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Peracetic acid: Effect on the Chicken Eggshell Cuticle and Decontaminating Action on Filamentous Fungi

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Abstract

Decontamination methods of egg change according to the different countries. However, this practice is extremly important, because there may be contamination by a wide range of micoorganisms. In free-range raising systems, in addition to being exposed to the pathogenic microorganisms, hens may be also exposed to a different kinds of fungy, both field and storage species, contaminating the eggs. The main fungi disseminators is the substrate used in nests, especially when forage or crop residues (straw or shavings) are used. Chemical sanitizers used in poultry production have in their formulation, peracetic acid (PAA) in several concentrations, which can eliminate pathogenic microorganisms resistant to other types of disinfectants. Therefore, this work evaluated the use of PAA solutions, with four different concentrations, applied on the eggs, on the eggshell estructure and on the control of fungy proliferation. The use os PAA solution in 75, 150 ppm centration reduced the development of fungi spores and hyphae, with a slight compromising of the external cuticle structure. On the other hand, PAA 300 ppm concentration can induce micro fragmentation in some regions of the membrane and compromise the egg quality.The mechanism of PAA sanitization occus from oxidation reactions of the product resulting in acetic acid, hydrogen peroxide and water. Probably the amout of acetic acid produced may interact with the eggshell compounds and cause the microfragmentations observed in the micrographs. Thus, further studies are necessary to establish an ideal concentration of PAA as antifungal agent to egg, preventing contamination by mycotoxins and without any kind of damage in eggshell structure.

Key words: Decontamination, Egg, Food safety, Free-range, Fungi, Microorganism.

1. Introduction

Peracetic acid (PAA) has been used as a decontaminant in several segments of animal production for many species of pathogenic microorganisms (Grasteau, Patrick Daniel, & Valérie Chesneau, 2015; Khater, Seddiek, El-Shorbagy, & Ali, 2013). An organic peroxide, a colorless liquid with a characteristic odor similar to acetic acid, its stability, depending on the concentration, provided effective disinfection for at least 4 days without corrosion capacity (Costa, Paula, Silva, Leão, & Santos, 2015). Considered one of the

disinfectants that best meet the requirements required in environments susceptible to contamination. A biodegradable compound, but the use of individual safety equipment provides greater security at the time of application (Svidzinski & Svidzinski, 2007).
In poultry establishments, compounds such as glutaraldehyde, quaternary ammonia, and sodium hypochlorite, are widely used in hygiene, however, they are potentially harmful to the environment (Grasteau et al., 2015). Fungi contamination in eggs occurs when hyphae pass through the pores and reach the inner membrane of the shell. Another characteristic of fungal contamination is the increase in pore diameter, which further facilitates internal contamination of the egg depending on the period of exposure to fungi (Soares, Maiorka, Dahlke, & Scussel, 2020).
The eggshell layers (cuticle; vertical crystal layer; palisade; mammillary; outer; inner layer) serve as a protection system for the egg interior (outer shell membrane), which also has protein matrices as a natural defense system (Solomon, Bain, Cranstoun, & Nascimento, 1994). Scanning electron microscopy is used in several analyzes in order to understand how the action of different types of chemical compounds on different food matrices (Abu-Saied et al., 2020; Lotfali, Valizadeh, Ghasemi, Amir, & Feghhi, 2021). The application of four concentrations of PAA on the eggshell and the evaluation of the action on the eggshell and fungi, by scanning electron microscopy, were tested to demonstrate potential safe forms of concentrations without interfering with the integrity of the eggshell.

2. Material and Methods

2.1 Material

Samples: the eggs (n=16) were collected from wooden nests, with straw as substrate, in a free range system . The layer hens were raised with water and feed *ad libitum* , (ration based with corn, soybean meal) and free access to pasture. Hens of the Hy-line Brown lineage with 88 weeks of age raised in the Laboratory - Avicultura UFSC, at the Experimental Farm.
Equipment: tweezers (Prolab, São Paulo, SP, Brazil); bacteriological incubator Sterilifer Sx1,3 (Dtmc, São Paulo, SP, Brazil); scanning electron microscope (SEM) (5000x), model JSM-6390LV (Jeol, Peabody, Mass., USA); SEM gold coating model EM- Scd500 (Leica, Leider, Illinois, USA); PAA hortoxi-150 (15%) (Alloxy, São Paulo, SP, Brazil); PAA concentration meter Spect Test-AP3 (50-5000 ppm) (Specol, São Paulo, SP, Brazil); pH meter (Kasvi, Chiang Ang, China); potentiometer (Tecnal, São Paulo, SP, Brazil). Other materials: stubs (small metal blocks, 9 in diameter and 10 mm in height).

2.2 Method

Fungal Incubation: the eggs were artificially and naturally contaminated with fungi strains (Figure 1), then stored in for 7 days at ± 25º C in the Laboratory of Mycotoxicology and Food Contaminants.
Preparation for microscopy: the eggs were prepared for SEM as follows: different external parts of the eggshells, however, in the same region, were obtained and immediately adhered to carbon tape a for SEM.
Application of peracetic acid: application of PAA with a volume of 100 µl over the eggshell ($1cm^2$): Group **I** - (GC) absence of PAA; groups **II**, **III**, **IV** application of three concentrations of PAA (75, 150 and 300 ppm) and **V** with the commercial product with a concentration of 15% diluted in distilled water (v/v), respectively. Figure 1 shows how PAA was applied to the eggshell.

