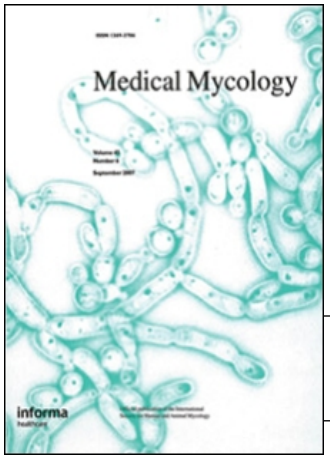

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Phospholipase activity of yeasts from wild birds and possible implications for human disease

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Phospholipase activity of yeasts from wild birds and possible implications for human disease

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Over the last decades, reports on yeast infections in humans have increased especially with respect to immunocompromised individuals. Phospholipases are enzymes which may be associated with pathogenic processes caused by opportunistic yeasts. Phospholipase activity (ph.a.) was investigated in 163 isolates of 13 species of yeasts. A total of 133 isolates were obtained through the screening of a total of 768 cloacae of wild birds (Group I: 182 birds of prey; Group II: 165 passeriformes and Group III: 421 other wild migratory birds), while 30 isolates were recovered from the droppings of birds housed in 32 distinct aviaries (Group IV). Phospholipase production was evaluated and quantified at 2 and 5 day pre-incubation (Pr.t) and incubation times (I.t). Isolates from cloacae (48.1%) and excreta (73.3%) produced ph.a. with the highest values registered after 5 days of I.t. *Candida albicans*, *C. tropicalis*, *C. glabrata*, *C. lusitaniae*, *C. pelliculosa*, *Cryptococcus albidus*, *C. laurentii*, *Trichosporon beigelii*, and *Saccharomyces cerevisiae* displayed the highest ph.a. after 2 days of Pr.t while *Candida famata*, *C. guilliermondii* and *Cryptococcus neoformans* after 5 days of Pr.t. Ph.a. was never found in *Rhodotorula rubra* isolates recovered from the cloacae of wild birds. Isolates (73.3%) from bird droppings showed a higher ph.a. than those from cloacae thus indicating that wild birds not only act as carriers but may also spread phospholipase-producing yeasts in the environment.

Keywords *Candida albicans*, *Cryptococcus neoformans*, phospholipase activity, wild birds, yeasts

Introduction

Yeasts are considered part of the normal biota of the gastrointestinal tract, skin and urinary system of animals and humans [1] but may induce cutaneous and/or systemic diseases [1]. Reports on yeast infections in humans (e.g., *Cryptococcus neoformans* and *Candida* spp.) have increased over the past few years especially in immunocompromised individuals and cancer patients [1], as well as in humans exposed to bird droppings [2–11].

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Human pathogenic fungi have been isolated from pigeon droppings [12–14], the cloacae of migratory birds [15,16] and birds of prey [17] thus suggesting that these animals could be potential carriers and spreaders of fungal infections. It has been reported that opportunistic fungi produce phospholipases [18] which may be involved in the development of disease [19]. Phospholipases are enzymes that hydrolyze one or more ester-linkages in glycerophospholipids and are involved in disruption of host cell-membranes [20]. They have been detected in opportunistic yeasts including *C. albicans* [21–23], non-*C. albicans Candida* species [24,25], *Rhodotorula rubra* [26], *C. neoformans* [27–29], *Malassezia furfur* [30] and *M. pachydermatis* [31].

Reports on the virulence factors of yeasts isolated from avian droppings and/or from the lower digestive tract of birds are scant and those available are limited

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to *C. neoformans* from pigeon (*Columba livia*) and psittacidae droppings [29,32].

Hence, the aim of this work was to investigate phospholipase activity (ph.a.) in different species of yeasts recovered from the cloacae and environment of wild birds to assess risks for human health via environmental contamination.

