University of Trás-os-Montes and Alto Douro

The impact of the topical exposure to *Cytinus hypocistis* extract on fungi of medical relevancy and in *Staphylococcus aureus* as a potential alternative treatment for bovine mastitis

Masters in Zootechnical Engineering

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Abstract

Bovine mastitis is defined as an intramammary inflammation. It can be caused by several factors like physical injuries but usually, the cause is infectious, which is most commonly bacterial in origin. It affects not only the milk production and quality but also the animal's welfare. Bovine mastitis is seen as one of the most financially damaging diseases in the dairy sector. The treatment employs the use of antibiotics however, the efficiency of these drugs has been decreasing due to the emergence of antibiotic-resistant species. Therefore, there is a strong demand for alternative treatments for bovine mastitis.

Cytinus hypocistis (L.) L. is a holoparasitic plant occurring in the Mediterranean region. Its tannin-rich extract has been found to have multiple beneficial properties, accredited to the presence of gallotannins.

The main objective of the present work was to study the effect of a *C. hypocistis* enriched topical formulation (EF) in mice's ears microbial isolates using routine mycological techniques, to access the potential of this extract as an alternative treatment for bovine mastitis.

For this study, three EF were prepared with different concentrations of the *C. hypocistis* extract: CH1, 3.1 mg of extract/g of cream; CH2, 6.2 mg /g; and CH3, 12.4 mg/g. Thirty samples were collected from each female mice's outer ears (strain FBV/n) and inoculated in Potato Dextrose Agar (PDA). The experiment procedure was approved by an Animal Ethics Committee-DGAV number 0421/000/000/2014, 24/09/2014 (020172). The bacterial and fungal species isolated were identified using API test and microscopy with Lactophenol with Cotton Blue staining, respectively. An antibiogram and identification of virulence factors were also performed on the bacterial species. To access the specific antifungal effect of the extract, the inhibition percentage (IP%) method was calculated using the growth of *Aspergillus fumigatus*. The statistical analysis was performed using the program SPSS® version 25.0. The *t* Student test with $p<0.05$ of significance was used to analyse the differences between the mean IP% for each extract concentration.

Four fungal genera were isolated: *Penicillium* (16.7%), *Mucor* (16.7%), *Cladosporium* (3.33%), *Aspergillus* (3.33%). In total, fungi were isolated in 14 (46.7%) animals. After the treatment, only one fungal colony was isolated, and the genus was not identified.

The bacterial colonies were identified as *Staphylococcus aureus* for both the collection instances. These showed resistance to penicillium G and ceftazidime, and presented the genes for *Staphylococcus* enterotoxins G and I, hemolysin-α and toxic shock syndrome toxin-1.

The higher IP% was for the concentration of 0.115 mg/mL on the third day after inoculation, being higher than 67%. The IP% for this concentration were always higher compared to the ones for the concentration of 0.230 mg/mL, on each day of assessment. However, the differences between the means of the IP% for both the concentrations were found to be not significant ($t=0.232$; $p=0.828$).

The results obtained suggest a high fungi biodiversity in the mice's ears and that the extract inhibits the growth of *A. fumigatus* in vitro. However, no assumptions could be made about the *in vivo* effects of this extract or about the potential to treatment mastitis.

Resume

A mastite bovina é definida como uma inflamação intramamária. Pode ser causada por vários fatores como lesões físicas, mas dentre estes a infeção bacteriana é o mais comum. Esta doença tem repercussões não só no leite, mas também no bem-estar do animal. Esta é das doenças que provocam mais perdas económicas no setor leiteiro. Geralmente, é tratada utilizando antibióticos, mas a sua eficácia tem vindo a diminuir devido ao emergente aparecimento de cada vez mais resistentes. Então existe um crescente interesse em formas alternativas de tratamento para a mastite bovina.

A planta *Cytinus hypocistis* (L.) L. presente em várias regiões Mediterrâneas apresenta múltiplas propriedades de interesse como atividade antimicrobiana devido ao seu elevando teor em taninos, especificamente gallotaninos.

Assim o principal objetivo deste trabalho foi analisar o efeito que uma formulação tópica enriquecida com extrato de *C. hypocistis* (EF) tem na microbiota das orelhas de murganho de modo a avaliar o potencial deste extrato como tratamento alternativo para a mastite.

Para isto foram utilizadas 3 EF com concentrações diferentes de extrato: CH1, 3.1 mg de extrato/g de creme; CH2 6.2 mg/g; e CH3, 12.4 mg/g. trinta amostras foram recolhidas dos pavilhões auriculares de 30 murganhos fêmea FBV/n, e inoculadas em meio Potato Dextrose Agar (PDA). O procedimento experimental foi aprovado pela Comissão de Ética Animal-DGAV number 0421/000/000/2014, 24/09/2014 (020172).

As espécies bacterianas e fúngicas foram isoladas e identificadas utilizando testes API e microscopia com lactofenol com azul de algodão, respetivamente. Adicionalmente, furam realizados um antibiograma e a deteção de fatores de virulência da bactéria.

Para avaliar o potencial antifúngico do extrato, foram ainda determinadas as percentagens de inibição (IP%) do extrato no crescimento de *Aspergillus fumigatus*. A analise estatística utilizou o programa SPSS® versão 25.0 e um teste *t* Student com $p<0,05$ de significância foi utlizado para avaliar a diferença entre as percentagens de inibição médias para as duas concentrações testadas.

Furam identificados 4 géneros fúngicos: *Penicillium* (16.7%), *Mucor* (16.7%), *Cladosporium* (3.33%), *Aspergillus* (3.33%). No total, 14 (46.7%) dos animais apresentaram fungos nas suas amostras. Após o tratamento, apenas uma amostra apresentou a presença de fungos, este não foi identificado. As colónias bacterianas de *Staphylococcus aureus* de ambos os períodos de

amostragem, mostraram-se resistentes a penicilina G e ceftazidime, e apresentaram os genes para as *Staphylococcus* enterotoxinas G e I, para a hemolisina-α e para a toxina da síndrome de choque toxico-1.

A concentração de 0.115 mg/mL apresentou a maior IP% (67%) 3 dias após a inoculação. As IP% para esta concentração registadas foram sempre maiores ao longo do procedimento comparativamente a concentração de 0.230 mg/mL. Mas, a diferença entre as médias de IP% para as duas concentrações de estrato não foi significativa (t=0.232; p=0.828).

Estes resultados sugerem a existência de uma grande biodiversidade de microrganismos nas no pavilhão auricular do murganho e que a exposição ao extrato de levou a inibição do crescimento de *A. fumigatus*. No entanto, não foi possível formular qualquer suposição acerca do efeito deste extrato *in vivo* e no tratamento de mastite bovino.

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List of Abbreviations

SCC – Somatic cell count

CM – Clinical mastitis

SCM – Subclinical mastitis

S. aureus – *Staphylococcus aureus*

S. agalactiae – Streptococcus agalactiae

S. uberis – *Streptococcus uberis*

E. coli – *Escherichia coli*

TSST-1 – Toxic shock syndrome toxin-1

SE – Staphylococcal enterotoxins

HLA – Hemofusin-α

CMT – California mastitis test

P. – *Prototheca*

PCR - Polymerase chain reaction

C. hypocistis – *Cytinus hypocistis*

C. ruber – *Cytinus ruber*

EF - *C. hypocistis* enriched topical formulation

HPV16 - Human papillomavirus 16

CH1 – EF with 3.1 mg of extract per g of cream

CH2 – EF with 6.2 mg/g of extract per g of cream

CH3 – EF with 12.4 mg/g of extract per g of cream

PDA – Potato dextrose agar

BHI - Brain Heart Infusion broth

CAN - Columbia naladixic acid agar

MIC – Minimal inhibitory concentration

NUC – Thermonuclease

S1 – *S. aureus* sample from the first collection instance

S2 – *S. aureus* sample from the first collection instance

M – Molecular weight marker

C- – Negative control

API – Analytical profile index

IP% - Inhibition percentage

S. xylosus – *Staphylococcus xylosus*

1. Introduction

The following work was done to assess the potential of a *Cytinus hypocistis* extract as an alternative treatment for bovine mastitis *in vivo*, as this disease is usually infectious in origin and the antimicrobial properties of this extract *in vitro* have been well documented by multiple authors. These was achieved by comparing the microbial populations present in the mouse's outer ear before and after applying a topical formulation enriched with this extract.

1.1. Bovine mastitis

Bovine mastitis is defined as the inflammation of the mammary gland, otherwise known as intramammary inflammation. Nowadays, it is recognized as one of the most prevalent and financially damaging diseases in the dairy sector worldwide. Having a major impact not only on the economic losses of dairy farms but also on the welfare of the animals (Ashraf and Imran, 2020; Rasheed et al., 2020).

Several published studies estimated the costs implicated with a single case of mastitis in order to better understand the effects of this disease, however, due to the complexity of this issue, the estimated values tend to vary. As multiple factors can be taken into account and these are also affected by regional differences (Halasa et al., 2007; Martins et al., 2019). Aghamohammadi et al. (2018) questioned 374 dairy producers to determine the mastitis-associated costs and the estimated value was 662 Canadian dollars for a milking cow per year for a standard Canadian dairy farm (Aghamohammadi et al., 2018). The costs are most often associated with the prevention, diagnosis and treatment of this disease as well as, the production losses, early culling and lower milk quality (Halasa et al., 2007). From which, the lower milk prices due to the decrease in quality, the reduction in production yields, the discarded milk and the premature culling are usually appointed as the most financially damaging aspects (Martins et al., 2019; Azooz et al., 2020).

A cow infected with mastitis presents a milk production decrease of 30% per quartile, which can suggest a decrease of 15% in milk production per dairy cow in a single lactation (Abutarbush, 2010). Another study estimated that the loss can be of 1.0 to 2.5 kg of milk on the first lactation after the onset of mastitis, with a total loss of 110 to 552 kg for the complete lactation, depending on the parity and time of occurrence (Sharun et al., 2021).

Mastitic milk cannot be used for human consumption as it suffered alterations to its chemical and physical properties that can be harmful to human health (Martins et al., 2019; Rasheed et al., 2020). This milk contains a higher somatic cell count (SCC) and total bacterial cell count, parameters taken in count upon milk commercialization, which contribute to the decrease of its commercial value or its discard if out of the legal limits. The bacterial cell count is particularly dangerous as these pathogens can induce diseases like food poisoning caused by the ingestion of some strains of *Staphylococcus aureus* (*S. aureus*). Although, thermal treatments of milk, like pasteurization, eliminate most of these pathogens, drinking raw milk and products produced using raw milk are more susceptible to be contaminated (Hameed et al., 2007). Furthermore, this milk is not suitable for cheese, butter or yoghurt production as its components negatively affect the manufacturing process and shelf time of these products (Martins et al., 2019). Moreover, mastitic milk can contain antibiotic residues if it is used to treat the animal, these residues can cause allergic reactions to sensitive individuals and even contribute to the emergence of resistant strains of bacteria, hence this milk must be decanted properly (Sharun et al., 2021).

In some cases the decrease in milk production by the cow can even be permanent depending on the severity of the case and damage caused to the mammary glands, this can lead to premature culling of the animal (Halasa et al., 2007). Having this in mind, mastitis also has a large impact on the welfare of the animal, as it causes pain and discomfort to the cow, and can even lead to its early death.

Other aspects to consider regarding the financial impact of mastitis are the additional veterinary services, labour and materials to treat and manage the herd. Proper management is essential for stopping the spread of the disease in the dairy farm, as infected animals can act like vectors if a contagious pathogen is the source of the infection. Recent studies emphasize that the type and species of the mastitis-causing pathogen can also have an impact on the costs related to this disease (Ashraf and Imran, 2020).

The rise in antibiotic-resistant microorganisms also stresses the interest in these disease as the use of antibiotics is the primary method of treatment for mastitis and the misuse and overuse of antibiotics in human and veterinary medicine are appointed as one of the main catalysts for the appearance of these resistant strains. There have been many reports of resistant bacterial strains isolated from animal infections, including mastitis cases in various species. Therefore, the treatment of mastitis cases became a public health issue as these microorganism can infect not only animal, but also human (Krömker and Leimbach, 2017).

