# Characterization of the gastrointestinal yeast microbiota of cockatiels (*Nymphicus hollandicus*): a potential hazard to human health

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Cockatiels are the world's second most popular psittacine pet bird, but no data characterizing their gastrointestinal microbiota have been found. Thus, the aim of this work was to characterize the yeast gastrointestinal microbiota of cockatiels and to evaluate the relevance of cockatiels as carriers of potentially pathogenic yeasts. A total of 60 cockatiels, from 15 different premises, were assessed. A thorough clinical examination was performed with each bird, and samples were collected from oral cavity, crop and cloaca. The stools were collected from cages where the birds were kept. The isolates were identified according to morphological and biochemical characteristics. Yeasts were isolated from at least one anatomical site of 65 % of the birds and 64.3 % of the stool samples. The oral cavity (53.3 %) and the crop (58.3 %) were the anatomical sites with the highest prevalence and the highest number of yeast isolates. Overall, 120 yeast isolates, belonging to 13 species, were obtained. The most frequently isolated species were *Candida albicans*, with 39 (32.5 %) isolates, followed by *Candida tropicalis* (20 %), *Trichosporon asteroides* (12.5 %), *Candida famata* (10 %) and others. Mixed yeast colonies were isolated from 23.3 % of the birds and *C. albicans* was seldom found in association with other species ($P<0.05$). The results of this work demonstrated that cockatiels harbour potentially pathogenic yeasts throughout their gastrointestinal tract and in stools, and are prone to disseminating them in the environment.

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## INTRODUCTION

The cockatiel (*Nymphicus hollandicus*) is a bird originated from Australia that belongs to the family *Psittacidae* and it represents the world's smallest species of cockatoo (according to the Integrated Taxonomic Information System). Currently, it is the world's second most popular psittacine pet bird [Birds in Backyards–Bird Finder–Cockatiel, http://birdsinbackyards.net/finder/display.cfm?id=49 (consulted 24 October 2009)].

It is known that yeasts are normal components of the gastrointestinal microbiota of birds, especially *Candida* spp., *Saccharomyces* spp., *Trichosporon* spp., *Rhodotorula* spp. and *Cryptococcus* spp. (Cragg & Clayton, 1971; Mancianti *et al.*, 2002; Melville *et al.*, 2004; Cafarchia *et al.*, 2006b). The microbiota may vary according to the bird species (Cafarchia *et al.*, 2006a), and no data on the characterization of cockatiel's gastrointestinal microbiota have been found.

Captive and free-ranging birds are also known to play a role as carriers and spreaders of yeasts that are potentially pathogenic for human beings, which contributes to environmental contamination and, possibly, to human and animal infection (Mancianti *et al.*, 2002; Cafarchia *et al.*, 2006a, b). This is a very important aspect, considering the increasing popularity of cockatiels as a pet bird, and the close relationship between human beings and their pets, which may expose people to potential pathogens that may belong to the normal microbiota of cockatiels. Children, elderly people and immunocompromised individuals are at a greater risk of developing diseases after exposure to pathogenic yeasts (Cafarchia *et al.*, 2006a).

Among the 25 species of emerging pathogen yeasts, 20 were *Candida* spp. (Hazen, 1995). In human beings, the most frequently isolated *Candida* species from clinical manifestations are *Candida albicans* (predominant worldwide),

 

*Candida parapsilosis* and *Candida tropicalis* (in Latin America) and *Candida glabrata* (in the USA) (Palacio *et al.*, 2009). The first three species are the same as those commonly found in bird infections (Velasco, 2000; Samour & Naldo, 2002).

Thus, due to the lack of knowledge on the role of cockatiels as carriers of pathogenic yeasts, the aim of the present study was to isolate yeasts from the gastrointestinal tract, in order to characterize the microbiota of cockatiels and evaluate the relevance of these birds as carriers of such micro-organisms.

## METHODS

**Animals.** Sixty apparently healthy cockatiels (*N. hollandicus*), from fifteen different places (five households, four breeders and six pet stores) in the city of Fortaleza, Ceará, Brazil, were assessed for this research. Thirty-eight were adults and twenty-two were young (2–8 months of age). The gender was not reliably identified for any of the individuals. This research was submitted and approved (protocol number 02/09) by the Ethics Committee of Animal Research of the Federal University of Ceará.