Material and methods

Study population and collection procedures

From October 2001 to May 2003, samples were collected directly from the cloacae, of different species of wild birds and from Passeriformes as well as droppings of birds in aviaries as previously reported [15,17]. Samples were stored at 4°C and delivered within 24 h to the mycological laboratory of the Faculty of Veterinary Medicine, University of Bari (Italy). Phospholipase activity was assessed in 163 yeast isolates collected from 768 birds and droppings in 32 aviaries. In particular, samples were obtained from four groups:

- Group I: 182 birds of prey belonging to different families (i.e., Falconidae, Accipitridae, Strigidae and Titonidae) and housed, due to their inability to fly, at the Apulian Regional Fauna Observatory, province of Bari, Southern Italy [17];
- Group II: 165 passeriformes of different families (i.e., Remizidae, Passeridae, Hirundinidae, Sylviidae, Motacillidae, Emberizidae, Turdidae, Aegithalidae) sampled in Matera (Southern Italy) during a period in which the Italian Society for Bird Protection (L.I.P.U.) captured wild birds to attach numbered metal or plastic rings to their legs or wings for later identification;
- Group III: 421 other wild migratory birds (i.e., families Scolopacidae, Phasianidae, Anatidae, Rallidae, Columbidae), which were among 1,726 wild birds illegally shot from September 2002 to January 2003 in the Balkans (i.e., Rumania, Hungary and Bulgaria). The carcasses were stored at −20°C until one day before sampling and were being usedfor research purposes following the issuance of a permit by the Provincial Judicial Authority [15];
- Group IV: bird droppings directly collected (within 3 days post-admission) from 32 aviaries in which birds of prey (Group I) were hospitalized [17]. Pooled droppings from one aviary were considered as a single sample.

Isolation of yeast-like organisms and their phospholipase production

Samples (i.e., cloacae swabs) from Groups I –III and 0.1 ml of droppings solution (i.e., 1.0 g of faeces suspended in 9 ml sterile saline solution containing 1000 μg/ml streptomycin and 500 UI penicillin/ml) from Group IV were inoculated onto Sabouraud dextrose agar with chloramphenicol 0.5 g/l (BioLife, Italiana s.r.l. Milano, Italy; SDA) and added biphenyl 0.1%, incubated at 30°C for 7 days and observed daily for growth. They were identified in a manner similar to those described elsewhere [15,17]. In particular, identification was based on microscopic morphology, urea hydrolysis and sugar assimilation. Sugar assimilation was tested by ID32C and the Vitek System (bioMérieux, Marcy-l'Etoile, France) and/or by API 20C Aux test (bioMérieux, Marcy-l'Etoile, France). The isolates of *C. neoformans* were also identified by observing dark colony development on Staib medium [33] and by the presence of capsules in India ink stained microscopic preparations. *C. albicans* was also identified through the germ tube production test [34]. Phospholipase production was evaluated from at least two isolates of each yeast species from each bird species that had previously tested positive [15,17]. Phospholipase activity (Ph.a.) was determined as previously reported using the egg-yolk (EY) plate method [31] with some modifications. Briefly, isolates were grown on SDA slants at 37°C for 2 and 5 days pre-incubation time (Pr.t.) to prepare the inocula. Then, the strains were inoculated onto EY at 37°C and readings were taken after 2 and 5 days incubation time (I.t.). Formation of zones of precipitation around the colony was considered as indicative for enzyme production. Phospholipase production (Pz) was expressed as a ratio of colony diameter (a) to diameter of the zone of precipitation (b). Therefore, the higher the Pz value, the lower the production of phospholipases. Each strain was tested in duplicate and the Pz represents an average of the two Pz values reported.

Statistical analysis

The number of isolates within each species producing phospholipase and the Pz value at different periods of incubation were statistically compared by Chi-square test and Student's *t*-test, respectively. A value of $P \leq 0.05$ was considered to be statistically significant.

Results

Eighty five (11.1%) of the samples collected from cloacae tested positive for yeasts (i.e., 17 (9.3%) from

Group I, 7 (4.2%) from Group II and 61 (14.5%) from Group III). Fourteen (43.7%) positive samples were obtained from Group IV. A total of 246 isolates belonging to 13 species of yeasts were identified (i.e., 190 from 768 birds and 56 from 32 aviaries). A total of 163 isolates of yeast species and two or three isolates of the same yeast species recovered from each positive bird or aviary (i.e., 133 from the cloacae and 30 from aviaries), were tested for the ph.a (Tables 1 and 2, Figs. 1 and 2).

