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RESEARCH ARTICLE OPEN ACCESS

Fungi Activity on Chicken (*Gallus gallus domesticus*) Eggshell and Their Pores Invasion by Scanning Electron Microscopy

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ABSTRACT
Eggshell exposure to both, field and storage toxigenic fungi, is of concern due to their possible metabolites transmission into the inner egg. The presence of fungi contamination in the nests environment, is expected due to its substrate, optimal temperature and humidity. In addition, the age of hens and eggshell increasing pore numbers together with egg storage conditions may influence their quality and safety. Diverse genera of fungi spores can get attached to the eggshell surface apart from the nest, also due to residual moisture coming from the natural moisture. This study reported the natural contamination by fungi of genera *Fusarium* and *Curvularia* (growth behavior) and activity through the eggshell pore canal investigated through scanning electron microscopy (SEM) being registered both surfaces (outer / inner) and along the pores (layers crossing –cuticle/ vertical crystal layer / palisade / mammillary / outer / inner layer) length (after 7 days incubation, at 23.5ºC and 82% environment relative humidity). As expected, natural contamination and improper storage provide conditions for fungi hyphae to pass through the eggshell pores and reach the outer shell membrane (inside the shell). SEM showed the presence of fungi from a thick mycelia with the reproductive structures. The genera of fungi identified in this study found in the pores, ideal places to develop their reproductive structures and contaminate the internal region, which affects the quality of eggs.
Key words: Contamination, Chicken, *Curvularia*, Eggshell, Fungi, *Fusarium*, Microscopy, Pores.

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I. INTRODUCTION

The poultry production chain plays an important role in several countries (Scanes, 2007). In the agricultural scenario, it stands for the ability to produce high-quality food and allows the population to access animal protein at an affordable cost(Nys *et al.* 2011).

Regarding eggs, several countries produce billions of them and thousands of laying hens per year (ABPA, 2017; UEP, 2019). Along with this large volume of production, there is some concern about food safety when it comes to filamentous fungi (*Fusarium*/*Aspergillus*/*Penicillium*) contamination, due to their possible mycotoxins production. Those toxins can affect different organs such as the central nervous system, embryonic malformation, kidney and liver problems in the birds as for humans (Hassan, 2010; Zain, 2011;Scussel et al., 2000,2018).

Hygiene processes against microorganisms (bacteria/yeasts/fungi) can increase contamination when the use of chemicals is applied incorrectly and the poor storage of eggs with temperatures and relative humidity are above 3.5ºC and 70%, respectively (Morgulus and Spinosa, 2005: Figueiredo 2008;Clímaco *et al.*, 2018).

In addition, both the shell surface (moisture and muco/protein) and its protection structure (which is well organized), divided into layers (cuticle/ vertical crystal layer -VCL/palisade, mammillary and internal and external membrane) composed by sequential deposition of 96.5 and

3.5% of mineral and organic fraction, respectively (Hincke *et al.* 2012a).

Nests material (dry straw, rice husk, etc.),utilized for eggs posture, must be clean, dry and free of microorganisms including fungi to prevent dirty/contaminants and cracks to the eggs, which would further increase the possibility of contamination (Soares *et al.* 2017).

Eggs (prior posture) are usually less contamination despite of types (Okamura *et al.* 2007). As expected, after laying (exposure to the environment), eggs may be contaminated by different sources, including soil, dust and poorly cleaned nesting materials(Goodenough and Stallwood 2012).

Several sanitizing compounds are tested to reduce microbial contamination, especially in wash water. On the other hand, often these sanitizers are ineffective in controlling eggshell fungi (Knape *et al.* 1999). Fungi spores an other contaminants can stick(surface) and/or reach(through pores)the edible part (egg white and yolk) and may lead to risks to human and animal health (Suleiman, 2018; Barbosa *et al.*, 2012).