Prior to specimen collection, cockatiels were physically restrained, as described for small sized psittacines (Harrison & Ritchie, 1994), and were individually evaluated, as follows. The body score was given based on the muscular mass over the sternum. The eyes were observed, and any signs of swelling and redness were considered an alteration. When examining the oral cavity, mucosal and choanal lesions, mucosal erythema and the presence of mucus or discharge were considered abnormal. For the nares, plugs or discharge were sought, and for the lungs and air sacs the animals were thoroughly auscultated and the presence of abnormal respiratory sounds was taken into account.

Individual records were written, containing all the obtained data, and each animal was scored based on the features mentioned above, losing one point for each one altered. The minimum was 0, when at least one alteration was observed for each evaluated feature, and the maximum score was 5, when no clinical alterations were observed (e.g. score 0, one clinical alteration for each of the five evaluated features; score 1, four altered features; score 2, three altered features; score 3, two altered features; score 4, one altered feature; score 5, no clinical alterations).

**Sample collection.** Samples were collected from three anatomical sites (oral cavity, crop and cloaca) and from droppings. Sterile cotton swabs were used to obtain samples from the oral cavity and cloaca. The swabs were inserted into the anatomical site and were rotated. Then, they were placed into sterile glass slants containing 1 ml sterile saline (0.9 % NaCl), keeping the cotton extremity in contact with the solution until processing, within 12 h.

In order to obtain crop content samples, a crop lavage was performed by injecting 3 ml sterile saline (0.9 % NaCl) into this anatomical site, through a siliconized PVC size 12 urethral catheter, attached to a 5 ml syringe. First, the catheter through which the saline solution was injected was introduced into the crop. Then, the crop was gently massaged and the solution was aspirated back into the syringe (Richardson, 2006). Afterwards, the catheter was removed, its final extremity was sealed and the content obtained through the lavage was used for mycological processing.

Stool samples were collected from the environment where the birds were maintained. When birds were kept in collective cages, a pool of at least 1 g faeces was collected. The samples were collected with a plastic spatula, previously disinfected by alcohol 70 %, and were kept in sterile Petri dishes until processing, within 12 h (Filiú *et al.*, 2002).

### Microbiological processing

**Isolation.** All samples were taken to the Specialized Medical Mycology Center from the Federal University of Ceará, in Fortaleza, Ceará, Brazil, where they were processed and the micro-organisms were properly identified.

For each collection site, two culture media were used for primary isolation: YEPD (yeast extract, peptone, dextrose) agar with chloramphenicol (0.5 g $l^{-1}$), and birdseed (*Guizotia abyssinica*) agar. Samples obtained from the oral cavity and cloaca were cultured in both media with the cotton swab used for collection. From the suspension obtained from the crop lavage, 100 µl were cultured in both media.

The faeces collected were ground in sterile Petri dishes, and approximately 1 g was added to a saline solution (0.9 % NaCl) containing chloramphenicol (0.4 g $l^{-1}$). The suspension was homogenized with a vortex for 3 min, and it was left to settle out into solid and liquid phases for 30 min, at 25 °C. Afterwards, aliquots of 100 µl from the supernatant (liquid phase) of each sample were cultured in both media (Filiú *et al.*, 2002).

Petri dishes containing the cultured media were incubated at 25 °C, for 10 days, and were observed daily. For the oral cavity and cloaca samples, the number of c.f.u. per swab was counted for each Petri dish after 48 h of growth, until the last day of incubation. The same was done for the crop samples expressed as c.f.u. (100 µl content)$^{-1}$, and for the stools, expressed as c.f.u. (g faeces)$^{-1}$.

**Identification.** Initially, for each positive sample, colonies were observed microscopically after Gram staining. Then, colonies were subcultured into YEPD agar slants and tests were performed for identification at the species level. Identification was based on microscopic morphology and biochemical parameters (urea hydrolysis and sugar assimilation), as described by Brito *et al.* (2009).

