results from amplification, the PCR products were sent to sequencing to STABVida® 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.

3.2. 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.

Production of 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. After incubation, the plates were observed and a positive result was measured by the appearance of a clear halo zone of precipitation around the colony (Elavarashi, E. et al., 2017).

Production of 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. A clear halo zone of clearance around the colony indicated lecithinase production (Arslan & Küçüksari, 2015).

Production of 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. Gelatin usually liquefies at 28°C and above and therefore to confirm gelatin liquefaction due to gelatinase production. The test gelatin tubes were refrigerated at 4°C for 30 minutes before observation. Later, the tubes were tilted to observe for gelatin liquefaction.

Production of DNase

The DNase medium was prepared and sterilized by autoclave. Afterwards, the toluidine blue reagent (0,1g/L) was added. After inoculation and incubation at 37ºC if the organism that

grows in the medium produces deoxyribonuclease, it breaks down DNA into smaller fragments. A positive result is the formation of pink halos around the colonies.

Haemolytic Activity

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 Production

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. If the isolate in fact has biofilm production, the medium will turn black.

3.3. 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.

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

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 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.

3.4. 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).

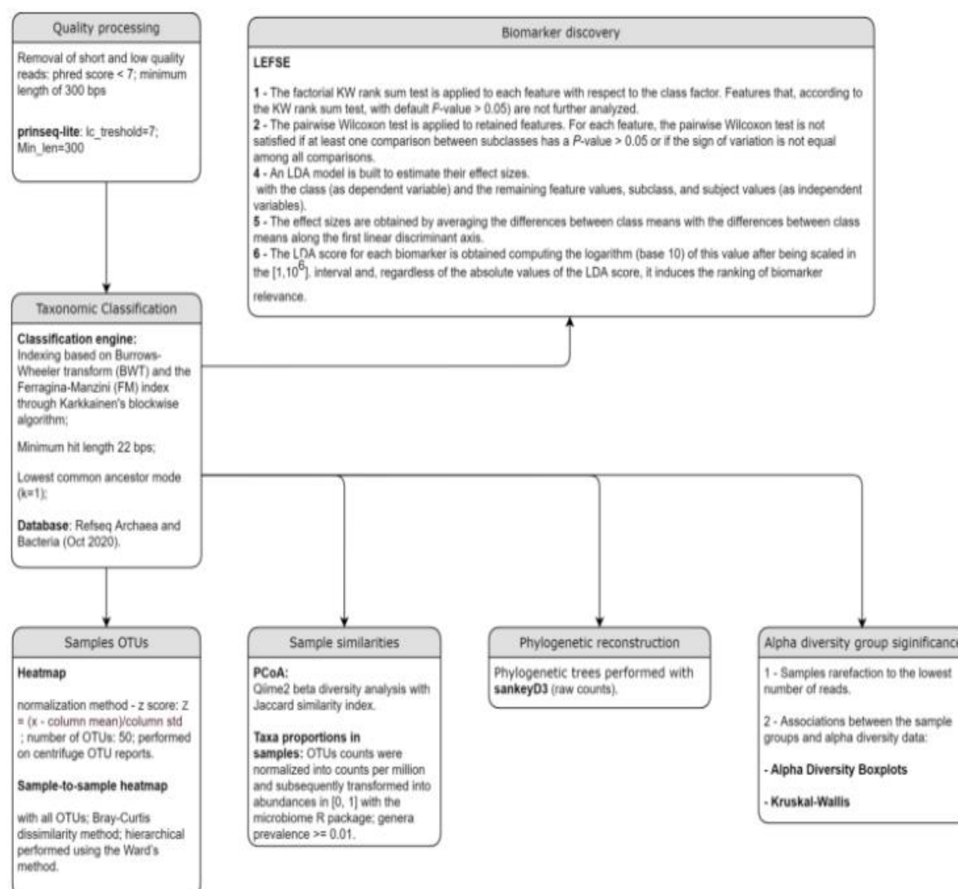


Figure 6- Analytical pipeline of the analysis developed by BioISIGenomics for taxonomic classification of 16S sequencing metagenomic data.

4. Results

4.1. *Mucor* spp. isolation and identification

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 7. C and D. Some *Mucor* species produce chlamydospores, Figure 7. A (Trovão & Pereira, 2019).

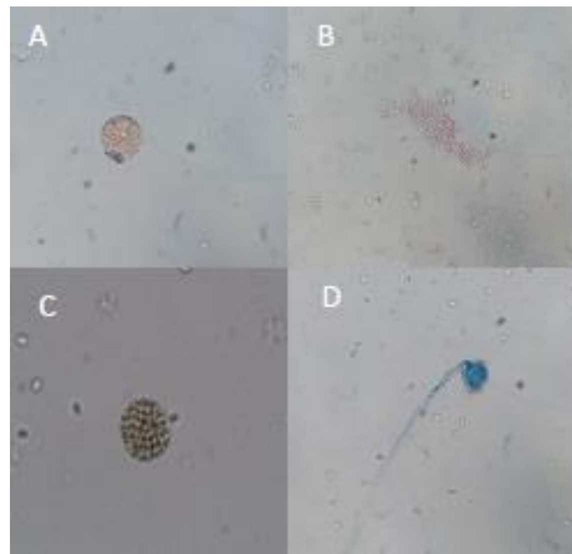


Figure 7- *Mucor* chlamydospore; B- *Mucor* conidia; C- *Mucor* sporangia; D- *Mucor* sporangia, columella, and conidiophore. 400x. Original.

4.2. Molecular identification

For the molecular identification, the DNA of the isolates was extracted for posterior amplification of the ITS region. After the products were separated through agarose gel eletrophoresis.

Observing Figure 8, it was possible to observe that the 7 samples of *Mucor* isolates produce amplicons with the expected size, 290bp (White et al., 1990).

Figure 8- PCR products after separation by 1,5% agarose gel electrophoresis. 1- *Mucor* isolate 1; 2- *Mucor* isolate 2; 3- *Mucor* isolate 3; 4- *Mucor* isolate 4; 5- *Mucor* isolate 5; 6- *Mucor* isolate 6; 7- *Mucor* isolate 7; NC- negative control; PC- positive control. Original.

4.3. Sanger sequencing

Before the PCR products were sent to sequencing, they were purified, and evaluated using nanodrop. The results of the amplification products sequences are shown in Table 3 below.

Table 3- ID number of the samples, scientific name, query cover, e-value, percentage of identity and sequence with ITS1 and ITS2 primers.