No differences in ph.a. were recorded ($P > 0.05$) among the same species of yeasts collected from members of Groups I–III, so Pz for each species has been reported as a mean value regardless of the family of birds from which it was recovered (Table 1, Fig. 1). A higher ph.a. was found in the yeasts recovered from Group IV samples (Table 2, Fig. 2). The number of phospholipase-producing yeasts and the Pz value changed as a function of both Pr.t. and I.t. In particular, after two days of Pr.t., the number of yeast isolates producing ph.a. were 51 and 64 at 2 and 5 days of I.t., respectively (Table 1). In contrast, after 5 days of Pr.t., the number of isolates producing ph.a. was 50 with no difference noted at 2 and 5 days of I.t. (Table 1). In general, the lowest value of Pz (i.e., highest ph.a.) was found after 5 days of I.t. (Fig. 1). In particular, *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. lusitaniae*, *C. pelliculosa*, *C. albidus*, *C. laurentii*, *T. beigelii*, and *S. cerevisiae* displayed the highest ph.a. after 2 days of Pr.t., while *C. famata*, *C. guilliermondii* and *C. neoformans* did so after 5 days of Pr.t. (Fig. 1). Ph.a. activity was never noted in *R. rubra* isolates recovered from cloacae (Table 1, Fig. 1).

Twenty two isolates (73.3%) from bird droppings produced ph.a., with the highest number found after 2 days of Pr.t. and 5 days of I.t. (Table 2). Yeasts from bird droppings showed a statistically higher ph.a. than did the yeasts from cloacae ($P < 0.05$) (Figs. 1, 2).

Discussion

A large number of yeast isolates recovered from the cloacae (48.1%) and excreta (73.3%) produced ph.a. with low Pz values, indicating a high enzymatic activity. As for the yeast species isolated from birds for which ph.a. was found, reports in the literature only deal with *C. neoformans* in pigeon and psittacidae droppings [29,32]. In the present study, ph.a. was noted in all the yeast species with the exception of *R. rubra* (from the cloacae). Our results indicate that birds of prey, migratory birds [15,17] and passeriformes not only act as carriers but may also spread phospholipase-producing yeasts through their droppings. It is also arguable that these yeasts may cause diseases to birds as has been demonstrated for *Candida* spp. and *Cryptococcus* spp. [35,36]. Temperature is likely to play a role in the ph.a. by influencing pathogenicity of the yeasts, whether from the cloaca and/or from the environment. Even if the number of isolates from the tested environment was smaller than that of the isolates from cloacae, the results of this study indicate that a greater number of isolates producing phospholipases

Table 1 Number of isolates of different species of yeasts isolated from cloacae of birds producing phospholipase. The enzyme activity after 2 and 5 days of pre-incubation (Pr.t.) and 2 and 5 days of incubation (I.t.) is reported. Statistically significant differences ($P < 0.05$) obtained with Chi-square test were marked with the same letters.

	+2 days of Pr.t.		+5 days of Pr.t.	
	+2 days I.t.	+5 days I.t.	+2 days I.t.	+5 days I.t.
Species	Pos/tot	Pos/tot	Pos/tot	Pos/tot
Candida albicans	5/16[a]	12/16 [a]	8/16	8/16
Candida guilliermondii	7/10	7/10	7/10	7/10
Candida tropicalis	6/8	6/8	6/8	6/8
Candida famata	3/6	3/6	3/6	3/6
Candida lusitaniae	0/2	1/2	1/2	1/2
Candida pelliculosa	3/4	4/4	2/4	2/4
Candida glabrata	0/3[b]	3/3[b]	0/3 [b]	0/3 [b]
Cryptococcus albidus	0/20[c]	5/20[c]	0/20 [c]	0/20 [c]
Cryptococcus neoformans	8/8	8/8	8/8	8/8
Cryptococcus laurentii	9/9	9/9	9/9	9/9
Trichosporon beigelii	2/10	2/10	2/10	2/10
Rhodotorula rubra	0/32	0/32	0/32	0/32
Saccharomyces cerevisiae	3/5	4/5	4/5	4/5
Total	51/133	64/133	50/133	50/133

[a,b,c] Chi-square test.

Table 2 Number of isolates of different species of yeasts isolated from bird droppings producing phospholipase. The enzyme activity after 2 and 5 days of pre-incubation (Pr.t.) and 2 and 5 days of incubation (I.t.) is reported. Statistically significant differences ($P < 0.05$) obtained with Chi-square test were marked with the same letters.