Regardless of the vast research done through the years to understand, prevent and treat mastitis, it still prevails as one the most financially damaging diseases in the dairy sector and the one with the most zoonotic potential in the world (Rasheed et al., 2020). Having this in mind, the study of alternative prevention and treatment methods for bovine mastitis continues to be a very relevant research subject nowadays.

1.2. Classification

Mastitis can be classified as clinical (CM) or subclinical (SCM) based on the level of inflammation diagnosed. Some authors also mention the denomination, chronic mastitis, in order to emphasize the differences between sudden onsets of the disease that appear in irregular intervals, corresponding to CM and SCM, and cases where the infection remains active for a longer period, sometimes several months (Cheng and Han, 2020).

1.2.1. Subclinical mastitis

In SCM visible clinical signs aren't present, neither in the animals' clinical evaluation nor in the milk observation, which hinders the detection of the disease (Cobirka et al., 2020). However, there is an alteration in the milk's chemical composition that can aid the uncovering of the SCM cases, for example, enzyme activity (an increase of lactate dehydrogenase activity), electrical conductivity, inflammatory cytokines, SCC and bacterial population, as well as the decrease of milk production (Khairullah et al., 2020). The SCC of a healthy animal must be under 200 000 cells per mL of milk, any amount above should be investigated as it can be a case of intramammary inflammation, an SCC of 400 000 cells/mL is normally identified as a mastitis case (Cobirka et al., 2020). SCM is the most prevalent form of mastitis found in dairy farms and persists for a longer period compared to CM, making it responsible for most of the financial losses (Sharun et al., 2021).

1.2.2. Clinical mastitis

On the other hand, CM can have a large impact on the animals' health and milk appearance. Some authors further categorize these cases according to the severity of the symptoms, as per-acute, acute and subacute (Ashraf and Imran, 2020), or as mild and severe CM. The symptoms

regarding the milk characteristics include a waterier consistency, presence of flakes and/or clots due to pathogenic enzymes (coagulases) and a rise of SCC (at a larger extent than in SCM) (Cobirka et al., 2020; Khairullah et al., 2020; Rasheed et al., 2020). Cows with CM commonly present swelling and redness of the udder, fever, pain, anorexia, abnormal udder secretions, appear lethargic and have a decrease in milk production. Herds with higher levels of production tend to present a larger incidence of CM, although these usually have proper management (Khairullah et al., 2020).

1.3. Etiology

Mastitis is most commonly the result of an interaction between multiple factors regarding pathogens, the animal and its environment (Benić et al., 2018; Kalińska et al., 2018). Therefore, the degree and severity of the inflammation depend on the causing agents, host factors like lactation stage, age, health and genetics, and environmental conditions such as cleanliness, humidity, temperature, and inadequate disinfection and use of milking equipment (Ashraf and Imran, 2020).

This disease is, most frequently, infectious in origin. However, it can also be caused by physical injuries, chemical irritation, or even thermal trauma (Benić et al., 2018; Kalińska et al., 2018; Ashraf and Imran, 2020). Physical injuries can damage the natural barriers of the teat, enabling the entry of pathogens into the mammary gland. In the teat canal, the teat orifice is made up of a smooth-muscle sphincter, that controls the release of milk as well as, prevents the invasion of pathogens. Moreover, the teat lining produces keratin, which has bacteriostatic properties and also serves as a physical barrier for unwanted microorganisms (Ashraf and Imran, 2020). The diameter of the teat orifice is also a factor to have in mind, as breeder selection takes into account milking characteristics like faster milk release, which is promoted by a wider teat canal yet, this also potentiates spontaneous milk leakage and the occurrence of mastitis (Benić et al., 2018). Physical lesions also stimulate the animal's immune system, resulting in intramammary inflammation.

Concerning pathogens responsible for mastitis, bacteria are the most common and vastest group, but various species of fungi, moulds, yeasts, algae and viruses have also been isolated from bovine mastitis cases (Sztachańska et al., 2016). These microorganisms that can be found in the animals' natural skin microbiota or its pathogen filled environment, invade the teat and reach the mammary gland where they use the nutrient-rich milk as a substrate in order to

multiply and colonize, releasing toxins that can damage the mammary epithelium (Khairullah et al., 2020). The immune response induced by the organism results in cytokines and proteases released from the blood strim into the mammary gland as well as, lysosomal enzymes and oxidative products by the macrophagocytes for pathogens phagocytosis, which can also damage the mammary cells (Kalińska et al., 2018). If the damage to the epithelium is severe it can led to permanent reduction in the yield of milk produced (Lange-Consiglio et al., 2019).

1.4. Pathogens

In terms of the origin of the pathogens and method of transmission, mastitis-causing pathogens can be divided into either contagious or environmental (Cobirka et al., 2020; Khairullah et al., 2020). Although some microorganisms' classification is ambiguous because of the multiple pathways they can use to infect the host (Klaas and Zadoks, 2018).

1.4.1. Contagious pathogens

Contagious pathogens are transmitted from cow to cow, and most can be found in the cow's udder skin and quartile lesions (Khairullah et al., 2020). However, the rectal, rumen and genital areas are where they can be found in larger quantities (Cobirka et al., 2020). The infection is frequently transmitted through the contact of a healthy teat with bacterial reservoirs during milking, such as used towels, unclean hands of the technician or milking equipment (Sharun et al., 2021).

Better management of infected animals such as reducing the contact of these with the rest of the herd, appropriate culling and the adequate treatment of dry cows, as well as, proper maintenance of milking equipment and milking protocol is very important for the control and prevention of contagious mastitis in every dairy farm (Cheng and Han, 2020).

1.4.2. Environmental pathogens

On the other hand, environmental pathogens originate from the animal's surroundings and, hence, are not normally found in the flora of the cow's udder. Instead, are in the soil, bedding, stationary water and, most frequently, faeces (Sharun et al., 2021). Therefore, conditions like

excessive humidity, mud and manure can potentiate infection (Khairullah et al., 2020). After milking, the teat canal remains open for up to two hours, during which the teat can come in contact with multiple environmental pathogens (Cobirka et al., 2020).

Some of these microorganisms can also be described as opportunistic, as the infection usually occurs when the integrity of the teat lining is compromised or the cow's immune system is weakened (Cheng and Han, 2020). This classification can also be considered as a separate category of pathogens, as it can be used to describe some contagious microorganisms as well (Nonnemann et al., 2019).

In these cases, the containment measures for contagious pathogens are not effective, rather it is best to employ procedures to boost udder and animal health, reduce the exposure to environmental pathogens and overall better management in the farm, which tend to have better results (Cheng and Han, 2020).

1.4.3. Bacteria

As was previously mentioned, bacterial pathogens are the most frequent causes of intramammary inflammation or mastitis. Krishnamoorthy et al. (2021) estimated the prevalence of mastitis pathogens in cattle worldwide by meta-analyses of reports from the years 1979 to 2019. *Staphylococcus* spp. was the most prevalent pathogen with 28%, followed by *Streptococcus* spp. (12%) and *Escherichia coli* (11%). This was also demonstrated by the number of countries where *Staphylococcus* spp. had a higher prevalence, which were 41 compared to the 6 and 2 for *Streptococcus* spp. and *Escherichia coli*, respectively. Furthermore, *Staphylococcus* spp. were found to cause mostly SCM cases whereas, *E. coli* most frequently caused CM cases, and *Streptococcus* spp. was isolated from both cases (Krishnamoorthy et al., 2021).

The most common contagious pathogens found in mastitis cases are *S. aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Mycoplasma bovis*, although the latter is less frequent (Nonnemann et al., 2019). However, some authors also consider *S. aureus* and *S. agalactiae* as environmental pathogens due to the many ways they can be transmitted. Although these are present in the natural microflora of the udder, which corresponds to the contagious classification, they can also be found and transmitted via the bedding, urine and faeces or other contaminants present in the animal's surroundings (Klaas and Zadoks, 2018). As for

opportunistic pathogens, coagulase-negative *Staphylococcus* is also a prevalent cause of mastitis (Krishnamoorthy et al., 2021).

Furthermore, bacteria species like *E. coli*, *Klebsiella pneumoniae*, *Trueperella pyogenes*, *Enterobacter aerogenes*, *Streptococcus uberis* and other bacteria from the genus *Streptococcus* are the most commonly found environmental mastitis-causing pathogens (Nonnemann et al., 2019; Cobirka et al., 2020; Khairullah et al., 2020). However, species like *Enterococcus* spp., *Bacillus* spp., *Lactococcus* spp. *Corynebacterium* spp., *Pasteurella multocida*, *Pseudomonas* spp. and multiple Enterobacteriaceae, can also be the cause of mastitis in cattle (Nonnemann et al., 2019).

It is important to note, that the frequency in which one of these pathogens appear can be, impacted by many factors as are examples, the country or region where the farm is located (Gao et al., 2017). For example, Latin America seems to have a higher prevalence of *Streptococcus* spp. whereas, in Oceania, *E. coli* seems to be the most prevalent mastitis pathogen (Krishnamoorthy et al., 2021). Climatic conditions can also influence the prevalence of the pathogens, such as humidity, temperature, and season. Studies in the dairy herds of China revealed that the incidence of CM is higher in summer, with coliforms as the most frequently isolated pathogen. In opposition, in winter the incidence of CM cases seems to be lower and most cases were caused by *Streptococcus* spp. (Gao et al., 2017). The direct surrounding of the animal is also impactful, as it can aid the proliferation of pathogens. The type of bedding used, for example, when organic material is used, it potentiates the growth of pathogens, especially when manure and urine are also present. Therefore, the standard material for bedding tends to be sand or another inorganic material, as it exposes animals to a lower bacterial count and reduced the incidence of mastitis by environmental pathogens (Gao et al., 2017).

1.4.3.1. Staphylococcus aureus

From all the bacteria species, *S. aureus* is the most prevalent mastitis cause worldwide, isolated mostly from SCM cases with a prevalence of 25%. Studies also show that in recent years, there has been a rise in the prevalence of this pathogen in Africa, North America and Oceania, whereas in Europe, Asia and Latin America it seemed to decrease (Krishnamoorthy et al., 2021).

S. aureus is a Gram-positive bacterium with capsule but no motility or spore formation yet presents catalase and coagulase activity (Pérez et al., 2020). The many strains of *S. aureus* that

have been retrieved from animals with mastitis have different genotypes and phenotypes that can have an impact on the severity and persistence of the intramammary inflammation as these affect the production of exoproteins and the formation of biofilms that induce the animal's immune response (Veh et al., 2015).

Biofilms can lessen the antibody opsonization and phagocytosis as well as, reduce the susceptibility to antibiotics by bacteria within, which could explain the persistence of some cases of *S. aureus* mastitis (Veh et al., 2015). There are various exoproteins potentially toxic to humans and animals produced by *S. aureus*, among which enterotoxins and the toxic shock syndrome toxin-1 (TSST-1), who are specifically known as superantigens for their ability to excessively activate T-cells and thus, the release of a large number of cytokines leading to the potentially lethal, toxic shock syndrome (Otto, 2014; Benkerroum, 2018). Staphylococcal enterotoxins (SE) are specially recognized for causing intoxication or Food Poisoning by dairy products, due to their action on intestinal cells that incite enteritis with emesis, clinical signs like diarrhoea and others, typically associated with enteritis aren't common in Food Poisoning by SE (Benkerroum, 2018). Furthermore, hemolysin-α or α-toxin (HLA), produced by *S. aureus* can provoke the cellular lysis of erythrocytes and leucocytes except for neutrophils (Grumann et al., 2014).

The links between these properties and the severity/persistence of intramammary inflammations aren't yet well known however, research found that strains that persisted during the dry period produced significantly more biofilms *in vitro* compared to the strains that weren't persistent (Veh et al., 2015).