ID number	Scientific name	Query cover	E value	Per. Ident.	Sequences
1	*Mucor* spp.	68%	5e-122	100 %	ccgggaaaacaaaattttatgggcttgtccattattatctatttactgtgaactgtattattacttgacg cttgagggatgctccactgctataaggataggcggtggggatgttaaccgagtcatagtcaagcttaggc ttggtatcctattattatttaccaaaagaattcagaattaatattgtaacatagacctaaaaaatctata aaacaacttttaacaacggatctcttggttctcgcatcgatgaagaacgcagcatcgatgcagctcccct cgtccatgtaccgaacagcgtccatccaccgaacggcatcgatgcacagcacagcggcgatgccccccgc acca
2	*Mucor circenelloides*	93%	8e-124	99.60%	cccgtaaaatcaataattttgggcttgtccattattatctatttactgtgaactgtattattacttgacg cttgagggatgctccactgctataaggataggcggtggggatgttaaccgagtcatagtcaagcttaggc ttggtatcctattattatttaccaaaagaattcagaattaatattgtaacatagacctaaaaaatctata aaacaacttttaacaacggatctcttggttctcgcatcgatgaagaaaacagcaaa
3	*Mucor circinelloides*	97%	2e-125	99.22%	nngtaaatcataattttgggcttgtccattattatctatttactgtgaactgtattattacttgacgctt gagggatgctccactgctataaggataggcggtggggatgttaaccgagtcatagtcaagcttaggcttg gtatcctattattatttaccaaaagaattcagaattaatattgtaacatagacctaaaaaatctataaaa caacttttaacaacggatctcttggttctcgcatcgatgaagaacgcagcaat
4	*Mucor circinelloides*	77%	1e-86	94.81%	nnnnnnnnnnnnnntnnnnanntntnntatnagttntgtttangtgtcgtatcgtaactagtatataattctcg aattcttttggtantaataataggatagcaagcctaagcttgactatgactcggttaacatccccaccgcctatcctta tagcagtggagcatccctcaggcggcaagtaataatactgttcacagtaaatagataataatggacaagccaaaat tattgattatttaatgatccttccgccggtncaactacggaaa
5	*Mucor circinelloides*	93%	2e-120	99.18%	nnnnnnnntcatattntggcttgtcattattatctatttactgtgaactgtattattacttgacgcttga gggatgctccactgctataaggataggcggtggggatgttaaccgagtcatagtcaagcttaggcttggt atcctattattatttaccaaaagaattcagaattaatattgtaacatagacctaaaaaatctataaaaca acttttaacaacggaactcttggttctcgcatcgatgaagaacacagccatc
6	*Mucor* spp.	83%	7e-65	85.59%	nnnnnnnnnanancatnatcgnntgcannntcttacgtatgactcatctctttttttagtgtagaccgatt tctttttcagagttggctgtctgtcttataaccaacgggatnngctactagaaagtaaaacgagctgaaa gtcaggcttaggcctggtatccnangnattatttaccataagaattccgtctnntaattgtaacataagc gtaaaaaacttataaaacaacttttaacaacggatctcttggttctcgcatcgatgaagaacgcagcatc
7	*Mucor circinelloides*	92%	6e-120	99.59%	gnnnannnannattatnggcttgtcattattatctatttactgtgaactgtattattacttgacgcttga gggatgctccactgctataaggataggcggtggggatgttaaccgagtcatagtcaagcttaggcttggt atcctattattatttaccaaaagaattcagaattaatattgtaacatagacctaaaaaatctataaaaca acttttaacaacggatctcttggttctcgcatcgatgaagaacgcagcatcg

Observing Table 3, it is possible to observe that all *Mucor* isolates were sequenced properly. Query coverage refers to the percentage of the contig length that aligns with the NCBI hit. Isolate 1 had the lowest query coverage (68%) meaning that its contig is less aligned with the reference genome than the rest of the isolates. The e-value parameter is the number of hits expected to be seen by chance and the closer to zero the better. Analysing all e-values obtained, it means low number of hits, but of high quality. Regarding the percent identity, it refers to the percentage of bases that are identical to the reference genome. A query sequence can have a low percentage identity, but still be a real hit. It is essential to take the e-value into account and look for homology between conserved regions (HelpCenter, 2020). All isolates had a percentage identify above 85% meaning that the sequence is very similar to the reference genome. All isolates were identified as *Mucor* spp., isolates 2, 3, 4, 5 and 7 being identified as *Mucor circinelloides*.

4.4. Phenotypic identification

In this part of the project the production of enzymes such as lipase, lecithinase, gelatinase, DNase and haemolysins were evaluated. Biofilm production by the isolates was also tested. All assays were monitored at 24, 48 and 72 hours.

Table 4- Summary of the results of the phenotypic identification after 24, 48 and 72 hours. First result at 24h, second at 48h and third at 72h. Example: --+ had a negative result at 24 and 48 hours but a positive result at 72h. * the results were the same at 24h, 48h and 72h.

	BIOFILM	LIPASE	HAEMOLYSIS	GELATINASE	DNASE	LECITHINASE
1	---	---	α-haemolysis*	--+	---	-++
2	--+	---	α-haemolysis*	---	--+	-++
3	---	+++	α-haemolysis*	---	-++	--+
4	+++	+++	-	---	---	---
5	---	---	α-haemolysis*	---	--+	-++
6	---	+++	α-haemolysis*	--+	-++	---
7	---	---	α-haemolysis*	---	--+	---

Biofilm production

Regarding biofilm production, is 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. In Figure 9 and 10, the difference between a negative, a weak positive and a clear positive is represented.

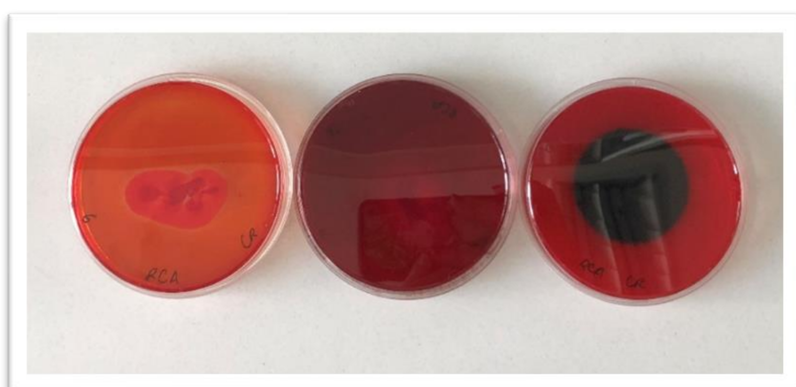


Figure 9- A negative result (isolate 6), a weak positive result (isolate 2) and a positive result (isolate 4) for biofilm production at 72 hours. Original.

Figure 10- Isolate 4. Positive result for biofilm production at 72 hours. Original.

A clear a positive result was indicated by black colonies with a dry crystalline consistency. A darkening of the colonies with the absence of a dry crystalline colonial morphology indicated a weak positive result.

Lipase production

Regarding lipase production, a positive result was measured by the appearance of a clear halo zone of precipitation around the colony (Elavarashi, E. et al., 2017). 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.

Figure 11- Positive result for lipase production. Isolate 3 at 72 hours Original.

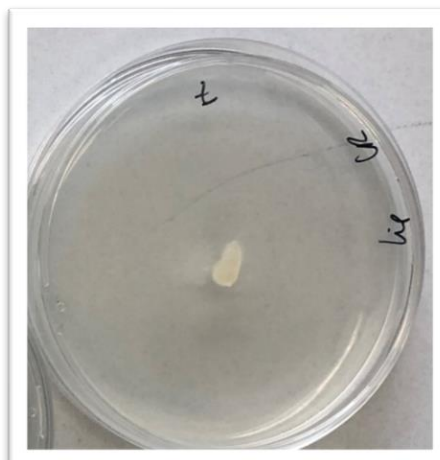


Figure 12- Negative result for lipase production. Isolate 7 at 72 hours. Original.

Comparing Figures 11 and 12, is possible to observe the difference between the two results. Figure 11 represents a positive result for lipase production in which a halo was formed around the *Mucor* colonies. In Figure 12 that is not observed as no halo was observed surrounding the colonies.

