Investigating the effects of fungal community structure and interactions on *Aspergillus fumigatus* from kākāpō (*Strigops habroptilus*) nest soils.

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Abstract

New Zealand's rare and endemic parrot species, kākāpō (*Strigops habroptilus*), have suffered aspergillosis outbreaks during their last two breeding seasons (2019 and 2022). The fungal causative agent, *Aspergillus fumigatus*, typically infects the airways of birds and mammals that are immunosuppressed or exposed to high spore loads. However, monitoring by the Department of Conservation found that *A. fumigatus* was almost undetected in 2019 nest soils, even in those linked to infected individuals. This prompted an investigation into other ecological drivers of disease proliferation in kākāpō. As microorganisms can co-infect, suppress host immunity, or alter metabolite production through competition, this thesis investigated how *A. fumigatus* may interact with other soil fungi within kākāpō nests and whether such interactions may help explain disease outcomes observed in kākāpō during recent aspergillosis outbreaks.

Aspergillus species and fungal communities within kākāpō nest soils were investigated using culture-based methods (viable counts), DNA community analysis (ITS amplicon sequencing), and experimental co-cultivation assays. Kākāpō nest soils were collected from two breeding islands, Whenua Hou (Codfish Island) and Pukenui (Anchor Island), across three nest microhabitats (cavity, entrance, and two metres away) during and after the 2019 and 2022 breeding seasons. Samples included soils from both aspergillosis-affected and unaffected nests, allowing comparisons between nests linked to infection and nests associated with healthy birds.

Across abundance analyses (viable abundance and DNA relative abundance), *A. fumigatus* was found at low abundances relative to other *Aspergillus* species and yeasts, despite being the confirmed disease-causing agent for the kākāpō aspergillosis outbreaks. Yeasts were highly abundant in 2022 nest soils (particularly within cavities and entrances), and their presence was negatively associated with *Aspergillus* spp. counts across both years, suggesting potential competitive interactions. Statistical modelling showed that nests associated with aspergillosis in 2019 contained higher overall *Aspergillus* loads, largely driven by *A. niger* and *A. flavus* rather than *A. fumigatus*, implying that infection may arise from complex community interactions rather than a single causal species.

Fungal community analyses revealed that richness and composition was more strongly driven by the nest microhabitat and island, than by disease status/outcome. Fungal richness increased with distance from nesting burrows on both islands, while fungal community composition differed between islands only within their nest microhabitat. This indicates that kākāpō nesting activity strongly effects fungal diversity and composition within nesting burrows. Dominant yeasts such as *Apiotrichum*, *Debaryomyces*, and *Kazachstania* were also prevalent in kākāpō faeces in a previous study, and their relative abundance decreased once chicks fledged and shifted diets, supporting the idea that diet influences the nest mycobiota.

Experimental co-cultivation assays confirmed that many of these nest-associated yeasts can suppress *A. fumigatus* and *A. niger* growth and conidiation (sporulation). Most isolates inhibited mycelial expansion and suppressed conidiation, with *Meyerozyma guilliermondii* showing the strongest inhibitory effect. These interactions may contribute to the low *A. fumigatus* loads observed in nest soils, though they also raise questions about the dual roles of yeasts as potential biological controls or contributors to disease risk (as opportunistic pathogens or enhancers of *A. fumigatus* pathogenicity).

Taken together, this thesis demonstrates that aspergillosis risk in kākāpō cannot be explained by *A. fumigatus* soil abundance alone, and that disease outcomes are likely shaped by complex ecological processes within the nest, including microbial interactions, host behaviour, and diet-driven changes. By integrating cultivation, sequencing, and competition assays, this work provides an ecological perspective on kākāpō nest fungi and delivers management-relevant insights for monitoring soils, dietary effects, and microbial interactions to help mitigate future aspergillosis outbreaks.

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor used artificial intelligence tools or generative artificial intelligence tools (unless it is clearly stated, and referenced, along with the purpose of use), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed by Amber Kanis

31/10/2025

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AI tools were occasionally utilised for structural feedback and refining R code for data visualisation.

1 Chapter 1: Introduction & Literature Review

1.1 Introduction

1.1.1 Emerging wildlife disease

It is estimated that wildlife extinction rates are 100-1000 times higher in recent years compared to theorized natural background extinction rates (Cowie et al., 2022; World Wildlife, 2024). This is largely attributed to the rise of anthropogenic activities such as population growth, globalisation, industrialization, and colonisation, ultimately resulting in large-scale habitat destruction. For example, one-third of the planet's original forests have been lost to anthropogenic activity (Ritchie, 2021), which has also led to the classification of more than 166,000 species worldwide as 'threatened', and over 46,300 species at risk of extinction (IUCN, 2024).

Other than direct biodiversity loss, anthropogenic change may also influence wildlife health by increasing disease emergence and transmission. Habitat modification or fragmentation associated with urbanisation can elevate disease risk, such as through facilitating pathogen transmission via increased host densities between wildlife, humans, and domestic animals, and by causing stress-induced immunosuppression (Brearley et al., 2010; Brearley et al., 2012; Brearley et al., 2013; Johnstone et al., 2011; Johnstone et al., 2012; Partecke et al., 2006). As animals are forced into smaller or marginal habitats, predation resulting from enhanced exposure, and competition for limited resources may intensify, inducing chronic stress. Stress responses, mediated by glucocorticoid hormones (Hussain, 2010; Talbott, 2007), can impair immune function and increase susceptibility to infection (Cohen & Williamson, 1991; Martin, 2009; McLean, 1982; Sapolsky et al., 2000). Human-induced stress in wildlife is often associated with direct human presence or handling (Gartrell et al., 2014). In New Zealand, for example, yellow-eyed penguins (*Megadyptes antipodes*) exposed to unregulated tourism show heightened physiological stress compared to less-disturbed colonies (Ellenberg et al., 2013; Ellenberg et al., 2007).

Large-scale human movement can also act as vectors for the spread of pathogens beyond their native range, which is a process termed "pathogen pollution" (Daszak et al., 2001; Williams et al., 2002). In New Zealand, this is exemplified by the likely introduction of non-endemic *Plasmodium* spp. strains (causing avian malaria) via the European blackbird (*Turdus merula*) (Alley & Gartrell, 2019; Schoener et al., 2013), a species deliberately introduced by early English settlers between 1867 and 1879 to evoke familiarity with their homeland, particularly through its song (Te Ara - the Encyclopedia of New Zealand, 2006). Since its arrival, 17 different lineages of avian malaria have been recorded in 37 indigenous New Zealand bird species (Schoener et al., 2024), including threatened taxa such as mohua (*Mohua ochrocephala*) (Alley et al., 2008), dotterel (*Charadrius obscurus*) (Schoener et al., 2013),

South Island saddleback (*Philesturnus carunculatus carunculatus*) (Alley et al., 2010), little penguins (*Eudyptula minor*) (Sijbranda et al., 2017), hihi (*Notiomystis cincta*), and two species of kiwi (*Apteryx* spp.) (Alley et al., 2012; Banda et al., 2013; Howe et al., 2012). Many of these species, however, have also faced habitat loss and predation from introduced mammals following human colonisation, which along with factors such as breeding stress and loss of genetic diversity, may have played a role in infection also.

As a more indirect influence, global warming is said to possibly increase wildlife disease by expanding pathogen ranges, intensifying outbreaks after climate extremes, and providing more favourable environmental conditions for infection. For example, warmer climates have enabled mosquitos and consequently *Plasmodium relictum* to invade higher elevations in Hawai‘i, driving avian malaria into previously cooler refuges for native birds (Atkinson et al., 2014; Kilpatrick et al., 2024). Warming is also projected to broaden the climatic niches of amphibian chytrid fungi (*Batrachochytrium dendrobatidis* and *B. salamandrivorans*) (Sun et al., 2023), and extreme sequences like drought followed by flooding have triggered sharp post-rain surges of *B. dendrobatidis* infections in frogs (Sasso et al., 2024). Another example is the white-nose syndrome fungus (*Pseudogymnoascus destructans*), which typically infects bats. This fungus is described as psychrophilic (cold loving) and are found in winter hibernation sites (Wibbelt, 2018). Although a warming climate may be expected to decrease the niche of psychrophilic fungi, recent research shows that in the United States of America (USA) many southern hibernacula are still within and even closer to the fungus's optimal growth range (Sirajuddin et al., 2024). Although this may suggest that the species is cold-tolerant rather than truly cold-loving, it raises concerns about how many other psychrophilic (or psychrotolerant) pathogens could experience increased growth and transmission under a gradually warming climate.

Overall, anthropogenic change can heighten wildlife immunosuppression through stress, facilitate pathogen introduction via human-mediated movement, and increase pathogen prevalence as wider regions become more favourable under climate change. These ecological processes are consistent with the disease triangle framework (Figure 1.1) used to describe disease incidence in wildlife and plants (Scholthof, 2007; Turner et al., 2011), where disease incidence is typically a result of three factors combined: pathogen presence, a favourable environment causing proliferation and a host that is susceptible (e.g., immunosuppressed and/or naive).

1.1.2 The threat of fungal disease

Fungal diseases pose a serious and growing threat, yet they remain understudied in infectious disease research compared to viral and bacterial diseases (Barroso et al., 2021; Dobson & Foufopoulos, 2001; Tompkins et al., 2015). Global surveillance data show that fungal disease alerts are increasing. ProMED alerts rose from 1% to 7% between 1995 and 2010, and HealthMap alerts from 0.1% to 0.3% between 2007 and 2011 (Fisher et al., 2012). Climate change is expected to expand the geographic ranges of many pathogens, likely amplifying this trend. However, due to a historic lack of research attention,

effective vaccines, and limited treatment options for fungi, we are poorly prepared to manage emerging fungal threats (Chechi et al., 2023). Human fungal infections have been historically neglected as a field of study (Brown et al., 2012; Denning, 2024) despite causing approximately 3.8 million deaths annually (Denning, 2024). This neglect is also tied to fundamental biological differences between viruses, bacteria and fungi, largely because the characteristics of viruses and bacteria make them more amenable to study and control.

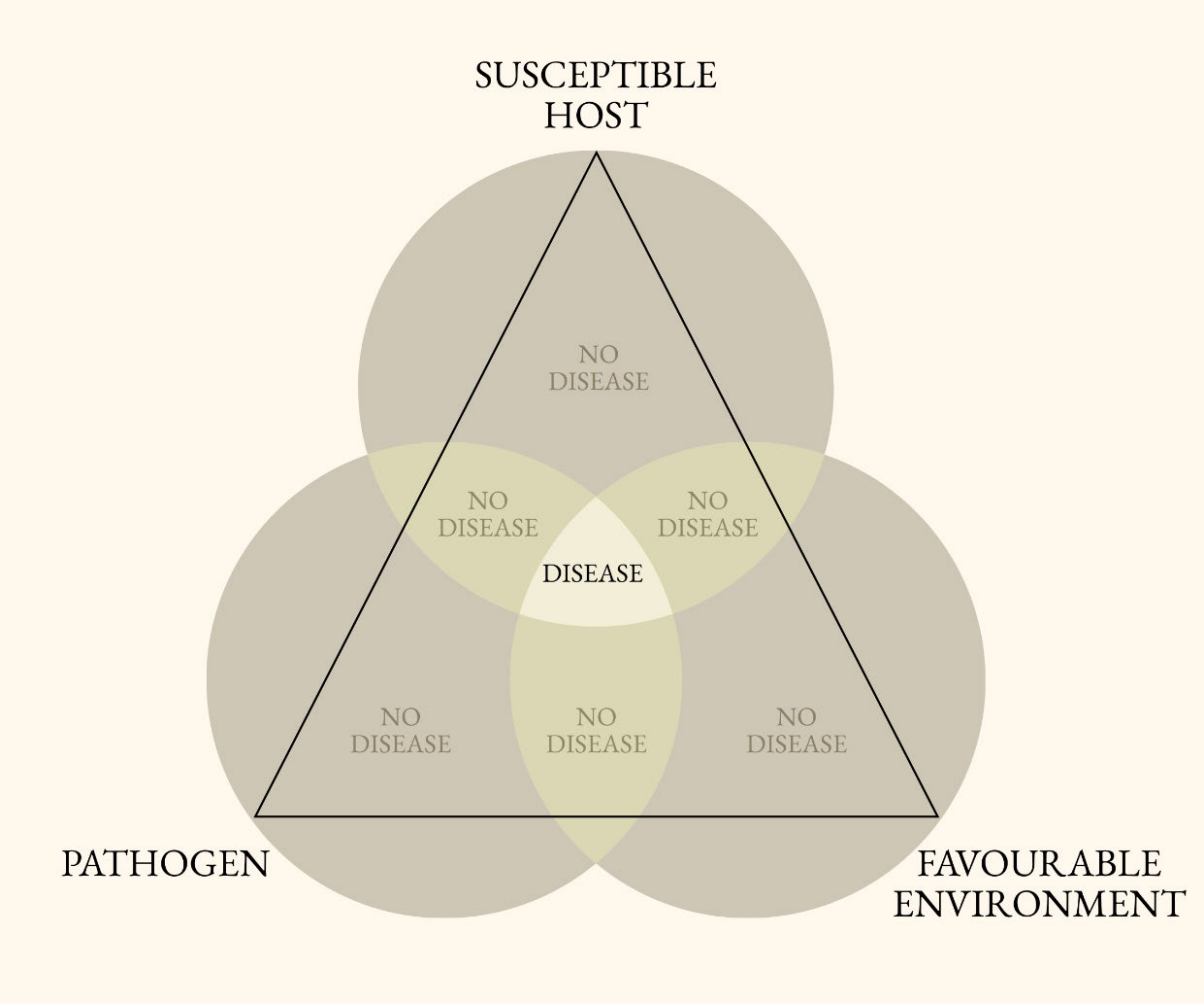

Figure 1.1 Disease triangle representing the contributing factors that must come together for a disease to proliferate. Made using Canva (2024).

This is likely because fungal diseases are considered less communicable and mostly associated with immunosuppressed individuals (Casadevall, 2018; Rodrigues & Nosanchuk, 2020). However fungal infections are increasingly recognised as major threats to both human and wildlife health. Approximately 6.5 million people are diagnosed annually with invasive fungal infections, and this number is likely higher due to under-reporting (Williams et al., 2024). In wildlife, fungal pathogens account for around 70% of known pathogen-driven animal extinctions worldwide (Fisher et al., 2012).

Viruses and bacteria differ markedly from fungi in ways that contribute to their dominance in disease research. Viruses are extremely small (20–100 nm), lack metabolic machinery, and rely entirely on host

cells for replication, producing hundreds of new virions per cell and often triggering acute, high-mortality infections (Chappell & Dermody, 2014; Lostroh, 2024; Weitzman & Fradet-Turcotte, 2018). Their rapid replication and transmission allow them to spread efficiently through healthy populations, causing more acute outbreaks with high mortality, while their relatively simple structure makes them more tractable for vaccine development (French et al., 2023; Kuhn et al., 2021). Bacteria are larger (0.4–3 μm^3) and more complex than viruses but are still structurally simpler than eukaryotes, lacking nuclei and specialised organelles (Levin & Angert, 2015; Starr et al., 2013). They are ubiquitous in the environment, reproduce rapidly (e.g., *E. coli* can divide every 20 minutes), and employ well-characterised virulence mechanisms including adhesion, invasion, and toxin production (Chappell & Dermody, 2014; Lostroh, 2024; Weitzman & Fradet-Turcotte, 2018). Their prokaryotic nature also makes them easier to study and target with treatments that spare host cells (Alberts et al., 2002). Together, these features have made viruses and bacteria more accessible to research and surveillance efforts, and more amenable to therapeutic intervention, helping explain their disproportionate representation in disease literature (Gibson et al., 2018; Levin & Angert, 2015; Wilson et al., 2002). In contrast, fungi are eukaryotic, with cells and genetic machinery similar to those of animals, which complicates research and therapy. Because fungal cells share many pathways and structures with animal cells, it is difficult to find drug targets that do not also cause collateral damage to the host (Segal & Elad, 2018). This similarity limits vaccine development (Chechi et al., 2023; Oliveira et al., 2021) and increases side effects of antifungals like Amphotericin B, which targets ergosterol (structurally similar to cholesterol in animal membranes) causing nephrotoxicity (Laniado-Laborín & Cabrales-Vargas, 2009). Overall, factors that may explain their underrepresentation in the literature are fewer treatment options, slower replication, and more complex biology.

Nonetheless, fungal diseases pose serious risks. Their slower growth is associated with chronic infections, thus prolonged treatment courses (French et al., 2019; Reddy et al., 2022), along with antifungal resistance in biofilms or during dormant states (Berman & Krysan, 2020; de Castro et al., 2023; Rosowski et al., 2018; Silva et al., 2017). Unlike bacteria, many fungi reproduce through the release of large numbers of airborne spores, contributing to widespread environmental persistence and continuous host exposure (Caillaud et al., 2018; Kauserud et al., 2011; Puhlmann et al., 2023). Like fungal spores, endospores produced by some species of bacteria are also capable of surviving harsh environmental conditions for extended periods (Dijksterhuis, 2019; Paul et al., 2019). However, spore-forming bacteria typically produce only a single endospore under unfavourable conditions (Cutting, 2011; Erkmen, 2021), compared to fungal spore production that is reproductive and prolific.

In summary, viral and bacterial infections are a large focus in scientific literature because they cause acute, high-impact diseases and offer clearer targets for intervention, whereas the similarity of fungal cells to that of animals, their subtler disease progression, and the relative scarcity of antifungal treatments have caused medical mycology to lag behind (Brown et al., 2012; Fisher et al., 2012).

However, the increasing incidence and severity of fungal infections worldwide make it clear that this imbalance in research needs to be addressed. Emerging fungal pathogens pose a growing threat in a world undergoing accelerated environmental change, where disturbance, habitat fragmentation, extreme climate events and expanded pathogen geographic ranges may cause unpredictable fungal outbreaks. Closing the knowledge and investment gap in fungal disease research is crucial to develop the diagnostics, treatments, and preventive measures (medical and environmental) needed to avert potentially devastating consequences of fungal epidemics.

1.1.3 Aspergillosis and *Aspergillus fumigatus*

Aspergillosis is one of the most prevalent fungal diseases worldwide and is increasingly recognised as a potential emerging threat under a changing climate. The primary agent, *Aspergillus fumigatus*, is a saprophytic, mesophilic, and thermotolerant fungus that often exhibits antifungal resistance (Beernaert et al., 2009; Burks et al., 2021; Meis et al., 2016; Nascimento et al., 2003; Toyotome et al., 2018; Ziółkowska et al., 2014) and remarkable ecological flexibility. These traits enable it to thrive in a wide range of environments (Kwon-Chung & Sugui, 2013), including disturbed habitats where other fungi fail to persist (Liu et al., 2024). Rising global temperatures, more frequent wildfires and droughts, and extensive agricultural azole use are expected to further favour its growth, dispersal, and evolution of resistance, ultimately expanding environmental reservoirs and increasing host exposure risk (Korfanty et al., 2023; Salazar-Hamm & Torres-Cruz, 2024; Seidel et al., 2024; van Rhijn et al., 2025). *A. fumigatus* is considered an opportunistic pathogen due to its natural role as a saprophyte yet can cause infection when hosts are immunocompromised or exposed to exceptionally high conidial (spore) loads. It is responsible for approximately 90–95% of human and avian aspergillosis cases (Arné & Lee, 2020; Marr et al., 2002; Tell, 2005), although other species such as *A. niger* and *A. flavus* are occasionally implicated.

Aspergillosis is a respiratory disease whereby infection by *A. fumigatus* typically occurs from inhaling airborne conidia (Latgé & Chamilos, 2019). Although *A. fumigatus* is primarily a saprophytic fungus that does not depend on a host for survival, its small (2–3 μm), buoyant, and highly abundant conidia make it an effective opportunistic pathogen, easily inhaled and capable of reaching the air sacs and lung alveoli (O'Gorman, 2011). Additionally, its thermotolerance and mesophilic nature (optimal growth range: ~37–40 °C) allow for frequent infection of warm-blooded animals (Chang et al., 2004; J. P. Latgé, 1999; McCormick et al., 2010).

In avian species, aspergillosis is arguably more prevalent as they are more susceptible to infection than mammals, mostly because birds lack certain anatomical defences and immune system functions which help prevent and combat infections (Tell, 2005) (see Section 1.2.3). In New Zealand, many native and endemic birds have been affected by aspergillosis, many of which can be attributed to disease susceptibility in populations that have experienced genetic bottlenecks (see Section 1.2.4). However, a

portion of these cases also took place in relatively healthy populations or individuals (e.g., kea and brown kiwi). As climate temperatures and environmental changes continue to increase, it is possible that diseases such as aspergillosis will become more prevalent, likely as a result of increased geographic range, environmental loads, or perhaps changes in overall microbial communities which can increase pathogenicity.

1.2 Literature Review

1.2.1 *Aspergillus fumigatus* pathogenicity

Aspergillus fumigatus derives from the class Eurotiomycetes (phylum Ascomycota), typically associated with microscopic fruiting bodies, unlike common mushrooms produced by species from the phylum Basidiomycota. The microscopic fruiting bodies specific to *Aspergillus* species are called "conidiophores" which produce "conidia" (spores). In comparison to those produced by other *Aspergillus* species, *A. fumigatus* produces the smallest conidia, about two to three microns in size (Sugui et al., 2014). This trait is a large contributor to pathogenicity. For instance, their conidia are able to: 1) access small spaces in the respiratory tracts of animals, such as the alveoli in human lungs, 2) travel large distances making it highly ubiquitous, and 3) remain airborne for longer periods of time, prolonging exposure and increasing the risk of inhalation (J. P. Latgé, 1999; Mullins et al., 1976; Mullins et al., 1984; Murali et al., 1993; Vesper et al., 2007). *A. fumigatus* also produces thousands of conidia per conidial head (Figure 1.2), which not only further increases its prevalence but can completely overwhelm the immune system with lack of intervention from wind, ventilation, or sufficient air filtration.

Aspergillus fumigatus along with other *Aspergillus* species also produces mycotoxins, primarily aflatoxins, within their conidia (Bennett & Klich, 2003; Perrone & Gallo, 2017; Taniwaki et al., 2018). Gliotoxin is the most common aflatoxin produced by *A. fumigatus*, which is 96% responsible for virulence in human *A. fumigatus* infections (Ghazaei, 2017). The mechanism of action for gliotoxin primarily depends on the intact disulfide bond within its molecular structure which allows for interaction with various host cell types, ultimately disrupting immune system functions (Scharf et al., 2012), such as inhibiting T-cell activation and macrophage phagocytosis (Bennett & Klich, 2003). Therefore, the production of gliotoxin by *A. fumigatus* further increases its pathogenicity and virulence by exacerbating immunosuppression, allowing for sustained growth within the airways.

A. fumigatus also prefers higher temperatures for optimal growth (35-40°C) compared to *A. flavus* (28-37°C) (Hernández-Benítez et al., 2025) and *A. niger* (30-35°C) (Hayden & Maude, 1994) allowing for more successful establishment in mammalian and avian respiratory tracts (Chang et al., 2004; J. P. Latgé, 1999; McCormick et al., 2010). The species can also tolerate temperatures of 46-55°C and has

been previously observed at 70°C (Haines, 1995; Raper & Fennell, 1965), which is why they are commonly described as thermophilic, although more accurately described as thermotolerant.

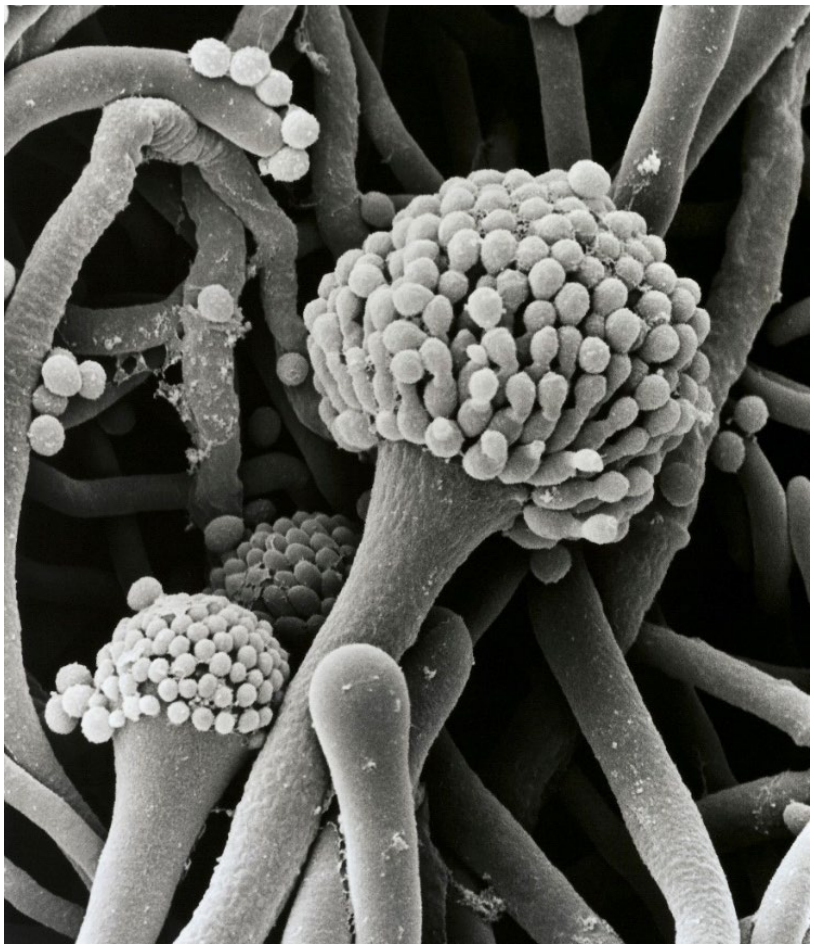

Figure 1.2 *Scanning electron micrograph of* Aspergillus fumigatus *showing conidial head structure.* Image by David Gregory & Debbie Marshall, *Aspergillus*: 3 stages of conidiophore (SEM), Wellcome Collection / JSTOR Open Artstor Collection. © Wellcome Collection, licensed under CC BY 4.0. https://www.jstor.org/stable/community.24713462

1.2.2 *Aspergillus fumigatus* environmental preferences & tolerances

Along with high temperatures, high levels of moisture are also a cause for proliferation of *A. fumigatus*. In a study of leaf litter in zoos across New Zealand (Glare et al., 2014), high soil levels of *A. fumigatus* (5,000-1,000,000 CFU g^{-1}) and two kiwi aspergillosis deaths in a brown kiwi (*Apteryx mantelli*) house were assumed to be due to prolonged storage of leaf litter in humid conditions (within plastic bags). Studies regarding aspergillosis outbreaks in hihi on Mokoia Island, New Zealand, showed that high levels of *A. fumigatus* in soils along with other factors were likely due to high humidity as well as higher soil temperatures (Perrott, 2001). In this same study, high *A. fumigatus* levels were also found in young-growth forests with canopy gaps and forest edges allowing for increased exposure of sunlight to soils, subsequently leading to higher soil temperatures. Additionally, the area encompassing the sampling

location (Rotorua, New Zealand) is also renowned for its geothermal activity. High *A. fumigatus* levels were also observed in hihi nest boxes on Mokoia Island, as well as tree cavities on Little Barrier Island, which were associated with higher humidity levels (Alley et al., 1999; Perrott, 2001; Perrott & Armstrong, 2011).

Although humidity is often linked to *A. fumigatus* proliferation, the species can also persist under dry conditions and has been isolated from arid and semi-desert soils (Giusiano et al., 2017). Its conidia have also been shown experimentally to withstand prolonged desiccation, remaining viable for extended periods and germinating rapidly when moisture returns, demonstrating a strong capacity for dormancy and environmental persistence (Segers et al., 2023). These small, lightweight, and hydrophobic spores are easily lifted by air currents or disturbance, meaning that dry, dusty environments can greatly enhance their dispersal and the likelihood of exposure (Kwon-Chung & Sugui, 2013; Paulussen et al., 2017).

Soil disturbance is also associated with increased *A. fumigatus* growth. A doctoral study by Perrott (2001) found high levels of *A. fumigatus* in young regenerating forests containing canopy gaps, forest edges, and areas where leaf litter was removed. This was also due to greater exposure of soil to sunlight, ultimately creating ideal temperatures for *A. fumigatus,* whilst simultaneously reducing competition if less thermotolerant species. Increased light was also found to encourage conidiation (sporulation) in other *Aspergillus* species (Hill, 1976; Rinaldi, 1983), which could have explained the high *Aspergillus* spp. levels found in previous work where nocturnal captive kiwi enclosures were exposed to extended periods of artificial lighting (Truter-Meyer, 2021). Additionally, substrate disturbance or agitation (e.g., of soil or decomposing matter) can cause increased aerosolization of conidia, which may ultimately increase colony establishment (O'Gorman, 2011; Williams et al., 2019). For example, it was identified that in compost heaps, aerial concentrations of *A. fumigatus* downwind from agitated compost piles were significantly higher than that downwind from stationary piles (Millner et al., 1980). Overall, the buoyancy and excessive abundance of released conidia along with its preference for high temperatures, makes *A. fumigatus* widespread across all habitats and one of the most prevalent facultative pathogens globally.

1.2.3 Avian susceptibility

Avian species lack certain anatomical defences and immune system functions (Figure 1.3), making them more susceptible to infection than mammals. For example, in humans (and other mammals), structures like the epiglottis block particulate matter from entering the airways, while the diaphragm facilitates the cough reflex to expel debris, providing critical protection against respiratory infections (Tell, 2005).

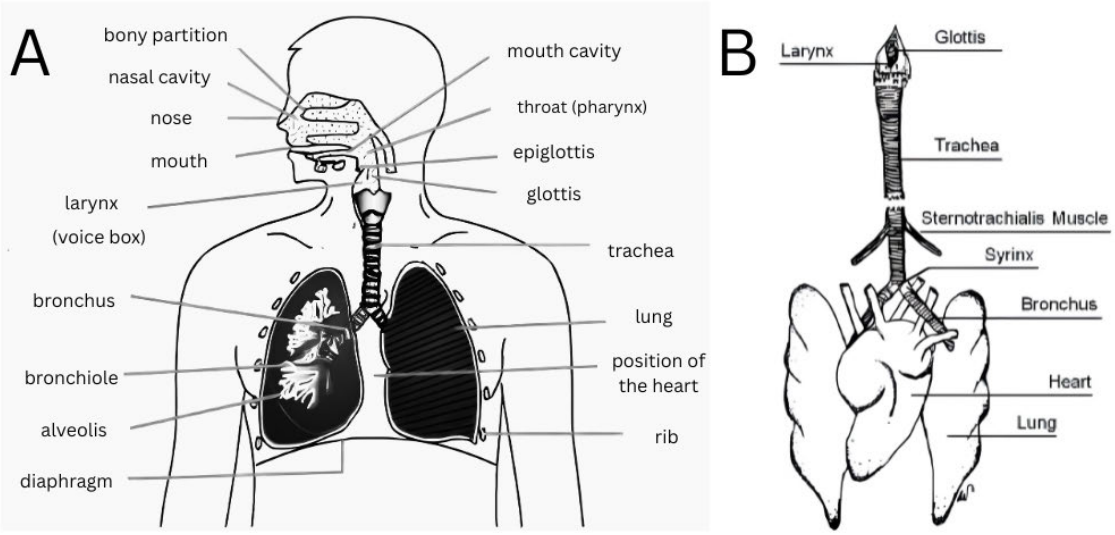

Figure 1.3 Comparison of the human and avian respiratory systems. Human respiratory anatomy (A) and avian respiratory anatomy (B). Adapted from Mawaishasan (Wikimedia Commons, CC BY-SA 4.0) and Dr. Jacquie Jacob, University of Kentucky (Wikimedia Commons, CC0 1.0). Additional labelling and figure composition by the author.

For immune system functioning, humans produce more abundant and advanced white blood cells for phagocytosis of foreign bodies and pathogens. Avian species lack surface macrophages for phagocytising *Aspergillus* species conidia, and they possess the avian equivalent of human neutrophils (heterophils), which are less effective against killing hyphae (Harmon, 1998; Klika et al., 1996). Human neutrophils have more effective mechanisms against hyphal invasion, which includes oxidative mechanisms, such as the production of myeloperoxidase (MPO). This an enzyme can form hypochlorous acid (HOCl) from chloride ions (Cl^-) and hydrogen peroxide (H_2O_2), allowing for effective ingestion of microbial pathogens. Avian heterophils in comparison demonstrate less efficient MPO activity and lower concentrations overall, along with simply being less effective at phagocytosis.

Aspergillus species can also sporulate in the bodies of avian species, which is not commonly observed in humans and other mammals (Arné et al., 2021; Richard et al., 1981; Stedham et al., 1968). This can increase the chances of mortality as sporulation accelerates growth and colonisation of the respiratory organs. Tell (2005) explains that sporulation may be possible in birds due to their cavernous air sacs and a warm core body temperature (38-45°C) which promotes conidial germination. They also suggest that birds are sensitive to gliotoxin, which could possibly result in tissue necrosis, creating a nutrient rich environment for further growth. The higher sensitivity of birds to gliotoxin is likely due to their inability to 'detoxify' gliotoxin to its less toxic, inactive form; bis(methyl)gliotoxin (bmGT), which is an ability humans possess (Reidy et al., 2022).

1.2.4 Susceptibility of New Zealand avian species to aspergillosis

Many New Zealand native and endemic avian species have been subject to aspergillosis outbreaks in the past few decades. Most of these case studies attribute these outbreaks to stress, which leads to

immunosuppression, whether it be from breeding stress or anthropogenic factors such as increased human handling and habitat changes (Gartrell et al., 2014; Perrott, 2001; Perrott & Armstrong, 2011). However, another common factor shared by New Zealand avian species is low genetic variation, which can be a contributing factor to the higher disease susceptibility observed in endangered species.

Low genetic variation in New Zealand's native birds is largely due to the mass declines and extinctions that began following human colonisation (Jamieson, 2009). As of 2021, around 83% of Aotearoa's native bird species were considered *Threatened* or *At Risk* of extinction under the New Zealand Threat Classification System (Robertson et al., 2021). These declines were largely caused by two major anthropogenic factors: 1) the introduction of mammalian predators and 2) habitat degradation. New Zealand harbours a unique ecosystem where endemic terrestrial vertebrates are predominantly avian (Gibb et al., 2015; Mitchell et al., 2014; Mitchell et al., 2016) due to the hypothesised absence of mammalian predators during the country's long isolation from Gondwana around 80 million years ago (Trewick & Gibb, 2010; Worthy et al., 2017). It has been theorized that the lack of mammalian predators allowed many endemic bird species to develop behavioural naïveté to mammalian predation (Duncan & Blackburn, 2004; Lawrence, 2018), as well as flightlessness, leaving them with a disadvantage for escape.

Human colonisation has also resulted in the loss of approximately three quarters of New Zealand's native forest cover, leading to major habitat degradation for other natives as well as avian (Ewers et al., 2006). These direct and indirect anthropogenic effects have resulted in the extinction of 51 native bird taxa, with a further 170 taxa currently listed as threatened or at risk under the 2021 New Zealand Threat Classification System (Robertson et al., 2021). Approximately 16 flightless species remain, including the renowned kiwi (*Apteryx* spp.), which is a national New Zealand icon and a taonga species (treasure to the indigenous Māori of New Zealand).

Past New Zealand native and endemic birds that have been affected by aspergillosis, include the North Island brown kiwi (*Apteryx mantelli*) (Glare et al., 2014), hihi (*Notiomystis cincta*) (Alley et al., 1999; Cork et al., 1999; Ewen et al., 2012; Perrott, 2001; Perrott & Armstrong, 2011), kākāpō (*Strigops habroptilus*) (Alley & Gartrell, 2019; Arné et al., 2021; Meda Gedara, 2021; West et al., 2025; Winter et al., 2022), yellowheads (*Mohoua ochrocephala*) (Arné et al., 2021), blue penguins (*Eudyptula minor*) (Hocken, 2000), New Zealand dotterels (*Charadrius obscurus aquilonius*) (Gartrell et al., 2014), the north island robin (*Petroica longipes*) (Low et al., 2005) and more recently a few kea (*Nestor notabilis*) from (Wellington Zoo Te Nukuao, 2024).

Studies have found hihi (*Notiomystis cincta*) to be particularly susceptible to aspergillosis. Alley, Castro, and Hunter (1999) found that aspergillosis was the confirmed cause of death in 19% of hihi examined post-mortem on Mokoia Island, New Zealand. A further 44% of deaths were likely attributable to the disease based on observed clinical signs prior to death or disappearance. Another

study found aspergillosis to be the most significant cause of death for hihi, affecting 35.5% of the sample population (Cork et al., 1999). It was speculated that aspergillosis outbreaks in hihi were a result of immunosuppression caused by the associated stress with their breeding season (Alley, 2002). In 2011, 60 New Zealand dotterels were rescued from the cargo vessel Rena oil spill, of which six individuals died and 100% of those deaths were attributed to aspergillosis (Gartrell et al., 2014). This study linked this outbreak to stress caused by the oil spill and increased handling when being rescued and treated. Kiwi have also experienced multiple aspergillosis outbreaks. In 2013, eight ōkārito brown kiwi (*Apteryx rowi*) died of aspergillosis, due to suspected immunosuppression from nematode infections, and two North Island brown kiwi (*Apteryx mantelli*), likely due to increased conidial loads within their habitat (Glare et al., 2014). Additionally, from 1997 to 2011, 17 kiwi died of airsacculitis and pneumonia as a result of aspergillosis infection (Glare et al., 2014).

In 2019, nine kākāpō died of aspergillosis, prompting several studies aimed at understanding the species' susceptibility to the disease (Alley & Gartrell, 2019; Arné et al., 2021; Meda Gedara, 2021; West et al., 2025; Winter et al., 2022). These studies suggest that kākāpō may be particularly vulnerable to infection due to factors such as immunosuppression from breeding season stress, higher seasonal temperatures, and potentially human-induced spread, especially in relation to the recent outbreaks in 2019 and 2022. However, no single cause has been definitively identified, highlighting the need for further research to safeguard this critically endangered species.

Although stress-induced immunosuppression may be a factor of aspergillosis contraction in our native birds, it has been theorised that (Arné et al., 2021) healthy birds can also contract aspergillosis (Perrott, 2001; Tell, 2005). For example, in an experimental model, Beernaert and colleagues (2008) found that immunocompetent pigeons, inoculated with *A. fumigatus* directly into the thoracic air sac, experienced rapid mortality, whereas immunosuppressed birds inoculated in the lung or trachea developed slower, chronic infections. The study suggested that this acute outcome in healthy birds was driven by an intense inflammatory response leading to disseminated infection, while immunosuppression dampened inflammation and delayed disease progression. Thus, while healthy birds are generally resistant under natural conditions, these experiments illustrate how breaches in physical barriers or extreme spore exposure can still trigger disease.

Altogether, many of the NZ avian species previously identified as vulnerable to aspergillosis are also considered threatened due decreased population sizes (BirdLife International, 2017a, 2017b, 2018, 2022a, 2022b). In some instances, this could result in low genetic variation and possibly higher susceptibility to infection or disease outbreaks. Aspergillosis, therefore, remains a significant concern for New Zealand conservation efforts, and may also impact the preservation of endangered species worldwide.

1.2.5 Aspergillosis in the critically endangered kākāpō

The kākāpō (*Strigops habroptilus*), one of Aotearoa's, and arguably the world's, most 'at risk' avian species, holds the status of critically endangered according to the International Union for Conservation of Nature (IUCN), with a population of only ~237 individuals remaining (BirdLife International, 2018; Department of Conservation, 2024). Kākāpō are unique as they are the heaviest and only nocturnal, flightless parrot in the world. Their population decline has been a result of habitat degradation, mammalian predation, low fecundity rates and periods of sex bias towards males (Clout et al., 2002; G. Elliott et al., 2001; Lloyd & Powlesland, 1994; Robertson et al., 2006). While their current population size is alarmingly low, it represents a significant recovery from the 1990s when the kākāpō population had dwindled to just about 50 individuals (Department of Conservation, 2024; Dussex et al., 2021; G. Elliott et al., 2001). Since then, translocations to predator-free islands, intensive recovery and breeding programs have been essential in their resurgence. In the 1860s, explorer and Fiordland land surveyor Charlie Douglas described the kākāpō as highly abundant:

"The birds used to be in dozens round the camp, screeching and yelling like a lot of demons, and at times it was impossible to sleep for the noise" (Grzelewski, 2002).

"You could shake a tree and the kākāpō could fall down like apples." (Douglas & Pascoe, 1957).

The scene described by Douglas, in stark contrast to the state of the kākāpō 100 years later, highlights the rapid impact of human colonisation in pushing a species to the brink of extinction. Unfortunately, the dire consequences that kākāpō have recently faced have therefore required human intervention for their subsequent recovery.

The Kākāpō Recovery programme, run by New Zealand's Department of Conservation, have implemented strict practices involving intensive management on mammalian predator free islands, and ex-situ wildlife management facilities such as Auckland Zoo (Department of Conservation). Although these practices have been successful thus far, the consequence of breeding after a genetic bottleneck will have led to low genetic variation, and possibly increased susceptibility to disease.

During the 2019 breeding season on Whenua Hou Island, kākāpō experienced an aspergillosis outbreak, affecting 21 individuals and causing death in nine (two adults and seven chicks) (Department of Conservation, 2022). In the following breeding season of 2022, an aspergillosis outbreak took place on another breeding island, Pukenui, with seven affected and one death. The following breeding season is expected to occur in 2026 and projected to be the biggest breeding season on record. With concerns of another aspergillosis outbreak, identifying environmental causes and drivers are vital for ensuring successful population recovery going forward.

Currently, it is speculated that kākāpō susceptibility to aspergillosis may be a result of low genetic diversity. Notably, the outbreaks occurred during two consecutive breeding seasons, where females and

their chicks were predominantly affected. Female kākāpō are solely responsible for raising their young (Cockrem, 2006), and chicks would have had limited time to develop a robust immune response. As such, it is reasonable to assume that stress-induced immunosuppression in the females, coupled with the underdeveloped immune systems in chicks, played a key role in disease occurrence. However, these factors alone may not fully account for the outbreaks, as they have only emerged in the two most recent breeding seasons, pointing to the possibility of other underlying factors contributing to the recent emergence of aspergillosis.

1.2.6 Current treatments and prevention strategies for avian aspergillosis

1.2.6.1 Clinical Treatment

Despite the availability of numerous antimycotic products and clinical methods for treating aspergillosis, the associated limitations of treating wildlife in-situ often lead to treatment failure. This includes difficulties with initial diagnosis (Ewen et al., 2012), identification of a suitable treatment, and detection of treatment success (P. Arné et al., 2011; L. A. Beernaert et al., 2010; Krautwald-Junghanns et al., 2015). Additionally, many of these treatments are also known for producing various side effects (commonly hepatotoxicity) (Bechert et al., 2010; Flammer et al., 2008; Lumeij et al., 1995; Orosz & Frazier, 1995; Sanchez-Migallon Guzman et al., 2010) and ineffectiveness due to acquired antimycotic resistance from *A. fumigatus* (Balfour & Faulds, 1992; Beernaert et al., 2009; Emery et al., 2012; Nascimento et al., 2003; Silvanose et al., 2006; Ziółkowska et al., 2014).

Successful antimycotic treatment of aspergillosis is rare, with efficacy largely dependent on the fungal strain. Notably, one of the few successes includes the 12 kākāpō that recovered from aspergillosis during the 2019 outbreak (a 57% success rate). However, *Aspergillus fumigatus* is globally recognized for its high potential to develop azole resistance (Auxier et al., 2023; Burks et al., 2021; Meis et al., 2016; Toyotome et al., 2018). While treatment success improved in the following outbreak (resulting in six survivors and one death, an 86% success rate), even a small number of losses represents a significant impact on a small population.

Other New Zealand native birds have also experienced low treatment success. A New Zealand study investigating the causes of mortality in hihi (Cork et al., 1999) found antimycotic clinical treatment (nebulisation of amphotericin b) ineffective against aspergillosis, although this was only trialled on one patient. Another New Zealand study on dotterels (Gartrell et al., 2014) performed three antimycotic treatments on seven aspergillosis infected birds. After not responding to another antimycotic (oral itraconazole), nebulised amphotericin was given to seven birds. Six failed to respond to these treatments, leading to the administration of oral voriconazole, which also, failed and the six birds died.

In some cases, clinical treatment of avian species may be counterproductive, potentially aggravating immunosuppression and reducing recovery success. For example, treatment for kākāpō requires

transferring birds from breeding sites (e.g., offshore islands near South Island) to ex-situ care facilities (e.g., Auckland Zoo, North Island), where repeated handling, diagnostic procedures, and prolonged captivity may induce stress, potentially exacerbating illness and reducing treatment efficacy. Overall, clinical treatment of aspergillosis often yields unpredictable outcomes, making it an unreliable option and generally recommended only once the disease is established. Therefore, the most effective approach to minimizing this issue in avian species may be to integrate environmental management-based approaches, particularly maintaining low exposure of *A. fumigatus* conidia and toxins whilst minimising factors that may exacerbate immunosuppression.

1.2.6.2 NZ environmental management of avian diseases

Current literature on the environmental management of avian diseases in New Zealand remains limited. Comprehensive reviews that consider multiple avian pathogens tend to focus on descriptive accounts of disease occurrence or treatment and translocation as management responses. Veterinary interventions such as health screening, prophylactic treatment, and emergency vaccination have played an increasingly important role in conservation programmes for native birds (Ewen et al., 2012; Gartrell et al., 2005). These practices have proven valuable for mitigating immediate disease risks, particularly during translocations and captive management. However, like antimycotic treatments for aspergillosis, the success of these interventions depends on early detection, specialist expertise, and repeated handling, which can be costly, impractical and stressful for small, free-living populations.

Health screening has proven vital for kākāpō, particularly for identifying and managing recurrent conditions such as cloacitis, as well as more acute infections like aspergillosis, by enabling early detection and targeted treatment before disease progression. These measures, however, cannot prevent initial pathogen exposure, particularly for environmentally acquired infections such as *Aspergillus fumigatus*, highlighting the need for complementary environmental management strategies to reduce infection risk at its environmental source. Additionally, there are currently no prophylactic or vaccine treatment options available for fungal diseases such as aspergillosis, largely due to the challenges associated with developing effective antifungal vaccines, as discussed earlier in this chapter (Section 1.1.2). These limitations emphasise that long-term disease mitigation in native birds must include both clinical care as well as environmental management practices that target the ecological conditions underpinning initial pathogen exposure.

In one of the earliest reviews on New Zealand avian diseases, Alley (2002) provided an overview of the major diseases affecting New Zealand's native birds and emphasised that effective disease management of wild avian populations will require the collaboration of veterinarians and ecologists. More specifically, that the challenge of diagnosis and control of emerging diseases will require full utilisation of skills in epidemiology, microbiology, and toxicology to conserve New Zealand's avian biodiversity. While not prescribing specific environmental management strategies, this review marked

an important shift in emphasis from solely clinical treatment toward its use together with ecosystem-level prevention and health monitoring.

A more recent review by Alley and Gartrell (2019) expanded on these ideas by proposing management strategies such as translocating individuals with greater genetic diversity to establish populations that may be more resilient to infectious disease and relocating species to habitats with climatic conditions less favourable to pathogens. These concepts highlight a trend toward proactive, population-based mitigation. However, such approaches face practical limitations for species with constrained genetic diversity, such as kākāpō. Moreover, *Aspergillus fumigatus* exhibits broad environmental tolerance, making exposure to at least low levels of this ubiquitous pathogen virtually unavoidable in forested habitats. Since climate change is expected to expand the geographic range of many fungi, including *A. fumigatus*, into previously cooler regions, this further complicates habitat-based strategies aimed at reducing exposure in the long-term.

1.2.6.3 Aspergillosis Management in New Zealand

In New Zealand, clinical treatment remains the only option following avian aspergillosis infection. However, increasing attention is being given to strategies preventing environmental *A. fumigatus* proliferation, likely because such measures are more feasible and cost-effective. Such control strategies in avian habitats generally aims to prevent fungal proliferation through measures such as regular replacement of litter or soil (Glare et al., 2014), improving ventilation, and removing decaying organic matter or nest material (Cork et al., 1999).