1.4.4. Fungi

Mycotic mastitis in bovines is usually the result of a yeast infection still, filamentous fungi have also been isolated, like *Aspergillus fumigatus* (Bakr et al., 2015; Seyedmousavi et al., 2018). *Cryptococcus*, *Rhodotorula* and *Trichosporum* yeasts, all have been reported to cause mastitis in cattle however, the most common yeasts found are from the *Candida* genera. Some *Candida* species isolated from mastitic milk are *Candida krusei*, *Candida rugosa*, *Candida kefyr*, *Candida albicans*, and *Candida. tropicalis* (ErbaŞ et al., 2017).

In recent years, the prevalence of mastitis cases caused by fungi has increased and, therefore, the interest in these microorganisms has risen (Zhou et al., 2013; Seyedmousavi et al., 2018).

The infection by fungi mostly happens due to the prolonged administration of antibiotics and poor hygienic conditions of the barns, milking area and equipment, as these pathogens tend to act opportunistically (Sztachańska et al., 2016). In China, a survey was made to study the occurrence of mycotic mastitis in cattle from the Heilongjiang Province, which determined that extensive production systems presented a higher percentage of infected milk as this type of systems frequently had less hygienic practices during milking and cleanliness of the equipment (Zhou et al., 2013).

1.4.5. Others

From the numerous microorganism that can cause mastitis in bovines, a species has been recently in focus, *Prototheca* is a genus of yeast-like algae that switched their photosynthetic aptitudes for parasitism, they can be found abundantly in the environment and animal´s intestine. The species most frequently isolated from mastitis infection are *P. zopfii* and *P. blaschkeae* (Kano, 2020).

Protothecal mastitis as long been reposted globally and its prevalence has been increasing for the past two decades, it is still understudied as *Prototheca*'s pathogenic potential is underestimated. Studies reveal that *Prototheca* spp. was the third most common mastitis-causing pathogen in Poland, following streptococci and staphylococci (Jagielski et al., 2019).

1.5. Detection methods

There are a variety of methods for mastitis detection. As previously mentioned, SCM are easily overlocked as neither the animal nor the milk shows visible signs of the disease. However, the milk can be quickly analysed to discover the infection.

The California Mastitis Test (CMT) is one of the most common methods used, as it provides rapid and accurate results and can be easily performed by the producer on-site for a low price. A reagent is added to a milk sample from each teat which results in the lysis of the cells' membranes causing its precipitation resulting in the change of viscosity of the mixture. The level of viscosity increases with the concentration of somatic cells present, in other words, a higher SCC will create a more viscous mixture. However, it doesn't provide any information regarding the pathogen at fault for the infection (Adkins and Middleton, 2018).

In order to identify the pathogen, it is necessary to perform cultures or Real-Time Multiplex Polymerase Chain Reaction (PCR), both methods present benefits and downfalls. Cultures provide an identification based manly in phenotype that can take 24 hours to 10 days for a low price whereas, PCR is a faster and more sensitive method because it analyses the pathogen's DNA but although commercial PCR assays to detect mastitis pathogens exist, these are still more expensive comparer to cultures (Adkins and Middleton, 2018).

The early detection and identification of the pathogen are important to determine a suitable treatment strategy of the animal as well as, the proper management of the herd. The early detection allows an early treatment of the animal, preventing permanent damage of the mammary epithelium, and limits the spread of the disease in the herd. On the other hand, the identification of the mastitis-causing pathogen is essential for the determination of the correct and most efficient treatment method (Krömker and Leimbach, 2017).

1.6. Treatment

Although mastitis is a treatable illness, it can have a permanent impact in the health and milk production of a cow as well as, have a significant contribution in financial losses of the dairy farm. Therefore, there are many preventive measures that can reduce the appearance and prevalence of mastitis in the herd, and need to be considered by the owners for these farms (Cheng and Han, 2020; Radzikowski et al., 2020b).

1.6.1. Antibiotics

Before the administration of any drug, there must be a visual examination of the udder and teats to detect possible injuries like teat fistulas, leaky teats or udder lesions, as these need to be immediately treated to prevent further contamination by the pathogens in the environment (Sharun et al., 2021).

Regarding antibiotics usage, however, it is typically done on lactating cows with CM, intramammary or parenterally, if the teat canal is obstructed. Antimicrobials can also be used in Dry Cow Treatment, where the administration happens then the cow initiates drying-off. This technic is used as a prophylactic measure for mastitis but, due to the recent efforts to reduce the misuse of antibiotics worldwide this practice became obsolete, even though it had positive

results (Sztachańska et al., 2016; Krömker and Leimbach, 2017). Therefore, bovine mastitis is the major reason for the usage of these drugs in dairy farms.

Yet the efficacy of antimicrobials seems to be decreasing due to the ever-increasing emergence of resistant bacterial strains. A study on cattle and buffaloes in the Ismailia Province, Egypt, reported that from the 84 strains of *S. aureus* isolated, 64.3% were resistant to penicillin, 59.5% to tetracycline and 35.7% to cefoxitin, making them methicillin-resistant *S. aureus* (MRSA). In opposition, the bacterial strains appeared to be most sensitive to amoxicillin-clavulanic acid (78.6%), ampicillin-sulbactam (72.6%), and erythromycin (63.1%) (Algammal et al., 2020). The misuse and overuse of these drugs in veterinary practice are the main reasons for the overwhelming appearance of these resistant strains, as antibiotics are prescribed regardless of the severity of the illness or any knowledge of the pathogenic agent. For more efficient usage of antimicrobials, cultures and sensibility testing must be performed. Although, these tests do not guarantee the efficacy of the treatment in clinical cases as the disparities between *in vitro* and *in vivo* can affect the performance of the drug (Sharun et al., 2021).

In recent years, various species of fungi have also been reported to have resistance to common antifungals, for example yeasts from the genus *Candida* which are commonly isolated from mastitis cases in bovines (ErbaŞ et al., 2017).

Another important aspect to consider about the usage of antibiotics is the contaminated milk produced. As previously mentioned, this milk is discarded as it can't be consumed or used for the production of dairy derivatives and is potentially harmful to human health, in addition it can also the appearance of more resistance microbial strains (Sharun et al., 2021).

1.6.2. Alternative treatments

To reduce the use of antibiotics and overcome their downfalls, various papers have proposed alternative methods to prevent and treat bovine mastitis.

The use of plants and herbs to treat both human and animal illnesses have long been recorded. Nowadays, various studies have documented the antibacterial, antifungal, and even anti-inflammatory properties of these compounds, yet their applicability for the control of bovine mastitis is still limited (Mushtaq et al., 2018).

Furthermore, their usage causes less resistance in the microorganisms, as plants have developed protective strategies to control these mechanisms and can be administrated in many forms

(included in the feed, injected intramammary or in the bloodstream, topically or in bolus) (Mushtaq et al., 2018; Radzikowski et al., 2020b). In farms that practice organic production, the usage of natural extracts is the main method of treatment for many illnesses in the animals, as antibiotics are only used as a last resort (Radzikowski et al., 2020b).

However, a major disadvantage of this method of treatment is the potential alteration of some organoleptic properties of the milk, like the colour, smell, and taste, causing the discard of the milk (Cheng and Han, 2020).

Hase et al. (2013) studied the effects of the topical application of Mastilep gel and the herbal spray (AV/AMS/15) on cows diagnosed with SCM. Mastilep gel and the herbal spray used contained phytochemicals from the plant species *Cedrus deodara*, *Curcuma longa*, *Glycyrrhiza glabra* and *Eucalyptus globulus* winch were selected for their antibacterial, anti-inflammatory, analgesic and immunomodulatory properties. Both the treatments were applied to the udder twice a day, after milking for 5 days. After the treatments, it was detected a decrease of the SCC in the milk from the cows treated with Mastilep gel as well as, the spray AV/AMS/15 compared to the control group, however, the later treatment produced a larger decrease. Also, an increase in milk yield and fat content of the milk from these animals. The author claimed that these results might indicate that the treatment had a boosting effect on udder immunity (Hase et al., 2013).

There are also studies in the antifungal activity of essential oils against mastitis-causing fungi. The activity of *Origanum floribundum* Munby, *Rosmarinus officinalis* L. and *Thymus ciliatus* Desf. essential oils against *Candida albicans* isolated from mastitic milk was evaluated by Ksouri et al. (2017). All the oils presented a significant antifungal activity (Ksouri et al., 2017).

Regarding plants and essential oils, these were two examples of many possible treatments for mastitis. However, many other treatment methods have been proposed and evaluated like nanoparticles, antimicrobial peptides, bacteriophage, bacteriocins and even tissue regenerative therapy using amniotic cells from the progenitor (Lange-Consiglio et al., 2019; Radzikowski et al., 2020a).

1.7. *Cytinus* spp.

Part of the Cytinaceae family, the plant genus *Cytinus* is characterized by rootless, stemless, leafless and nonchrophyllic holoparasitic plants that have a vegetative body limited to an

endophytic system that exclusively grows within the roots of its host. The only visible part of these plants are the flowers, that emerge from the host's tissue in the reproductive period. The host provides a source of nutrients and water. This form of parasitism is regarded as one of the most extreme in this family (Silva et al., 2019). Only six species comprise this genus, they can be found in South Africa, Madagascar, and the Mediterranean Basin. In the latter, the most common species are *Cytinus hypocistis* (C. *hypocistis*) and *Cytinus ruber* (*C. ruber*). However, recent studies have shown that *C. ruber* is not genetically differentiated from the three subspecies of *C. hypocistis* (Roquet et al., 2016).

Cytinus hypocistis (L.) L. includes four subspecies that can be differentiated by the plant which they parasitize - subsp. *hypocistis* can be found in a variety of *Cistus* and *Halimium* spp., subsp. *macranthus* parasitizes on *Halimium* spp. plants, subsp. *orientalis* infects the plant species *Cistus parviflorus*, and, finally, subsp. *pityusensis* parasitizes on *Cistus clusii* (Silva et al., 2019). They can be recognized by their bright yellow flowers, which usually contrast with the flowers of the host (Maisetta et al., 2019).

Figure 1: *Cytinus hypocistis* (L.) L. subsp. *macranthus* Wettst (image obtained fromhttps://jb.utad.pt/especie/Cytinus_hypocistis_subesp_macranthus).

This species has long been used in European popular medicine to treat dysentery and inflammation of the eyes and throat, due to their astringent and haemostatic properties (Maisetta et al., 2019). Studies about the consumption of wild plants by humans customarily in the Iberian Peninsula, reported that *C. hypocistis* was also used as a famine food, the nectar would help workers to evade hunger pains as it could be sucked as a sweet or spread on rye bread (Silva et al., 2019).

Previous studies in the composition and properties of extracts of this species have had promising developments. Silva et al. (2020) studied the antibacterial activity of *C. hypocistis* hydroethanolic extracts made from the stalks, the petals and the nectar of the plant, as well as its entirety. All the extract presented activity against the Gram-positive and Gram-negative bacteria tested, which included strain of *E. coli*, *Klebsiella pneumoniae*, methicillin-sensitive and methicillin-resistant *S. aureus*. The stalk extracted appeared to be the most effective in the inhibition of bacterial growth, with MIC (minimal inhibitory concentration) that ranged from 0.625 to 1.25 mg/mL and 0.625 to 2.5 mg/mL, for the Gram-negative and Gram-positive bacteria respectively. Revelling being even more effective against Gram-negative bacteria than ampicillin, a commonly used antibiotic (Silva et al., 2020).

Furthermore, Maisetta et al. (2019) reported that both aqueous and ethanolic extracts of *C. hypocistis* and *C. ruber* presented bactericidal activity against *S. aureus* ATCC 33591 and *Staphylococcus epidermidis* with an approximated reduction of 3 Logs in viable cells for a concentration of 62.5 to 250 μg/mL. It was also determined that the Gram-positive bacteria strain (*Staphylococcus aureus* (ATCC 33591); *Staphylococcus epidermidis* (ATCC 35984); *Enterococcus faecium* VanR 1) tested were all sensitive to both the extracts, with MIC that ranged from 125 to 500 μg/mL for the aqueous extract and from 31.25 to 250 μg/mL for the ethanolic extract. However, neither of the extracts was able to inhibit the bacterial growth of the Gram-negative strain used (*Klebsiella pneumoniae* (ATCCBAA-1706); *Pseudomonas aeruginosa* (ATCC 27853), which is not corroborated by the previous manuscript. Furthermore, these author reported the biofilm suppressive effect of these extract against *Staphylococcus epidermidis* (Maisetta et al., 2019).