For *Candida* spp., the colonies were initially grown on chromogenic media (HiCrome Candida differential agar; HiMedia) for identification of mixed colonies. Afterwards, individual colonies were subcultured into slants containing YEPD agar and Christensen's urea agar, and after 24 to 48 h the micro-organisms were grown on cornmeal Tween 80 agar in order to perform a morphological analysis. A sugar-assimilation test was also performed for each isolate and the results were interpreted according to information published by De Hoog *et al.* (2000).

*Trichosporon* species were, initially, identified macroscopically. Then, a little piece of the colony was observed microscopically on glass slides, after being stained with cotton blue lactophenol. Afterwards, the micro-organisms were subcultured into slants containing YEPD agar and Christensen's urea agar, and 24 to 48 h later slide cultures on 2 % malt extract agar were made for morphological evaluation. Finally, a sugar-assimilation test was performed for each isolate. The obtained results were interpreted according to Guého *et al.* (1994).

For *Rhodotorula* spp., colonies were initially identified based on their colour. Then, the micro-organism was subcultured into slants containing YEPD agar and Christensen's urea agar, and after 24 to 48h it was grown on 2 % malt extract agar for morphological evaluation. Sugar-assimilation tests were performed for each isolate and they were crucial for species identification. The results were interpreted according to De Hoog *et al.* (2000).

*Cryptococcus* spp. isolates were initially grown on cornmeal Tween 80 agar and on Christensen's urea agar for microscopic and biochemical

evaluation, which suggested the genus. Afterwards, an automated analysis was performed using Vitek 2 (bioMérieux) in order to determine the species.

**Statistical analysis.** Data were analysed by descriptive variable analysis. Variance analysis and Pearson $\chi^2$ tests were used to analyse clinical scores and frequency distribution among the studied categories, respectively. The smallest significance level was $P<0.05$.

## RESULTS AND DISCUSSION

### Clinical scores

Out of the examined cockatiels, 2 (3.3 %) presented a clinical score of 0, 4 (6.7 %) presented a score of 1, 11 (18.3 %) presented a score of 2, 16 (26.7 %) presented a score of 3, 17 (28.3 %) presented a score of 4 and 10 (16.7 %) presented a score of 5. A high percentage of birds presenting high c.f.u. count (>50 c.f.u.) was observed in clinical score groups 0 ($n=2$) and 1 ($n=2$) (Table 1). No statistical differences were observed between clinical scores and age, c.f.u. counts, diet of the birds or isolated yeast species.

Even though the number of birds fed exclusively on extruded bird food was small ($n=7$), it was observed that four (57.1 %) of them presented high c.f.u. counts, a high percentage, when compared to the cockatiels fed on mixed diets (5.5 %) and those fed on all-seed diets (17.1 %).

The most common clinical signs of avian gastrointestinal candidiasis are pseudomembranous whitish lesions in the oral cavity, crop and oesophagus, depression, delayed crop emptying, regurgitation, lack of appetite and poor digestion of food (Dahlhausen, 2006). Panigrahy *et al.* (1979) reported the occurrence of gastrointestinal *C. albicans* infection in cockatiel nestlings. The main lesions observed in the necropsied birds were pseudomembranes, and ulcers in the oral cavity, oesophagus and crop.

In the present study, all evaluated cockatiels were apparently healthy and they did not present any sign of systemic compromise. Through clinical examination, it was observed that 21 (35 %) individuals presented lesions in the oral cavity, which are suggestive of oral candidiasis.

The majority of the analysed birds presented a satisfactory clinical status. However, the six birds that presented low clinical scores ($\leqslant 1$) presented high prevalence with high c.f.u. counts. Such a finding is probably related to the occurrence of immune impairment due to concomitant infectious, metabolic and/or nutritional disorders, which are considered risk factors for the development of candidiasis (Vieira & Acqua-Coutinho, 2009).

### Prevalence

A total of 39 (65 %) out of 60 cockatiels were positive for yeast growth, considering all 3 evaluated anatomical sites, which was higher than the prevalence (25 %) obtained from the gastrointestinal tract of necropsied raptors (Cafarchia *et al.*, 2006b). A total of 13 (59.1 %) out of 22 young birds and 26 (68.4 %) out of 38 adult birds were positive for yeast growth, presenting no statistical differences.