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 incubation.

Figure 13- α-haemolysis of isolate 6 at 48 hours. Original.

Figure 14- Negative result for haemolytic activity of isolate 4 at 72 hours. Original.

As may be observed in Figure 13, isolate 6 had a clear alpha haemolysis which is characterized by the partial destruction of red blood cells in the blood and can turn the agar under the colony dark and greenish. Regarding isolate 4, no haemolytic activity was registered (Figure 14).

Gelatinase production

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. Gelatin usually liquefies at 28°C and above, so a positive result is detected when there is gelatin liquefaction due to gelatinase production and the medium liquifies.

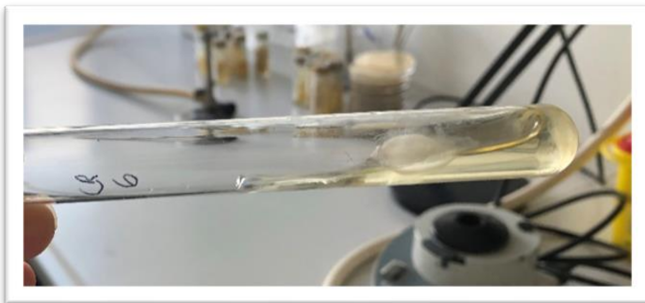


Figure 15- Positive result for gelatinase production by isolate 6 at 72 hours. Original.

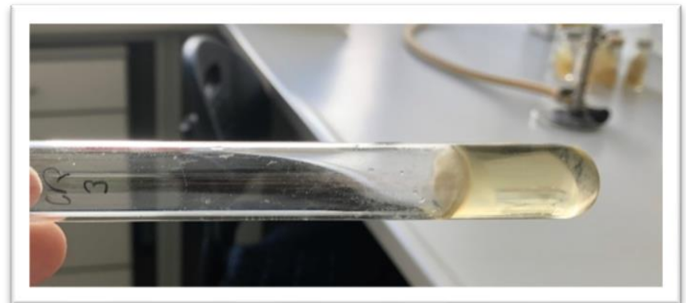


Figure 16- Negative result for gelatinase production by isolate 3 at 72 hours. Original.

Observing Figures 15 and 16, it is possible to see the clear difference between a positive and a negative result, as a positive result results in the liquefaction of the gelatinase medium as mentioned above.

DNase 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.

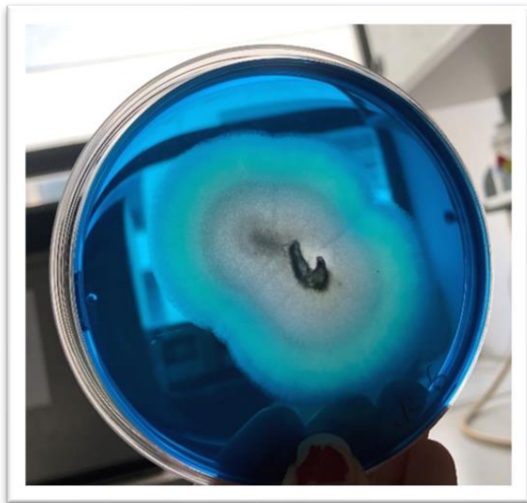


Figure 17- Positive result for DNase production from isolate 6 at 72 hours. Original.

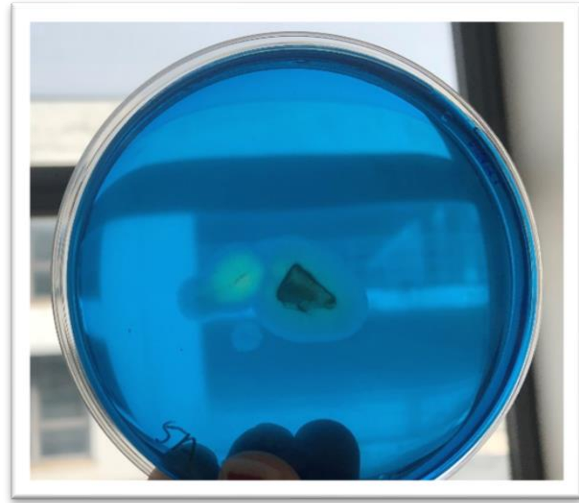


Figure 18- Negative result for DNase production from isolate 5 at 24 hours. Original.

A positive result for DNase production corresponds to the formation of pink halos around the colonies, Figure 17. On the opposite, in Figure 18, corresponds to the absence of a halo, therefore a negative result. Furthermore, this virulence factor was the strongest after the haemolysis as 5 out of the 7 isolates showed a positive result for DNase production by the end of the experience.

Lecithinase production

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.

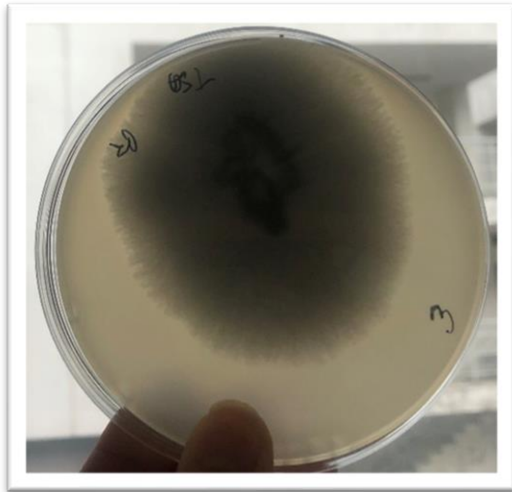


Figure 19- Negative result for lecithinase production from isolate 3 at 48 hours. Original.

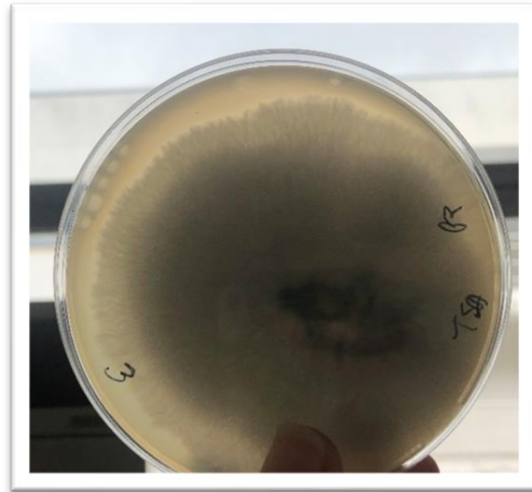


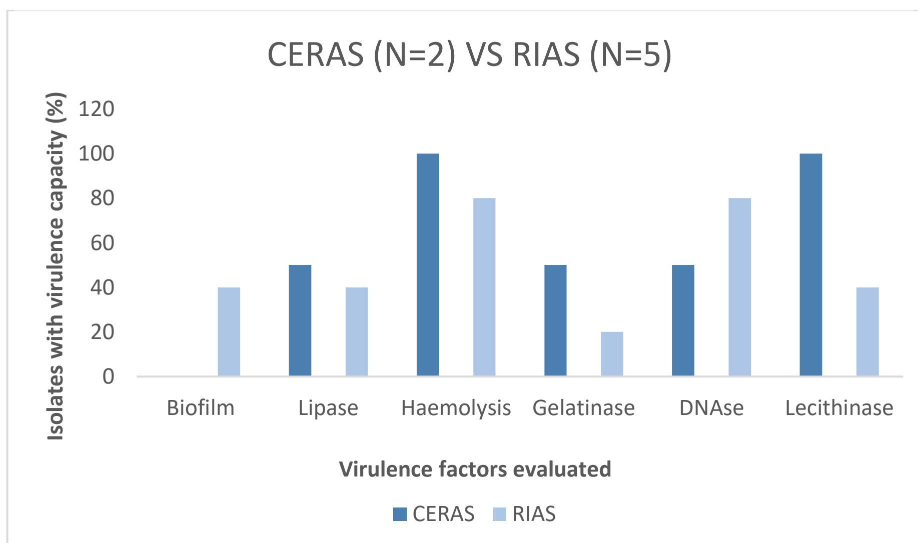
Figure 20- Positive result for lecithinase production from isolate 3 at 72 hours. Original.