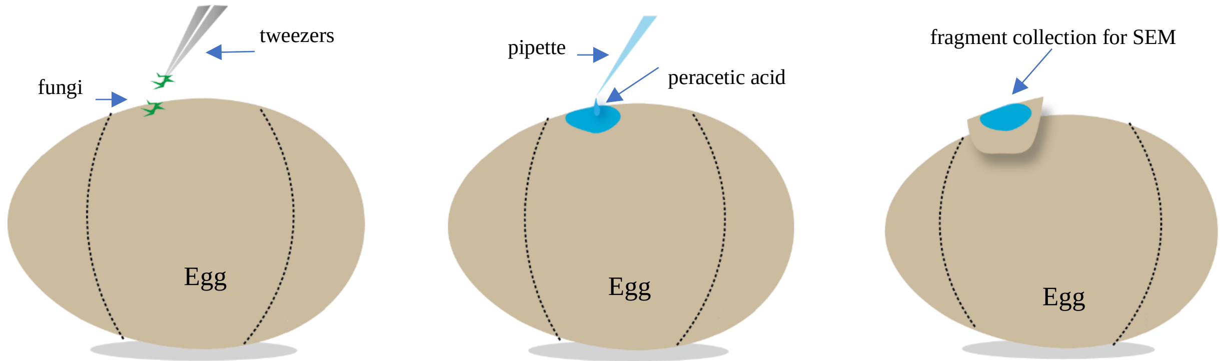

Figure 1. Infographic demonstrating the fungi contamination and peracetic acid application on the eggshell. SEM: scanning electron microscope.

3. Results and Discussion

Several microorganisms become resistant to the disinfectants used in the poultry industry, further intensifying the quantity and concentration during application. PAA has been utilized for a long time, however, there are some limitations (concentration, half-life, and presence of organic matter) in its use. The application of four concentrations of PAA on the eggshell and the evaluation of the action on the shell and fungi, by SEM, were used to demonstrate potential safe concentrations without interfering with the integrity of the eggshell.

The use of scanning electron microscopy to evaluate the action of several decontaminants in many food matrices (Feroze et al., 2020; Ruangwong et al., 2021). The applicability of clean, safe, and effective methods of decontamination such as ultraviolet light, pulsed light, and ozone are essential and promising in the production of eggs and for the production of food today, where there is a great concern with food safety (Clímaco et al., 2018; Lasagabaster, Arboleya, & De Marañón, 2011). However, a few concerns about the action of fungi on eggs, with unsatisfactory packaging (high humidity and temperature) are ideal conditions for fungal development and the change in their protective structure (cuticle) facilitates the penetration of fungi (Soares et al., 2020).

3.1 Natural microfissures and microfragments

In the samples of the Group - GC- we observed the integral shape of the eggshell without the action of fungi and PAA at room temperature (Figure 2). In figures (2.a, b) we can see the structure of the outer surface of the peel (pores and cuticles). In these collected samples, the pores are intact and others are completely open, without the protection of the cuticle. The cuticle, on the other hand, had naturally occurring microfissures. Sparks and Board (Sparks & Board, 1985) show by SEM that the cuticle has natural microfissures in different regions of the shell surface, in our research the amount of microfissures is possibly related to the age of the hens (88 weeks).