When comparing specific sites individually, in this research, the prevalence of yeast in the oral cavity was 53.3 % (32/60), higher than that observed for four wild columbid species (14–24 %) (Kocan & Hasenclever, 1972), similar to that for ostriches (50 %) (Melville *et al.*, 2004) and white crowned pigeons (56 %), and lower than that found for two columbid species, including feral pigeons (95 and 100 %) (Kocan & Hasenclever, 1972).

The prevalence found in the crop was 58.3 % (35/60), similar to the result obtained in a study with baby Amazon parrots (*Amazona aestiva* and *Amazona amazonica*) during which *Candida* spp. was isolated from 57.5 % of the analysed birds (Vieira & Acqua-Coutinho, 2009). The tested parrots, however, were nestlings, apprehended from illegal trade, submitted to poor management conditions, and 45 % of them presented clinical signs of ingluvitis, making them more prone to the isolation of *Candida* spp. In spite of that, the apparently healthy cockatiels still presented a higher prevalence of yeasts isolated from the crop.

The cloaca presented a prevalence of 23.3 % (14/60), higher than that observed for ostriches (8 %) (Melville *et al.*, 2004), migratory birds (15.7 %) (Cafarchia *et al.*, 2006a) and raptors (9.9 %) (Cafarchia *et al.*, 2006b). In addition, out of the analysed stool samples, 64.3 % (9/14) were positive for yeast growth, which was higher than the prevalence observed by others (Mancianti *et al.*, 2002;

**Table 1.** Population size of yeasts expressed as c.f.u.

| Clinical score | <50 c.f.u. count [*n* (%)] | >50 c.f.u. count [*n* (%)] | Negative [*n* (%)] | Total |
|---|---|---|---|---|
| 0 | – | 2 (100) | – | 2 |
| 1 | – | 2 (50) | 2 (50) | 4 |
| 2 | 7 (63.6) | 1 (9.1) | 3 (27.3) | 11 |
| 3 | 10 (62.5) | 2 (12.5) | 4 (25) | 16 |
| 4 | 9 (52.9) | 2 (11.8) | 6 (35.3) | 17 |
| 5 | 2 (20) | 2 (20) | 6 (60) | 10 |
| Total | 28 (46.7) | 11 (18.3) | 21 (35) | 60 |

Cafarchia *et al.*, 2006b), who isolated yeast from 49.2 and 43.7 % of analysed psittacine and raptors stool samples, respectively.

Ten birds presented yeasts in only one anatomical site, six of them in the oral cavity, three in the crop and one in the cloaca. Isolates were obtained simultaneously from the oral cavity and crop of 16 cockatiels, from the crop and cloaca of 3 birds, and from all 3 anatomical sites of 10 individuals. Interestingly, a relationship of dependence was observed for the distribution of yeasts throughout the anatomical sites, which means that it was more likely to recover yeasts from more than one anatomical site than from only one (Table 2).

## Isolated yeast species

Overall, 120 isolates, belonging to 4 genera and 13 species, were obtained (Table 2). A total of 41 (34.2 %), 44 (36.7 %), 19 (15.8 %) and 16 (13.3 %) isolates were obtained from oral cavity, crop, cloaca and stools, respectively (Fig. 1).

*Candida* spp. was the most frequently isolated genus and *C. albicans* the most frequently isolated species from the gastrointestinal tract, with 39 isolates (32.5 %), as also described in some other works (Kocan & Hasenclever, 1972; Moretti *et al.*, 2000; Mancianti *et al.*, 2002; Samour & Naldo, 2002; Garcia *et al.*, 2007). *C. tropicalis* was the second most frequently isolated species (20 %), followed by *Trichosporon asteroides* (12.5 %), *Candida famata* (10 %), *Rhodotorula mucilaginosa* (8.4 %), *C. parapsilosis* (6.7 %) and *C. glabrata* (4.2 %). *C. albicans* was the most commonly isolated species from the oral cavity, crop and cloaca, while *C. tropicalis* was the most commonly isolated species from stools (Table 2).