A cloudy halo and/or clear zone around the colonies indicated lecithinase activity, thus a positive result. On the other hand, a lack of such cloudy halo and/or clear zone around the colonies corresponded to a negative result. Comparing Figures 19 and 20, it is possible to understand the difference between a positive result (Figure 20) and a negative result (Figure 19) from the same isolate after 48h and 72h of incubation. In Figure 20, a clear halo around the *Mucor* colonies is visible, whereas in Figure 19 there is not a halo surrounding the colonies.

4.4.1. Descriptive analysis

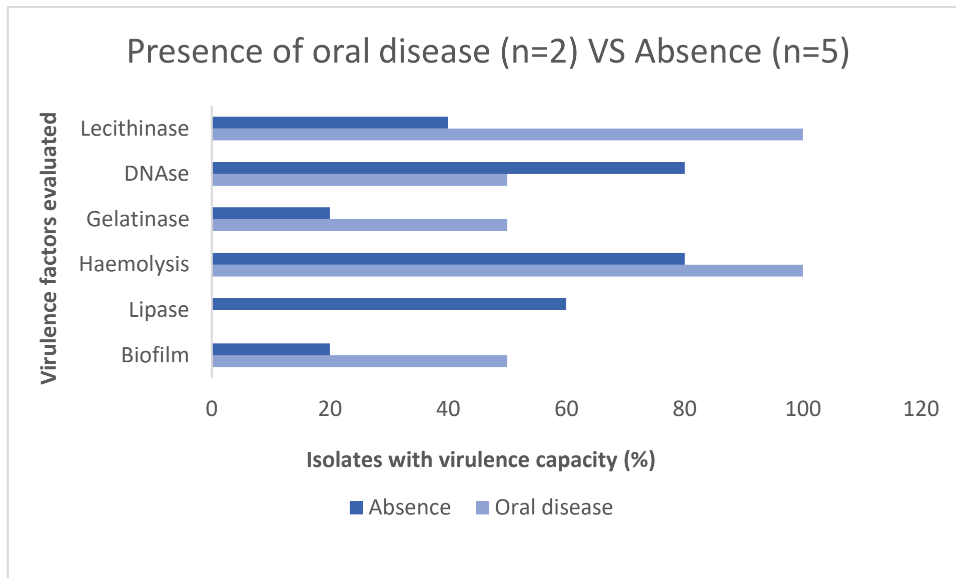
In this analysis the goal is to understand the prevalence of *Mucor* isolates with the ability to produce virulence factors in the two vulture recovery centres in which the samples were collected and if the presence of an oral disease had any influence on the virulence of the samples.

Graphic 1- Percentage of isolates with virulence capacity. CERAS vs RIAS.



In graphic 1, the percentage of isolates with virulence capacity in both rehabilitation centres (RIAS and CERAS) was analysed. Observing the graphic, it is possible to understand that the isolates retrieved from CERAS (1 and 3) seem to present a higher virulence ability regarding haemolysis and lecithinase production. The isolates from RIAS, were stronger biofilm producers.

Graphic 2- Percentage of isolates with virulence capacity considering the health status of the sampled vulture.



The samples from which isolates 1 and 2 were retrieved were obtained from vultures that had been diagnosed with an oral disease. As may be observed, these isolates seem to have a higher ability to produce virulence determinants except regarding DNase and lipase production. The most predominant virulence factor is the haemolysis and lecithinase production, as both isolates were strong producers.

4.5. Inhibitory ability

As mentioned above in Material and Methods section, this step consisted in the evaluation of the inhibitory ability of the *Mucor* isolates using a collection of potentially pathogenic bacteria from the Bacteriology Laboratory of FMV/ULisboa, as well as two yeasts species isolated from the oral cavity of the vultures.

The results of this study obtained at 24h, 48h and 72h are summarized on Table 5.

Table 5- Results of the inhibitory ability assays after 24, 48 and 72 hours respectively. The first +/- result refers to the mean between the results from three rounds at 24h, the second to the mean at 48h and the third at 72h. For the results of all rounds, consult Annex I.

	N. zoodegmatis **CCUG 52598T**	***P. aeruginosa*** **413/18**	***P. aeruginosa*** **ATCC 27853**	***P. aeruginosa*** **Z25.1**	***E. coli*** **ATCC 25922**	***E. faecium*** **CCUG 36804**	***S. aureus*** **ATCC 29213**	***S. aureus*** **Z25.2**	***Candida*** **spp. S2-1**	***Rhodotorula*** **spp. S2-2**
1	---	---	---	---	---	---	---	---	---	---
2	--+	---	---	---	+++	-+-	---	---	---	---
3	-+-	---	---	---	---	---	---	---	---	---
4	--+	---	---	---	---	-+-	---	---	---	---
5	---	---	---	---	---	---	---	---	---	---
6	---	---	---	---	---	---	---	---	---	---
7	---	---	---	---	-+-	---	---	--+	---	---

Inhibitory ability towards *N. zoodegmatis* CCUG 52598T

Regarding the inhibition ability of the *Mucor* isolates towards *N. zoodegmatis,* the extracts from all isolates did not present inhibitory potential, except for the extract of isolate 6, which showed a weak positive inhibitory activity in the second round.

Concerning the *Mucor* isolates with 48 hours of incubation, the extract of isolate 3 showed an inhibitory potential, as a positive inhibitory halo was registered in the first two assays of results. The extracts from isolates 2, 4 and 6 showed a positive result in only one of the rounds.

Analysing the results of the inhibitory activity of the extracts from the isolates with 72 hours of growth, it is possible to observe that more isolates showed an inhibitory ability. The isolates with better results were isolates 2 and 4 as a positive result was detected for these extracts in two of the rounds. Also, isolates 5 and 6 exhibited a positive result in the first assay.

Figure 21- Inhibitory ability of isolate 3 and 4 in *N. zoodegmatis* CCUG 52598T at 48 hours. Second round of results. Original.

Inhibitory ability towards *P. aeruginosa* 413/18

Regarding the power of inhibition of the *Mucor* extracts towards *P. aeruginosa* isolated from an otitis of a dog at 24 hours, all the extracts showed a negative result throughout the 3 rounds of results.

Concerning the *Mucor* isolates with 48 hours of incubation, the results were also negative except for isolate 7 that presented a positive result in the second round of results.

Lastly, for *Mucor* extracts with 72 hours of incubation, the extract of isolate 4 showed a positive result in one round of results. All the other isolates exhibited a negative result in all three rounds.