These study also assessed the antibacterial activity of the synthetic penta-O-galoy-β-D-glucose and 1-O-galloyl-β-D-glucose, both present in the *C. hypocitis* extracts, and determined that the first was affective against the Gram-positive bacteria and the second wasn't active for concentrations of less than 500 μg/mL (Maisetta et al., 2019). These compounds can be classified as tannins, more specifically gallotannins, and are part of many plants' defence mechanisms against pathogens and others. Recently, these compounds have been the aim of various studies due to their many interesting properties. Moreover, galloyl moieties were identified as the most bioactive components in tannin-rich plants as they presented antimicrobial, anti-inflammatory, antidiabetic and antioxidant activities (Silva et al., 2020). The antimicrobial activity of these compounds have been allocated to the interaction with the pathogens membrane and/or the ability to complex metal ions and, therefore, the low

effectiveness of tannins against Gram-negative bacteria can be explained by the strong negative charge of the lipopolysaccharides (Maisetta et al., 2019; Silva et al., 2020).

Having in mind the properties of the extract and its compounds, it would be pertinent to ask if it could be a natural form of prevention and/or treatment for diseases caused by microorganisms like bovine mastitis, resulting in the improvement of the overall health of the tissue, not only by having an effect in the organism but also on its microbiota (Silva et al., 2020).

1.8. Mice as models of Bovine Mastitis

As previously mentioned, mice were used as the *in vivo* model in this work, in order to study how the treatment with a *C. hypocistis* enriched cream would affect the microbiota of their outer ear.

Nowadays, mice are the animal model used to study many conditions in larger mammals, including humans, as the use of larger animals tends to be more costly and more difficult to manage (Notebaert and Meyer, 2006). Furthermore, according to Notebaert et al. (2006) the murine model is suitable to study intramammary inflammation due to the anatomical and physiological similarities between both animals.

Another very relevant aspect that needs to be taken account is the similarity in the microbiome of the cow´s udder and the mouse's outer ear. To the authors' knowledge, there haven't been studies describing the specific microbiota of the HPV16 transgenic strain of mice used in this study or any significant difference in comparison to the Wildtype strains described in other articles. Therefore, many of the microorganism species found in the mouse's ear microbiota can also be isolated from the cow's udder, some of which are bovine mastitis-causing pathogens, these is also verified by our result.

Bacterial species like *Streptococcus* spp. and *Staphylococcus* spp., can be widely found in many animals' skin microbiota and, for example, *S. aureus* and *S. uberis* are some of the most common mastitis-causing pathogens (Derakhshani et al., 2018; Belheouane et al., 2020). Regarding fungal species, although infections by yeast like *Candida* spp. are more commonly responsible for mastitis in bovines. Filamentous fungal genus like *Aspergillus* and *Penicillium* have also been isolated from mastitis cases. Both the yeast and fungi species can be present in natural microbiota of the mouse's ear (Couto et al., 2010; ErbaŞ et al., 2017; Andrews et al., 2019). Many of these fungal and bacterial species are vastly available in the environment and

grow in foodstuff utilized to produce livestock feed thus, the isolation of these microorganisms from the skin of animals is very common (Seyedmousavi et al., 2018; Yadav et al., 2018).

In conclusion, it is possible to access the potential of the *C. hypocistis* extract as a preventive measure/ treatment by analysis the microbiota samples in the mouse's outer ear.

2. Objectives

The following objectives were taken into account:

- The characterization of the fungal diversity in the mice's outer ear using traditional microbiology procedures before and after the treatment with a *C. hypocistis* enriched topical formulation (EF);
- Accessing the inhibition effect of the EF on the fungus *A. fumigattus*;
- The isolation and identification of *S. aureus* before and after the treatment with an EF, applying traditional microbiology procedures;
- The phenotypical characterization of the strains of *S. aureus* from before and after the treatment with an EF, using traditional microbiology procedures;
- The genetic characterization of the strains of *S. aureus* from before and after the treatment with an EF, using PCR techniques;
- Comparing the variability of the results of the case groups and the control group.

Allowing for a better assessment of the potential utilization of EF in dairy herds as a way of preventing and/or treating mastitis,

3. Materials and Methods

The following work was done in parallel to another, that evaluated the evolution of lesions in the ears of HPV+ mice treated with creams enriched with a *C. hypocistis* extract, hence some parts of the protocol refer to this other work and will not be presented.

3.1. *C. hypocistis* enriched topical formulation preparation

The extract and the topical formulations of *Cytinus hypocistis* (L.) L. subsp. *macranthus* Wettst used in this work were prepared by a research group in Instituto Politécnico de Bragança (IPB).

The plants were harvested in July 2018 from three different locations in Castro Daire (Portugal) from the host *Halimium lasianthum* subsp. *alyssoides (Lam.) Greuter*. The identification and characterization were performed applying Flora Europaea botanical criteria and the flora.on an online platform managed by the Portuguese Botanical Association.

After the fresh plant was cleaned properly with deionized water and carefully dried with absorbent tissue, it was frozen at -30ºC and lyophilized (FreeZone 4.5 model 7750031, Labconco, KS, USA). The lyophilized plant was then separated into two groups, whole plant and nectar, and reduced to a fine powder (20 mesh), which was stored away from light at -30ºC, as described in (Silva et al., 2019).

For the three different EF used in this study, different amounts of the powder from the whole plant were incorporated into a base cream to achieve the required concentrations for the essay. CH1 had 3.1 mg of extract per g of cream, CH2 6.2 mg/g and CH3 12.4 mg/g. The base cream was also used for the positive and negative controls. The creams were stored at 4ºC during the experiments.

3.2. Animals

For this procedure, 30 female mice of the strain FBV/n were used. Twenty of these were transgenic and were carriers for human papillomavirus (HPV16) and the remaining 10 were wild-type and did not carry HPV16.

The mice were divided into six groups of 5 mice each. Two groups of wild-type and the other four HPV16 mice. In the wild type mice groups, one was treated with the base cream (G6), to assist as a negative control, and the other with the CH3 (G4), to access toxicity. The remaining

four groups, one was a positive control on which was applied the base cream (G5) and the rest were treated with three creams with rising concentrations of the extract CH1, CH2 and CH3 (G1, G2 and G3, respectively), in order to determine the most efficient concentration for the treatment. Which leads to the following groups:

- G1: HPV16, CH1;
- G2: HPV16, CH2;
- G3: HPV16, CH3;
- G4: Wild-type, CH3;
- G5: HPV16, positive control;
- G6: Wild-type, negative control.

The evaluation of the correct dosage and toxicity of the EF for the mice weren't accessed in this work.

The experiment procedure was approved by the local Animal Ethics Committee-DGAV number 0421/000/000/2014, 24/09/2014 (020172).

The animals were kept in hard polycarbonate cages with rounded edges covered in corn cobs, the change and clean-up of the cages was performed once a week. The mice were fed in an *ad libitium* regimen and had always had access to water.

The mice were kept in appropriate conditions that were always assured by the university's bioterium. The relative humidity at 50±10%, temperature at 23±3ºC, light cycle with 12 hours of light and 12 h of dark and the health state of the mice was checked daily. The welfare of the animals was examined at the end of every week using a punctuation chart with various related variables (End point chart). The mice that scored 4 or more were euthanized. However, this was never recorded, and all mice survived till the end of the essay. There were no mortalities registered in the experiment period.

3.3. Application protocol

In order to apply the EF on the mice, the creams were previously mixed to guarantee sample homogeneity. The creams were then applied uniformly, using a cotton swab to both outer ears of the mice of the corresponding groups. This protocol occurred daily, from the 10th of November 2020 to the 17th of December 2020, excluding weekends.

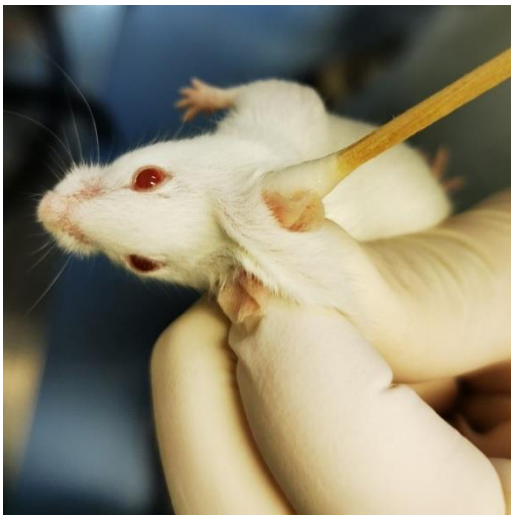

Figure 2: Application of the *C. hypocistis* cream on the mouse´s ear.

3.4. Sample collection

Samples were collected on day 1 of the assay, before the first application, and on day 17, before the seventeenth application.

The samples were collected by rubbing the surface of the ear for 1 minute, with a cotton swab that was previously emerged in saline solution. The samples were inoculated in Petri dishes (9 cm) with the culture medium PDA (FRILABO), followed by an incubation period of 15 days at 37 °C. This procedure followed routine mycological techniques.

Figure 3: Sample inoculation procedure.

3.5. Fungal isolation and identification

From the main samples, fungal isolates were prepared by cutting a portion of the medium with the fungus in the main dish and placing it in a new dish with PDA medium. After the incubation period of 15 days at 37 °C, the isolates were analysed in the optic microscope.

The microscopic identification of the isolated fungi specimens was carried out using the Lactophenol with Cotton Blue technique for staining the filamentous fungi and identification of the genera.

3.6. Isolation and identification of *Staphylococcus aureus* and *Staphylococcus xylosus*

The bacterial colonies found in the sample were inoculated in Brain Heart Infusion broth (BHI) medium for 18 to 24 hours at 37 °C to be isolated and posteriorly identified.

The identification of the bacterial genera was performed using gram staining, the catalase test by the slide or drop method, the coagulase and DNase tests. Furthermore, to identify the bacterial species it was used an API STAPH 20500 (BioMérieux).

3.7. *Staphylococcus aureus* antibiotic susceptibility test

To assess the antibiotic susceptibility of the bacteria, it was used the Kirby-Bauer disk diffusion method.

Both the *S. aureus* samples from the two collection instances were inoculated in thioglycolate with resazurin and CAN (Columbia naladixic acid agar) with sheep's blood mediums for 24h at 37 °C, to isolate the cultures before the protocol. Then a bacterial solution with BHI medium was made using the isolated colonies, the turbidity was confirmed by spectrophotometry (0.5 McFarland Turbidity Standard).

Afterwards, the cultures were inoculated in Petri dishes with PDA medium and paper disks impregnated with different antibiotics were placed accordingly across the surface of the medium. The antibiotics used were penicillin G (P10), ampicillin (AM10), amoxicillin (AMC30), florfenicol (FFC30), cefoxitin (FOX30), cephalothin (CLT30), cefazolin (CZ30), cefotaxime (CTX30), ceftazidime (CAZ30), chloramphenicol (C30), doxycycline (DO30), erythromycin (E15), clindamycin (DA2), enrofloxacin (ENR5), lincomycin (L15), marbofloxacin (MAR5), tetracycline (TE30), meropenem (MEM10). The diameter of the inhibition zone was measured after an incubation period of 24h at 37 °C.

Figure 4: Inhibition zone measurement.

3.8. Inhibition effect of EF in *Aspergillus fumigatus*

To study the inhibition percentage (IP%) of *C. hypocistis*, it was used the fungus *Aspergillus fumigatus* isolated from the first sampling instance and posteriorly identified (the sample identification was erased and therefore, the sample origin of the fungus was not identified).