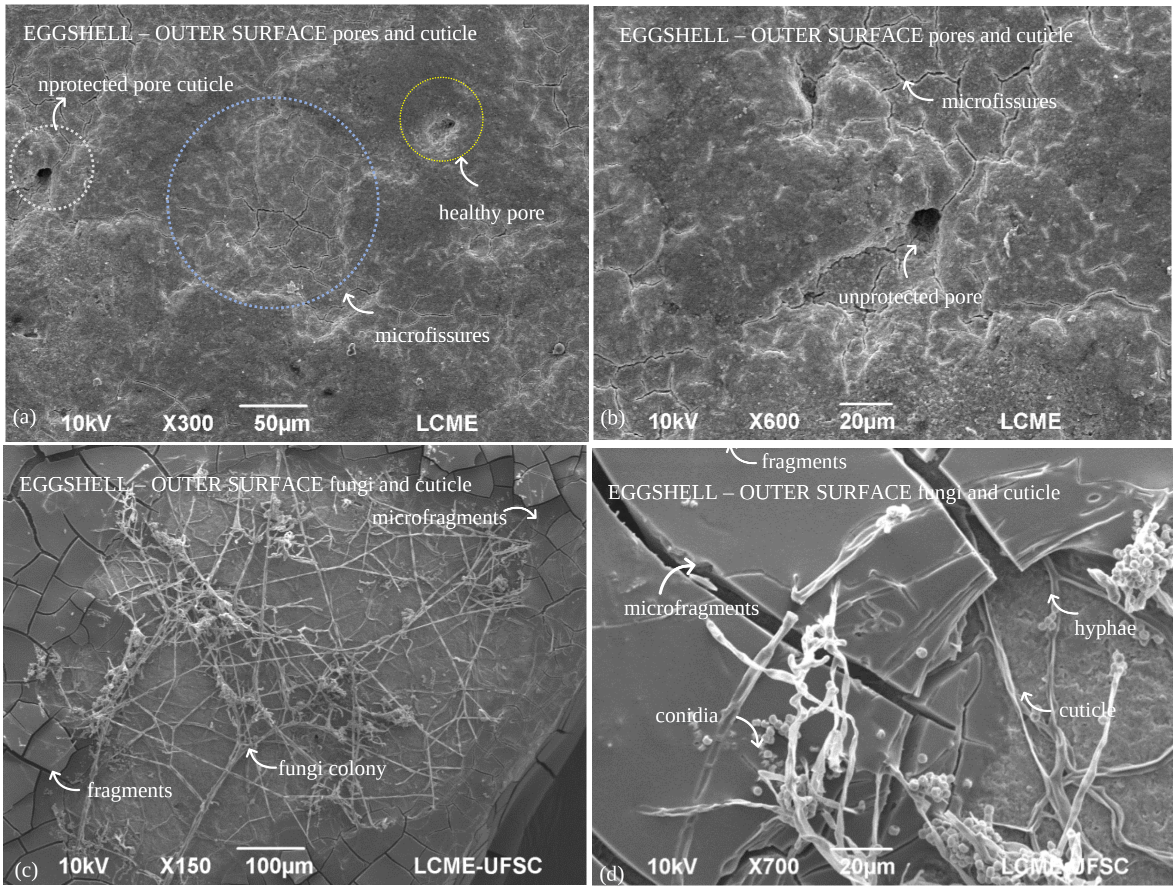

Figure 2. SEM micrographs of healthy external surfaces (without structure damage) of the eggshell (a and b) and the presence of fungi and fragments after application of peracetic acid in low concentrations (c and d).

The absence of the cuticle makes the pore canal an ideal site for fungal development and the microfragments facilitate the penetration of fungi and other pathogenic microorganisms into the eggs (Soares et al., 2020). The application of low or high concentrations of PAA solutions on the eggshell can cause microscopic damage to the cuticle. Depending on the integrity of the eggshell and the poor storage conditions, the use of water for disinfection or even low concentrated PAA solution can cause microfragments in the cuticle and vertical crystal layer (Figure 2c and 2d).

With ideal conditions for the development of fungi (temperature and humidity) even after the application of PAA in concentration of 600 ppm, they propel the hyphae to propagate under the microfragments, invading easily, and find an ideal place to develop in the vertical crystal layer of the eggshell (Figure 2c and 2d). Where there was the action of the PAA solution, hyphae and conidia were not viable. Soares et al. (Soares et al., 2020) observed the invasion of deteriorating fungi carried to the inner layer of the egg carried by the pore canal.

The use of water for washing eggs is not recommended, even in eggs younger layer hens (45 weeks of age), as it results in damage to the surface of the shell, give rise to microfissuras, which facilitate the entry of pathogenic microorganisms (Gole et al., 2014). PAA can stop the development of fungi and prevent the hyphae from branching across the surface of the eggshell. According to Liu et al. (Liu, Chen, Wu, Lee, & Tan, 2016), the storage temperature critically influences the quality of the egg, and washing the eggs can reduce the cover of the cuticle, compromising even more its quality.

3.2 Action of natural fungal contamination on the eggshell

In figures 3a and 3b it can see contamination by deteriorating fungi on the eggshell and its ramification behavior on the surface without the application of PAA. Some pores are without their protection from the cuticle, exposing the interior of the egg to these microorganisms. The search for new alternatives to maintain egg quality such as covering the shell by solution with a protein concentrate of powdered rice associated with essential oils can influence the internal quality of commercial eggs, protecting them during the storage period (P. G. S. Pires et al., 2020).

