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ORIGINAL ARTICLE

Determination of fungi and their aflatoxins in embryonated eggs a production batch

Determinación de hongos y sus aflatoxinas en huevos embrionados en un lote de producción

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A B S T R A C T

The fungal load of origin and the presence of aflatoxins were determined in a batch of embryonated egg production, for this, aliquots of dissolution medium were seeded after a gentle rubbing of the eggshells of a batch, were incubated and then the total load of fungi of origin was quantified. Subsequently, the presence of any of the four main aflatoxins (B_1, B_2, G_1 and G_2) was determined using the ELISA method following the kit recommendations. All of the embryonated eggs had the presence of fungus, the fungal loads varied between 100 and 10 520 MFU/egg. 76 % (38/50) of the samples were positive for any of the four main aflatoxins and the estimated concentration of aflatoxins was 5.4 ± 2.9 µg/egg. It was possible to demonstrate the fail to the quality and safety of the food, both for human or animal consumption, or for animal production, since there is evidence of the high embryonic mortality generated by the mycotoxins that manage to enter the egg, in addition, it is evident the need to develop "organic" strategies for fungal control in the embryonic eggshell.

Keywords: Mycotoxins, Aflatoxin, Embryonated egg

R E S U M E N

Se determinó la carga fúngica de origen y la presencia de aflatoxinas en un lote de producción de huevo embrionado, para ello se sembraron alícuotas de medio de disolución tras un frotado suave de las cascaras de huevo de un lote, se incubaron y se realizó la cuantificación de la carga fúngica total de origen. Posteriormente se determinó la presencia de alguna de las cuatro aflatoxinas principales (B_1, B_2, G_1 y G_2) empleando el método de ELISA siguiendo las recomendaciones del kit. Todos los huevos embrionados tuvieron presencia de hongo, las cargas fúngicas variaron entre 100 y 10 520 UFM/huevo. El 76 % (38/50) de las muestras resultaron positivas a alguna de las cuatro aflatoxinas principales y la estimación de la concentración de aflatoxinas fue de 5.4 ± 2.9 µg/huevo. Se logró evidenciar el compromiso de la calidad e inocuidad del alimento, tanto para consumo humano o animal, o para la producción animal, ya que existen evidencias de la alta mortalidad embrionaria que generan las micotoxinas que logran ingresar al huevo, además, se hace evidente la necesidad de desarrollar estrategias "orgánicas" para el control fúngico en la cáscara del huevo embrionado.

Palabras clave: Micotoxinas, Aflatoxina, Huevo embrionado

INTRODUCTION

Impact of the presence of toxigenic fungi in the embryonic egg

In practice, 100 % yield can seldom be achieved in egg incubation for "broiler" chicken production (Manders *et al.,* 2021). There are many factors that affect the incubation process and it is essential to know how to do a good analysis of all the available data to know what the problem is and to propose "organic" alternatives that do not modify the percentage yield of the product.

The commercial incubation rate is the number of births of "first-class chicks" (healthy, with good vitality and suitable for rearing), calculated on the number of eggs placed in the incubator, expressed in percent; this is affected by losses caused by different causes, the main ones are listed in Table 1. The authors point out that the frequency values that appear in Table 1 may increase as incubation conditions worsen (genetic quality, nutrition, management, conservation, climate, avian health, among others) (Manders *et al.,* 2021).

Table 1. Main causes of losses in the commercial incubation rate.
Tabla 1. Principales causas de pérdidas en la tasa de incubación comercial.

Cause	Frequency (%)
Broken shell / with movable inner tube	26.2
Presence of fungi / yeasts in the shell	22.6
Not fertilized	6.3
Dead embryo due to heterogeneous heat distribution in the incubation chamber	3.1
Other*	41.8

*Causes with frequencies less than 1.6 %. Source: Manders *et al.,* 2021.

Alternatively, the most common environmental factors that affect the industrial incubation process and cause losses in the commercial incubation rate are described in Table 2. In the egg shell it is common to find microorganisms, including non-pathogenic bacteria that are found in the oviduct and ovary of hens, such as *Lactobacillus* and *Micrococcus*, however, it is also possible to find pathogenic bacteria such as *Salmonella, Staphylococcus, Pasteurella, Listeria* and *Pseudomonas* of environmental origin and of the oviduct and ovary of hens, as well as toxigenic fungi (Neira-Solís, 2016).