The fungus was inoculated in three sterile Petri dishes (9 cm) for each cream concentration and controls, proceeding in triplicate. Due to the lack of cream, only CH1 and CH2 were used in the experiment.

The dishes with EF had 6 mL of PDA medium, 4 mL of sterile water where was previously diluted 1 g of cream. Then for the two control experiments, one had 6 mL of PDA medium and 4 mL of sterile water, and the other only had 9 mL of PDA medium,

The inoculation of the fungus was done in a laminar flow chamber, following every necessary biosafety protocol, with the help of a 4 mm agar cutter, portions of the fungal colony were placed in the geometric centre of each dish, having in mind that the side with the colony is directly in contact with the new medium. All the dishes were then incubated at 37°C.

With the purpose of analysing the antifungal effects of the EF against the *A. fumigatus*, the diameter of the fungal colonies was measured in the mornings of the third, fifth and seventh days after the inoculation. Two perpendicular measurements were noted every day for each dish, to calculate the mean between them.

The results for both the controls were then compared with the inoculated dishes. The variation was then presented in a form of an IP% $[(C - T) \times 100/C]$, where C and T represent respectively, the radial diameters of the control and of the inoculated dishes' colonies, according to the methodology described by Zhang et al. (2006).

The statistical analysis was performed using the program SPSS® (Statistical Package for the Social Sciences) version 25.0. The *t* Student test, with $p < 0.05$ of significance, was utilized to analyse the difference between in the mean IP% for both the extract concentrations tested.

3.9. Virulence genes analysis for *Staphylococcus aureus*

3.9.1. DNA extraction

The two samples of *S. aureus*, one from each collections instance, were inoculated in 5 mL of BHI medium and then incubated for 24h hours at 37°C, from which total DNA was extracted using the kit GF1- Extraction of Bacterial DNA (Vivantis, Malaysia), as recommended by the manufacturer protocol.

Initially, 1.5 mL of the bacterial solution were subjected to a first centrifugation at 10 000 g for 5 minutes, with following discard of the supernatant. Then 1.5 mL of the bacterial solution were added again, and the centrifugation protocol was repeated, in order to isolate the bacterial cells and discard the medium.

The resulting pellet was resuspended in 100 μL of the R1 Buffer. To induce cellular lysis, 20 μL of lysozyme (50 mg/mL) and 15 μL of lysostaphin (0.1 mg/mL) were added to the cellular suspension, this mixture was left to incubated for at least 1 hour in a water bath at 37°C, followed by centrifugation at 10 000 g for 5 minutes and the disposal of the supernatant.

Next for the protein denaturation, the pellet was resuspended in 180 μL of the R2 Buffer and 20 μL of proteinase K, then incubated in a water bath at 65°C for 1 hour with agitation or mixing every 5 minutes. The obtained lysate presented a light colour, to which was added 2 x 400 μL of the buffer BG and the mixture was mixed by inversion in order to obtain a homogeneous solution, which was then incubated at 65°C for 10 minutes. Subsequently, it was added 200 μL of ethanol absolute and immediately mixed to prevent the uneven precipitation of the nucleic acids.

This mixture (maximum 650 μL) was transferred to a column allied to a collector tube and centrifugated at 10 000 g for 1 minute. After the discard of the filtrate, the centrifugation was repeated with the rest of the mixture. Next, to wash the column, 650 μL of the Wash buffer was added, centrifugated at 10 000 g for 1 minute and the filtrate was discarded. To dry the column, the excess of buffer and ethanol was removed by a centrifugation at 10 000 g for 2 minutes.

The column was then transferred to another tube for the elution. For this, 100 μL of Elution buffer, previously heated, was deposited directly on the membrane and, after 2 minutes, centrifugated at 10 000 g for 1 minute. The DNA extracted was kept at 4°C.

After the extraction, electrophoresis in 1% agarose gel was performed to access the quantity and quality of the DNA, as well as the determination of the sample's purity by

spectrophotometry. The molecular weight marker used was Thermo Scientific GeneRuler DNA Ladder Mix #SM0331 (Thermo Fisher).

3.9.2. Genus confirmation

The thermonuclease (*NUC*) gene (270 bp) was detected using a PCR technic. The mix for the procedure comprised 10.0 µL of MyGo HS Taq Mix (MyGo), 0.5 µL of Primer Forward (10 µM), 0.5 µL of Primer Reverse (10 µM), 8.0 µL of DNA (10ng/µL) and 1.0 µL of ddH2O, totalizing 20 µL. Table 1 describes the conditions for the PCR reaction.

After the reaction, the samples were then submitted to electrophoresis at 1% agarose gel, stained with GreenSafe Premium (Nzytech), for 40 minutes. The gel was visualized using the Molecular Imager Gel DOC™ XR+ with image Lab™ software (Bio-rad).

Table 1: PCR conditions for the *NUC* gene amplification.

Cycles	Time	Temperature
1	5 '	95 °C
40	15 "	95 °C
	20"	60 °C
	20"	72 °C
1	5'	72 °C
…	∞	4 °C

3.9.3. Virulence factors gene detection

1.1.1.1. Genes for *Staphylococcus* enterotoxins D, G and I

For the detection of *SED*, *SEG* and *SEI* genes,171 or 385, 287 and 466 bp, respectively, it was used the multiplex PCR protocol described in Table 2. The mix utilized was made up of 10.0 µL of MyGo HS Taq Mix (MyGo), 6.4 µL of DNA (10ng/µL), 0.6 µL of Primer Forward (10 µM) and 0.6 µL of Primer Reverse (10 µM) for each gene totalizing 20 µL. Afterwards, the samples were submitted to electrophoresis at 1.5% agarose gel, stained with GreenSafe Premium (Nzytech). The molecular weight marker used was Thermo Scientific GeneRuler DNA Ladder Mix #SM0331 (Thermo Fisher). The gel was visualized using the Molecular

Imager Gel DOC™ XR+ with image Lab™ software (Bio-rad). The same procedure was used to visualize the following gels.

Table 2: PCR conditions for amplification of *SED*, *SEG* and *SEI* genes.

Cycles	Time	Temperature
1	5 '	95 °C
40	15 "	95 °C
	20"	59 °C
	90"	72 °C
1	5'	72 °C
…	∞	4 °C

1.1.1.2. Gene for the hemolysin-α

The PCR protocol utilized to amplify the *HLA* gene (209 bp) is shown in Table 3. The mix comprised of 0.4 µL of Taq Polymerase (Bioron), 0.8 µL of Forward Primer (10 µM), 0.8 µL of Reverse Primer (10 µM), 2.0 µL Buffer solution (10x), 1.6 µL $MgCl_2$ (25 mM), 2.0 µL dNTPs (2 mM), 4.4 µL of ddH2O and 8.0µL of DNA (10 ng/µL), adding up to a total of 20 µL. Afterwards, the samples were submitted to electrophoresis at 1% agarose for 40 minutes.

Table 3: PCR conditions for the amplification of the *HLA* gene.

Cycles	Time	Temperature
1	5 '	94 °C
35	30 "	95 °C
	45"	58 °C
	1'	72 °C
1	10'	72 °C
…	∞	4 °C

1.1.1.3. Gene for toxic shock syndrome toxin-1

In order to identify the *TSST-1* gene (326 bp), a mixture of 0.4 µL of Taq Polymerase (Bioron), 0.7 µL of Forward Primer (10 µM), 0.7 µL of Reverse Primer (10 µM), 2.0 µL Buffer solution (10x), 1.6 µL $MgCl_2$ (25 mM), 2.0 µL dNTPs (2 mM), 4.6 µL of ddH2O and 8.0 µL of DNA (10 ng/µL), making up a total of 20 µL, using the PCR protocol presented in Table 4. Afterwards, the samples endured electrophoresis at 1% agarose for 40 minutes.

Table 4: PCR conditions for the amplification of the *TSST-1*gene.

Cycles	Time	Temperature
1	5 '	94 °C
40	30 "	95 °C
	45"	58 °C
	1'	72 °C
1	10'	72 °C
…	∞	4 °C

4. Results

4.1. Fungal biodiversity for the collected samples

In order to do a proper mycological identification, both the macro and micro characteristics of the fungi have to be considered. Figures 5 to 8 are some of the fungal specimens analysed in this essay.

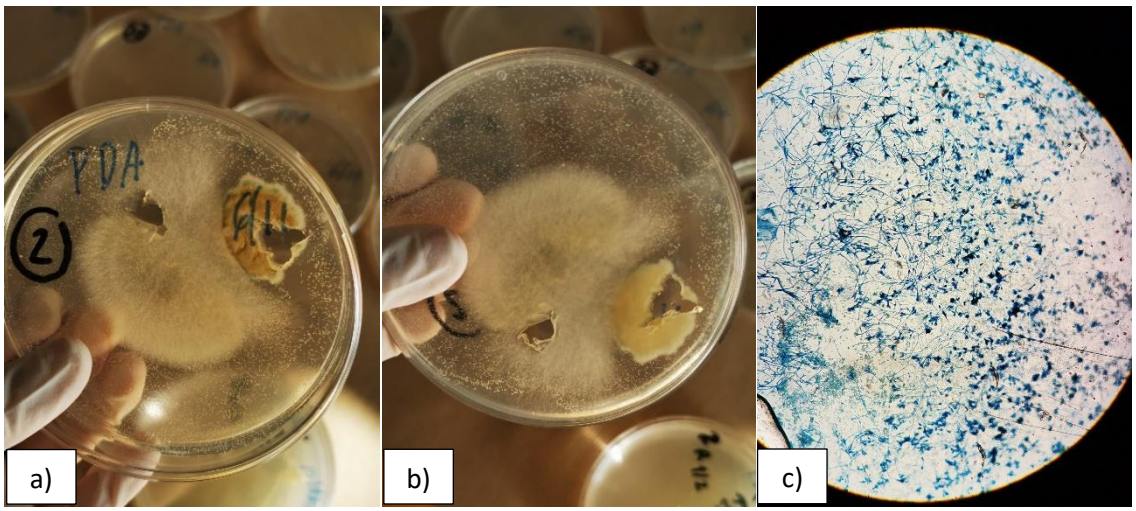

Figure 5: Sample 2, fungal growth of the genus *Mucor* (left) and *Penicillium* (right): a) View of the Petri dish; b) View of the flipside of the Petri dish; c) Microscopic image of *Penicillium* at 40x magnification.

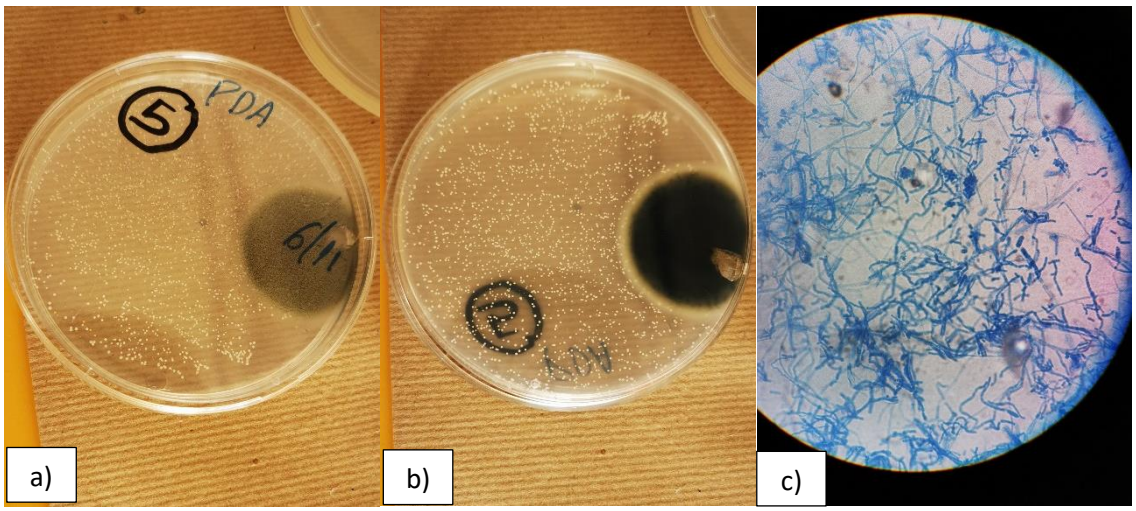

Figure 6: Sample 5, fungal growth of the genus *Cladosporium*: a) View of the Petri dish; b) View of the flipside of the Petri dish; c) Microscopic image of *Cladosporium* at 40x magnification.