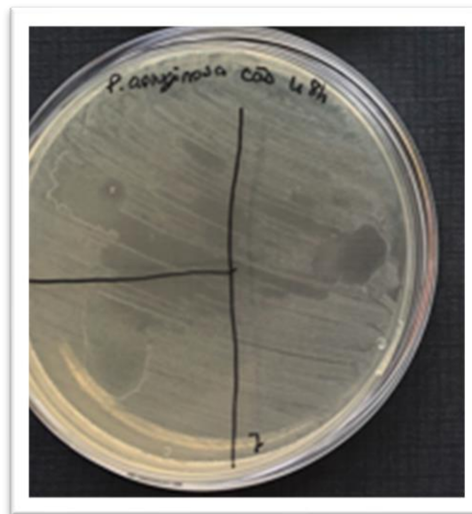


Figure 22- Inhibitory ability of isolate 7 in *P. aeruginosa* 413/18 at 48 hours. Second round of results. Original.

Inhibitory ability towards *P. aeruginosa* ATCC 27853

Considering the inhibition ability of the *Mucor* extracts on *P. aeruginosa* strain type, the only positive result obtained was for isolate 7 in one round at 24 hours of growth of the *Mucor* isolates. All the other isolates showed a negative result throughout all three rounds.

Figure 23- Inhibitory ability of isolate 4 in *P. aeruginosa* ATCC 27853 at 72 hours. First round of results. Original.

Inhibitory ability towards *P. aeruginosa* Z25.1

The results obtained from the inhibitory capacity of the extracts of *Mucor* on *P. aeruginosa* isolated from a patient diagnosed with diabetic foot, were negative for all isolates throughout all assays.

Inhibitory ability towards *E. coli* ATCC 25922

Regarding the power of inhibition of the *Mucor* extracts on *E. coli* ATCC 25922 at 24 hours of growth of the isolates showed a positive result for isolate 2 in two of the rounds as well as a positive result for isolate 3, 4, 5 and 7 in one of the rounds.

Concerning the *Mucor* isolates with 48 hours of incubation, isolate 2 still presented a positive result in the first two rounds and isolate 7 showed an inhibition ability on the 3 rounds of results. Furthermore, isolate 1, 3 and 4 also presented a positive result in only one round of results.

Finally, the *Mucor* isolates with 72 hours of growth showed again a positive result for isolate 2 in the first two rounds and isolates 5 and 6 presented a positive result only in one round.

Figure 24- Inhibitory ability of isolates 1, 2 and 4 in *E. coli* ATCC 25922 at 48 hours. Second round of results. Original.

Inhibitory ability towards *E. faecium* CCUG 36804

At 24 hours of incubation of the *Mucor* extracts, it was tested their inhibition capacity towards *E. faecium* CCUG 36804. All extracts of the isolates failed at presenting any inhibition on the *E. faecium* CCUG 36804 growth in the three rounds studied.

Regarding the extracts of *Mucor* isolates with 48 hours of incubation, extracts from isolates 2 and 4 showed signs of inhibiting *E. faecium* CCUG 36804 growth in two of the three rounds of results.

Lastly, for *Mucor* extracts with 72 hours of incubation, the results were negative for all isolates throughout all three rounds.

Figure 25- Inhibitory ability of isolates 2 and 4 in *E. faecium* CCUG 36804 at 48 hours. Second round of results. Original.

Inhibitory ability towards *S. aureus* ATCC 29213

Regarding the power of inhibition of the *Mucor* extracts towards *S. aureus* ATCC 29213, at 24 hours of incubation the results revealed the presence of inhibitory abilities for extracts of isolates 3, 4, 5 and 6 in one of the rounds of results.

At 48h of incubation of the *Mucor* extracts, the results showed repressive capacities from extract of isolate 4 in only one round.

Finally, analysing the results of the isolates with 72 hours of growth, it is possible to understand that only isolate 6 showed inhibitory abilities in the first round of results.

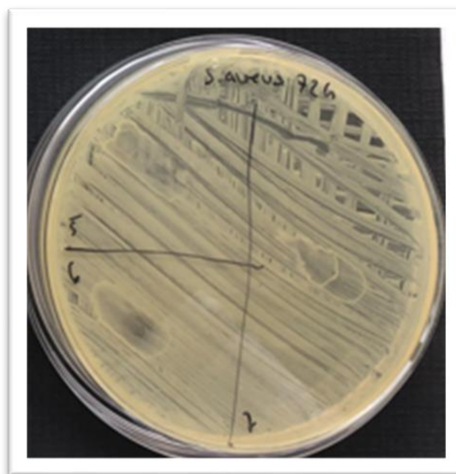


Figure 26- Inhibitory ability of isolates 6 in *S. aureus* ATCC 29213 at 48 hours. First round of results. Original.

Inhibitory ability towards *S. aureus* Z25.2

Considering the inhibition ability of the *Mucor* extracts towards this strain of *S. aureus* Z25.2*,* the results from the 24 and 48 hours of *Mucor* isolates growth showed an inability to produce any inhibitory proprieties as all isolates showed a negative result in all three rounds.

In the end, at 72 hours of growth, the *Mucor* isolates 2, 4 and 7 proved to inhibit the growth of *S. aureus.* Isolate 7 registered a positive result in two rounds whereas isolates 2 and 4 only in one.

Figure 27- Inhibitory ability of isolates 6 in *S. aureus* Z25.2 at 72 hours. First round of results. Original.

Inhibitory ability towards *Candida* spp. S2-1

The results obtained from the inhibitory capacity of the extracts of *Mucor* towards *Candida* spp. S2-1, were negative for all isolates extracts throughout all three assays.

Inhibitory ability towards *Rhodotorula* spp. S2-2

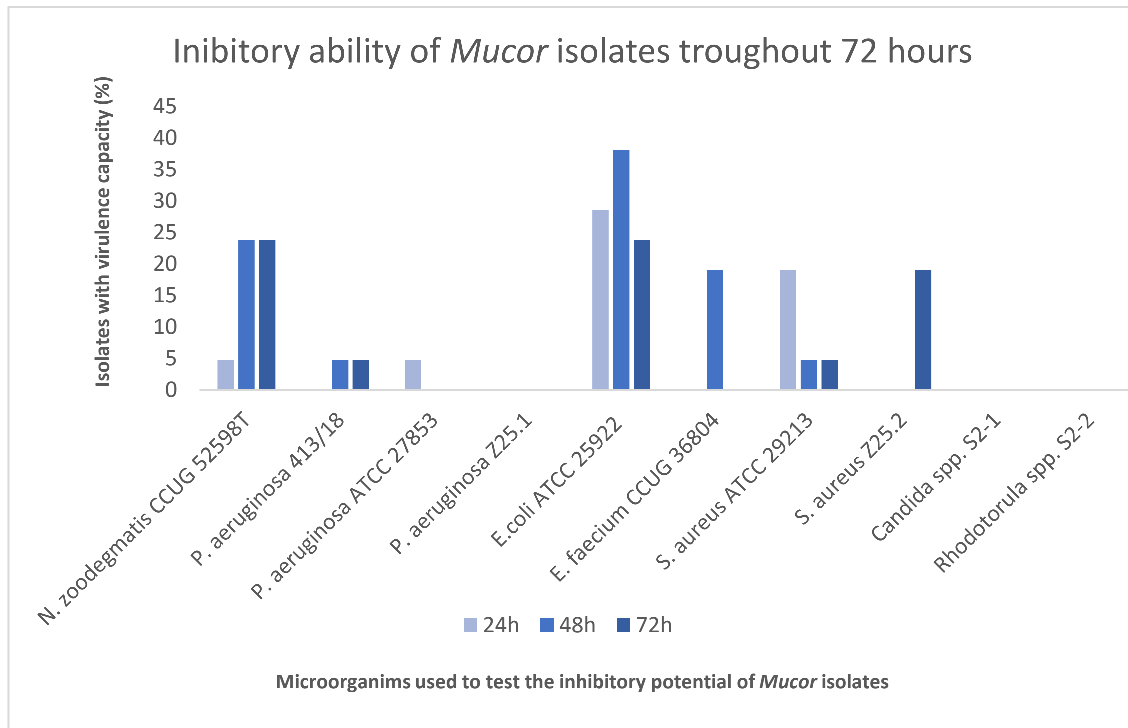
The results obtained from the inhibitory capacity of the extracts of *Mucor* towards *Rhodotorula* spp. S2-2, were negative for all isolates throughout all three assays.