Regular replacement of litter and soil in captive avian environments to avoid high growth and conidial loads of *A. fumigatus* have been suggested (Glare et al., 2014) because of two brown kiwi deaths (*Apteryx mantelli*) likely attributed to prolonged interim storage of leaf litter in plastic bags (48 hours) possibly creating humid conditions for *A. fumigatus* proliferation. Similarly, an aspergillosis outbreak in dotterels (Cork et al., 1999) lead to the suggestion of regular nest box cleaning to also avoid *A. fumigatus* proliferation. Cork and colleagues (1999) also suggested that moist soils causing *A. fumigatus* proliferation could be prevented by increasing sunlight exposure to soils (e.g., opening the surrounding canopies of open aviaries). Although the opening of canopies may reduce soil moisture and leaf litter providing a less favourable environment for *A. fumigatus*, increasing sunlight exposure to soils has previously been associated with higher *A. fumigatus* levels, likely due to increased soil temperature (Perrott, 2001).

These studies present a common trend, that infection by *A. fumigatus* can be minimised by preventing its proliferation in avian habitats. However, such measures are primarily effective in captive or closely managed environments, where interventions and regular monitoring is more feasible. In contrast, applying similar manipulation of abiotic factors (e.g., removing nest litter or drying soils) is far more challenging in free-living populations, where larger spatial scales can make sustained management

impractical. For example, in free-living hihi populations on Mokoia, young-growth forests with canopy gaps and forest edges were associated with higher *A. fumigatus* soil levels, sparking the argument that the reduction of *A. fumigatus* spore loads in wild habitats is not feasible unless these forests are left to mature naturally (Armstrong et al., 2007).

One strategy that was proposed for management in free-living or wild avian populations is avoidance-based, but currently the most effective strategy that has been implemented. In response to the lack of feasibility in reducing *A. fumigatus* soil levels, hihi on Mokoia have been translocated away from areas found to have high loads (Armstrong et al., 2007). This strategy was reactive and was implemented following aspergillosis outbreaks in hihi. However, Ewen and colleagues (2012) made an additional suggestion which could be applied as a preventative strategy; that prior to avian translocations, environmental *A. fumigatus* (viable spore abundance) of the destination habitat should be measured also, and that spore counts should be below 100,000 colony forming units per gram of soil ($CFUg^{-1}$) (Perrott & Armstrong, 2011). However, since birds' own activity (e.g., defecation, movement within the nest and increased temperatures from body heat) can frequently alter microbial communities, moving populations every time high loads are identified could cause further stress and immunosuppression. Furthermore, in response to the safe limit of *A. fumigatus* soil counts; New Zealand's native avian species may have varying levels of disease susceptibility depending on their histories and ecological niches, and therefore it may be difficult to accurately estimate what is a safe level of exposure.

1.2.6.4 Aspergillosis management in kākāpō

By 2019, DOC's Kākāpō Recovery Group had already implemented strict management protocols to minimise pathogen transfer to breeding islands, such as the in-between disinfection of clothing and materials. However, the 2019 and 2022 outbreaks were examples of how these practices, although they likely minimised *A. fumigatus* loads, was not enough to prevent total exposure to kākāpō.

The 2019 outbreak prompted research into how kākāpō were infected, such as identifying potential sites of initial exposure, and what could be facilitating movement of the pathogen. Winter and colleagues (2022) found that the kākāpō *A. fumigatus* outbreak strain (unnamed) was detected in nests, bowl display sites, tracks, feeding stations and buildings used to store supplementary feed, on both Whenua Hou and Pukenui Island. The study therefore suggested that supplementary feed and human-facilitated movement could have been linked to *A. fumigatus* exposure at the time.

In response, the recovery group began to store supplementary feed at freezing temperatures to avoid *A. fumigatus* growth and potential aflatoxin contamination (A. Digby, personal communication, September 23, 2022). This may prevent further fungal growth but may not be effective for preventing aflatoxin contamination. Since aflatoxin remains stable at freezing temperatures (Kutasi et al., 2021), feed would need to be screened prior to freezing for existing aflatoxins. Notably, regular PCR screening

of the feed was also suggested, particularly for *A. fumigatus* growth (Winter et al., 2022), but also for aflatoxin contamination (A. Digby, personal communication, September 23, 2022).

Following the 2019 outbreak and the resulting changes in kākāpō monitoring and management, another aspergillosis outbreak occurred the following breeding season (in 2022) on Pukenui Island. The decrease in disease incidence was possibly owed to the enhanced management practices and tightened restrictions. However, in small bird populations, the loss or illness of even a few individuals, especially during the breeding season, can significantly hinder recovery efforts. Following the second outbreak, the Kākāpō Recovery Programme had increased monitoring: of nests, the health and weights of the chicks as well as adult bird activity (A. Digby, personal communication, September 23, 2022). Precautionary measures have also been implemented, such as selecting "at risk' chicks for CT scans and further examination at Dunedin Wildlife Hospital. The above information suggests that management of aspergillosis in kākāpō is largely experimental, since so little is known and understood about these outbreaks. To address this knowledge gap, the Kākāpō Recovery Group has initiated and supported research to inform future management strategies, recognising that effective action depends on identifying the root causes.

Overall, current management of avian aspergillosis in New Zealand is centred on veterinary intervention and mitigation of *Aspergillus fumigatus* within captive or semi-captive habitats. These strategies offer clear advantages for reducing disease incidence in managed populations but remain limited by the absence of an effective means to prevent infection, particularly in free-living birds. Environmental control of *A. fumigatus* appears to be the most practical management approach for captive populations, whereas translocation to lower-risk habitats may serve as the most feasible (albeit short-term) avoidance strategy for free-living species. Collectively, these findings highlight that aspergillosis management in New Zealand's avifauna is highly context-dependent, shaped by each species' physiology, nesting ecology, and genetic susceptibility. For example, kākāpō on breeding islands may present as an in-between of free-living and captive populations due to close tracking and monitoring, meaning *A. fumigatus* mitigation strategies may be feasible, likely more than translocations. However, early reports of low *A. fumigatus* levels in kākāpō nests suggest that infection risk persists even where environmental abundances are minimal (see Section 1.3.1). This reinforces Alley's (2002) earlier argument that effective disease mitigation requires an integrated understanding of both the host (e.g., immunosuppression, genetic susceptibility) and its environment (e.g., *A. fumigatus* exposure, presence of other pathogens, shared pathogen reservoirs), which is achievable only through close collaboration between veterinarians and ecologists.

1.2.7 Interactions between *A. fumigatus* and other fungi

To date, research on the causes of *A. fumigatus* infection in birds has focused primarily on abiotic factors that promote its environmental proliferation and consequently increase exposure risk. In contrast, few

studies have investigated how biotic factors influence *A. fumigatus* growth, sporulation, or pathogenicity, or the potential contribution of other microorganisms to aspergillosis incidence in avian species.

Of the literature that is available regarding fungal interactions with *A. fumigatus*, one study showed that *A. fumigatus* growth can be inhibited by other *Aspergillus* species and strains (Losada et al., 2009). In this study, two strains of *Aspergillus fumigatus* (Af293 and FGSC1163) were co-cultivated with other *Aspergillus* spp. strains, where one *A. fumigatus* strain (Af293) was outcompeted by strains of *A. niger*, *A. flavus*, *A. terreus*, *A. oryzae*, *A. clavatus*, *A. nidulans*, and *A. fisheranus*. The second *A. fumigatus* strain (FGSC1163) was also outcompeted by those *Aspergillus* spp. strains, excluding *A. flavus* and *A. terreus*. Despite the apparent biological control potential of these non-pathogenic *Aspergillus* species, several co-culture extracts were found to enhance conidiation (increase sporulation) in *A. fumigatus* Af293. The same strain also produced different toxins or secondary metabolites in co-culture compared to when grown in monoculture. Although this response may represent a stress-induced defence mechanism, abundant airborne conidia and toxic secondary metabolites (e.g., gliotoxin) are key virulence factors for *A. fumigatus* infections, raising the question of whether other competing microorganisms could enhance *A. fumigatus* pathogenicity.

A more recently emerging area of study is the antagonistic potential of yeasts, such as *Debaryomyces hansenii* against *A. fumigatus*. *D. hansenii* is one of relatively abundant yeasts isolated from kākāpō nests (see Chapter 2 and 3) (West et al., 2025). This particular species has demonstrated the ability to reduce *A. fumigatus* growth (Medina-Córdova et al., 2016; Núñez et al., 2015) and as well as reduce the growth and sporulation of other pathogenic *Aspergillus* species (Chacón-Navarrete et al., 2022; de Melo Pereira et al., 2016; Dikmetas et al., 2023; Fan et al., 2023; Iacumin et al., 2020; Iacumin et al., 2017; Liu & Tsao, 2009; Peromingo et al., 2019). Studies have also proven the antagonistic potential of the yeast *Meyerozyma guilliermondii* (see Chapter 2 and 3) against pathogenic *Aspergillus* species through the reduction of growth and sporulation (Choińska et al., 2020; Dikmetas et al., 2023; Kasfi et al., 2018). However, the mechanism of action for both species (*D. hansenii* and *Meyerozyma guilliermondii*) have been shown or are suspected to be through the production of volatile organic compounds (VOCs) of which the effects on birds and mammals is unknown (Chacón-Navarrete et al., 2022; Choińska et al., 2020; Kasfi et al., 2018; Medina-Córdova et al., 2016).

Although these studies highlight the potential of other fungi as biological controls against *A. fumigatus*, the ability of these competitive interactions to enhance pathogenic traits (e.g., enhanced sporulation and altered toxin profiles) in *A. fumigatus* or the competing microorganism, raises the question of whether these interactions may play a role in disease incidence or susceptibility.

1.2.8 Methods for estimating *Aspergillus* spp. abundance and microbial effects on *Aspergillus* spp. growth.

1.2.8.1 Viable counts

The viable count method is a traditional approach for quantifying living microorganisms in environmental samples (Pasrija & Kumari, 2025). It involves serially diluting a sample, plating the dilutions, and calculating the original abundance of the target organism. Because only viable cells can grow on synthetic media (e.g., agar, broth), this technique specifically measures active microbial occurrence. For *A. fumigatus*, it provides an accurate estimate of exposure risk, as only viable cells (e.g., spores) are capable of causing infection.

Viable counts have been implemented in estimating *A. fumigatus* abundance in soils to gauge the level of pathogen exposure to avian species. In hihi on Mokoia, and brown kiwi in New Zealand captive enclosures, this method was used to estimate *A. fumigatus* exposure, where high abundances aligned with aspergillosis incidence (Glare et al., 2014; Perrott, 2001). The cultivation method adopted by cultivation method adopted by Glare et al. (2014) using BST (Beauveria Streptomycin Tetracycline) agar (a semi-selective medium: potato dextrose agar amended with antibiotics) and higher temperature incubation for mesophilic microorganisms (35°C), particularly estimates *A. fumigatus* abundance by eliminating bacterial colonies and selecting for the fungus's preferred temperature range for optimal growth. However, since only a portion of microorganisms can grow in-situ on synthetic media, this method does not provide an accurate estimate of whole microbial communities that may be present environmentally (Jeewon & Hyde, 2007; O'Brien et al., 2005).

1.2.8.2 DNA fungal community analysis (amplicon sequencing)

In contrast to the viable count method, DNA-based community analysis provides a broader and more comprehensive view of whole microbial communities and diversity. Microbial community analysis typically involves amplicon sequencing, which detects microbial DNA in a sample following PCR (polymerase chain reaction) amplification. For fungi, highly conserved regions within the ribosomal DNA, such as the internal transcribed spacer (ITS) regions, including ITS1 and ITS2 (Gardes & Bruns, 1993; White et al., 1990), are commonly targeted (Bazzicalupo et al., 2013; Schoch et al., 2012). These regions can be selectively amplified to enable effective sequencing and taxonomic identification.

However, this method is not effective for accurate quantification of a particular species, such as *A. fumigatus*. For instance, the resulting data does not represent absolute abundance (as with viable counts), rather, its abundance relative to the total community structure identified (proportion). DNA-based community analysis also does not distinguish between living and dead cells (relic DNA) (Carini et al., 2016; Lennon et al., 2018), which could lead to an overestimation of relative abundance for some taxa. Conversely, taxa can be underestimated when they occur alongside others with higher abundance, or when the total biomass between samples varies. PCR primers may not bind to all sequences equally,

and differences in binding efficiency can distort amplification, producing proportions that deviate from the original template proportions (Polz & Cavanaugh, 1998). As a result, low-abundance or 'rare' taxa are especially prone to underrepresentation in mixed communities (Brooks et al., 2015; Silverman et al., 2021). Therefore, if *A. fumigatus* is both rare within a sample and a poor match to primers, it may be severely underrepresented or even undetected. Together, these biases mean that DNA-based community analysis provides an unreliable sole measure of *A. fumigatus* exposure and is more suited to providing a broad estimate of microbial diversity (species richness and evenness) and community composition.

1.2.8.3 Co-cultivation techniques

While DNA-based community analysis can reveal patterns in composition and diversity, highlighting potential broad-scale interactions, co-cultivation techniques enable direct examination of interactions between selected fungal species, providing a more accurate assessment of competitive dynamics. Different competition assay types typically reflect varying mechanisms of microorganisms' antagonistic potential. For example, radial inhibition assays (Chacón-Navarrete et al., 2022; Choińska et al., 2020; Medina-Córdova et al., 2016; Núñez et al., 2015) performed on solid media (e.g., PDA) are used to test the ability of a yeast to suppress the radial growth of filamentous fungi like *Aspergillus* species. Because yeasts form colonies more similar to bacteria than filamentous fungi, the method accounts for the differences in fungal growth and morphology by introducing yeasts to the co-culture as a lawn. The *Aspergillus* species is then inoculated into the centre of the plate, and its outward radial growth is observed overtime in comparison to its growth in the absence of the competitor (control). The purpose of this method is to assess growth inhibition by the yeast via the uptake of space and potentially nutrients. As a broader estimate of growth inhibition capabilities, it however does not narrow down the mechanism behind the yeast's antagonism.

Another method which assesses fungal growth inhibition by placing two competitors side-by-side is the method commonly perceived as a 'co-culture', sometimes referred to as a dual-culture (Alanzi et al., 2023; Cui et al., 2023; Du et al., 2022), antagonistic activity assay or in-vitro confrontation assay (Bolívar-Anillo et al., 2024). For co-cultures of *Aspergillus* spp. and yeasts following the same approach, these have been referred to as 'diffusible antifungal' assays (Medina-Córdova et al., 2016; Núñez et al., 2015). This assumes the production of antifungal compounds, because unlike the radial inhibition method, observed inhibition is typically attributed to molecules diffusing through the medium rather than through physical obstruction by the competitors. However, for confirmation of antifungal compound production (e.g., secondary metabolites, toxins, VOCs), the preferred methods are VOC assays (Chacón-Navarrete et al., 2022; Kasfi et al., 2018; Medina-Córdova et al., 2016) or metabolite identification methods such as GC/MS (Choińska et al., 2020; Núñez et al., 2015; Ruiz et al., 1998) or LC/MS (England, 2023).

1.3 Research Gaps & Thesis Overview

1.3.1 Study significance

Past aspergillosis outbreaks in New Zealand avian species (e.g., hihi on Mokoia Island) have been linked to high *A. fumigatus* environmental exposure (Armstrong et al., 2007; Glare et al. 2014; Perrott, 2001). However, determining the causes for kākāpō infection has proven far less straightforward. The kākāpō outbreak *A. fumigatus* strain responsible for the 2019 aspergillosis outbreak was identified (Winter et al., 2022), yet initial reports of its levels in kākāpō nest burrow soils specify that they were minimal (further analysed in Chapter 2). This may mean that environmental *A. fumigatus* spore loads may no longer be an effective measure of aspergillosis risk in vulnerable species, or at least that minimal nest abundances do not ensure prevention of disease incidence. This presents a significant challenge, as avoidance of *Aspergillus fumigatus* may be the most promising approach available for prevention of avian aspergillosis. However, with continued environmental and climate change, ecological processes and interactions may become more complex, making the causes of events like disease outbreaks more difficult to identify or narrow down to one specific cause.

Coupled with the limitations of clinical interventions, this underscores the urgent need to investigate all ecological interactions and environmental processes that may be contributing to these outbreaks; factors that traditional approaches may have overlooked. Since traits associated with pathogenicity in *A. fumigatus* can be enhanced by competitive interactions with other microorganisms, and because the presence of other potential opportunistic pathogens in kākāpō nest soils are currently unknown, this highlights the need to assess soil microbial communities in kākāpō nests and its potential impact on host health.

1.3.2 Research gaps

Overall, little has been studied on the interactions that occur between *A. fumigatus* and other fungal species, and how these interactions taking place within avian habitats may influence or play a role in disease incidence. If the abundance of *A. fumigatus* and other fungal types contribute to infection in kākāpō, understanding the ecological drivers of these abundances becomes critical.

Because kākāpō aspergillosis outbreaks have only recently been observed, there are currently no published data on the viable abundance of *Aspergillus fumigatus* within kākāpō nest environments. Quantifying exposure to environmental loads of this pathogen represents an essential first step in determining potential causes of aspergillosis. Preliminary observations have suggested that *A. fumigatus* abundances in nest soils are low, even in cases where infection has occurred. Although aspergillosis incidence may depend on more than just high levels of *A. fumigatus* exposure, this may still be a factor contributing to infection risk. Therefore, all nest soils from both recorded outbreaks (2019 and 2022) require further quantitative analysis to confirm that exposure risk within nests is minimal (see Chapter

2). In addition, the spatial distribution of *A. fumigatus* within and among nests remains unknown. Identifying spatial patterns (e.g., across microhabitats) could reveal environmental factors that promote or inhibit *A. fumigatus* proliferation, providing insights for future management strategies.

Fungal community analysis of kākāpō nest soils have recently been performed (West et al., 2025), demonstrating that there is no obvious link between the mycobiota of the gut or soil with aspergillosis incidence. However, fungal communities across spatial scales (e.g., microhabitats) have not yet been characterised, which may provide insight into spatial variation in fungal diversity, potential pathogen reservoirs, and the specific ecological conditions that may facilitate *A. fumigatus* proliferation, particularly when interpreted alongside viable count measurements.

Lastly, no studies have experimentally examined how *A. fumigatus* responds when co-cultivated with other fungi isolated from the same environment, or whether such interactions suppress or enhance its growth or sporulation. Understanding these dynamics is crucial, as microbial competition may alter *A. fumigatus* pathogenicity and may also help explain why *A. fumigatus* abundances appear low in nest soils despite repeated disease outbreaks. Investigating these ecological interactions through controlled co-culture assays will help clarify whether other nest-derived fungi act as natural inhibitors or facilitators of *A. fumigatus*, thereby contributing to observed patterns of abundance and potentially influencing infection risk.

1.3.3 Thesis aim

The overarching aim of this thesis is to investigate interactions between *Aspergillus* spp. and other mesophilic fungi (e.g., yeasts) in kākāpō breeding sites to assess its potential role in disease proliferation or susceptibility (i.e., high aspergillosis incidence within a population, or the vulnerability of kākāpō to infection respectively). These insights will be critical for understanding causative mechanisms and informing potential prevention strategies.

1.3.4 Thesis outline

The results from this study will be reported in three experimental chapters (Figure 1.4). Chapter Two quantifies the viable soil abundance of *Aspergillus* spp. and other mesophilic fungi across kākāpō nest microhabitats, islands, and in nests linked to aspergillosis incidence. This aims to assess spatial distributions and potential associations of both fungal groups with aspergillosis susceptibility, while also examining broader patterns of competition. Chapter Three characterises baseline soil fungal community composition (beta diversity) and richness (alpha diversity) across kākāpō nest microhabitats, islands, and nests associated with aspergillosis incidence. Using amplicon sequencing, this chapter provides a more precise identification of prevalent fungal taxa, examines how aspergillosis incidence may influence community structure, and evaluates spatial patterns in fungal assemblages to identify potential drivers of fungal proliferation. Chapter Four investigates the direct effects of kākāpō

nest yeast isolates on the growth and conidiation of *A. fumigatus* and *A. niger*; two causative agents for aspergillosis and also isolates from kākāpō and their nests. Results from these in-vitro experiments may broadly reflect fungal interactions within kākāpō nest soils and provide a foundation for further investigation of its potential contribution to disease susceptibility. As the concluding chapter, Chapter Five will synthesise these findings and discuss implications on future kākāpō management and research.

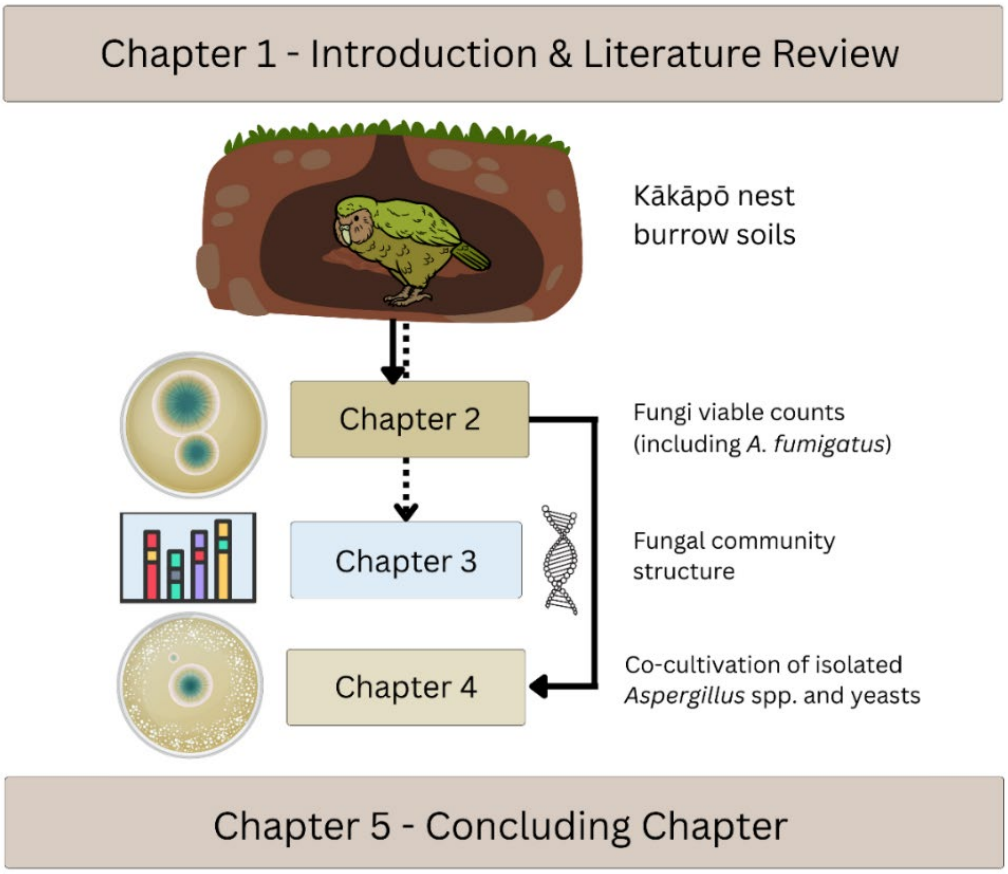

Figure 1.4 Infographic overview of thesis. Made using Canva (2024).

2 Chapter 2: Prevalence of *Aspergillus* spp. and environmental yeasts from kākāpō nest burrow soils during an aspergillosis outbreak on breeding islands in New Zealand.

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2.1 Abstract

New Zealand's critically endangered kākāpō have recently faced two outbreaks (2019 and 2022) of aspergillosis, a respiratory disease primarily caused by *Aspergillus fumigatus*. This study investigated whether environmental spore loads were associated with infection risk by quantifying viable *Aspergillus* spp. and other fungi from kākāpō nest microhabitat soils (cavities, entrances and two metres away) on breeding islands Whenua Hou and Pukenui. Fungi were cultured on potato-dextrose agar (PDA) supplemented with streptomycin sulfate and tetracycline hydrochloride and incubated at 35 °C to promote mesophilic growth. The zero-inflated negative binomial model identified that nests associated with infected birds were more likely to contain higher *Aspergillus* spp. counts (ZINB, estimate = -2.71 ± 0.10, z = -2.70, $p < 0.01$), although this was mostly composed of *Aspergillus flavus* and *Aspergillus niger*, with only one affected nest containing *A. fumigatus*. Mean *A. fumigatus* nest cavity counts were 60,000 ± 603,000 CFU g^{-1} in 2019 and 930.23 ± 2, 939.03 CFU g^{-1} in 2022, however no *A. fumigatus* was cultured from 90-92% of sampled nest cavities in both years. Mesophilic yeasts, including *Debaryomyces* spp., *Meyerozyma guilliermondii*, and *Candida palmioleophila*, were cultivated alongside *A. fumigatus*, and were significantly higher in nest cavities (ZINB, estimate = 3.30 ± 0.89, z = 3.71, $p < 0.001$) and entrances (ZINB, estimate = 3.17 ± 0.90, z = 3.54, $p < 0.001$) than soils two metres away. Both 2019 and 2022 datasets showed significant negative associations between yeasts and *Aspergillus* species. In 2019, yeast presence was associated with a ~95% reduction in *Aspergillus* spp. abundance (ZINB, estimate = 3.07 ± 0.83, z = 3.68, $p < 0.001$), and in 2022, yeast presence was associated with a ~33% reduction in *A. fumigatus* abundance (ZINB, estimate = 0.41 ± 0.16, z = 2.48, $p < 0.05$). These consistent negative effects suggest strong competitive interactions between yeasts and *Aspergillus* spp. in kākāpō nests. Some of these yeasts were also isolated from kākāpō carcasses following death by aspergillosis, therefore further investigation is required to assess its potential associations with aspergillosis incidence in kākāpō.

Keywords: *Aspergillus fumigatus*, aspergillosis, kākāpō, *Strigops habroptilus*.

Abbreviations: CFU = colony-forming units; BST = Beauveria Streptomycin Tetracycline (based on BSM: Beauveria Selective Medium); PDA = potato dextrose agar.

Highlights:

- Low levels of *Aspergillus* spp. found across kākāpō nests during aspergillosis outbreaks.
- Kākāpō nest soils during recent aspergillosis outbreaks showed a consistent inverse relationship between yeast presence/abundance and *Aspergillus* spp. abundance, suggesting potential interspecific competition.

- Suggestions for management include continual monitoring of kākāpō nest soils, air, faecal samples and supplementary feed/feeding sites for *Aspergillus* spp., yeasts and mycotoxin levels.

2.2 Introduction

Aotearoa New Zealand's kākāpō (*Strigops habroptilus*) is currently classified as critically endangered by the IUCN (International Union for Conservation of Nature) (IUCN, 2024). Kākāpō are the heaviest, only nocturnal and only flightless parrot in the world. Many New Zealand endemic birds have evolved flightlessness as a result of hypothesised isolation from mammalian predators during the separation from Gondwana approximately 80 million years ago (Duncan & Blackburn, 2004; Ericson et al., 2002). It is this flightlessness that has exacerbated the vulnerability of New Zealand's native birds to introduced mammalian predators, driving extinctions or near extinctions upon human colonisation (Lawrence, 2018). Mammalian predation combined with habitat loss are primary reasons for the dwindling numbers of kākāpō, which thanks to the Department of Conservation's Kākāpō Recovery Programme, has risen to approximately 237 individuals from only 50 in the 1990's (Department of Conservation, 2024; Dussex et al., 2021; G. Elliott et al., 2001). As a part of this programme, populations were translocated to New Zealand predator-free offshore islands that were eradicated of any exotic mammalian predator (G. Elliott et al., 2001). Only recently, a small population has been reintroduced to the mainland (Sanctuary Mountain, North Island, New Zealand) in a strictly controlled environment (G. Elliott et al., 2001). While these translocations aimed to shield kākāpō from their primary threat; predation, they now face a new emerging challenge to their survival.

2.2.1 Kākāpō aspergillosis outbreaks

Kākāpō have recently faced two outbreaks of aspergillosis; a respiratory disease typically caused by the fungus *Aspergillus fumigatus*. These outbreaks have coincided with the past two kākāpō breeding seasons, affecting breeding islands Whenua Hou in 2019 (Winter et al., 2022) and Pukenui in 2022 (Department of Conservation, 2022). The three-year gap between breeding seasons reflects the typical three-to-five-year reproductive cycle of kākāpō, which is closely tied to rimu (*Dacrydium cupressinum*) mast fruiting events (periodic mass production of fleshy fruits that kākāpō consume when breeding) (Cottam, 2010; Harper et al., 2006; Von Hurst et al., 2016). During the 2019 outbreak on Whenua Hou, 21 individuals were diagnosed with aspergillosis, resulting in nine deaths. During the 2022 outbreak, seven kākāpō were confirmed with aspergillosis, resulting in one death (Department of Conservation, 2022).

Aspergillosis is a respiratory disease primarily caused by the fungus *Aspergillus fumigatus* (sometimes *Aspergillus niger* or *Aspergillus flavus*) (Machado et al., 2024; Saad, 2025; Tell, 2005), and infection occurs through inhalation of *A. fumigatus* conidia (spores) into the airways of (typically) endothermic animals (mammals and birds). The warm conditions provided by endothermic or warm-blood animals

(37-43°C) fit the temperature range required for optimum growth in *A. fumigatus* (Paulussen et al., 2017; Tell, 2005; van de Veerdonk et al., 2017). *A. fumigatus* does not require a host to survive and reproduce, deeming it a facultative pathogen (Kamei et al., 2002; Misslinger et al., 2021). More specifically, its primary ecological role is as a 'harmless' saprophyte, but since it can cause infection when immune system defences are low (Blatzer & Latgé, 2017; O'Gorman et al., 2009), it is also seen as opportunistic pathogen. Notably, healthy individuals exposed to high spore counts can also become infected (Pascal Arné et al., 2011; L. Beernaert et al., 2010).

2.2.2 Kākāpō disease susceptibility

Birds in general, are particularly susceptible to *Aspergillus* infections as they have less advanced immune systems compared to mammals and have anatomical traits that increase their vulnerability to respiratory diseases. For example, birds lack the anatomical defences that mammals possess, such as the epiglottis that reduce particulate matter entering the airways, and the diaphragm, which facilitates the cough reflex to expel debris (Tell, 2005). For immune system functioning, mammals produce more abundant white blood cells for phagocytosis of pathogens compared to birds, and possess heterophils instead of mammalian neutrophils, which are less effective against killing hyphae (Harmon, 1998; Klika et al., 1996).

Unlike in mammals, *Aspergillus* spp. can occasionally sporulate *in vivo* within the respiratory tract of birds (notably in air sacs or lung lesions), thereby accelerating local colonization and increasing spore dissemination from infected birds (Arné et al., 2021; Richard et al., 1981; Stedham et al., 1968). This is likely due to the large, well-aerated air sacs, as well as the high and thermally stable core body temperature of birds (≈ 38–45 °C), supporting both conidial production and germination (L. Beernaert et al., 2010). Furthermore, birds are more sensitive to gliotoxin; an aflatoxin commonly produced within *A. fumigatus* spores for which is responsible for 96% of virulence in human infections by *A. fumigatus* (Tell, 2005). One study suggested that the increased sensitivity of gliotoxin in avian species is due to their inability to 'detoxify' gliotoxin to its less toxic, inactive form; bis(methyl)gliotoxin (bmGT); an ability mammals possess (Reidy et al., 2022).

Kākāpō may also be vulnerable to diseases, due to their low genetic diversity resulting from experiencing major population bottlenecks. Since both aspergillosis outbreaks have taken place during the past two kākāpō breeding seasons, affecting only mothers and their chicks, this leaves the third vulnerability as immunosuppression caused by breeding stress (as a mother) or a developing immune system (as a juvenile).

2.2.3 Aspergillosis treatment

Clinical treatments for avian aspergillosis are well studied. Aspergillosis is typically treated with antimycotics, such as azoles, and has demonstrated some success for kākāpō, specifically those that

have recovered from the outbreaks (Winter et al., 2022). However, many avian species across the world have not shared this successful experience. It is well documented that *A. fumigatus* is highly resistant to antimycotic treatments, and therefore aspergillosis is typically associated with treatment failure. Notably, the kākāpō outbreak strain possessed the cyp51A gene which has been linked to azole resistance, despite its effectiveness in the surviving kākāpō (Winter et al., 2022). Therefore, although studying azole resistance may be helpful for identifying virulence in *A. fumigatus*, the outcomes for aspergillosis infection are still variable.

2.2.4 Current kākāpō aspergillosis research

The recent aspergillosis outbreaks, along with other kākāpō diseases, marked the beginning of an investigation into the gut microbiome of kākāpō. The identification of the kākāpō gut microbiome has provided many valuable insights, including its putative association with immune system response (West et al., 2023), that supplementary feeding alters gut microbiome diversity (West et al., 2022), and that the presence of infected chicks in nests did not influence the fungal composition of the gut or the leaf litter (West et al., 2025). Based on these studies, we can determine that although the immune response is linked to the gut microbiome, the diversity of the gut microbiome itself is not directly linked to aspergillosis infection. Additionally, the presence of infected chicks did not influence the fungal alpha diversity of nest leaf litter either. However, there is currently no available literature linking the presence of infected kākāpō and the viable abundance of *A. fumigatus* in kākāpō nest soils.

Exposure to high spore abundances in forest floor soils and nest material, has been associated with aspergillosis in New Zealand native hihi (*Notiomystis cincta*) (Perrott, 2001). Hihi and kākāpō are both forest-dwelling, cavity-nesting species, where hihi typically occupy arboreal tree cavities (sometimes ground cavities or nest boxes) (Perrott, 2001), and kākāpō nest at the base of hollow trees, within fallen logs, or in ground cavities (Clout & Merton, 1998). The limited ventilation characteristic of cavity nests, particularly compared with other nesting types (e.g., open-cup nests), may facilitate the accumulation of *Aspergillus fumigatus* spores. This pattern was observed in hihi nests, where *A. fumigatus* loads in natural hihi cavity soils and internal air of hihi nest boxes were higher inside than outside (Perrott, 2001). If similar conditions occur in kākāpō nests, this could further increase exposure to *A. fumigatus*, especially given their proximity to soil where this fungus commonly resides. Therefore, quantifying *A. fumigatus* abundance in kākāpō nest soils may be an important step toward understanding potential environmental sources of exposure.

2.2.5 Fungal interactions with *Aspergillus* spp.

While the contribution of high *A. fumigatus* environmental loads to avian aspergillosis is well-studied, little has addressed the effects of *A. fumigatus* interactions with other microorganisms on disease susceptibility. For example, interactions between *Aspergillus fumigatus* and other *Aspergillus* spp. can alter the production of secondary metabolites or stimulate increased conidiation in *A. fumigatus*,

potentially enhancing its pathogenicity (Losada et al., 2009). Similarly, some yeast species release volatile organic compounds (VOCs) when competing with *Aspergillus* spp. (Chacón-Navarrete et al., 2022; Choińska et al., 2020; Kasfi et al., 2018; Medina-Córdova et al., 2016), which, if occurring within wildlife habitats, could have important consequences for host health. To date, no research has examined the role of microbial interactions in avian aspergillosis, particularly among New Zealand endemic species that nest or forage near forest floors where *Aspergillus fumigatus* is typically more prevalent. To evaluate how other microorganisms, especially fungi, may influence *A. fumigatus*, it is first necessary to determine whether the soil fungal communities exhibit competitive potential against the disease-causing agent.

Overall, understanding kākāpō susceptibility to aspergillosis, particularly regarding the role of immune function, may take years before it yields effective management strategies. Additionally, clinical treatment remains unreliable and can worsen immunosuppression due to the stress of human handling (Gartrell et al., 2014). Preventative strategies involving the environmental management of *Aspergillus fumigatus* exposure may offer the most feasible line of defence. However, to develop such strategies, we need to understand the ecology of *A. fumigatus* in kākāpō nest soils, whether abundance alone remains a contributing factor to infection, and whether interactions with other fungi may influence kākāpō susceptibility to disease.

This study therefore aims to quantify the soil abundance of: (1) *A. fumigatus* and other mesophilic species across spatial scales (kākāpō nest microhabitats), determining whether kākāpō nest occupation may be a factor influencing abundance, (2) *A. fumigatus* and other mesophilic species between two breeding islands, providing insight for whether geographic location is a factor influencing abundance, and (3) *Aspergillus* spp. between nests associated and not associated with confirmed aspergillosis, determining whether high *Aspergillus* spp. abundance remains a contributing factor to infection. Identifying the factors that drive elevated *A. fumigatus* loads will help guide kākāpō management by informing site selection and, where possible, preventing fungal proliferation. Assessing *A. fumigatus* exposure across nests will either support its role in infection or indicate the need to investigate alternative causes.

2.3 Methods

2.3.1 2019 outbreak kākāpō nest soils

During the 2019 aspergillosis outbreak, the Kākāpō Recovery Programme, Department of Conservation (DOC) New Zealand, collected soils from Summer to Autumn from 43 kākāpō nest burrows on Whenua Hou (Codfish Island; 46°46.07′ S, 167°37.23′ E) located near Rakiura (Stewart Island) (Figure 2.1), of which 15 were associated with nests that harboured infected birds (Appendix A, Table S1). In this study, these nests will be referred to as “affected nests”, while nests that were not associated with infection

will be referred to as “unaffected nests”. Sampling collection times for affected nests ranged from four months prior to one month following associated kākāpō deaths. Lincoln University’s Dr. Travis Glare performed serial dilutions and colony count measurements on these soils following a similar protocol described below (see Section 2.3.3). The viable abundance of *Aspergillus* species: *A. fumigatus*, *A. niger* and *A. flavus* was recorded, along with the presence and absence of yeast colonies.

2.3.2 2022 outbreak kākāpō nest soils

During the 2022 aspergillosis outbreak, the Kākāpō Recovery Programme collected soil samples from kākāpō nest areas, which included soils from the nest cavities, entrances and two metres away from the nest. Sampled nests were also from Whenua Hou and Pukenui, and the samples that were viable for cultivation were collected from May to June (Autumn-Winter season). Only two nests (belonging to kākāpō breeding females: Jemma and Roha) were associated with infected birds in the 2022 breeding season. However, these two samples (along with some other healthy nest samples) were no longer viable for accurate quantification in cultivation studies and therefore were excluded from the sample pool. Therefore, since the remaining 2022 soil samples were not associated with affected nests, fungal counts from these soils could not be analysed for association with confirmed aspergillosis. However, since these samples were collected from other nest microhabitats (entrances and two metres away from nests) as well as nest cavities, the 2022 kākāpō nest soils were used for viable count analyses across a spatial scale to assess broad fungal distributions. Due to a more limited sample pool, fifteen nests were selected at random resulting in total of 45 soil samples (Appendix A, Table S2). Samples were then stored at -20°C. Cultivation and colony count measurements took place at Manaaki Whenua (Landcare Research), St Johns, Auckland, New Zealand. All *Aspergillus* species (e.g., *A. fumigatus* and *A. niger*) observed were recorded for counts, along with other mesophilic fungi (e.g., yeasts).

2.3.3 *Aspergillus fumigatus* isolation from soil and leaf litter

One gram of each sample (the top one centimetre of soil and debris) was weighed in a sterilised plastic weigh boat, poured into a plastic sterile falcon tube, and suspended with nine millilitres of sterilised water. Each suspension was vortexed for 30 seconds and serial diluted in four times. One hundred microlitres of each dilution was plated in triplicate on pre-poured potato dextrose agar plates amended with antibiotics: 50 mg/L tetracycline hydrochloride and 350 mg/L streptomycin sulfate (both from Sigma Chemical Co., St. Louis, MO), replicating the BST agar (semi-selective medium) described by Glare and colleagues (Glare et al., 2014). Suspensions were spread using sterile L-shaped plastic spreaders. The previously described antibiotics were used to suppress bacterial growth, allowing for only fungal growth and thus a clearer observation of *A. fumigatus* colonies. Plates were incubated at 35°C for 48 hours, and colonies resulting from the 10^{-2} and 10^{-3} dilutions were counted. Only counts from the 10^{-3} dilutions will be included for analyses. The selected incubation temperature was within the range for optimal growth of *A. fumigatus*, thus selecting for its growth over other fungal species.

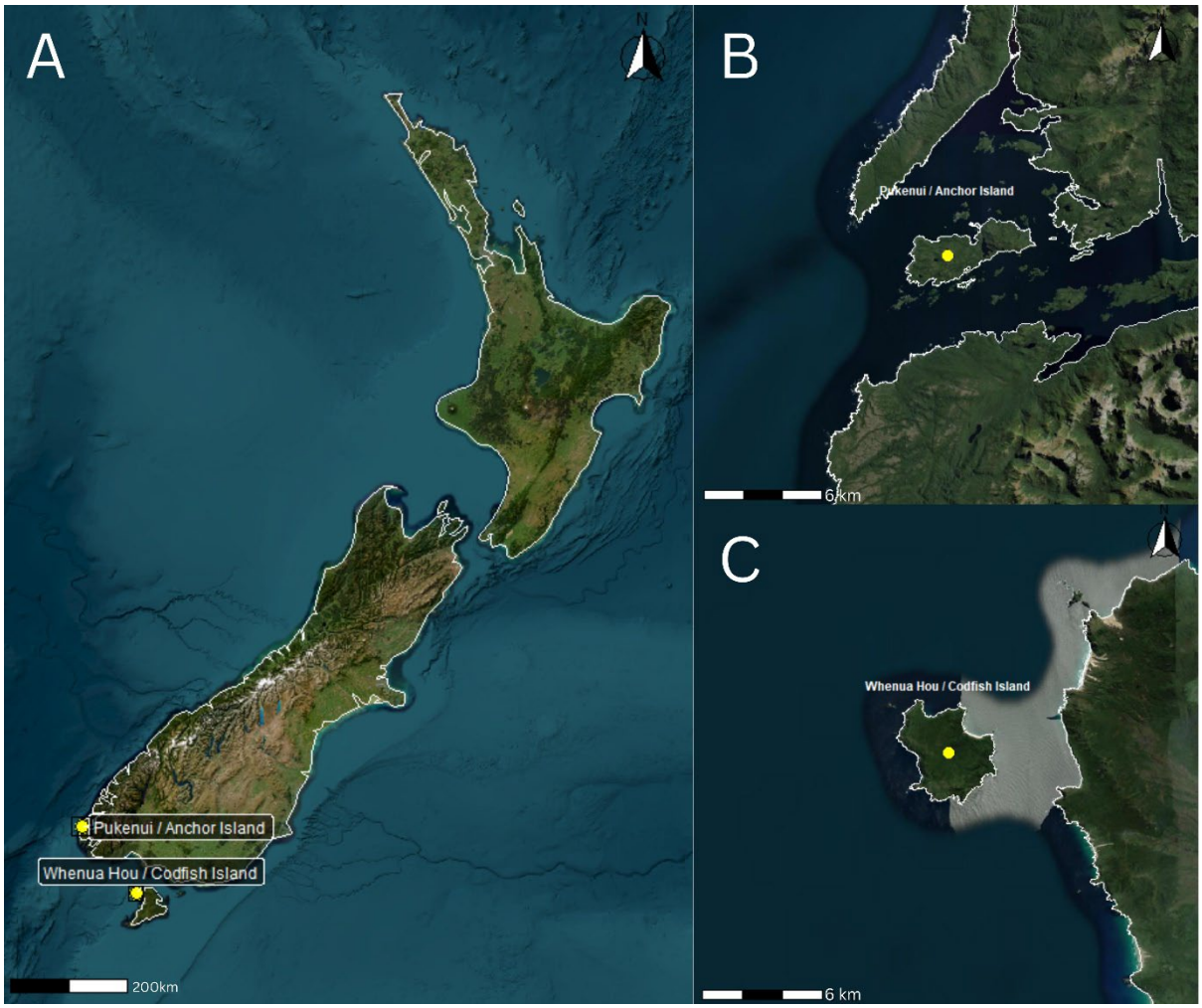

Figure 2.1 Satellite map of New Zealand (A) showing the locations of the two kākāpō breeding islands sampled in this study: (B) Pukenui / Anchor Island and (C) Whenua Hou / Codfish Island. Insets show equal-scale close-up views of each island within Fiordland and Rakiura (Stewart Island) regions, respectively. Basemap imagery: Esri World Imagery; coastlines: Natural Earth (public domain).

2.3.4 *Aspergillus fumigatus* identification

Identification of the *A. fumigatus* was performed using colony and cellular morphology and was confirmed through NCBI BLASTn search using the ITS region sequence data (DNA sequencing by Manaaki Whenua Landcare Research, New Zealand), aligning with 100% sequence identity, and an e-value of 0.0 against *Aspergillus fumigatus* strain DTO 402-H1 (MT316338.1). Due to sequencing of the ITS region only, identification to strain level was not obtained, therefore direct comparison with the strain identified during kākāpō outbreaks (Winter et al., 2022) was not possible.

2.3.5 Statistical analysis

All statistical analyses were conducted in RStudio (RStudio Team, 2024). To investigate potential relationships between *Aspergillus* spp. colony counts, nest location, island, and nests associated with aspergillosis infection, we used a zero-inflated negative binomial (ZINB) regression model, appropriate for over dispersed count data with excess zeros. This model groups zeros into 'structural zeros', where the result will always be a zero, and 'sampling zeros', representing a zero that could have been a count. The ZINB model combines a count component (negative binomial), which estimates expected counts where the organism could be present (not in the structural zero group), with a logit (zero-inflation) component, which estimates the probability that a sample is a structural zero where the organism is absent. This model was used for statistical analyses both within the 2019 and 2022 datasets. Since the ZINB model uses a log link, regression coefficients (β or "Estimate") from the count component are on the log scale.

Fold changes in abundance were calculated as:

$$\text{Fold change} = \exp(\text{Estimate})$$

Percentage changes in abundance were calculated as:

$$\text{Percentage change} = (\exp(\text{Estimate}) - 1) \times 100$$

Predictors were included in the count component only, while the zero-inflation component was specified with an intercept. Therefore, only results from the count component are interpreted in the text (full model outputs are provided in Appendix A; Tables S4-8).

2.4 Results

This study quantified the abundance of *Aspergillus spp.* between nests that were linked and not linked to aspergillosis cases (2019 outbreak), as well as the *A. fumigatus* and other mesophilic fungi across kākāpō nest microhabitats and breeding islands (2022 outbreak). Other mesophilic fungi that were cultivated from these soils were all yeasts, which were then assessed for their distribution and potential interactions with *A. fumigatus*. Together, these datasets provide a broad observation of the fungal influence on pathogen abundance in kākāpō nesting environments.

2.4.1 Aspergillus spp. abundance in affected and unaffected nests (2019 outbreak)

On average, kākāpō nests that had harboured infected individuals (affected nests) in 2019 had *Aspergillus* abundances of ~85,000 ± 258,000 CFU g^{-1}, although this was strongly influenced by a single nest with exceptionally high counts (Hoki_2: 5.4 million CFU g^{-1}) (Figure 2.2). Excluding this outlier, mean *Aspergillus* spp. abundances across all nests were 37,800 ± 70,300 CFU g^{-1}, which is under the 100,000 CFU g^{-1} threshold commonly cited for other New Zealand native birds for infection

risk (Ewen et al., 2012; Glare et al., 2014). Affected nests contained significantly higher *Aspergillus* spp. abundances than unaffected nests, with a 93% increase estimated by the ZINB model ($p = 0.007$; Table S4). Of the affected nests with detectable *Aspergillus* spp. counts (8 nests total), only one showed detectable *A. fumigatus*, whilst the remaining nests were attributed to *A. niger* (5 nests) and *A. flavus* (3 nests) (Appendix A, Table S1). Because *A. fumigatus* was detected in only three samples during the 2019 breeding season (limiting statistical power) and the majority of *Aspergillus* counts were driven by *A. flavus* and *A. niger*, effects such as aspergillosis status and yeast presence were analysed using total *Aspergillus* spp. abundance rather than *A. fumigatus* alone. Overall, forty-three percent of affected nests yielded no detectable *Aspergillus* spp. counts, indicating that infection may not always be associated with high *Aspergillus* spp. exposure.