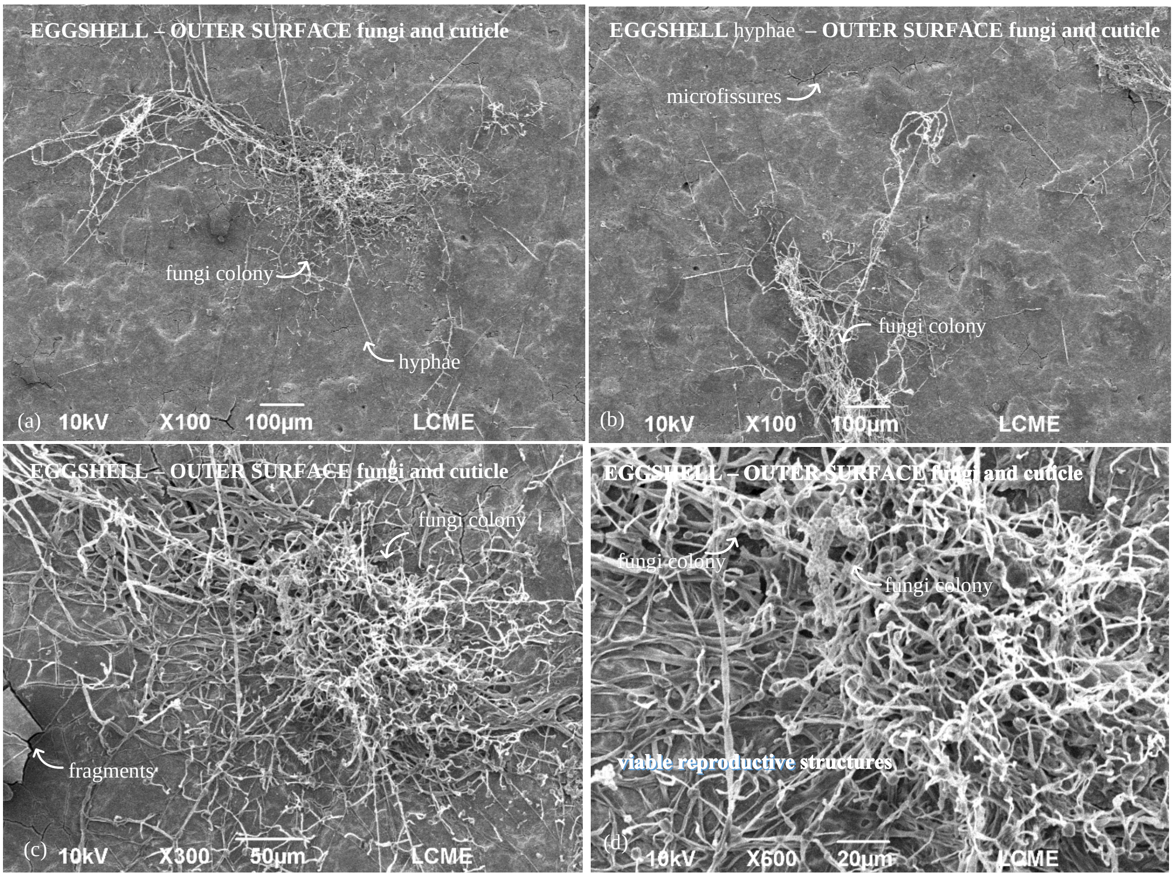

Figure 3. SEM micrographs of external surfaces (without structure damage) of the eggshell (microfissures) (a) fungi behavior on the shell without the use of peracetic acid (b), and the presence of viable fungi and reproductive structures (c and d).

In the figures 3c and 3d, it did not observe hyphae invading the small natural microfissures on the surface of the eggshell. For fungi, the microfissures do not seem to provide an ideal site for their development, even with colonies and viable reproductive structures. The search for strategies such as pore protection, some compounds used can favor fungal contamination due to the physical structure of the substrate and the high starch content present in its composition (Pires, 2019).

75 ppm

Several microfissures may have been created on the surface of the eggshell by the presence of the PAA solution (Group II- 75 ppm). There are no fungal reproductive structures as hyphae and conidia to evaluate the action of this concentration of PAA on fungi (Figure 4.a, b). However, we can infer that the mere presence of the solution on the surface interferes with the external quality of the egg, creating microfisurres on the cuticle. Extending the magnitude of the images, depigmentation of the cuticle is observed where there is deterioration of the cuticle, further increasing the exposure of the pores to pathogens (Figure 4 c, d).

Egg washing may have deteriorated the cuticle coverage resulting in less protection and greater susceptibility to contamination (Liu et al., 2016). However, genetic selection can increase cuticle deposition in commercial birds, reducing the transmission of microorganisms and also preventing potentially harmful ultraviolet wavelengths from reaching the embryo (Bain et al., 2013; Liliana D'Alba;, Torres;Roxana, Waterhouse, Eliason, & Mark E. Hauber, 2017).

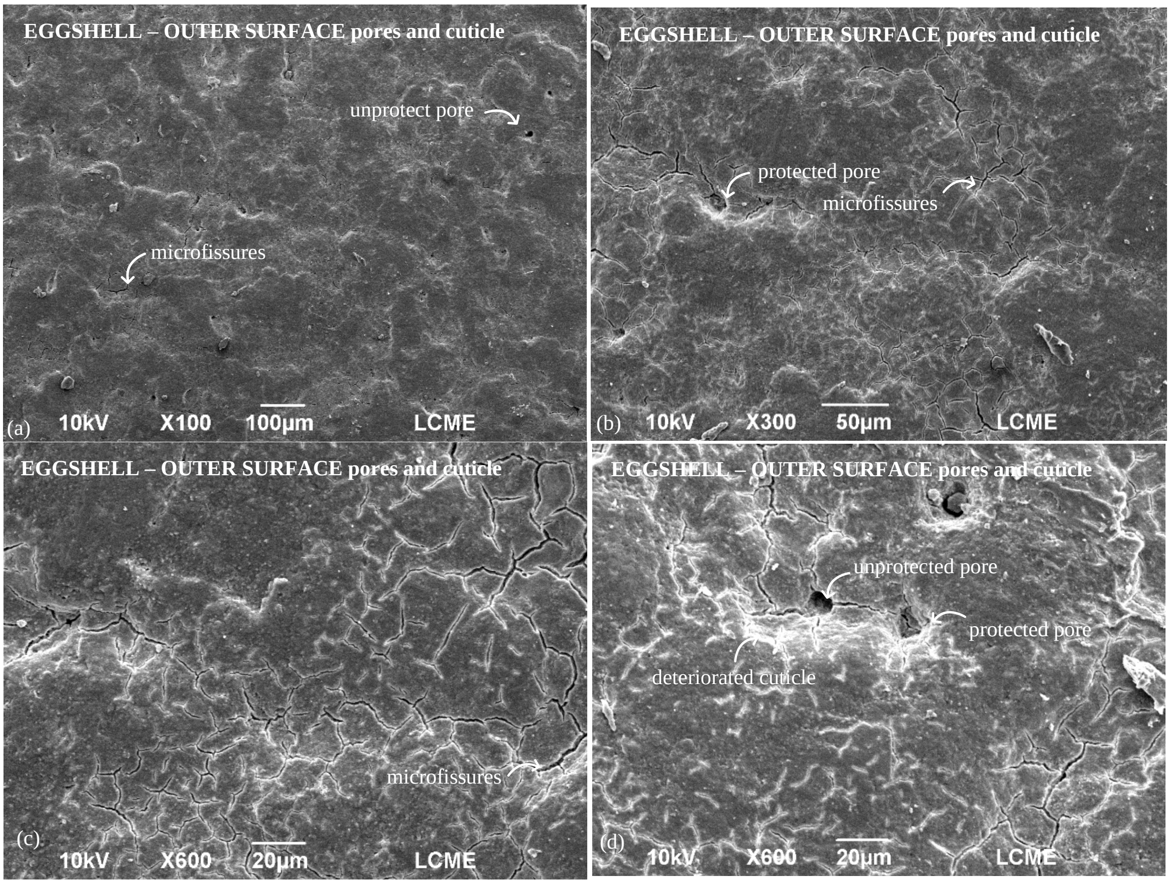

Figure 4. SEM micrographs of external surfaces of the eggshell with the cuticle deteriorated and the pores protected and unprotected, after the application of PAA (75 ppm) [100 -600x].

