







Original Articles

## Mold species and fungi load of washed and unwashed table eggs

Espécies de bolores e contaminação fúngica de ovos de galinha lavados e não lavados

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### ABSTRACT

Egg quality has been widely studied, mainly because defects in quality can pose risks to public health, as well as economic losses. Nevertheless, studies about fungi in eggs are scarce. The objective was to compare the fungal microbiota from washed and unwashed eggs in the rainy season and dry season of the year. This exploratory research consisted in the analysis of large size white table eggs acquired from 48 different lots. Two manufacturers were sampled considering the main characteristic of washed or unwashed eggs. From each lot, a 30-egg pack were purchased and six of those eggs were used for mycological analyzes. The eggs were analyzed externally with 0.1% peptone salt solution wash of the eggshells and internally with aliquots being sampled from a pool made from the six eggs content. Samples were inoculated in Potato Dextrose Agar and isolated colonies were passed to test tubes. When sporulated, the isolates were subjected to decimal dilutions using 0.1% Tween 80 to dissociate the conidia. Microcultures were carried out for optical microscopy observation of the reproductive structures of fungi, stained with lactophenol. *Aspergillus* spp. was the most frequently isolated fungi isolated, with *A. niger* and *A. flavus* predominant in the dry season, while *A. fumigatus* and *A. terreus* in the rainy season. Low numbers of fungi were identified from egg shells, with a higher amount from unwashed eggs. The season did not influence the number of fungi in eggs, despite having influenced the fungal diversity.

### RESUMO

A qualidade do ovo já foi largamente estudada, especialmente por causa de defeitos na qualidade que podem significar riscos para a saúde pública, além de perdas econômicas. Entretanto, estudos sobre fungos em ovos são escassos. O objetivo foi comparar a microbiota fúngica de ovos lavados e ovos não lavados nas estações chuvosa e seca do ano. As amostras consistiram de ovos comerciais, do tipo branco e tamanho grande, com caráter exploratório pela análise de 48 lotes. Dois fabricantes foram amostrados considerando a característica principal de ovos lavados ou não lavados. De cada lote, foram adquiridos 30 ovos, sendo seis ovos utilizados nas análises micológicas. Os ovos foram analisados externamente fazendo lavagem com solução salina peptonada e internamente com alíquotas retiradas de pools de seis ovos. Amostras foram inoculadas em Ágar Dextrose Batata e os isolados foram passados para tubos de ensaio. Posteriormente as colônias foram replicadas para tubos de ensaio contendo meio Ágar Dextrose Batata. Quando já esporulados, os isolados foram submetidos a diluições utilizando Tween 80 0,1% para desagregar os conídios. Foram realizados microcultivos para observação em microscópio óptico das estruturas de reprodução dos fungos, corados com lactofenol. *Aspergillus* spp. foi a espécie mais frequentemente isolada, com predominância de *A. niger* e *A. flavus* na estação seca e *A. fumigatus* e *A. terreus* na chuvosa. Baixas quantidades de fungos foram observadas nas cascas dos ovos, com quantidades maiores em ovos não lavados. A estação não influenciou no número de fungos em ovos, apesar de ter influenciado em sua diversidade.

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## INTRODUCTION

The chicken egg has proteins whose nutritional characteristics meet almost all human requirements, due to its content rich in vitamins, minerals and fatty acids. These substances together are capable of preventing various diseases of interest to the consumer population, which is why their consumption has been boosted in recent years (SANTOS et al., 2015). Despite all its beneficial characteristics, the egg is a highly perishable food and begins to lose its quality right after laying (LANA et al., 2017; SANTOS et al., 2015).

In view of the greater demand from consumers for quality, the producer has shown concern about the adequacy of the products. Washing the eggs before they are packaged is very common, however, despite the unquestionable improvement in the appearance of the product, this process can speed up the contamination of the egg content, due to the removal of the protective film that covers it, the cuticle (GOLE et al., 2014). The washing procedures are regulated by government to ensure its efficiency and prevent problems such as content contaminations. The eggs must be washed only by mechanic methods, in a continuous and fast process, using water at a maximum of 10°C higher than egg temperature and with a maximum of 50 ppm chlorine, being dried immediately after washing (BRASIL, 1990).