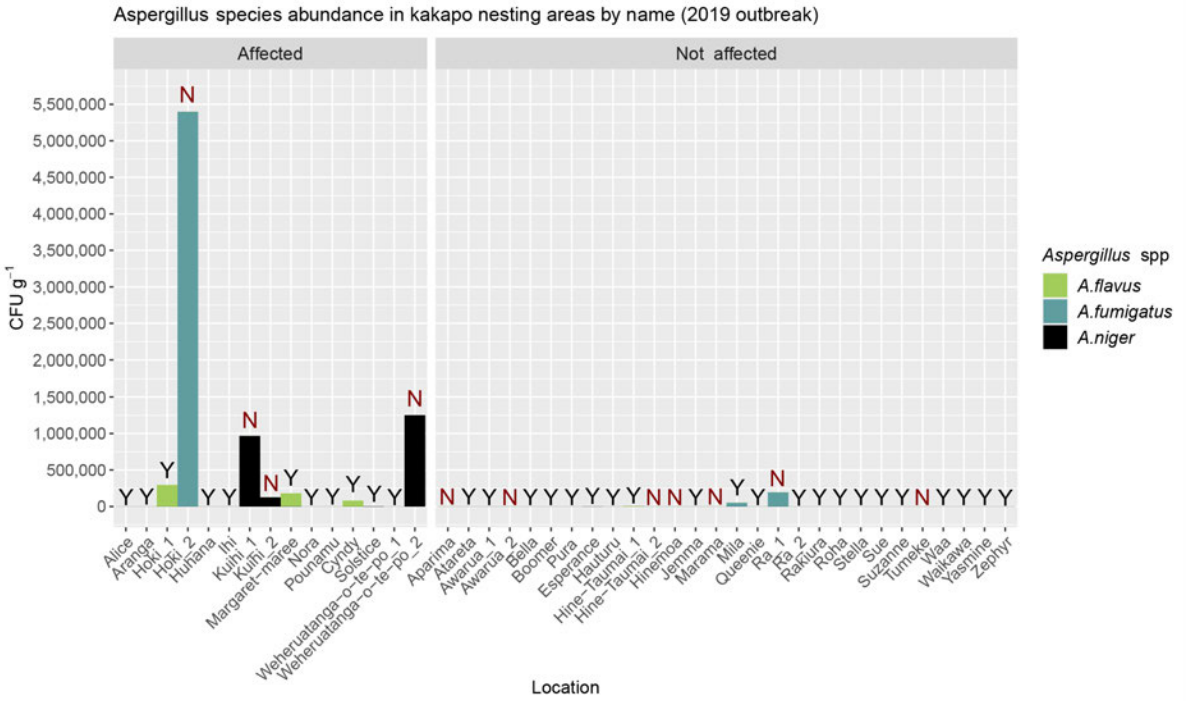

Figure 2.2 Viable abundance of *Aspergillus* spp. during the 2019 aspergillosis outbreak between affected and unaffected nests. N (red) = no presence of yeasts, Y = presence of yeasts. Samples were collected by the Kākāpō Recovery Group (Department of Conservation, New Zealand) and processed by Dr. Travis Glare (Lincoln University).

2.4.2 *Aspergillus fumigatus* viable abundance between nest locations and islands (2022 outbreak)

Across all nest soils in the 2022 dataset, mean *A. fumigatus* abundance was 2,652 ± 14,508 CFU g^{-1}. One entrance sample (Cyndy's nest entrance) exceeded 50,000 CFU g^{-1} (Appendix A, Table S2). *Aspergillus niger* was also detected, only in two nest microhabitat samples on Pukenui Island (Marian's nest cavity: 3,333 ± 5,774 CFU g^{-1}, Marama's nest entrance: 40,000 ± 17,321 CFU g^{-1}). Therefore, ZINB models for 2022 were fitted using *A. fumigatus* counts rather than total *Aspergillus* spp., as *A. niger* (n = 2) was insufficiently represented and would disproportionately influence the combined metric

without adding meaningful statistical power. Excluding Cyndy's nest entrance as an outlier, *A. fumigatus* counts slightly increased with distance from the nest cavities. On average, nest cavities were 930 ± 2, 939 CFU g^{-1}, entrances were 1,707 ± 4, 417 CFU g^{-1} and two metres away were 1,777 ± 5,756 CFU g^{-1} (Figure 2.3). The zero-inflated negative binomial (ZINB) model supported this pattern, showing that counts in cavities were 46% lower than other microhabitats (ZINB, estimate = -0.61 ± 0.18, z = - 3.3, $p < 0.01$; Appendix A, Table S6). Including Cyndy's nest entrance and another outlier (Marama's nest entrance), *Aspergillus* spp. together (*A. fumigatus* and *A. niger*) was highest at the nest entrances (7,955 ± 26 021 CFU g^{-1}). Between islands, Whenua Hou exhibited significantly higher *A. fumigatus* counts than Pukenui, with a 59% increase predicted by the model (ZINB, estimate = 0.47 ± 0.16, z = 2.9, $p < 0.01$; Appendix A, Table S6). When *A. niger* counts were included alongside *A. fumigatus* (i.e., total *Aspergillus* spp. abundance), no significant effects were detected.

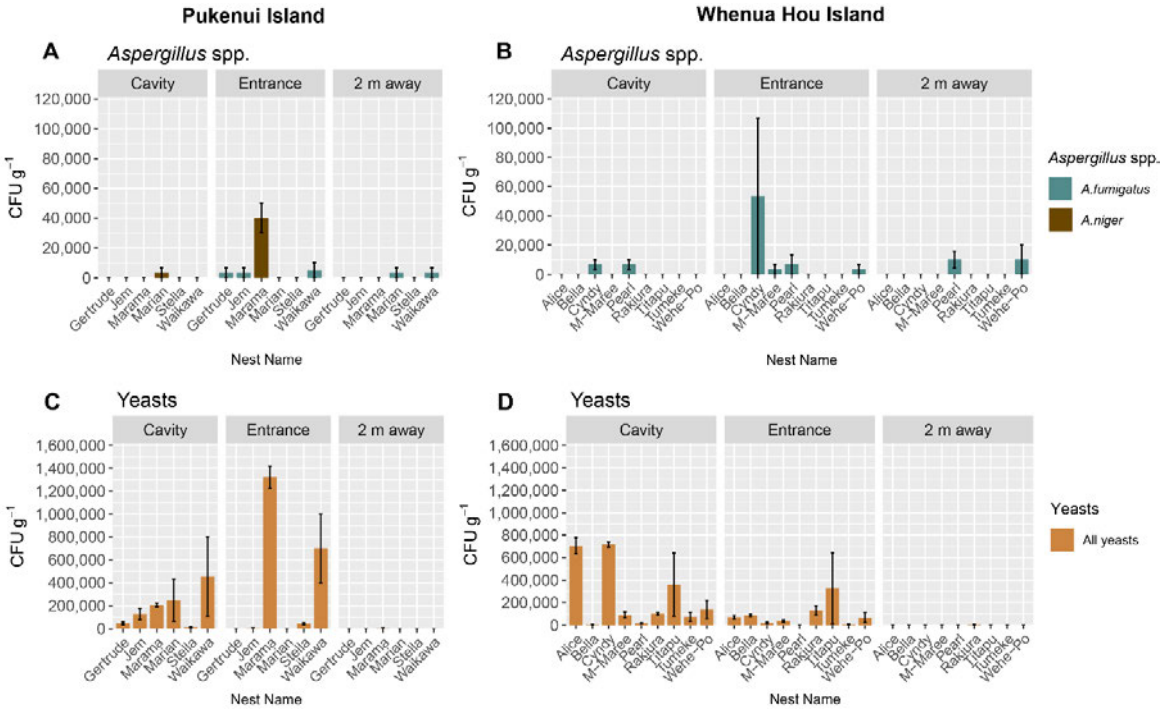

Figure 2.3 Viable abundance of *Aspergillus fumigatus, Aspergillus niger* and mesophilic yeasts during the 2022 outbreak across nest microhabitats. Pukenui Island (A & C) and Whenua Hou Island (B & D), across kākāpō nest microhabitats: cavity, entrance and two metres away (2m away). $CFUg^{-1}$ = colony forming units per gram of wet soil.

2.4.3 Yeast viable abundance between nest locations and islands (2022 outbreak)

From the 2022 samples, thirteen distinct yeast types (categorised by their colony morphology) were identified mostly as *Debaryomyces* and *Candida* species through DNA sequencing (Appendix A, Table S3). Mean yeast counts across all nest microhabitats were 129, 167 ± 284, 678 CFU g^{-1}. Yeasts decreased with distance from the nest (cavities: 216,047 ± 279, 542 CFU g^{-1}, entrances: 175, 909 ± 378, 733 CFU g^{-1}, 2m away: 444 ± 2,084 CFU g^{-1}), following the opposite pattern observed for *A. fumigatus*

counts (Figure 2.3; Appendix A, Table S2). Abundances ranged from zero to more than 1.3 million CFU g^{-1}, with the highest counts recorded in Marama's entrance sample. The ZINB model confirmed that yeast abundances were ~27-fold higher in cavities (ZINB, estimate = 3.30 ± 0.89, $z = 3.71$, $p < 0.001$) and ~24-fold higher at entrances (ZINB, estimate = 3.17 ± 0.90, $z = 3.54$, $p < 0.001$) compared to soils two metres away (Appendix A, Table S7) while island differences were not significant.

2.4.4 Yeast-*Aspergillus* spp. interactions

Although yeasts were cultivated alongside *Aspergillus* spp. from both 2019 and 2022 kākāpō nest soil samples, samples from 2019 only included presence and absence data of yeasts (Figure 2.2). The ZINB model showed that, in 2019 samples, yeast presence was associated with significantly lower *Aspergillus* spp. counts, with an estimated 95% reduction compared to nests without yeasts (ZINB, estimate = -3.07 ± 0.84, $z = -3.68$, $p < 0.001$; Appendix A, Table S5). Following a similar pattern, ZINB results from 2022 demonstrated an estimated 33% decrease in *A. fumigatus* abundance when yeasts were present (ZINB, estimate = -0.41 ± 0.16, $z = -2.48$, $p < 0.01$; Appendix A, Table S8).

2.5 Discussion

2.5.1 *Aspergillus* spp. abundance in affected and unaffected nests (2019 outbreak)

The 2019 samples collected by the Department of Conservation (DOC) and processed by Lincoln University, included nests that harboured infected individuals (confirmed aspergillosis). Although the overall abundance of *Aspergillus* spp. (*A. flavus*, *A. niger* and *A. fumigatus*) was mostly low, affected nests were still significantly associated with higher overall *Aspergillus* spp. counts than unaffected nests (Appendix A, Table S4).

Despite the link between affected nests and higher *Aspergillus* spp. abundance, ~42% of affected nests harboured no *Aspergillus* species, and only one of the 15 affected nests had detectable *A. fumigatus,* specifically. *Aspergillus fumigatus* was confirmed as the disease-causing agent of the 2019 outbreak, and therefore its near absence in nests with infected birds suggests that initial spore exposure may have occurred outside of kākāpō nests, or from other nest substrates that were not tested (e.g., dust in air, food remains). Winter and colleagues (2022) also proposed the possibility of *A. fumigatus* exposure external from nests, as the outbreak strain of *A. fumigatus* was also isolated from feeding stations, bowl display sites, and the buildings where their supplementary feed was prepared and stored.

Since a single *A. fumigatus* strain was responsible for infections across multiple kākāpō nests in 2019 (Winter et al., 2022), this could either mean multiple sources of exposure but a common origin or a shared external source of exposure. Kākāpō breeding females, which occasionally leave the nest to feed, may have encountered contaminated feed or airborne spores at the sites where this strain was detected. However, this does not explain how chicks, who mostly remain within the nest (until fledging) became

infected, unless kākāpō breeding females transferred these spores to the nesting environment. However, consequently, one would expect more frequent detection of *A. fumigatus* detection within these soils, unless soil conditions prevented substantial growth or establishment. Therefore, we need to consider even rare scenarios, such as transfer of spores from kākāpō female feathers to the air during certain movements (e.g., rustling feathers, self-grooming), or through contaminated food brought back to the nest.

Although avian aspergillosis is not contagious or typically transmitted between individuals, direct vertical transference of *A. fumigatus* spores from birds to their eggs has been deemed a possibility (Alhassani et al., 2025; Hamza et al., 2022; Shaapan & Girh, 2024). For example, Wright et al. (1961) demonstrated that *A. fumigatus* spores can penetrate embryonated eggs and infect developing embryos under certain conditions (mould growth was evident inside eggs by candling). They recovered *A. fumigatus* from both live embryos and dead-in-shell chicks before hatching, indicating that infection can occur inside the egg. Contaminated feed has also been the source of *A. fumigatus* exposure for many birds (Arné et al., 2011; Krnjaja et al., 2014), but it is yet to be tested whether spore exposure can occur from contaminated feed from the female to their chicks.

2.5.1.1 Potential Pathogenicity of Other Aspergillus spp.

Of the affected nests during the 2019 outbreak, only one contained *A. fumigatus*: the species confirmed as the disease-causing agent. Affected nests that had *Aspergillus* spp. counts, were mostly attributed to *A. niger* and *A. flavus*. Intriguingly, five of the nine aspergillosis deaths either showed signs indicative of aflatoxin exposure or had *A. flavus* cultured alongside *A. fumigatus* post-mortem.

Aflatoxin, which is a mycotoxin typically produced by *Aspergillus flavus*, was recorded as a possible contributor to the poor physiological conditions of infected kākāpō. One of the nine deaths experienced liver changes indicative of aflatoxin exposure (Huhana), and another two (Bella-1-A-19 and Hoki) experienced unspecified physiological changes that were considered possibly due to aflatoxin exposure (A. Digby, personal communication, September 23, 2022). However, *A. flavus* was not cultured from these three individuals, although Hoki's nest harboured 295,000 CFU g^{-1} of *A. flavus*. This demonstrates that environmental exposure of aflatoxins can occur without requiring infection by *A. flavus*. Contrastingly, another two of the nine kākāpō (Queenie-4-A and Margaret-Maree-2-B) were culture positive for *A. flavus*, with no sign of aflatoxin exposure. Margaret-Maree's nest however harboured 180,000 CFUg^{-1} of *A. flavus*, whilst Queenie's did not. Overall, these findings suggest that both aflatoxin exposure and infection by *A. flavus* may independently contribute to poor health conditions. Further research is needed to assess the possible contribution of *A. flavus* to immune function and aspergillosis susceptibility in kākāpō.

2.5.2 *Aspergillus fumigatus* viable abundance between islands (2022 outbreak)

Aspergillus spp. abundance in 2022 was significantly higher on Whenua Hou Island (Codfish Island) compared to Pukenui Island (Anchor Island), despite no aspergillosis cases occurring on Whenua Hou during this time. The *A. fumigatus* strain associated with the 2019 outbreak on Whenua Hou was also detected on Pukenui during the same year, even though no cases of aspergillosis were recorded (Winter et al., 2022). Winter and colleagues (2022) argued that this could indicate that strain presence alone is insufficient to explain infection risk. Therefore, together our findings suggest that the presence of the outbreak *A. fumigatus* strain (Winter et al., 2022), and broad-scale *A. fumigatus* abundance (overall island averages from the current study) alone may not explain aspergillosis incidence in kākāpō.

Although the presence of the outbreak strain alone did not determine whether infection occurred (Winter et al., 2022), it remained the causative agent of the kākāpō aspergillosis outbreaks, and therefore the prevention of its introduction to other populations are necessary for kākāpō conservation going forward. The presence of this strain on both islands suggests potential facilitation of pathogen movement or transfer. Since Whenua Hou and Pukenui are approximately 142 kilometres apart, and because kākāpō are flightless, it is possible that spores may have been dispersed via environmental factors (e.g., wind and ocean currents), transference via human movement (e.g., translocating birds) or other terrestrial animals (e.g., bird species with flight ability, and/or flying insects). Winter and colleagues (2022) suggested that kākāpō supplementary feeding practices may have facilitated *A. fumigatus* spread due to detection of the strain at feeding sites and buildings which stored feed. This strain was also able to persist through the Kākāpō Recovery Programme's hygiene practices (anti-fungal disinfection of equipment and clothing between islands), highlighting the resilience and ubiquity of this species. Because human movement between islands is essential for kākāpō population recovery, we recommend identifying disinfectants and hygiene protocols that effectively target and remove *A. fumigatus* hyphae and resilient spores from workers and transferred equipment.

2.5.3 *Aspergillus fumigatus* viable abundance between nest microhabitats (2022 outbreak)

In 2022, raw data shows that *Aspergillus* spp. (*A. fumigatus* and *A. niger*) were higher at the kākāpō nest entrances, which could have been attributed to repeated soil disturbance through the inward and outward movement of kākāpō from their nests. In captive brown kiwi (*Apteryx mantelli*) at Auckland Zoo (Truter-Meyer, 2020), *Aspergillus* spp. abundance was higher at enclosure entrances where soils received constant disturbance by the treading of zookeepers and movement of enclosure gates. Previous investigations on aspergillosis in endemic New Zealand hihi (*Notiomystis cincta*) have found that soil disturbance (primarily removal of surface leaf litter) increases *A. fumigatus* abundance in the soil, likely due to the exposure of the bottom soil layers to the sunlight, increasing the soil temperature and thus increased spore germination (Perrott, 2001; Perrott & Armstrong, 2011). Additionally, the forced

release of spores from the soil and into the air may increase distribution and establishment of fungal colonies.

Since mean *Aspergillus* spp. counts in entrances were likely driven by two outliers, excluding these, mean *A. fumigatus* and overall *Aspergillus* spp. counts increased with distance from nest cavities. A similar pattern was observed in a study measuring viable *A. fumigatus* abundance in hihi nests, where 62.5% of surveyed nest boxes on Mokoia Island were associated with lower *A. fumigatus* viable abundances compared to outside soils (Perrott, 2001). This study attributed these patterns to abiotic factors, for example, water content in nest box materials was 1.6 times lower than in soils taken from the outside, emphasising that *A. fumigatus* may have preferred the higher moisture content within external soils. Contrastingly, in natural hihi cavities on Little Barrier Island, all surveyed cavities contained higher *A. fumigatus* abundances compared to outside soils, despite those cavities also demonstrating drier nest material compared to outside. However, the cavities showed much more stable microclimate conditions, with relative humidity varying by only 9.2% (versus 22.6% outside) and mean temperatures fluctuating by just 2.3 °C (versus 5.4 °C outside). Additionally, *A. fumigatus* abundances in chitinous materials (e.g., decaying cicadas) in hihi nest boxes on Mokoia, were on average 3.1 times higher than in nestbox materials and outside soils, indicating that specific organic substrates, can influence *A. fumigatus* abundance. Airborne spore densities in Mokoia nest boxes were also 13 times higher inside compared to outside, emphasising that ventilation may also play a role in *A. fumigatus* abundance. Overall, the present study highlights that minor abiotic differences between nests microhabitats and island locations are possible contributors to variation in *A. fumigatus* abundances.

2.5.4 Yeast viable abundance between nest locations and islands (2022 outbreak)

Yeast abundances did not differ significantly between the two islands. However, on both islands, abundances were significantly higher in nest cavities and entrances compared to two metres away. Mean yeast counts were observed to decrease with distance from the nest, which was the opposite pattern observed for *Aspergillus* spp., excluding outliers. Overall, significant differences across nest microhabitats but not islands, suggest that elevated yeast levels are a consistent feature of kākāpō nesting areas rather than a result of island-specific conditions.

Since the yeast species identified in this study were also detected via DNA analysis in kākāpō faecal samples and nest litter (e.g., *Debaryomyces hansenii*, *Candida palmioleophila*, and *Spencermartinsiella* spp.) (West et al., 2025), it is likely that kākāpō defecation is a key driver of yeast proliferation in kākāpō nest cavities. Some of these yeast species (e.g., *Debaryomyces* and *Candida* spp.) were amongst the most abundant taxa (via DNA analysis) in Auckland Zoo's nocturnal kiwi nest boxes (Truter-Meyer, 2020). Worldwide, high yeast abundances are typically found in avian faecal matter or droppings (Partridge & Winner, 1965; Simi et al., 2018), specifically yeasts such as *Cryptococcus* spp. (Baroni et

al., 2006; Caicedo et al., 1999; Elhariri et al., 2015; López-Martínez & Castañón-Olivares, 1995; Lugarini et al., 2008) and *Candida* species (Mendes et al., 2014). Naturally, avian nests experience a build-up of faecal matter overtime, and therefore high yeast abundances are also found within avian nests (Chen et al., 2015; Hubalek et al., 1973; Kew et al., 2014; Kornillowicz-Kowalska & Kitowski, 2018). Simi and colleagues (2018) explain that dried faecal matter provides favourable substrates for fungal growth, particularly due to high concentrations of nitrogenous bases. Supporting this mechanism, unpublished data on the physicochemical properties of kākāpō nest areas (Cameron, unpublished data) show that nitrogen levels were significantly higher in kākāpō nest cavities and decreased with distance from the nest ($p < 0.001$), reinforcing the likelihood that faecal enrichment contributes to the enhanced yeast proliferation observed in these sites. Intriguingly, yeasts detected in kākāpō faecal matter, which aligned with our study, declined when kākāpō switched from supplementary feed to predominantly plant material, indicating that yeast proliferation in kākāpō nests may be influenced by diet (West et al., 2025).

2.5.5 Yeast-*Aspergillus* spp. interactions

Results from both the 2019 and 2022 datasets showed that the presence of yeasts were associated with a significant reduction in abundance of *Aspergillus* species in kākāpō nest soils. This was supported by observed patterns in counts, where yeasts decreased and *Aspergillus* spp. (excluding outliers) increased with distance from the nest, suggesting potential competitive interactions between these fungal groups. One of the yeasts detected in this study; *Debaryomyces hansenii*, is known to inhibit the growth of *Aspergillus fumigatus* through the production of secondary metabolites or VOCs (Medina-Córdova et al., 2016; Núñez et al., 2015). Additionally, *D. hansenii* produces toxic proteins, commonly referred to as 'killer' toxins or 'mycocins', which exhibit antifungal activity (Al-Qaysi et al., 2017; Buzzini & Martini, 2001; Marquina et al., 2001; Santos et al., 2002). Killer toxins produced by *D. hansenii* (and another identified yeast: *Meyerozyma guilliermondii*) have also demonstrated antagonistic effects against *Aspergillus niger* (Bleve et al., 2006; Çorbacı & Uçar, 2017). Given that *D. hansenii* was a commonly occurring species, its presence may have contributed to the observed reduction in *A. fumigatus* levels.

Although the potential inverse relationship observed in our study may have been influenced by other factors, (e.g., abiotic factors, competition through nutrient and space uptake), visual observations from subsequent co-culture experiments (Kanis et al., in prep; Ch. 4) using isolates recovered during the present study, frequently revealed partial inhibition of conidiation (sporulation) between the two fungi (Figure 2.4). In previous co-cultivation studies, yeasts such as *D. hansenii* and *M. guilliermondii* (also isolated in the current study) have been shown to suppress sporulation in *A. fumigatus* and *A. niger*, while producing volatile organic compounds (VOCs) (Choińska et al., 2020; Kasfi et al., 2018; Chacón-Navarrete et al., 2022). These findings suggest that antagonistic interactions observed in this study may be partially mediated, by the production of secondary metabolites.

Aspergillus fumigatus itself produces the mycotoxin, gliotoxin, which is 96% responsible for virulence in human *A. fumigatus* infections (Ghazaei, 2017). Its toxic activities mainly depend on the intact disulfide bond within its molecular structure that allows for interaction with various host cell types, ultimately disrupting immune system functions (Scharf et al., 2012). Consequences of these activities include inhibition or disruption of key cellular processes, which ultimately suppress the immune response, facilitating *A. fumigatus* invasion and spread of the airways (Ben-Ami et al., 2009; Coméra et al., 2007; Kroll et al., 1999; Pardo et al., 2006).

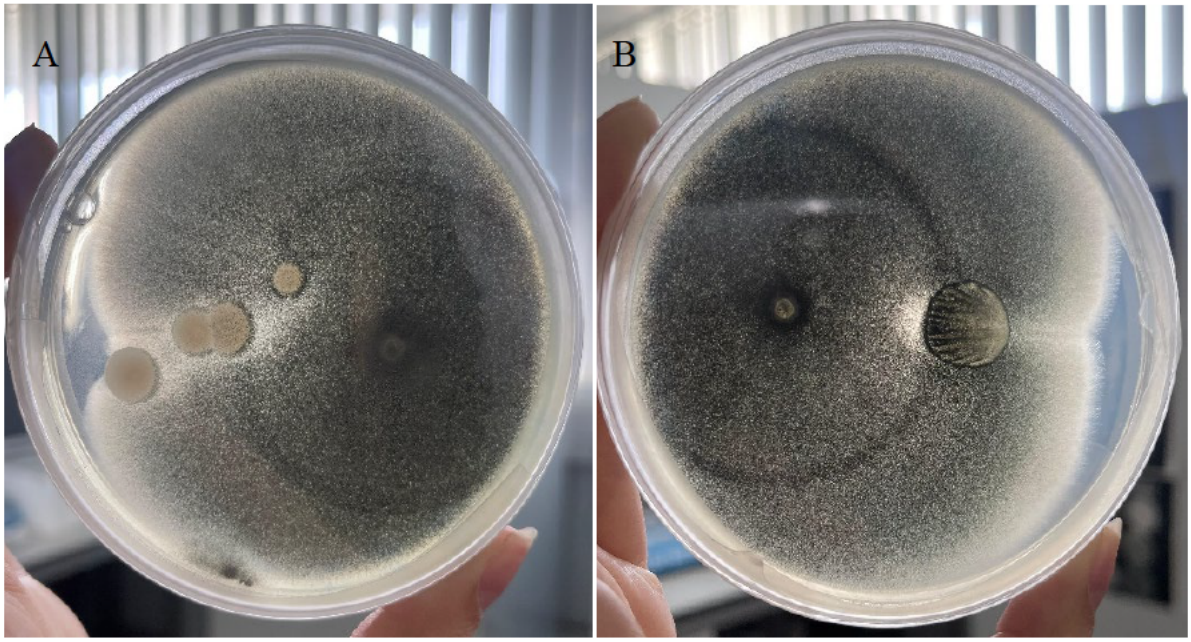

Figure 2.4 Co-cultivation of *Aspergillus fumigatus* (ICMP 24469) with (A) *Meyerozyma guilliermondii* and (B) *Debaryomyces hansenii.*

Additionally, avian species are likely more susceptible to gliotoxin than mammals. A study by Reidy and colleagues (2022) found gliotoxin (GT) in 87% of samples from birds diagnosed with aspergillosis, whereas its breakdown product, bis(methyl)gliotoxin (bmGT), was not detected. Mammals can detoxify GT through methylation, converting it into the less toxic, inactive form of bmGT. The absence of bmGT in the study's samples (Reidy et al., 2022) suggests that birds may lack this methylation process, leaving them exposed to the active and toxic form of GT for a longer period. In a recent analysis of kākāpō nest soils, bmGT was detected in only two of 48 samples (England, unpublished data), and since GT concentrations were not tested, this raises the question of whether GT may have been higher or detected in a larger number of samples. However, since only healthy nests were included in that dataset, no accurate links could have been made to disease incidence, if GT was tested for. Overall, the difference between bmGT and GT highlights the importance of screening kākāpō soils specifically using a GT standard, to fully account for GT exposure and its potential role in the aspergillosis outbreaks.

2.5.5.1 Potential pathogenicity of yeasts

During the 2019 outbreak, one of the nine deceased kākāpō (Hoki) had contracted yeast ventriculitis (presumptive candidiasis) whilst receiving treatment for aspergillosis (L. Uddstrom, personal communication, March 3, 2023). The yeast infection was suspected to be secondary to immunosuppression caused by aspergillosis as well as hepatopathy. During the 2022 outbreak, *Debaryomyces* spp. and *Kazachstania* spp. were identified post-mortem in Jemma (the only death resulting from aspergillosis in the 2022 outbreak), and *Yarrowia bubula* was identified post-mortem in an isolated case of a male kākāpō (Jester) with aspergillosis on Hauturu Island, separate to the outbreak that targeted breeding females and chicks on Pukenui. Additionally, another isolated case of candidiasis occurred in the same year; a 10-day old kākāpō chick (Waa). The causal agent confirmed by the histopathology results was *Candida albicans* (L. Uddstrom, personal communication, March 3, 2023). Since Jester was a male, there were no nest soil samples to acquire. Soil samples also could not be obtained from Waa's nest due to its immediate closure. Jemma's nest soils were not viable for accurate colony counts in this study.

Of these individuals, Hoki was the only kākāpō whose nest cavity soils were available for analysis shortly after the 2019 outbreak. Two samples were collected from Hoki's nest: in May and December of 2019 (Appendix A, Table S1). Hoki's cavity soil in May harboured high abundances of *A. flavus* (295,000 $CFUg^{-1}$ wet material), no *A. fumigatus* or *A. niger*, but yeasts were present. Contrastingly in December, Hoki's cavity soil harboured extremely high levels of *A. fumigatus* (5,400,000 CFU g^{-1} wet material) whilst other *Aspergillus* species and yeasts were absent.

Many species of yeast are considered pathogenic and can cause various types of infections in animals. *Debaryomyces hansenii* was the most observed yeast in this study and had been isolated from kākāpō Jemma post-mortem after suffering from aspergillosis. Pathogenicity of *D. hansenii* has only been recorded in human patients, causing infection in both the immunosuppressed and immunocompetent (Desnos-Ollivier et al., 2008; Karapetsa et al., 2019; Wong et al., 1982). *D. hansenii* has also caused candidemia in a patient receiving antimycotic treatment for aspergillosis (Wagner et al., 2005). However, since *D. hansenii* has also demonstrated probiotic properties (Angulo et al., 2023; Angulo et al., 2020; Jeong et al., 2022), this suggests if pathogenic, it is likely opportunistic (West et al., 2025).

Aspergillosis is often considered a secondary infection in humans that arises following immunosuppression caused by other diseases. Contrastingly, New Zealand avian aspergillosis outbreaks generally report *A. fumigatus* as the primary pathogen, potentially facilitated by non-disease factors such as stress or inbreeding. Since yeasts were isolated from two aspergillosis-infected kākāpō and given their capacity to produce potentially harmful metabolites during competition, it is important to consider whether these yeasts may contribute to immunosuppression or act as opportunistic pathogens that exploit immunosuppression induced by *A. fumigatus*. If nest-associated yeasts are

demonstrated to pose negative effects on kākāpō health, and since diet may influence yeast proliferation in kākāpō nest soils, future research could assess dietary alternatives that minimise abundance but sustain kākāpō nutrient requirements; recognising that supplementary feed was implemented for enhanced breeding outcomes (Houston et al., 2007; Von Hurst et al., 2016).

2.6 Conclusion

Our study showed that *Aspergillus* spp. abundances in kākāpō nests during the 2019 aspergillosis outbreak were generally low, with mean counts close to or below the 100,000 CFU g^{-1} threshold commonly reported for New Zealand avian translocations (Ewen et al., 2012; Glare et al., 2014). Some nests that had harboured birds with confirmed aspergillosis yielded no detectable *Aspergillus* spp., while others contained only moderate abundances (e.g., *A. flavus* ~37,000 CFU g^{-1}; *A. niger* ~156,000 CFU g^{-1}). One outlier nest (Hoki_2) exhibited exceptionally high counts (~5.4 million CFU g^{-1}), strongly influencing the overall mean, and was the only affected nest that had harboured the disease-causing agent; *A. fumigatus*. Thus, while affected nests on average had higher *Aspergillus* soil abundances than unaffected nests, the presence of infected birds was not always associated with high *Aspergillus* spp. soil loads, let alone the presence of *A. fumigatus* (instead *A. niger* and *A. flavus*), indicating that additional factors beyond nest soil abundance likely contribute to aspergillosis risk. This finding suggests that the initial exposure to the pathogenic species *A. fumigatus* could have occurred outside many nests, or from nest substrates other than soil (e.g., air or airborne dust), highlighting that investigating sources of *A. fumigatus* exposure should extend beyond soil counts and also the nesting area alone.

To investigate the potential role of proliferating yeasts in kākāpō nests as an additional factor in aspergillosis susceptibility, our first step was to broadly assess whether there was an inverse relationship between yeasts and *Aspergillus* spp. abundance. Statistical modelling (ZINB regression model) of viable counts suggested that an inverse relationship did occur between the two fungal types. Our results therefore suggest further research for nest-associated yeasts as potential biological controls. However, because these species have previously demonstrated pathogenic potential or enhancement of pathogenic traits in *A. fumigatus* (e.g., enhanced conidiation), further study is required to assess its effects on kākāpō health and whether this could be a contributing factor to aspergillosis susceptibility since nest soil abundance alone is not clear driver.

The spatial distributions of *Aspergillus* spp. and yeasts may provide valuable insights into causes of proliferation. Raw *Aspergillus* spp. counts were highest at kākāpō nest entrance soils, with potential links to soil disturbance caused by kākāpō movements. Yeasts were most abundant within kākāpō nest cavities, decreasing with distance from the nest, whilst there were no significant differences between the two breeding islands. Prior research indicates that kākāpō defecation is a key driver of these yeast communities.

2.6.1 Future research recommendations

We recommend regular monitoring of microbial communities in kākāpō nest soils (microbial community structure and viable counts) before and after each breeding season and any future outbreaks. By tracking changes in microbial communities and pathogen abundance, this could reveal whether infections stem from baseline microbes or from shifts caused by kākāpō activity. We also recommend monitoring of *A. fumigatus* loads in kākāpō nest air, dropped feed in nests, shared sites (e.g., feeding stations), and further research on potential spore transfer from mothers to their chicks, to confirm routes of initial pathogen exposure.

Since supplementary feeding can alter gut microbial diversity in kākāpō, and faecal microbiota may reflect the microbial composition of nest soils (West et al., 2025), it is possible that diet may indirectly shape the kākāpō nest microbial community. Comparative studies of environmental microbiota (e.g., nest soil, leaf litter, air), gut microbiota, and diet in healthy versus unhealthy birds could help uncover complex ecological processes leading to disease risk and clarify whether this risk is influenced by supplementary feeding or natural foraging. Lastly, we suggest genomic analysis comparing kākāpō that have been previously diagnosed with aspergillosis and those who have not, to help identify genetic susceptibility to aspergillosis and other commonly diagnosed diseases in kākāpō.

2.7 Acknowledgements

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3 Chapter 3: Variation in kākāpō nest-associated mycobiota reveals defecation as a potential driver

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3.1 Abstract

New Zealand's endemic kākāpō (*Strigops habroptilus*) have suffered two recent outbreaks of aspergillosis, a fungal respiratory disease that threatens population recovery efforts. Although *Aspergillus fumigatus* was confirmed as the causal agent, its near absence in nest soils during the 2019 outbreak suggests that other factors may influence disease risk. Using ITS1 (Internal Transcribed Spacer 1) amplicon sequencing, this study examined whether broader fungal communities may associate with aspergillosis susceptibility by characterising soil fungal diversity (alpha and beta) between affected and unaffected nests, breeding islands and across nest microhabitats. Fungal diversity did not differ significantly between aspergillosis-associated and unaffected nests, indicating no link between community richness or composition and disease outcome. However, diversity varied strongly among microhabitats: alpha diversity increased with distance from the nest cavity (Kruskal-Wallis, $H = 13.7–19.8$, $p \leq 0.001$), and beta diversity differed significantly across microhabitats (PERMANOVA, pseudo-$F = 7.62$, $R^2 = 0.20$, $p = 0.0001$). Island-level differences appeared only in nest-proximate soils (cavities and entrances), suggesting shared background forest communities but local modulation of nest microbiota. Nest cavities and entrances were dominated by yeasts - *Apiotrichum porosum* (100% prevalence), *Debaryomyces hansenii* (86.8%), and *Kazachstania telluris* (60.3%), overlapping with taxa known from kākāpō faecal microbiota. These patterns indicate that kākāpō presence strongly shapes nest fungal assemblages, likely through defecation and diet by proxy, producing yeast-enriched communities near nests. Given that several of these yeasts are known opportunists and may interact competitively with *Aspergillus* species, diet-derived yeasts could indirectly influence aspergillosis risk. Further research should therefore investigate dietary sources and their contribution to gut and nest mycobiota, ensuring management strategies consider potential dietary influences on exposure to pathogenic fungi.

Keywords: *Aspergillus fumigatus*, avian aspergillosis, kākāpō, mycobiota, soil, *Strigops habroptilus*.

3.2 Introduction

3.2.1 Kākāpō aspergillosis

Within the past decade, kākāpō have experienced diseases such as cloacitis (Jayasinghe et al., 2022; West, Ayriss, et al., 2025), aflatoxicosis (Meda Gedara, 2021), and candidiasis (Gartrell et al., 2021). However, a respiratory disease, aspergillosis, has begun to affect portions of the kākāpō population, coinciding with the past two breeding seasons in 2019 and 2022. Unlike these other conditions, aspergillosis, caused by the saprophytic fungus *Aspergillus fumigatus* (sometimes *A. flavus* and *A. niger*), is an acute and often fatal respiratory disease with no reliable treatment in birds (Hauck et al., 2020; R. M. Shaapan & Z. M. Girh, 2024; Tell, 2005; Tell et al., 2019). Respiratory infection by *A. fumigatus* typically occurs by the inhalation of its conidia (spores) (Arné et al., 2021; Moldoveanu et al., 2021). Avian species are likely more susceptible to this disease than humans, due to fewer defence mechanisms in their anatomy and immune systems when compared to mammals (Figure 3.1). For example, birds possess less-effective phagocytic heterophils and lack mammal-associated anatomical features that allow for expulsion (diaphragm) and trapping of particulate matter (epiglottis) from their airways (Tell, 2005). Gliotoxin, produced by *A. fumigatus,* is said to be 96% responsible for human aspergillosis (Ghazaei, 2017), and also a key virulence factor in avian aspergillosis (Reidy et al., 2022). However, unlike birds, humans can convert this compound to an inactive non-toxic product; bisdethiobis(methylthio)gliotoxin (bmGT), which means birds may be exposed to the active form for longer periods.

3.2.2 Biology of *A. fumigatus*

A. fumigatus is a saprophytic mould but also an opportunistic pathogen, mostly infecting immunocompromised mammals (e.g., humans) and birds (Hauck et al., 2020; R. M. Shaapan & Z. M. Girh, 2024; Tell, 2005; Tell et al., 2019). However, when exposed to large conidial abundances, infection can also occur in healthy individuals (Anne-Laure, 2021; Arné et al., 2021). *A. fumigatus* mostly infects through the respiratory tract of endotherms (i.e., mammals and birds), as conidia are airborne and therefore inhaled. Since *A. fumigatus* is considered mesophilic (optimal growth at 35-40°C) and thermotolerant (Chang et al., 2004), the warm core body temperatures of endotherms provide optimal growth conditions for *A. fumigatus*.

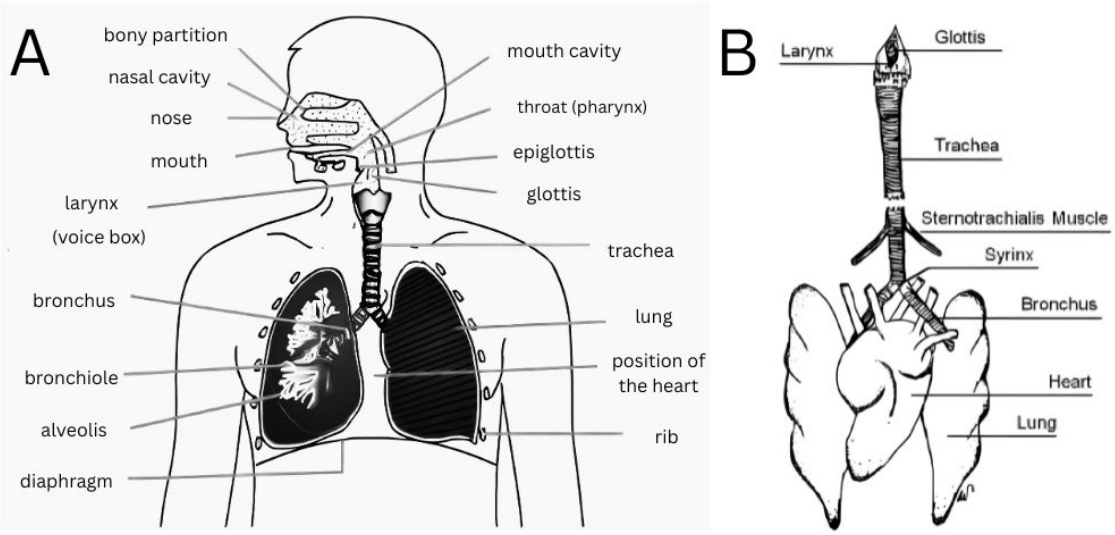

Figure 3.1 Comparison of the human and avian respiratory systems. Human respiratory anatomy (A) and avian respiratory anatomy (B). Adapted from Mawaishasan (Wikimedia Commons, CC BY-SA 4.0) and Dr. Jacquie Jacob, University of Kentucky (Wikimedia Commons, CC0 1.0). Additional labelling and figure composition by the author.

3.2.3 Disease susceptibility in kākāpō

In 2019, a kākāpō aspergillosis outbreak occurred on Whenua Hou Island (Figure 3.2), affecting 21 individuals and resulting in nine deaths. A second, smaller outbreak occurred on Pukenui Island in 2022, during which seven individuals were affected, and one died. Because kākāpō exist in such a small, intensively managed population, each aspergillosis death represents a significant setback to recovery efforts, making this disease uniquely threatening to the species' survival (Foster & Robertson, 2022; Meda Gedara, 2021; West et al., 2022; West et al., 2025; Winter et al., 2022).

Due to the alignment of outbreaks with the recent breeding seasons, it is suspected that the immunosuppression of mothers caused by breeding-stress is a large contributing factor to infection, along with the immature or underdeveloped immune systems of chicks. In New Zealand, kākāpō, along with other endemic bird species likely harbour even less advanced immune responses due to low genetic variation following major bottlenecks (Perrott & Armstrong, 2011; Tompkins & and Gleeson, 2006; Winter et al., 2022). These bottlenecks were a result of sudden population declines caused by habitat degradation and introduced mammalian predators, which were estimated to have been absent from the landmass for millions of years prior to human colonisation (Crisp, 2008; Fea et al., 2021; Tennyson, 2010). A well-known theory amongst ecologists and ornithologists is that the absence of mammalian predators may have been a contributing factor to the evolutionary trait loss of flight (referred to as flightlessness) as an escape mechanism in some New Zealand birds (Bromham et al., 2012; Johnston & Mitchell, 2021; McNab, 1994; Wright et al., 2016). However, upon the later introduction of mammalian predators, the flightlessness of many New Zealand birds, combined with their lack of evolved defences against such predators, make them particularly susceptible to predation.

Of these vulnerable avian species, the kākāpō (*Strigops habroptilus*) was one of the most severely affected, as their numbers declined to about 51 individuals in 1995 (G. Elliott et al., 2001; Stone et al., 2017). Since the establishment of the Kākāpō Recovery Programme in the mid 1990's, translocations to predator-free islands and intensive breeding programmes have successfully facilitated population recovery (Fischer et al., 2025; Meda Gedara, 2021; Urban et al., 2023). However, the kākāpō population remains low at 237 individuals (Department of Conservation, 2025). Due to the severe population bottleneck, increasing population numbers would have required inbreeding, thereby limiting genetic diversity and increasing susceptibility to diseases such as aspergillosis.

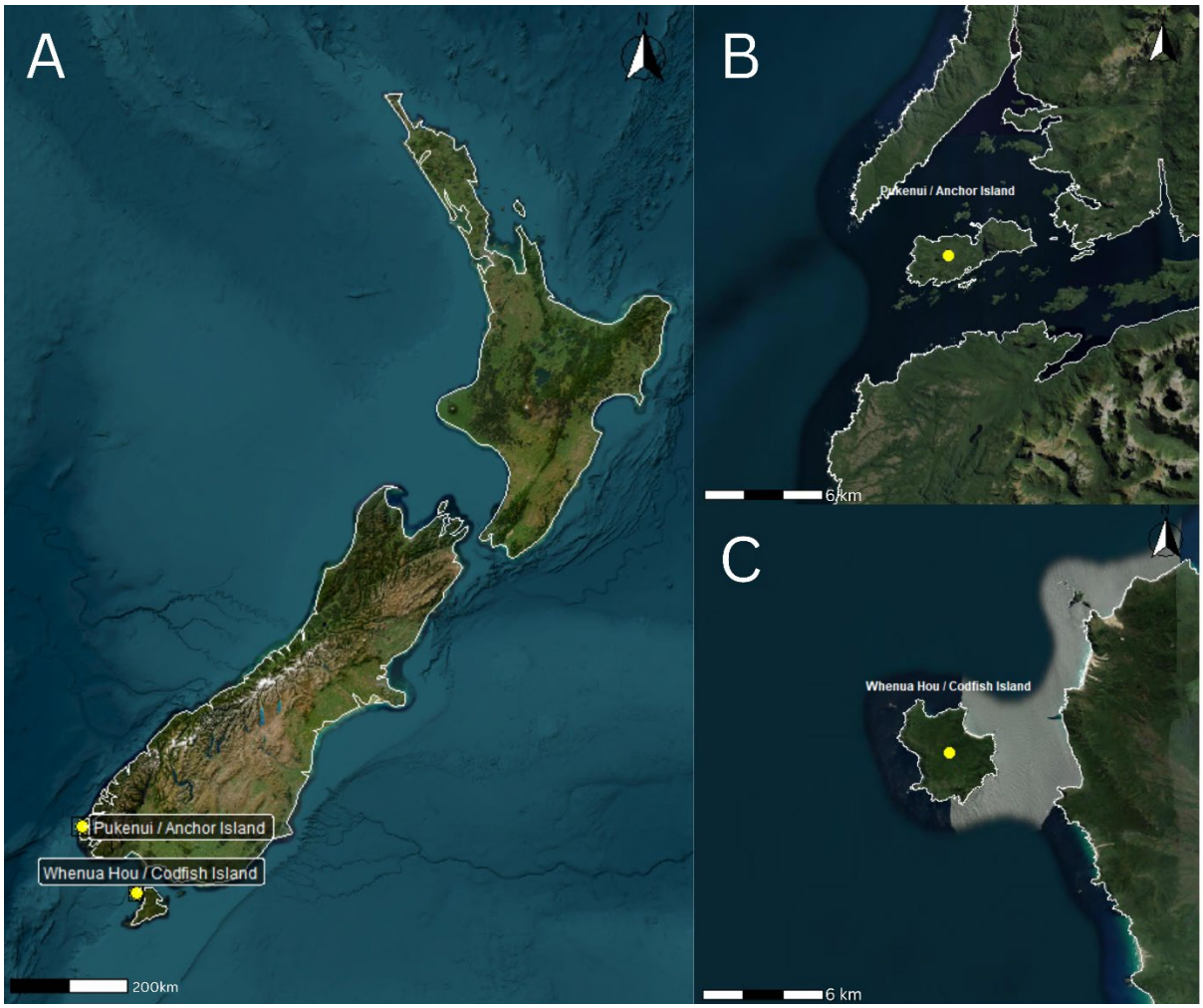

Figure 3.2 Satellite map of New Zealand showing the locations of the two kākāpō breeding islands sampled in this study: (B) Pukenui / Anchor Island and (C) Whenua Hou / Codfish Island. Insets show equal-scale close-up views of each island within Fiordland and Rakiura (Stewart Island) regions, respectively. Basemap imagery: Esri World Imagery; coastlines: Natural Earth (public domain).

3.2.4 Importance of environmental microbiomes

Environmental microbiomes form the foundation of ecosystem processes, playing a key role in nutrient cycling, decomposition of organic matter and providing nutrients for plants (Chen et al., 2024). In wildlife ecosystems, microbiomes influence the exposure of commensal and pathogenic microbes to

hosts (West et al., 2022). For kākāpō, the forest soil microbiomes of their breeding sites would arguably play a large role in health, conservation management and population recovery outcomes. By studying the microbial communities in kākāpō nest soils, researchers can identify beneficial taxa that maintain healthy and diverse soil communities (e.g., preventing proliferation of potential pathogens) and levels of pathogen exposure (e.g., *A. fumigatus*), providing a more holistic view of what may be influencing disease susceptibility.

Abiotic factors which drive forest soil microbial communities are typically include soil physicochemical conditions such as pH, moisture, temperature, oxygen availability, and nutrient content (Baldrian, 2016). Spatial and temporal (seasonal) variations also play a large role as it is tightly linked to microclimate (Wan et al., 2024). However, abiotic factors such as nutrient content and pH are also partially driven by biotic factors. For example, kākāpō nesting environments likely experience nutrient enrichment and pH changes through inputs such as faecal material, feathers, and food remains (Zwolicki et al., 2013), in addition to contributions from invertebrate activity and plant matter (e.g., leaf litter, roots) (Perrott, 2001). Beyond these inputs, interactions take place within microbial communities themselves. Competitive dynamics, including competition for nutrients and space, as well as producing secondary metabolites, ultimately affect the relative abundances of different taxa (Boddy & Hiscox, 2016; Kennedy, 2010; Losada et al., 2009; Wang & Kuzyakov, 2024).

In the context of *A. fumigatus*, abiotic factors that encourage higher abundances include warmer temperatures (Kwon-Chung & Sugui, 2013), higher water availability (Pasanen et al., 1991) and nutrient availability (Perez-Cuesta et al., 2021). However, little research has been conducted on the effects other microorganisms may have on *A. fumigatus* abundance in wildlife habitats, and whether these interactions lead to positive or negative outcomes on host health. For example, if competition causes the production of harmful secondary metabolites, or if other surrounding fungi pose as potential opportunists under immunosuppression caused by established infections. Therefore, capturing a broad view of avian environmental microbiomes is critical, as subtle changes in these environments may alter pathogen risk, thereby influencing population recovery efforts.