150 ppm

The egg surfaces of (Group III-150 ppm) after 7 days of application of peracetic acid showed small microfissures identical to those of healthy shells. We observed that on the surface of the shell there was a dehydrated fungi colony with few viable reproductive structures (5.c,d). However, the low efficiency of PAA on fungi in this concentration, allowed the development to be less accentuated. In figures 5.a, b the fungi colonies are retracted. However, they found an ideal environment for their development and to propagate hyphae across the surface of the eggshell.

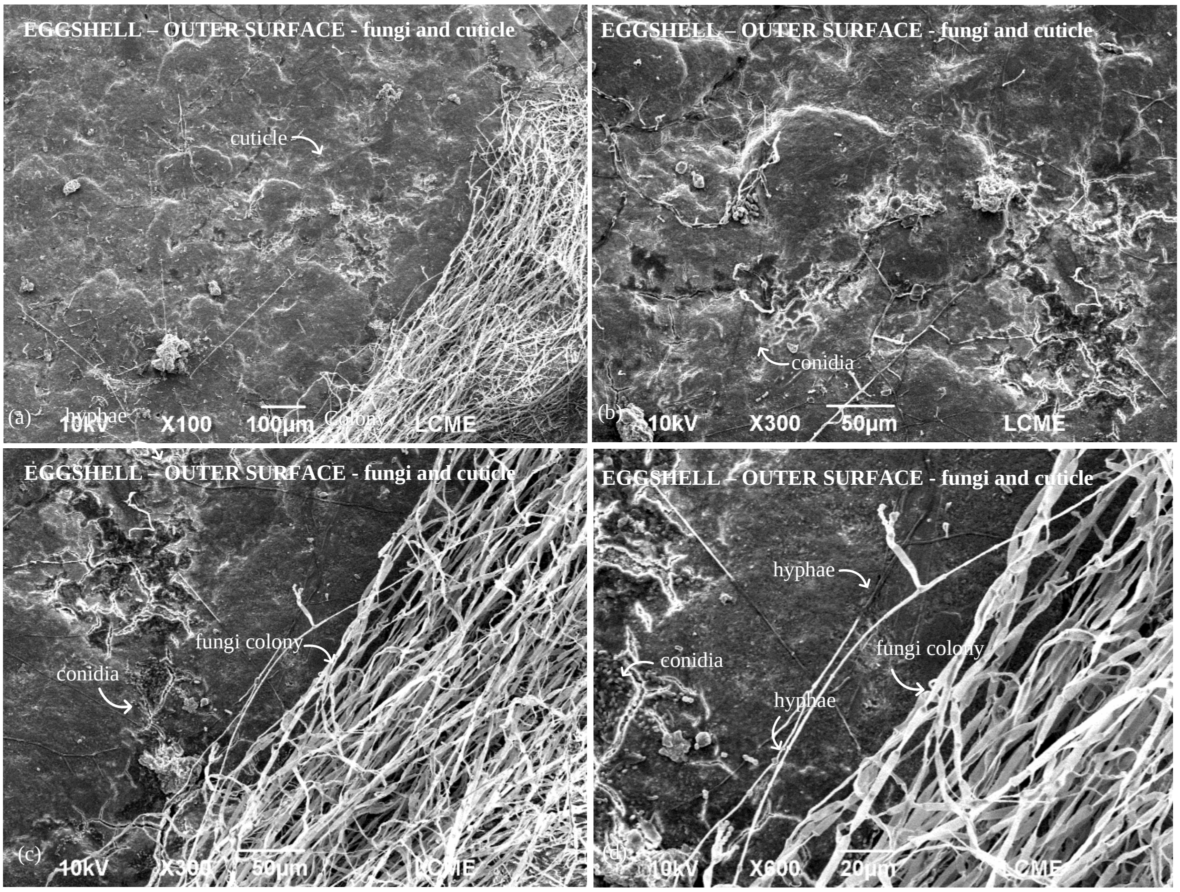

Figure 5. SEM micrographs of external surfaces of the eggshell, with application of PAA (150 ppm) on fungi present in the eggshell (a, b) formation of colony and propagation of hyphae and (c, d) dehydrated reproductive structures [100 -600x].

Regarding the antifungal capacity, PAA at this concentration (150 ppm), did not present sanitizing efficacy in the species of deteriorating fungi observed in the images. Figure 4 shows conidia and hyphae that are apparently viable, which is worrying, since any precarious storage condition can provide an ideal condition for fungal development. However, fungal strains showed low resistance to intermediate and higher concentrations of peracetic acid, which shows the variation in sensitivity between species (Bernardi, Stefanello, Lemos, Garcia, & Copetti, 2019).

300 ppm

The application of PAA (Group IV - 300 ppm) apparently may have altered the cuticle layer in several places on the eggshell surface as well as in other solutions (75 and 150 ppm) applied for the tests. Microfragments (Figure 6.a, b) in some regions of the cuticle are possibly the result of the action of peracetic acid, as these microfragments were not observed in other work results. Thirty seconds after laying, the cuticle presents micro-cracks and fragments, these natural damages were observed at different moments after laying eggs (Board et al., 1994).

