

Yeast isolated from cloacal swabs, faeces and eggs of laying hens

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Claudia Cafarchia¹, Roberta Iatta¹, Patrizia Danesi², Antonio Camarda¹, Gioia Capelli², Domenico Otranto¹

¹Dipartimento di Medicina Veterinaria, Università degli Studi di Bari “Aldo Moro”, Bari, Italy;

²Istituto zooprofilattico Sperimentale delle Venezie, Legnaro, Italy

Short title: *Candida albicans* and other yeasts in laying hens

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Address for correspondence: Claudia Cafarchia, Dipartimento di Medicina Veterinaria, Università degli Studi di Bari “Aldo Moro”, Str. Prov. per Casamassima km 3, 70010 Valenzano, Bari, Italy; tel +39 080 4679834; fax +39 080 4679839; email: claudia.cafarchia@uniba.it

Abstract

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Domestic and wild birds may act as carriers of human pathogenic fungi, though the role of laying hens in spreading yeasts has never been investigated. We evaluated the presence of yeasts in the cloaca (Group I, n=364), faeces (Group II, n=96) and eggs (Group III, n=270) of laying hens. The occurrence and the population size of yeasts on the eggshell, as well as in the yolks and albumens, were assessed at the oviposition time and during storage of eggs at 22±1°C and 4±1°C.

A statistically higher prevalence and population size of yeasts were recorded in Group I (49.7% and 1.3x10⁴ CFU/ml) and II (63.8% and 2.8x10⁵ CFU/ml) than in Group III (20.7% and 19.9 CFU/ml). *Candida catenulata* and *Candida albicans* were the most frequent species isolated. *Candida famata* and *Trichosporon asteroides* were isolated from the eggshells, whereas *Candida catenulata* was also isolated from yolks and albumens. During storage, the yeast population size on the shell decreased (from 37.5 to 8.5 CFU/ml) in eggs at 22±1°C and increased (from 4.6 to 35.3 CFU/ml) at 4±1°C.

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The laying hens harbour potentially pathogenic yeasts throughout their gastrointestinal tract, and are prone to disseminating them in the environment through the faeces and eggs. Eggshell contamination might occur during the passage through the cloaca or following deposition and yolk and albumen contamination might depend on yeast density on eggshell

Introduction

Amongst yeasts, *Candida* spp., *Cryptococcus* spp., *Trichosporon* spp., *Rhodotorula* spp., *Malassezia* spp., *Sporobolomyces* spp. and *Saccharomyces* spp. induce cutaneous and/or systemic diseases in animals and humans. ¹⁻³ Arendrup et al., 2014. These infections are usually characterised by an endogenous source, as the yeasts commonly colonize the skin and the intestinal mucosa of humans and animals. However, an exogenous origin (i.e., indirect transmission between patients and health care workers' hands and food consumption, via contact) for *Candida lusitanae*, *Candida parapsilosis*, *Candida pelliculosa* and *Candida famata* infections has been suggested. ⁴⁻⁶ Domestic and wild birds are **reservoirs of potentially pathogenic yeasts** and they can easily spread yeasts in the environment via their faeces. ⁷⁻¹¹ Pathogenic yeasts were isolated from the faecal or cloacal content of psittacidae, passeriformes, columbiformes and falconiformes. ^{10,12-14} Nonetheless, despite their importance as egg producers, scientific data concerning laying hens as infection source of yeasts is lacking. ^{15,16} In hens, egg contamination by microorganisms may occur before oviposition with a direct contamination of the yolk, albumen and eggshell membranes ¹⁷⁻²¹ or after oviposition, via contact with infected faeces or cloacal mucosa. ^{16,22,23} In the latter case, environmental factors, during egg storage, may affect the load of microorganisms on the eggshell as well as inside the eggs (i.e., albumen and yolk). ^{15,24-26} Studies on fungal egg contamination are scant and those available focus mainly on the filamentous fungal species isolated from the eggshell. ²⁶ In addition, no data is available in literature on the relationships between egg storage and yeast population.