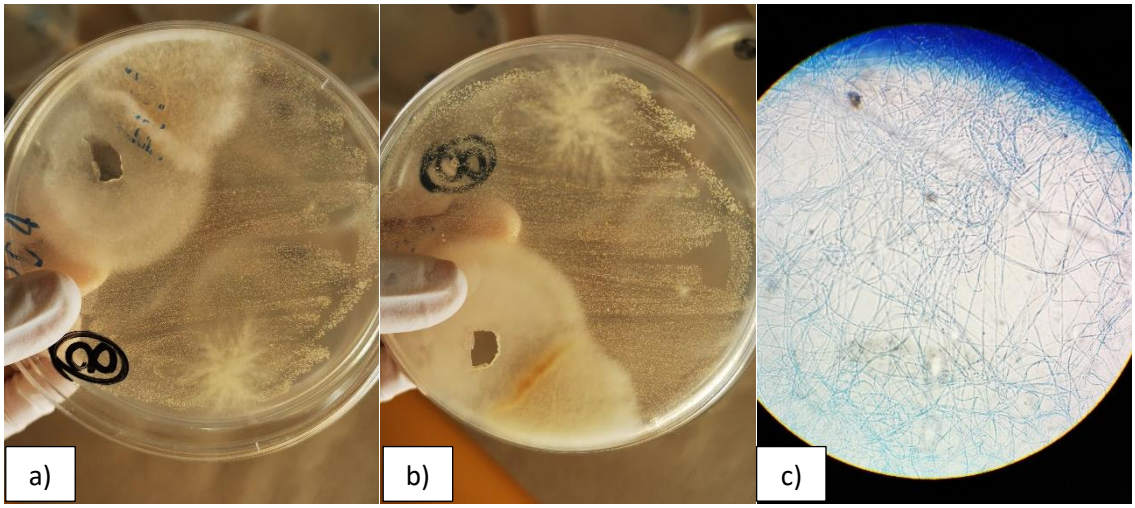

Figure 7: Sample 8, fungal growth of the genus *Mucor* (bottom): a) View of the Petri dish; b) View of the flipside of the Petri dish; c) Microscopic image of *Mucor* at 400x magnification.

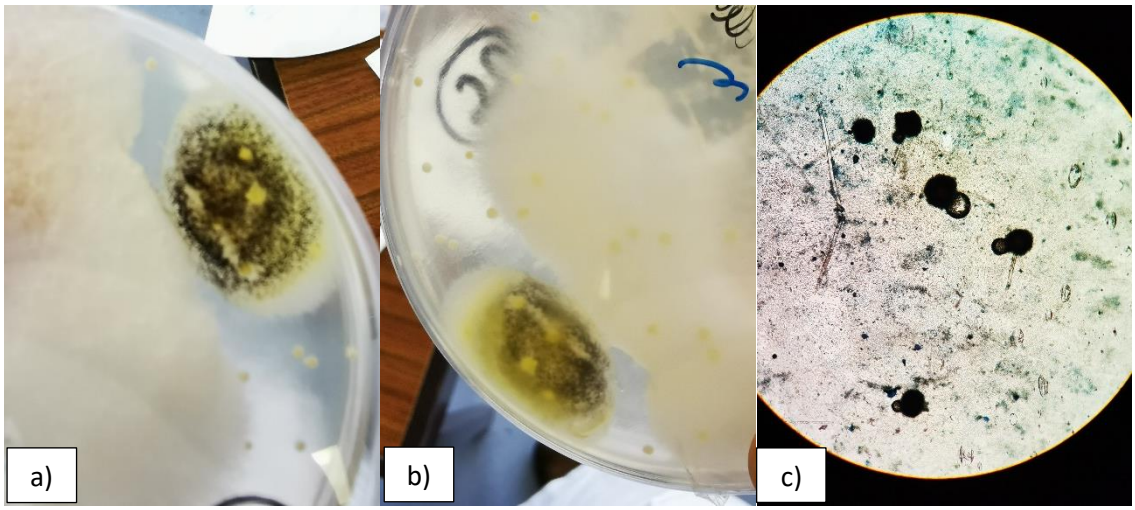

Figure 8: Sample 20, fungal growth of the genus *Aspergillus*: a) View of the Petri dish; b) View of the flipside of the Petri dish; c) Microscopic image of *Aspergillus* at 400x magnification.

In the entirety of samples from the first collection, before the application of the cream, 6 filamentous fungal genera with medical relevancy were isolated: *Penicillium* (16.7%), *Mucor* (16.7%), *Cladosporium* (3.33%), *Aspergillus* (3.33%), *Alternaria* (3.3%) and *Fusarium* (6.7%) (Table 5). In total, fungi were isolated in 14 animals (46.7%) of the 30. In two of the samples, fungal identification was not possible. Posteriorly, the *Aspergillus* species was identified as, *Aspergillus niger*.

Additionally, yeast cultures were also identified. *Rhodotorula mucilaginosa* (10,0%) in group 6 and another species in groups 4 and 6 that was not identified.

Table 5: Number of fungal genus samples and overall frequency in percentage, for the first collection instance.

Fungal Genus	**N(%)**
Penicillium	5 (16.7%)
Mucor	5 (16.7%)
Cladosporium	1 (3.3%)
Aspergillus	1 (3.3%)
Alternaria	1 (3.3%)
Fusarium	2 (6.7%)
Rhodotorula mucilaginosa	1 (3.3%)
Non identified Yeast	3 (10.0%)

After the application of *C. hypocistis* cream for 17 days in the ears, only one fungal colony was isolated, the genus was not identified.

4.2. Bacterial biodiversity for the collected samples

In the first collection of samples, bacteria colonies were isolated in 12 (40.0%) of the 30 mice. The colonies were white and yellow with a circular form and had a uniform distribution throughout the medium. The preliminary identification tests results can be found in Table 6. These indicate that the genus of both cultures is possibly *Staphylococcus*. Furthermore, the API STAPH tests scores implies that the colonies were *Staphylococcus aureus* and *Staphylococcus xylosus*.

Bacteria from the species *Staphylococcus aureus* were isolated in 6 mice (20.0%).

Table 6: Bacterial genus identification tests and respective results.

Identification Tests	**Results**
Gram Staining	Gram positive bacilli
Catalase	Positive
Coagulase*	Positive (clot formation)
DNase	Positive

*2 to 4 hours at 37 °C.

In the samples collected after the treatment, the growth of bacterial colonies with the same characteristics was again spotted, however, they were now in 27 of the 30 samples (90.0%). The identifications tests were performed and indicated the presence of *Staphylococcus aureus* and *Staphylococcus xylosus*.

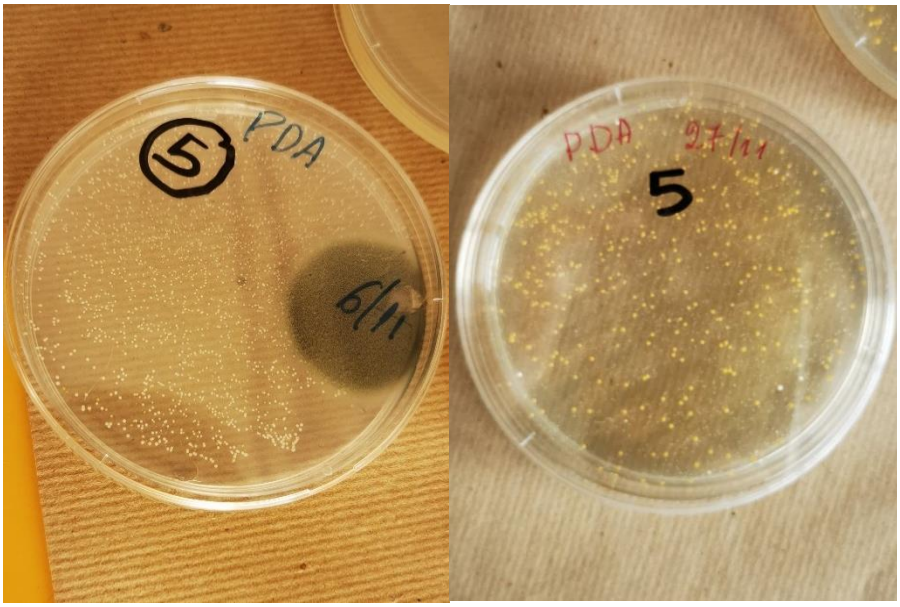

Figure 9: Sample 5 with multiple bacterial colonies in the first collection instance (left) and second (right), after treatment.

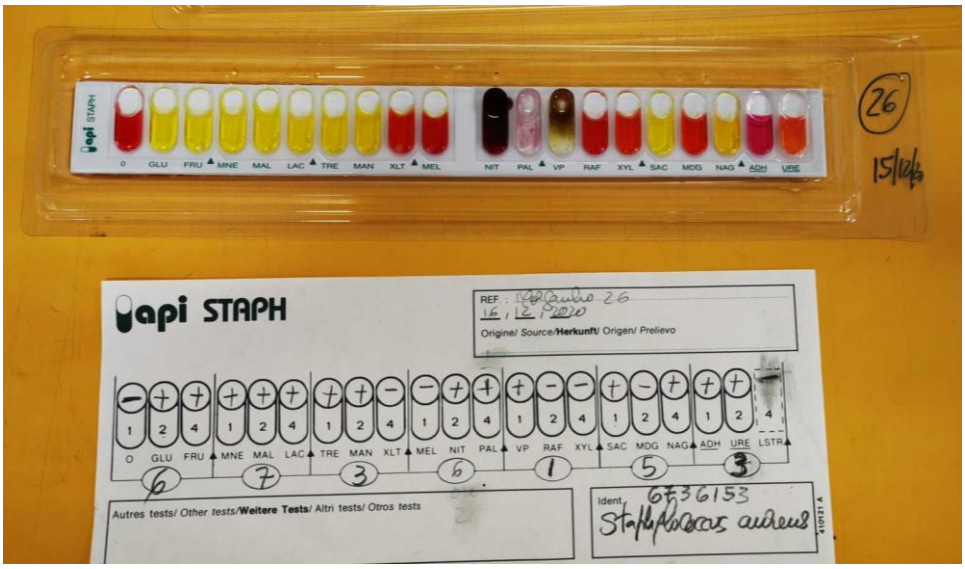

Figure 10: API test result for *Staphylococcus aureus*.

4.3. Phenotypical profile of the fungal and bacterial isolates

The phenotypical profile of the fungal isolates from the first collection can be seen in Table 7. *Mucor* was the only genus of filamentous fungi that was isolated in junction with another fungi genus.

Table 7: Phenotypical profile of the fungal isolates from the first collection instance.

Phenotypical profile of the fungal isolates	**N.º**
Cladosporium	1
Mucor	1
Penicillium	3
Non identified Yeast	1
Penicillium *Mucor*	2
Mucor *Fusarium*	1
Fusarium Non identified Yeast	1
Mucor *Aspergillus niger*	1
Alternaria *Rhodotorula mucilaginosa* Non identified Yeast	1

In Table 8, the fungal isolates are organized according to the group of animals where they were found. As shown, there were no fungal genera isolated from group G3.

The fungal isolates according to the groups where they were found were *Penicillium* in G1, G4 and G5, *Mucor* in G1, G2, G4 and G5, *Cladosporium* in G1, *Aspergillus* in G4, *Alternaria* in G6 and *Fusarium* in G2 and G4. Fungal genera were not identified in three mice, from G1 and G5. There was no growth detected in three mice of G6. The group with the biggest fungal diversity was G4 and the one with the lowest was G6, with only *Alternaria* being isolated.