	+2 days Pr.t.		+5 days Pr.t.	
	+2 days I.t.	+5 days I.t.	+2 days I.t.	+5 days I.t.
Species	Pos/tot	Pos/tot	Pos/tot	Pos/tot
Candida albicans	4/4	4/4	0/4	0/4
Candida tropicalis	2/2	2/2	0/2	0/2
Cryptococcus neoformans	8/8	8/8	8/8	8/8
Trichosporon beigelii	4/4	4/4	4/4	4/4
Rhodotorula rubra	0/12[a]	4/12[a]	0/12[b]	4/12[b]
Total	18/30	22/30	12/30	16/30

[a-b] Chi-square test.

came from the environment than from cloacae and their ph.a. was also higher. Further support for this hypothesis comes from findings that have demonstrated that the lower the temperature (as in the environment) the higher the ph.a. [37]. Again, ph.a. was not found with *R. rubra* recovered from the cloaca but it was noted in the isolates collected from droppings in the environment.

Other factors such as the physiological and chemical characteristics of excreta (e.g., pH, protein, lipid and glucide composition) may influence phospholipase gene expression, or protein activity/ies. Overall, excreta may represent an enriched medium in which yeasts may easily grow and produce phospholipases.

The Pz values of *Candida* spp. isolated both from cloacae (Groups I–III) and excreta (Group IV) do not differ from those reported for the same species in diseased humans [23–25]. Conversely, *C. neoformans* from cloacae and from bird excreta had a higher ph.a. (i.e., Pz = 0.46 and 0.32, respectively) than the values found in isolates from pigeons and psittacidae excreta (Pz = 0.83) and from AIDS patients (Pz = 0.67) [30,33]. A likely explanation for these results is that conditions in cloacae or excreta may favour yeasts with a higher

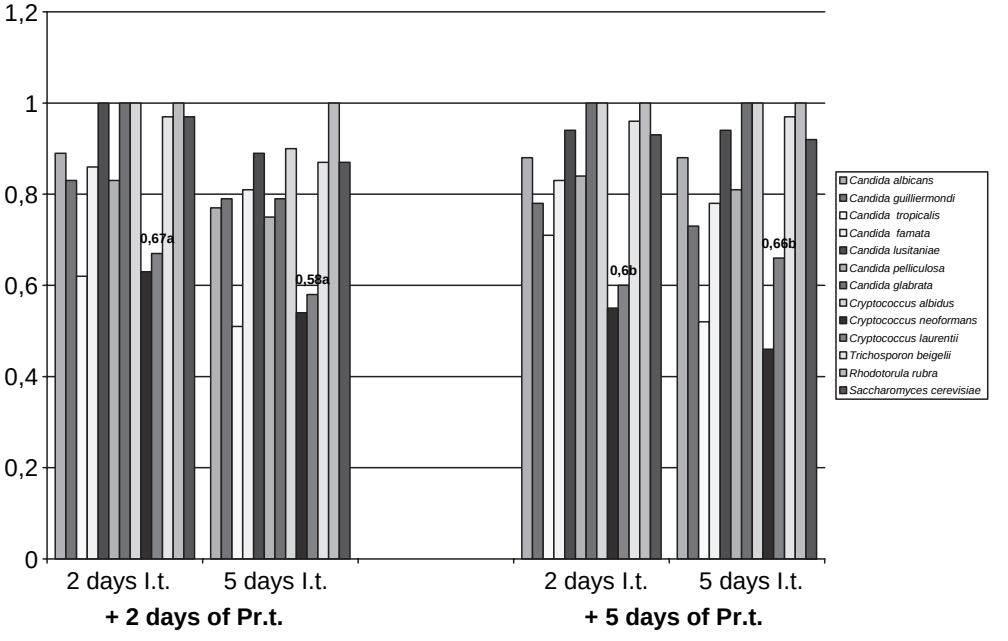

Fig. 1 Phospholipase activity (expressed as mean Pz value) of different species of yeasts isolated from cloacae of birds. The enzyme activity after 2 and 5 days of pre-incubation (Pr.t.) and 2 and 5 days of incubation (I.t.) is reported. Statistically significant differences ($P < 0.05$) obtained with Student's *t*-test were signed and marked with the same letters.