Scanning electron microscopy (SEM) was used to evaluate the quality of the hen's eggshell, mainly to obtain information about the mineralization of the layers that make up the shell. Regarding microorganisms, it is utilized as a tool for identification and investigation of reproductive structures of filamentous fungi (Eduard et. al 1988; Choi *et al.*, 2010;Soares, et al., 2018).

This work aimed to evaluate the behavior of filamentous fungi by natural contamination of chicken eggs (*Gallus gallus domesticus*) and shell invasion activity through the pores utilized scanning electron microscopy (SEM) techniques.

II. MATERIAL AND METHODS

2.1 MATERIAL

Samples:chicken whole fresh eggs – collected* two days after laying on nests (mix of wood shavings and straws).*from a poultry farm where birds (over two years old) are reared free, with *ad libitum* water and a controlled diet, formulated with corn and soybean meal and pasture.

Culture media and reagents*:* potato dextrose agar (PDA) and peptone bacteriology media were from Himedia (Curitiba, Parana, Brazil) and chloramphenicol from Vetec (Duque de Caxias, RJ, Brazil), phenolphthalein and sodium hydroxide, from Merck (Darmstadt,Germany).

Equipment: micropipette(1ml), Kasvi, (Curitiba, PR, Brazil), laminar flowcabinet, Veco (Campinas, SP, Brazil); fume cabinet, Quimis (Diadema, SP, Brazil) colonies counter, Phoenix (Araraquara, SP, Brazil), bacteriological incubator, Model Sterilifer Sx13 Dtmc (São Paulo, SP, Brazil); scanning electron microscope(5000x), model JSM- 6390LV, Jeol (Peabody, Mass., USA) and gold coating machine, model EM- SCD 500, Leica (Leider, IL, USA). Other materials: stubs (small metal blocks), 9 and 10 mm for diameter for height, rescpectivelly.

2.2 METHODS

Egg incubation: briefly, 12 whole eggs were stored in a microbiological incubator at an average temperature of 23.5 ºC for a period of 7 days. The samples were observed daily through the glass door to monitor the natural development of fungi.

Eggshells preparation for microscopy (SEM): Briefly, different fragments (5-10 mm) of eggshells (where the colony of fungi grew). Then, these fragments were immediately prepared for SEM observation (surfaces and cross-sections) after being fixed in stubs (containing double-sided carbon tape) were coated with gold (with a layer of gold at 40 nm under vacuum).

III. RESULTS AND DISCUSSION

It was possible to observe colonies of fungi established on the eggshell, and after the shell has broken, we can observe the dark coloration on the inner membrane of the shell (inner region of the egg), possibly caused by the fungi. However, when analyzed under SEM, it was not possible to observe with visual quality hyphae or other vegetative structures of the fungi in the inner membrane of the shell.

The network of interlacing fibres in the egg membrane (Ketta and Tůamová 2016) resembles filamentous fungal hyphae, which implies further studies to assess the behavior of the hyphae inside the egg. Probably protected against microbial attacks, by the chemical defense system present in the membranes of the shell and the albumen (Stadelman, William J.; Cotterill 2013).

3.1 FUNGI GROWTH ON EGGSHELL – OUTER AND INNER SURFACE

From the data obtained on the chicken eggshell, it was possible to observe its growth behavior through the eggshell (both, surfaces and pores) by applying microscopy techniques (SEM). figures 1-5, respectively, shows SEM micrographs of eggshell surfaces (inner and outer) and pores infected by their hyphae/mycelia and reproductive structures.

As expected, different genera of fungi were able to grow on the eggshell (Fig1a,b).Some with intense development and propagate due to

temperature and humidity conditions and eggshell substrate (dos Santos *et al.* 2009).In the literature, fungi belonging to the genera: *Penicillium*, *Alternaria*, *Mucor*, *Rhizopus* sp. were reported by Szablewski and Tomczykin commercial fresh eggs shell. Authors implied that the contamination came from the storage conditions egg were exposed (Szablewski and Tomczyk, 2015).