Thus, the aims of this study were i) to evaluate the occurrence of yeasts in the cloaca, faeces and eggs of laying hens, ii) to assess the presence of yeasts on eggshells and in the yolks and albumens and iii) to investigate the effects of storage temperature on egg-yeast populations of eggs.

Material and Methods

Study population and farm management

From February to May 2016, a study was carried out on egg-producing poultry farms in the Apulia region in southern Italy (latitude 39°57' and 41°47' North, longitude 18°17' and 15°43' East). Overall, 12 egg-laying intensive poultry farms housing a total of ~250.000 animals were examined. All the farms were located in rural and/or sub-urban areas, and fully complied with the Council Directive 1999/74/EC that lays down minimum standards for the protection of laying hens.

These farms were routinely examined for the adequate husbandry practice by veterinarian of the Department of Veterinary Medicine, University of Bari Aldo Moro. The veterinarians reported that the reared animals were healthy

Farms were arranged with one and/or two sheds with 2-4 blocks of cages each. A total of 15 sheds were enrolled in the study. Birds were housed in enriched cages (n=11), or reared in a free run housing system (n=1). In all cages, the feeding and watering containers were sanitized every four weeks by using a commercial solution (i.e., sodium hypochlorite, quaternary salts or iodine compounds) and faeces were removed weekly.

Sampling collection and processing procedures

Three groups of samples were investigated:

Group I: swabs from cloaca (n=364). For each poultry farm, at least 24 animals (2-3 animals/row) were randomly swabbed from the cloaca by using a sterile cotton swab.

Group II: droppings (n=96). In each single shed, a volume of 50 gr of droppings was collected from the conveyor belt of each row. Then, 1 gram of droppings was suspended in 9 ml sterile saline solution (NaCl 0.9%) containing 1000 mg/ml streptomycin and 500 UI penicillin/ml.

Group III: eggs (n=270). Eggs were collected automatically through a conveyor belt and immediately following oviposition (T0).

A set of 30 eggs was separated for mycological investigations at time zero (T0), whereas 120 eggs were stored at room temperature (i.e., 22±1°C) and an equal number preserved at refrigeration

conditions (i.e., $4\pm 1^{\circ}\text{C}$) for 28 days. For both groups, 6 eggs from both room and refrigeration temperature were analysed every 7 days from T_0 to the 28th day of storage (i.e., T_0 , T_{+7} , T_{+14} , T_{+21} and T_{+28}).

In order to evaluate the presence of yeasts on eggshells, each egg was placed in a sterile Stomacher bag containing 10 ml of buffered peptone water, and washed for 1 minute. Then, a volume of 100 μl of the washing solution was cultured in an appropriate medium .

For the mycological evaluation of albumens and yolks, each washed egg was immersed in 70% ethylic alcohol for 5 minutes and then passed over a flame. The shell was opened using sterile scissors and the albumen and yolk were placed in separate sterile bags. The contents were homogenized for 30 seconds at normal speed in a Stomacher 400 Circulator (Seward, London, UK) and 100 μl cultured onto an appropriated medium. All specimens were processed within few hours from collection.

Mycological culture and identification procedures

Swabs from Groups I, 100 μl of dropping solution from Group II, 100 μl of eggshell washing solution and 100 μl of albumen and yolk homogenate from Group III were cultured onto Sabouraud dextrose agar with chloramphenicol (0.5 gr/l) (SAB, BioLife®), incubated at 30°C for 7 days and observed daily. In addition, samples were serially diluted in sterile saline solution until reaching a 10^{-4} dilution. One hundred microliters of each dilution were cultured as reported above.