The oral cavity (53.3 %) and crop (58.3 %) were the anatomical sites with the highest prevalence and the highest number of yeast isolates, 41 and 44, respectively, and crop was the site from where the highest number of species was isolated ($n$=9), followed by the cloaca ($n$=8), and the oral cavity ($n$=7) and stools ($n$=7).

Only four out of eight yeast species isolated from the cloaca were also recovered from stools, which suggests the occurrence of sample contamination with environmental micro-organisms. However, such observation demonstrates the role of bird excreta as an adequate environment for fungal growth and maintenance, and also as an important source for human and animal infection.

Even though yeasts are considered commensal organisms and are part of the normal biota, they are capable of causing disease whenever an impairment of the immune system is established. In recent years, after a worldwide increase in the number of immunocompromised individuals, the incidence of opportunistic mycosis, caused primarily by yeasts, has increased (Pfaller & Diekema, 2007). *Candida* spp. and *Cryptococcus neoformans* are the most common micro-organisms involved in human yeast infections (Cafarchia *et al.*, 2006b, 2008). *Trichosporon* spp. and *Rhodotorula* spp. are not as commonly reported as infecting agents, but recently they have been more frequently implicated in systemic infections, especially in neutropenic patients (Araujo Ribeiro *et al.*, 2008; Tuón & Costa, 2008). The results of our research show that cockatiels may act as carriers of potentially zoonotic yeasts, as demonstrated by the isolation of *Candida* spp., *Cryptococcus* spp., *Trichosporon* spp. and *Rhodotorula* spp.

## Growth of mixed colonies

From 26 samples, obtained from 14 (23.3 %) birds, it was possible to isolate more than one yeast species from at least one collection site. Eight samples from the oral cavity, ten from the crop, four from the cloaca and four from stools

**Table 2.** Number and percentage of positive sites of collection from cockatiels listed according to the species of yeasts isolated

| Yeast species | Collection site | | | | |
|---|---|---|---|---|---|
| | Oral cavity [*n* (%)] | Crop [*n* (%)] | Cloaca [*n* (%)] | Stool [*n* (%)] | Total [*n* (%)] |
| *Candida albicans* | 16 (39) | 15 (34.1) | 7 (36.8) | 1 (6.3) | 39 (32.5) |
| *Candida famata* | 7 (17.1) | 3 (6.8) | – | 2 (12.5) | 12 (10) |
| *Candida glabrata* | 1 (2.4) | 2 (4.5) | 1 (5.3) | 1 (6.3) | 5 (4.2) |
| *Candida krusei* | – | 1 (2.3) | – | – | 1 (0.8) |
| *Candida parapsilosis* | 3 (7.3) | 4 (9.1) | – | 1 (6.3) | 8 (6.7) |
| *Candida tropicalis* | 9 (22) | 8 (18.2) | – | 7 (43.8) | 24 (20) |
| *Cryptococcus albidus* | – | 2 (4.5) | – | – | 2 (1.7) |
| *Cryptococcus laurentii* | – | – | 1 (5.3) | – | 1 (0.8) |
| *Trichosporon asteroides* | 3 (7.3) | 5 (11.4) | 5 (26.3) | 2 (12.5) | 15 (12.5) |
| *Trichosporon inkin* | – | – | 1 (5.3) | – | 1 (0.8) |
| *Trichosporon ovoides* | – | – | 1 (5.3) | – | 1 (0.8) |
| *Rhodotorula minuta* | – | – | 1 (5.3) | – | 1 (0.8) |
| *Rhodotorula mucilaginosa* | 2 (4.9) | 4 (9.1) | 2 (10.5) | 2 (12.5) | 10 (8.3) |
| Total | 41 | 44 | 19 | 16 | 120 |

presented mixed yeast species (Table 3). Out of these birds, seven presented mixed colonies in two or more different sites.

