**Running title**: Proteolytic Activity and Health Impact of feathers mycobiont

**Proteolytic Activity and Health Impact of Fungi Isolated from Chickens Feathers in House Breeding Cages**

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**Abstract**

The vast majority of fungi are saprophytic, they have the ability to utilize dead plant and animal tissues. Keratinophilic fungi are a specific group that decomposed keratinized structures of humans and animals viz skin, hair, feathers etc., and they may cause mycoses as opportunistic or true pathogens. The house breeding cages of chickens are a source of saprophytic fungi including keratinophilic group. The present study aims to isolate fungi from feathers in house breeding cages, explain the composition of the fungal community, examine the proteolytic activity of fungal taxa via skimmed milk agar test, as well as clarify their health risk on breeders. A total of fifty feather samples were collected from urban sites in Erbil city. Samples were cultured on Sabouraud’s dextrose agar and Typha pollen agar. Petri dishes were incubated at 25oCand the developed fungi were checked after three days and for four weeks.

Eighteen fungi were recorded belonging to 14 genera viz *Aspergillus,* *Chrysosporium sp., Fusarium sp., Chaetomium sp., Emericella sp, Papulospora sp.*, *Cunninghamella sp.,* *Rhodotorula sp.*, *Candida sp., Acremonium sp., Cladosporium sp., Mucor sp., Myceliophthora sp.,* and *Phoma sp.* The *Chrysosporium* *sp.* showed the highest occurrence (60%), and it also had the most proteolytic activity followed by *Myceliophthora* *verrucosa*. All isolates were reported as opportunistic pathogens, they are responsible for several fungal infections viz allergy, dermatomycosis, and deep infections.

**Keywords**: poultry; proteolytic; feathers; keratinase; health impact

**Introduction**

Feathers were described as a carrier of fungi that can infect other animals and humans when prompting factors are present. Feather structures are compos of dead keratin-filled corneocytes (Camin*et al.* 1998; Wang *et al*. 2016). The keratinized tissues such as feathers are not easily broken down in the environment. Keratinolytic and keratinophilic fungi- a distinguished group of fungi- had the ability to grow on keratin substrates and decompose them into simple primary components (Călin *et al*. 2017).

Keratinophilic fungi inhibit feathers and featherless sites (Deshmukh 2004). They include pathogenic isolates and may cause human diseases viz superficial, cutaneous, and onychomycosis, as well as pulmonary disorders. They also cause several types of infections in poultry and domestic animals. Fungi associated with feathers are one of the means to get rid of feather waste at the level of the poultry industry. Note that, biodegradation is the best method to deal with this huge amount of pollutants, and several studies aimed to identify active proteolytic fungi have been carried out globally on this subject (Kumar and Kushwala 2014).

Simona and coworkers Nardoni and Mancianti (2021) listed 11 Keratinophilic fungi from feathers, viz *Scopulariopsis brevicaulis, Chrysosporium keratinophilum, Trichophyton terrestre, Microsporum gypseum, Sepedonium sp., Chrysosporium pannorum, Myriodontium sp., Chrysosporium tropicum, Chrysosporium pruinosum, Chrysosporium luteum,* and *Aphanoascus fulvescens* .

From chickens in Nigeria, twelve keratinophilic were isolated beside two dermatophytes viz *Microsporum*, and *Trichophyton*. *Chrysosporium* showed the highest incidence (Efuntoye 2002)

There is a lack of information about fungi associated with poultry in Iraq. Feather and featherless parts of the hen’s bodies as well as their house’s environment not been fully studied. The few studies focused on fungi in poultry feed and their capability to produce mycotoxins (Sharee 2010), and Aspergilloses outbreaks in commercial broiler chickens (Eassa 2017).

The present study aims to explain the fungal community associated with chicken feathers in house breeding cages and test the proteolytic activity of identified taxa. The health hazards of isolates and their human infections were reviewed.

**Materials and methods**

**Sample collection** 50 feather samples were collected in December 2022 from several urban locations in Erbil city. All samples were without dropping or moistened soil materials, they were kept separately in nylon bags at 4oC.

**Culturing and identification**  Three types of culture media were used, Sabouraud’s dextrose agar (SDA=61gm/L), and the lab-made Typha pollen agar (TPA=5gm/L) (Al-Bader 2018) for the primary culturing, as well as diluted skim milk agar (SMA=20gm/L) for proteolytic activity test. The culture media were supplemented by Chloramphenicol (15 mg/L) to prevent bacterial growth. The direct plate method was followed, and pieces of feathers were fixed on the surface of the culture media by sterile forceps. The Petri-dish plates then were incubated at 25± 2.