Overall, we can understand 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.

4.5.1. Descriptive analysis

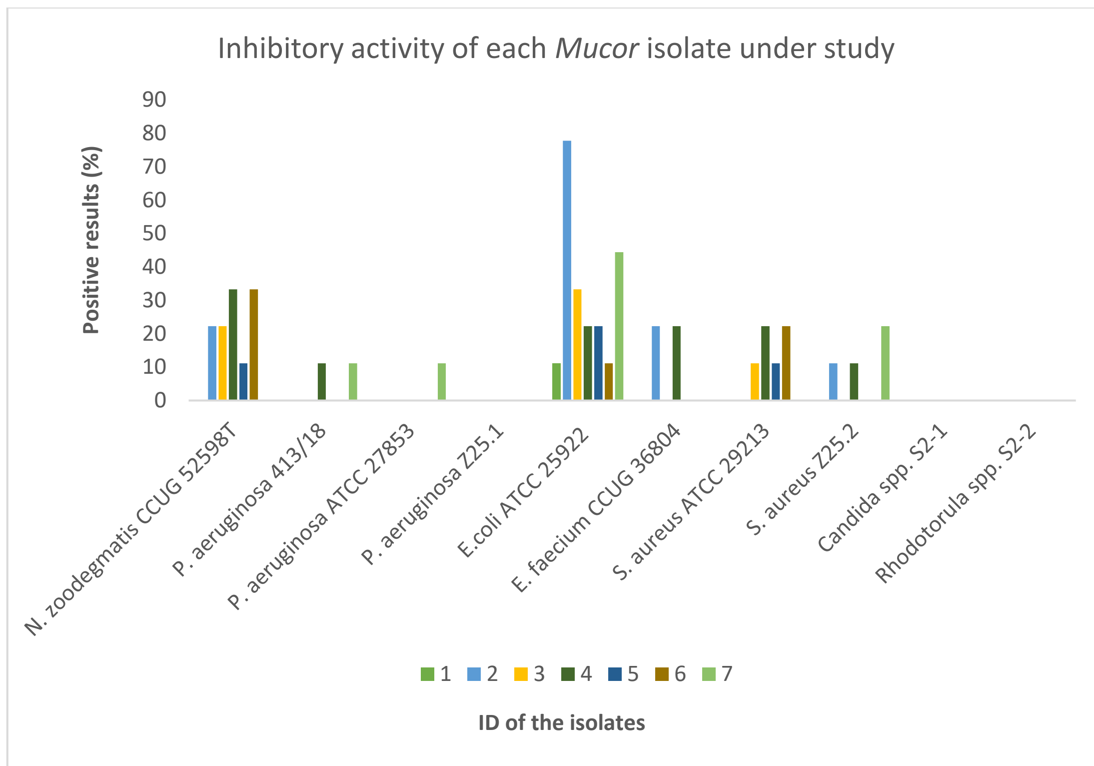
In this analysis, the inhibitory potential of *Mucor* isolates in study was evaluated having in consideration their time of incubation as well as prevalence of each isolate on the microorganisms used in this study.

Graphic 3- Percentage of *Mucor* isolates with inhibitory ability at 24, 48 and 72 hours.



Observing Graphic 3, it is possible to comprehend that 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. Again, it is possible to evaluate that both yeasts (*Candida* spp. S2-1 and *Rhodotorula* spp. S2-2) as well as *P. aeruginosa* isolated from a dog (Z25.1) were not susceptible to any isolate. Regarding the incubation period, it was observed that at 48 hours of incubation was when more isolates showed a broader spectrum of action.

Graphic 4- Inhibitory capacity of the *Mucor* isolates in the microorganisms in study.



Observing Graphic 4, it is possible to evaluate that isolate 4 produce 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 25922and *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.

4.6. 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.

In Table 6 below is represented the first step of this metagenomics analysis in which it was possible to evaluate the number of raw reads as well as the percentage of classified and unclassified reads. The percentage of bacterial and archaeal reads is also represented.

Table 6- Raw reads classification statistics in percentage and in raw read numbers and with sample information.

Animal ID	Number of raw reads	Classified Reads	Unclassified reads	Archaeal reads	Bacterial reads
2	119,631	99.92%	0.008%	0%	100%
3	217,012	99.99%	0.013%	0%	100%
4	318,729	99.99%	0.014%	0%	100%

In Table 6, the percentages of classified and unclassified reads from the total number of raw reads and the percentage of classified reads belonging to each taxa are shown per each sample. Regarding sample 2, 99.92% of the reads were classified and 100% of the reads corresponded to bacterial reads. For samples 3 and 4, 99.99% of the reads were correctly classified and, all of those reads correspond to bacterial reads.

After, it was analysed the read counts percentages per each genus on each sample. Tables 7, 8 and 9 represented the top 4 genus found on each sample. Graphic 5 represents all genus found on the three samples analysed.

Table 7- Table showing the read counts percentages per each Genus (row) on each sample (column) for sample 2.

Genus	Max	Sample 2
Clostridium	394431	57.79%
Paeniclostridium	283843	31.84%
Dysgonomonas	14487	1.084%
Yersinia	87954	1.018%

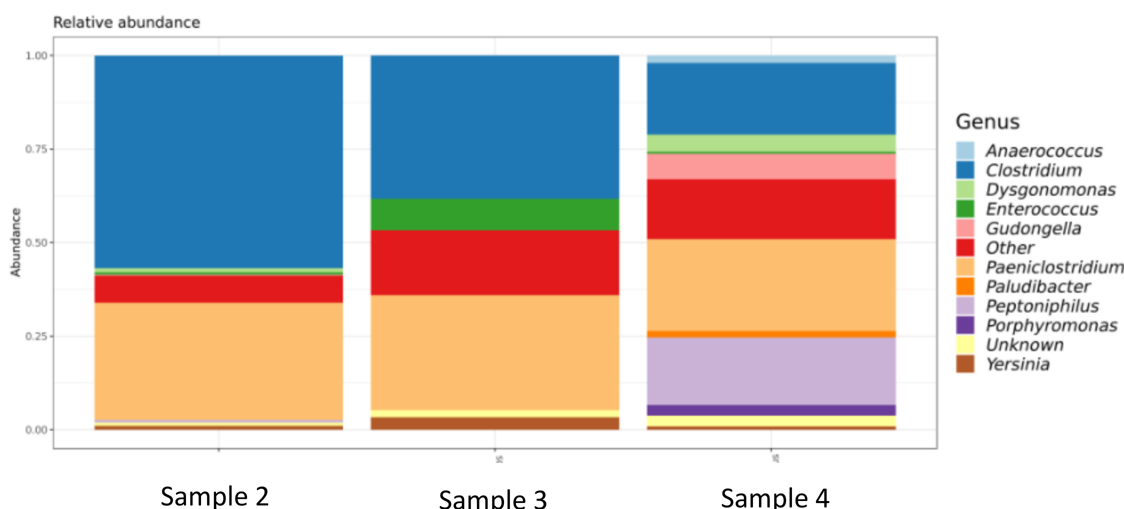
Table 8- Table showing the read counts percentages per each Genus (row) on each sample (column) for sample 3.