Table 2. Most common environmental causes of alteration in the commercial incubation rate.
Tabla 2. Causas medioambientales más comunes de alteración en la tasa de incubación comercial.

Environmental cause	Signs
More 10 days in conservation at temperature > 18 °C of the eggs during the pre-incubation period (aging of the eggs).	1. High mortality from the first hours of incubation. Most embryonic eggs do not have blood rings. 2. Increases evaporation of water in eggs during weighing. 3. Embryonic development is uneven, some eggs delayed, others early.
High temperature (> 30 °C)	Increase in embryonic and hatched chick mortality. Most of the unhatched urchins are dead.
Low temperature (< 28.8 °C)	Extension of the incubation period (> 504 h). Unhatched semi-active live chicks.
High humidity (> 60 % RH)	High embryonic mortality due to poisoning due to the presence of pathogenic fungi in the cuticle, intermediate membrane and inner membrane of the egg shell.
Low humidity (< 56 % RH)	Live, semi-active chicks without hatching, due to dryness of the membranes and lung.
Alterations in the air circulation regime and in the turning of the eggs	Dramatically increases embryonic mortality due to anoxia.
Nutrient deficiency (vitamins and minerals) in the egg	If the feed of the laying hens is deficient in vitamins and minerals for some time prior to the evacuation of the egg (> 2 weeks), a high embryonic mortality occurs, in semi-active chicks without hatching and even after hatching, during the first periods breeding.

Source: Manders *et al.,* 2021.

The main genera of toxigenic fungi found as contamination of origin in the egg are: *Aspergillus, Fusarium* and *Penicillum,* as well as yeasts of the genus *Torula* (Neira-Solís, 2016).

The high humidity (56 – 60 % Relative Humidity, RH) and the incubation temperature (28.8 - 30 °C) at which embryonated eggs must be incubated favors the development of fungal microorganisms in their shell (Nyholm, 2020). From the moment of oviposition, the egg presents a high load of fungi and yeasts on its surface (> 10^3 "CFU" and "MFU", respectively per egg) (Bunker *et al.,* 2021). According to Chousalkar and McWhorter (2020), the horizontal microbial contamination of eggs (different from that of origin) depends on the cleanliness of the laying sites and the way they are handled after being obtained. If the shell remains intact, the only way for microorganisms and their mycotoxins to penetrate into the egg is through the pores (Flórez-Valencia, 2020).

In the storage of food of livestock origin, a major problem is the deterioration and contamination with mycotoxins produced by fungi such as *Aspergillus, Fusarium* and *Penicillium* that cause great economic losses throughout the world (Alonso *et al.,* 2013). In addition, these fungi produce allergenic spores and mycotoxins that cause serious potential health hazards (Egbuta *et al.,* 2017).

Impact of mycotoxins on the embryonic egg

Mycotoxins are considered toxic secondary metabolites produced by microscopic fungi during the stationary phase of their growth on food and often cause food poisoning (Peivasteh-Roudsari *et al.,* 2021; Ráduly *et al.,* 2020). In addition, the fungal allergens produced mainly by the genera *Alternaria, Aspergillus, Cladosporium* and *Penicillium* are ingested with foods such as cheeses processed by fungi, gross fungi, vegetables, dehydrated fruits, eggs, foods containing yeast, soy sauce or vinegar and produce respiratory allergies in susceptible subjects (Rodríguez-Orozco *et al.,* 2008). In embryonated eggs, it generates a high embryonic mortality due to intoxication, loss of osmoselective porous structure of the cuticle, intermediate membrane and internal membrane of the egg shell (Manders *et al.,* 2021).

The reduction of the fungal and yeast load in the eggshell is achieved by exposing the fertilized egg to a low penetration ultra-violet light system, with diluted solutions of 0.5 % formaldehyde, by exposing the water vapor at 40 °C and use of antifungal chemicals such as posaconazole, voriconazole, fluconazole and itraconazole, however, all strategies reduce the effect of altering the commercial incubation rate (Manders *et al.,* 2021). Adequate control measures to prevent the growth of fungi in the embryonic egg are of primary importance to avoid contamination with mycotoxins (Alaniz-De La O *et al.,* 2016).