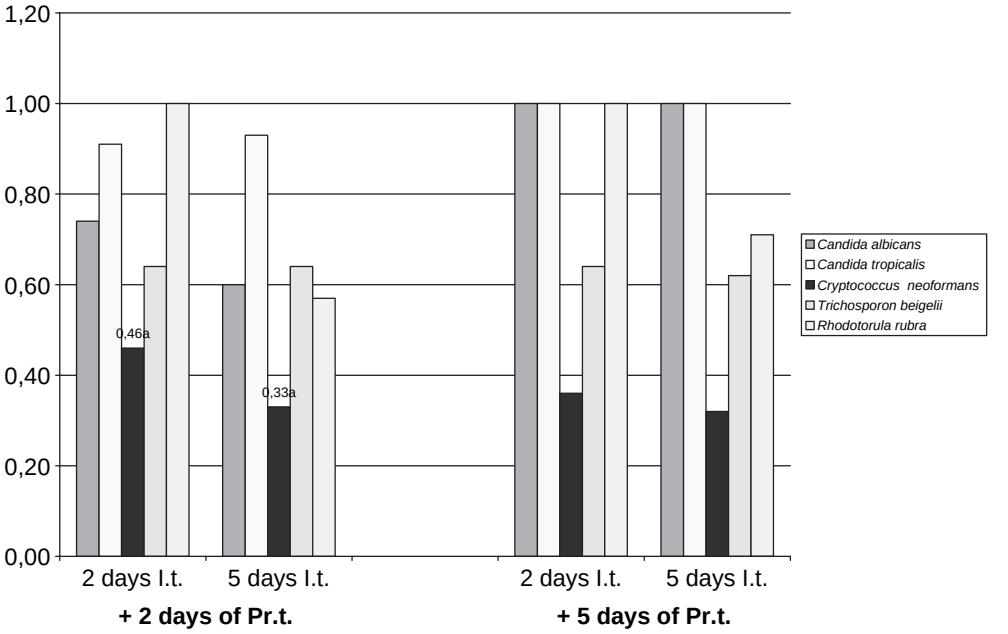

Fig. 2 Phospholipase activity (expressed as mean Pz value) of different species of yeasts isolated from bird droppings. The enzyme activity after 2 and 5 days of pre-incubation (Pr.t.) and 2 and 5 days of incubation (I.t.) is reported. Statistically significant differences ($P < 0.05$) obtained with Student's *t*-test were signed and marked with the same letters.

tendency towards pathogenicity. It was initially hypothesized that extracellular phospholipases were produced only by *C. albicans* [24], but they were also found in other *Candida* species (i.e., *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. lusitanie*, *C. krusei* and *C. kefyr*) [18,24,25]. To our knowledge, this is the first time that extracellular phospholipases have been detected in *C. guilliermondii*, *C. albidus*, *C. laurentii* and *T. beigelii*. No doubt the Pr.t. and I.t. affected the species of yeasts and the number of isolates with ph.a. as well as the Pz values. This finding could also be accounted for by the biochemical type of phospholipases produced. Furthermore, previous evidence has indicated that the yeast growth phase (i.e., Pr.t.) is relevant to phospholipase production by modulating gene and phospholipase expression [37]. Finally the evidence that *C. albicans* and *C. tropicalis* from cloacae had negative results if they were incubated after 5 days of Pr.t. could be due to the fact that in that condition they lose the potential virulence.

Overall, wild birds harbour yeasts in their cloacae and spread them in the environment. These yeasts are potential pathogens for mammals and represent a significant zoonotic concern due to the vast distances birds cover between different areas. Because of bird resting during migration in and around towns (i.e, small artificial lakes in urban parks), migratory birds as well as passeriformes and birds of prey are of importance for human health. The resting sites of these birds are commonly frequented by children and the elderly as well as immunocompromised patients who are at increased risk of contracting opportunistic diseases.

Since reports of human cryptococcosis and candidosis have been described both in immunocompromised and immunocompetent humans exposed to bird droppings [2–11], specific recommendations for the risk reduction of such infections are needed. Indeed, immunosuppressed individuals, and specifically those who are at high risk of contracting opportunistic diseases (e.g., HIV/AIDS) should be informed of the potential hazards of living or working in dirty environments infested by pigeons or other wild birds where guano is accumulated.

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