The cultures were checked daily from the third day over a period of 4 weeks. The observed developing fungal growths were directly transferred to Sabouraud’s dextrose agar to prepare a pure culture. The identification was conducted via macroscopic and microscopic characteristics based on (De Hoog and Guarro 2001; Aravinitis and Mylonakis 2015 ; Domsch *et al* 1989).

**Fungal community analysis**

In order to identify the predominant taxa, the occurrence% and frequency % were calculated via the following equations: O% = (No. of times fungal appear X100) /No. of collected samples. F%= (No. of fungal isolates X100)/ No. of total fungal isolates (Al-Bader and Zefenkey 2023).

**Detection of protease activity:** The proteolytic activity was examined for the predominant isolates. The diluted skim milk agar medium (SMA) was used (Kanchana 2013). Petri plates with 2% skim milk agar were prepared and inoculated with active growth from the edge of 7 days of pure fungal cultures. Plates were incubated at 25±2°C, and after 4 days, the clear zone around colony growth was observed and measured. The mean diameters of three replicates were calculated.

**Results**

**The fungal community structure:** A total of 298 isolates were counted from fifty feather samples, they belong to fourteen fungal genera. The identified taxa included 5 species of *Aspergillus* viz*A. fumigatus, A. flavus, A. nidulans, A. niger, A. parasiticus* as well as 13 genera belonging to several taxonomic groups including *Acremonium  hyalinulum, Cladosporium cladosporoidis, Chrysosporium sp., Myceliophthora verrucosa,* *Papulospora sp.,Fusarium sp., Mucor miehie, Cunninghamella elegans, Emericella nidulans, Chaetomium sp., Candida sp., Rhodotorula mucilagenosa,* and *Phoma herbarum* . *Chrysosporium sp*. was the predominant taxa. (Table 1).

Table 1- The isolated fungi with occurrence% (O%) and frequency%(F%). H=Hyphomycetes, A=Ascomycetes, Z=Zygomycetes, C= Coelomycetes, B=Basidiomycetes.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| F% | O% | T.G. |  Fungi |  |
| 5.3 | 26 | H |  *Acremonium  hyalinulum* | **1** |
| 10.0 | 50 | H | *A. flavus*  | **2** |
| 6.0 | 28 | H | *A. fumigatus* | **3** |
| 3.0 | 14 | H | *A. nidulans*  | **4** |
| 10.0 | 44 | H | *A. niger*  | **5** |
| 5.0 | 22 | H | *A. parasiticus* | **6** |
| 3.3 | 14 | A |  *Candida sp.* | **7** |
| 2.3 | 4 | A |  *Chaetomium sp.* | **8** |
| 20.8 | 90 | H | *Chrysosporium sp.*  | **9** |
| 10.0 | 46 | H | *Cladosporium cladosporioides* | **10** |
| 1.3 | 8 | Z |  *Cunninghamella elegans*  | **11** |
| 2.0 | 12 | A |  *Emericella nidulans* | **12** |
| 6.3 | 34 | H | *Fusarium sp.* | **13** |
| 4.6 | 24 | Z | *Mucor miehei* | **14** |
| 2.6 | 16 | H | *Myceliophthora verrucosa* | **15** |
| 1.0 | 4 | H |  *Papulospora sp.* | **16** |
| 2.6 | 16 | C |  *Phoma herbarum* | **17** |
| 3.0 | 10 | B | *Rhodotorula mucilaginosa* | **18** |

Hyphomycetes represented the highest occurrence (46 samples, 92%), they are commonly predominant in feather habitats ( Deshmukh 2004 and Mandeel *et al.* 2011) followed by Zygomycetes (10samples, 20%), Basidiomycetes (6 samples, 12%), Ascomycetes and Coelomycetes (4 samples, 8%) (Figure 1). .

Figure-1 The taxonomic groups % of isolated genera

**The biodegradable activity of isolated fungi:** *Aspergillus* was the predominant genus in the present study,it is a well-known saprophytic fungus, and it can utilize different synthetic and organic substrates including plant and animal tissues. *Aspergillus flavus* showed measuring clear zone only, while the other species with very weak proteolytic activity. Several studies mentioned the role of Aspergillus in the biodegradation of feathers (Derhab *et al*. 2022; Kim 2003; Kumar and Yadav 2020).