Cultures were defined “positive” when fungal colonies were confirmed microscopically by Gram staining. Colonies were counted and the yeast population size was expressed as mean of colony forming units (CFU)/plate. According the different group, CFU were referred as CFU/swab (Group I), CFU/gram of dropping (Group II) and CFU/ml of wash egg solution (Group III). At least six colonies, for each positive culture were sub-cultured in SAB agar slants for yeast identification at species level, by microscopic morphology, urea hydrolysis and sugar assimilation by ID32C (bioMerieux®).^{10,12}

Molecular identification

Yeast identification was confirmed molecularly for all yeast species isolated from eggs, cloaca swabs and faeces from animals in the shed. DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen, Courtaboeuf, France) following the manufacturer's instructions and stored at -20°C until further processing. A negative control was included in each series of DNA extractions. The molecular identification of isolates was achieved by polymerase chain reaction (PCR) and ~~sequencing~~ [sequencing of internal transcribed spacer \(ITS\) regions \(including ITS-1, 5.8S rRNA, and ITS-2\) of ribosomal DNA using primers ITS1 \(5'-TCCGTAGGTGAACCTGCGG-3'\) and ITS4 \(5'-TCTTTTCCTCCGCTTATTGATATGC-3'\) and PCR conditions as previously reported.](#)²⁷ ~~of internal transcribed spacer (ITS) regions (including ITS-1, 5.8S rRNA, and ITS-2) of ribosomal DNA following protocol and PCR conditions as previously reported.~~²⁷

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Sequences were edited with SeqScape software v2.5 (Applied Biosystem, Foster City, CA, USA) and aligned with Muscle integrated in the MEGA6 package²⁰. The BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST/>) program allowed comparison of sequences with those available from International Society for Human and Animal Mycology ITS Database (ISHAM ITS Database - <http://its.mycologylab.org/>) to confirm taxonomy identification and absence of contamination. A rooted tree of ITS sequences was built using the Neighbor joining algorithm with 1000 bootstrap replicates in MEGA6. *Schizosaccharomyces pombe* was used as outgroup (AN????). All nucleotide sequences obtained in this study have been deposited in the GenBank database under accession numbers MF370975 - MF370983.

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Statistical analysis

The prevalence of yeasts in different groups was compared using the *Chi-square* test. The *t-student* was used to compare the mean value of CFU recorded in the samples from different groups. A value $p < 0.05$ was considered statistically significant.

Results

Yeasts were isolated with a statistically higher prevalence and population size in Group I (49.7% and 1.3×10^4 CFU/swab, respectively) and II (63.8% and 2.8×10^5 CFU/gr) than in Group III (21.8% and 16.1 CFU/ml). The occurrence and the population size of yeasts recovered from yolk and albumen samples were 1.1% and 1 CFU/ml, respectively (**Table 1**). A total of 2091 yeast strains belonging to 19 yeast species was isolated and identified (**Table 2**). *Candida catenulata* and *C. albicans* were the most frequently isolated yeast species in all groups (**Table 2**).

All species isolated from eggs (Group III) were also isolated from cloacal samples (Group I) ~~or~~ and faeces (Group II), with exception of *C. famata* (1.5%) and *Trichosporon asteroides* (0.7%) (**Table 2**). However, *C. catenulata* was the only species isolated from yolk and albumen, with a prevalence of 1.1% and had the same ITS profile of those isolated from the corresponding eggshell (**Figure 1**).