3.2.5 Kākāpō nest microbiome

Studies have performed microbial community analysis on avian nest materials to assess microbial influences on bird health, such as gut colonisation, hatchling survival and exposure to beneficial or pathogenic bacteria (Diez-Méndez et al., 2023; Song et al., 2023; Xin et al., 2023). In kākāpō nesting areas, the identification of soil and gut microbiota has only recently become available, encompassing the diversity and composition of both bacterial and fungal communities (West et al., 2022; West et al., 2023; West et al., 2025). Both studies on bacterial and fungal communities in kākāpō nests, found no significant effect on aspergillosis incidence, supporting the idea that kākāpō aspergillosis may be caused by a combination of ecological processes, rather than a singular driver.

However, although microbial community structure was not significantly different between collective affected and unaffected nests, it does not mean the nest microbiome has no effect at an individual level. For example, during the 2022 outbreak, yeasts such as *Debaryomyces* and *Kazachstania* spp. were isolated post-mortem from the only resulting aspergillosis death (kākāpō Jemma) (A. Digby, personal communication, March 20, 2023), which were the second and third most prevalent fungal taxa in kākāpō nest and faecal matter (West et al., 2025). Although, nest microbial communities as a whole may not directly link to aspergillosis, they may still contribute to disease progression or susceptibility of certain individuals, possibly based on genetic differences (e.g., immune-system functioning). Therefore, understanding what drives baseline kākāpō nest microbiomes may help inform future research and management strategies, especially if proliferating microbes prove to enhance or facilitate infection by *Aspergillus fumigatus*.

3.2.6 Aim

This study therefore aims to assess soil fungal community structure across spatial and temporal scales in kākāpō nesting areas to identify possible drivers of these communities. Research questions are whether fungal community structure and diversity differ between: (1) nest microhabitats (nest cavity, entrance and two metres away from the nest) (2) breeding islands (Pukenui and Whenua Hou), (3) aspergillosis-affected and unaffected nests of the 2022 outbreak (aspergillosis status), and (4) seasons (Summer, Autumn & Winter).

3.3 Methods

3.3.1 Soil Collection

Kākāpō nest soil samples were collected by the Kākāpō Recovery Group, Department of Conservation (DOC) New Zealand. These samples were collected from two offshore islands (Figure 3.2); Whenua Hou (Codfish Island; 46°46.07′ S, 167°37.23′ E) located near Rakiura (Stewart Island) and Pukenui (Anchor Island; 45.7587° S, 166.5093° E) located in Fiordland. Two sampling periods took place in 2022, one from the period of February to April (16 nests) and the other from May to June (15 nests) (Table 3.1). During the 2022 aspergillosis outbreak, only two kākāpō nests were associated with confirmed aspergillosis (associated with kākāpō Jemma and Roha). For further analyses, these nests will be referred to as affected nests (and nests with no confirmed aspergillosis cases as ‘unaffected nests’). Jemma’s soil was collected approximately one month prior to her death (the only 2022 outbreak death). In total, 93 soil samples were analysed, of which six were sampled from affected nests.

Soil samples were placed in plastic resealable bags, containing approximately 50-70 grams of combined topsoil and leaf litter. Each kākāpō nest included samples from three sublocations (nest microhabitats): the nest cavity, cavity entrance and two metres away from the cavity. Samples were then stored at -20°C until further analyses.

Table 3.1 Two sampling periods showing the associated kākāpō nests, island and whether those nests were affected or unaffected by aspergillosis (harboured an individual with diagnosed/confirmed aspergillosis). Wehe-Po = Weheruatanga- o te pō, M-Maree = Margaret-Maree.

February-April 2022			May-June 2022		
Breeding Kākāpō female	**Island**	**Aspergillosis**	**Breeding Kākāpō female**	**Island**	**Aspergillosis**
*Jemma	Pukenui	Affected	*JEM	Pukenui	Unaffected
Ra	Pukenui	Unaffected	Cyndy	Whenua Hou	Unaffected
Titapu	Whenua Hou	Unaffected	Titapu	Whenua Hou	Unaffected
Roha	Pukenui	Affected	M-Maree	Whenua Hou	Unaffected
Pearl	Whenua Hou	Unaffected	Pearl	Whenua Hou	Unaffected
Bella	Whenua Hou	Unaffected	Bella	Whenua Hou	Unaffected
Gertrude	Pukenui	Unaffected	Gertrude	Pukenui	Unaffected
Rakiura	Whenua Hou	Unaffected	Rakiura	Whenua Hou	Unaffected
Stella	Pukenui	Unaffected	Stella	Pukenui	Unaffected
Marama	Pukenui	Unaffected	Marama	Pukenui	Unaffected
Alice	Whenua Hou	Unaffected	Alice	Whenua Hou	Unaffected
Marian	Pukenui	Unaffected	Marian	Pukenui	Unaffected
Tiwhiri	Pukenui	Unaffected	Waikawa	Pukenui	Unaffected
Waikawa	Pukenui	Unaffected	Tumeke	Whenua Hou	Unaffected
Tumeke	Whenua Hou	Unaffected	Wehe-pō	Whenua Hou	Unaffected
Wehe-pō	Whenua Hou	Unaffected			

3.3.2 DNA extraction and amplicon library construction

Microbial DNA was extracted from the soil samples using the DNeasy PowerSoil Pro Kit (Qiagen, USA) following the standard manufacturer's protocol. Recovered DNA were quantified using QubitTM DNA HS Assay kit (ThermoFisher Scientific, Massachusetts, USA) and a Qubit 2.0 Fluorometer (Invitrogen; USA) (see Appendix B, Table S1 for results).

Five nanograms per microlitre of DNA was used for PCR amplification targeting the entire ITS region (ITSI-5.8S-ITSII) using primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') with Illumina adapter overhang sequences appended to the 5' ends for sequencing library preparation. Library preparation was completed following as per manufacturer's protocol (Fungal Metagenomic Sequencing Protocol; Document #1000000064940 v00, Illumina, San Diego, CA, USA, 2018) with PCR purification completed using homemade Carboxyl-modified Sera-Mag Magnetic Speed-beads as described in (Rohland & Reich, 2012). Amplicon libraries were sequenced using Illumina MiSeq V3 kit (600 cycle) following the manufacturer's protocol. Negative

controls were used throughout extractions (Appendix B, Table S1), PCR library prep and DNA sequencing (see Section 3.4.1 for results).

3.3.3 Data analysis

Due to the poor quality of reverse reads, only forward reads covering the ITS1 subregion were used for downstream analysis. This approach is supported by literature demonstrating that single forward reads using ITS1F alone could provide sufficient or even higher taxonomic resolution for fungal community studies than assembled reads (Pauvert et al., 2019) and is an effective alternative for when reverse read quality is limiting (Hoggard et al., 2018).

All raw forward sequencing reads were processed in R (version 4.2.2) (Team, 2024). Primer sequences were trimmed using cutadapt (version 4.2) (Martin, 2011) with the ITS1F primer (5′-CTTGGTCATTTAGAGGAAGTAA-3′) as the only target. Using the DADA2 pipeline (version 1.28.0) (Callahan et al., 2016), reads were filtered with the following thresholds: no ambiguous bases (maxN = 0), minimum length of 50 bp, maximum expected errors of 5 (maxEE = 5), and minimum quality score of 2 (truncQ = 2). PhiX sequences were removed, and filtered reads were compressed for downstream analysis. Denoising was performed using DADA2's pseudo-pooling method, with error rates learned from the filtered reads. Amplicon sequence variants (ASVs) were inferred and compiled into a sequence table. Chimeras were identified and removed using the consensus method. Read counts were tracked at each processing step for all samples to assess data retention. Taxonomy was assigned to non-chimeric ASVs using the DADA2 naïve Bayesian classifier against the UNITE database (version 04.04.2024). A phyloseq object (McMurdie & Holmes, 2013) was created from the ASV table and taxonomy assignments for downstream ecological analyses.

The DADA2-denoised fungal ITS1 amplicon data were imported as a phyloseq object using R. Sample metadata were independently loaded from an Excel spreadsheet and formatted with sample IDs as row names. Metadata were then merged with the phyloseq object to ensure alignment between sequence data and sample information. The resulting combined phyloseq object was validated for integrity before downstream analyses.

3.3.4 Statistical analyses

R was used for all visual and statistical analyses. The dataset was filtered to retain only fungal sequences (using the genefilter package) (Gentleman et al., 2005) and decontaminated using the decontam package's prevalence method (Davis et al., 2018). ASVs identified as contaminants were removed prior to analysis. For relative-abundance visualisations, samples with fewer than 500 total reads were excluded to reduce low-depth noise, resulting in the removal of 21 samples. The remaining 75 samples were transformed to relative abundance by scaling counts within each sample to sum to 1.

All data visualizations were generated using ggplot2 (Wickham, 2016). For fungal community structure plots, ggplot2, ggforce (Pedersen, 2019) for facet wrapping by nest microhabitat, and RColorBrewer (Neuwirth, 2014) to provide distinguishable palettes. Relative abundance percentages were measured at both phylum and genus levels using phyloseq. Taxa were aggregated and normalised to relative abundance, filtering out taxa below 1% using genefilter. Mean relative abundances were summarized by nest microhabitat and converted to wide format for easier comparison. Mean relative abundances were summarised by nest microhabitat and presented at both broad (phylum) and finer (top 15 genera) taxonomic resolutions (Appendix B, Table S4).

For diversity analyses, samples with fewer than 1,000 total reads were excluded, and the remaining samples were rarefied to 1,000 reads (phyloseq rarefy_even_depth) prior to alpha- and beta-diversity analyses. Alpha diversity (Observed, Chao1, Shannon) was computed with estimate_richness (phyloseq). Because distributions were non-normal, Kruskal–Wallis tests (base R) were used for group comparisons (nest microhabitat, island, season, aspergillosis status). Post-hoc pairwise tests for multi-level factors (microhabitat, season) used Dunn's test with Benjamini–Hochberg (BH) correction, and adjusted *p*-values are reported. Box–jitter plots were assembled with patchwork (Pedersen, 2020), with adjusted *p*-values (or stars) annotated.

Beta diversity was quantified as Bray–Curtis dissimilarity and visualised by PCoA (Oksanen et al., 2022). Group differences were tested by PERMANOVA (adonis2, vegan) using a fixed seed (set.seed(42)) and 9,999 permutations. To verify assumptions, homogeneity of multivariate dispersion was assessed with PERMDISP (betadisper & permutest) within microhabitats and within islands. Non-significant dispersion results support interpretation of PERMANOVA effects as centroid (composition) differences rather than unequal spread. The full dataset was used to assess alpha and beta diversity across spatial and temporal scales, however, only February and March samples were included in analyses of aspergillosis status (months corresponding to affected samples) to remove potential variability associated with later months.

3.4 Results

3.4.1 Fungal community structure of kākāpō nest areas

The total number of raw forward reads obtained from MiSeq sequencing was 684,669. After primer trimming, quality filtering, denoising, and chimera removal, potential contaminants were identified using the decontam prevalence method (threshold = 0.10) with 4 negative controls. This flagged 1 of 1,126 fungal ASVs (0.09%), representing less than 0.01% of reads, and the flagged ASV was removed prior to downstream analyses. Samples with fewer than 500 reads were then excluded from downstream analyses, reducing the dataset from 96 to 68 samples and yielding 684,669 high-quality forward reads across the retained samples. Only 4.62% of amplicon sequence variants (ASVs) could not be classified to a known fungal phylum. Sequencing depth was highly variable (10,068.66 ± 17,289.14 SD reads per

sample); therefore, samples were rarefied to 1,000 reads to standardise sequencing effort and improve the reliability of diversity estimates while retaining as many samples as possible (Appendix B, Figure S1). Although rarefaction curves did not visibly plateau, Good's coverage ($1 - f_1/N$) indicated high sequencing completeness across all samples (mean = 0.998 ± 0.002) (Appendix B, Table S2). Even after standardizing to 1,000 reads per sample, coverage remained 0.991 ± 0.008, suggesting that most fungal taxa present were captured at the chosen depth and that remaining unseen diversity was confined to extremely rare ASVs. In total, 1,046 unique fungal ASVs were detected across all samples. The number of ASVs per sample ranged from 6 to 197, averaging at 45 ASVs.

3.4.1.1 Lack of Aspergillus spp. detection

Only six ASVs were assigned to the genus *Aspergillus*. They occurred in four samples and were always rare (less than 3% relative abundance). None could be confidently identified to species level by NCBI BLAST. Of the two nest areas with confirmed aspergillosis, only Jemma's nest area showed detectable *Aspergillus* (a single ASV at 0.03% RA) while all other *Aspergillus* ASVs were found in nest areas without aspergillosis (Appendix B, Table S3).

3.4.1.2 Overall relative abundance

Basidiomycota, Ascomycota and Mortierellomycota were the most abundant fungal phyla across all nest microhabitats (cavity, entrance and two metres away from the nest) (Figure 3.3; Appendix B, Table S4). Although Basidiomycota was the dominating phylum across all nest microhabitats, it was most abundant within the nest cavities and gradually decreased with distance, whilst Mortierellamycota increased with distance from the nest cavities. Mean relative abundance of Ascomycota remained somewhat consistent across all nest microhabitats, ranging from 18-26%.

Apiotrichum was dominant genus across all nest microhabitats but was on average the highest (79.38%) within nest cavities (Figure 3.4; Appendix B, Table S4). Compared to the other nest microhabitats, the nest cavities harboured substantially higher mean relative abundances (MRAs) of genera *Arthrographis* (6.23%), *Debaryomyces* (5.39%), and *Penicillium* (2.34%). Nest entrances harboured the highest relative abundances of *Ilyonectria* (3.94%) and *Sporothrix* (3.34%), and soils from two metres away harboured the highest *Podila* (16.39%), *Trichoderma* (10.54%), *Candida* (6.3%), *Tausonia* (4.41%), and *Mortierella* (3.25%).

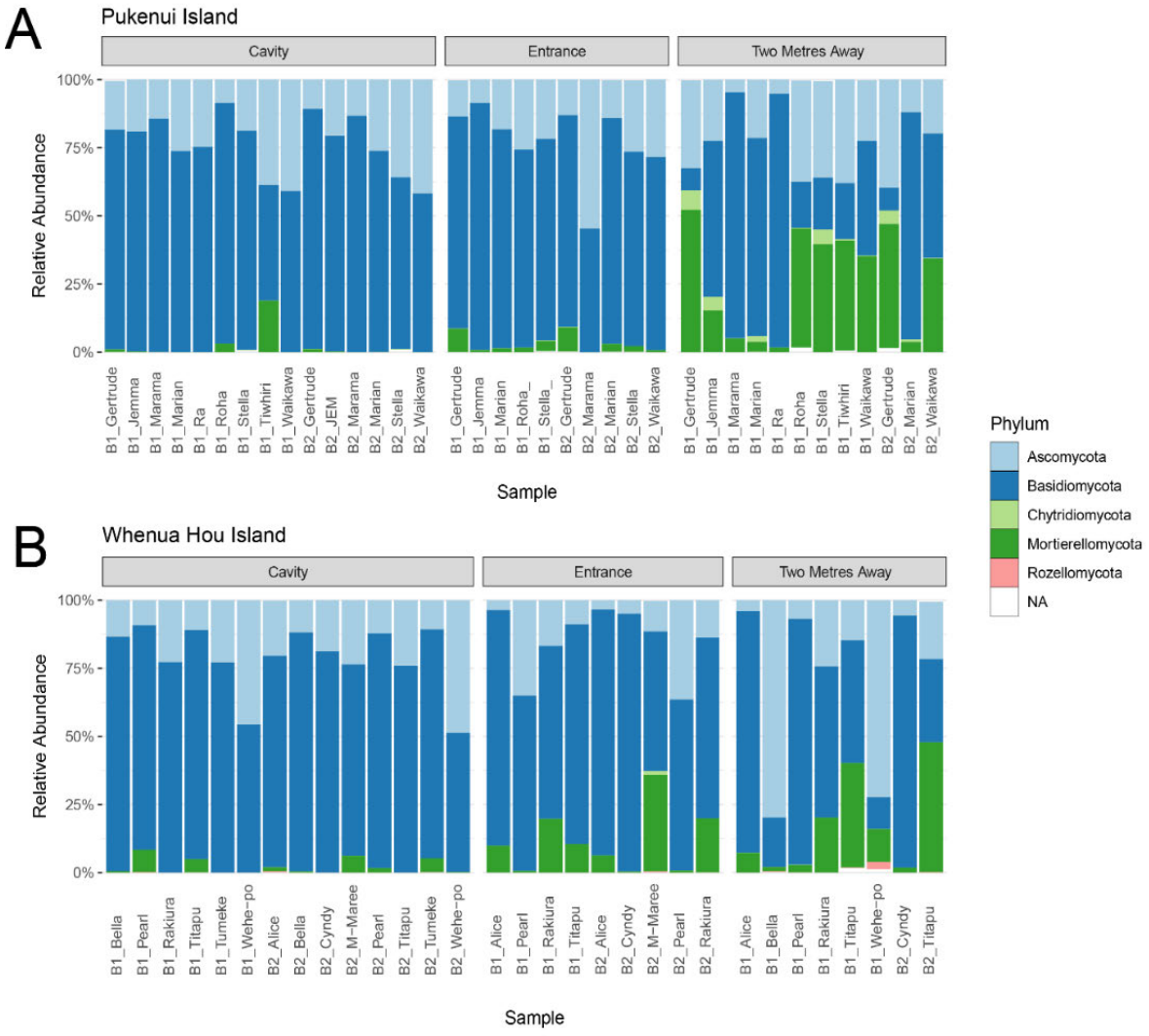

Figure 3.3 Relative abundance (RA) of fungal (ITS1) phyla higher than 1% in at least one sample, across nest microhabitats, and between breeding islands: (A) Pukenui/Anchor Island and (B) Whenua Hou/Codfish Island. Each sample (x-axis) corresponds to a kākāpō nest (named by kākāpō breeding female). B1 = February to April, B2 = May to June.

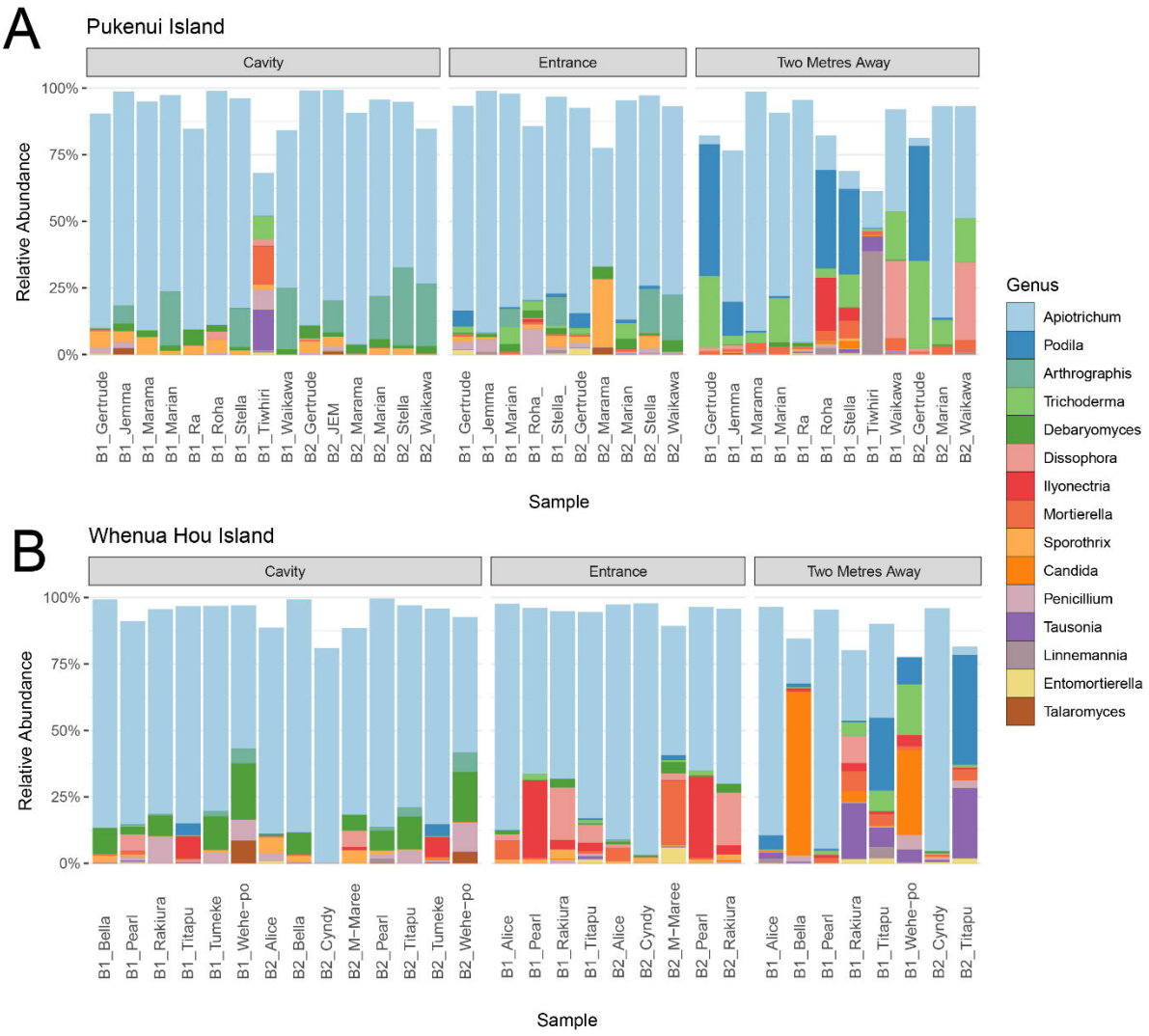

Figure 3.4 Fifteen most abundant (RA) fungal genera higher than 1% in at least one sample, across nest microhabitats and between breeding islands: (A) Pukenui/Anchor Island and (B) Whenua Hou/Codfish Island. Each sample (x-axis) corresponds to a kākāpō nest (named by kākāpō breeding female). B1 = February to April, B2 = May to June.

Of the seven most common ASVs, three were assigned to species via NCBI BLAST (Appendix B, Table S5); ASV4–ASV7 were resolvable only to genus. ASV1 matched *Apiotrichum porosum* (100% prevalence). ASV2 matched *Debaryomyces hansenii* (87% prevalence). ASV3 matched *Kazachstania telluris* (61% prevalence) but remained consistently low in relative abundance and did not rank among the 15 most abundant genera. Genus-level assignments for ASV4–ASV7 included *Ilyonectria*, *Podila*, *Apiotrichum*, and *Penicillium*.

Prevalence of ASVs were calculated as the proportion of samples in which an ASV was detected:

$$Prevalence\ (\%) = \frac{number\ of\ samples\ with\ ASV}{number\ of\ total\ samples} \times 100$$

3.4.1.3 Between-island relative abundance

Although both Pukenui and Whenua Hou showed similar proportions of *Apiotrichum* spp. (Appendix B, Table S4), minor differences in relative abundance of other genera were observed. Nest cavities of Pukenui showed higher relative abundances of *Arthrographis* spp. (~10%) compared to Whenua Hou (~1.5%), whilst the Whenua Hou's cavities harboured a greater proportion of *Debaryomyces* species (~8%) than Pukenui (~2.5%). Pukenui's nest entrances had a similar composition to their nest cavities, whilst Whenua Hou's entrances showed a slightly different composition to its cavities, with greater proportions of *Ilyonectria* (~8%), *Dissophora* (~6%) and *Mortierella* (~4.5%). On Pukenui, soils from two metres away showed high relative abundances of *Podila* (~16%) and *Trichoderma* (~12%), whereas on Whenua Hou, higher proportions of *Candida* (~13%) and *Tausonia* (~8%) were observed.

3.4.2 Alpha and beta diversity of fungal ASVs

3.4.2.1 Nest microhabitat

Significant differences in alpha diversity were observed between nest microhabitats (Table 3.2), increasing with distance from nest cavities (Figure 3.5). Dunn's pairwise tests showed broad differences in observed richness among microhabitats (Figure 3.3), with Chao1 and Shannon differing significantly only between cavity and two metres away.

Table 3.2 Kruskal–Wallis test results for alpha diversity metrics (Observed richness, Chao1 richness, and Shannon index) across aspergillosis status, island, season, and nest microhabitat. Test statistics are reported as H with corresponding degrees of freedom (df) and *p*-values.

Observed Richness			
Covariate	H	df	*p*-value
Aspergillosis Status	0.12	1	0.73
Island	0.73	1	0.39
Season	5.53	2	0.06
Nest microhabitat	19.8	2	0.00005****
Chao1 Richness			
Covariate	H	df	*p*-value
Aspergillosis Status	0.09	1	0.77
Island	1.44	1	0.23
Season	5.03	2	0.08
Nest microhabitat	16.6	2	0.00025***
Shannon Index			
Covariate	H	df	*p*-value
Aspergillosis Status	0.12	1	0.73
Island	0.03	1	0.86
Season	1.80	2	0.41
Nest microhabitat	13.7	2	0.00106**

Significant *p*-values are denoted with asterisks ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, $p < 0.0001$ = ****).

For beta diversity (Bray–Curtis PERMANOVA), the overall effect of nest microhabitat was highly significant ($p < 0.001$; Figure 3.4). Pairwise PERMANOVA (Benjamini–Hochberg adjusted) indicated

no difference between cavity and entrance, whereas two metres away differed from both cavity (p(adj) < 0.001) and entrance (p(adj) < 0.001). However, PERMDISP showed heterogeneous dispersion across microhabitats overall ($p < 0.001$) and dispersion differences for comparisons involving two metres away (cavity vs two metres away, $p < 0.001$; entrance vs two metres away, $p < 0.001$), while cavity vs entrance showed no dispersion difference ($p = 0.155$). Therefore, the non-significant cavity–entrance result may reflect similar community centroids with comparable spread, whereas the strong differences involving two metres away may reflect a combination of location (centroid) and dispersion effects. These findings indicate that microhabitat patterns in beta diversity are likely driven by the distinct and more variable communities found two metres from nests, while cavity and entrance communities are comparatively similar.

Table 3.3 Dunn's post-hoc pairwise comparisons of alpha diversity metrics (Observed richness, Chao1 richness, and Shannon index) between nest microhabitats (Cavity, Entrance, Two metres away). Test statistics are reported as Z, with corresponding raw and Benjamini–Hochberg-adjusted p-values. Sample sizes for each microhabitat were: cavity (n = 26), entrance (n = 18), and two metres away (n = 17).

Measure	Comparison	Z	p - value	p (BH-adjusted)
Observed	Cavity – Entrance	1.98	0.0473*	0.0473*
	Cavity – Two Metres Away	4.44	0.00000879****	0.0000264****
	Entrance – Two Metres Away	2.3	0.0214*	0.032*
Chao1	Cavity – Entrance	2.01	0.0447*	0.0549
	Cavity – Two Metres Away	4.05	0.0000501****	0.00015***
	Entrance – Two Metres Away	1.92	0.0549	0.0549
Shannon	Cavity – Entrance	1.8	0.0718	0.0774
	Cavity – Two Metres Away	3.68	0.000229***	0.000688***
	Entrance – Two Metres Away	1.77	0.0774	0.0774

Significant p-values are denoted with asterisks ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, $p < 0.0001$ = ****).

Table 3.4 Beta-diversity (Bray–Curtis) PERMANOVA results for fungal community dissimilarities across all nest microhabitats (Cavity, Entrance, Two metres away). including overall effects, interactions, within-microhabitat comparisons, and pairwise contrasts. Test statistics are reported as pseudo-F, with corresponding R^2 values and raw and adjusted p-values.

Comparison	F	R^2	p - value	p (BH-adjusted)
Island	1.66	0.02	0.0996	0.1727
Island: Nest microhabitat	1.50	0.04	0.1036	0.1727
Nest microhabitat	7.62	0.20	0.0001 ***	0.0005 ***
Season	0.73	0.02	0.7621	0.9526
Aspergillosis Status	0.41	0.01	0.9589	0.9589
Cavity: Island differences	3.46	0.13	0.0054 **	0.0162 *
Entrance: Island differences	1.97	0.11	0.0221 *	0.0332 *
2 m away: Island differences	1.02	0.06	0.3655	0.3655
Cavity vs Entrance	1.78	0.04	0.0666	0.0666
Cavity vs 2 m away	10.56	0.20	0.0001 ***	0.0003 ***
Entrance vs 2 m away	6.04	0.15	0.0002 ***	0.0003 ***

Notes. PERMANOVA used 9,999 permutations with a fixed seed (set.seed(123)). Homogeneity of multivariate dispersion (PERMDISP) checks were non-significant within the relevant strata, supporting interpretation as centroid (compositional) differences. For aspergillosis status (affected vs. not affected), only February–March samples were analysed. F = pseudo-F; R^2 = variance explained. Significance: $p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, $p < 0.0001$ = ****.

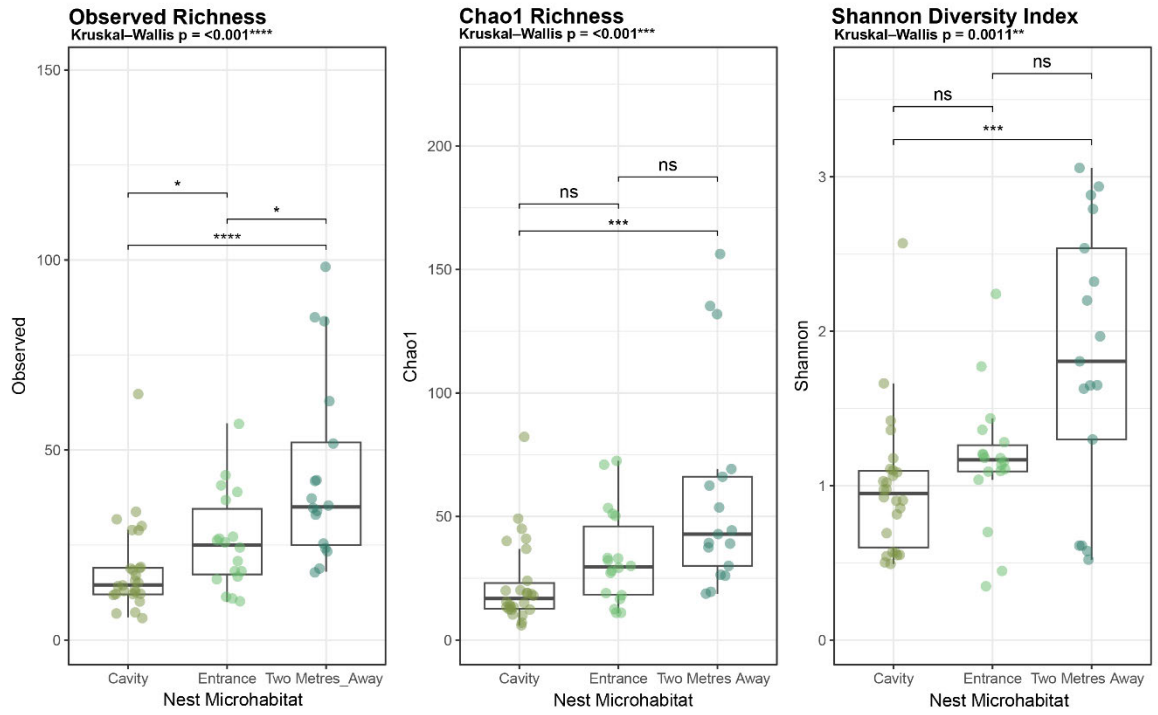

Figure 3.5 Fungal alpha diversity in kākāpō nest soil samples across the nest cavity, entrance and two metres away. Alpha diversity metrics were measured using observed richness, Chao1 estimated richness, and the Shannon Diversity Index. Kruskal–Wallis test *p*-values demonstrate statistical differences in alpha diversity between nest microhabitats. Significant P-values are denoted with asterisks ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***, $p <$ 0.0001 ****).

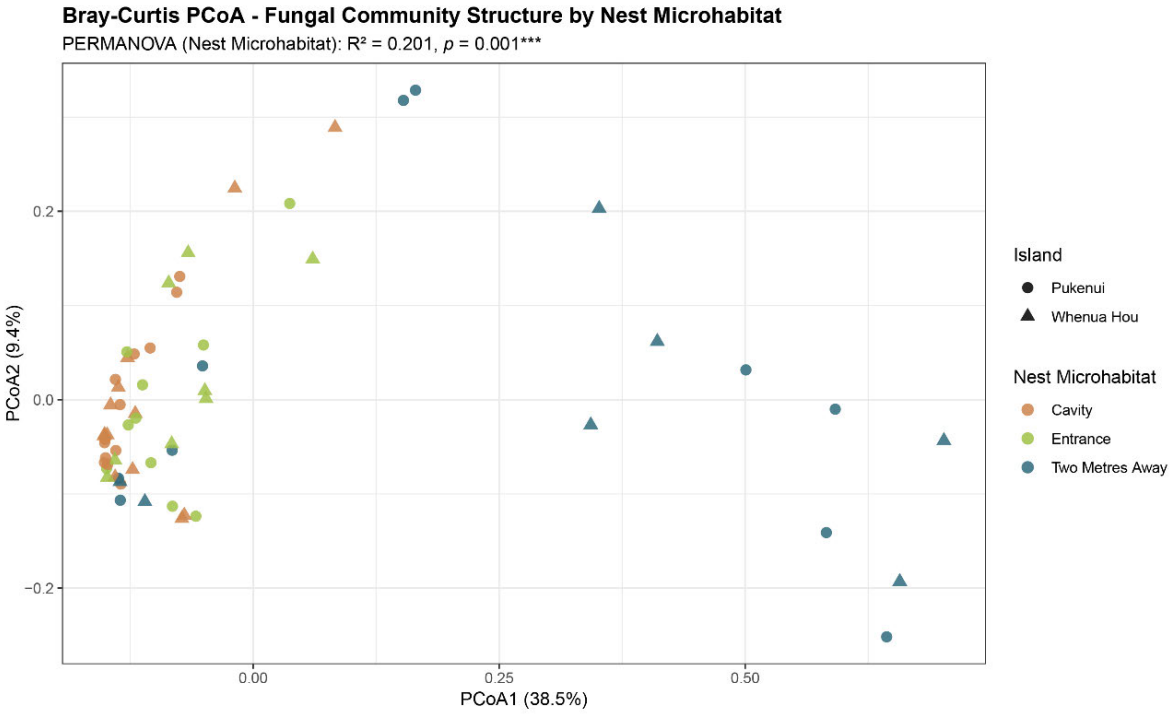

Figure 3.6 Principal coordinate analysis (PCoA) of rarefied ITS1 sequences from kākāpō soil samples using Bray-Curtis dissimilarity. Each dot represents the fungal community structure of each soil sample, coloured by nest microhabitat type and shaped by island. Samples were rarefied to 1,000 reads (Appendix B, Figure S1).

3.4.2.2 Island

Island location had no significant effect on fungal alpha diversity (Figure 3.7; Table 3.2). For beta diversity across all microhabitats, there was no island effect and no 'island × nest-microhabitat' interaction (Table 3.4). Within microhabitats, between-island differences were detected in cavities (p(adj) < 0.05) and entrances (p(adj) < 0.05), but not at two metres away samples. Dissimilarity between islands was observed in PCoA when two metres away samples were removed (Figure 3.8). Homogeneity of dispersion (PERMDISP) within each microhabitat was not violated (Cavity: $p = 0.742$; Entrance: $p = 0.544$; Two metres away: $p = 0.856$), supporting interpretation as centroid (composition) differences. Together, these results indicate that fungal composition differs between islands inside nests and at entrances, while richness and evenness do not.

3.4.2.3 Aspergillosis status and season

No significant differences were observed in fungal alpha diversity and beta diversity between affected nests and unaffected nests, and between seasons (Table 3.2; Table 3.4) (Appendix B, Figure S2-S5). Additionally, no significant effects were observed when samples from two metres away were removed.

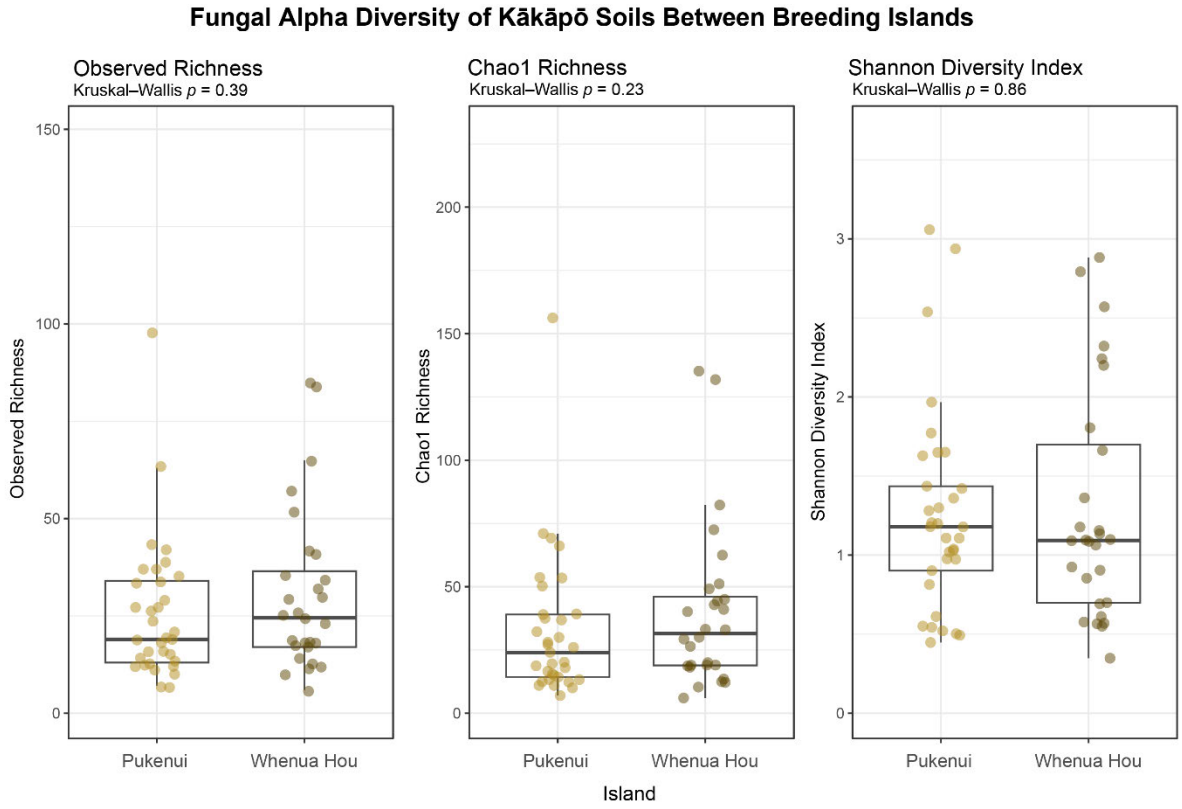

Figure 3.7 Fungal alpha diversity in kākāpō nest soil samples from Pukenui and Whenua Hou, measured using observed richness, Chao1 estimated richness, and the Shannon Diversity Index. Kruskal–Wallis test p-values demonstrate no statistical differences in alpha diversity between islands.

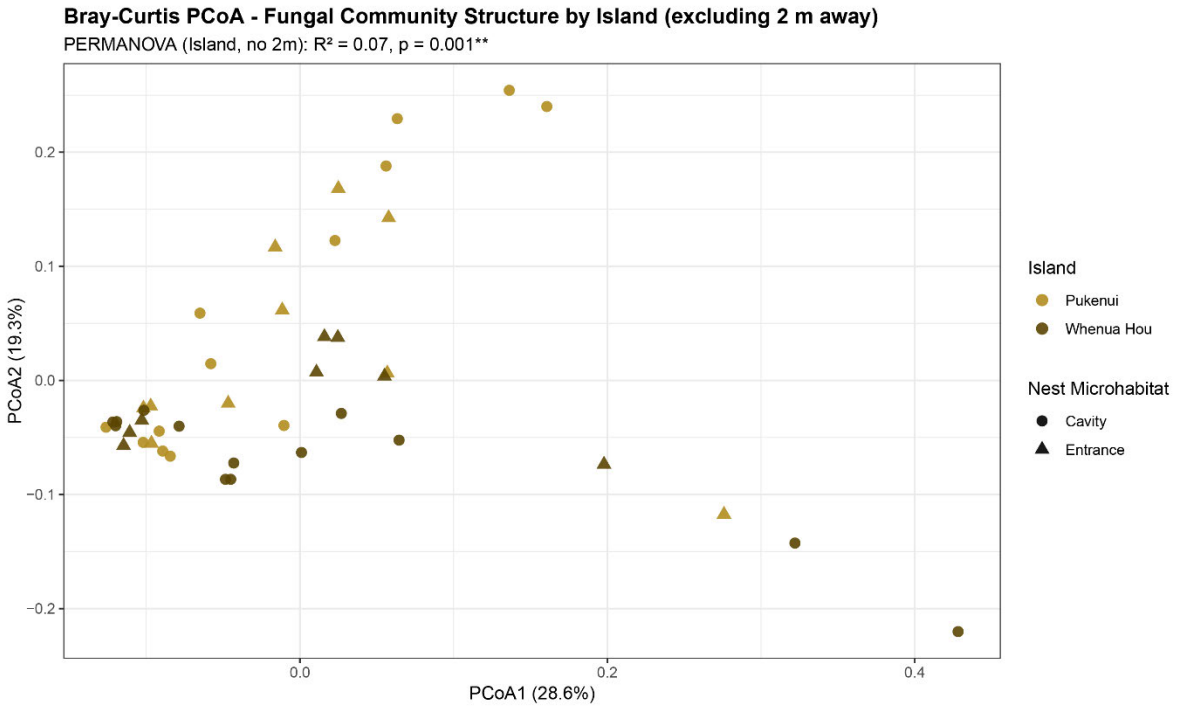

Figure 3.8 Principal coordinate analysis (PCoA) of rarefied ITS1 sequences from kākāpō soil samples using Bray-Curtis dissimilarity. Plot shows island differences with cavity and entrances only. Each dot represents the fungal community structure of each soil sample, coloured by island, and shaped by nest microhabitat. Samples were rarefied to 1,000 reads (Appendix B, Figure S1).

3.5 Discussion

3.5.1 Kākāpō defecation as a potential driver of nest mycobiota

Fungal community composition (beta diversity) across kākāpō nest microhabitats differed significantly, whereas pairwise comparisons of cavities and entrances alone did not. Pairwise comparisons also revealed that soils two metres away were more species rich, making them highly dissimilar to cavities and entrances. Overall, this demonstrates that fungal composition of kākāpō nest-proximate soils (cavities and entrances) varied strongly from natural forest soil communities, consistent with observations in other bird species where nest environments support microbial communities distinct from surrounding soils (Xin et al., 2023; Yang et al., 2025). In parallel, richness and evenness (alpha diversity) increased with distance from cavities but did not differ between islands. Together, these patterns highlight a localized nest effect, where in kākāpō nests (regardless of island location), certain taxa are introduced to the nest or enriched, leading to yeast-dominated, uneven and less diverse communities.

Island comparisons support this view, for example, the community structures of soils two metres away from nest sites were consistent across samples collected at Whenua Hou and Pukenui respectively, implying broadly similar background forest communities. In contrast, nest-proximate soils differed between these islands, indicating that while a common nest effect occurred, local environmental factors

may have further driven changes in fungal composition. Examples could be differences in abiotic factors (e.g., temperature, moisture, vegetation type) or biotic factors (e.g., diet via faecal deposition or kākāpō breeding female behaviour) (Castaño et al., 2018; Muneer et al., 2021; Zhou et al., 2017).

Previous analysis of viable counts in kākāpō nest soils (Kanis et al., in prep., Ch. 2) showed that yeasts proliferate in cavities and entrances and are scarce at two metres away. The present DNA-based analysis corroborates this, where *Apiotrichum* spp. dominated nest cavities (~79% RA overall), and Whenua Hou cavities also presented large proportions of *Debaryomyces* (~8% RA). Both taxa decreased in RA with distance from the nest. Across methods, the conclusion is the same, kākāpō nests consistently harbour yeast-rich communities.

This study's results align with West et al. (2025), who reported similar fungal composition in kākāpō faecal and nest-litter samples. In both studies, *Apiotrichum* (often unclassified to species), *Debaryomyces hansenii*, and other yeast taxa (e.g., *Candida palmioleophila*, *Spencermartinsiella* spp.) were prominent. Both studies' most prevalent ASVs were also *Apiotrichum porosum*, *D. hansenii*, and *Kazachstania telluris* (Table 3.5).

Table 3.5 Results comparison of the three most prevalent amplicon sequence variants (ASVs) with West and colleagues (2025).

Species Name of ASV	Current Study (Soil)	(West et al., 2025) (Nest Litter)	(West et al., 2025) (Faecal Matter)
Apiotrichum spp.	100% (*A. porosum*)	100% (unclassified)	86.1% (unclassified)
Debaryomyces hansenii	86.76%	96.1%	87%
Kazachstania telluris	60.29%	-	88.9%

The overlap in fungal composition of kākāpō faecal matter and nest litter samples, along with the spatial patterns observed (across nest microhabitats and islands), indicates that kākāpō defecation is a likely driver of their nest fungal community structure. Considering the likely warm and nutrient rich environment of a kākāpō nest, this also may be contributing to the high relative abundances of mesophilic taxa observed.

3.5.2 The role of diet in avian nests

According to West and colleagues (2025), kākāpō mothers feed their chicks a mixture of rimu berries and supplementary feed (Harrison's High Potency Coarse pellets) (Foods, 2025) until fledging (~10 weeks). Afterwards, chicks begin to shift to seasonal plant material, coinciding with a marked decline in the relative abundance of yeasts such as *Apiotrichum*, *Debaryomyces hansenii*, and *Candida palmioleophila* in the gut mycobiota (faecal samples). This pattern suggests that dietary composition is a major contributor to the yeast-rich mycobiota observed in kākāpō nests. At the same time, nest microclimate likely reinforces these effects, where the warm, humid, nutrient-rich conditions of active nests may promote proliferation of introduced yeasts and amplify background environmental taxa. For instance, *Apiotrichum* occurs in soils two metres away but reaches much higher relative abundances

within nest cavities, suggesting environmental amplification, whereas *Debaryomyces* appears largely confined to nest-proximate soils, implying dietary or host-mediated introduction. Overall, yeast communities in kākāpō nest soils appear shaped by both dietary inputs and environmental selection operating within kākāpō nest microhabitats.

Although rimu berries cannot be ruled out as a potential contributor to yeast proliferation, yeasts that were found in kākāpō nests and gut mycobiota (e.g., *Apiotrichum*, *Debaryomyces*, *Kazachstania*, *Candida*, *Meyerozyma* and *Spencermartinsiella*) (Kanis et al., in prep., Ch. 2; West et al., 2025), were also detected in the habitats and gut of captive or partially synanthropic birds. Particularly, those that occasionally consume human food waste or processed feed.

In Russia (Glushakova & Kachalkin, 2024b), partially synanthropic birds showed gut mycobiota rich in *Candida* spp., *Debaryomyces hansenii*, and *Meyerozyma* spp., whereas wild birds had little to none of these taxa. Principal component analysis (PCA) showed that the gut mycobiota of partially synanthropic birds differed significantly from those of wild birds and closely resembled their respective diets. More specifically, three partially-synanthropic species were observed feeding at an urban waste storage site, which was reflected in PCA as their gut mycobiota clustered together. However, faecal samples of one partially-synanthropic species (not observed feeding at waste sites) aligned more closely with fruit samples.

In another Russian study on Mew gulls (Glushakova & Kachalkin, 2024a), faecal samples from natural nesting sites were associated with higher fungal diversity, whilst urban nesting sites were associated with mostly pathogenic antifungal resistant species (e.g., *C. parapsilosis*, *C. tropicalis*, *D. hansenii*). Faeces of gulls from natural nesting sites were linked to yeast species found in flowers, fruits, seeds, insects and topsoil, whereas urban gulls were observed to consume more human food waste products from urban sites.

Studies on captive and domesticated birds further support this link. In broiler chickens, gut mycobiota shifted rapidly after the introduction of a soybean–corn diet, with *Trichosporon* (*Apiotrichum*) and *Meyerozyma* among the dominant taxa (Robinson et al., 2022). Urban rock pigeons (Nualmalang et al., 2023) and captive teal ducks (Sakda et al., 2023) similarly exhibited gut communities dominated by *Candida*, *Debaryomyces*, or *Kazachstania* species associated with grain-based or processed diets.