Emerging technology for the control of fungi and their mycotoxins

The chemical and physical control of fungi on living and inherent surfaces is widely documented, recently, "organic" strategies have been described that can be used on living surfaces and foods, such as the use of bacteriocins from lactic acid bacteria, competitive yeasts such as *Streptomyces*, secondary metabolites of plants, among others (Takaya, 2002; Shah and Pell, 2003; Thines *et al.,* 2004; Prapagdee *et al.,* 2008; Smaoui, *et al.,* 2010). Regarding the control of the production of mycotoxins, the control of the temperature, relative humidity and biochemical environment of the substrate is the most effective strategy, however, there are novel methodologies for the destruction of the chemical structure of mycothixin such as ionizing and non-ionizing irradiation, high-pressure processing, pulsed electric field, pulsed light, cold plasma, and ultrasound (Magan and Olsen, 2004; Weaver *et al.,* 2020; Alizadeh *et al.,* 2020).

The rationale for this study

In the egg shell it is common to find microorganisms, including non-pathogenic bacteria, pathogenic bacteria and toxigenic fungi that are found as contamination of origin in the egg (*Aspergillus, Fusarium* and *Penicillum*), as well as yeasts of the *Torula* genus (Neira-Solís, 2016). In addition to the presence of these, the high humidity and the incubation temperature at which the embryonated eggs must be incubated favors the development of fungal microorganisms in their shells (Nyholm, 2020).

The presence of the fungus, its mycotoxins and its allergens in a food compromise the quality and safety of the food for human or animal consumption. Likewise, there is evidence of high embryonic mortality generated by mycotoxins that are introduced into the egg with fungal presence in the shell (Peivasteh-Roudsari *et al.,* 2021).

The purpose of this study

Determine the fungal load of origin and the presence of aflatoxins in an embryonated egg production lot.

MATERIALS AND METHODS

For the quantification of the total fungal load of egg origin in a production batch

From a batch of embryonated eggs (100 units), each egg was aseptically introduced one by one into a sterile bag with 15 mL of peptone diluent (DP, Difco™). Each of them independently rubbed the eggshell in circular motions for two minutes (by soft rub). Thee aliquots of 3.33 mL (for increase the method sensibility) and another of one milliliter were seeded in the potato dextrose agar culture medium (APD, Difco™), adding rose bengal (60 mg/L) and ampicillin (100 mg/L) using the technique of pouring in plate, to later be incubated for 5 d at 25 °C. Colonies with typical fungus and yeast morphology were counted. The report of the fungal load of origin was reported in MFU/egg.

To determine the presence of any of the four main aflatoxins (B_1, B_2, G_1, and G_2)

The ELISA (Enzyme-Linked Immunosorbent Assay) method was used, which is a direct competitive enzyme immunoassay in solid phase, provided by Sigma-Aldrich™. That it has an optimized specific antibody that allows the four subtypes of aflatoxin to be cross-determined. To carry out the method according to the supplier's recommendations, it is necessary to extract one milliliter of allantoic fluid from the embryonic egg and dissolve it in 2 mL of 70 % methanol. A 50 µL aliquot of this methanol solution is deposited into an antibody coated microwell on the ELISA plate. Subsequently, it had to be incubated for 15 min at 22 - 25 ºC. Once the time had elapsed, the methanol solution was decanted and 100 µL of the enzymatic substrate (Reagent A, Redoxy-specific immunoprotein conjugated with methylene blue) will be added. It was incubated for 60 min; the intensity of the blue color will decrease according to the concentration of any of the types of aflatoxin present. Therefore, the intensity of the color is directly proportional to the amount of conjugate bound and inversely proportional to the aflatoxin concentration in the sample or standard. Therefore, as the aflatoxin concentration in the sample or standard increases, the intensity of the blue color will decrease. The presence or absence of aflatoxin in the sample and its concentration with respect to the standard were reported.

Statistical data analysis

In this study the determinations are made in duplicate. The responses recorded as results have a dichotomous character (present or absent). The frequency is presented as a percentage of positivity or occurrence of the response variable. The T-student test (*alpha* = 0.05) was used to describe statistical differences between the replicas. Dispersion graphs of means and quartiles represented by box and whisker graphs were generated, both for the concentration of fungi present in the egg shell, and for the quantification of aflatoxins in the allantoic liquid.