The occurrence and the population size of yeasts on eggshell stored at different temperatures (i.e., $4 \pm 1^\circ\text{C}$ and $22 \pm 1^\circ\text{C}$) varied throughout storage (**Figures 3 and 4**). The occurrence of yeasts was significantly higher ($p < 0.05$) on eggshell stored at $4 \pm 1^\circ\text{C}$, than at $22 \pm 1^\circ\text{C}$ throughout storage (**Figure 2**). In particular, from T_0 to T_{+14} , the occurrence of yeasts increased from 33.3 to 50% in eggs stored at $4 \pm 1^\circ\text{C}$ and decreased from 33.3% to 13.3% in eggs stored at $22 \pm 1^\circ\text{C}$ (**Figure 2**). From T_{+7} to T_{+21} , the population size of yeasts on eggshell decreased significantly (from 37.5 to 8.5 CFU/ml) at $22 \pm 1^\circ\text{C}$, and increased (from 4.6 to 35.3 CFU/ml) at $4 \pm 1^\circ\text{C}$ (**Figure 3**). The yeasts were isolated from yolk and albumen only at T_{+7} when the eggs were stored at $4 \pm 1^\circ\text{C}$ and at T_{+14} when eggs were stored at $22 \pm 1^\circ\text{C}$ (**Figures 2 and 3**).

Discussion

Yeasts, and mainly *Candida* spp., are present in the cloaca of laying hens and contaminate the environment through faeces and eggs. The prevalence of yeasts in cloaca swabs (49.7%) and in the faeces (63.8%) was higher than those previously recorded in bird of pray (i.e., 9.9%)¹² and migratory birds (25%)¹⁰ indicating the role that laying hens play as carrier and spreaders of these microorganisms.

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The prevalence of yeasts in cloaca swabs (49.7%) and in the faeces (63.8%) was higher than that previously recorded in wild (9.9%) and migratory birds (25%),^{10,12} indicating the role that laying hens play as carrier and spreaders of these microorganisms. The more frequent occurrence and larger population size of yeasts in the faeces, when compared to those in cloaca swabs or on eggs, confirms that excreta are a suitable enriching medium, which favours the growth of yeasts.^{7,8,10,12}

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Additionally the fungal population size of faecal and cloaca samples as determined by our study was higher than those documented in similar studies (Shokri et al.,2011) thus confirming a greater change of laying hens in disseminating yeasts to other animals, and environment.

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Interestingly a total of 15 yeast species were isolated from the cloaca of laying hens and majority of them were represented by *C. catenulata* and *C. albicans*. All these yeast species were known as the commensal organisms of gut microbioma of bird.^{28,10} However the present study demonstrated a higher colonization by *C. catenulata* than *C. albicans* and this is contrary to that observed in gut microbioma of poultry (Shokri et al. 2011; Subramanya et al., 2017), or migratory birds (i.e. Woodcock and Collared-doves)¹² and bird of pray (Scops Owl)¹⁰, thus confirming that the gut fungal species composition might vary accordingly to dietetic, hygienic or other ecological factors [Subramanya et al., 2017]. As far *C. parapsilosis*, its presence in the cloaca might be due to an accidental contamination of swab from the bird anal mucosa being this species usually considered as commensal organism of animal and human skin²⁹. The finding that *Candida albicans*, *C. catenulata*, *C. parapsilosis* and *Rhodotorula rubra* were the yeast species isolated in samples from faeces and eggs other than cloaca suggests that laying hens might spread the yeasts in the environment by faeces and eggs. Since ITS sequence analysis is not useful enough to genotyping *Candida* strains, more accurate analysis (i.e., RAPD analysis, PCR fingerprinting or microsatellite typing) should be performed in order to demonstrate the genetic identity of strains from different sources (Güzel et al., 2013).

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The isolation of *Candida albicans*, *C. catenulata*, *C. parapsilosis* and *Rhodotorula rubra* from the cloaca, faeces and eggs, suggest that these yeasts are part of the physiological commensal flora of gastrointestinal tract of laying hens and might be spread in the environment by faeces and eggs. However, while *Candida albicans*, *C. catenulata* and *R. rubra* are known as commensal microorganisms of the gastrointestinal mucosa of pigeons²⁸ or migratory birds¹⁰, *C. parapsilosis* is