Genus	Max	Sample 3
Clostridium	394431	39.41%
Paeniclostridium	283843	31.63%
Enterococcus	107685	8.726%
Yersinia	87954	3.278%

Table 9- Table showing the read counts percentages per each Genus (row) on each sample (column) for sample 4.

Genus	Max	Sample 4
Paeniclostridium	283843	25.43%
Clostridium	394431	19.85%
Peptoniphilus	56989	18.67%
Gudongella	21793	7.14%

Graphic 5- Genera composition plot of samples corresponding to samples 2, 3 and 4, respectively.



Observing Table 7, 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, observing Table 8, 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 and observing Table 9, 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.

Also evaluating Graphic 5, it is possible to understand in a more schematic form what it is exhibited in Tables 7, 8 and 9. The genus most present in the bacterial reads of the 3 samples are *Clostridium,* followed by *Paeniclostridium* genus. Furthermore, *Enterococcus* and *Yersinia* genus also represent a part of the reads of sample 3 as *Peptoniphilus* represents part of the bacterial reads of the sample 4.

5. Discussion

Vultures are often exposed to infectious diseases which can affect their survival or their reintroduction in the wild as it is the case of some of the vultures from which some of the samples were collected.

This analysis of the results allows the evaluation of the pathogenic profile of oral *Mucor* species from Eurasian Griffon Vultures trough phenotypic characterization as well as the inhibitory capacities of the extracts of the *Mucor* isolates. Thus, this study focusses on the presence of the pathogenic species present in the oral cavity of vultures that may be responsible infections development leading to a better understanding of this problematic. Hence, the present work hopes to bring awareness for this endangered animal species and contribute for the development of adequate management programmes for protection of the vultures.

5.1. Phenotypical identification

Most fungi exist in the environment as saprophytes. In some cases, the fungus may grow on or in living tissues especially in an immune suppressed individual. This may provide an opportunity for colonization and infection. Regarding the virulence factors demonstrated by the *Mucor* isolates, the results obtained revealed that the strongest virulence factor observed was the haemolysis activity as 6 of the 7 samples showed a strong alpha haemolysis throughout the 72 hours of experience. Expression of a protein that can lyse a competitor's cells could help provide a survival advantage for the fungal species (Nayak, A. et al., 2013).

Lipase was expected to have a higher production as most of the isolates investigated in previous studies showed a capacity to produce lipase production. Thus, in this study 42,9% of the isolates revealed to be able to produce lipase. It was expected that more isolates showed that capacity (Nagaoka, K. et al., 1969; Coca-Armas, J. et al., 2008; Perraud, R. et al., 1995). 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) in which all *Mucor* isolates in study showed a potential of DNase production.

As it concerns the biofilm-forming capacity, several zygomycetes were studied before and revealed that some species as *Rhizopus oryzae*, *Lichtheimia corymbifera* and *Rhizomucor pusillus*, produced robust, highly intertwined, filamentous, adherent structures that were encased in an extracellular matrix. 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* (Singh A., et al., 2011).

Referring to the results of the gelatinase production, no previous studies using specifically *Mucor* spp. were found, but studies were performed in that area using other filamentous fungi as *Candida, Cryptococcus and Geomyces*, among others. In these studies, gelatinase production was one of the most frequently found enzymatic activities. In comparison, the present study did not show a high virulence capacity regarding the production of gelatinase (Souza, P., 2015).

The production of lecithinase was one of the most predominant virulence factors recorded. Although the mechanism(s) through which lecithinase modulates fungal virulence is still under investigations, early data suggest that direct host cell damage and lysis are the main mechanisms contributing to fungal virulence (Ghannoum, M., 2000). Thus, the results obtained in this study are similar to previous studies developed in several filamentous fungi (Ghannoum, M., 2000; Sharaf E. et al., 2014), which detected the production of lecithinase as one of the most predominant enzymatic activities.

Furthermore, it was evaluated if the recovering centre from which they were collected had any effect on the virulence of the isolates as well as the presence of a previously diagnosed oral disease. The results lead to believe that the isolates retrieved from CERAS were more virulent. Moreover, the presence of oral disease seems to have an influence on the virulence activity of the isolates, mostly on haemolysis activity and in the lecithinase production. The presence of thrush-like lesions was confirmed in fledglings several weeks after leaving the nests. These fledglings were generally reluctant to feed themselves in rehabilitation centres, apparently due to pain during food ingestion (Pitarch et al., 2017). Thus, the presence of an oral disease may contribute to weaken the vulture's immune system leaving them more susceptible to other infections.

The strong presence of these virulence factors on the *Mucor* isolates may suggest that the recovery of the vultures may be delayed. The presence of oral mycoses is alarming because of the delicate conservation status of several of the affected species. These fungi could opportunistically contribute as concurrent disease agents to exacerbate the impact of the lesions on host health (Vela et al., 2015).

5.2. Inhibitory ability

The *Mucor* extracts presented inhibitory ability 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. These results were expected, as previous studies already showed the antibacterial properties of *Mucor* spp (Aziz, N. et al., 2016).

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. were proved to have antifungal activity (Aziz, N. et al., 2016).

In these previous investigations, the isolates have shown more activity against Gram-negative bacteria. Studies by Rhim et al. (2006) and Huang et al. (2011) have spawned similar observations, presumably owing to the thinner cell walls of the Gram-negative strains leading to easy perforation and more rapid absorption. However, in the present study antifungal activity was not verified as *Mucor* extracts did not show any inhibitory activity towards *Candida* spp. and *Rhodotorula* spp. Moreover, it was observed a higher inhibitory activity towards Gram-negative bacteria as the previous studies mentioned above stated.

5.3. Microbiome characterization

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. The presence of *Mucor* spp. in the oral cavity of vultures may have contributed to the decreasing of the presence of bacteria, as our results indicate that the *Mucor* extracts may 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.

Thus, the presence of *Mucor* spp. on the oral cavity of the vultures is problematic as their virulence potential turns the vultures more susceptible to infections as it weakens their immune system. Additionally, there is a possibility that the presence of species of *Mucor* contributes for the disturbance of the oral microbiome of the host, possibly by eliminating competing bacteria leading to a possible invasive mycosis.