In New Zealand, *Apiotrichum porosum* and *Candida* spp. dominated the gut mycobiota of captive Ōkārito kiwi in brooders but decreased sharply after transfer to soil-based holding pens (Rowe, 2022), demonstrating how environmental exposure can restructure gut fungal communities despite diet. The 'standard diet' for captive kiwi was also outlined: beef mince, oxheart, oats, wheat germ, cat biscuits, fruit, vegetables, vitamins, and occasional invertebrates. It was confirmed by Manaaki Whenua Landcare Research (Manaaki Whenua, 2025) that some of these components were used implemented in this experiment. This diet was carefully considered to maintain stability between kiwi translocations,

as well as for maintaining known baselines for calorie and nutrient intake. However, the study also refers to this diet as more artificial, with unknown long-term consequences.

In a past study on captive brown kiwi (*Apteryx mantelli*) at Auckland Zoo (Truter-Meyer, 2021), one soil sample from the entrance of a nocturnal kiwi enclosure was similar in fungal composition of kākāpō nest soils (e.g., high RAs of *Apiotrichum*, *Debaryomyces*, *Candida* spp.). Samples from other substrates (e.g., air, leaves, wooden surfaces, concrete) presented substantially different community compositions (Appendix B, Supplementary Figure S6). Diet details were unknown, aside from noted provision of *Galleria* grubs. The soil sample was taken from a disturbed substrate (regular zookeeper foot traffic), and a nearby mouse carcass (ruru feed) was observed, both of which are examples of what could have influenced the local mycobiota.

Overall, our data combined with known studies implicate diet as an important modulator of the avian gut and nest mycobiota, but that environmental conditions and background communities may also play a role fungal community structure. Regarding kākāpō, the yeast-rich communities in nest soils are therefore likely driven by both dietary introduction and subsequent proliferation under nest-specific conditions.

3.5.3 Potential role of fungal communities in kākāpō aspergillosis incidence

Our results together with previous observations of low *A. fumigatus* viable counts in nest soils of infected kākāpō (Kanis et al., in prep., Ch. 2), indicate that there is no clear link between kākāpō aspergillosis incidence and *A. fumigatus* viable or relative abundance in their nests. During the 2019 outbreak only one of the 15 affected nest samples harboured the disease-causing agent, *A. fumigatus*. In this study, although *Aspergillus* spp. was detected, no ASVs could be accurately identified to species level, therefore *A. fumigatus* specifically was not identified. Regardless, there was also no significant effect of aspergillosis status (affected vs unaffected nests) on soil fungal composition and diversity. However, it should be noted that only two affected nests were included in this analysis, as they were the only nests affected by the 2022 outbreak. Therefore, definitive explanations are limited by sample size, however, a similar study which had analysed the soils of 11 affected kākāpō nests (West et al., 2025), also found no significant relationship between fungal diversity (alpha and beta) of nest litter mycobiota and aspergillosis infection. West and colleagues also did not detect *A. fumigatus*, and this lack of detection could be due to insufficient extraction, PCR bias, primer bias or low template yield. Because of differences in PCR primer binding efficiencies, rare taxa are likely more prone to underrepresentation in mixed communities, which can lead to slightly distorted community proportions (Brooks et al., 2015; Polz & Cavanaugh, 1998; Silverman et al., 2021).

Since there are currently no clear links between kākāpō aspergillosis and *A. fumigatus* soil exposure, there is an on-going investigation into other environmental factors which could be increasing the vulnerability of kākāpō to infection. For example, other fungal communities could be potential

contributors to poor kākāpō health and immunity, enhancing their sensitivity to minimal levels of *A. fumigatus*. Previous analysis showed that although *A. fumigatus* was almost absent in affected nests of 2019, these nests were still associated with significantly higher *Aspergillus* levels overall (Kanis et al., in prep., Ch. 2). These species were mostly *Aspergillus flavus* and *Aspergillus niger*, indicating that they may be potential contributors to aspergillosis incidence. Although they were not identified as the disease-causing agent, *A. flavus* was isolated from two of the nine deceased kākāpō from aspergillosis in 2019, while another four presented signs of aflatoxin exposure; a mycotoxin that *A. flavus* typically produces (L. Uddstrom, personal communication, March 3, 2023).

Another notable finding from previous work (Kanis et al., in prep., Ch. 2), was that during the 2022 outbreak, yeast viable abundances were significantly higher in nest cavities and entrances than in soils from two metres away, and an inverse relationship was observed between yeasts and *Aspergillus fumigatus* counts. The proliferation of yeasts within kākāpō nests, along with its potential competitive dynamic with *A. fumigatus*, lead to the hypothesis that this interaction may have an effect on kākāpō health or vulnerability to *A. fumigatus* infection, particularly through: 1) enhanced or altered secondary metabolite secretion from either yeast species or *Aspergillus* spp. (e.g., mycotoxins) or 2) inhalation of prolific yeasts which may also act as opportunistic pathogens. Since yeasts like *D. hansenii* and *K. telluris* were isolated post-mortem from the only kākāpō death (Jemma) in 2022 (L. Uddstrom, personal communication, March 3, 2023), the contribution of these yeasts to poor kākāpō health is a possibility.

Overall, our study indicates that kākāpō defecation is a key factor driving yeast-dominant communities within nests. The consistent inverse relationship between yeasts and *A. fumigatus* observed in previous analyses (Kanis et al., in prep., Ch. 2) raises the question whether competitive interactions within nest soils influences host susceptibility to infection, either by suppressing *A. fumigatus* growth or altering secondary metabolite production in ways that affect host health. Although further work is needed to determine causality, these results highlight the importance of considering broader microbial interactions, rather than *A. fumigatus* viable abundance alone, when assessing aspergillosis risk in kākāpō populations.

3.6 Conclusion

Soils from the 2022 kākāpō aspergillosis outbreak showed that aspergillosis status had no effect on soil fungal alpha or beta diversity. Instead, proximity to nests did, where richness increased with distance from nest cavities, and community composition differed among all nest microhabitats (cavities, entrances, and soils two metres away). Nest cavities showed high relative abundances of yeasts *Apiotrichum* spp. (both islands) and *Debaryomyces* spp. (Whenua Hou Island), aligning with the high yeast viable abundance within cavities and entrances observed in prior analyses (Kanis et al., in prep., Ch. 2). The absence of alpha diversity differences between islands, coupled with similar community profiles at two metres away, points to kākāpō activity as a major driver of nest-associated mycobiota,

superimposed on a broadly shared forest-floor background. Island-level differences restricted to nest-proximate soils further suggest that local abiotic or vegetative factors may modulate this overarching nest effect.

A similar kākāpō study (West et al., 2025) detected these yeasts in both kākāpō nest and gut mycobiota, while key yeasts such as *Apiotrichum* spp. and *Debaryomyces* spp. decline in chick guts after switching from supplementary feed and rimu to a more plant-based diet. Together with the present study, this indicates that the kākāpō activity shaping nest soils is most likely linked to defecation and diet, which together may introduce gut-associated fungi into the nest environment. Introduced taxa coexisting with background environmental fungi may be amplified under the warm, humid, nutrient-rich nest conditions. For example, *Apiotrichum* appears widespread in island soils but reaches greatest abundances in nest cavities, suggesting environmental amplification, whereas *Debaryomyces* is largely confined to nest-proximate soils, implying dietary or host-mediated introduction. Comparisons with synanthropic and captive birds reinforce this pattern, where less natural, processed diets can favour proliferation of similar yeast taxa (*Candida*, *Debaryomyces*, *Meyerozyma*).

Supplementary feed has been critical for improving kākāpō breeding success and enhancing female nutrient status (Houston et al., 2007; Von Hurst et al., 2016). Possible links between kākāpō diet, gut composition, and nest soil microbiomes suggests that nutrient inputs from diet and faecal deposition shape the microbial environment of nests (Kanis et al., in prep., Ch. 3). These yeasts may influence host health either positively or negatively, depending on host susceptibility, pathogen abundance and interactions with other fungi, including *Aspergillus fumigatus* (Kanis et al., in prep., Ch. 2). Understanding how "natural" nests without or alternative supplementary feeding differ in microbial composition and health outcomes is therefore a key research priority. However, rather than complete feed removal, future management may need to balance nutritional requirements for breeding with maintenance of a gut and nest microbiome that supports optimal health.

Across studies, a consistent "nest effect" emerges in which kākāpō activity enriches yeast-dominated fungal communities within nests relative to surrounding soils. Combined with prior viable abundance data showing an inverse relationship between yeasts and *A. fumigatus* (Kanis et al., in prep., Ch. 2), these results indicate that *A. fumigatus* nest soil abundance alone does not explain aspergillosis incidence. Instead, aspergillosis risk may be influenced by broader microbial interactions (e.g., competition enhancing pathogenicity in yeasts or *Aspergillus* species). Additionally, since only kākāpō nest soils were sampled for community analyses, it is also possible that sources of initial *A. fumigatus* exposure could have occurred from other nest substrates (e.g., air or airborne dust particles), warranting further investigation. To disentangle these mechanisms, future research should (1) investigate dietary and gut mycobiota links, (2) characterise island-specific and abiotic contributors to fungal dynamics, (3) screen soils, feed, and co-cultures for secondary metabolites such as mycotoxins and VOCs, and (4) undertake longitudinal sampling of nest litter, soils, air and airborne dust before and after aspergillosis

events. This integrated approach will clarify whether managing both diet and nest substrates can mitigate disease risk while sustaining the ongoing recovery of kākāpō populations.

3.7 Acknowledgements

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4 Chapter 4: Antagonistic Effects of Kākāpō-Associated Yeasts on *Aspergillus fumigatus* and *Aspergillus niger*

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4.1 Abstract

New Zealand's critically endangered kākāpō (*Strigops habroptilus*) have experienced two outbreaks of aspergillosis, a fungal respiratory disease, during 2019 and 2022 breeding seasons, posing a threat to their population recovery. Contrary to previous studies, in 2019 nests that harboured infected birds, there were little to no viable *Aspergillus fumigatus* – the disease-causing agent, suggesting additional contributors to disease susceptibility. From kākāpō nest soils of the 2022 outbreak, low *A. fumigatus* levels in nests were associated with the presence of (nest-prolific) mesophilic yeasts (e.g. *Debaryomyces hansenii*, *Meyerozyma guilliermondii* and *Candida palmioleophila*), suggesting competitive dynamics between fungal types (*A. fumigatus* and yeasts). Since these yeasts have potential as opportunistic pathogens or to enhance pathogenic traits in *A. fumigatus*, our study aimed to confirm these competitive interactions by observing in vitro growth and conidiation suppression of *A. fumigatus* and *A. niger* by six kākāpō nest yeast species (*Debaryomyces hansenii*, *Debaryomyces* spp., *Meyerozyma guilliermondii*, *Candida palmioleophila*, *Spencermartinsiella ligniputridi* and *Clavispora* spp.) using co-cultivation techniques. Radial inhibition assays showed that all six yeasts significantly suppressed *A. fumigatus* and *A. niger* mycelial growth, of which four also suppressed conidiation at varying levels. *Meyerozyma guilliermondii* was the most effective at reducing both mycelial growth (by 80-88%) and conidiation (by 76-86%) of *A. fumigatus* and *A. niger*. The second most prevalent yeast in kākāpō nest and gut mycobiota, *Debaryomyces hansenii*, reduced mycelial growth by 22-26% and conidiation by 19-35% of both *A. fumigatus* and *A. niger*. Diffusible-compound assays confirmed moderate, uniform suppression of *A. fumigatus* mycelial growth (9–25% inhibition) across five tested yeasts. Our results support that kākāpō yeast isolates demonstrate antagonism against mycelia and conidiation of *A. fumigatus* and *A. niger*, although the mechanism of action is not yet understood. Further research on these interactions is needed to determine whether yeasts play a positive role (e.g., as biological controls), or a negative role (e.g. enhancing *Aspergillus* species pathogenicity) in kākāpō disease susceptibility.

Keywords: *Aspergillus fumigatus*, *Aspergillus niger*, competition, kākāpō, biological control, yeasts

4.2 Introduction

New Zealand's kākāpō (*Strigops habroptilus*) is a rare parrot species that has suffered a major population decline, primarily due to habitat loss and predation by introduced mammalian predators (G. Elliott et al., 2001; Lloyd & Powlesland, 1994). Since their decline to approximately 50 individuals in the 1990's, New Zealand's Kākāpō Recovery Programme (Department of Conservation or DOC) have made significant advances towards population recovery (Department of Conservation, 2024). Kākāpō breeding females (age 5-10 years old) (Department of Conservation; Eason et al., 2006) have been translocated to offshore predator-free islands (Lloyd & Powlesland, 1994), such as Whenua Hou and Pukenui, and intensive conservation strategies have been implemented to protect all kākāpō populations. The resulting outcome of these efforts within the past three decades is a population number of approximately 237 individuals today (Department of Conservation, 2024).

Despite the success of these efforts, kākāpō have recently faced two outbreaks of aspergillosis; in 2019 on Whenua Hou island (Winter et al., 2022), and in 2022 on Pukenui Island (Department of Conservation, 2022), posing a major threat to recovering numbers. Aspergillosis is a respiratory disease which commonly affects the airways of mammalian and avian species, and the disease-causing agent is primarily *Aspergillus fumigatus*, which is generally a saprophytic fungus that plays a key ecological role in decomposing plant matter (Latgé & Chamilos, 2019). As a result, it is commonly found in soil, especially in forested environments where organic debris accumulates (De Linares et al., 2023; Nji et al., 2023; Perrott, 2001; Perrott & Armstrong, 2011; Riedling et al.). However, the ability of *A. fumigatus* to cause infection is largely based on favourable growth conditions provided by the bodies of endotherms, typically requiring the host to be immunosuppressed, which deems *A. fumigatus* an opportunistic pathogen. Among *Aspergillus* species*, A. fumigatus* prefers the highest temperatures for optimal growth (35-40°C) and is also thermotolerant, which means the warm core body temperatures of mammals (37°C) and birds (40°C) (Chang et al., 2004; J.-P. Latgé, 1999; McCormick et al., 2010), paired with reduced immune defences undoubtedly provides favourable conditions.

During the 2019 kākāpō aspergillosis outbreak, seven of 15 nests that were affected by aspergillosis showed no *Aspergillus* spp. colony counts, and of the seven nests containing over 100,000 CFU g^{-1} of *Aspergillus* spp., (a limit set for safe soils in hihi translocations; (Ewen et al., 2012; Glare et al., 2014)) only one nest was associated with *A. fumigatus* specifically (Kanis et al., in prep., Ch. 2). During the 2022 aspergillosis outbreak, *A. fumigatus* counts were on average ~930 CFU g^{-1} of wet soil in kākāpō nest cavities, whilst mesophilic yeasts counts were on average ~216 047 CFU g^{-1} (Kanis et al., in prep., Ch. 2). Fungal community analysis of kākāpō nest soils showed high relative abundances of yeasts *Apiotrichum* spp., and *Debaryomyces* spp., which was also identified dominant genera in kākāpō gut mycobiota (West et al., 2025).

Previous analyses of outbreak data (Kanis et al., in prep., Ch. 2) showed that yeast presence was inversely proportional to *A. fumigatus* abundance. Based on results from a zero-inflated negative binomial model (ZINB), we found a significant reduction in *Aspergillus* spp. abundance when yeasts were present (2019 outbreak: 95% reduction, ZINB, estimate = –3.07, $p < 0.001$; 2022 outbreak: 33% reduction, estimate = –0.41, $p < 0.01$). Since kākāpō contracted aspergillosis in 2019 despite low *A. fumigatus* exposure in nests, this led to investigating whether nest-associated yeasts are a contributing factor in the complex web of ecological interactions leading to aspergillosis susceptibility. Some of the yeasts isolated from these soils (e.g., *Debaryomyces hansenii* and *Meyerozyma guilliermondii*) have been well studied for their potential as a biocontrol against *Aspergillus* spp. along with other filamentous or pathogenic fungi (Chacón-Navarrete et al., 2022; Choińska et al., 2020; Droby et al., 1989; Herrera-Balandrano et al., 2023; Kasfi et al., 2018; Medina-Córdova et al., 2016; Núñez et al., 2015). However, *Aspergillus* spp. can also alter toxin profiles or enhance conidiation when in competition with another species (Losada et al., 2009). Of these yeasts, *D. hansenii* has been found to produce killer toxins (Al-Qaysi et al., 2017; Banjara et al., 2016; Morales-Menchén et al., 2018; Nascimento et al., 2020) and both *D. hansenii* and *M. guilliermondii* have been the cause of rare infections in immunocompromised humans (Chaves et al., 2021; Chen et al., 2020; Desnos-Ollivier et al., 2008).

During aspergillosis outbreaks, kākāpō have also contracted yeast infections or had yeasts isolated from post-mortem tissue samples (A. Digby, personal communication, March 20, 2023). One of the nine deceased kākāpō (Hoki) in 2019 had contracted yeast ventriculitis (presumptive candidiasis) whilst receiving treatment for aspergillosis (L. Uddstrom, personal communication, March 3, 2023). It was suspected that the infection was secondary to immunosuppression by aspergillosis and treatment-caused hepatopathy. *Debaryomyces* spp. and *Kazachstania* spp. was identified post-mortem in Jemma, the only death resulting from aspergillosis in the 2022 outbreak, which were two of the most prevalent ASVs within kākāpō nest soils (Kanis et al., in prep., Ch. 3), litter and gut mycobiome (West et al., 2025). Additionally, *Yarrowia bubula* was identified post-mortem in an isolated case of a male kākāpō (Jester) with aspergillosis on Te Hauturu-o-Toi Island, separate to the outbreak that targeted breeding females and chicks on Pukenui.

Together with the ability of some nest-associated yeasts to cause infection in or colonise tissue of both humans and kākāpō, this raises the question whether these yeasts alone have potential as opportunistic pathogens. Since toxin profiles of *Aspergillus* spp. can be altered in during competition, this also raises the question whether the production of secondary metabolites can change or increase in concentration during competition. If yeasts residing in kākāpō nests influence disease susceptibility through competitive interactions, it is first necessary to confirm competition dynamics between yeasts and *Aspergillus* species. This study's aim was to measure the antagonistic potential of yeast isolates through performing two competition assay techniques: 1) biotic radial inhibition assays to measure the effect of

yeast abundance on *Aspergillus* spp. colony growth, and 2) diffusible antifungal compound assays to measure the effect of yeasts through competitive exclusion of *Aspergillus* spp. colony growth.

4.3 Methods

4.3.1 Soil sample collection

Kākāpō nest soil samples were collected by the Kākāpō Recovery Programme, Department of Conservation (DOC) New Zealand, from two offshore islands; Whenua Hou (Codfish Island; 46°46.07′ S, 167°37.23′ E) and Pukenui (Anchor Island; 45.7587° S, 166.5093° E) from May to June 2022. Samples were collected in plastic resealable bags containing approximately 50-70 grams of topsoil. Each kākāpō nest, named after the breeding kākāpō female, included soils from three nest microhabitats: the nest cavity, cavity entrance and two metres away from the cavity, of which were stored at -20°C until further analyses at Auckland University of Technology (AUT). A total of 15 nests were included in this study (Appendix C, Table S1).

4.3.2 Yeast isolation and identification

Soil samples from the nest microhabitats corresponding to each of the 15 nests were serially diluted. One gram per soil sample was suspended in 9 millilitres (mL) of sterilised water and diluted 10-fold to a final dilution of 10^{-3}. One hundred microlitres (µL) of the final dilutions were each plated in triplicate on potato dextrose agar (PDA) amended with antibiotics: 50 mg/L tetracycline hydrochloride and 350 mg/L streptomycin sulfate (both from Sigma Chemical Co., St. Louis, MO), replicating the BST agar (semi-selective medium) described by Glare and colleagues (Glare et al., 2014). The integration of antibiotics was to suppress bacterial growth, allowing for only fungal growth and thus a clearer observation of fungal colonies. Plates were incubated at 35°C for 48 hours to account for optimal growth of *Aspergillus fumigatus*, and other mesophilic fungi. Plates containing dilutions of 10^{-2} and 10^{-3} were observed for isolation and identification.

Yeasts from all plates were grouped based on morphological differences, such as colony colour and texture. Representative colonies were selected for further isolation, which involved collecting cells with a sterile plastic loop, performing five-streak plating, and incubating at 35 °C for 48 hours. This process was repeated a total of three times to obtain pure cultures suitable for co-cultivation experiments. Pure isolates were sent for amplicon sequencing (fungal ITS and LSU regions) by Manaaki Whenua Landcare Research (MWLR) and for taxonomic assignment by MWLR's Research Leader for mycology and bacteriology, Dr. Bevan Weir.

4.3.3 *Aspergillus* spp. isolates

Four different *Aspergillus* spp. isolates were included in this study, three of which were obtained by Manaaki Whenua Landcare Research's International Collection of Microorganisms from Plants (ICMP): *Aspergillus fumigatus* Fresen. (ICMP 23423), *Aspergillus fumigatus* Fresen. (ICMP 24469), and *Aspergillus niger* Tiegh. (ICMP 23509). The two isolates of *A. fumigatus* Fresen. were isolated from kākāpō lung tissue, while *A. niger* Tiegh was isolated from a nest associated with confirmed aspergillosis. Another isolate of *Aspergillus fumigatus* was obtained from cultivation of this study's soils, specifically from kākāpō JEM's nest entrance. Species-level identity was confirmed by PCR amplification and sequencing of the fungal internal transcribed spacer (ITS) region. The resulting sequence was 100% identical to multiple *A. fumigatus* ITS entries in GenBank. The two isolates of *A. fumigatus* Fresen. were similar in colony morphology when grown on potato dextrose agar (PDA), characterised by outer white mycelial ring and an inner blue-green zone of sporulation (conidiation zone) (Figure 4.1). *A. fumigatus* JEM slightly different in morphology, with an inner white mycelial ring additional to its outer mycelial ring. The *A. niger* strain was morphologically identifiable by its dark brown to black conidiation zone, with a thinner outer mycelial ring.

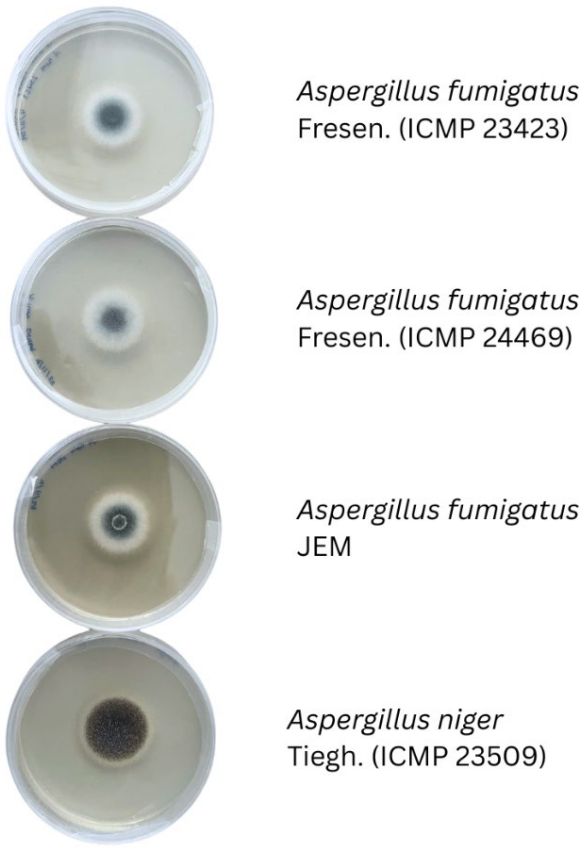

Figure 4.1 Colony morphology of the four *Aspergillus* spp. isolates included in this study. ICMP number = Isolate number stored in the International Collection of Microorganisms from Plants at Manaaki Whenua Landcare Research, New Zealand. Image photographed with an iPhone 12 Pro Max, edited using Canva.

4.3.4 Culture preparation

Yeast cells from each of the 11 isolates (see Section 4.4.1; Table 4.1), were collected with a sterile plastic loop (one loopful) and inoculated into 15 mL falcon tubes each containing 3 mLs of potato dextrose broth. The same was performed for the four *Aspergillus* spp. isolates. The resulting suspensions were then vortexed for 30 seconds and incubated at 35°C for one week. Yeast isolate *Spencermartinsiella ligniputridi* (Figure 4.5J) required a longer incubation period of two weeks due to a slower observed doubling time likely attributed to the difference in its morphology compared to the other yeast isolates. Suspensions were then diluted to 10^6 cells/mL (yeast) and 10^6 spores/mL (*Aspergillus* spp.). Cell and spore concentrations were determined using a haemocytometer (Neubauer chamber) sand a compound microscope. Since *S. ligniputridi* was the only yeast observed to produce hyphae or pseudohyphae, cell quantification was limited to the observation of only circular yeast cells to maintain consistency across species and to enable comparison with strictly yeast-form isolates. Once the desired concentrations were achieved, the suspensions were stored in a refrigerator at 4°C until co-cultivations could be performed. Two competition assays were implemented in this study: radial inhibition assays and diffusible antifungal compound assays described by Medina-Córdova et al. (2016) and Núñez et al. (2015). For radial inhibition assays, the prepared suspensions of both yeast cells and *Aspergillus* spp. spores were used. For diffusible antifungal compound assays, *Aspergillus* spp. mycelia were used in place of spore suspensions. To prepare mycelial cultures, spores were collected using a sterile plastic loop and dotted onto the centre of PDA plates. These were incubated at 28°C for 48 hours and stored in a refrigerator at 4°C until co-cultivations could be performed shortly after.

4.3.5 Biotic radial inhibition of *Aspergillus* spp. by yeast isolates

To test the antagonistic potential of the 11 yeast isolates by suppressing entire colony growth of the four *Aspergillus* spp. isolates, 'radial inhibition assays' were performed following methods described by Medina-Córdova et al. (2016) and Núñez et al. (2015) (Figure 4.2). One hundred microlitres of each yeast suspension was spread on PDA using a sterile plastic spreader. After drying, a sterile filter paper disc was placed in the centre of the plate, which was then loaded with 10 µL of each *Aspergillus* spp. suspension. In total, 132 co-cultures were performed (triplicates of 11 yeasts x 4 *Aspergillus* isolates) with 12 *Aspergillus* spp. controls (triplicates of 4 *Aspergillus* isolates). Plates were incubated at 35°C for 72 hours, and the diameters of both *Aspergillus* spp. colony mycelia and conidiation zones were measured for comparison (see Figure 4.3 for example). Mycelial and conidiation zone diameters were compared between controls (*Aspergillus* spp. isolates in monoculture) and treatments (*Aspergillus* spp. isolates in co-culture with yeast isolates). Percentage inhibition of *Aspergillus* spp. by yeasts was calculated as:

$$\%\ inhibition = \frac{C - T}{C} \times 100$$

where C is the mean diameter in control (monoculture) and T is the mean diameter in treatment (co-culture).

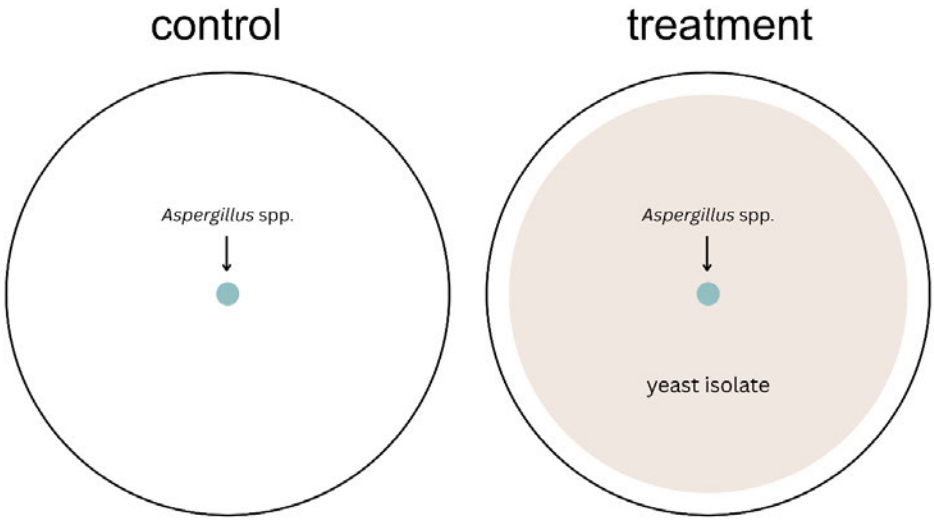

Figure 4.2 Diagram visually demonstrating the radial inhibition assay method described by Medina-Córdova et al. (2016) and Núñez et al. (2015), with control and treatment setup. The two large circles indicate potato-dextrose agar (PDA) plates. Light beige colouring on the treatment plate indicates the 100µL yeast cell suspension spread onto the agar. Blue-green dots in the centre of both plates indicate the filter discs infused by 10µL of the *Aspergillus* spp. spore suspension. Controls are *Aspergillus* spp. growing in monoculture and treatments are the co-cultures of *Aspergillus* spp. with yeast isolate lawns. Diagram was made using Canva.

Figure 4.3 Example image/diagram of *Aspergillus* spp. colony growth, showing the diameters of mycelial growth (white outer ring; hyphae), and the conidiation zone (centre green-blue zone; conidia and conidiophores). Example plate was of *A. fumigatus* from kākāpō JEM's nest entrance after 72 hours incubation at 35℃.

4.3.6 Competitive exclusion of *Aspergillus* spp. growth by yeast species

Following radial inhibition assays, yeasts that had demonstrated little antagonistic potential were excluded from further co-cultivations (i.e., *Spencermartinsiella ligniputridi*). Therefore, antagonistic potential of the remaining 10 yeast isolates to inhibit *Aspergillus* spp. growth was also tested through 'diffusible antifungal compound assays', following a similar method to Medina-Córdova et al. (2016). This method involves growing both yeast and *Aspergillus* spp. isolates on two opposite sides of a PDA plate (Figure 4.4), resulting in growth suppression of *Aspergillus* spp. colonies. Mycelial agar discs (5mm) were taken from the prepared *Aspergillus* spp. solid cultures and placed 2.5 cm from the edge of the new co-culture (PDA) plate using a sterile metal cork-borer. Yeast suspensions used for the previous method were checked and adjusted to 10^6 cells/mL where needed. After adjustments, 10µL of the yeast suspension was pipetted 3 cm on the opposite side of the co-culture plate. In total, 132 co-cultures were performed (triplicates of 11 yeasts x 4 *Aspergillus* isolates) with 12 *Aspergillus* spp. controls (triplicates of 4 *Aspergillus* isolates). Plates were incubated at 35°C for 6 days, and the radius of *Aspergillus* spp. colony mycelia (control vs yeast treatment) were measured for comparison. Using a ruler placed over the back of the petri dish, measurements were taken from colony centre points horizontally to the opposite end of the petri dish (control) or towards the yeast colony (treatment).

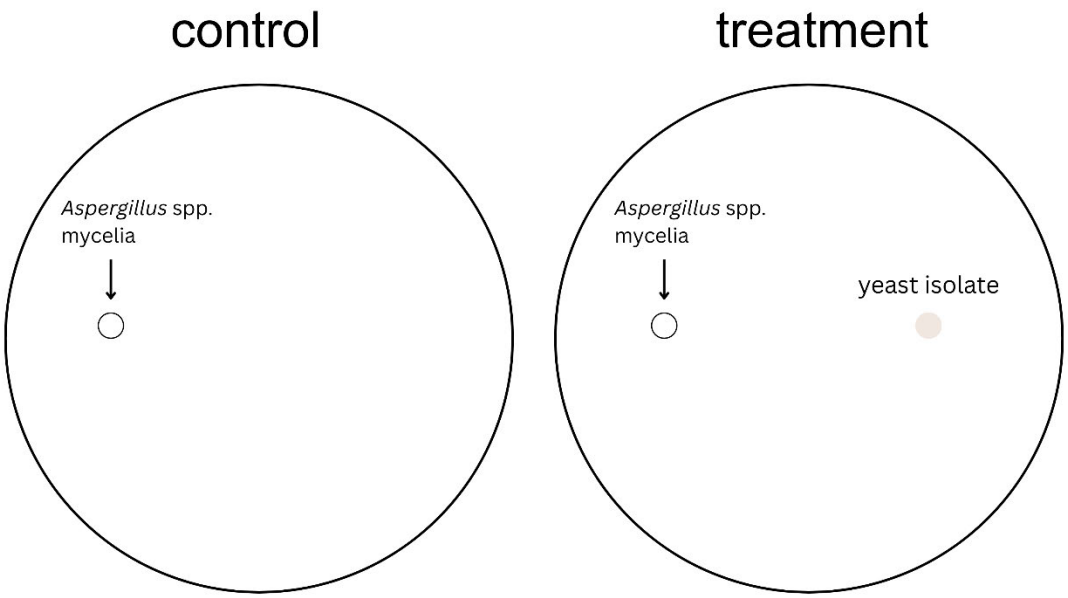

Figure 4.4 Diagram visually demonstrating the diffusible antifungal compound assay method described by Medina-Córdova et al. (2016). The two large circles indicate potato-dextrose agar (PDA) plates, left = control, right = treatment. Small white circles on both plates indicate a 5 mm mycelial disc of Aspergillus species. The small beige circle on the treatment plate indicates a 10µL yeast cell suspension pipetted directly onto the agar. Controls are *Aspergillus* spp. growing in monoculture and treatments are the co-cultures of *Aspergillus* spp. with yeast isolates. Diagram was made using Canva.

4.3.7 Data analysis

The R dplyr package (Wickham et al., 2023) was used for data manipulation, primarily to group yeast isolates by their species or genus identification. To assess differences in *Aspergillus* spp. growth between control and treatment groups, Welch's *t*-tests were performed using R's built-in *t*-test function, which is appropriate for comparing groups with unequal variances and for both normally and non-normally distributed data. Mean and standard deviation values were calculated using base R functions. Grouped bar plots used for data visualisation were produced using the ggplot2 package (Wickham, 2016).

4.4 Results

4.4.1 Yeast isolation and identification

A total of 11 yeast morphological types were isolated and identified to either species or genus level, some of which were assigned to the same taxa despite differences in colony morphology (Table 4.1; Figure 4.5). Co-cultivations experiments, as well as the recorded data, were based on these morphological isolates but since multiple were identified as the same species or strain, morphological isolates were grouped by these for data analysis.

Table 4.1 List of yeast isolates grown on BST agar based on distinct morphology (colour and texture).

Isolate Name	Kākāpō nest	Nest Microhabitat	Dilution	ICMP no.	Species identification
1A	Cyndy	Cavity	10^{-3}	24917	*Debaryomyces hansenii* (Zopf) Lodder & Kreger 1952
1B	Cyndy	Cavity	10^{-3}	24918	*Meyerozyma guilliermondii* (Wick.) Kurtzman & M. Suzuki
1D	Cyndy	Cavity	10^{-3}	24919	*Spencermartinsiella ligniputridi* G. Péter & Dlauchy
2A	Bella	2m away	10^{-2}	24933	*Debaryomyces hansenii* (Zopf) Lodder & Kreger 1952
3A	M-Maree	Cavity	10^{-2}	24920	*Debaryomyces hansenii* (Zopf) Lodder & Kreger 1952
5A	Gertrude	Cavity	10^{-2}	NA	*Debaryomyces* Lodder & Kreger ex Kreger 1984
5B	Gertrude	Cavity	10^{-2}	24922	*Clavispora* Rodr. Mir. 1979
5C	Gertrude	Cavity	10^{-2}	24935	*Clavispora* Rodr. Mir. 1982
6A	JEM	Cavity	10^{-2}	24923	*Debaryomyces hansenii* (Zopf) Lodder & Kreger 1952
6C	JEM	Cavity	10^{-2}	24924	*Clavispora* Rodr. Mir. 1979
10B	Wehe-po	Cavity	10^{-2}	24925	*Candida palmioleophila* Nakase & Itoh

Note: ICMP no. = Manaaki Whenua Landcare Research ICMP number (International Collection of Microorganisms from Plants) and species identification by fungal ITS sequencing. Wehe-po = Weheruatanga-o-te-po. M-Maree = Margaret-Maree.

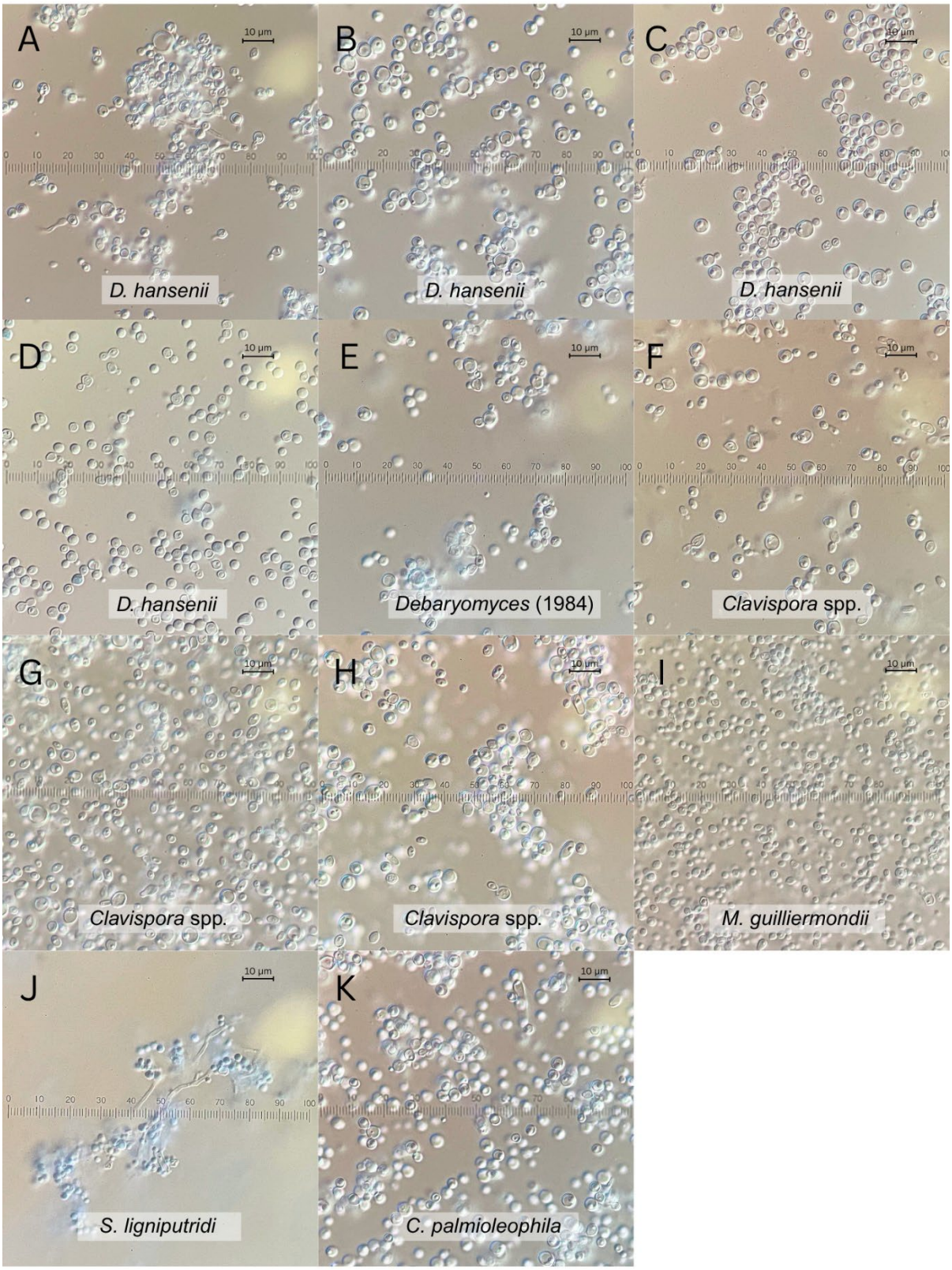

Figure 4.5 Microscopy images of yeast isolates grown on PDA and viewed at 1000× magnification (*M. guilliermondii* viewed at 600 x magnification) using oil immersion and phase contrast optics. (A–D) *Debaryomyces hansenii* (Zopf) Lodder & Kreger 1952, (E) *Debaryomyces* Lodder & Kreger ex Kreger 1984, (F–H) *Clavispora* Rodr. Mir. 1979., (I) *Meyerozyma guilliermondii* (Wick.) Kurtzman & M. Suzuki, (J) *Spencermartinsiella ligniputridi* G. Péter & Dlauchy, (K) *Candida palmioleophila* Nakase & Itoh. Images were cropped for clarity.

4.4.2 Taxonomic assignment of yeast isolates

A total of six yeast species were identified among the 11 yeast isolates (Table 4.1; Figure 4.5). Four yeast isolates were identified to the species level: *Spencermartinsiella ligniputridi* G. Péter & Dlauchy, *Debaryomyces hansenii* (Zopf) Lodder & Kreger 1952, *Candida palmioleophila* Nakase & Itoh and *Meyerozyma guilliermondii* (Wick.) Kurtzman & M. Suzuki. Two yeast isolates in this study could not be identified to species level and were therefore retained at the genus level: *Debaryomyces* (Lodder & Kreger ex Kreger 1984) and *Clavispora* (Rodr. Mir. 1979). The *Debaryomyces* (Lodder & Kreger ex Kreger 1984) isolate will be referred to as *Debaryomyces* 1984.

4.4.3 Biotic radial inhibition of *Aspergillus* spp. by yeast isolates

All six yeast treatments significantly suppressed colony growth (mycelia) and conidiation zones of *Aspergillus* spp. isolates, except *Spencermartinsiella ligniputridi* in suppressing conidiation zones of all *Aspergillus* spp. isolates and mycelial growth for *A. fumigatus* 23423 (Figure 4.7; Appendix C, Table S2 & S3). The yeast that had the greatest suppressive effect was *Meyerozyma guilliermondii*, which significantly reduced all *Aspergillus* spp. mycelial growth by 80-88% and conidiation zones by 76-86% ($p \leq 0.001$) (Appendix C, Table S4, Figure S1). Compared to *M. guilliermondii*; *D. hansenii*, *C. palmioleophila*, *Debaryomyces* spp., and *Clavispora* spp. all demonstrated higher suppression of the conidiation zones. The highest observable difference between the suppression of mycelial growth and conidiation zones was seen in *Clavispora* spp., where mycelia across all *Aspergillus* spp. isolates were suppressed by 35-50%, whilst conidiation zones were suppressed by 46%-84% (see Figure 4.6 for example).

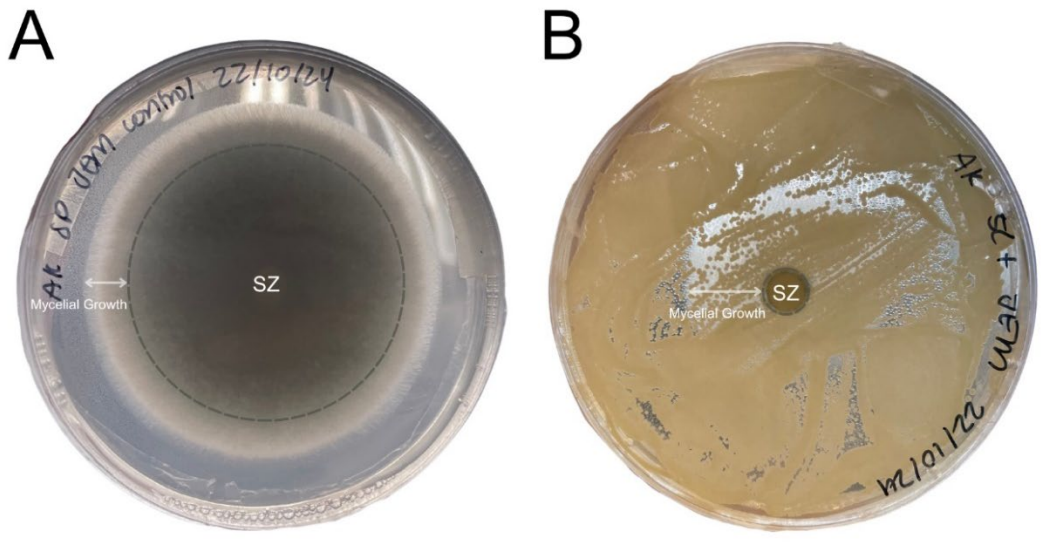

Figure 4.6 Image of *Clavispora* spp. (yeast isolate 5C) demonstrating higher suppression of conidiation zone than mycelial growth in *A. fumigatus* JEM. A) control, B) = yeast treatment. SZ = conidiation zone, indicated by dark green dotted lines. Mycelial growth = growth of mycelia without conidiation (blue-green colouring, conidiophore structures).

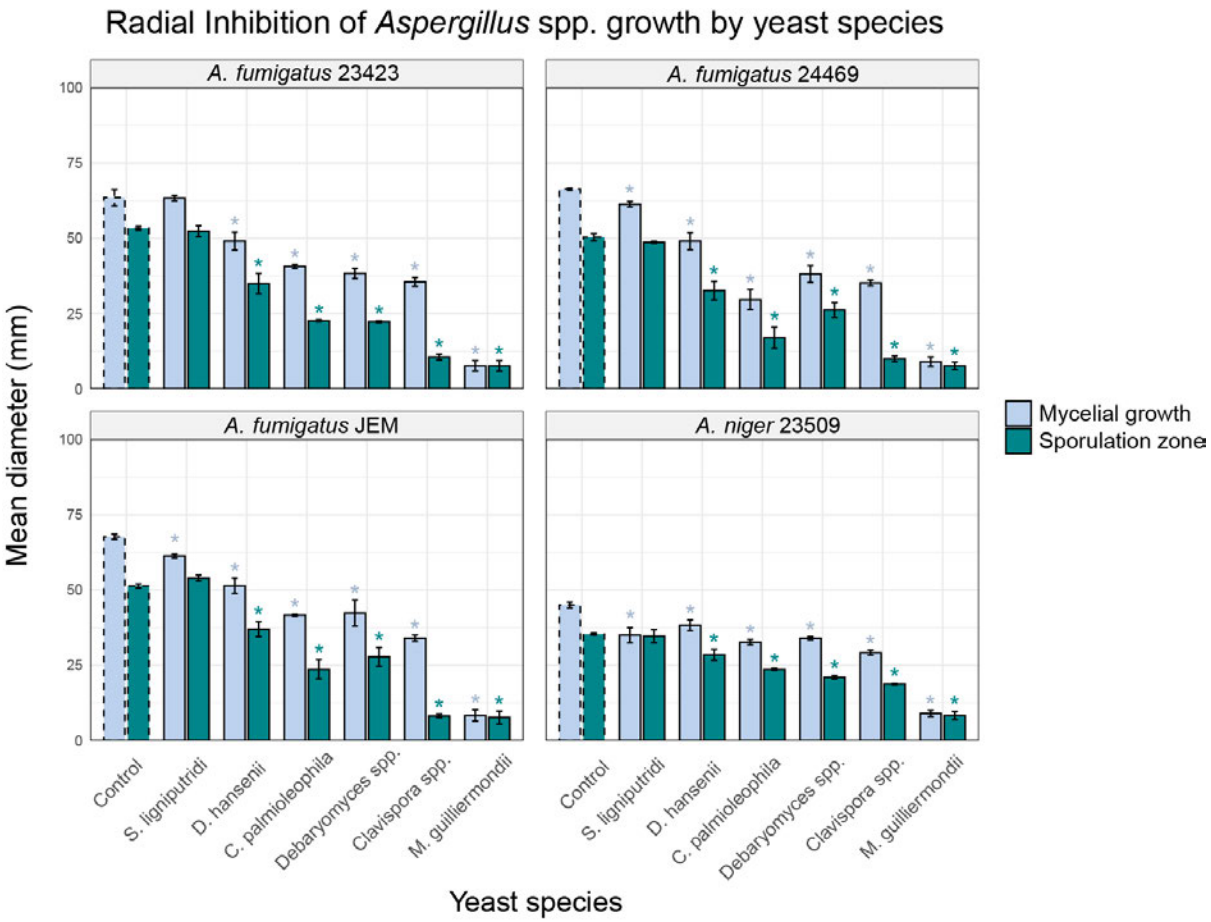

Figure 4.7 Radial inhibition of *Aspergillus* spp: *A. fumigatus* Fresen (ICMP 23423 & 24469), *A. fumigatus* from kākāpō JEM's nest entrance, and *A. niger* Tiegh. (ICMP 23509) by yeasts: *S. ligniputridi*, *D. hansenii*, *C. palmioleophila*, *Debaryomyces* spp. (1984), *Clavispora* spp. and *M. guilliermondii*. *Aspergillus* spp. controls bars are indicated with dotted borders. Asterisks (*) indicate significant differences ($p < 0.05$) of treatments from controls.