usually considered a commensal of animal and human skin²⁹, and thus its presence in the cloaca might be due to an accidental contamination of swab from the bird anal mucosa.) On the other hand, the absence of some species of yeast in faeces or in eggs (i.e., *C. colliculosa*, *C. krusei*, *C. rugosa* and *S. cerevisiae*) might be due to the fact that these species do not find a suitable environment outside the cloaca. On the contrary, the absence of *C. pelliculosa* and *T. asahii* in cloaca swabs might indicate that these species are not commensal of the gastrointestinal tract of birds, being epi- or esoprophytes of skin of healthy subjects and plants.⁹ All of the yeast species retrieved in the cloaca, with the exception of *C. famata* and *T. asteroides*, were also isolated from the eggshell and/or yolk and albumen, suggesting that these yeasts contaminate the whole egg during the passage through the cloaca or post-deposition.

Besides *T. asteroides*, which is an episaprophytic yeast colonizing different parts of the human body (i.e., oral cavity, gastrointestinal system, urinary tract or skin), *Candida famata* was isolated from a large range of sources (e.g., ground, seeds, etc.) suggesting that eggs might be contaminated from environmental sources.^{30,31} However, the small number of yeast colonies obtained from the eggshell (19.6 CFU/ml) confirms that this microhabitat is not favourable to the yeast growth.³² In addition, the physical and chemical barriers of the eggshell most likely prevent the contamination of yolk and albumen during oviposition, as demonstrated for other microorganisms (i.e., enterobacteria, *Salmonella* serotypes and *Pseudomonas* spp..^{16, 31} However, during storage of eggs, the prevalence and the population size of yeasts increased, mainly at T₊₁₄, indicating that the egg protective factors above (i.e., cuticle, shell inner membranes, proteins and albumen pH) lose their ability to inhibit microorganisms over time.^{15,16} The storage temperature seems to play a pivotal role in increasing the population size of yeasts on eggshell^{14,24} favouring the growth of yeasts at 22±1°C, rather than at 4±1°C. Consequently, the high density of yeasts on eggshell recorded on eggs at 22±1°C might have favoured the penetration of yeasts in the eggs.²⁴ On the contrary, the observation of yeasts inside eggs stored at 4±1°C might be due to temperature and humidity variation caused by the fridge door

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opening (i.e., frost effect). The finding that only *C. catenulata* was retrieved inside the eggs during storage might be due to the high population size of this yeast on the shell or to its higher survival in the environment than other yeasts. Future studies are needed to address this finding.

All the species of yeast isolated in the present study are considered opportunistic pathogens or emerging yeasts causing infections in immunocompromised patients. ³ With the exception of *C. albicans*, the main agent of severe fungal infections in humans ^{3,33}. Interestingly, *C. catenulata* and *C. famata* were recognized as agent of food borne have been seldom reported in cases of septicaemia in cancer patients, with food being suspected as source of infection. ^{2,33,34} Therefore, *Candida* non-*albicans* isolated from food could represent a risk since these yeasts produce virulence factors (i.e., phospholipase, protease and haemolysins) ^{3,35,36} that would allow them to cause infections; thus, contaminated eggs may represent a potential source of pathogenic yeasts and they should be carefully handled and properly consumed by immunocompromised patients.

Conclusion

Laying hens are reservoirs of potentially pathogenic yeasts and might spread these organisms in the environment through faeces and eggs. The contamination of eggshell might occur during the passage through the cloaca or post deposition but it is very low immediately after oviposition. Laying hens are carriers and spreaders of potentially pathogenic yeasts for humans and animals with the contamination of eggshell most likely occurring during the passage through the cloaca or post deposition. On the other hand, the storage temperature affects both the prevalence and the population size of yeasts on eggshell, favouring their penetration inside the eggs. Proper management of poultry farm and/or correct sanitification procedures should be implemented to reduce contamination of eggs by pathogenic yeasts. At the same time, consumer education about good sanitary practices in handling eggs is important to reduce the risk of animal and human infection.

Declaration of interest

All authors declare to have no conflict of interest.

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