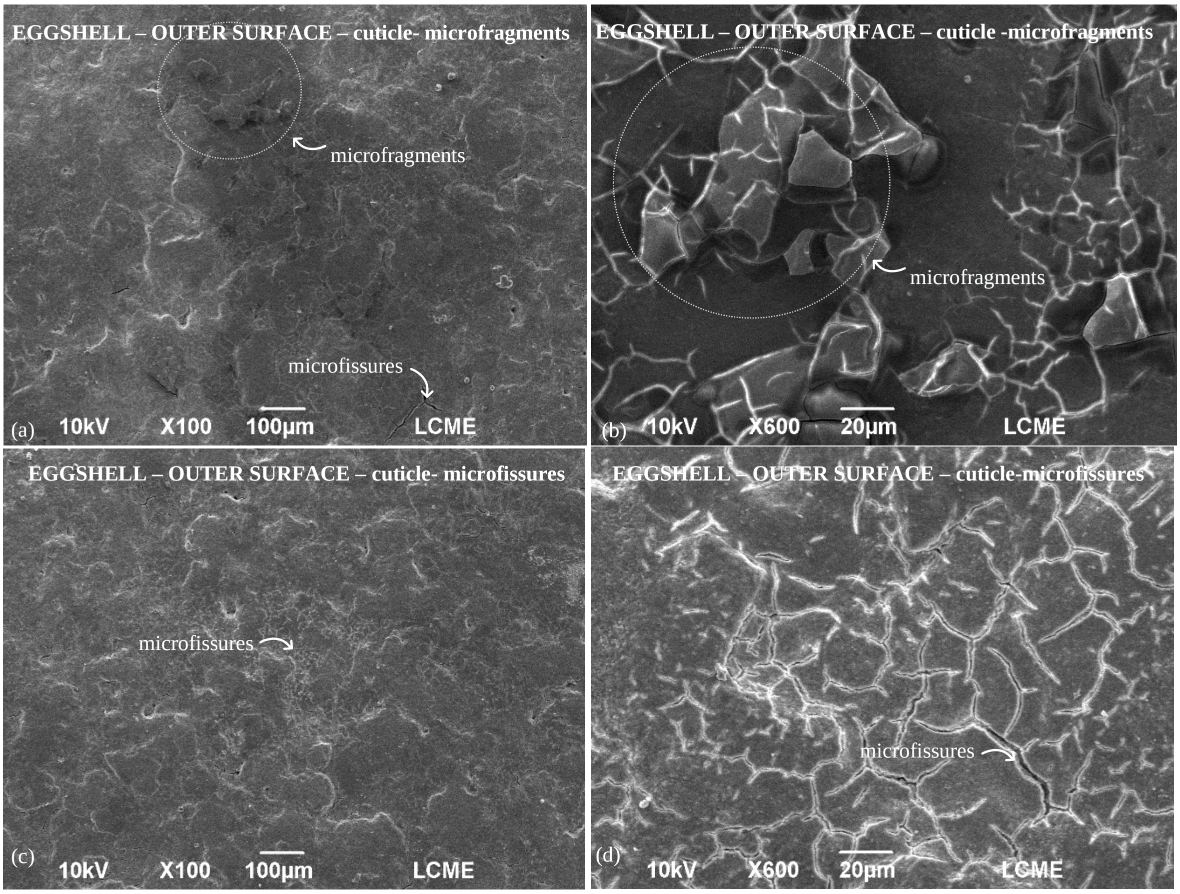

Figure 6. SEM micrographs of external eggshell surfaces with PAA application (330 ppm) on the shell surface (a) formation of cuticle microfragments (b) characteristics of cuticle microfragments [100 -600x].

These microfragments in specific places created by the application of the PAA propose that in some regions of the cuticle they are susceptible to the use of any sanitizing agent (Figure 6.a, b). Other images obtained after the application of PAA in the same concentration (300 ppm) in other regions of the egg shell, it was not possible to observe the fragmentation of the cuticle of the shell (6.c, d).

Eggs for chick production (incubation), were exposed to a product developed with a non-toxic characteristic ("artificial cuticle"), composed of chitosan soluble in (PAA 1%) and other components such as hydrogen peroxide (H_2O_2), nanometric calcium carbonate ($CaCO_3$) to prevent contamination, both by fungi and by other contaminating agents that interfere in the hatchability of eggs (Bordunova et al., 2021).

3.5 Behavior of fungi colonies under 300 ppm

Currently, some products with PAA in different concentration, are used as sanitizing to eliminate pathogenic bacteria on the surface of objects used in food production (Gurung, Ancona, Campos, & Choppakatla, 2020; Kunigk & Almeida, 2001; Moustafa Gehan, 2009).

Its sanitizing action has been effective in controlling fungi both in storage and in the field in various food matrices, it has been reported in several research(Bernardi et al., 2018; Gurung et al., 2020; Marín et al., 2002). In this context, the susceptibilities of some fungal species such as *Aspergillus* and *Penicillium* to some concentrations of PAA are described, resulting in a reduction in the fungal count.

Some species of fungi of the genus *Chaetomium* involved in food spoilage and environmental contamination are resistant to PAA because the cell walls are thicker (Nakayama et al., 2013). The mechanism of action of PAA ceases the chemosmotic function of the lipoprotein cytoplasmic membrane and transport through the displacement or rupture of cell walls (Baldry, 1988).