Fungal contamination often occurs in the nest, due to the substrate inserted to accommodate eggs and hens(Cumeras *et al.* 2016). The type of nest is often related to the fungal growth of eggs, nesting eggs in boxes exhibited less fungi growth than open cup nest eggs (Godard *et al.* 2007).

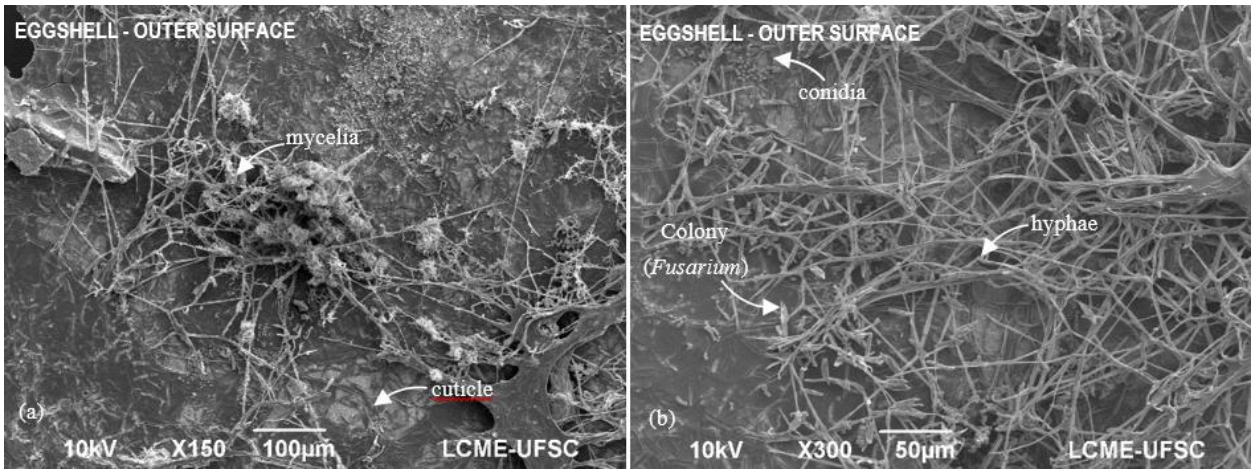

Figure 1. SEM micrographs of eggshell OUTER surfaces: (a) different genera and STRUCTURES of fungi on eggshells and (b) *Fusarium* natural growth and other conidia [150 -300x] -7 days incubation, 23,5ºC.

The propagation by *Fusarium* hyphae (mycelia) on the eggshell is relevant, as it increases the concern with the possible formation of its metabolites in the eggs, both in the external or internal region of the egg. Studies suggest that there may be bioaccumulation of mycotoxins from *Fusarium beauvericina* and *enniatins* in egg yolk (Jestoi *et al.* 2009).

Fungal genera, such as *Fusarium*, are able to grow on egg shells)(Tomczyk *et al.* 2018).. The study carried out by (Tomczyk *et al.*(2019)isolated toxigenic pathogenic species (*F.culmorum*) and (*F. equiseti*) and under controlled storage conditions (moisture: 35-95% and temperature: 8-25ºC). Fungi crossed through the shell and found ideal conditions to produce toxins. These conditions can favor storage or promote the development of fungi on the eggshell and compromise quality (Tomczyk *et al.* 2019).

The recommended consumption of eggs is around two weeks or refrigerated for 2 months, after which the eggs reduce the quality parameters (Feddern *et al.* 2017). Eggs stored for 40 days in ambient and humid conditions, showed internal contamination by fungi (dark coloration on the outer shell membrane), in addition to identifying two fungi species *Cladosporium macrocarpum* and *Botrytis cinereal* (Cumeras *et al.* 2016).

SEM micrographs show in the figures (2a,b) adhered to the inner region of the egg (outer shell membrane), the reproductive structures of the fungi (conidias and hyphae), respectively. The reduced proliferation may be related to the short storage period (7 days) or the defense system that albumen proteins have against organisms that can invade immediately before the structural changes in the shell membranes have been completed(Burley and Vadehra, 1989; Hincke *et al.* 2011).