4.4.4 Competitive exclusion of *Aspergillus* spp. growth by yeast species

Most yeasts demonstrated antagonistic potential against the four *Aspergillus* spp. isolates (Figure 4.8). *Aspergillus* spp. colony growth (including mycelia) was moderately suppressed by all yeasts, of which most reductions were statistically significant, ranging from 9-25% inhibition (Appendix C, Table S5-S7). Data for *C. palmioleophila* against *A. fumigatus* 23423 was not included in this analysis as two of the three replicates demonstrated no growth and was likely a technical error. Each yeast produced a clear and approximate 2–4 mm non-sporulating gap at the interface with *Aspergillus* spp. colonies (Figure 4.9). In further discussion, these will be referred to as conidiation suppression halos, as underlying mycelia were not inhibited and therefore did not constitute genuine inhibition zones. Measurements of these conidiation suppression halos were excluded from our analysis, as zone lengths were uniform across all yeasts.

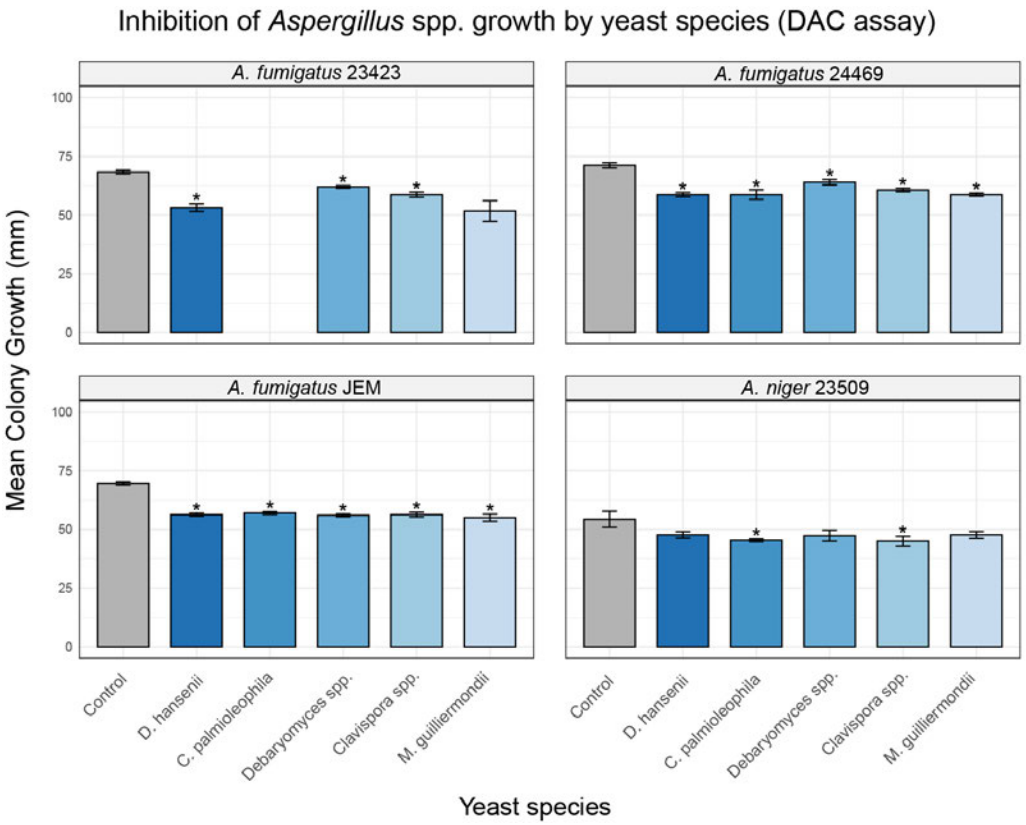

Figure 4.8 Inhibition of *Aspergillus* spp. growth by yeast species using the diffusible antifungal compound (DAC) assay. *Aspergillus* spp. isolates: *A. fumigatus* Fresen (ICMP 23423 & 24469), *A. fumigatus* from kākāpō JEM's nest entrance, and *A. niger* Tiegh. (ICMP 23509). Yeasts species: *D. hansenii*, *C. palmioleophila*, *Debaryomyces* spp. (1984), *Clavispora* spp. and *M. guilliermondii*. Asterisks (*) indicate significant differences ($p < 0.05$) of treatments from controls. *C. palmioleophila* has been omitted from *A. fumigatus* 23423 due to insufficient replicates (2/3 replicates had no growth).

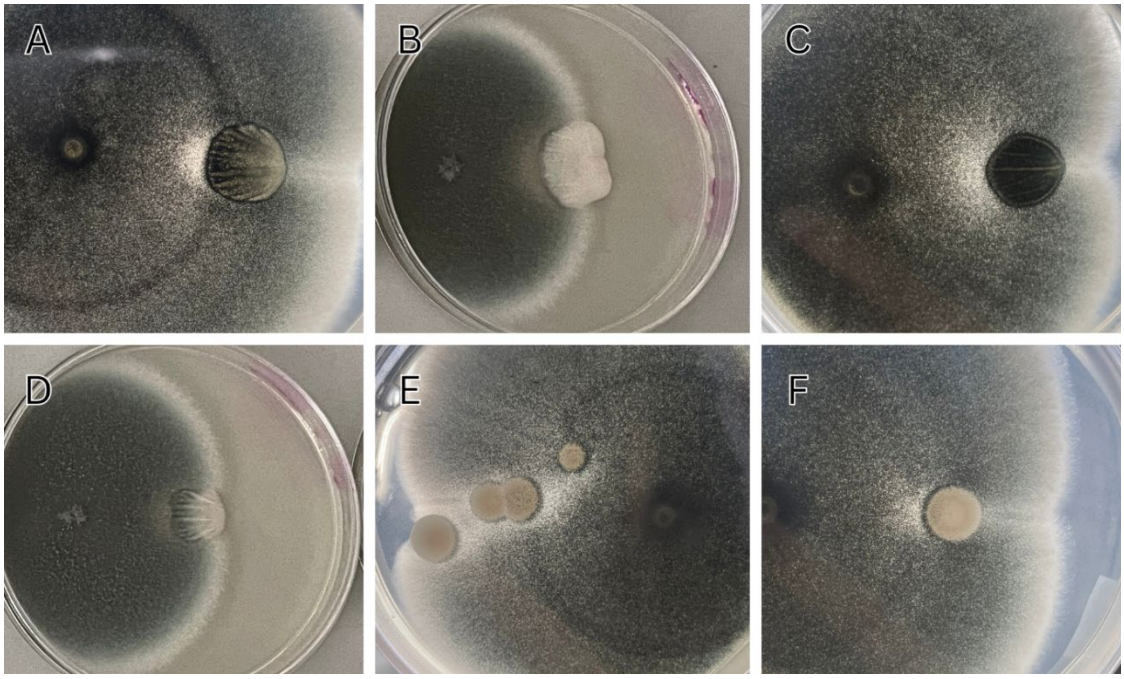

Figure 4.9 Conidiation suppression halos caused by yeasts against *A. fumigatus* isolates. A) *D. hansenii*, B) *C. palmioleophila*, C) *Debaryomyces* spp., D) *Clavispora* spp., E) *M. guilliermondii* as multiple colony forming units (CFU), and F) *M. guilliermondii* as one CFU.

4.5 Discussion

Yeasts used in this study were isolated from five kākāpō nests; of which four isolates were prevalent (among top amplicon sequence variants or ASVs) in fungal community analyses of nest soils, litter, and gut mycobiota (*Debaryomyces* spp., *D. hansenii*, *Candida palmioleophila*, and *Spencermartinsiella ligniputridi*) (Kanis et al., in prep., Ch. 3) (West et al., 2025). In previous analysis of fungal communities in kākāpō nest soils, *Debaryomyces* spp. were one of the 15 most relatively abundant fungal genera and *Debaryomyces hansenii* specifically was the second most prevalent ASV (86.76%) across kākāpō nesting areas. In a study on kākāpō gut mycobiota and nest litter (West et al., 2025), *D. hansenii* was also the second most prevalent ASV in both kākāpō faecal and litter samples (identified in 87% and 96.1% of nests respectively). This study had also found *Spencermartinsiella* spp. to be among the 15 most relatively abundant fungi across faecal samples, and together with *Candida palmioleophila* as two of the 20 most relatively abundant fungi within nest litter samples. Therefore, the yeasts tested in this study may represent a large portion of yeast communities previously identified in kākāpō nests.

4.5.1 Biotic radial inhibition and competitive exclusion of *Aspergillus* spp. by yeast isolates

Radial inhibition assays showed that all six yeast isolates significantly suppressed all *A. fumigatus* isolate and *A. niger* colony growth, with *Meyerozyma guilliermondii* the most effective at reducing mycelial diameter (80-88%) and conidiation zones (76–86%). Diffusible-compound assays confirmed moderate, uniform suppression of *A. fumigatus* radial growth (9–25% inhibition) across the five tested yeasts, all of which produced 2–4-millimetre conidiation-suppression halos despite no change to underlying mycelia. Overall, these results confirm that kākāpō nest yeasts have antagonistic potential against *A. fumigatus* and *A. niger*, implicating yeast proliferation as a contributing factor for low *Aspergillus* loads observed in nest soils.

4.5.2 Possible mechanisms of yeast antagonism

The mechanisms responsible for yeast antagonism against fungal pathogens have been identified as competition for nutrients and space (Guo et al., 2014; Medina-Córdova et al., 2016; Nally et al., 2015), antagonistic metabolite production (Choińska et al., 2020; Herrera-Balandrano et al., 2023) and mycoparasitism (Morath et al., 2012). Antagonism of yeasts against fungal pathogens using radial inhibition assays may reflect the yeasts' ability to compete for nutrients and space (Medina-Córdova et al., 2016). For example, among the yeasts tested in our study, *S. ligniputridi* exhibited the weakest inhibition of *Aspergillus* spp. radial growth. Its comparatively slow proliferation, which may be attributable to hyphal development (Figure 4.5J), likely affected its ability to compete effectively for space. Although yeasts may be producing antifungal compounds, performing volatile organic compound (VOC) assays or the actual screening for secondary metabolites are more definitive techniques for detecting antifungal production. The diffusible antifungal compound assay method

described by Medina-Córdova et al. (2016) has been suggested to reflect the secretion of suppressive substances. The clear inhibition zones (conidiation suppression halos) observed in this assay may be indicative of antifungal secondary metabolites, however when live yeast cultures are used, it becomes difficult to distinguish inhibition by secreted suppressive metabolites from effects of resource competition. To directly assess yeast-derived antagonistic metabolites, future assays should employ cell-free yeast supernatants (also tested by Medina-Córdova et al. (2016)), thereby eliminating resource competition and isolating the effects of secreted compounds. While our assays demonstrated the antagonistic potential of all tested yeast species, they do not clarify the underlying mechanisms responsible for *Aspergillus* spp. colony suppression, therefore further investigation employing screening methods and assays specific for identifying antifungal compounds are needed.

4.5.3 Current literature on yeast-*Aspergillus* competition

To investigate possible mechanisms behind these suppressive effects, we reviewed relevant literature on yeast-mediated suppression of *Aspergillus fumigatus* and *A. niger* (Table 4.2). Several studies attributed the antifungal activity of *Meyerozyma guilliermondii* and *Debaryomyces hansenii* to the release of volatile organic compounds (VOCs) and some have reported visible suppression of conidiation. This aligns with our findings, where yeasts *Debaryomyces* spp., *Clavispora* spp., and *Candida palmioleophila* suppressed *Aspergillus* spp. conidiation.

Meyerozyma guilliermondii, the most effective competitor in our study, has previously inhibited the growth of multiple fungal pathogens (Herrera-Balandrano et al., 2023) including *A. fumigatus* through the production of VOCs (Table 4.2) (Choińska et al., 2020). Using two different assay methods, *M. guilliermondii* suppressed *A. fumigatus* mycelial growth at similar levels (83 and 84%) to those resulting from our radial inhibition assays (80-88%). In another study, *M. guilliermondii* suppressed mycelial growth of *A. niger* by 45% in grapes, and when grown in potato dextrose broth (PDB), conidiation was inhibited by 14-28%. A VOC assay was also performed, demonstrating mycelial growth suppression of approximately 20-65% across three different *M. guilliermondii* strains. Based on these studies, it is a possibility that the production of VOCs is one of the mechanisms responsible for *M. guilliermondii* antagonism against *A. fumigatus* and *A. niger*, but that the level of inhibition is strain dependent.

Debaryomyces hansenii has been thoroughly studied for its biological control potential, but literature regarding the effect of *D. hansenii* on *Aspergillus* spp. is minimal (Table 4.2). Medina and colleagues (2016) performed five different assays, examining the effect of *D. hansenii* on an unclassified *Aspergillus* species. Three of these assays demonstrated the antagonistic potential of *D. hansenii* on the *Aspergillus* isolate, by radial suppression of mycelial growth (97-98%) and by potential secretion of diffusible antifungal compounds (56%).

Table 4.2 Reported antagonistic potential of our study's yeasts against *Aspergillus* species. VOCs = Volatile organic compounds.

Yeast	Pathogen	Assay Type	Substrate	Cell concentration	Mechanism of action	Identified VOCs	Mycelial Growth Inhibition (%)	Reduced conidiation	Treatment Conditions	Citation
M. guilliermondii	*A. fumigatus*	Radial Inhibition Assay	YGC	1×10^6 cells/mL	Unknown	NA	83	Not noted.	10 days; 25 °C	(Choińska et al., 2020)
M. guilliermondii	*A. fumigatus*	Volatile Compounds Assay	YGC	1×10^6 cells/mL	VOCs	tetradecanal; 2-decen-1-ol; phenylethyl alcohol; isogeraniol 9-octadecenol; isoamyl butanoate; phenethyl acetate; citronellyl formate; ethyl dodecanoate; ethyl nonanonate; methyl dihydrojasmonate; acetophenone; 2-nonanone; o-cymene; α-limonene; α-bisabolol; β-farnesene; α-himachalene; 1,3, -bis(1,1-dimethylethyl) benzene.	84	Not noted.	5 days; 25 °C	(Choińska et al., 2020)
M. guilliermondii	*A. niger*	Wounded berry test	cv. Thompson Seedless Grapes	1×10^7 cells/mL	Unknown	NA	45	Not noted.	7 days; 25 °C	(Kasfi et al., 2018)
M. guilliermondii	*A. niger*	Spore germination inhibition method	PDB	1×10^7 cells/mL	Unknown	NA	14-28	Yes	3 days; 25 °C	(Kasfi et al., 2018)
M. guilliermondii	*A. niger*	Volatile Compounds Assay	PDA	1×10^7 cells/mL	Possible VOCs	Not tested	20-65	Yes	7 days; 28 °C	(Kasfi et al., 2018)
D. hansenii	*Aspergillus* spp.	Radial Inhibition Assay	PDA	1×10^8 cells/mL	Unknown	NA	~97–98	Not noted.	7 days; 28 °C	(Medina-Córdova et al., 2016)

D. hansenii	*Aspergillus* spp.	Diffusable Antifungal Compounds Assay	PDA; 1µL/mL chloramphenicol (50 mg/mL of 70% alcohol)	1 x 10^8 cells/mL	Possible VOCs	NA	56	Not noted.	7 days; 28 °C	(Medina-Córdova et al., 2016)
D. hansenii	*Aspergillus* spp.	Volatile Compounds Assay	PDA	1 x 10^8 cells/mL	Possible VOCs	Not tested	0	Not noted.	7 days; 28 °C	(Medina-Córdova et al., 2016)
D. hansenii	*Aspergillus* spp.	Yeast Cell-Free Supernatant Assay	PDA	Initial yeast suspension concentration unknown.	Unknown	NA	0	Not noted.	7 days; 28 °C	(Medina-Córdova et al., 2016)
D. hansenii	*Aspergillus* spp.	Mycelial growth in maize grain	Maize grain	1 x 10^8 cells/mL (spray)	Unknown	NA	Growth delayed by 24hrs	Not noted.	7 days; 25 ± 1 °C	(Medina-Córdova et al., 2016)
D. hansenii	*A. niger*	Streak Inhibition Assay	YPDA	150 µL, 1 A_{600}	Possible VOCs	NA	~5-55	Not noted.	5 days; 28 °C. NaCl: 0 M, 0.5 M, 1 M	(Chacón-Navarrete et al., 2022)
D. hansenii	*A. niger*	Radial Inhibition Assay	PDA	1 x 10^8 cells/mL	Unknown	NA	~7-25	Yes	7 days; 28 °C	(Chacón-Navarrete et al., 2022)
D. hansenii	*A. niger*	Volatile Compounds Assay	PDA	1 x 10^8 cells/mL	Possible VOCs	Not tested	Visible reduction by *D. hansenii* CBS767.	Yes	7 days; 28 °C	(Chacón-Navarrete et al., 2022)

Additionally, when treated with *D. hansenii*, initial *Aspergillus* spp. growth on maize grain was delayed by 24 hours. However, the other two assays which investigated the potential production of VOCs and secondary metabolites remaining in cell-free supernatants, presented no mycelial growth suppression in the *Aspergillus* isolate. Results from this study may indicate resource competition as the main mechanism of action, or other non-VOC producing mechanisms that are associated with active yeast cell growth.

Debaryomyces hansenii was also tested against *A. niger*, with a radial inhibition assay, a streak inhibition assay (similar to diffusible antifungal compound assay), and a VOC assay (Table 4.2). *A. niger* mycelial growth was suppressed within the first two assays, ranging from 5-55% and 7-25% inhibition across strains, respectively. The VOC assays, however, demonstrated minimal effect (variable outcomes leading to exclusion of results). Upon observing VOC assay supplementary imagery, the treatment of only one strain (*D. hansenii* CBS767) visibly reduced *A. niger* mycelial growth, whilst other strains did not, indicating that the production of VOCs by *D. hansenii* may be strain specific. Intriguingly, the suppression of conidiation resulting from radial inhibition and VOC assays were mentioned and visibly proven through supplementary imagery.

There is a current lack of study on the antagonistic potential of yeasts *C. palmioleophila*, *Clavispora* spp., and *S. ligniputridi* against *A. fumigatus* and *A. niger*. Other than for its isolation from rotting wood, *S. ligniputridi* is not a well-studied yeast (Barros et al., 2024; Guo et al., 2024), and therefore there are currently no reports of its antagonistic potential. Due to its slight to moderate inhibition of mycelial growth, we note that the antagonistic potential of this particular species may not be highly significant. *C. palmioleophila* has been studied for its oil-degrading capabilities (Trevino-Trejo et al., 2024), but also for its role as a rare opportunistic pathogen causing candidemia and candidiasis (Aboutalebian et al., 2023; Costa et al., 2023; Oliveira et al., 2025; Wang et al., 2021; Wu et al., 2023). Since this yeast was high in relative abundance within kākāpō nest litter and faecal samples (West et al., 2025), it's abundance should be monitored due to its pathogenic potential. Although this study's *Clavispora* spp. isolate could not be identified to species level, *Clavispora lusitaniae* (146 and AgL21) has been noted for its 'killer yeast' antagonism against the plant-pathogenic mould, *Penicillium digitatum*, by suppressing sporulation (Pereyra et al., 2024; Perez et al., 2019). Mechanisms of action have been described as nutrient competition and the confirmed production of VOCs (Pereyra et al., 2022).

In our study, *C. palmioleophila* and *Clavispora* spp. demonstrated great antagonistic potential against both mycelial growth and conidiation of pathogenic *Aspergillus* species. We recommend further investigation of these interactions, to determine mechanisms of action, potential use as a biocontrol, but also to assess any effects on *Aspergillus* species secondary metabolite production.

4.5.4 Ecological relevance for kākāpō nesting environments

This study showed that most yeast species, including yeasts from fungal community analysis, such as *D. hansenii* and *C. palmioleophila* (Kanis et al., in prep., Ch. 3) (West et al., 2025), demonstrated significant growth suppression of kākāpō nest and lung-isolate strains; *A. fumigatus* and *A. niger*. Based on current literature, it is apparent that *M. guilliermondii*, and *D. hansenii* (one of the most prevalent yeasts in kākāpō nest cavities), may produce VOCs when competing with *A. fumigatus* and *A. niger*. This study's results, together with evidence from other co-cultivation studies suggest that the proliferation of kākāpō nest yeasts are likely contributing to low viable abundances of *A. fumigatus*.

These low observed *A. fumigatus* abundances in kākāpō nest areas despite the occurrence of two major aspergillosis outbreaks in these kākāpō populations, caused confusion surrounding the true cause of these outbreaks. Earlier work (Kanis et al., in prep., Ch. 2) began exploring the proliferation of yeasts in kākāpō nests and their inverse relationship with *A. fumigatus* as a potential factor contributing to aspergillosis susceptibility in kākāpō. Due to prior infections by some of these nest yeasts (e.g, *Debaryomyces* spp., *Kazachstania* spp.) in deceased kākāpō following aspergillosis, it is possible that these species can be opportunistic pathogens. In another study, *A. fumigatus* toxin profiles and conidiation were enhanced when in competition with another *Aspergillus* spp. (Losada et al., 2009), raising the possibility that yeasts may also enhance pathogenic traits in *A. fumigatus*. However, as the yeast isolates in this study largely inhibited *A. fumigatus* conidiation, enhancement of pathogenicity via increased conidiation can be reasonably ruled out.

Taken together, our results and prior studies indicate that kākāpō nest yeast isolates could function as opportunistic pathogens, with the possibility of producing secondary metabolites with uncharacterised impacts on host health. Nonetheless, the consistent suppression of *A. fumigatus* conidiation observed here suggests that conidiation enhancement is not a likely mechanism by which these yeasts would increase *A. fumigatus* pathogenicity.

Previous fungal community analyses (Kanis et al., in prep., Ch. 3) showed that high yeast relative abundances in kākāpō cavities were attributed to kākāpō presence, particularly faecal inputs, based on patterns in fungal diversity across spatial scales. Another independent study had reported declines in yeast relative abundance (e.g., *Apiotrichum* spp., *Candida palmioleophila* and *D. hansenii*) after chicks fledged and shifted from supplementary feed and rimu berries to a largely plant-based diet (West et al., 2025), implicating diet in shaping yeast proliferation. Notably, fledging coincides with changes in location occasional supplementary feeding by some fledged chicks. Therefore, further study is required to disentangle diet from co-occurring environmental factors. However, if diet is a driving factor behind yeast proliferation in kākāpō nests, and if yeasts prove to have a negative impact on kākāpō health, future studies could investigate dietary alternatives that meet similar nutrient requirements while lowering yeast viable abundance in kākāpō nest soils.

4.6 Conclusion

This study demonstrated that culturable yeast species isolated from kākāpō nesting soils possess measurable antagonistic potential against both *Aspergillus fumigatus* and *Aspergillus niger* growth. The yeast *Meyerozyma guilliermondii* emerged as the most effective suppressor, reducing mycelial growth by 80–88% and conidiation by 76–86% in radial inhibition assays. *Debaryomyces hansenii*, unclassified *Debaryomyces* spp., *Candida palmioleophila*, and unclassified *Clavispora* spp. each achieved moderate inhibition of *A. fumigatus* and *A. niger* (22–58% of mycelial growth and 28–84% of conidiation). Diffusible compound assays further confirmed moderate (9–25%) reduction of radial growth by all yeast species excluding *S. ligniputridi*, accompanied by consistent 2 to 4 mm conidiation-suppression halos.

Together with prior observations of yeast enrichment in nest cavities (likely fuelled by kākāpō faecal inputs and diet) (Kanis et al., in prep., Ch. 2 & 3), this study's results suggest that naturally proliferating yeasts may suppress *Aspergillus* growth and sporulation *in situ*. The most plausible mechanisms are resource competition and the production of antifungal secondary metabolites. To clarify the mechanisms and implications of these interactions for kākāpō health, future research should characterise the secondary metabolites produced during competition between kākāpō-associated yeasts and *Aspergillus* strains, including volatile organic compounds (VOCs) and potential mycotoxins. Since *A. fumigatus* can produce gliotoxin as a competitive stress response (Carberry et al., 2012; Curtis et al., 2023; Reece et al., 2018) and this toxin can cause immunosuppressive and cytotoxic effects in birds (de Oca et al., 2017; Latif et al., 2015; Richard et al., 1994; Richard et al., 1996; Wei et al., 2024), screening for both *Aspergillus* conidial and yeast-derived mycotoxins will be essential to determine whether yeast proliferation in kākāpō nests contributes to aspergillosis susceptibility, or conversely, whether certain yeasts can be explored as biological control agents.

Priority next steps therefore include identifying the specific antagonistic mechanisms employed by yeasts (e.g., through VOC and cell-free supernatant assays), linking in vitro interactions to in-nest exposure and bird health outcomes, and assessing whether yeast activity offers protective antagonism or, under some conditions, increases disease risk.

4.7 Acknowledgements

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5 Chapter 5 – General Discussion & Conclusions

5.1 Background

The kākāpō (*Strigops habroptilus*) is a flightless, nocturnal parrot unique to Aotearoa New Zealand. Following severe habitat loss and predation by introduced mammals after human colonisation, the population plummeted to about 50 individuals in the 1990's (Department of Conservation, 2024; Dussex et al., 2021; G. Elliott et al., 2001). Thanks to the Department of Conservation's Kākāpō Recovery Programme, numbers have rebounded to 237 birds by 2025 (Department of Conservation, 2024). However, this conservation success remains vulnerable to emerging diseases, notably aspergillosis (Department of Conservation, 2022; West et al., 2025); a respiratory infection caused by the ubiquitous fungus *Aspergillus fumigatus* (Winter et al., 2022).

Aspergillus fumigatus is a prevalent mesophilic, thermotolerant saprophyte (L. A. Beernaert et al., 2010; J. P. Latgé, 1999; Ostfeld et al., 2008) and therefore has ecological value as an organic matter decomposer. However, it is also a facultative pathogen, where warm core body temperatures (Paulussen et al., 2017; Tell, 2005; van de Veerdonk et al., 2017) and lowered immune responses in some animals (Blatzer & Latgé, 2017; O'Gorman et al., 2009) provide a favourable environment for growth. As a mesophile with thermotolerance, gradual climate change is predicted to expand the range of such fungi (Atkinson et al., 2014; Kilpatrick et al., 2024; Sun et al., 2023). This means that understanding its ecology is critical for managing exposure in wildlife and humans alike.

Most avian aspergillosis outbreaks are linked to stress-induced immunosuppression combined with heavy spore loads (Tell et al., 2019). For kākāpō, driving causes have been more difficult to identify, as New Zealand's Kākāpō Recovery Programme initially reported low detection of *A. fumigatus* in kākāpō nests even in those that had harboured infected birds (A. Digby, personal communication, March 20, 2023). Therefore, this thesis investigated an overlooked dimension: the role of nest-associated yeasts and other microbes in shaping *Aspergillus* growth and pathogenic potential, which would ultimately provide new insights into disease ecology and conservation management.

5.2 Integrated Summary and Significance

This thesis examined how nest-associated yeasts interact with *Aspergillus* species in kākāpō (*Strigops habroptilus*) breeding habitats, with the overarching goal of understanding drivers of aspergillosis outbreaks and informing conservation management. Across all chapters, a consistent narrative emerged: kākāpō nest soils are yeast-rich environments in which *Aspergillus* is naturally scarce, and many of these yeasts can directly inhibit *Aspergillus* growth. These findings challenge the assumption that aspergillosis risk in avian species is predominantly influenced by high exposure to *A. fumigatus* spore loads, highlighting the need to consider the role of broader ecological interactions.

This study highlights the value of combining multiple approaches to investigate potential microbial influences on aspergillosis risk in kākāpō breeding habitats. Viable colony counts (Chapter Two) quantified metabolically active fungi, identifying those most likely to interact with the nest environment, other microbes, and the host. DNA-based community profiling (Chapter Three) provided finer taxonomic resolution and, together with viable counts, revealed how fungal communities shift with nest microhabitat and host activity. Co-cultivation assays (Chapter Four) demonstrated competitive interactions between *A. fumigatus* and nest-associated yeasts, offering potential explanations for observed viable abundance patterns. Together, these complementary methods provide further understanding of pathogen dynamics in nest soils and point to ecological factors that may guide management strategies to reduce aspergillosis risk.

By integrating data from Chapters Two, Three, and Four, a consistent pattern emerges across the subset of samples with both viable colony counts and DNA-based community profiles. Yeast pseudo-abundances (Figure 5.1A) were calculated as total yeast CFU g^{-1} (Chapter Two) multiplied by the relative abundance (%) of yeasts (Chapter Three), predominantly the genera associated with yeast isolates identified from environmental samples in Chapter Four (*Candida*, *Debaryomyces*, *Meyerozyma*, *Spencermartinsiella*). The genus *Clavispora* was not detected in Chapter Three's analysis and therefore was not included in this integrated data visualisation. *Aspergillus fumigatus* abundances (Figure 5.1B) came directly from colony counts. In Figure 5.1C, yeast pseudo-abundances were further scaled by mean radial mycelial inhibition percentages from Chapter Four co-cultivation assays, yielding an inhibition-weighted metric of CFU × (% inhibition). Together, these calculations show that *A. fumigatus* was usually present in samples with low yeast pseudo-abundance and inhibition-weighted scores. This integration provides a quantitative and mechanistic estimate of *A. fumigatus* and yeast interactions, showing that common nest-associated yeasts can suppress or contribute to low *A. fumigatus* growth in kākāpō nest soils.

This opens new directions for management, such as considering the dietary and behavioural drivers of yeast proliferation, and how these may either buffer or exacerbate disease occurrence and prevalence. Rather than viewing nests as passive environments in which pathogens accumulate, the findings suggest they are dynamic microbial systems shaped by host activity and fungal interactions.

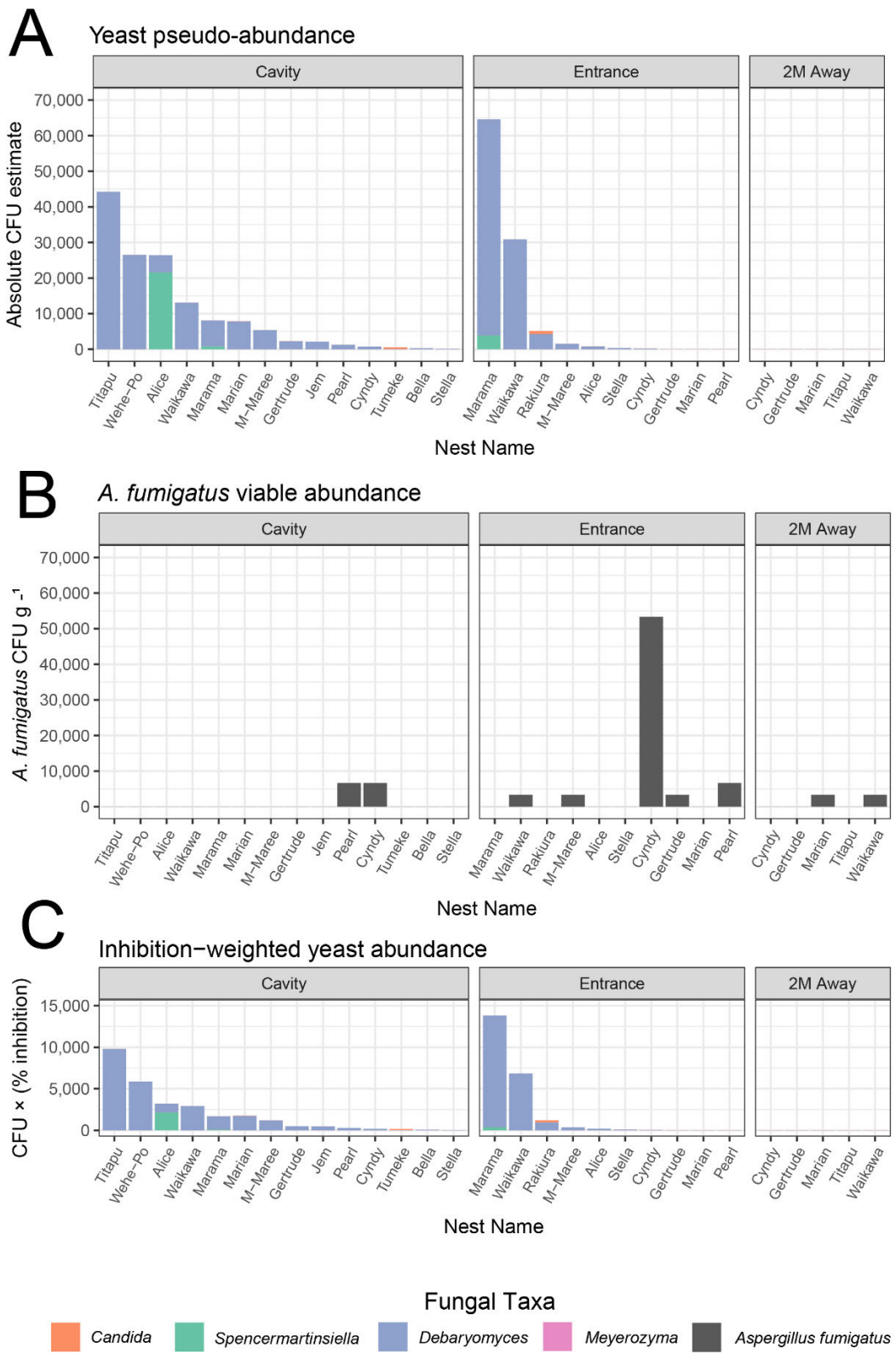

Figure 5.1 Data synthesis of each working chapter (Chapter Two, Three and Four), showing the potential inverse relationship between kākāpō nest yeasts and *A. fumigatus*. (A) yeast pseudo-abundance calculated using total yeast CFU from Chapter Two and relative abundances of genera (Chapter Three) associated with yeast isolates used in Chapter Four (only *Candida*, *Debaryomyces*, *Meyerozyma* and *Spencermartinsiella* spp. were detected, whilst *Clavispora* spp. was not). (B) CFU of *A. fumigatus*. (C) Inhibition pressure against *A. fumigatus* from yeasts, calculated with the yeast-pseudo abundance and inhibition percentages from Chapter Four's radial inhibition of *A. fumigatus* mycelia.

Addressing aspergillosis in kākāpō conservation remains an issue because of the many interacting drivers; from host immunity and diet to environmental disturbance and microbial ecology. Each breeding season brings urgency as new chicks are at risk, yet research funding has not kept pace with the complexity of the challenge. This thesis contributes evidence that ecological interactions deserve greater attention, but it also raises more questions than it answers. Without consistent, long-term investment into understanding these microbial dynamics, conservationists are forced to act with partial knowledge, making it difficult to design sustainable strategies for preventing future outbreaks.

Therefore, after summarising key thesis findings and implications, this chapter will discuss areas in need of further research, which will improve our understanding of disease risk in protected species like kākāpō (discussed in Section 5.4). Integrated suggestions for monitoring, sampling and management will also contribute to designing sustainable strategies for future outbreak prevention.

5.2.1 Chapter Two

In Chapter Two, cultivation techniques and zero-inflated negative-binomial modelling were used to quantify viable *Aspergillus* spp. in kākāpō nest soils from cavities, cavity entrances, and adjacent forest (two metres away from cavities) on Whenua Hou and Pukenui. Although nests associated with aspergillosis occasionally contained higher general *Aspergillus* counts, *A. fumigatus* was rarely detected and mean abundances from 2019 and 2022 remained below 100,000 CFU g^{-1} wet soil, indicating that soil viable counts alone is an unreliable predictor of disease risk. However, since the disease-causing agent was confirmed as a single strain of *A. fumigatus*, this indicates that infected kākāpō would have been exposed to this pathogen at some initial point. As mentioned before, *A. fumigatus* was mostly absent in affected kākāpō nest soils, however, since kākāpō chicks (which remain within the nest) were also infected, this may suggest introduction of spores to the nest via kākāpō breeding females or spore exposure from other nest substrates (e.g., air or airborne dust particles). However, since affected nests were significantly associated with higher *Aspergillus* counts overall (mostly *A. niger* and *A. flavus*), it is possible these species interactions play a role in kākāpō infection risk. In contrast to *Aspergillus* spp., yeasts (yeasts including *Debaryomyces hansenii*, *Meyerozyma guilliermondii*, and *Candida palmioleophila*) dominated all nest cavities and entrances, and yeast presence showed a strong negative association with *A. fumigatus* and *Aspergillus* spp. abundance, suggesting potential competitive interactions.

It was also found that yeast abundances were highest in nest cavities yet did not significantly differ in abundance between islands, suggesting the possibility of kākāpō driving yeast communities more than factors specific to island location. This cultivable representation of key fungal groups highlighted the possible competitive role of yeasts, guiding the broader community-level analyses in Chapter Three and the targeted yeast–*Aspergillus* interaction experiments in Chapter Four.

Overall, these findings highlight that recent kākāpō aspergillosis outbreaks are likely caused by, (1) a shared external source of *A. fumigatus* spore exposure (regardless of the level) and (2) multiple immunosuppressive factors, such as stress, low genetic diversity and presence of other potential opportunistic pathogens (e.g., other *Aspergillus* species, proliferating yeasts and mycotoxin exposure). When considering the two common causes identified as drivers of aspergillosis; high spore exposure and immunosuppression, we are left with the assumption that immunosuppression is likely a stronger driver of infection for kākāpō.

5.2.2 Chapter Three

Chapter Three broadened the investigation to the entire fungal community using ITS amplicon sequencing. Fungal alpha and beta diversity did not differ between aspergillosis-associated and unaffected nests, indicating that overall community structure is not a primary driver of disease. Instead, communities varied strongly with nest microhabitat, where richness and evenness (alpha diversity) increased with distance from the cavity, and composition (beta diversity) differed significantly across cavities, entrances, and surrounding forest. Island-level differences in beta diversity were detected within cavities and entrances but not in soils two metres away, suggesting shared background forest communities with local, island-specific modulation of the nest microbiota. Yeasts previously identified in the kākāpō gut and nest litter, such as *Apiotrichum porosum* and *D. hansenii* (West et al., 2025), dominated cavity soils and declined with increasing distance from nests, possibly implicating kākāpō defecation and diet as factors shaping the nest mycobiota. In West and colleagues' study, gut-associated yeasts like *D. hansenii* decrease when kākāpō juveniles transition from a rimu and supplementary-feed diet to a predominantly plant-based diet. Due to the consistent occurrence of these yeast isolates in birds associated with partial consumption of processed foods (Glushakova & Kachalkin, 2024a, 2024b; Nualmalang et al., 2023; Robinson et al., 2022; Sakda et al., 2023; Simi et al., 2018), it is likely that supplementary feed contributes to these proliferating yeast species. If supplementary feeding is shown to pose greater risks (through increased exposure to both yeasts and mycotoxins) than benefits in meeting nutritional demands and population recovery, this would highlight that even necessary human intervention may have unintended consequences. Even minor alterations to an animal's natural diet may have cascading effects on its health, where diet may shape environmental microbiomes, forming a feedback loop that can ultimately influence animal health (Figure 5.2). Collectively, these results highlight a strong host–environment link in which diet and defecation drive the spatial organisation of nest fungal pathogen communities.

5.2.3 Chapter Four

Chapter Four provided experimental confirmation that kākāpō nest yeasts can antagonise kākāpō lung and nest isolates of *Aspergillus fumigatus* and *A. niger*. In vitro co-culture assays showed that all six tested yeast isolates (i.e., *D. hansenii*, *Debaryomyces* spp. unidentified, *Clavispora* spp. unidentified, *Spencermartinsiella ligniputridi*, *M. guilliermondii*, and *Candida palmioleophila*) suppressed mycelial

growth of the tested *Aspergillus* spp., with *M. guilliermondii* reducing both growth and conidiation by up to ~80%. Additionally, percentage inhibition of *Aspergillus* isolate conidiation by yeasts such as *D. hansenii*, *C. palmioleophila* and *Clavispora* spp. were higher than percentage inhibition of mycelial growth, indicating that together with *M. guilliermondii*, these yeasts have the potential to effectively reduce conidiation of *A. fumigatus* and *A. niger*. Suppression of conidiation by most yeasts (*S. ligniputridi* excluded) was also apparent in diffusible antifungal compound assays, along with observations of moderate inhibition. If these in vitro assays accurately reflect fungal interactions *in situ* (natural kākāpō nests), the consistently high proportions and prevalence of *D. hansenii* in kākāpō nests may have a positive effect in reducing *A. fumigatus* spore exposure.

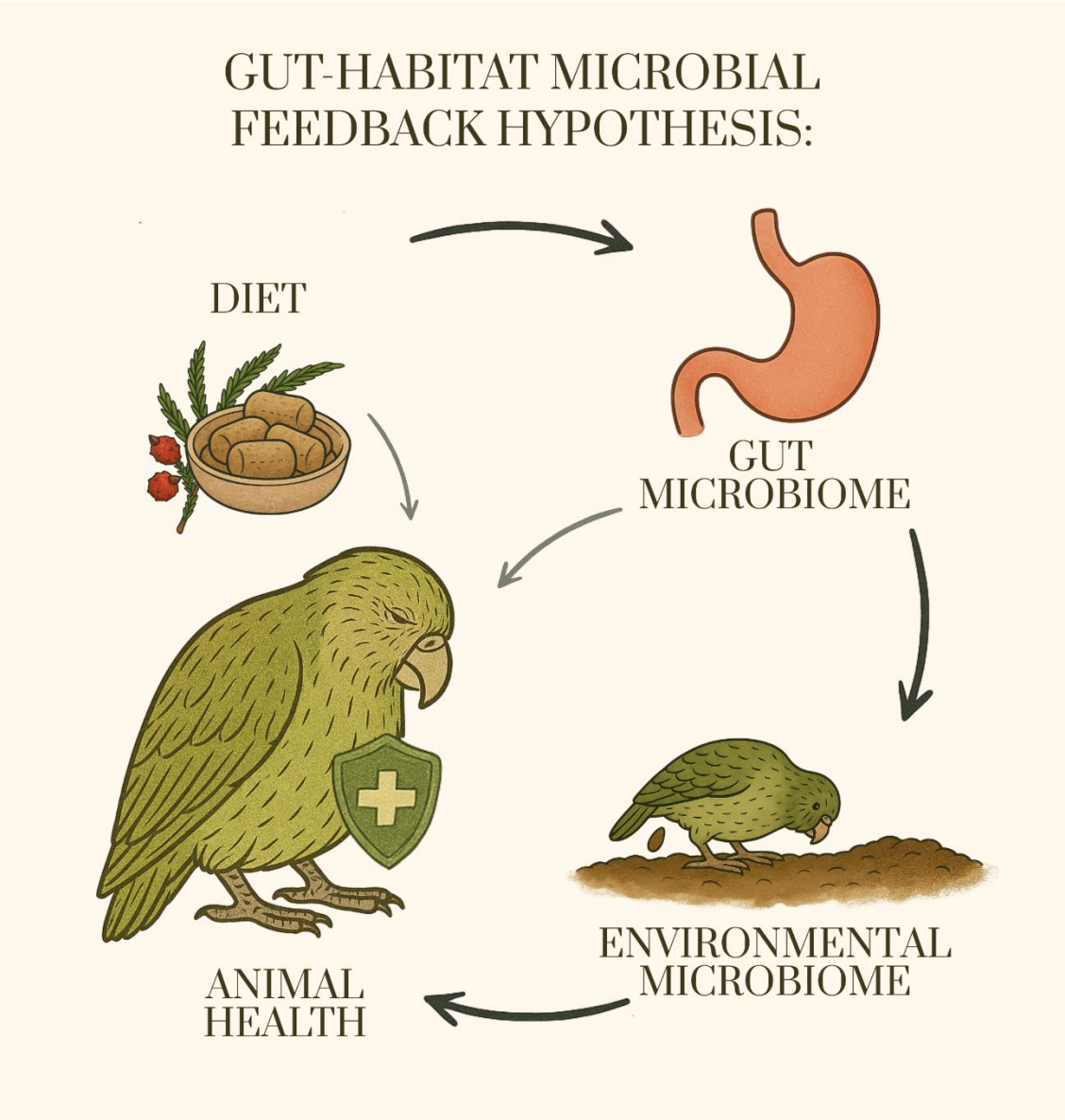

Figure 5.2 Gut-Habitat Microbial Feedback hypothesis, describing how diet influences animal gut microbiota and consequently their habitats and overall health. Image created using Canva.

Whilst acknowledging that aspergillosis infection occurred despite low *A. fumigatus* spore loads for kākāpō, exposure to high spore loads remains a contributing factor to avian aspergillosis worldwide and would likely exacerbate kākāpō aspergillosis outbreaks if not controlled. Together, findings support the hypothesis that yeasts help maintain the low *Aspergillus* abundances observed in kākāpō nest soils. However, the metabolites responsible for yeast antagonism have not yet been identified, and therefore it cannot be confirmed whether the antagonistic potential of kākāpō nest yeasts result in a positive or negative effect on kākāpō health. Whether these interactions alter toxin production in *A. fumigatus* also, is yet to be investigated, and should be considered for its potential impact on kākāpō health.

Collectively, these chapters suggest that the microbiome of kākāpō nests may be shaped by diet-driven yeast enrichment and that these yeasts can directly suppress the growth of *A. fumigatus*. The work highlights two key implications: (1) management strategies that rely solely on environmental *A. fumigatus* spore counts, specifically soils, will underestimate disease risk, and (2) supplementary feeding, and other (although required) human interventions may partially influence gut and nest microbiomes, potentially creating feedbacks that affect pathogen dynamics. By linking host diet, microbial interactions, and disease ecology, this thesis provides a foundation for future research on microbial competition, mycotoxin monitoring, and management of *A. fumigatus* in critically endangered kākāpō and other vulnerable wildlife.

5.3 Limitations

5.3.1 Methods for fungal quantification in kākāpō nest soils

In Chapter Two, serial dilutions and colony count techniques were used to estimate fungal abundance, which quantifies viable and culturable microorganisms. This approach offers a more relevant measure of living species capable of growing with these conditions, particularly when studying interspecies interactions. However, a key limitation is that many fungi are unculturable under standard conditions, and media selection can bias results toward certain taxa (Jeewon & Hyde, 2007; O'Brien et al., 2005). However, this method was appropriate for estimating *Aspergillus* spp. abundance given its broad environmental tolerance and ease of cultivation. Nonetheless, *Apiotrichum* spp., which was the most relatively abundant genus identified in Chapter Three, was not recovered in our cultures, highlighting the inability of culturing methods to capture full community diversity.

In contrast, Chapter Three employed high-throughput DNA sequencing, which provides a more comprehensive view of fungal community composition and confirms taxa through genetic identification (O'Brien et al., 2005). While this method overcomes culturing biases, it cannot differentiate between live and dead cells. *A. fumigatus* was also not detected using this method but was cultured from the same soils in Chapter 2, albeit at low concentrations. West and colleagues (2025) also could not detect *A. fumigatus* while specifically targeting the ITS2 region. Possible explanations for this lack of detection

include insufficient DNA extraction, PCR or primer bias, or that the ITS sequences of *A. fumigatus* lacked species-level specificity (could not be assigned to species level using NCBI BLAST).

Because only two nests were affected by aspergillosis during the 2022 outbreak, Chapter Three could not accurately compare fungal community structure and diversity between nests that were affected and nests that were not. West's kākāpō study (2025), however, analysed nest soils from the 2019 outbreak with a higher number of affected nests, yet observed no effect between fungal communities on aspergillosis-associated nests.

For Chapters 2 and 3, measurements of abiotic factors within kākāpō nests (e.g., temperature, relative humidity, and soil nutrient content) were not available at the time of analysis. As a result, it was not possible to evaluate how these environmental variables may have contributed to observed patterns in fungal abundance or community structure. Future work incorporating concurrent abiotic measurements would therefore help clarify the extent to which microclimatic or physicochemical conditions influence nest-associated mycobiomes.

5.3.2 Competition assays

Compared to simply observing an inverse relationship between the competitors' viable abundances (Chapter Two), the competition assays used in Chapter Four allowed us to more accurately observe the competitive potential of kākāpō nest yeasts by minimising confounding effects by other soil fungi. Competition assays also ensure a more level playing field for fungal competition by standardising initial suspensions (cell and spore concentrations). However, it is important to consider that these techniques only demonstrate the competitive outcome (e.g., reduced growth) but do not clarify the mechanisms of action employed by the competitor. Therefore, it could not be confirmed whether the antagonistic potential of yeasts against *A. fumigatus* was through rapid growth, more efficient nutrient uptake or the production of antifungal compounds.

5.3.3 Mycotoxin concentration analysis

Toxin analysis on co-cultivation assays and kākāpō soils would have clarified whether kākāpō nest yeasts play a role in aspergillosis susceptibility. However, due to time constraints and the inability to obtain a gliotoxin standard (mycotoxin produced by *A. fumigatus*; see following section) needed for liquid chromatography – mass spectrometry (LC-MS), toxin analyses could not be performed.