As a food rich in nutrients, the proliferation of pathogenic and deteriorating microorganisms is enhanced, posing risks to the consumer's health. Several fungi can cause spoilage of foods but the biggest concern about the contamination with these microorganisms is related to the production of mycotoxins, which are substances with potential for acute toxicity and carcinogenicity (FORSYTHE, 2019). Five fungal toxins were considered to be at greatest risk to human and animal health: aflatoxins, ochratoxin A, zearalenone, deoxynivalenol and fumonisins (OLIVEIRA et al., 2017).

Despite of potential risk associated with fungal contamination on eggs, only a few studies have identified its occurrence to the genus or species level (COOK et al. 2003; GRIZARD et al., 2014; RAJMANI et al., 2011; TOMCZYK et al., 2018)

The objective of this work was to compare the fungal microbiota from washed and unwashed eggs in the rainy season and dry season of the year.

## MATERIAL AND METHODS

The mycological analyzes were performed at the Laboratory of Princípios Bioativos de Origem Microbiana, Faculdade de Ciências Agrárias, Universidade Federal do Amazonas. The samples consisted of table eggs of the white type and large size, purchased from supermarkets of Manaus, Amazonas, Brazil. These eggs were always acquired in the morning, where the temperature of the egg temperature was

measured with an infrared thermometer (Incoterm® ST-620). and the environment and the humidity of the environment were measured with a thermo hygrometer (Incoterm® 7666.02.0.00).

The research had an exploratory character, with methodologies that characterize a case study with the analysis of 48 lots. The lots were sampled considering a manufacturer that does the egg wash process, according to an official procedure (BRASIL, 1990), and another that does not do this process. The period of the year in which the eggs were produced was also considered for collections: the rainy season, between November 2016 and May 2017; and dry season, between June and October 2017. To ensure representative sampling, collections were carried out in the four regions of the city: North, Midwest, South and East, respecting the proportionality between the regions. Cartons containing 30 eggs were purchased for each lot sampled. Six eggs from each carton were chosen for mycological analysis, using the first eggs from top to bottom and from the left to the right side of the package, excluding those with cracked or broken shells.

The selected eggs were grouped to form a pool in a sterile plastic bag. The sterile bag was weighed without the eggs and weighed again with the eggs, for later calculations of the results per gram of sample. Eggs were washed with 60mL of 0.1% peptone salt solution added to the bag, enough to cover the entire surface of the eggs. The eggs were washed with manual agitation for 10 minutes in order to recover fungi from the eggshells. The wash solution was placed in a sterile container, considered the 10<sup>0</sup> solution. Subsequent dilutions of 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup> were made from the wash solution. Then, the eggs were immersed in 150mL of 70% ethyl alcohol solution for approximately five minutes. After drying on absorbent paper, the eggs were opened aseptically, their contents deposited in a sterile container, and homogenized for 60 seconds to compose the analytical sample of the egg content. Subsequent dilutions up to 10<sup>-2</sup> were made in 0.1% peptone salt solution.

Each serial dilution solution had 0.1mL (10<sup>0</sup>, 10<sup>-1</sup>, 10<sup>-2</sup> and 10<sup>-3</sup>) inoculated into sterile Petri dishes containing solidified Potato Dextrose Agar (PDA) with the addition of 1.5mL of 10% tartaric acid solution for each 100mL of medium. The aliquot was carefully spread over the medium with the aid of a previously sterilized Drigalski loop until its complete absorption. The plates were incubated at room temperature, approximately 25°C, for a period of five to seven days. Only the dilutions with 15 to 150 colonies were considered for counting, using the dilution factor and expressing the result in Colony Forming Units per 1.0 g of sample (CFU/g) (BRASIL, 2003).