RESULTS AND DISCUSSION

Microbial safety is an important factor contributing to the egg quality. During egg acquisition, there is significant risk of contamination of the eggshell surface with microscopic fungi. Mycelial hyphae may grow on the eggshell surface and penetrate into the egg content. However, there is no information on the populations of microscopic fungi on the eggshell surface and, consequently, on possible production of mycotoxins. Therefore, the aim of the study was to identify the species of microscopic fungi present on the eggshell surface acquired from different breeding systems and to measure the number of selected mycotoxins.

In our study, the composition of the fungal species in the egg shell was not determined, a total micellar count was carried out. The fungal load in the embryonic egg shell was determined, resulting in levels between 100 MFU/egg (limit of quantification) and 10 520 MFU/egg, with an average of 4 746 MFU/egg and a standard deviation of 2 058 MFU/egg (see Figure 1). All of the embryonated eggs presented the presence of fungi.

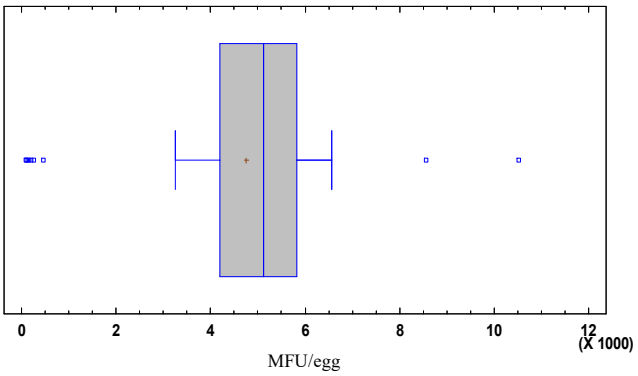

Figure 1. Distribution of the fungal load in the shell of the embryonic egg.
Figura 1. Distribución de la carga fúngica en la cáscara del huevo embrionado.

Tomczyk *et al.* (2018), reported that the composition of the species of fungi isolated from egg shells differed according to the housing system of laying hens, in such

a way that our study may show considerable differences against other similar. In the aforementioned study, a predominant prevalence of species of the genus *Alternaria* is shown, followed by *Fusarium, Scopulariopsis, Purpureocillium, Aspergillus, Botrytrichum* among others.

The diversity of potentially pathogenic fungal species on the eggshell surface is related to the unique microclimate inside the henhouse with the deep litter system, that is, high air humidity and temperature, poor ventilation, exogenous contamination, (litter, feed) and endogenous contamination (dust) (Gros *et al.,* 2015). In addition, the possibility to control hen breeding conditions in the free-range system is limited. As hens have a free access to full-value feed or green forage, litter and the range, there is higher risk of contact with pathogenic organisms of different origin (Piskorska-Pliszczynska, *et al.,* 2014). However, adequate humidity and temperature reduces the risk of the extensive growth of pathogenic fungi (SCIENCE, 2021).

The presence of fungi on the eggshell surface involves the potential risk of their presence and production of mycotoxins in the egg content. Szablewski *et al.* (2010), showed that there were no fungi in the yolk after two weeks of storage at high relative humidity, however, they evidenced the presence of mycotoxins in the egg white. In addition, the study by Tomczyk *et al.* (2018), did not show any correlation between the mycotoxin content in the egg and the laying hen rearing system.

In this studio, the presence of any of the four main aflatoxins (B_1, B_2, G_1 and G_2) was determined, resulting in a positivity of 76 % (38/50) (Figure 2).

The aflatoxin concentration was determined by spectrophotometry (ELISA reader light/UV, Thermo-Scientific™ Multiskan™ FC) with respect to the standard provided by Sigma-Aldrich® (Figure 3). Estimates of aflatoxin concentration per egg were 5.4 ± 2.9 µg/egg (Figure 4). It should be noted that the level of mycotoxins in embryonated eggs is below the level allowed for human or animal consumption, it becomes relevant in the context of embryonic production of farm chickens. However, attention should be paid to the possibility of mycotoxin bioaccumulation in animal tissue that could be consumed by humans (Escrivá *et al.,* 2017). Our study reports on the new potential source of chemical and microbial hazards in the poultry farming industry.