*Chrysosporium* is the second predominant taxon here, it is commonly isolated from the feathers. The current results showed that *Chrysosporium* is the most active keratinophilic fungi, and due to its high proteolytic activity, *Chrysosporium*, was used in important industrial and agricultural applications (Kumar *et al*. 2020; Maruthi *et al*. 2011).

*Cladosporium* in the third level is highly isolated from animals and plant surfaces as well as the objects made from them. The assessment of *Cladosporium* as a biodegradation eco-friendly agent for keratin waste materials was examined in several studies (Călin *et al*. 2017; Constantin *et al*.2022)

*Fusarium* was regarded initially as a plant pathogen while studies in later years have shown a wide range of animal infections including feathers and other keratinous tissues (Preczeski *et al*. 2020; Kumar and Kushwaha 2021).

*Acremonium* and *Mucor* showed moderate abundance, they were commonly isolated from feather samples (Singh and Kushwaha 2010).

The rest genera with low abundance can hydrolyze keratin and several organic substrates. They have been recorded in several studies as a part of the feather’s fungal community or associated with other keratinous tissues. (Kumar and Kushwaha 2021; Mohammed *et al*. 2017; Al-Bader 2018; Najwa and Abu-Majded 2013).

 Results of the SMA test showed that only 6 taxa exhibited clearing zones (fig. 2), the tested isolates grew on diluted skimmed milk agar as deformed colonies except for *Aspergillus flavus* which showed normal macroscopic characteristics. The maximum clearing zone for *Chrysosporium* *sp*. (no. 3) proves the highest proteolytic activity, followed by *Myceliophthora* *verrucosa* (no.4), *Papulospora sp*.(no.6), *Acremonium hyalinum* (no.5), *Chaetomium sp.* (no.2), and *Asp. flavus* (no.1). The other fungal taxa showed weak proteolytic activity with narrow zones (<3mm) and were not easy to measure.



Figure 2- The result of proteolytic activity by SMA culture medium. *Asp. flavus* (no.1). *Chaetomium sp.* (no.2),  *Chrysosporium* *sp*. (no.3),  *Myceliophthora* *verrucosa* (no.4), *Acremonium hyalinum* (no.5), and *Papulospora sp*.(no.6).

**The health hazards of isolated fungi:** Even though pathogenicity is a trait of species or strain, the keratinophilic genera include dermatophytes and opportunistic taxa and they are probably agents of disease ( Torres-Rodríguez and López-Jodra 2000). The predominant genera in the present study have been isolated from several types of human infection. *Aspergillus* and *Cladosporium* are among the most air bioaerosols and have high relation with pulmonary disorders (Wang *et al*. 2022). Furthermore, *Aspergillus* is a common wound contamination leading to cutaneous aspergillosis also (Mousa *et al*.1999), and members of *Cladosporium* are causative agents of [Phaeohyphomycosis](https://academic.oup.com/mmy/article-abstract/39/1/135/1034416) (Sandoval-Denis *et al*. 2016).

*Chrysosporium sp.* is an infectious agent for animals and humans, it includes mostly keratinophilic species that live on the remains of hair and feathers in soil, and most infections were reported in immunocompromised patients ( Anstead *et al.*2012; Cabañes *et al*. 2014 ).

*Fusarium* and *Mucor* are well-known agents of several types of opportunistic mycosis including keratomycosis, besides skin and nail infections (Preczeski *et al.* 2020).

*Acremonium sp*., *Candida sp*., *Chaetomium* *sp.*, *Cunninghamella sp.*, *Myceliophthora sp*., *Papulospora sp*., *Phoma sp. , Rhodotorula sp*. can cause a wide range of infections ranging from superficial to deep mycoses (De Hoog and Guarro 2001)

**Discussion**

The studied habitat showed high fungal diversity, which is highly affected by temperature and humidity. The recorded fungi in the present study seem to be a heat/drought-tolerant group besides their affinity to the feather structure. Moorthy *et al*. (2011) confirmed this finding, they reported that the prevalence of keratinophilic fungi is significantly higher in bird feathers than in other animals.