Subsequently, the colonies were differentiated macroscopically and triplicate seeded into test tubes containing PDA. When already sporulated, the isolates were subjected to decimal dilutions up to 10<sup>-3</sup> in 0.9%

saline and 0.1% tween 80 used to dissociate the conidia. To obtain monosporic colonies, 100 µL of the last dilution was inoculated in Petri dishes containing Sabouraud agar, pH 6.9 ± 0.2. For the fungi identification, microcultures were performed for observation of the reproductive structures under an optical microscope, stained with lactophenol (BARNETT; HUNTER, 1972). After identification, the CFUs of each genus/species of fungus grown in the primary samples were quantified, based on the colony morphology.

The results for counting of molds and yeasts were analyzed using the Kruskal-Wallis test to compare results from different seasons and Mann-Whitney to compare between washed and unwashed eggs means. Statistical analyzes were performed using the InStat 3.1 software (Graphpad®), all at a 5% significance level.

Table 1. Colony forming units (CFU), number of days from production, environment temperature and humidity on the moment of sample acquisition in relation to type of processing and season of the year.

	Unwashed eggs			Washed eggs		
	Rainy <sup>1</sup> n=12	Dry <sup>1</sup> n=12	Mean <sup>2</sup> n=24	Rainy <sup>1</sup> n=12	Dry <sup>1</sup> n=12	Mean <sup>2</sup> n=24
Fungi count (CFU/g)	14,59 <sup>a</sup>	37,25 <sup>a</sup>	25,92 <sup>A</sup>	1,46 <sup>a</sup>	0,77 <sup>a</sup>	1,11 <sup>B</sup>
Days from production	7,66 <sup>a</sup>	9,75 <sup>ab</sup>	8,71 <sup>A</sup>	21,58 <sup>bc</sup>	33,50 <sup>c</sup>	27,54 <sup>B</sup>
Environment temperature (°C)	25,54 <sup>a</sup>	27,17 <sup>a</sup>	26,35 <sup>A</sup>	25,41 <sup>a</sup>	26,87 <sup>a</sup>	26,14 <sup>A</sup>
Environment humidity (%)	57,17 <sup>a</sup>	52,08 <sup>a</sup>	54,62 <sup>A</sup>	58,75 <sup>a</sup>	50,83 <sup>a</sup>	54,79 <sup>A</sup>

<sup>1</sup> Means followed by equal lower-case letters on the same line do not differ statistically from each other, using the Kruskal-Wallis test ( $p>0,05$ ). <sup>2</sup> Means followed by equal capital letters on the same line do not differ statistically from each other, using the Mann-Whitney test ( $p>0,05$ ).

Unwashed eggs showed the highest average ( $p<0,05$ ) for the number of molds and yeasts in the samples. The fungi higher number in these eggs may be related to the washing process at the farm, demonstrating the efficiency of washing process in reducing fungi from table eggs. Jones et al. (2004) showed that, after washing the eggs, the yeast and mold populations remained low for the 10-week period of the study, resulting in significant difference on counts ( $p<0,0001$ ) at all data points. Musgrove et al. (2005) also found a low count of yeasts and molds with a mean of 57,54 CFU/egg. Even with low numbers, the washing process was able to significantly reduce contamination by 1 log on average ( $p<0,001$ ).

The contamination of eggs by microorganisms most of the time occurs through the shell and the storage time and temperature are fundamental factors for them to be able to pass from the surface of the shell to the internal structures of the egg, causing risks to the consumer's health (TOMCZYK et al., 2018). Extrinsic factors such as temperature, moisture on the eggshell, the number of microorganisms and the storage conditions may influence eggshell penetration (GOLE et al., 2014). It was demonstrated the growth of fungi inside old eggs stored in humid conditions and the possibility of detecting a passage of volatile organic compounds produced by these fungi through the shell (CUMERAS et al., 2016). Therefore, mycotoxin production inside the egg or its