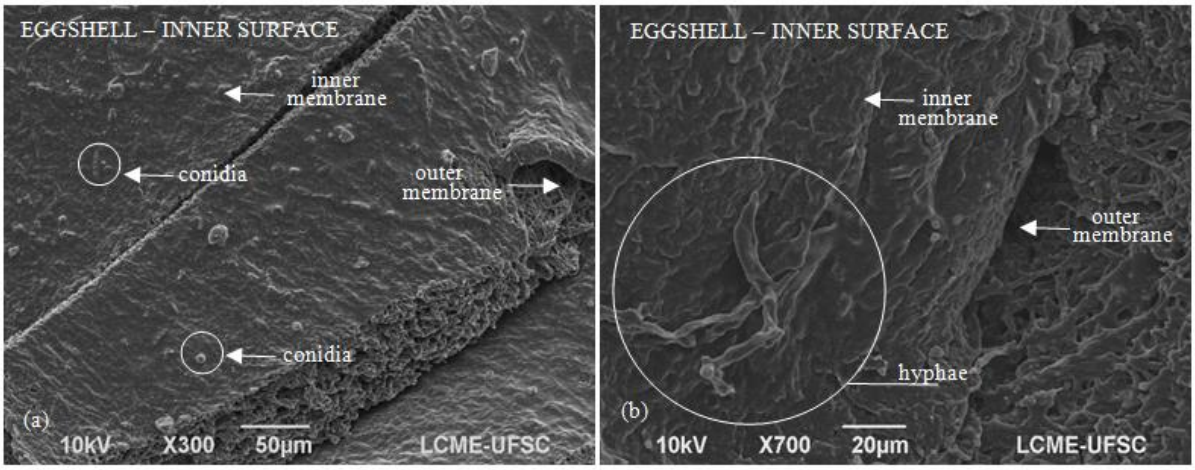

Figure 2. Eggshell INNER surface: (a) CONIDIAS adhered to the OUTER MEMBRANE [300x]and (b)presence of HYPHAE in the OUTER MEMBRANE of the eggshell [700x].

Tomczyk *et al.*, (2019)observed that after three weeks, regardless of storage conditions, fungi and significant reproductive growth on the eggshell surface were isolated and that the caged birds had lower concentrations of mycotoxins in the albumen.

The nests can contain conidia and contaminate the egg during the time it remains in the nest substrate (Andreatti Filho *et al*. 2009).Utilizing diets contaminated with Zearalenone, Dänicke *et al.* (2002) reported a loss in chicken performance. However, no residues were found in yolk, albumen and follicles greater (Dänicke *et al.* 2002).

3.2 FILAMENTOUS FUNGI AND THEIR ACTION ON THE SHELL

Eggshell microstructures versus fungi development/transfer/propagation

Chicken eggs have natural porosity that allows contamination by filament fungi in the environment and even after cleaning if improperly stored(Lacerda, 2011). However, hyphae can increase & pore diameter and developed over the canal to reach the albumen (egg edible part).

One of the first studies on the eggshell structures reported the presence of 7000 to 17000, and that in older mother eggs had a higher pore size per egg area (cm^2), inferring that egg quality (< pores numbers/ porosity/ impurities/passage versus safety) decreases with age (Tyler 1953, Barbosa *et al.* 2012).

It is important to know the living organisms in food, including fresh eggs. Through SEM, it is possible to identify insects, mites, fungal genera, and better observe reproductive structures, filamentous fungus genera such as *Aspergillus* and *Rhizopus*, in addition to their morphological differences and spore concentrations(Soares *et al*.2018,2019;Eduard, 1988; Da Cunha *et al.*, 2009).