Interestingly, it was observed that out of 39 isolates of *C. albicans*, only 5 (12.8 %; $P<0.05$) were associated with other yeast species: 3 (7.7 %) were associated with either *C. famata* or *C. glabrata* and 2 (5.1 %) were found in coexistence with *Trichosporon* spp. On the other hand, out of 24 isolates of *C. tropicalis*, 17 (70.8 %; $P<0.05$) were associated with other species: 8 (33.3 %) were isolated from mixed *Candida* spp. colonies (*C. famata*, *C. parapsilosis*, *C. glabrata*), 4 (16.7 %) were found to be coexisting with *Candida* spp., *Trichosporon* spp. and/or *Rhodotorula* spp., and 5 (20.8 %) were found associated with *Trichosporon* spp. and/or *Rhodotorula* spp. For *C. parapsilosis*, *C. glabrata* and *C. famata*, 4 (50 %), 3 (60 %) and 11 (91.7 %; $P<0.05$) isolates were recovered associated with other species, respectively. Based on these observations, it seems like *C. albicans* is more likely to grow as the only species within the same site.

Additionally, *C. albicans* was the only species recovered from all three anatomical sites of six out of seven analysed cockatiels that fed exclusively on extruded bird food ($P<0.05$). It is important to consider that the extruded food offered to the cockatiels contains prebiotics, which might have influenced the isolation of this yeast species.

Another possibility to explain this observation is the contamination of the offered food with *C. albicans*. However, it is unlikely considering the high temperature required in order to complete the process of extrusion. Besides, such a hypothesis would not explain the statistically significant difference between the different analysed diets, especially when compared to birds that fed on mixed diets (extruded food and seeds).

A maximum of three different species was recovered from the same site, particularly from the oral cavity ($n=4$) and the crop ($n=2$) (Table 3). Out of the four oral cavities from which three different species were isolated, two presented two species of *Candida* spp. and *Trichosporon* spp. or *Rhodotorula* spp., and the other two presented three species of *Candida* spp. simultaneously, such as *C. famata*, *C. parapsilosis* and *C. tropicalis*, and *C. famata*, *C. glabrata* and *C. tropicalis*.

The presence of two species of yeasts in six raptors (10 %) has been observed, out of which three presented *C. famata* and *R. mucilaginosa* in the cloaca and the other three presented *C. famata* and *C. parapsilosis* in the crop (Cafarchia *et al.*, 2006b). Mixed *Candida* spp. infections in the crop of two (5 %) Amazon parrots were also observed, presenting *C. guilliermondii* in coexistence with *C. parapsilosis* or *C. humicola* (Vieira & Acqua-Coutinho, 2009).

From the stool samples, 4/14 (28.6 %) resulted in mixed yeast colonies, which was also often observed in a study with psittacine droppings (Mancianti *et al.*, 2002), but the authors did not specify the species involved. In our study, from one of the stool samples, four different species were recovered (two *Candida* spp., one *Rhodotorula* spp. and one *Trichosporon* spp.), but this observation was probably related to the occurrence of contamination with environmental micro-organisms.

In this research and in the other studies mentioned above, *C. albicans* was seldom seen in association with other yeast species. Considering that it is a normal inhabitant of the avian gastrointestinal tract, we hypothesize that this species might be capable of inhibiting mucosal colonization by other yeast species, especially *Candida* spp. *C. albicans* was isolated from 20 individuals, out of which 7 presented high c.f.u. counts at any of the evaluated sites, and from these birds, only this species was recovered, suggesting its capacity to overcome microbial competition.

## Final considerations

Even though other yeast species were recovered from cockatiels, special attention must be given to *Candida* spp. as they represented 74.2 % of the isolates (89/120), and it has been suggested that animals should be considered as potential sources of *Candida* spp. infections in humans, especially for immunocompromised individuals (Edelmann *et al.*, 2005). The results of this work demonstrate that cockatiels harbour potentially pathogenic yeasts throughout their gastrointestinal tract and are prone to disseminating them in the environment. Therefore, it is the veterinary clinician's responsibility to warn owners that their pet might represent a hazard to human health, especially to immunocompromised individuals, children and the elderly.

**Table 3.** Number of yeast species isolated from different collection sites

| Collection site | No. of samples with multiple yeast species | | | Total |
|---|---|---|---|---|
| | Two species | Three species | Four species | |
| Oral cavity | 4 | 4 | 0 | 8 |
| Crop | 8 | 2 | 0 | 10 |
| Cloaca | 4 | 0 | 0 | 4 |
| Stools | 3 | 0 | 1 | 4 |
| Total | 19 | 6 | 1 | 26 |

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