## RESULTS AND DISCUSSION

There was no difference ( $p> 0,05$ ) for temperature and ambient humidity recorded in supermarkets during the egg acquisition period. These variables showed averages of 26.24 °C and 55%, respectively. The highest temperature recorded was 30.1 °C and the lowest 23.3 °C. In the data referring to ambient humidity, the highest observed value was 75% and the lowest 44% (Table 1). Most molds and yeasts grow best at temperatures between 25 °C and 30 °C (CAFARCHIA et al., 2014), therefore, the temperatures verified in the present work are at the ideal range for the growth of these microorganisms.

passage through the shell is a possibility (COOK et al., 2005; LACIAKOVÁ et al., 2001).

Considering the number of days the eggs were produced, washed eggs showed a significant difference, being stored for the longer period. However, it did not show differences in relation to the period of the year the eggs were produced (Table 1). Unlike what would be expected, the storage time did not influence the fungi count and the numbers were lower for eggs stored for longer. Probably the washing process was efficient and able to reduce contamination in numbers that did not allow significant subsequent growth. Also, this process removed dirt that could serve as substrate for these microorganisms.

In the content, the presence of these microorganisms was not identified, reinforcing the importance of the natural egg barriers to prevent contamination, since only eggs without cracks or cracks were used in this study. Jones et al. (2004) also found low concentrations (<0.3 log CFU/ml) of yeasts and molds in the contents of shell eggs with no difference ( $p>0,05$ ) between unwashed and washed eggs.

Regardless if the eggs were washed or not the contamination observed was low and, therefore, the risks to consumers' health were small. There is no legal limit for this group of microorganisms for eggs in shell, but for preserved or brined eggs, there is a maximum

limit of  $10^4$  CFU/g, number much higher than that found in this study (BRASIL, 2019).

From growth in plates obtained for fungi counts, 622 colonies were characterized morphologically. From these, 96 isolates were selected for microscopic characterization of the colonies, representing others that had the same aspect of size, shape and color. A part of the isolates (0.96%) could not be identified. The filamentous fungi identified were *Aspergillus niger* (37.62%), *A. flavus* (23.47%), *A. fumigatus* (13.50%), *A. terreus* (9.16%), *Penicillium* spp. (10.77%), *Fusarium* spp. (0.64%) and yeasts (3.85%) (Table 2). In this study, an important predominance of *Aspergillus* spp. was observed, different results were obtained by others (COOK et al., 2003; GRIZARD et al., 2014; RAJMANI et al.,

2011; TOMCZYK et al., 2018). Cook et al. (2003) found frequencies of *Aspergillus* spp., *Candida* spp. and *Fusarium* spp. of 1-5% and *Ovulariopsis* spp. and *Trichoderma* spp. of less than 1%. Tomczyk et al. (2018) observed as the most prevalent genera in eggshells *Alternaria* (28%), *Penicillium* (23%), *Chaetomium* (13%) and *Fusarium* (11%). Rajmani et al. (2011) found high frequencies of different fungi, identified as *Aspergillus* spp. (42%), *Rhizopus* spp. (26%), *Mucor* spp. (16%), *Penicillium* spp. (14%), *Fusarium* spp. (12%) and yeasts (9%). Grizard et al. (2014) observed a very singular mycobiota from incubation eggs, identified using molecular techniques. Most of the species identified were environmental or plant pathogens fungi, with the exception of *Cryptococcus* spp., corresponding 7.7% of affiliated sequences from early incubation eggs.

Table 2. Number and percentage of colonies morphologically characterized and microscopically identified obtained from unwashed (UW) and washed (W) eggs in two periods of the year.