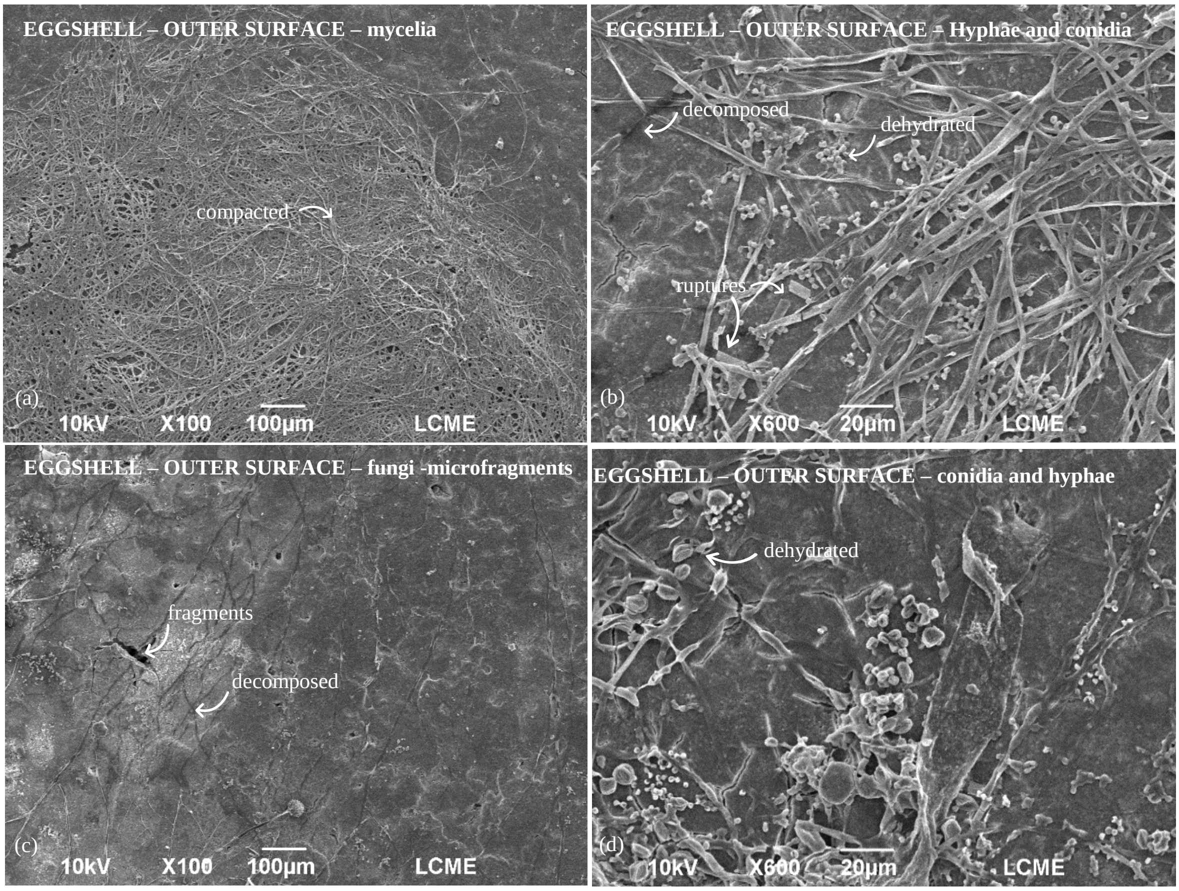

Figure 7. SEM micrographs show the sanitizing action of PAA (a) compacted mycelia (b) hyphae and conidia with ruptures and dehydration (c) microfragments and decomposed hyphae (d) conidia and hyphae that are possibly non-viable [100-600x].

The fungi found an ideal environment for the development of their reproductive structures in the eggshell, However, PAA, in concentrations of 75 ppm as well as 300 ppm, restrict the growth of hyphae, preventing them from ramification throughout the shell. The application of the 2% peracetic acid solution, it was sufficient to sterilize it in eggs and not compromise hatchability (Harrison, 2007). Through steroscopy we can observe that *A. flavus* was limited to the site of application of the acid (area with microscopic fissures). When inserting the PAA in the eggshell, we can observe that a fragmentation process begins in some places of the eggshell cuticle, as shown in the figure (7.c).

The sanitizing action of peracetic acid demonstrated by SEM on mycelia and conidia shows that the reproductive structures of the fungi were inactivated and collapsed in a compacted, dehydrated and with ruptures in their filaments (Figure 7). They present anomalous hyphae and conidia decomposed by the use of PAA on the cuticle of the eggshell. Similar effect with the use of 150 ppm on hyphae that dehydrated (Figure 5).

Similar changes were recorded by Fahselt et al. (Fahselt, Madzia, & Alstrup, 2001) in free living filamentous fungi and after evaluating the effects of antifungals (Ghannoum, Isham, Henry, Kroon, & Yurdakul, 2012; Simionato et al., 2017). PAA for disinfecting pathogens in eggs is promising. However, it is necessary to evaluate the optimum concentration of the product that promotes the disinfection of fungi without changes or damages in the physical and chemical characteristics of the cuticle and eggshell.

Solution 15%

This mechanism of action can be seen in micrographs of eggshells contaminated. The microorganisms appear to be dehydrated and inactive. On the other hand, it is possible to verify the occurrence of microfragments in the peel and the possible interference in the cuticle, aspects that make the use of PAA as a sanitizing agent unsafe (Figure 8).