Figure 3 shows colony development, dissemination and movement of filamentous fungi of the genera *Fusarium* sp. and *Curvularia* sp., where they find the pores and lodge. The fungal colony in figure 3a.1 is beginning to pore invasion, where there are no conidia, making it difficult to identify the genus. However, in previous figure 3.a2, we can see that a large amount of hyphae from inside the pores, indicating an ideal site for fungal development. We can also observe the presence of conidia, hyphae, with the growth of their extremities (sprout), which causes an increase in pore diameter when compared to the adjacent pore.

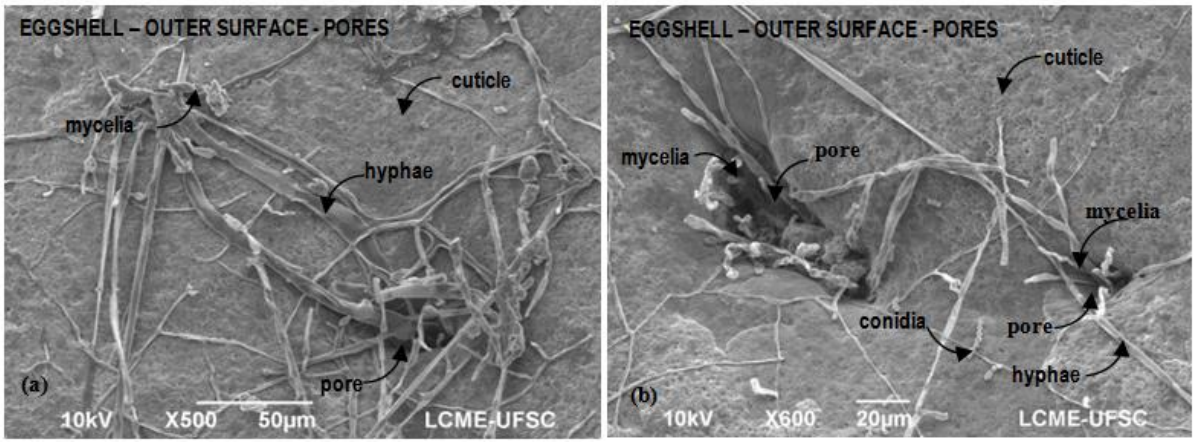

Figure 3. SEM micrographs of eggshell OUTER surface: (a) presence of fungi and their vegetative structures (MYCELIA and HYPHAE) inside the eggshell PORE [500x] and (b) difference in size between the pores and the presence of mycelia and CONIDIA adhered to the CUTICLE [600x].

In evaluating the microbiological quality of the shell, Village *et al.*, (2016) reported the presence of fungi pathogens of food origin. Fungi genera such as *Mucor* sp., *Rhizopus* sp., *Aspergillus* sp., *Fusarium* sp. and *Penicillium* sp., which constitutes a serious risk to consumer health (Scussel et al. 2014). As we can see in figures 3a.1,2 there are at least 3 genera of fungi, *Fusarium*, *Curvularia* and *Aspergillus* sp. who have found an ideal place for their development(Venkatesh Babu *et al.* 2018).