6. Conclusion

The Eurasian Griffon Vulture (*Gyps fulvus*) and Cinereous Vulture (*Aegypius monachus*) are extremely important for our ecosystem and have been suffering dramatic declines mainly due to poisoning or decreased food sources. Also, 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 leading to a small recuperation rate of the vultures in recovering centres. These opportunistic mycoses could therefore have a major impact on populations because they may promote fledgling emaciation and dehydration caused by pain during food swallowing, subsequent difficulty with eating and eventual death by starvation. In addition, these mycoses can be life-threatening if they become invasive.

The aim of this study 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. 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. Also, further testing of the inhibitory activity of *Mucor* spp. *in vivo* would ultimately lead to a better understanding of how new approaches could be taken to help the recovering of vultures in the centres.

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8. Annex I

Here it is represented the results of the 3 rounds of results of the studies on the inhibitory activity.

1st Round

Table A1- Inhibitory potential of *Mucor* isolates towards *Neisseria zoodegmatis* CCUG 52598T throughout 72h. 1st round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***NEISSERIA ZOODEGMATIS* CCUG 52598T**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	+	-
4	-	-	+
5	-	-	+
6	-	-	+
7	-	-	-

Table A2- Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* 413/18 throughout 72h. 1st round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***PSEUDOMONAS AERUGINOSA* 413/18**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	+
5	-	-	-
6	-	-	-
7	-	-	-

Table A3- Inhibitory potential of *Mucor* isolates towards *Enterococcus faecium* CCUG 36804 throughout 72h. 1st round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***ENTEROCOCCUS FAECIUM* CCUG 36804**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A4- Inhibitory potential of *Mucor* isolates towards *Escherichia coli* ATCC 25922 throughout 72h. 1st round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***ESCHERICHIA COLI* ATCC 25922**	**24H**	**48H**	**72H**
1	-	-	-
2	+	+	+
3	-	-	+
4	-	-	-
5	+	-	-
6	-	-	+
7	-	+	-

Table A5- Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* ATCC 29213 throughout 72h. 1st round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***STAPHYLOCOCCUS AUREUS* ATCC 29213**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	+	-	-
6	+	-	+
7	-	-	-

Table A6- Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* Z25.2 throughout 72h. 1st round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***STAPHYLOCOCCUS AUREUS* Z25.2**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	+

Table A7- Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* ATCC 27853 throughout 72h. 1st round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***PSEUDOMONAS AERUGINOSA* ATCC 27853**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-

4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A8- Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* Z25.1 throughout 72h. 1st round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***PSEUDOMONAS AERUGINOSA* Z25.1**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A9- Inhibitory potential of *Mucor* isolates towards *Candida* spp. S2-1 throughout 72h. 1st round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***CANDIDA* SPP. S2-1**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A10- Inhibitory potential of *Mucor* isolates towards *Rhodotorula* spp. S2-2 throughout 72h. 1st round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***RHODOTORULA* SPP. S2-2**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

2nd Round

Table A11- Inhibitory potential of *Mucor* isolates towards *Neisseria zoodegmatis* CCUG 52598T throughout 72h. 2nd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***NEISSERIA ZOODEGMATIS* CCUG 52598T**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	+-	-
4	-	+-	-
5	-	-	-
6	+ -	+-	-
7	-	-	-

Table A12- Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* 413/18 throughout 72h. 2nd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***PSEUDOMONAS AERUGINOSA* 413/18**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	+-	-

Table A13- Inhibitory potential of *Mucor* isolates towards *Enterococcus faecium* CCUG 36804 throughout 72h. 2nd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***ENTEROCOCCUS FAECIUM* CCUG 36804**	**24H**	**48H**	**72H**
1	-	-	-
2	-	+-	-
3	-	-	-
4	-	+-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A14- Inhibitory potential of *Mucor* isolates towards *Escherichia coli* ATCC 25922 throughout 72h. 2nd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***ESCHERICHIA COLI* ATCC 25922**	**24H**	**48H**	**72H**
1	-	+-	-
2	+-	+-	+
3	-	-	-
4	-	+-	-
5	-	-	+-
6	-	-	-
7	-	+	-

Table A15- Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* ATCC 29213 throughout 72h. 2nd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

STAPHYLOCOCCUS AUREUS ATCC 29213	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A16- Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* Z25.2 throughout 72h. 2nd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***STAPHYLOCOCCUS AUREUS* Z25.2**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A17- Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* ATCC 27853 throughout 72h. 2nd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***PSEUDOMONAS AERUGINOSA* ATCC 27853**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	+	-	-

Table A18- Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* Z25.1 throughout 72h. 2nd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***PSEUDOMONAS AERUGINOSA* Z25.1**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A19- Inhibitory potential of *Mucor* isolates towards *Candida* spp. S2-1 throughout 72h. 2nd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***CANDIDA* SPP. S2-1**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A20- Inhibitory potential of *Mucor* isolates towards *Rhodotorula* spp. S2-2 throughout 72h. 2nd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***RHODOTORULA* SPP. S2-2**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-

7	-	-	-

3rd Round

Table A21- Inhibitory potential of *Mucor* isolates towards *Neisseria zoodegmatis* CCUG 52598T throughout 72h. 3rd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***NEISSERIA ZOODEGMATIS* CCUG 52598T**	**24H**	**48H**	**72H**
1	-	-	-
2	-	+	+
3	-	-	-
4	-	-	+
5	-	-	-
6	-	-	-
7	-	-	-

Table A22- Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* 413/18 throughout 72h. 3rd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***PSEUDOMONAS AERUGINOSA* 413/18**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A23- Inhibitory potential of *Mucor* isolates towards *Enterococcus faecium* CCUG 36804 throughout 72h. 3rd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***ENTEROCOCCUS FAECIUM* CCUG 36804**	**24H**	**48H**	**72H**
1	-	-	-
2	-	+	-
3	-	-	-
4	-	+	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A24- Inhibitory potential of *Mucor* isolates towards *Escherichia coli* ATCC 25922 throughout 72h. 3rd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***ESCHERICHIA COLI* ATCC 25922**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	+	+	-
4	+	-	-
5	-	-	-
6	-	-	-
7	+	+	-

Table A25- Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* ATCC 29213 throughout 72h. 3rd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***STAPHYLOCOCCUS AUREUS* ATCC 29213**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	+-	-	-
4	+-	+	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A26- Inhibitory potential of *Mucor* isolates towards *Staphylococcus aureus* Z25.2 throughout 72h. 3rd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***STAPHYLOCOCCUS AUREUS* Z25.2**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	+-
3	-	-	-
4	-	-	+-
5	-	-	-
6	-	-	-
7	-	-	+-

Table A27- Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* ATCC 27853 throughout 72h. 3rd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***PSEUDOMONAS AERUGINOSA* ATCC 27853**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A28- Inhibitory potential of *Mucor* isolates towards *Pseudomonas aeruginosa* Z25.1 throughout 72h. 3rd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***PSEUDOMONAS AERUGINOSA* Z25.1**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A29- Inhibitory potential of *Mucor* isolates towards *Candida* spp. S2-1 throughout 72h. 3rd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***CANDIDA* SPP. S2-1**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-

Table A30- Inhibitory potential of *Mucor* isolates towards *Rhodotorula* spp. S2-2 throughout 72h. 3rd round. + indicates a positive result; - indicates a negative result; +- indicates a weak positive result.

***RHODOTORULA* SPP. S2-2**	**24H**	**48H**	**72H**
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	-	-
7	-	-	-