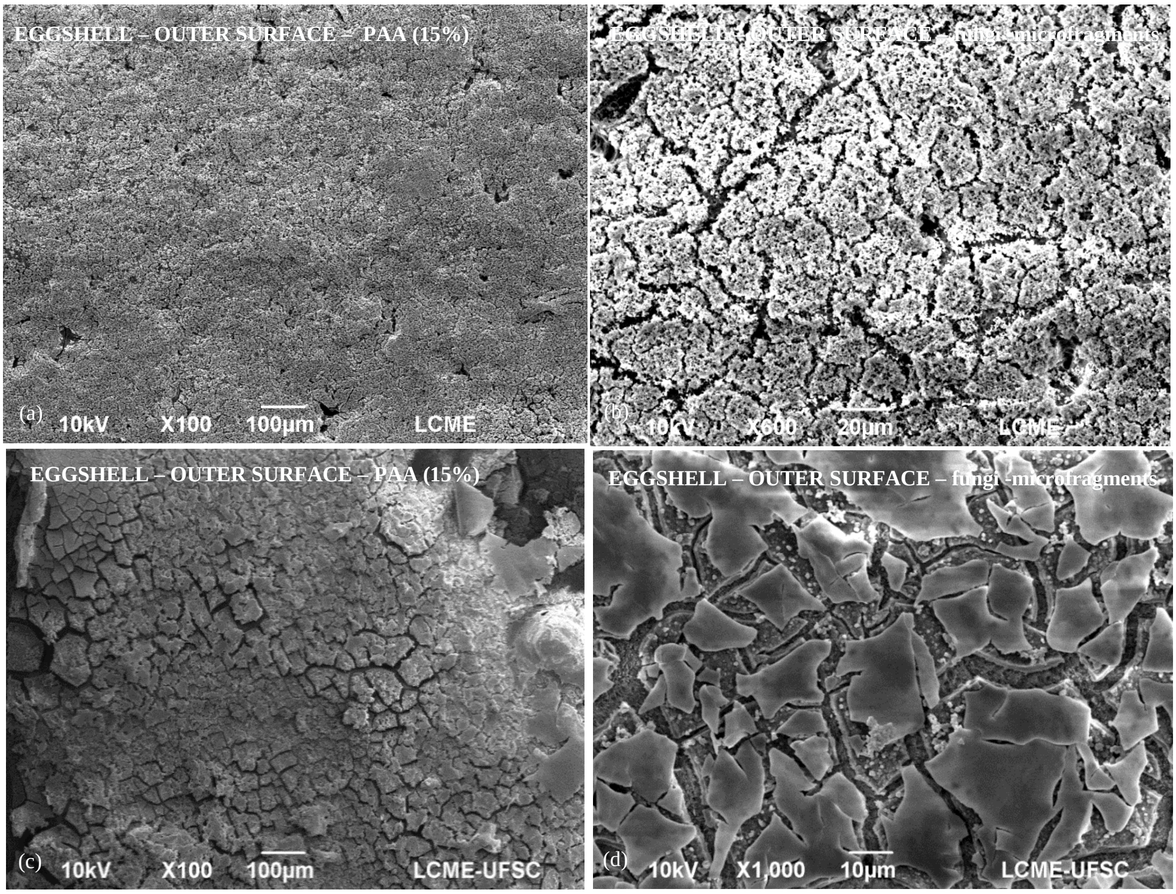

Figure 8. SEM micrographs show differences in the action of PAA (15%) on the cuticle showing that each region presents variation in microfragments [100-1000x].

The use of sanitizing agents such as PAA throughout post-harvest processes is extremely important for the disinfection of food products and equipment. Peracetic acid can be defined as a chemical agent, translucent, with high oxidizing power at low pH. It can damage some metals through redox reactions. It has a sanitizing action due to its antimicrobial power. Furthermore, its use is environmentally safe because PAA is considered biodegradable (Zhao, Zhang, Zhou, & Liu, 2007).

The PAA sanitizing solution has in its composition a combination of peracetic acid (C_2H4O_3), hydrogen peroxide (H_2O_2), in addition to acetic acid (CH_3COOH) and an agent to stabilize the reaction. The sanitization reaction promoted by the PAA relies on the action of a *singlet* oxygen molecule, which is reactive and supplied by the presence of the compound's H_2O_2, including CH_3COOH as a product of its decomposition (Leggett et al., 2016).

In this context, the action of PAA can be similar to the action of H_2O_2, with the difference due to the presence of an acid molecule that acts to increase the power of sanitization. PAA promotes the oxidation reaction of disulfide bonds and thiol bonds present in the cytoplasmic membrane in the internal components of microorganisms, causing cell lysis and inactivation of vital functions, also acting on fungal spores (Leggett et al., 2016).

In this case, the CH_3COOH molecules supplied by the PAA that are free and have not acted on microorganisms may interact with the egg shell components. The eggshell is composed mainly of calcium carbonate ($CaCO_3$). The reaction between two CH_3COOH molecules and a CaCO3 molecule releases calcium acetate ($Ca(CH_3COO)_2$), water and carbon dioxide (Teir; Eloneva; Zevenhoven, 2005). This reaction may be related to the microfragments at certain eggshell sites that were observed in the micrographs, especially when using PAA 15% (Figure 8).

These evaluations reinforce the need for new studies capable of indicating the optimal concentration of PAA to be used as a sanitizing agent for laying eggs. In addition, other variables such as the exposure time of the sanitizer for total fungi inactivation, including differences in the quality of laying eggs influenced by the age, must be evaluated.

4 Conclusion

SEM enabled us to accurately evaluate the sanitizing action of PAA on the eggshell and some genera of fungi tested. Many exposed pores and microfissres are a result of the advanced age of chickens. However, microfissures do not seem to be a site with ideal conditions for fungi development. Low concentrations of the PAA solution can intensify the formation of new microfissures, create microfragments and compromise the quality of the egg. The utilized of PAA for disinfecting pathogens in eggs is promising. However, it is necessary to evaluate the optimum concentration of the product that promotes fungal decontamination without altering or damaging the cuticle and shell characteristics of the eggs.

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