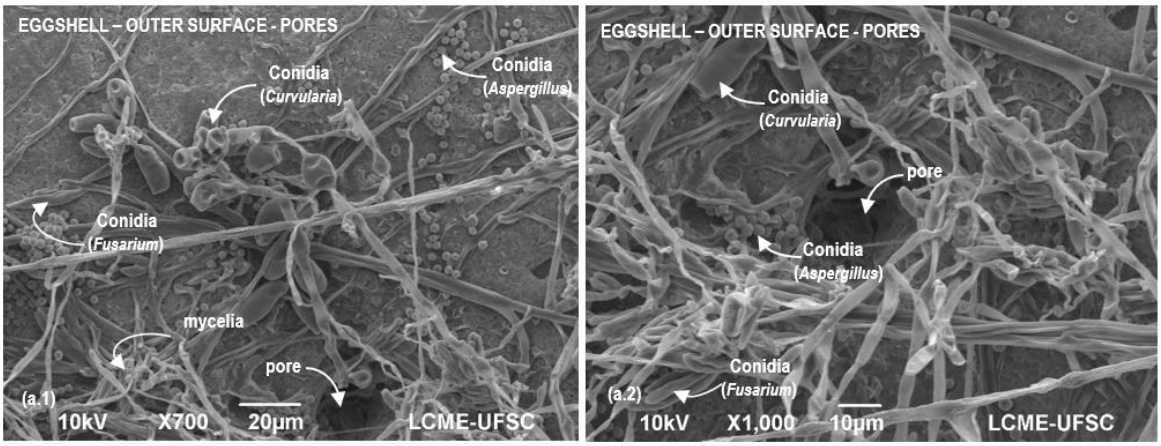

Figure 4. Presence of filamentous fungi and the formation of colonies in the CUTICLE and PORES of EGGSHELLS SURFACE (a.1) fungi growth INSIDE and AROUND the pores [700x] (b) presence of THREE FUNGI GENERA and total PORE INVASION [1000x].

Even with their wide distribution in the environment, *Curvularia* filamentous fungal species associated with allergic syndrome, but uncommonly cause infection in humans, cause damage to grain crops(Kerbelker *et al.*, 2019;Hussaini *et al.*, 2007;Bheemanagouda et al, 2014).

Several studies highlight the importance of endophytic fungi with *Curvularia* sp as a source of bioactive secondary metabolites and their antimicrobial action(Avinash *et al.* 2015, Kaaniche *et al.* 2019).*Fusarium* can produce mycotoxins and contaminate both table and hatching eggs for chick production. Generally, foods that are highly contaminated with mycotoxins (zearalenone, trichothecenes, fumonisins and deoxynivalenol) expose a serious risk to human and animal health(Scussel et al. 2018; Magan and Olsen, 2004; Asam, et al., 2017).

3.3 CONTAMINATION BY FUNGI THROUGH PORE CANAL

Previous micrographs show the outer region with the pores and cuticle, which plays an important role in preventing microbial penetration into the eggshell (Samiullah and Roberts 2014). However, the fungi were able to cross the shell through the pore canal and reach the eggs external

membrane under conditions established by the experiment, simulating inadequate storage (Figure 5).

The pore canal was completely invaded by hyphae and easily crossed, reaching the interior of the egg (outer shell membrane), which has protein matrices as a natural defense system (Solomon *et al.* 1994).

The shell has 5 layers described from outer to inner (cuticle, vertical crystal layer, palisade, mammillary and shell membrane), the pores that allow the diffusion of metabolic gases and water vapor (Hincke *et al.* 2012b).

Figure 5.a,b it is possible to observe the layers of the eggshell where the fungi reach the inner membrane of the shell that shows a protrusion, which possibly triggered the natural defense mechanism of the egg that is present in the inner membrane of the shell (Mine *et al.* 2003). Higher magnification of the cuticle region and vertical crystal layer shows more clearly the behavior of the fungi in the pore canal, with formation of mycelia inside (Figure 5.c,d).