5.4 Future Research

5.4.1 Aflatoxin screening

5.4.1.1 Kākāpō nests

A. fumigatus produces gliotoxin (GT), a type of aflatoxin that is said to be 96% responsible for virulence in human *A. fumigatus* infections and also has immunosuppressive and cytotoxic effects in birds (de

Oca et al., 2017; Latif et al., 2015; Richard et al., 1994; Richard et al., 1996; Wei et al., 2024). Intriguingly, the human immune system has the ability break GT down into a non-toxic form, known as bisdethiobis(methylthio)gliotoxin (bmGT). However, it has been reported that avian species do not possess this ability, allowing for longer exposure of the active, toxic GT form (Reidy et al., 2022). Given that GT is a major virulence factor of *A. fumigatus* in humans, the inability of birds to inactivate it may render them more vulnerable to its pathogenic effects than humans. This is supported by evidence that persistent GT detection in infected penguins was associated with poor prognosis (Reidy et al., 2022), and that experimental exposure in ducklings caused multi-organ damage and oxidative stress (Jin et al., 2023).

In this thesis, GT is of concern because the effect of kākāpō nest yeasts on *A. fumigatus* GT production remains unknown. Although these yeasts suppress *A. fumigatus* growth, the persistence of aspergillosis despite yeast proliferation suggests that secondary metabolites produced during competition could influence disease susceptibility or immunity. If yeasts suppress fungal growth but not GT production or induce GT as a stress response, they could inadvertently increase toxin exposure, warranting management to limit their abundance in kākāpō nests (see Section 5.4.2).

In general, infection by *A. fumigatus* is likely to result in GT production or exposure, for example, Reidy et al. (2022) found that in plasma samples of infected birds, 85.7% were positive for GT. A study by Cray et al. (2025) showed that 50% of blood samples taken from infected common goons tested positive for GT, whilst those from healthy birds did not. The remaining 50% of blood samples that were not positive for GT was assumed to be influenced by timing of infection (e.g., early or late testing) or infection by other *Aspergillus* species, presumably those that do not produce gliotoxin. In an older study (Richard & DeBey, 1995) which injected two different strains of *A. fumigatus* into turkey poult tissue, one strain produced GT in 100% of poults and the other produced GT in 70% of poults.

Together this information highlights the importance of gliotoxin as a virulence factor in avian aspergillosis and therefore should be accounted for as a predictor for disease risk moving forward. Furthermore, screening for gliotoxin may be a more accurate indicator of disease risk than measuring *A. fumigatus* viable abundances. This is because *A. fumigatus* presence or spore loads may not fully correlate with GT detection. For example, in the turkey poult study mentioned previously (Richard & DeBey, 1995), there was no correlation between the level of *A. fumigatus* conidial concentrations injected (high: 5×10^7, low: 5×10^6) and resulting GT concentrations. In another study, *A. fumigatus* was isolated from the tissue of six turkeys (Richard et al., 1996), which although produced GT in-vitro, the tissue of three of those turkeys were negative for GT. Furthermore, GT was detected in two turkeys of which *A. fumigatus* was not isolated from.

Intriguingly, a similar observation has already been observed in post-mortem kākāpō during the 2019 outbreak, but with *Aspergillus flavus* and aflatoxin (see Section 5.4.1.2). As previously mentioned

throughout this thesis, aflatoxin exposure was confirmed via histology in two kākāpō: one without infection by *Aspergillus* spp. at all, and one only infected by *A. fumigatus* with no isolation of *A. flavus*. Another two kākāpō had *A. flavus* isolated post-mortem yet no signs of aflatoxin exposure.

A possible explanation for the detection of aflatoxin exposure despite the absence of the pathogen, could be exposure from an external source or growth. For example, studies have found that *A. fumigatus* isolated from cereals, animal feed and hay can produce high levels of GT (Gareis & Wernery, 1994; Soleiro et al., 2013), which may negatively impact animal health regardless of infection by *A. fumigatus*.

These studies show that aflatoxin (including gliotoxin) concentrations can be independent of *Aspergillus* spp. abundance, suggesting that toxin exposure either depends on the strain and growth conditions of the pathogen, or the presence of a nearby external source. Therefore, further investigation of gliotoxin concentrations in kākāpō nests are warranted, as low *A. fumigatus* abundances does not guarantee low gliotoxin exposure. Considering its immunosuppressive effects, high levels of gliotoxin in kākāpō nests may be the 'tipping point' for effective establishment of *A. fumigatus* in already immunosuppressed kākāpō mothers and juveniles.

5.4.1.2 Supplementary feed

Of the sick kākāpō during the 2019 outbreak, almost half of those that had died of aspergillosis demonstrated signs of aflatoxin exposure post-mortem (commonly produced by *A. flavus*). Aflatoxin is a mycotoxin produced primarily by *A. flavus* and *A. parasiticus*, with potent hepatotoxic and carcinogenic effects across many vertebrates (Frisvad et al., 2019), including birds (Lawson et al., 2006). Unlike gliotoxin, which is mostly encountered as an immunosuppressive virulence factor secreted by *A. fumigatus* during infection (Kwon-Chung & Sugui, 2009), aflatoxin is more typically encountered as a contaminant of stored cereals and grain-based feeds (Jallow et al., 2021).

Signs of (confirmed) aflatoxin exposure in infected kākāpō post-mortem (2019), and a previous case of aflatoxicosis in a male kākāpō linked to contaminated supplementary nuts (Jakob-Hoff & Gartrell, 2011) shows that aflatoxin exposure poses a credible risk to this species. Given that kākāpō are provisioned with supplementary pellets composed primarily of plant-derived ingredients, screening of supplementary feed for aflatoxins (B1, B2, G1, G2 and M1) during the 2019 outbreak was an essential step to rule out dietary toxin exposure. However, no concerning results were reported (Chapter Two). Although aflatoxin B1 was detected in one unopened, unused bag at a low concentration (0.62 ppb), levels were well below the maximum tolerated level for young poultry (≤ 50 ppb). This suggests the supplementary feed was unlikely to be the primary source of exposure, however, some uncertainty remains over whether all batches of feed in use at the time were included in toxin testing. Given that aflatoxins can arise independently of infection and are common contaminants of stored cereals and plant-derived feeds, continued screening of supplementary feed remains an important management measure.

A preventative measure proposed for kākāpō management going forward was consistent freezer storage of supplementary feed. While effective in preventing *Aspergillus* growth, freezing does not degrade aflatoxins, meaning any toxin present prior to freezing will remain stable under frozen conditions (Kutasi et al., 2021). Therefore, routine aflatoxin screening should continue alongside gliotoxin (and other mycotoxin) monitoring in kākāpō nesting environments to better assess both dietary and environmental toxin exposure risks. Additionally, since *A. fumigatus* was isolated from a food-storage facility on Whenua Hou of 2019, another recommendation would be to screen for mycotoxins in feed also.

5.4.1.3 Co-cultivations

To confirm the mechanism of action for kākāpō nest yeast antagonism against *Aspergillus* spp., future co-cultures should be measured for mycotoxins, secondary metabolites and volatile organic compounds (VOCs). If these yeasts do not produce harmful metabolites or do not enhance production of harmful metabolites via competition with *A. fumigatus*, they could be further studied as a potential biological control against *Aspergillus* species. Conversely, if such metabolites are detected, this would provide a better understanding of disease dynamics in kākāpō nests

5.4.2 Kākāpō diet and yeast origins

Supplementary feed has been implemented in the kākāpō diet to meet nutritional demands and to ensure that females reach the weight threshold required for breeding (Clout et al., 2002; G. P. Elliott et al., 2001; Jakob-Hoff & Gartrell, 2011; Robertson et al., 2006). Although supplementary feed has been reported as not sufficient to trigger breeding (Wilson et al., 2006), one study has observed a significant increase in clutch size and concluded that egg production was limited by lack of nutrients (provided by formulated pellets) rather than energy (nut supplements) (Houston et al., 2007). Therefore, it is important to recognise the value of supplementary feed (currently Harrison's High Potency Fine Pellets) for kākāpō population recovery. However, if kākāpō nest yeasts are driven by supplementary feed and link to disease susceptibility, this may also threaten kākāpō recovery.

Parallel findings from (West et al., 2025) showed that yeasts such as *Apiotrichum*, *Debaryomyces hansenii*, and *Candida palmioleophila* dominate kākāpō nest and gut samples but decline sharply after fledging, when chicks transition from a diet of predominantly supplementary feed and rimu berries to a more natural, plant-based diet. This pattern, together with the present study's evidence that nest-proximate soils differ from surrounding forest soils, supports a model where diet and defecation introduce gut-associated fungi that subsequently proliferate within nests.

Patterns observed in this study suggest that different yeasts may have distinct ecological origins and roles. For example, although *Apiotrichum* spp. was high in relative abundance (RA) within nest cavities, it was also moderately relatively abundant two metres away, which may suggest environmental origins that were amplified under favourable nest conditions (e.g., warmth, nutrient rich substrates etc.). In

contrast, *Debaryomyces* spp. showed strong cavity-specific enrichment and is well known from plant and grain materials, suggesting possible introduction through supplementary feed. *Kazachstania* spp. was less dominant overall, but its increased RA in fledging chicks that shift to a plant-based diet points to an association with a more natural diet rather than predominantly pellets (West et al., 2025).

Although commercial feed is produced hygienically, low-level fungal contamination in animal feed is common (Laouni et al., 2024; Tesfaye et al., 2024) and spores can persist through storage and processing (Placinta et al., 1999). Even minimal contamination could seed fungal growth within kākāpō nests. The strong beta-diversity differences between islands for nest soils, but not for more natural forest soils, further imply that yeasts in nests are shaped by dietary introduction (and microenvironmental amplification) rather than by island-specific background communities.

To determine whether these yeast-rich communities are a modern consequence of supplementary feeding or reflect a long-standing dietary pattern, it is important to consider the natural, pre-supplementary diet of kākāpō. Analyses of kākāpō coprolites (fossilised faeces) indicate that the modern diet (Best, 1984; Butler, 2006; Wilson et al., 2006) has not changed substantially since before human arrival (Horrocks et al., 2008). Because kākāpō are herbivorous, coprolite analyses revealed a predominance of native New Zealand plant material (e.g., flowers, leaves, mosses, and fern fronds), broadly resembling the modern diet. Despite this, many other native plants were identified in coprolites as not part of this modern diet, and it had been concluded that fern fronds (e.g., *Cyathea smithii* and *Hymenophyllum* spp.) were the most preferred food at the time. Given that this coprolite study was conducted nearly two decades ago using microscopy-based methods, reassessment with modern metabarcoding techniques could reveal previously undetected fungal or plant components, providing an updated baseline for understanding the natural kākāpō diet and its potential microbial associations.

Future research should prioritise clarifying the dual role of diet and environment in shaping kākāpō nest mycobiota. Targeted screening of supplementary feed for viable yeasts and fungal DNA (across production, storage, and deployment stages) will determine whether certain taxa, such as *Debaryomyces*, are introduced via feed or are natural background taxa that simply proliferate under favourable nest conditions. Integrating these analyses with longitudinal monitoring of gut and nest soils will help establish whether supplementary feed functions primarily as a source or as a substrate that amplifies yeast growth.

At the same time, re-examining the natural kākāpō diet through modern metabarcoding of historical coprolites and preserved nest material would provide a pre-supplementary baseline for comparison. Such work could reveal whether yeast-rich gut or nest communities existed prior to pellet feeding, and whether changes in diet composition have altered microbial ecology over time. Together, these approaches will disentangle the relative contributions of diet, defecation, and habitat in driving yeast

proliferation, informing feed formulation, handling, and management practices that maintain breeding success while minimising microbial risks to kākāpō health and recovery.

5.4.3 Shotgun metagenomic sequencing and quantitative PCR (qPCR)

In addition to targeted metabolite work, more detailed molecular analysis will be essential for fully understanding host–pathogen–microbiome dynamics. Methods such as shotgun metagenomic sequencing would enable comprehensive community characterisation of the soil microbial community beyond the ITS region, capturing functional genes and secondary metabolite pathways that may influence pathogenicity. Additionally, precise quantification of *A. fumigatus* should be performed using qPCR or ddPCR, allowing researchers to measure true DNA abundance of *A. fumigatus*, using primers that are effective for targeting this specific species. Combining these approaches with toxin screening from competition assays would provide a more integrated framework to predict aspergillosis risk and to disentangle the respective contributions of fungi, yeasts, and their metabolites to aspergillosis susceptibility.

5.4.4 Abiotic factors

Microbial community dynamics are shaped by abiotic factors such as temperature, humidity, soil moisture, pH, and nutrient availability, all of which regulate fungal growth, spore production, and secondary metabolite (e.g., gliotoxin) synthesis. Although kākāpō diet and defecation are likely key factors introducing yeast communities to nest soils, measuring abiotic factors within the nest will provide an indication of what makes the nest a favourable environment for yeast proliferation, and if some of these factors can be mitigated without disturbing kākāpō and if these yeasts are proven to negatively affect kākāpō health.

Quantifying dust concentrations inside kākāpō nest burrows, for instance using a portable particulate matter ($PM_{2.5}$) sensor, could also provide valuable insight into potential sources of initial *A. fumigatus* exposure. Elevated dust levels are often associated with *A. fumigatus* spore inhalation, as dust particles provide both substrate and dispersal surfaces (O'Gorman, 2011). In addition, kākāpō behaviour (e.g., dustbathing, rustling feathers, preening, or movement in and out of burrows) may re-suspend fine soil particles and organic debris, increasing airborne dust and spore concentrations within enclosed nesting environments. Thus, measurement of dust load and airborne *A. fumigatus* counts would indicate how much inhalable spore exposure occurs and whether dust accumulation is a key contributing factor to infection. Furthermore, monitoring female kākāpō behaviour using nest cameras, alongside measurements of dust and airborne spore levels, could help determine whether bird behaviour within the nesting environment contributes to increased spore inhalation rates.

Additionally, the distinct microclimates in nest-proximate soils observed between Pukenui and Whenua Hou suggest that island-specific environmental conditions may contribute to differences in fungal community structure and pathogen pressure. Investigating soil physicochemical parameters alongside

microbial data would help disentangle whether the observed patterns in yeast–*Aspergillus* dynamics are primarily biotic (competition) or environmentally mediated.

5.4.5 Sampling periods

For future monitoring, viable counts of *Aspergillus* spp. and yeasts should be complemented with DNA-based approaches (fungal and bacterial community profiling, shotgun metagenomics, and qPCR) as well as toxin analysis of kākāpō nest soils and faecal material. Sampling should take place at the beginning (or once nests are identified) and end of the breeding season, which could capture dynamic shifts in microbial abundances and interactions that may predispose birds to disease. Air sampling should also be incorporated, as it would provide direct insight into the abundance of airborne (and thus more directly inhaled) *Aspergillus* species. Since air samples were not collected during the aspergillosis outbreaks, it remains uncertain whether the pathogenic agent was entirely absent from nest environments.

5.5 Concluding remarks

Our findings suggest that the abundance of *A. fumigatus* in kākāpō nest soils may not be the sole driver of infection. This thesis, together with other kākāpō studies, suggests that initial exposure of kākāpō to *A. fumigatus* spores may have occurred external to the nesting environment, while factors within the nest could have elevated host susceptibility and airborne dust/spore levels. One theory is the transfer of spores acquired externally by kākāpō females into nest burrows, where behaviours such as transfer of contaminated feed, preening, feather shaking, and dustbathing could have resuspended dust and spores into the confined nest air. Meanwhile, *A. fumigatus* could have remained at low detectable levels in nest soils due to competitive suppression by abundant yeasts that inhibit its growth and sporulation. Factors inside the nest, such as mycotoxin interactions between opportunistic pathogens may have further elevated host susceptibility, enabling inhaled airborne spores to colonise respiratory tracts of both kākāpō females and chicks.

Studying pathogen interactions in kākāpō nests provides a valuable model for understanding disease risk in cavity-nesting birds, such as hihi (*Notiomystis cincta*) and other species known to be susceptible to aspergillosis. As environmental changes intensify globally, sudden disease events in rare or endangered species like the kākāpō may also serve as early indicators of what lies ahead for other endangered wildlife populations. In response, it is important to acknowledge the complexity of disease ecology and to look beyond simple measures of pathogen abundance when assessing infection risk. A comprehensive understanding requires consideration of all components of the disease triangle, each of which typically must align for infection to occur. These include: (1) host susceptibility, such as immunosuppression arising from mycotoxin exposure or co-infections; (2) pathogen presence, encompassing potential sources and pathways of exposure across different habitat substrates; and (3) environmental conditions that favour pathogen growth and persistence, including warm temperatures,

high humidity, and nutrient-rich substrates, as well as factors that promote spore accumulation and inhalation, such as substrate disturbance and elevated dust concentrations within confined nest environments.

As a concluding remark, wildlife managers, ecologists, conservationists, veterinarians, microbiologists, and indigenous communities (e.g., mana whenua Māori) should continue to work together to better understand and bring attention to disease threats within taonga species on mammal predator-free islands in Aotearoa.

Appendix A – Chapter 2 Supplementary Material

Table S1 Colony forming units ($CFUg^{-1}$) wet material of *Aspergillus* spp. (*A. fumigatus*, *A. flavus* and *A. niger*) recovered from kākāpō nest cavity soil/litter material in 2019. Affected nests = nests associated with individuals diagnosed with aspergillosis. Unaffected nests = nests not associated with individuals diagnosed with aspergillosis. Yeast = yeast presence, Y = yes/present, N = no/not present.

Date	Island	Kākāpō Name	*A. fumigatus*	*A. flavus*	*A. niger*	Yeast
Affected nests						
18/05/2019	Whenua Hou	Margaret-Maree	-	180000	750	Y
16/05/2019	Whenua Hou	Ihi	-	-	-	Y
16/05/2019	Whenua Hou	Huhana	-	-	-	Y
15/05/2019	Whenua Hou	Pounamu	-	-	-	Y
15/05/2019	Whenua Hou	Cyndy	-	81000	-	Y
19/05/2019	Whenua Hou	Hoki_1	-	295000	-	Y
18/05/2019	Whenua Hou	Kuihi_1	-	-	965000	N
19/05/2019	Whenua Hou	Alice	-	-	-	Y
14/05/2019	Whenua Hou	Aranga	-	-	-	Y
19/05/2019	Whenua Hou	Wehe-po	-	-	-	Y
14/05/2009	Whenua Hou	Kuihi_2	-	-	125000	N
23/05/2019	Whenua Hou	Wehe-po	-	-	1250000	N
28/05/2019	Whenua Hou	Nora	-	-	-	Y
23/05/2019	Whenua Hou	Solstice	-	-	3000	Y
2/12/2019	Whenua Hou	Hoki_2	5400000	-	-	N
Unaffected nests						
19/05/2019	Whenua Hou	Sue	-	-	-	Y
19/05/2019	Whenua Hou	Esperance	-	-	1800	Y
15/05/2019	Whenua Hou	Queenie	-	-	-	Y
17/05/2019	Whenua Hou	Rakiura	-	-	-	Y
15/05/2019	Whenua Hou	Awarua_1	-	-	-	Y
15/05/2019	Whenua Hou	Zephyr	-	-	-	Y
17/04/2019	Whenua Hou	Pura	-	-	-	Y
19/05/2019	Whenua Hou	Suzanne	-	-	-	Y
21/05/2019	Whenua Hou	Tumeke	-	-	-	N
16/05/2019	Whenua Hou	Bella	-	-	-	Y
23/05/2019	Whenua Hou	Awarua_2	-	-	-	N
25/05/2019	Pukenui	Hinemoa	-	-	-	N
25/05/2019	Pukenui	Ra_1	195000	-	-	Y
27/05/2019	Pukenui	Roha	-	-	-	Y
25/05/2019	Pukenui	Hine-Taumai_1	-	7000	-	Y
25/05/2019	Pukenui	Atareta	-	-	-	Y
25/05/2019	Pukenui	Waikawa	-	-	-	Y
26/05/2019	Pukenui	Aparima	-	-	-	N
28/05/2019	Pukenui	Stella	-	-	-	Y
27/05/2019	Pukenui	Boomer	-	-	-	Y
27/05/2019	Pukenui	Marama	-	-	-	N
27/05/2019	Pukenui	Mila	50000	-	-	Y

29/05/2019	Pukenui	Yasmine	-	-	-	Y
28/05/2019	Pukenui	Hauturu	-	-	-	Y
28/05/2019	Pukenui	Jemma	-	-	-	Y
28/05/2019	Pukenui	Waa	-	-	-	Y
6/12/2019	Pukenui	Hine-Taumai_2	-	-	-	N
3/12/2019	Pukenui	Ra_2	-	-	-	N

Table S2 Abundance of *Aspergillus fumigatus* and yeasts as colony forming units per gram (CFU g^{-1}) of wet soil in kākāpō nest habitats of 2022 (across nest locations, and islands). SD = standard deviation values. Nest MH = nest microhabitat, WH = Whenua Hou Island, *A.fum* = *A. fumigatus*, *A.nig* = *A. niger*.

Date Collected	Island	Nest Name	Nest MH	Mean *A fum*	SD *A.fum*	Mean *A.nig*	SD *A.nig*	Mean Yeast	SD Yeast
19/06/2022	WH	Cyndy	Cavity	6,666.67	5,773.50	-	-	716,666.67	41,633.32
	WH	Cyndy	Entrance	53,333.33	92,376.04	-	-	20,000.00	10,000.00
	WH	Cyndy	2m	-	-	-	-	-	-
23/06/2022	WH	M-Maree	Cavity	-	-	-	-	90,000.00	45,825.76
	WH	M-Maree	Entrance	3,333.33	5,773.50	-	-	36,666.67	15,275.25
	WH	M-Maree	2m	-	-	-	-	-	-
22/06/2022	WH	Pearl	Cavity	6,666.67	5,773.50	-	-	16,666.67	5,773.50
	WH	Pearl	Entrance	6,666.67	11,547.01	-	-	-	-
	WH	Pearl	2m	10,000.00	10,000.00	-	-	-	-
23/06/2022	WH	Bella	Cavity	-	-	-	-	3,333.33	5,773.50
	WH	Bella	Entrance	-	-	-	-	86,666.67	15,275.25
	WH	Bella	2m	-	-	-	-	-	-
22/06/2022	WH	Rakiura	Cavity	-	-	-	-	100,000.00	17,320.51
	WH	Rakiura	Entrance	-	-	-	-	130,000.00	75,498.34
	WH	Rakiura	2m	-	-	-	-	3,333.33	5,773.50
27/06/2022	WH	Alice	Cavity	-	-	-	-	703,333.33	124,230.97
	WH	Alice	Entrance	-	-	-	-	70,000.00	26,457.51
	WH	Alice	2m	-	-	-	-	-	-
24/05/2022	WH	Tumeke	Cavity	-	-	-	-	73,333.33	66,583.28
	WH	Tumeke	Entrance	-	-	-	-	6,666.67	5,773.50
	WH	Tumeke	2m	-	-	-	-	-	-
26/02/2022	WH	Wehe-Po	Cavity	-	-	-	-	140,000.00	113,137.08
	WH	Wehe-Po	Entrance	3,333.33	5,773.50	-	-	66,666.67	73,711.15
	WH	Wehe-Po	2m	10,000.00	17,320.51	-	-	-	-
24/06/2022	WH	Titapu	Cavity	-	-	-	-	360,000	485,077
	WH	Titapu	Entrance	-	-	-	-	326,667	548,574
	WH	Titapu	2m	-	-	-	-	-	-
6/05/2022	Pukenui	Marian	Cavity	-	-	3,333.33	5,773.50	246,666.67	315,013.23
	Pukenui	Marian	Entrance	-	-	-	-	-	-
	Pukenui	Marian	2m	3,333.33	5,773.50	-	-	-	-
4/06/2022	Pukenui	Waikawa	Cavity	-	-	-	-	455,000.00	487,903.68
	Pukenui	Waikawa	Entrance	5,000.00	7,071.07	-	-	700,000.00	424,264.07
	Pukenui	Waikawa	2m	3,333.33	5,773.50	-	-	-	-

3/06/2022	Pukenui	JEM	Cavity	-	-	-	-	126,666.67	85,049.01
	Pukenui	JEM	Entrance	3,333.33	5,773.50	-	-	3,333.33	5,773.50
	Pukenui	JEM	2m	-	-	-	-	-	-
9/06/2022	Pukenui	Gertrude	Cavity	-	-	-	-	46,666.67	20,816.66
	Pukenui	Gertrude	Entrance	3,333.33	5,773.50	-	-	-	-
	Pukenui	Gertrude	2m	-	-	-	-	-	-
31/05/2022	Pukenui	Stella	Cavity	-	-	-	-	10,000.00	10,000.00
	Pukenui	Stella	Entrance	-	-	-	-	43,333.33	15,275.25
	Pukenui	Stella	2m	-	-	-	-	-	-
3/06/2022	Pukenui	Marama	Cavity	-	-	-	-	206,666.67	28,867.51
	Pukenui	Marama	Entrance	-	-	40,000.00	17,321.51	1,323,333.33	167,431.58
	Pukenui	Marama	2m	-	-	-	-	3,333.33	5,773.50

Table S3 Yeasts isolated from kākāpō nest soils. Isolate name = each morphologically distinct isolate (determined by colour and texture). ICMP number = associated accession number stored in Manaaki Whenua Landcare Research's International Collection of Microorganisms from Plants (ICMP) database. Species and species strain identified by Dr. Bevan Weir (Manaaki Whenua Landcare Research).

Isolate Name	ICMP number	Species	Species strain
ATM1A, 2A, 3A, 6A	ICMP 24917, 24933, 24920, 24923	*Debaryomyces hansenii*	*Debaryomyces hansenii* (Zopf) Lodder & Kreger 1952
ATM1B	ICMP 24918	*Meyerozyma guilliermondii*	*Meyerozyma guilliermondii* (Wick.) Kurtzman & M. Suzuki
ATM1D	ICMP 24919	*Spencermartinsiella ligniputridi*	*Spencermartinsiella ligniputridi* G. Péter & Dlauchy
ATM3B, 5A	ICMP 24921, unknown	*Debaryomyces* spp.	*Debaryomyces* Lodder & Kreger ex Kreger 1984
ATM5B, 5C, 6C	ICMP 24922, 24935, 24924	*Clavispora* spp.	*Clavispora* Rodr. Mir. 1979
ATM10B	ICMP 24925	*Candida palmioleophila*	*Candida palmioleophila* Nakase & Itoh

Table S4 Results of the zero-inflated negative binomial model examining the effects of affected and unaffected nests on *Aspergillus* species abundance from the 2019 dataset (*Aspergillus* spp = *A. fumigatus*, *A. niger*, *A. flavus*).

Predictor	Estimate	Std. Error	*z* value	*p*-value
(Intercept)	13.7322	0.5581	24.604	< 2e-16 ***
Aspergillosis_Unaffected	-2.7046	1.0007	-2.703	0.00688 **
Log(theta)	-1.0323	0.3625	-2.847	0.00441 **
Zero-Inflation Model (Binomial with Logit Link)				
(Intercept)	2.1763	0.2934	7.416	1.2e-13 ***

The table shows the estimated model coefficients, their standard errors, *z* values (the coefficient divided by its standard error), and associated *p*-values. Asterisks denote statistical significance ($p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***). Positive estimates indicate an increase in *Aspergillus* abundance relative to the reference category, while negative estimates indicate a decrease. The reference category is "Affected" nests.

Table S5 Results of the zero-inflated negative binomial model examining the effects of yeast presence and absence on *Aspergillus* species counts from the 2019 dataset (*Aspergillus* spp = *A. fumigatus*, *A. niger*, *A. flavus*).

Predictor	Estimate	Std. Error	*z* value	*p*-value
(Intercept)	14.4753	0.6937	20.867	< 2e-16 ***
Yeast_presenceY	-3.0654	0.8336	-3.677	0.000236 ***
Log(theta)	-0.6550	0.3375	-1.940	0.052320 .
Zero-Inflation Model (Binomial with Logit Link)				
(Intercept)	2.1869	0.2925	7.476	7.69e-14 ***

The table shows the estimated model coefficients, their standard errors, *z* values (the coefficient divided by its standard error), and associated *p*-values. Asterisks denote statistical significance ($p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***). Positive estimates indicate an increase in *Aspergillus* spp. abundance relative to the reference category, while negative estimates indicate a decrease. The reference category is "N" (No) for Yeast Presence.

Table S6 Results of the zero-inflated negative binomial model examining the effects of nest location and island on *Aspergillus fumigatus* abundance from the 2022 dataset. A replicate of Cyndy's nest entrance was removed as an outlier.

Predictor	Estimate	Std. Error	*z* value	*p*-value
(Intercept)	9.3531	0.1565	59.76	< 2e-16 ***
IslandWhenua_Hou	0.4655	0.1605	2.9	0.003730 **
LocationCavity	-0.6082	0.183	-3.324	0.000888 ***
LocationEntrance	-0.2277	0.1605	-1.419	0.155973
Log(theta)	2.6965	0.3615	7.46	8.66e-14 ***
Zero-Inflation Model (Binomial with Logit Link)				
(Intercept)	2.0455	0.2744	7.455	8.99e-14 ***

The table shows the estimated model coefficients, their standard errors, z values (the coefficient divided by its standard error), and associated *p*-values. Asterisks denote statistical significance ($p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***). Positive estimates indicate an increase in *A. fumigatus* abundance relative to the reference category, while negative estimates indicate a decrease. The reference categories are "Pukenui" for Island and "Two metres away" for Location.

Table S7 Results of the zero-inflated negative binomial model examining the effects of nest location and island on yeast abundance from the 2022 dataset.

Predictor	Estimate	Std. Error	*z* value	*p*-value
(Intercept)	9.4957	0.8893	10.677	< 2e-16 ***
IslandWhenua_Hou	-0.5831	0.3283	-1.776	0.075727 .
LocationCavity	3.2991	0.8875	3.717	0.000201 ***
LocationEntrance	3.1667	0.8959	3.535	0.000408 ***
Log(theta)	-0.4159	0.1464	-2.841	0.004503 **
Zero-Inflation Model (Binomial with Logit Link)				
(Intercept)	-0.2022	0.1759	-1.15	0.25

The table shows the estimated model coefficients, their standard errors, *z* values (the coefficient divided by its standard error), and associated *p*-values. Asterisks denote statistical significance ($p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***). Positive estimates indicate an increase in yeast abundance relative to the reference category, while negative estimates indicate a decrease. The reference categories are "Pukenui" for Island and "Two metres away" for Location.

Table S8 Results of the zero-inflated negative binomial model examining the effects of yeast presence and absence on *A. fumigatus* abundance from the 2022 dataset (Only *A. fumigatus* cultivated). A replicate of Cyndy's nest entrance was removed as an outlier.

Predictor	Estimate	Std. Error	*z* value	*p*-value
(Intercept)	9.6158	0.1116	86.201	< 2e-16 ***
Yeast_presenceY	-0.4055	0.1633	-2.483	0.013 *
Log(theta)	2.3078	0.3595	6.420	1.37e-10 ***
Zero-Inflation Model (Binomial with Logit Link)				
(Intercept)	2.0455	0.2744	7.455	8.99e-14 ***

The table shows the estimated model coefficients, their standard errors, *z* values (the coefficient divided by its standard error), and associated *p*-values. Asterisks denote statistical significance ($p < 0.05$: *, $p < 0.01$: **, $p < 0.001$: ***). Positive estimates indicate an increase in *A. fumigatus* abundance relative to the reference category, while negative estimates indicate a decrease. The reference category is "N" (No) for Yeast Presence.

Appendix B – Chapter 3 Supplementary Material

Table S1 DNA concentrations of extracted DNA from kākāpō nest soil samples in nanograms per microlitre (ng/µL). Neg_Control = Negative controls (highlighted in orange).

SampleID	Display Name	Island	Aspergillosis Status	DNA Concentration (ng/µL)
Batch1_ITS_1	B1_Jemma_Cavity	Pukenui	Affected	>50
Batch1_ITS_2	B1_Jemma_Entrance	Pukenui	Affected	6.750725
Batch1_ITS_3	B1_Jemma_2m	Pukenui	Affected	9.483449
Batch1_ITS_4	B1_Ra_Cavity	Pukenui	Not_affected	>50
Batch1_ITS_5	B1_Ra_Entrance	Pukenui	Not_affected	10.20044
Batch1_ITS_6	B1_Ra_2m	Pukenui	Not_affected	4.704841
Batch1_ITS_7	B1_Titapu_Cavity	Whenua_Hou	Not_affected	6.024401
Batch1_ITS_8	B1_Titapu_Entrance	Whenua_Hou	Not_affected	14.92951
Batch1_ITS_9	B1_Titapu_2m	Whenua_Hou	Not_affected	0.147978
Batch1_ITS_10	B1_Roha_Cavity	Pukenui	Affected	5.190924
Batch1_ITS_11	B1_Roha_Entrance	Pukenui	Affected	2.173981
Batch1_ITS_12	B1_Roha_2m	Pukenui	Affected	8.152613
Batch1_ITS_13	B1_Pearl_Cavity	Whenua_Hou	Not_affected	1.390917
Batch1_ITS_14	B1_Pearl_Entrance	Whenua_Hou	Not_affected	5.521948
Batch1_ITS_15	B1_Pearl_2m	Whenua_Hou	Not_affected	6.411797
Batch1_ITS_16	B1_Bella_Cavity	Whenua_Hou	Not_affected	8.30038
Batch1_ITS_17	B1_Bella_Entrance	Whenua_Hou	Not_affected	5.876892
Batch1_ITS_18	B1_Bella_2m	Whenua_Hou	Not_affected	>50
Batch1_ITS_19	B1_Gertrude_Cavity	Pukenui	Not_affected	2.69356
Batch1_ITS_20	B1_Gertrude_Entrance	Pukenui	Not_affected	12.2149
Batch1_ITS_21	B1_Gertrude_2m	Pukenui	Not_affected	8.353261
Batch1_ITS_22	B1_Rakiura_Cavity	Whenua_Hou	Not_affected	5.99264
Batch1_ITS_23	B1_Rakiura_Entrance	Whenua_Hou	Not_affected	12.02217
Batch1_ITS_24	B1_Rakiura_2m	Whenua_Hou	Not_affected	8.513873
Batch1_ITS_25	B1_Stella_Cavity	Pukenui	Not_affected	12.64703
Batch1_ITS_26	B1_Stella_Entrance	Pukenui	Not_affected	3.788569
Batch1_ITS_27	B1_Stella_2m	Pukenui	Not_affected	10.56984
Batch1_ITS_28	B1_Marama_Cavity	Pukenui	Not_affected	>50
Batch1_ITS_29	B1_Marama_Entrance	Pukenui	Not_affected	3.664562
Batch1_ITS_30	B1_Marama_2m	Pukenui	Not_affected	>50
Batch1_ITS_31	B1_Alice_Cavity	Whenua_Hou	Not_affected	>50
Batch1_ITS_32	B1_Alice_Entrance	Whenua_Hou	Not_affected	26.49151
Batch1_ITS_33	B1_Alice_2m	Whenua_Hou	Not_affected	27.4005
Batch1_ITS_34	B1_Marian_Cavity	Pukenui	Not_affected	>50
Batch1_ITS_35	B1_Marian_Entrance	Pukenui	Not_affected	26.87232
Batch1_ITS_36	B1_Marian_2m	Pukenui	Not_affected	6.775114
Batch1_ITS_37	B1_Tiwhiri_Cavity	Pukenui	Not_affected	10.71552
Batch1_ITS_38	B1_Tiwhiri_Entrance	Pukenui	Not_affected	11.05771
Batch1_ITS_39	B1_Tiwhiri_2m	Pukenui	Not_affected	11.0665

Batch1_ITS_40	B1_Waikawa _Cavity	Pukenui	Not_affected	>50
Batch1_ITS_41	B1_Waikawa _Entrance	Pukenui	Not_affected	>50
Batch1_ITS_42	B1_Waikawa _2m	Pukenui	Not_affected	10.41044
Batch1_ITS_43	B1_Tumeke_Cavity	Whenua_Hou	Not_affected	>50
Batch1_ITS_44	B1_Tumeke_Entrance	Whenua_Hou	Not_affected	12.15507
Batch1_ITS_45	B1_Tumeke_2m	Whenua_Hou	Not_affected	2.182693
Batch1_ITS_46	B1_Wehe-po_Cavity	Whenua_Hou	Not_affected	8.266829
Batch1_ITS_47	B1_Wehe-po_Entrance	Whenua_Hou	Not_affected	>50
Batch1_ITS_48	B1_Wehe-po_2m	Whenua_Hou	Not_affected	>50
Batch1_ITS_49	B1_Neg_Control_	Lab	Not_affected	1.801949
Batch1_ITS_50	B1_Neg_Control	Lab	Not_affected	0.101739
Batch2_ITS_1	B2_JEM_Cavity	Pukenui	Not_affected	25.28931
Batch2_ITS_2	B2_JEM_Entrance	Pukenui	Not_affected	12.36758
Batch2_ITS_3	B2_JEM_2m	Pukenui	Not_affected	13.99153
Batch2_ITS_4	B2_Cyndy_Cavity	Whenua_Hou	Not_affected	32.2633
Batch2_ITS_5	B2_Cyndy_Entrance	Whenua_Hou	Not_affected	11.83077
Batch2_ITS_6	B2_Cyndy_2m	Whenua_Hou	Not_affected	8.741541
Batch2_ITS_7	B2_Titapu_Cavity	Whenua_Hou	Not_affected	0.00615
Batch2_ITS_8	B2_Titapu_Entrance	Whenua_Hou	Not_affected	0.238492
Batch2_ITS_9	B2_Titapu_2m	Whenua_Hou	Not_affected	0.51718
Batch2_ITS_10	B2_Margaret-Maree_Cavity	Whenua_Hou	Not_affected	4.480035
Batch2_ITS_11	B2_Margaret-Maree_Entrance	Whenua_Hou	Not_affected	1.622102
Batch2_ITS_12	B2_Margaret-Maree_2m	Whenua_Hou	Not_affected	7.268675
Batch2_ITS_13	B2_Pearl_Cavity	Whenua_Hou	Not_affected	2.598126
Batch2_ITS_14	B2_Pearl_Entrance	Whenua_Hou	Not_affected	5.742433
Batch2_ITS_15	B2_Pearl_2m	Whenua_Hou	Not_affected	14.90902
Batch2_ITS_16	B2_Bella_Cavity	Whenua_Hou	Not_affected	6.015846
Batch2_ITS_17	B2_Bella_Entrance	Whenua_Hou	Not_affected	19.29867
Batch2_ITS_18	B2_Bella_2m	Whenua_Hou	Not_affected	26.96063
Batch2_ITS_19	B2_Gertrude_Cavity	Pukenui	Not_affected	>50
Batch2_ITS_20	B2_Gertrude_Entrance	Pukenui	Not_affected	14.76764
Batch2_ITS_21	B2_Gertrude_2m	Pukenui	Not_affected	>50
Batch2_ITS_22	B2_Rakiura_Cavity	Whenua_Hou	Not_affected	6.522664
Batch2_ITS_23	B2_Rakiura_Entrance	Whenua_Hou	Not_affected	9.349689
Batch2_ITS_24	B2_Rakiura_2m	Whenua_Hou	Not_affected	>50
Batch2_ITS_25	B2_Stella_Cavity	Pukenui	Not_affected	>50
Batch2_ITS_26	B2_Stella_Entrance	Pukenui	Not_affected	>50
Batch2_ITS_27	B2_Stella_2m	Pukenui	Not_affected	36.9481
Batch2_ITS_28	B2_Marama_Cavity	Pukenui	Not_affected	>50
Batch2_ITS_29	B2_Marama_Entrance	Pukenui	Not_affected	>50
Batch2_ITS_30	B2_Marama_2m	Pukenui	Not_affected	>50
Batch2_ITS_31	B2_Alice_Cavity	Whenua_Hou	Not_affected	15.20205
Batch2_ITS_32	B2_Alice_Entrance	Whenua_Hou	Not_affected	23.00563
Batch2_ITS_33	B2_Alice_2m	Whenua_Hou	Not_affected	20.05818
Batch2_ITS_34	B2_Marian_Cavity	Pukenui	Not_affected	30.60787
Batch2_ITS_35	B2_Marian_Entrance	Pukenui	Not_affected	20.26612

Batch2_ITS_36	B2_Marian_2m	Pukenui	Not_affected	11.7368
Batch2_ITS_40	B2_Waikawa_Cavity	Pukenui	Not_affected	0.015735
Batch2_ITS_41	B2_Waikawa_Entrance	Pukenui	Not_affected	19.24609
Batch2_ITS_42	B2_Waikawa_2m	Pukenui	Not_affected	7.568718
Batch2_ITS_43	B2_Tumeke_Cavity	Whenua_Hou	Not_affected	11.0913
Batch2_ITS_44	B2_Tumeke_Entrance	Whenua_Hou	Not_affected	4.396403
Batch2_ITS_45	B2_Tumeke_2m	Whenua_Hou	Not_affected	2.833012
Batch2_ITS_46	B2_Wehe-po_Cavity	Whenua_Hou	Not_affected	18.68695
Batch2_ITS_47	B2_Wehe-po_Entrance	Whenua_Hou	Not_affected	>50
Batch2_ITS_48	B2_Wehe-po_2m	Whenua_Hou	Not_affected	>50
Batch2_ITS_49	B2_Neg_Control	Lab	Not_affected	0.074246
Batch2_ITS_50	B2_Neg_Control	Lab	Not_affected	0.063203

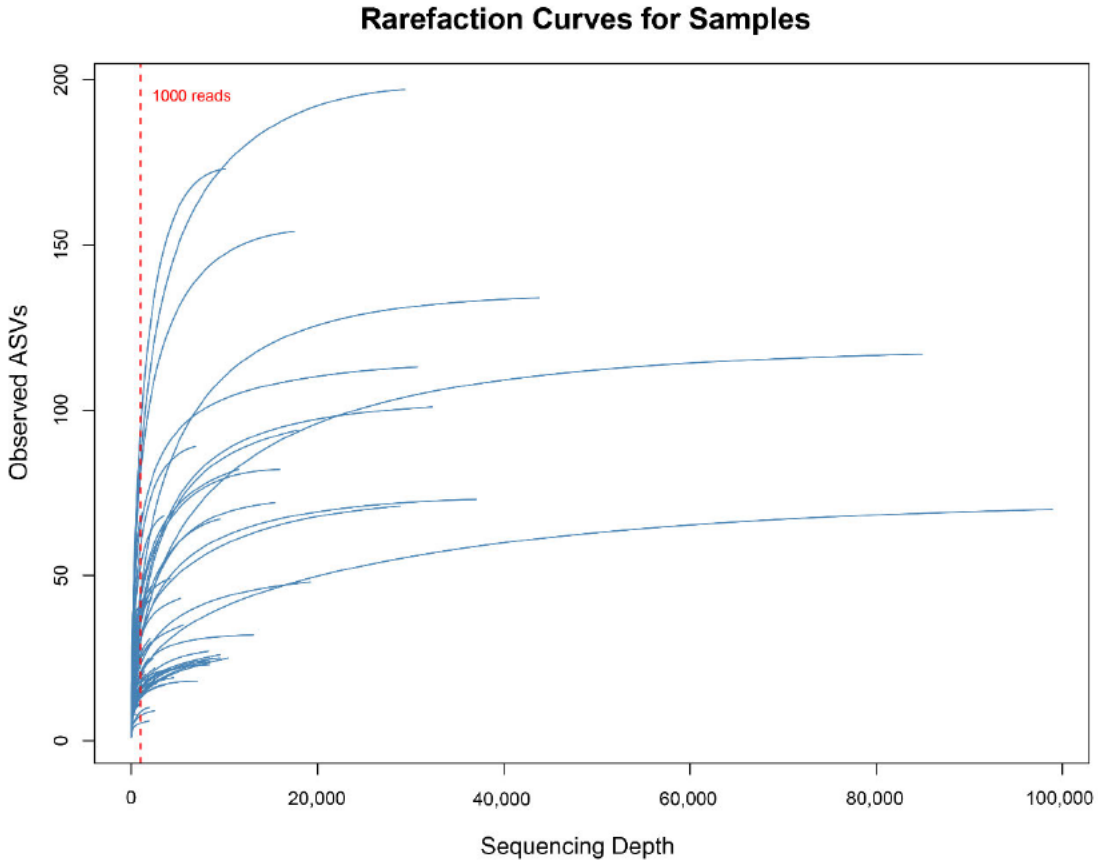

Figure S1 Rarefaction curves for each kākāpō soil sample, rarefied to 1000 reads to retain samples.

Table S2 Sequencing completeness statistics for each kākāpō nest-soil sample. "Coverage (observed)" ($1 - f_1/N$) represents the proportion of reads belonging to previously observed ASVs, and "Coverage (1000)" denotes coverage after rarefaction to 1, 000 reads. "Extra reads to 95 %/99 %" indicate the number of additional reads required to achieve those target completeness levels. Good's coverage ($1 - f_1/N$) estimates the proportion of reads belonging to ASVs already detected within the sample (i.e., not singletons), providing a measure of sampling completeness.

SampleID	Nest Name	Nest Microhabitat	Coverage (Observed)	Coverage (1000)	Extra reads to 95%	Extra reads to 99%
Batch2_ITS_31	Alice	Cavity	0.9991	0.9904	0	0
Batch1_ITS_32	Alice	Entrance	0.998	0.9946	0	0
Batch2_ITS_32	Alice	Entrance	0.9979		0	0
Batch1_ITS_33	Alice	Two_Metres_Away	0.9941		0	0
Batch2_ITS_16	Bella	Cavity	1	0.9963	0	0
Batch1_ITS_16	Bella	Cavity	0.9991	0.9956	0	0
Batch1_ITS_18	Bella	Two_Metres_Away	0.999	0.9886	0	0
Batch2_ITS_4	Cyndy	Cavity	0.9995	0.999	0	0
Batch2_ITS_5	Cyndy	Entrance	0.9977	0.9956	0	0
Batch2_ITS_6	Cyndy	Two_Metres_Away	0.9948	0.9932	0	0
Batch2_ITS_19	Gertrude	Cavity	0.9996	0.9958	0	0
Batch2_ITS_20	Gertrude	Entrance	0.996	0.996	0	0
Batch2_ITS_21	Gertrude	Two_Metres_Away	0.9962		0	0
Batch1_ITS_19	Gertrude	Cavity	0.9998	0.9887	0	0
Batch1_ITS_20	Gertrude	Entrance	0.9999	0.9772	0	0
Batch1_ITS_21	Gertrude	Two_Metres_Away	0.9978	0.9898	0	0
Batch2_ITS_1	JEM	Cavity	1	0.9967	0	0
Batch1_ITS_1	Jemma	Cavity	0.9999	0.9934	0	0
Batch1_ITS_2	Jemma	Entrance	0.9997	0.9956	0	0
Batch1_ITS_3	Jemma	Two_Metres_Away	0.9999	0.9841	0	0
Batch2_ITS_28	Marama	Cavity	0.9995	0.9973	0	0
Batch2_ITS_29	Marama	Entrance	1	1	0	0
Batch1_ITS_28	Marama	Cavity	0.9996	0.998	0	0
Batch1_ITS_30	Marama	Two_Metres_Away	0.9962	0.9938	0	0
Batch2_ITS_10	M-Maree	Cavity	1		0	0
Batch2_ITS_11	M-Maree	Entrance	0.9993	0.98	0	0
Batch2_ITS_34	Marian	Cavity	0.9999	0.9954	0	0
Batch2_ITS_35	Marian	Entrance	0.9998	0.9823	0	0
Batch2_ITS_36	Marian	Two_Metres_Away	0.9969		0	0
Batch1_ITS_34	Marian	Cavity	0.9982	0.9967	0	0
Batch1_ITS_35	Marian	Entrance	0.9993	0.997	0	0
Batch1_ITS_36	Marian	Two_Metres_Away	0.9994	0.9864	0	0
Batch2_ITS_13	Pearl	Cavity	0.9995	0.9954	0	0
Batch2_ITS_14	Pearl	Entrance	0.9966	0.9954	0	0
Batch1_ITS_13	Pearl	Cavity	0.9982	0.99	0	0
Batch1_ITS_14	Pearl	Entrance	0.9989	0.9935	0	0
Batch1_ITS_15	Pearl	Two_Metres_Away	0.9997	0.9926	0	0
Batch1_ITS_22	Rakiura	Cavity	0.9992	0.9962	0	0

Batch2_ITS_23	Rakiura	Entrance	0.9979	0.9952	0	0
Batch1_ITS_23	Rakiura	Entrance	0.9999	0.9858	0	0
Batch1_ITS_24	Rakiura	Two_Metres_Away	0.9998	0.9649	0	0
Batch1_ITS_4	Ra	Cavity	0.9994	0.9964	0	0
Batch1_ITS_6	Ra	Two_Metres_Away	0.9999	0.9876	0	0
Batch1_ITS_10	Roha	Cavity	0.9996	0.994	0	0
Batch1_ITS_11	Roha	Entrance	0.9956	0.9924	0	0
Batch1_ITS_12	Roha	Two_Metres_Away	0.9997	0.9578	0	0
Batch2_ITS_25	Stella	Cavity	0.9996	0.9962	0	0
Batch1_ITS_25	Stella	Cavity	0.9958		0	0
Batch2_ITS_26	Stella	Entrance	0.9965	0.9927	0	0
Batch1_ITS_26	Stella	Entrance	0.9953	0.9947	0	0
Batch1_ITS_27	Stella	Two_Metres_Away	0.9914	0.988	0	0
Batch2_ITS_7	Titapu	Cavity	0.9996	0.9978	0	0
Batch2_ITS_9	Titapu	Two_Metres_Away	0.9964	0.9931	0	0
Batch1_ITS_7	Titapu	Cavity	0.9997	0.9835	0	0
Batch1_ITS_8	Titapu	Entrance	0.9991	0.9831	0	0
Batch1_ITS_9	Titapu	Two_Metres_Away	0.9986	0.9851	0	0
Batch1_ITS_37	Tiwhiri	Cavity	0.9908		0	0
Batch1_ITS_39	Tiwhiri	Two_Metres_Away	0.9995	0.9955	0	0
Batch2_ITS_43	Tumeke	Cavity	0.9993	0.9811	0	0
Batch1_ITS_43	Tumeke	Cavity	0.9992	0.9979	0	0
Batch2_ITS_40	Waikawa	Cavity	0.9996	0.9973	0	0
Batch2_ITS_41	Waikawa	Entrance	0.9999	0.9935	0	0
Batch2_ITS_42	Waikawa	Two_Metres_Away	0.9965	0.9953	0	0
Batch1_ITS_40	Waikawa	Cavity	0.9974	0.9971	0	0
Batch1_ITS_42	Waikawa	Two_Metres_Away	0.9972	0.9925	0	0
Batch2_ITS_46	Wehe-po	Cavity	0.9999	0.9826	0	0
Batch1_ITS_46	Wehe-po	Cavity	0.9983	0.9973	0	0
Batch1_ITS_48	Wehe-po	Two_Metres_Away	0.9997	0.97	0	0

Table S3 Six ASVs assigned to the *Aspergillus* genus, with their associated kākāpō nest microhabitat.