Bacteria from the *Micrococcus* genus were isolated from groups G2, G3, G4 and G5.

Whereas bacteria from the *Staphylococcus aureus* species were isolated from groups G1, G2 and G3.

Table 8:Fungal isolates and bacteria from the first collection instance, according to the animal group.

Treatment Groups	**Fungal Genus**	**Bacterial Genus**
G1 - HPV16 +, CH1 (lowest extract concentration)	*Penicillium* (n=2) *Mucor* (n=1) *Cladosporium* (n=1)	*Staphylococcus aureus* (n=1)
G2 – HPV16 +, CH2	*Mucor* (n=1) *Fusarium* (n=1)	*Micrococcus* (n=4) *Staphylococcus aureus* (n=2)
G3 – HPV16 +, CH3 (highest extract concentration)		*Micrococcus* (n=5) *Staphylococcus aureus* (n=3)
G4 – Wild-type, CH3 (toxicity)	*Penicillium* (n=2) *Mucor* (n=2) *Aspergillus niger* (n=1) *Fusarium* (n=1)	*Micrococcus* (n=1)
G5 – HPV16 +, positive control	*Penicillium* (n=1) *Mucor* (n=1)	*Micrococcus* (n=2)
G6 – Wild type, negative control	*Alternaria* (n=1)	

4.4. Antibiogram

The diameters of the inhibition zone for the possibly resistances are in Table 9. These imply that both the strains of *S. aureus* from the two collection instances are likely resistant to the treatment with penicillium G and ceftazidime as the guidelines are ≥ 29 mm and ≥ 17 mm, respectively.

Table 9: Inhibition zone diameter in mm for penicillin G and ceftazidime, for *S. aureus*.

		Inhibition Zone (mm)	
		Penicillin G	Ceftazidime
First collection	*Staphylococcus aureus*	23	14
Second collection	*Staphylococcus aureus*	26	15

4.5. Inhibition effect of the *C. hypocistis* cream in *A. fumigatus*

For this protocol, only cream CH1 and CH2 were available and, therefore, the concentration of the extract in the medium made with these creams were 0.115 mg/mL and 0.230 mg/mL,

respectively. The Figures 11 to 13 show the growth evolution of *A. fumigattus* during the experiment.

The mean percentages of inhibition for the third, fifth and seventh days after inoculation can be found in Table 10. The largest IP% was for the concentration of 0.115 mg/mL on the third day, being higher than 67%. The IP% for this concentration was always higher compared to the IP% recorded for the concentration of 0.230 mg/mL, for itch day of assessment.

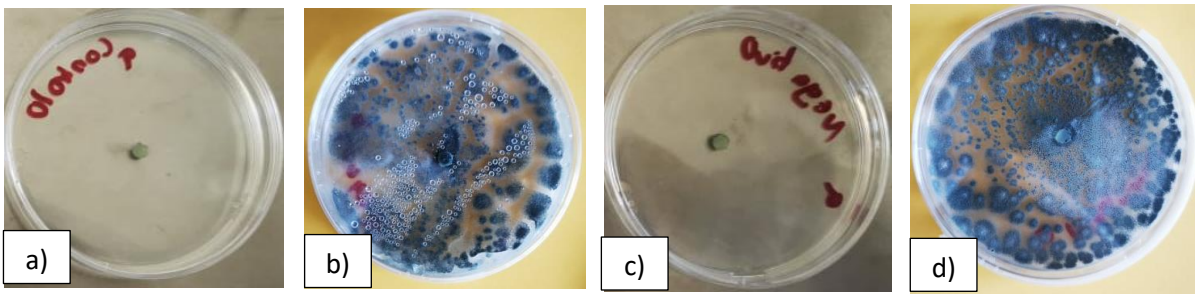

Figure 11: Fungal growth of *A. fumigatus* in both the control experiments after 3 days of incubation at 37°C. a) and b) the control dish with only PDA in the medium and, c) and d) the control dish with PDA and sterile water in the medium.

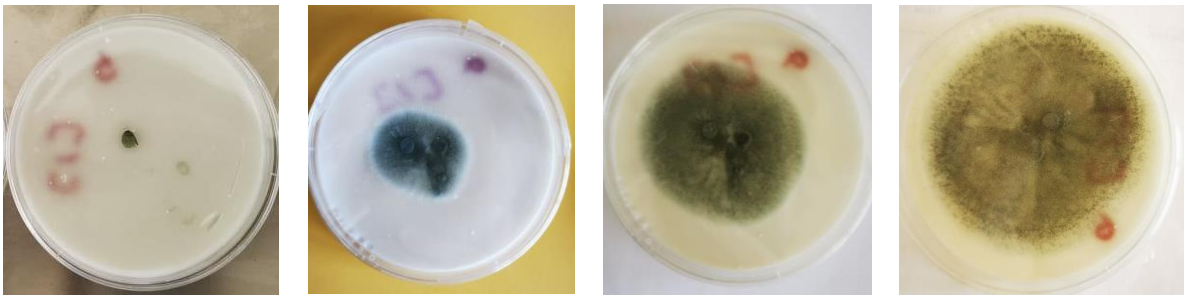

Figure 12: Fungal growth of *A. fumigatus* in PDA with CH1 right after inoculation and with three, five and seven days of incubation at 37°C, from left to right.

Figure 13: Fungal growth of *A. fumigatus* in PDA with CH2 right after inoculation and with three, five and seven days of incubation at 37°C, from left to right.

Table 10: Measurements for the fungal growth of *A. fumigatus* in the medium with different concentrations of *C. hypocistis* extract three, five and seven days after inoculation, at 37 °C.

	Inhibition percentage (%)		
	3rd-day measurement	**5th-day measurement**	**7th-day measurement**
0.115 mg/mL	67.22	42.41	22.59
0.230 mg/mL	61.85	39.81	17.96

In Table 11, it can be seen the final means for the IP%. In both the concentration mycelial inhibition occurred. The highest IP% happened for the concentration of 0.115 mg/mL.

Regarding the mean, the concentration of 0.115 mg/mL had an inhibition of the mycelial growth 1.1 times higher than the concentration of 0.230 mg/mL. The differences between the mean IP% for both the concentrations were found to be not significant (t=0.232; p=0.828).

Table 11: Mean and standard deviation of the measurements for the fungal growth of *A. fomigatus* in the medium with different concentrations of *C. hypocistis* extract.

C. hypocistis concentration in PDA (mg/mL)	**Mean**	**Standard Deviation**
0.115	44.0733	22.3615
0.230	39.8733	21.9451

4.6. Genetic confirmation of the bacterial genus

Regarding the quality of the extraction, the mean A260/A280 ratio for both the samples was 2.1, with a DNA concentration of 251.4 and 210.0 ng/μL for the first and second samples, respectively.

For the genus confirmation procedure, the first sample labelled as S1 relates to the collection before the start of the treatment and the second sample, S2, to the collection after the treatment. The translucency in S2 indicates a lower concentration of the gene possibly due to an error in the procedure. Also, the lack of a band in the negative control (C-) indicates the absence of contaminates.

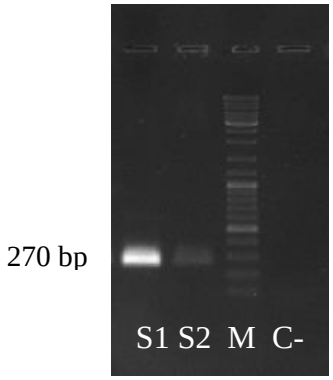

Figure 14: Electrophoresis gel with 1% agarose stained with GreenSafe Premium, for the confirmation of the bacterial genus from the samples of both collection instances. S1 corresponds to the sample from the first collection instance and S2 to the secund, after the treatment, M is the molecular weight marker and C- is the negative control.

In the gel (Figure 14) it can be identified an amplification corresponding to a band with 270 bp which represents the *S. aureus NUC* genes, these look to be coherent for both S1 and S2. Further corroborating the genus identification earlier mentioned.

4.7. Virulence genes identification

4.7.1. Genes for *Staphylococcus* enterotoxins D, G and I

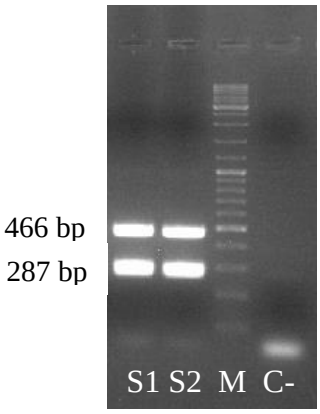

Figure 15: Electrophoresis gel with 1,5% agarose stained with GreenSafe Premium, for the identification of the genes for *SED*, *SEG* and *SEI* in the *Staphylococcus aureus* samples from both the collection instances. S1 corresponds to the sample from the first collection instance and S2 to the secund (after the treatment), M is the molecular weight marker and C- is the negative control.

The results (Figure 15) showed two bands for each sample, the first band possibly corresponds to the *SEG* gene with 287 bp, and the second band to the *SEI* gene with 466 bp. Indicating the absence of the *SED* gene.

4.7.2. Gene for the hemolysin-α

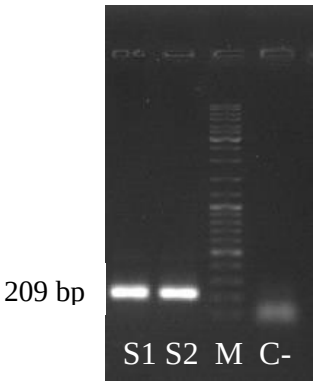

Figure 16: Electrophoreses gel with 1% agarose stained with GreenSafe Premium, for the identification of the genes for HLA in the *Staphylococcus aureus* samples from both the collection instances. S1 corresponds to the sample from the first collection instance and S2 to the secund (after the treatment), M is the molecular weight marker and C- is the negative control.

This procedure resulted in a bright short band which is consistent with the *HLA* gene which has 209 bp (Figure 16). Both S1 and S2 seem to have this band.

4.7.3. Gene for toxic shock syndrome toxin-1

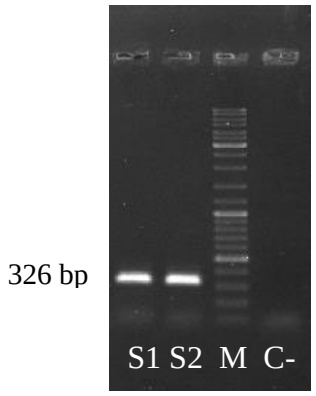

Figure 17: Electrophoreses gel with 1% agarose stained with GreenSafe Premium, for the identification of the genes for *TSST-1* in the *Staphylococcus aureus* samples from both the collection instances. S1 corresponds to the sample from the first collection instance and S2 to the secund (after the treatment), M is the molecular weight marker and C- is the negative control.

For the detection of the *TSST-1* gene, both the samples S1 and S2 resulted in a band with 326 bp, most likely corresponding to this gene.

5. Discussion

5.1. Biodiversity of the mice's outer ear microbiota

Regarding the biodiversity of the microbiota found in the samples, most of the fungi genera identified are commonly found in the skin and udder of the cow, as well as of many other animals (Seyedmousavi et al., 2018; Andrews et al., 2019). Analyses to mastitic milk revealed the presence of filamentous fungi of the genera *Penicillium*, *Mucor, Cladosporium* and *Fusarium*, although there was no evidence that these caused the infection (Ksouri et al., 2015; Bekele et al., 2019). These fungi are commonly found in harvested crop used to produce animal feed or in the environment and, therefore, are usually present in the microbiota of the cow's skin, yet some species of these fungi genera can be pathogenic causing a variety of conditions in bovines and other animals (Lawrence et al., 2016; Seyedmousavi et al., 2018; Yadav et al., 2018; Summerell, 2019; Chmielowiec-Korzeniowska et al., 2021).

Penicillium is one of the most known and researched fungi genera, due to the many applications of its species like the production of penicillin or as a fermentation agent in the production of some cheeses. However, in nature its principal role is the decomposition of organic material. This is also one of the easiest fungus to find as they are vastly disperse thru out the environment, in the soil, air and many food products like plants and fruits, even in extreme conditions like high or low temperatures (Yadav et al., 2018).