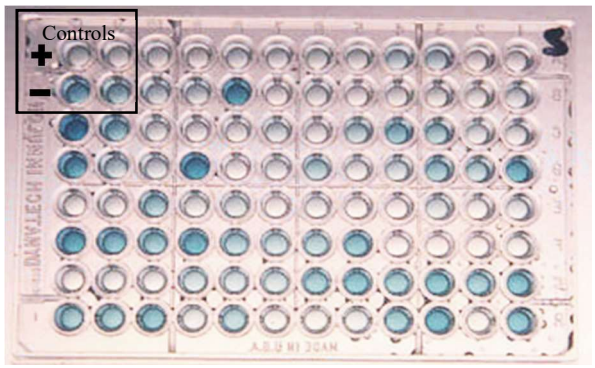

Figure 2. Results of the ELISA plate for the determination of the presence of any of the four main aflatoxins (B_1, B_2, G_1 and G_2).

Note: Wells not colored blue show positivity for the presence of aflatoxins.

Figura 2. Resultados de la placa de ELISA para la determinación de presencia de alguna de las cuatro aflatoxinas principales (B_1, B_2, G_1 y G_2).

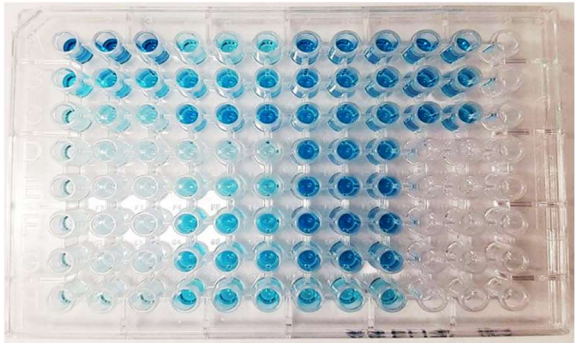

Figure 3. Results of the ELISA plate for the quantification of aflatoxins in embryonated egg samples.

Note: In rows A-C the absence of aflatoxin in the sample is confirmed. In rows D-H the concentration of aflatoxin in the sample was determined by dilution curve.

Figura 3. Resultados de la placa de ELISA para la cuantificación de aflatoxinas en las muestras de huevo embrionado.

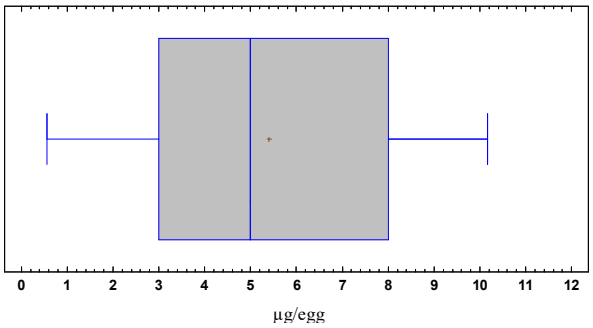

Figure 4. Distribution of aflatoxin quantification in the embryonic egg shell.

Figura 4. Distribución de la cuantificación de aflatoxinas en la cáscara del huevo embrionado.

CONCLUSION

It was possible to detect a fungal load of origin, all the units evaluated presented fungi in their shells. Definiteness, the quantitative analysis of the fungi isolated showed that the shell of embryonated eggs was a potential substrate for the growth of numerous fungi, including pathogenic and toxin-producing species, for example, *Aspergillus, Fusarium* and *Alternaria*. Moreover, the diversity of the fungal population differed according to the egg-laying hen housing system. The fungal species present on the eggshell surface may occur in the environment of the henhouse. The presence of aflatoxins in the embryonated egg production lot, compromises the quality and safety of the food, both for human or animal consumption, or for animal production, since there is evidence of the high embryonic mortality generated by the mycotoxins that manage to enter the egg, in addition, it is evident the need to develop "organic" strategies for fungal control in the embryonic eggshell.

Author Contributions

Conceptualization, C-F,TK, P-F,JJ, and N-V, CL; Methodology, C-F,TK, P-F,JJ, G-L. AY and O-P, LE; Investigation, C-F,TK, P-F,JJ, M-E,AL and R-G,MA; Data Curation, C-F,TK and P-F,JJ; Writing-Original Draft Preparation, C-F,TK and P-F,JJ.

Conflict of interest

The authors declare that they have no conflict of interest.

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