Nest Name	**Nest Microhabitat**	**Batch**	***Aspergillus* species**	**RA**
Jemma	Entrance	1	*Aspergillus* spp	0.03%
Tiwhiri	Two Metres Away	1	*Aspergillus* spp	1.19%
Tiwhiri	Two Metres Away	1	*Aspergillus* spp	0.89%
Cyndy	Two Metres Away	2	*Aspergillus* spp	0.17%
Wehe-Po	Cavity	2	*Aspergillus* spp	0.10%
Wehe-Po	Cavity	2	*Aspergillus* spp	0.04%

Table S4 Fifteen most abundant (relative abundance) fungal genera and their respective phyla associations, from kākāpō nest microhabitats in 2019 and 2022 (between two breeding islands: Pukenui and Whenua Hou). Values are presented as mean relative abundances and standard deviations (% RA ± SD). "2m Away" = Two metres away from kākāpō nests.

Fungal Taxa	Cavity		Entrance		2m Away	
	Pukenui	Whenua Hou	Pukenui	Whenua Hou	Pukenui	Whenua Hou
Basidiomycota	**74.50 ± 13.20**	**76.84 ± 11.29**	**74.49 ± 11.86**	**73.41 ± 14.97**	**46.52 ± 32.30**	**54.15 ± 33.15**
Apiotrichum	74.55 ± 19.00	78.16 ± 12.53	75.43 ± 9.61	72.46 ± 14.66	43.06 ± 34.04	44.70 ± 39.73
Tausonia	1.06 ± 4.09	0.07 ± 0.13	0.00 ± 0.01	0.12 ± 0.26	0.85 ± 2.29	8.30 ± 10.66
Ascomycota	**23.81 ± 11.00**	**21.04 ± 12.30**	**22.35 ± 13.13**	**14.87 ± 12.67**	**24.27 ± 12.66**	**28.78 ± 30.47**
Arthrographis	9.98 ± 11.02	1.49 ± 2.34	5.21 ± 7.17	0.07 ± 0.21	0.01 ± 0.02	0.00 ± 0.00
Debaryomyces	2.53 ± 1.63	7.83 ± 6.84	2.52 ± 1.78	1.73 ± 1.53	0.41 ± 0.57	0.18 ± 0.33
Penicillium	1.06 ± 1.99	3.28 ± 3.72	2.34 ± 2.98	0.35 ± 0.47	0.23 ± 0.47	1.60 ± 2.07
Sporothrix	2.88 ± 2.16	1.38 ± 2.03	4.82 ± 9.67	1.37 ± 1.01	0.11 ± 0.27	0.06 ± 0.16
Ilyonectria	0.06 ± 0.10	1.28 ± 2.90	0.19 ± 0.39	7.86 ± 12.71	2.25 ± 6.20	1.49 ± 1.58
Trichoderma	0.63 ± 2.30	0.04 ± 0.06	2.14 ± 2.42	0.68 ± 0.82	12.34 ± 11.04	4.57 ± 7.00
Candida	0.00 ± 0.01	0.09 ± 0.20	0.00 ± 0.00	0.11 ± 0.23	0.40 ± 0.93	13.24 ± 24.55
Talaromyces	0.25 ± 0.66	1.00 ± 2.48	0.35 ± 1.02	0.00 ± 0.01	0.05 ± 0.19	0.00 ± 0.00
Mortierellomycota	**1.66 ± 4.85**	**2.06 ± 2.80**	**3.08 ± 3.18**	**11.49 ± 11.72**	**26.88 ± 19.37**	**16.55 ± 17.83**
Mortierella	1.05 ± 3.83	0.26 ± 0.39	0.48 ± 0.38	4.51 ± 8.31	2.99 ± 1.94	2.66 ± 2.74
Entomortierella	0.09 ± 0.24	0.10 ± 0.26	0.49 ± 0.90	0.87 ± 2.05	0.08 ± 0.18	0.75 ± 0.91
Dissophora	0.41 ± 0.98	0.95 ± 2.37	0.09 ± 0.13	5.85 ± 8.18	5.28 ± 11.19	1.43 ± 3.71
Podila	0.03 ± 0.05	0.67 ± 1.63	1.70 ± 2.30	0.32 ± 0.64	15.64 ± 20.85	11.48 ± 16.25
Linnemannia	0.10 ± 0.25	0.14 ± 0.40	0.37 ± 0.43	0.13 ± 0.26	5.08 ± 15.72	0.87 ± 1.58

Table S5 Most prevalent ASVs with species and family identification across all samples.

ASV ID	Fungal Taxa	Prevalence
	Trichosporonacae	
ASV1	*Apiotrichum porosum*	100.00%
	Saccharomycetaceae	
ASV2	*Debaryomyces hansenii*	86.76%
ASV3	*Kazachstania telluris*	60.29%

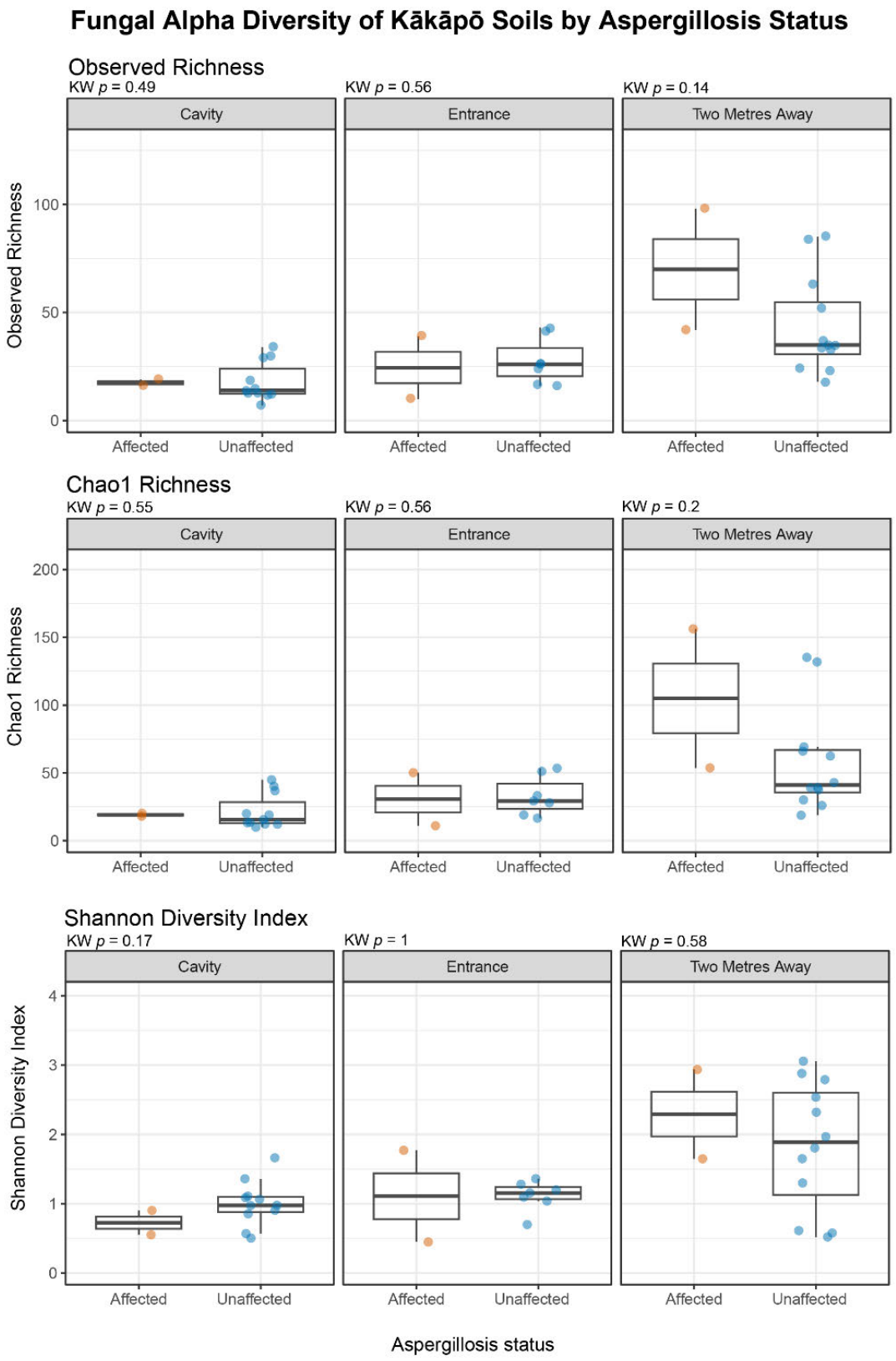

Figure S2 Fungal alpha diversity in kākāpō nest soil samples (February and March) between nests that harboured individuals with confirmed aspergillosis (affected) and nests that did not (unaffected), across nest microhabitats (cavity, entrance and two metres away from the nest). Alpha diversity metrics were measured using observed richness, Chao1 estimated richness, and the Shannon Diversity Index. Kruskal–Wallis test *p*-values (KW) demonstrate no statistical differences in alpha diversity between affected and unaffected nests, across nest microhabitats.

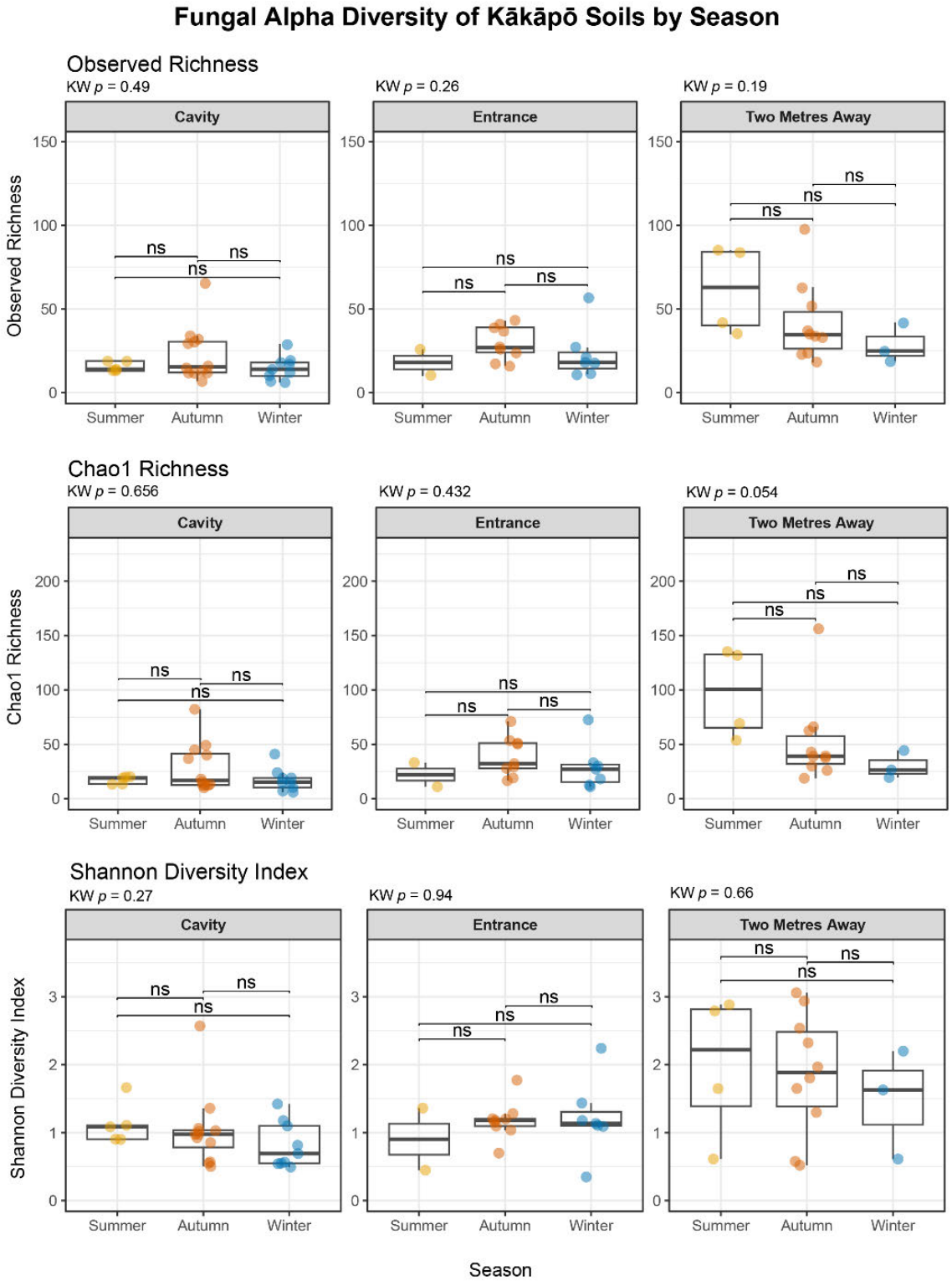

Figure S3 Fungal alpha diversity in kākāpō nest soil samples between Summer, Autumn and Winter, measured using observed richness, Chao1 estimated richness, and the Shannon Diversity Index. Kruskal–Wallis (KW) test *p*-values demonstrate statistical differences in alpha diversity between seasons. Post-hoc pairwise comparisons were performed using Dunn's test with Benjamini–Hochberg false-discovery-rate correction. NS = No significance.

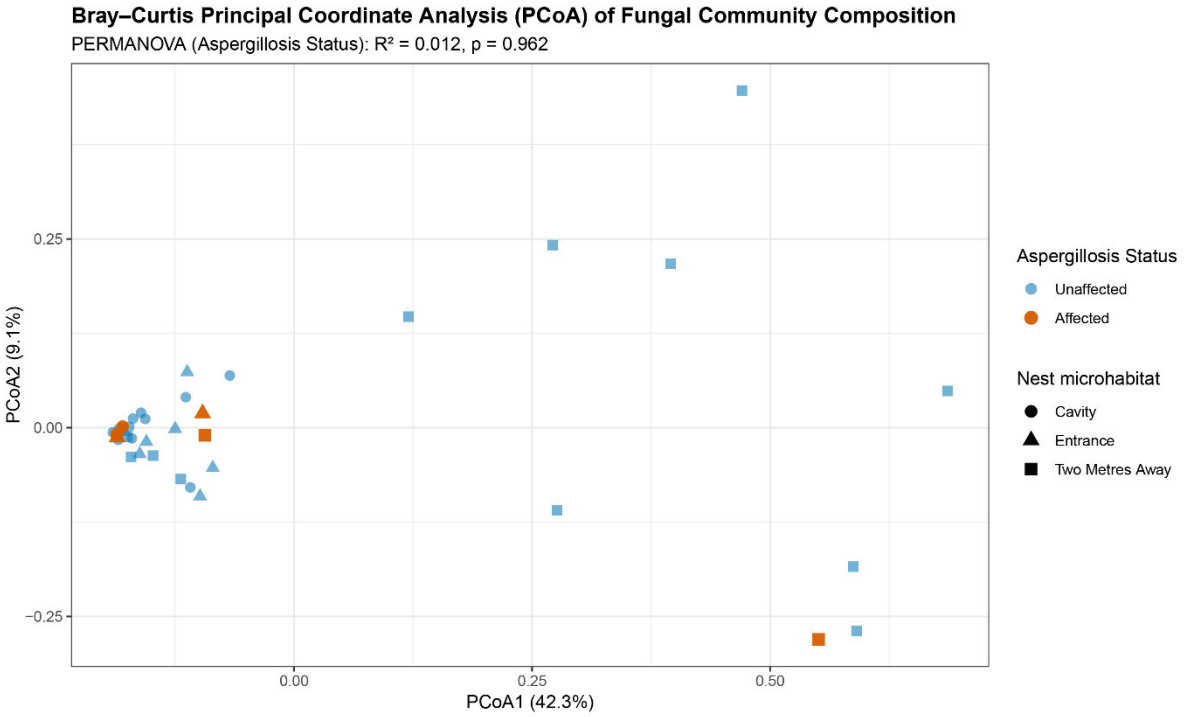

Figure S4 Principal coordinate analysis (PCoA) of rarefied ITS1 sequences from kākāpō soil samples using Bray-Curtis dissimilarity. Each dot represents the fungal community composition of each soil sample, coloured by aspergillosis status, and shaped by nest microhabitat. Samples were rarefied to 1,000 reads (Appendix B, Figure S1).

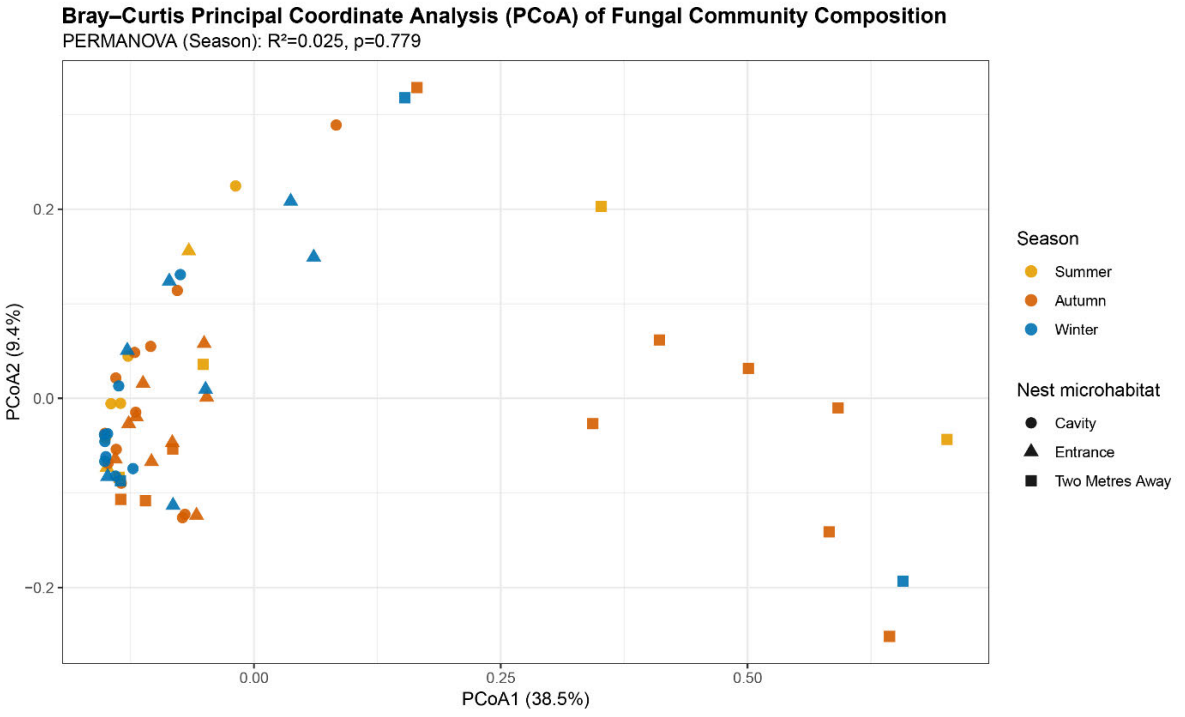

Figure S5 Principal coordinate analysis (PCoA) of rarefied ITS1 sequences from kākāpō soil samples using Bray-Curtis dissimilarity. Each dot represents the fungal community composition of each soil sample, coloured by season and shaped by nest microhabitat. Samples were rarefied to 1,000 reads (Appendix B, Figure S1).

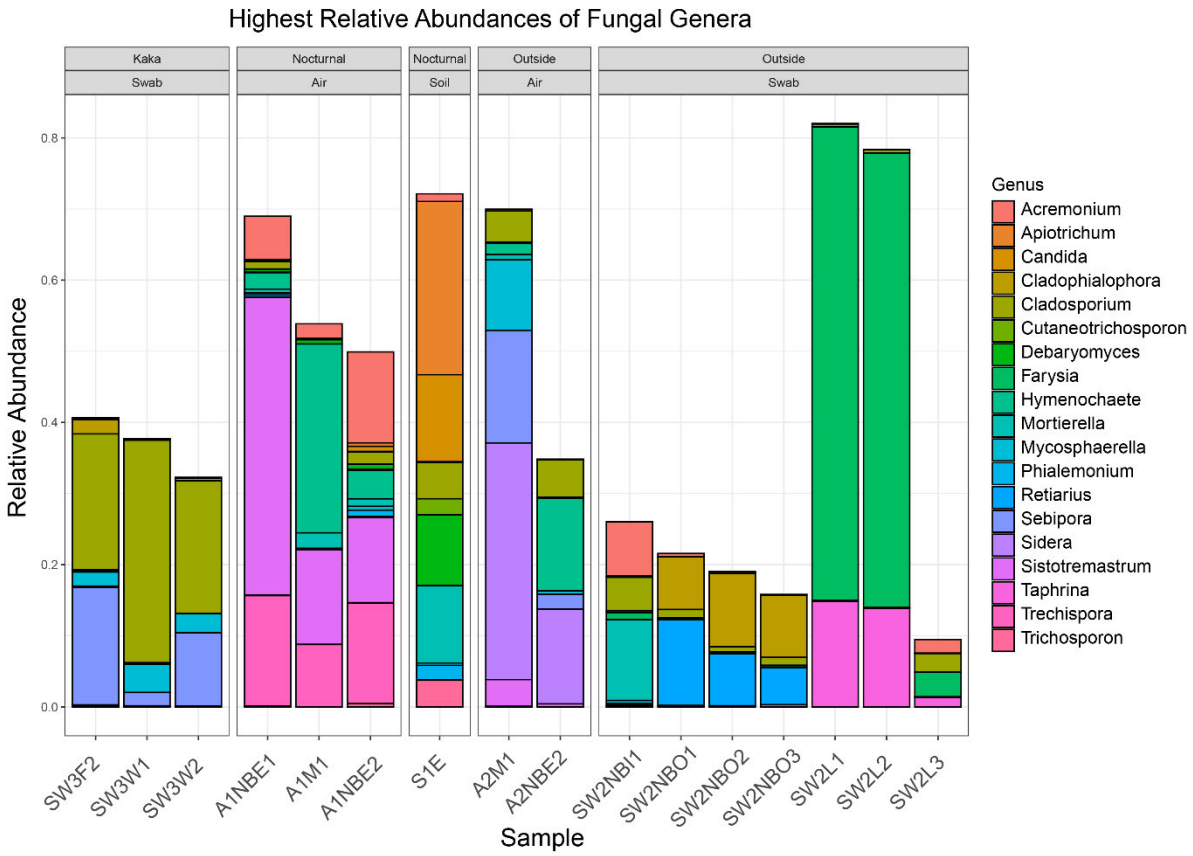

Figure S6 Highest relative abundances of fungal genera (ITS1) from nocturnal and captive avian enclosures at Auckland Zoo, New Zealand. Nocturnal soil sample "S1E" was taken at the entrance of a brown kiwi (*Apteryx mantelli*) nocturnal enclosure. Reproduced from Truter-Meyer, A. (2021). Isolation, identification, and estimation of *Aspergillus* spp. from an indoor brown kiwi (*Apteryx mantelli*) nocturnal house and outdoor avian enclosures at Auckland Zoo [Unpublished honours dissertation, Faculty of Health & Environmental Science, Auckland University of Technology]. © Amber Truter-Meyer. Reproduced with permission.

Appendix C – Chapter 4 Supplementary Material

Table S1 Fifteen kākāpō nests sampled and their associated island.

Breeding kākāpō female	Island
JEM	Pukenui
Cyndy	Whenua Hou
Titapu	Whenua Hou
Margeret-Maree	Whenua Hou
Pearl	Whenua Hou
Bella	Whenua Hou
Gertrude	Pukenui
Rakiura	Whenua Hou
Stella	Pukenui
Marama	Pukenui
Alice	Whenua Hou
Marian	Pukenui
Waikawa	Pukenui
Tumeke	Whenua Hou
Weheruatanga-o-te-pō	Whenua Hou

Table S2 Mean radial growth (diameter in mm ± SD) of *Aspergillus* isolates grown alone (control) and in co-culture with yeast species.

Yeast Species / Treatment	**23423**		**24469**		**JEM**		**23509**	
	Mycelia (mm ± SD, n)	Sporulation (mm ± SD, n)	Mycelia (mm ± SD, n)	Sporulation (mm ± SD, n)	Mycelia (mm ± SD, n)	Mycelia (mm ± SD, n)	Radial (mm ± SD, n)	Mycelia (mm ± SD, n)
Control	63.5 ± 4.77 (3)	53.3 ± 1.15 (3)	66.3 ± 0.58 (3)	50.3 ± 2.08 (3)	67.7 ± 1.53 (3)	51.3 ± 1.15 (3)	45.0 ± 1.73 (3)	35.3 ± 0.58 (3)
S. ligniputridi	63.3 ± 1.53 (3)	52.3 ± 3.21 (3)	61.3 ± 1.53 (3)	48.7 ± 0.58 (3)	61.3 ± 1.15 (3)	54.0 ± 1.73 (3)	35.0 ± 4.36 (3)	34.7 ± 3.79 (3)
D. hansenii	49.1 ± 10.35 (12)	35.0 ± 11.63 (12)	49.1 ± 9.81 (12)	32.7 ± 10.71 (12)	51.4 ± 8.90 (12)	37.0 ± 8.68 (12)	38.3 ± 6.24 (12)	28.5 ± 6.30 (12)
C. palmioleophila	40.7 ± 1.15 (3)	22.7 ± 0.58 (3)	29.7 ± 5.77 (3)	17.0 ± 6.08 (3)	41.7 ± 0.58 (3)	23.7 ± 5.51 (3)	32.7 ± 1.53 (3)	23.7 ± 0.58 (3)
***Debaryomyces* spp.**	38.3 ± 2.89 (3)	22.3 ± 0.58 (3)	38.2 ± 6.79 (6)	26.2 ± 6.11 (6)	42.3 ± 10.60 (6)	27.8 ± 7.60 (6)	34.0 ± 1.00 (3)	21.0 ± 1.00 (3)
***Clavispora* spp.**	35.6 ± 4.39 (9)	10.6 ± 2.92 (9)	35.2 ± 3.07 (9)	10.0 ± 2.83 (9)	34.0 ± 2.96 (9)	8.2 ± 1.79 (9)	29.2 ± 2.49 (9)	18.8 ± 0.67 (9)
M. guilliermondii	7.7 ± 2.89 (3)	7.7 ± 2.89 (3)	9.0 ± 2.65 (3)	7.7 ± 2.08 (3)	8.3 ± 3.21 (3)	7.7 ± 3.79 (3)	9.0 ± 1.73 (3)	8.3 ± 2.31 (3)

Mycelia = diameter of entire colony including mycelia, Sporulation = diameter of sporulating zone. Values are mean ± standard deviation. *n* indicates the number of replicates per yeast treatment. *Aspergillus* species isolates: 23423, 24469 = *Aspergillus fumigatus* Fresen, JEM = *Aspergillus fumigatus* isolated from kākāpō JEM's nest entrance soils, 23509 = *Aspergillus niger* Tiegh

Table S3 Welch's t-test results for radial inhibition assays.

***Aspergillus* spp. isolate**	**Yeast**	**Metric**	**Δ Diameter (mm)**	**95% CI**	***p*-value**	**Signif**
23423	*S. ligniputridi*	myc	0.17	[-10.5, 10.8]	0.958	-
	S. ligniputridi	spor	1.00	[-6, 8]	0.653	-
	D. hansenii	myc	14.42	[5, 23.9]	0.008	**
	D. hansenii	spor	18.33	[10.9, 25.8]	<0.001	***
	C. palmioleophila	myc	22.83	[11.8, 33.9]	0.011	*
	C. palmioleophila	spor	30.67	[28.3, 33.1]	<0.001	***
	Debaryomyces spp.	myc	25.17	[15.4, 34.9]	0.003	**
	Debaryomyces spp.	spor	31.00	[28.6, 33.4]	<0.001	***
	Clavispora spp.	myc	27.94	[18.4, 37.5]	0.002	**
	Clavispora spp.	spor	42.78	[40.1, 45.4]	<0.001	***
	M. guilliermondii	myc	55.83	[46.1, 65.6]	<0.001	***
	M. guilliermondii	spor	45.67	[39.5, 51.9]	<0.001	***
24469	*S. ligniputridi*	myc	5.00	[1.7, 8.3]	0.019	*
	S. ligniputridi	spor	1.67	[-3.1, 6.4]	0.298	-
	D. hansenii	myc	17.25	[11, 23.5]	<0.001	***
	D. hansenii	spor	17.67	[10.5, 24.8]	<0.001	***
	C. palmioleophila	myc	36.67	[22.5, 50.8]	0.008	**
	C. palmioleophila	spor	33.33	[19.9, 46.7]	0.006	**
	Debaryomyces spp.	myc	28.17	[21, 35.3]	<0.001	***
	Debaryomyces spp.	spor	24.17	[17.6, 30.8]	<0.001	***
	Clavispora spp.	myc	31.11	[28.7, 33.5]	<0.001	***
	Clavispora spp.	spor	40.33	[36.3, 44.3]	<0.001	***
	M. guilliermondii	myc	57.33	[51.1, 63.5]	<0.001	***
	M. guilliermondii	spor	42.67	[37.9, 47.4]	<0.001	***
JEM	*S. ligniputridi*	myc	6.33	[3.2, 9.5]	0.006	**
	S. ligniputridi	spor	-2.67	[-6.2, 0.9]	0.101	-
	D. hansenii	myc	16.25	[10.4, 22.1]	<0.001	***
	D. hansenii	spor	14.33	[8.7, 20]	<0.001	***
	C. palmioleophila	myc	26.00	[22.7, 29.3]	<0.001	***
	C. palmioleophila	spor	27.67	[14.7, 40.6]	0.010	*
	Debaryomyces spp.	myc	25.33	[14.2, 36.4]	0.002	**
	Debaryomyces spp.	spor	23.50	[15.5, 31.5]	<0.001	***
	Clavispora spp.	myc	33.67	[30.6, 36.8]	<0.001	***
	Clavispora spp.	spor	43.11	[40.9, 45.3]	<0.001	***
	M. guilliermondii	myc	59.33	[52.6, 66.1]	<0.001	***
	M. guilliermondii	spor	43.67	[35.2, 52.2]	0.001	**
23509	*S. ligniputridi*	myc	10.00	[0.6, 19.4]	0.043	*
	S. ligniputridi	spor	0.67	[-8.5, 9.8]	0.790	-
	D. hansenii	myc	6.67	[2.2, 11.1]	0.007	**
	D. hansenii	spor	6.83	[2.8, 10.9]	0.003	**
	C. palmioleophila	myc	12.33	[8.6, 16.1]	<0.001	***
	C. palmioleophila	spor	11.67	[10.4, 13]	<0.001	***
	Debaryomyces spp.	myc	11.00	[7.5, 14.5]	0.002	**

	Debaryomyces spp.	spor	14.33	[12.3, 16.4]	<0.001	***
	Clavispora spp.	myc	15.78	[12.5, 19.1]	<0.001	***
	Clavispora spp.	spor	16.56	[15.4, 17.7]	<0.001	***
	M. guilliermondii	myc	36.00	[32.1, 39.9]	<0.001	***
	M. guilliermondii	spor	27.00	[21.7, 32.3]	0.001	**

Δ diameter (mm) is the mean reduction in colony diameter, calculated as control − treatment; positive Δ indicates inhibition by the yeast. Each row reports one Welch's t-test comparing monoculture (control) versus co-culture (treatment) for a given yeast × *Aspergillus* spp. isolate combination. Metric: myc = mycelial diameter; spor = sporulation-zone diameter. *Aspergillus* spp. isolates: 23423, 24469 (*A. fumigatus* Fresen); JEM (*A. fumigatus* from kākāpō "JEM" nest soils); 23509 (*A. niger* Tiegh.). Significance (Signif): *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Table S4 Radial inhibition assays as percentage inhibition (Mean ± SE).

***Aspergillus* spp. isolate**	**Yeast**	**Metric**	**Mean ± SE (%)**
23423	*S. ligniputridi*	Mycelial	0.3 ± 1.4
	S. ligniputridi	Sporulation	1.9 ± 3.5
	D. hansenii	Mycelial	22.7 ± 4.7
	D. hansenii	Sporulation	34.4 ± 6.3
	C. palmioleophila	Mycelial	36.0 ± 1.0
	C. palmioleophila	Sporulation	57.5 ± 0.6
	Debaryomyces spp.	Mycelial	39.6 ± 2.6
	Debaryomyces spp.	Sporulation	58.1 ± 0.6
	Clavispora spp.	Mycelial	44.0 ± 2.3
	Clavispora spp.	Sporulation	80.2 ± 1.8
	M. guilliermondii	Mycelial	87.9 ± 2.6
	M. guilliermondii	Sporulation	85.6 ± 3.1
24469	*S. ligniputridi*	Mycelial	7.5 ± 1.3
	S. ligniputridi	Sporulation	3.3 ± 0.7
	D. hansenii	Mycelial	26.0 ± 4.3
	D. hansenii	Sporulation	35.1 ± 6.1
	C. palmioleophila	Mycelial	55.3 ± 5.0
	C. palmioleophila	Sporulation	66.2 ± 7.0
	Debaryomyces spp.	Mycelial	42.5 ± 4.2
	Debaryomyces spp.	Sporulation	48.0 ± 5.0
	Clavispora spp.	Mycelial	46.9 ± 1.5
	Clavispora spp.	Sporulation	80.1 ± 1.9
	M. guilliermondii	Mycelial	86.4 ± 2.3
	M. guilliermondii	Sporulation	84.8 ± 2.4
JEM	*S. ligniputridi*	Mycelial	9.4 ± 1.0
	S. ligniputridi	Sporulation	-5.2 ± 1.9
	D. hansenii	Mycelial	24.0 ± 3.8
	D. hansenii	Sporulation	27.9 ± 4.9
	C. palmioleophila	Mycelial	38.4 ± 0.5
	C. palmioleophila	Sporulation	53.9 ± 6.2
	Debaryomyces spp.	Mycelial	37.4 ± 6.4
	Debaryomyces spp.	Sporulation	45.8 ± 6.0

	Clavispora spp.	Mycelial	49.8 ± 1.5
	Clavispora spp.	Sporulation	84.0 ± 1.2
	M. guilliermondii	Mycelial	87.7 ± 2.7
	M. guilliermondii	Sporulation	85.1 ± 4.3
23509	*S. ligniputridi*	Mycelial	22.2 ± 5.6
	S. ligniputridi	Sporulation	1.9 ± 6.2
	D. hansenii	Mycelial	14.8 ± 4.0
	D. hansenii	Sporulation	19.3 ± 5.1
	C. palmioleophila	Mycelial	27.4 ± 2.0
	C. palmioleophila	Sporulation	33.0 ± 0.9
	Debaryomyces spp.	Mycelial	24.4 ± 1.3
	Debaryomyces spp.	Sporulation	40.6 ± 1.6
	Clavispora spp.	Mycelial	35.1 ± 1.8
	Clavispora spp.	Sporulation	46.9 ± 0.6
	M. guilliermondii	Mycelial	80.0 ± 2.2
	M. guilliermondii	Sporulation	76.4 ± 3.8

Metric: Mycelial = mycelial diameter; Sporulation = sporulation-zone diameter. *Aspergillus* spp. isolates: 23423, 24469 (*A. fumigatus* Fresen); JEM (*A. fumigatus* from kākāpō "JEM" nest soils); 23509 (*A. niger* Tiegh.). Percentage inhibition = [(C − T)/C] × 100, where C is the mean diameter in control (monoculture) and T is the mean diameter in treatment (co-culture).

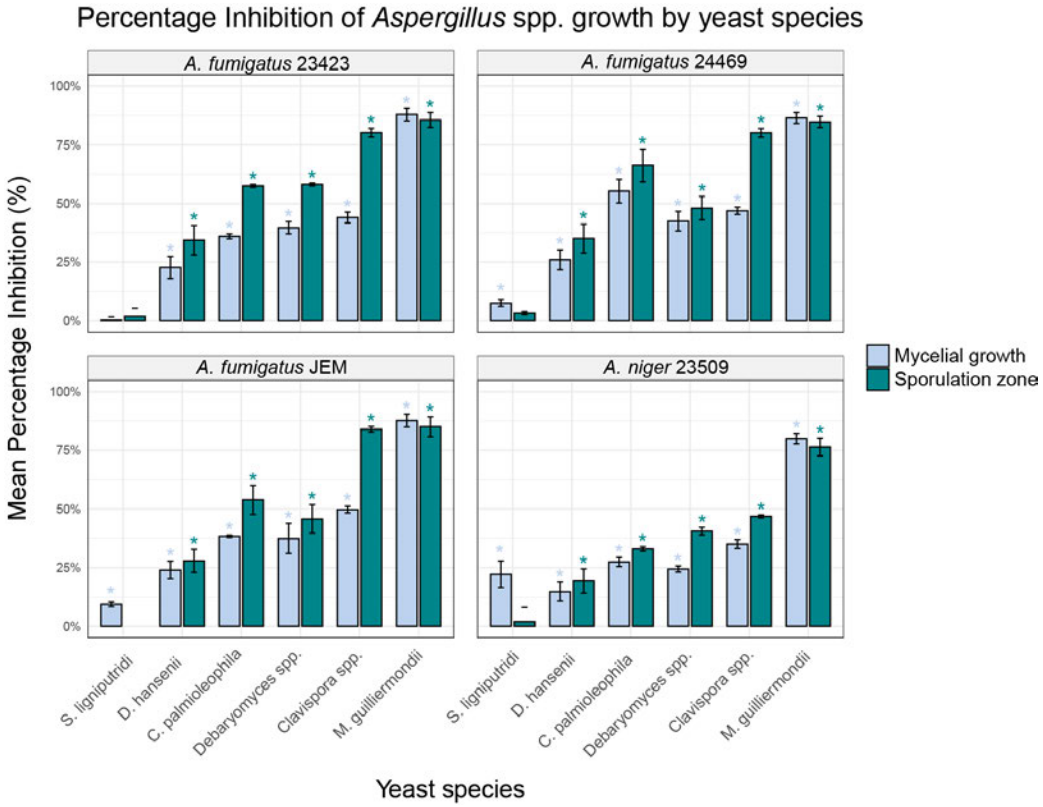

Figure S1 Percentage radial inhibition of *Aspergillus* spp: *A. fumigatus* Fresen (ICMP 23423 & 24469), *A. fumigatus* from kākāpō JEM's nest entrance, and *A. niger* Tiegh. (ICMP 23509) by yeasts: *S. ligniputridi*, *D. hansenii*, *C. palmioleophila*, *Debaryomyces* spp. (1984), *Clavispora* spp. and *M. guilliermondii*. *Aspergillus* spp. controls bars are indicated with dotted borders. Asterisks (*) indicate significant differences ($p < 0.05$) of

treatments from controls. Percentage inhibition = [(C − T)/C] × 100, where C is the mean diameter in control (monoculture) and T is the mean diameter in treatment (co-culture).

Table S5 Radius (mm ± SD) of *Aspergillus* isolates grown alone (control) and in co-culture with yeast species using the diffusible compound method. Values are mean ± standard deviation. *n* indicates the number of replicates per yeast treatment. *Aspergillus* species isolates: 23423, 24469 = *Aspergillus fumigatus* Fresen, JEM = *Aspergillus fumigatus* isolated from kākāpō JEM's nest entrance soils, 23509 = *Aspergillus niger* Tiegh. Results of *C. palmioleophila* with 23423 has been emitted from results due to technical errors.

Yeast Species/ Treatment	**23423 (n)**	**24469 (n)**	**JEM (n)**	**23509 (n)**
Control	68.3 ± 1.86 (6)	71.2 ± 2.71 (6)	69.5 ± 1.76 (6)	54.3 ± 8.36 (6)
D. hansenii	53.1 ± 5.01 (9)	58.7 ± 2.60 (9)	56.3 ± 1.87 (9)	47.7 ± 4.06 (9)
C. palmioleophila	-	58.7 ± 3.51 (3)	57.0 ± 1.00 (3)	45.3 ± 1.15 (3)
***Debaryomyces* spp.**	62.0 ± 1.00 (3)	64.0 ± 2.00 (3)	56.0 ± 1.00 (3)	47.3 ± 4.04 (3)
***Clavispora* spp.**	58.7 ± 2.73 (6)	60.7 ± 1.86 (6)	56.3 ± 2.42 (6)	45.0 ± 4.98 (6)
M. guilliermondii	51.7 ± 7.57 (3)	58.7 ± 1.15 (3)	55.0 ± 2.65 (3)	47.7 ± 2.52 (3)

Table S6 Welch's t-test results for diffusible antifungal compound assays.

Yeast Species/Isolate	***Aspergillus* spp. Isolate**	**Δ radius (mm)**	**Estimate 95% CI**	***t*-value**	***p*-value**
23423	*D. hansenii*	15.22	[-19.27, -11.18]	-8.29	<0.001***
	Debaryomyces spp.	6.33	[-8.60, -4.06]	-6.64	<0.001***
	Clavispora spp.	9.67	[-12.73, -6.60]	-7.16	<0.001***
	M. guilliermondii	16.67	[-34.74, 1.41]	-3.76	0.059
24469	*D. hansenii*	12.5	[-15.61, -9.39]	-8.89	<0.001***
	C. palmioleophila	12.5	[-19.54, -5.46]	-5.41	0.01*
	Debaryomyces spp.	7.17	[-11.17, -3.16]	-4.48	0.005**
	Clavispora spp.	10.5	[-13.55, -7.45]	-7.81	<0.001***
	M. guilliermondii	12.5	[-15.56, -9.44]	-9.67	<0.001***
JEM	*D. hansenii*	13.17	[-15.25, -11.08]	-13.84	<0.001***
	C. palmioleophila	12.5	[-14.70, -10.30]	-13.56	<0.001***
	Debaryomyces spp.	13.5	[-15.70, -11.30]	-14.64	<0.001***
	Clavispora spp.	13.17	[-15.93, -10.41]	-10.77	<0.001***
	M. guilliermondii	14.5	[-19.95, -9.05]	-8.59	0.004**
23509	*D. hansenii*	6.67	[-15.46, 2.12]	-1.82	0.115
	C. palmioleophila	9	[-17.76, -0.24]	-2.59	0.046*
	Debaryomyces spp.	7	[-16.79, 2.79]	-1.69	0.134
	Clavispora spp.	9.33	[-18.46, -0.20]	-2.35	0.046*
	M. guilliermondii	6.67	[-15.59, 2.26]	-1.8	0.119

Δ radius (mm) is the mean reduction in colony diameter, calculated as control − treatment; positive Δ indicates inhibition by the yeast. Each row reports one Welch's t-test comparing monoculture (control) versus co-culture (treatment) for a given yeast × *Aspergillus* spp. isolate combination. Metric: myc = mycelial radius; spor = sporulation-zone radius. *Aspergillus* spp. isolates: 23423, 24469 (*A. fumigatus* Fresen); JEM (*A. fumigatus* from kākāpō "JEM" nest soils); 23509 (*A. niger* Tiegh.) Significance: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

Table S7 Diffusable antifungal compound assays as percentage inhibition (Mean ± SE).

Aspergillus spp. isolate	Yeast	Metric	Mean ± SE (%)
23423	*D. hansenii*	Mycelial	22.3 ± 2.4
	Debaryomyces spp.	Mycelial	9.3 ± 0.8
	Clavispora spp.	Mycelial	14.1 ± 1.6
	M. guilliermondii	Mycelial	24.4 ± 6.4
24469	*D. hansenii*	Mycelial	17.6 ± 1.2
	C. palmioleophila	Mycelial	17.6 ± 2.8
	Debaryomyces spp.	Mycelial	10.1 ± 1.6
	Clavispora spp.	Mycelial	14.8 ± 1.1
	M. guilliermondii	Mycelial	17.6 ± 0.9
JEM	*D. hansenii*	Mycelial	18.9 ± 0.9
	C. palmioleophila	Mycelial	18.0 ± 0.8
	Debaryomyces spp.	Mycelial	19.4 ± 0.8
	Clavispora spp.	Mycelial	18.9 ± 1.4
	M. guilliermondii	Mycelial	20.9 ± 2.2
23509	*D. hansenii*	Mycelial	12.3 ± 2.5
	C. palmioleophila	Mycelial	16.6 ± 1.2
	Debaryomyces spp.	Mycelial	12.9 ± 4.3
	Clavispora spp.	Mycelial	17.2 ± 3.7
	M. guilliermondii	Mycelial	12.3 ± 2.7

Metric: Mycelial = mycelial diameter (whole colony diameter). *Aspergillus* spp. isolates: 23423, 24469 (*A. fumigatus* Fresen); JEM (*A. fumigatus* from kākāpō "JEM" nest soils); 23509 (*A. niger* Tiegh.). Percentage inhibition = $[(C - T)/C] \times 100$, where C is the mean diameter in control (monoculture) and T is the mean diameter in treatment (co-culture).

Table S8 Nine kākāpō deaths by aspergillosis in 2019, *Aspergillus* spp. cultured from individuals post-mortem and potential signs of aflatoxin exposure. Juv = juvenile.

Age Class	Name	Sex	Hatch date	Death date	Hosp. time	Age	Aspergillus species	Aflatoxin?
Juv	Pura-3-A-19	M	16/02/2019	7/04/2019	0	50	None	Yes
Juv	Bella-1-A-19	M	22/02/2019	18/04/2019	2	55	*A. fumigatus*	Less likely
Adult	Hoki	F	6/04/1992	21/04/2019	5	9876	*A. fumigatus*	Less likely
Juv	Bella-2-A-19	F	25/02/2019	7/05/2019	0	71	*A. fumigatus*	No
Juv	Tumeke-4-A	M	27/02/2019	8/05/2019	0	70	*A. fumigatus*	No
Juv	Queenie-4-A	F	6/03/2019	11/05/2019	5	66	*A. fumigatus* & *A. flavus*	No
Adult	Huhana	F	16/03/2009	23/05/2019	7	3720	*A. fumigatus*	Yes
Juv	Nora-1-A	F	10/02/2019	11/06/2019	27	121	*A. fumigatus*	No
Juv	Margaret-Maree-2-B	M	28/03/2019	11/09/2019	26/0	167	*A. fumigatus* & *A. flavus*	No
Juv	Kuihi-3-B-19	F	9/04/2019	19/09/2019	80/0	163	*A. fumigatus*	No

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