Many of these species have beneficial properties, none the less, many other can cause the rapid degradation of food crops and infectious diseases due to the production of mycotoxins (Yadav et al., 2018). In bovines, *Penicillium* spp. have been reported to cause endometritis and infections of the respiratory tract (Al-Khalidi et al., 2012; Saini et al., 2019). Jameel and Yassein (2021) also pointed to the possibility of *Penicillium chrysogenum* being a mastitis-causing pathogen, since this species was isolated in 34.8 % of the mastitic milk samples tested (26 of the 66 fungal isolates found) and showed multiple virulence factor like proteolytic action against albumin and casein (Jameel and Yassein, 2021).

Furthermore, the moulds of the *Mucor* genus, part of the order *Mucorales*, can originate multiples conditions in humans and animals alike. They can be found all around the environment (soil, air, food and decaying organic matter) therefore, isolating fungi from this genus is not unexpected in mice. Like many of its fungi counterparts, *Mucorales* infection

occur in an opportunistic fashion, in immune-compromised individuals or when there is an overwhelming exposure to the mould. In large ruminants, it can cause mucormycotic ruminitis, an infection of the rumen caused by a *Mucorales* colonization after antibiotic treatment, and lymphadenitis, an infection of the lymph nodes due to ingestion of *Mucorales* contaminated food (Seyedmousavi et al., 2018). However, moulds from this genus have not been reported to cause bovine mastitis.

Regarding the genus *Alternaria*, it is mainly recognised for being a plant pathogen, as it can cause the loss of crops for human and animal consumption and their contamination by mycotoxins which can be very economical damaging. However, these fungi can also cause disease in animals and humans like allergic reactions and asthma (Lawrence et al., 2016). Fungi from this genus can be found ubiquitously in natural, as well as, in urban environments, moist buildings and predominantly in plant derivatives (paper and food, for example) or in living plants mostly in senescent or damaged tissue (Aichinger et al., 2021). These can grow on a variety of substrates, in various conditions of moisture and temperature (Aichinger et al., 2021).

Concerning *Fusarium* fungi, these are also phytopathogens, as they can cause devastating diseases in many agricultural and horticultural crops, like head blight on wheat or wilt and stem rot diseases (Summerell, 2019). They are wildly dispersed in nature, on both the superficial and underground parts of plants, on soil, air and even in aquatic environments (Sáenz et al., 2020). Moreover, the mycotoxins produced by some of these species can gravely impact animals' and humans' heath, especially in immunocompromised conditions. For example, trichothecenes can be absorbed topically by the animal and metabolized, resulting in break down products that can affect the immune system, gastrointestinal tract, skin, kidney and liver (Sáenz et al., 2020). There have been reports of *Fusarium* infection in a myriad of aquatic animal, dogs and horses. In cattle there was a case reported of a *Fusarium* and *Aspergillus* fungal infection resulting in corneal stromal abscess and secondary anterior uveitis however, keratomycosis is very rare in bovine (Elligott et al., 2006).

The fungi from the *Cladosporium* genus are some of the most common fungi isolated in indoor spaces although, these can be found all over the environment, establishing various symbiotic interactions with both plants and animals (Salvatore et al., 2021). These can be abundantly found in the atmosphere, being commonly isolated from air samples of barns or other livestock facilities (Chmielowiec-Korzeniowska et al., 2021). The large availability of these fungi can result in allergic and even pathogenic reactions in humans and animals. *Cladosporium* infection can result in a large array of diseases, from a light allergic reaction like skin redness and

irritation, to a cerebral abscess that can led to the death of the animal (Menezes et al., 2017). However, there is no evidence that it is an aetiological agent of mastitis.

As previously mentioned, the *Aspergillus* genus, specifically *Aspergillus fumigatus* and *Aspergillus niger*, have been identified as a cause for bovine mastitis. These fungi can be found in the soil or rotten vegetation, seeds and grains. The infection by *A. fumigatus* can also result in pneumonia, gastroenteritis, placentitis, and even abortions in ruminants, which can have a large impact on the financial losses of a dairy farm (Pachauri et al., 2013; Seyedmousavi et al., 2018). The pathogens take advantage of animals who have their immune system compromised due to environmental stress, like inappropriate temperature and humidity conditions or poor husbandry management and cleanliness, to grow and produce mycotoxins that initiate an immune response leading to infection. This genus is part of the human skin microbiome and, therefore, its presence in the udder can be the result of lack of hygiene of the technicians during routine husbandry practices or milking (Seyedmousavi et al., 2018).

5.2. Antifungal activity of *C. hypocistis*

Pertain to the effect of the *C. hypocistis* extract in the fungi genera isolated, there was a reduction in the number and diversity of fungi isolated in the second collection period compared to the first. However, the samples from the control groups (treated only with the base cream) presented the same decrease therefore, no significant differences were found between the groups treated with the cream enriched with the extract and the control groups which prevents us to make any assumption about the effect that this extract had on the microbiome present in the mice's ears.

As far as the authors' knowledge, this is one of the first works that analysed the effect of this extract *in vivo*. This condition adds multiple variables like the interaction with other microorganism and the host that don't exist in an *in vitro* study and that can greatly impact the action of the extract, and therefore the outcome of the study.

However, to have a better understanding of the effect of this extract *in vivo*, it would have been useful to have had more sampling periods, allowing for the comprehension of how the microbiota evolved throughout the experiment. The study could also have a more uniform microbiota thru out all the mice's which could also led to better results and more conclusions.

The latter *in vitro* procedure with *A. fumigstus* exposed the potential antifungal action of the extract against this fungal species. The IP% calculated suggest that the extract of *C. hypocistis* hindered the growth of the fungal species *Aspergillus fumigatus*, which corroborates the results by Silva et al. (2022). This study also reported that the hydroethanolic extract of *C. hypocistis* exhibited antifungal activity against other species of *Aspergillus* spp. (*Aspergillus niger* and *Aspergillus versicolor*), *Penicillium* spp. and *Trichoderma viride* (Silva et al., 2022).

However, the limitations in the quantity and physical state of the cream did not allow for proper distribution of the extract throughout the medium and the determination of a MIC.

5.3. *Staphylococcus aureus* profile

Although, bacteria from the species *S. xylosus* were identified in the samples, no further analysis was performed to these as they weren't relevant as a mastitis-causing pathogen.

Regarding the identification of the bacterial species, the genetic identification by the detection of the *NUC* genes corroborates the results from the biochemical analyses and API test.

The antibiogram suggested that the strains isolated from both the collection periods are resistant to penicillin G, which is an β-lactam antibiotic. These types of antibiotics have been widely used to treat bovine mastitis for decades and penicillin being one of the first β-lactam to be utilized clinically, many *S. aureus* resistant strains have been isolated from cows with mastitis (Aslantaş and Demir, 2016; Pérez et al., 2020). Studies in China, Iran and Brazil all reported a high prevalence of these resistance *S. aureus* strains isolated from bovine mastitis (Yadav et al., 2018). Thru the years, these bacteria have been able to develop various mechanisms to avoid the effect of these antibiotics, for example, producing penicillinase (β-lactamase), the enzyme responsible for the inactivation of these antibiotics (Aslantaş and Demir, 2016).

The bacterial strains isolated also showed resistance to the antibiotic ceftazidime, which is considered a third-generation cephalosporin. This synthetic β-lactamase inhibitor, binds to penicillin-binding proteins of Gram-negative bacteria, inhibiting the production of β-lactamase in the cell wall (Wang et al., 2020). It can be used in bovine mastitis cases caused by Gram-negative bacteria like *E. coli* (Liu et al., 2018). Although, ceftazidime-resistant strains are not very common, a study conducted in China reported a 41% prevalence of *S. aureus* strains resistant to this cephalosporin. These bacteria were isolated from mastitic milk samples from 288 dairy cows during the years 2005 to 2006 (Shi et al., 2010).

The resistance to antibiotics is certainly an important factor to considerate in the infection process, as it aids in the invasion and colonization of the microbe in the intramammary space (Pérez et al., 2020). However, the bacterial virulence factors also assist the pathogens by promoting the adhesion to the host extracellular matrix components, damaging the host's cells, and fight the immune system (Monistero et al., 2018).

In both S1 and S2, it was possible to detect the absence of the *SED* gene, yet these bacterial strains presented the *SEI* and *SEG* genes. Most of the *S. aureus* strains isolated from mastitis cases were stated to have one or more *SE* genes, being *SEA, SEI* and *SEH* the most frequent ,although, there is a great variability of results due to the large distribution of these genes among these bacterial species in cattle (Monistero et al., 2018; Pérez et al., 2020). Furthermore, it has been reported a significant difference in the frequency of *SE* detection between mastitic and health cows suggesting that these enterotoxins play a role in mastitic infection, possibly in establishing colonizing conditions by compromising the immune response in the mammary gland and the susceptibility to antibiotics of the bacteria (Monistero et al., 2018). Particularly, *SEH* has been showed to cause apoptosis of bovine mammary epithelial cells *in vitro*, and *SEC* induces the release of proinflammatory cytokine, inflammation and therefore, generate mammary tissue damage (Pérez et al., 2020).

The *HLA* and *TSST-1* genes were also detected. The latter superantigen leads to a nonspecific polyclonal activation of several immune cells and release of high levels of cytokines, resulting in a perturbance of the host's immune system. The frequency in which *TSST-1* gene is identified in bacteria from mastitic bovine tends to vary a lot between countries and herds (Pérez et al., 2020).

Regarding *HLA*, it is the most frequently cytotoxin produced by *S. aureus* and is very important in chronic mastitis cases, as it contributed to the persistence of the pathogen in the mammary gland by aiding the bacterial invasion and avoidance of the host's immune system. This gene is also one of the most commonly harboured from *S. aureus* isolated from mastitic cows (Pérez et al., 2020). The linkage between *SEA, SED, SEG* and *SEH* reported by previous studies in strains isolated from bovine was not detected. However, other correlation like *SEC, SEI* and *SEG* or *SEC, SEI* and *TSST-1* might be present, yet further analysis needs to be performed in order to confirm this theory (Monistero et al., 2018). Furthermore, the latter correlation between *SE* and *TSST-1* has been linked to more harsh cases of bovine mastitis due to a more severe inflammation (Pérez et al., 2020).

In previous works, the extracts of *C. hypocistis* were reported to have antibacterial activity against various medically relevant Gram-positive and Gram-negative bacteria, including strains of *S. aureus*, even methicillin-resistant strains (Zucca et al., 2015; Silva et al., 2020). The concentrations of extract present in the creams of 3.1 mg/g of cream for CH1, 6.2 mg/g for CH2 and 12.4 mg/g for CH3, are well above de MIC recorded for these bacteria, 0.625 to 2.5 mg/mL. However, in this study after the application of the cream, it was recorded an increase in the number of samples that presented bacterial colonies of *S. aureus*.

These incongruences can be due to the many variables of the *in viv*o models, like the possible interactions with other microorganisms and/or the environment that aren't present in the *in vitro* experiment previously reported. Moreover, the strain of *S. aureus* isolated isn't the same as the one used in these other works, as this one was isolated from mice and not humans.

6. Conclusion

In this study, it was possible to verify the high fungal biodiversity of the mouse's outer ear and detect the growth inhibition effect of the *C. hypocistis* extract on the fungus, *A. fumigatus*. However, it was impossible to evaluate the properties of this extract *in vivo*, as the reduction of biodiversity observed after the treatment was also evident in the control groups samples, and therefore no assumptions could be done over the potential of these extract as an alternative preventive measure/ treatment against bovine mastitis.

On the other hand, the multiple bioactive properties of the extract *in vitro* have been vastly reported making the extract of *C. hypocistis* a very interesting product and with potential to be used in many scientifical fields, like animal science. However, not many *in vivo* studies have been made so far and hence, must be done to properly understand the impact this extract can have when applied in living organisms.

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