Several researchers discussed and warned about the health problems of a feather–associated fungi, and others focused on using the active isolates them to get rid of millions of tons of poultry feathers waste. They suggested using active proteolytic fungal isolates instead landfilling or burning, since the biodegradation method is an eco-friendly process and creates useful organic products for agricultural use. *Chrysosporium sp.* which showed the highest proteolytic activity in the present study was pointed out by several workers as the best decomposer for feathers (Koutb *et al.* 2023; Kumar and Kushwala 2014).

 On the other hand, the current results showed that feathers are a rich source of opportunistic fungi, and the health hazards increase due to their proteolytic activity. They are potential agents of allergy and/or mycotic infections. They are easily aerosolized and cause different types of respiratory disorders, furthermore, they are probable agents of superficial, cutaneous, and systemic mycoses.

*Aspergillus* is predominant in the poultry environment including feathers, feed, soil, and wastes ( Mandeel *et al*. 2011; Alhassan *et al*. 2021; Imran and Ali 2014; Alkhursan *et al*.2021). *Aspergillus spp.* have diverse extracellular enzymes and they can colonize several substrates (Kim 2003). The dry conidia of *Aspergillus* are easily aerosolized and thus increasing their health hazards, especially for respiratory system infections. The fungus *Aspergillus fumigatus* is the major cause of invasive aspergillosis while *A.flavus* is regarded as one of the most important species that can cause both noninvasive and invasive systematic aspergillosis in immunocompromised individuals( Liu *et al.* 2021). Roohi *et al*. (2023) reported that *A. niger* is the most infectious member of otitis.

*Chrysosporium*is a large genus of saprophytic, it is commonly found in soils, air, the surface bodies of animals and birds, high-humidity bird nests, etc (Gurung *et al*. 2018).

 The fungus has a high activity to utilize keratinous tissues, and over the past 20 years, it was recorded as an emerging pathogen. The *Chrysosporium* cutaneous infection may be fatal in types of reptiles (Cabañes *et al*. 2014). Species of *Chrysosporium* are isolated from skin and nail scrap samples, especially from feet and a rare subcutaneous infection.(Mijiti *et al*.2017).

*Cladosporium* which occurred in the third level is a well-known worldwide genus, and commonly isolated from soil and organic matter. It represents among the most frequently culturable airborne fungi. The genus includes thermotolerant ‘true human-pathogenic species (Bensch *et al*. 2012). Long-term exposure to a large amount of *Cladosporium* structures can cause adverse health effects, including allergies and asthma symptoms, as well as infections of the eye, ear, sinus, and skin problems (Ogórek *et al.*2012).

*Fusarium* is the causative agent of Fusariosis, the infection of plants, animals, and humans caused by various fungi of the genus. Human infections are not easily treated, this may be related to the antifungal resistance of several environmental strains (Ribas *et al.* 2016).Most infections in humans range from superficial, and locally invasive to distributed, with the most common infections being onychomycosis, skin infections, and keratitis (van Diepeningen *et al*. 2015).

The genus *Mucor*contains about 50 taxa, they are widespread on decaying food, soil, and animal excrement. Taxa with health importance include a few thermotolerant species which are the agents of several types of infections such as pulmonary mucormycosis, the most occurrence one, which develops after inhalation of spores into the bronchioles and alveoli (Agrawal *et al*. 2020).

The other taxa in (table-1) have been reported as agents of several types of mycoses ranging from superficial to deep infections. The health risks of *Acremoium sp., Candida sp., Chaetomium sp., Cunninghamella sp., Emericella sp., Myceliophthora sp., Paulospora sp., Phom asp.,* and *Rhodotorula sp*. increases remarkably for immunodepleted individuals (Das *et al*. 2010; Talapko *et al*. 2021; Cronin *et al*. 2021; Hallur *et al*. 2021; Baho *et al*. 2022; Selvin *et al*. 2014; Rai *et al.* 2021; Kim *et al*. 2021).

**Conclusion**

Depending on the findings, the feathers of chickens were colonized by several fungi. 30% of the isolated taxa had remarkable proteolytic activity. Keratinophilic fungi are very common and active in cycle feathers. We suggest *Chrysosporium sp*., the most active taxon for further studies.

 The keratinophilic and saprophytic fungi in the feathers can pose a risk to the health of chicken breeders, which is worse for children and the oldies. Working with this profile should be with a guide’s actions related to health surveillance. The use of a nose mask and gloves, as well as sterilizers, reduces the expected health risks in chicken breeding places.

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