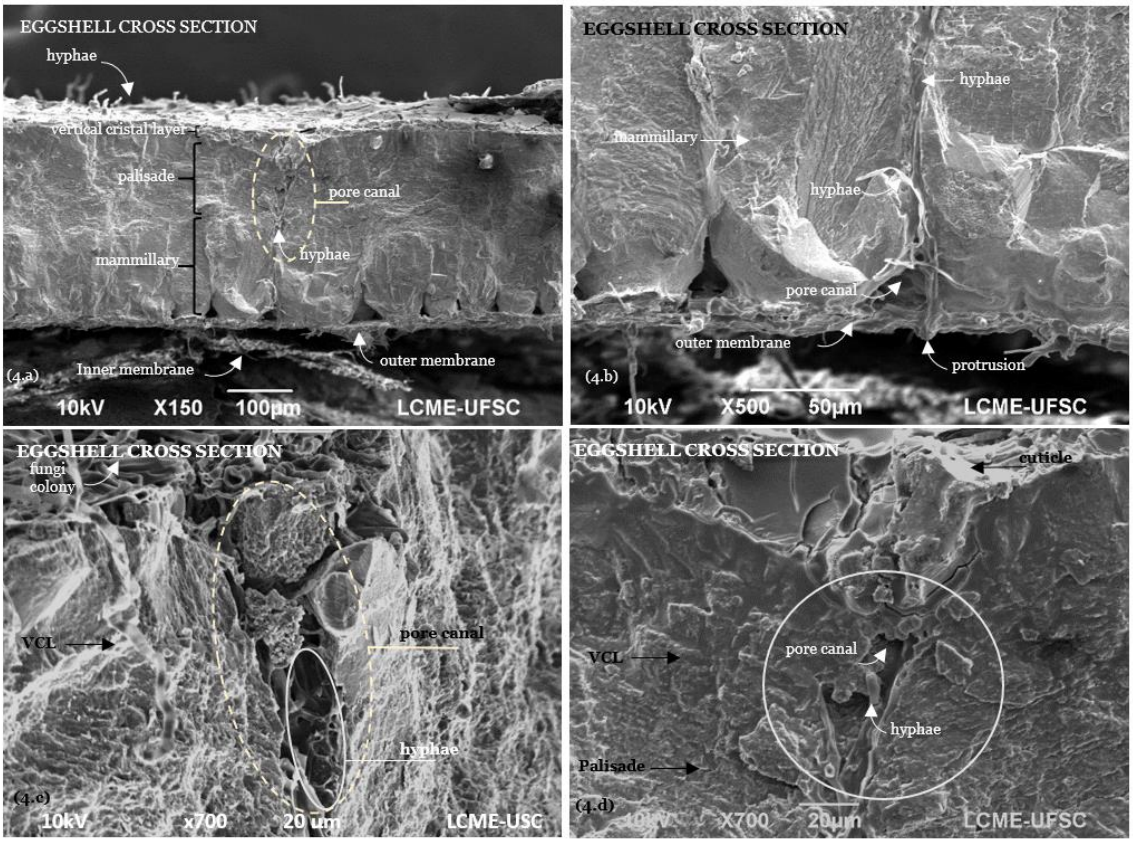

Figure 5. Scanning electron micrographs of the presence of filamentous fungi and the formation of colonies in the CUTICLE and PORES of EGGSHELL SSURFACE (a.1) PORE invasion and shell layers (a.2) fungal development to the outer membrane of the egg (b)hyphae and the formation of PROTRUSION in the inner membrane (c/d) BEHAVIOR of hyphae in the PORE CANAL [150 to 700x].

The quality or structure of the eggshell during the reproductive life of the chicken is influenced by the genetic constitution, feeding, climate, production environment and age (Nascimento *et al.* 1992). Mycotoxins produced by *Fusarium* sp as deoxynivalenol and zearalenone and aflatoxins by *Aspergillus flavus*, in addition to having an adverse effect on eggshell quality, may affect performance and immunity in broiler and broiler hens (Yegani et al., 2008;Devegowda and Ravikiran, 2008).

IV. CONCLUSION

It was possible to register / observe / show its invasion (the hyphae managed to cross through the pores of the eggshell and reach the inner membrane of the shell.

Another characteristic of the fungal invasion is the increase in the pore diameter, which further facilitates the internal contamination of the egg depending on the period of exposure to fungi.

Fungi of the genera *Fusarium* and *Curvularia* found ideal environmental conditions for their development. Circumstances of improper

egg storage that favor the growth of field toxigenic fungi can lead to contamination of the internal content of the eggs and compromise their quality / safety within a few days of exposure.

New research on decontaminants that do not leave toxic residues should be tested in nests and cages and in the egg storage room, as some regions have climatic conditions conducive to fungal development.

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