Fungi	Period of the year				Total
	Rainy season		Dry season		
	UW	W	UW	W	
<i>Aspergillus niger</i>	17 (10.9%)	9 (40.9%)	208 (46.8%)	-	234
<i>Aspergillus flavus</i>	-	11 (50.0%)	135 (30.4%)	-	146
<i>Aspergillus fumigatus</i>	80 (51.3%)	2 (9.1%)	2 (0.5%)	-	84
<i>Aspergillus terreus</i>	44 (28.2%)	-	13 (2.9%)	-	57
<i>Fusarium</i> spp.	1 (0.6%)	-	3 (0.7%)	-	4
<i>Penicillium</i> spp.	2 (1.3%)	-	65 (14.6%)	-	67
Yeasts	9 (5.8%)	-	15 (3.4%)	-	24
NI	3 (1.9%)	-	3 (0.7%)	-	6
Total	156	0	444	0	622

NI – not identified. Values with “-” means no fungi growth.

Regarding the number of isolates, only 30% came from eggs acquired during the rainy season and 70% from the dry season, although the absolute quantities were low and there was no significant difference between seasons (Table 1).

The predominant genus observed in the isolates was *Aspergillus*, followed by *Penicillium* and *Fusarium*. Environmental isolation of *Aspergillus* spp. in laying hen farms was always positive when humidity was higher than 50%. Also, showed that with temperatures between 26 and 29°C, positivity was 44.4%, while it was 100% for temperatures higher than 29°C. (CAFARCHIA et al., 2014). The fungi isolated in this work are commonly cited in the literature and can have an impact on public health. Fungi of these genera are found in grains, feed and food. During storage, under favorable conditions of temperature, humidity, water activity and relative humidity, they produce harmful mycotoxins for those who consume them (OLIVEIRA et al., 2017).

Some species of the *Aspergillus* genus produce the mycotoxin called aflatoxin, with the B1, B2, G1 and G2 types being the ones of major importance in terms of

toxicity. Among the identified species in this study, *A. flavus* is able to produce B1 and B2 toxins and B1 is considered the most toxic for animal and human species. It is potentially carcinogenic, categorized as a group I carcinogen by the International Agency for Research on Cancer (IARC), particularly affecting the liver. It is noteworthy that this genus is widely disseminated in nature and the excessive consumption of food contaminated by aflatoxins in developing countries is a concern, which is why researchers are focused on identification and prevention techniques (KUMAR et al., 2017). Another important mycotoxin is Ochratoxin A (OTA), produced by genus *Aspergillus niger* and *Penicillium verrucosum* (MURUGESAN et al., 2015). It can lead to an increased risk of nephritic syndrome at very high exposures to OTA in humans (BUI-KLIMKE; WU, 2015).

*Penicillium* was the second most isolated genus in this study and is an important producer of mycotoxins. It is also able to accelerate the process of rotting eggs (TOMCZYK et al., 2018). *Fusarium* was the least identified genus in this study. These molds can produce trichothecene mycotoxins and also have capacity to

penetrate the eggshell (TOMCZYK et al., 2018), therefore, can pose risk to consumers' health.

Different fungi could produce several types of mycotoxins and contamination of eggs with more than one mycotoxin has already been reported. Interactions between mycotoxins present in food can occur, causing synergistic or antagonistic effects (SMITH et al., 2016). However, detection of mycotoxins does not necessarily mean negative effects, but only when in high amounts and which vary according to the mycotoxin in question (MURUGESAN et al., 2015).

Despite the diversity of fungi found and the identification of species that are often related to health risks, a small number of these microorganisms was present in eggs, regardless of being washed or not and season of sampling. Thus, this study corroborates the research by Tomczyk et al. (2018), who concluded that the risk of contamination by mycotoxins and microorganisms in eggs can be considered negligible and that it is unlikely to pose a threat to human health.

## CONCLUSIONS

*Aspergillus* spp. was the most frequently isolated fungi isolated from table eggs, with *A. niger* and *A. flavus* predominant in the dry season, while *A. fumigatus* and *A. terreus* in the rainy season. Low numbers of fungi were identified from egg shells, with a higher amount isolated from unwashed eggs. The period of the year did not influence the amount of fungi in eggs, despite having influenced the fungal diversity.

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