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Product Quality and Safety

Evaluation of bacterial contamination of laying hen eggshells by using a classic and fast method: first report in Algeria

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Abstract. *Chicken eggs are considered an important source of high-quality protein for humans. Many types of germs can contaminate eggshells, some of which are pathogenic. This survey aims to study the bacterial contamination of eggshells from ISA Brown laying hens of 56 weeks old. The study concerned a total of 100 eggs randomly collected. Two methods were used for investigation and enumeration of bacteria: a classical method and a rapid method Rida®Count. The classical method concerned: total bacteria, total and fecal coliforms, fecal streptococci, Staphylococcus aureus and Salmonella spp.. The rapid method RIDA®COUNT concerned: Total bacteria count, total coliforms, Staphylococcus aureus and Salmonella spp. In both methods, all eggshells were found to be contaminated with aerobic mesophilic bacteria. The average number revealed by the rapid method was 2.95 ± 1.06 Log CFU/cm² and by the classical method it was 2.85 ± 0.99 Log CFU/cm². The rapid method revealed a higher number of eggshells infected with total coliforms (90%) and Staphylococcus aureus (43%) compared to the classical method (56% and 19%, respectively). The average number of total coliforms (2.47 ± 0.95 Log CFU/cm²) and Staphylococcus aureus (1.67 ± 0.86 Log CFU/cm²) revealed by the classical method was close to the rapid method (2.35 ± 1.01 Log CFU/cm² and 1.43 ± 0.83 Log CFU/cm², respectively). Bacterial counts were not significantly different between the two diagnostic methods. The total absence of Salmonella spp. was confirmed. However, the presence of two eggs infected by Raoultella planticola (2%), and two eggs by Escherichia coli (2%) were found. This investigation provided the first partial description in Algeria of the bacterial contamination of laying hen eggshells using two methods: classic and rapid. The good hygiene and management can avoid contamination with dangerous bacteria represented mainly by Salmonella spp.. However, it is necessary to study the bacterial contamination inside the egg and to extend the research to other bacteria.*

Keywords: Bacterial counts, eggshells, hens, Algeria

Introduction

The transmission of pathogens to humans through food is a major public health problem worldwide because of the mortality, morbidity and costs associated with treatment and prevention (WHO, 2015). Chicken eggs are considered an important source of high-quality protein for humans and one of the most versatile products in the food industries due to their foaming, emulsifying, gelling, and flavoring properties (Bhat et al., 2021). The nutritional value of egg proteins is ranked among the best and is just behind that of human mother's milk. It contains all nine essential amino acids and all nine non-essential amino acids (Founou et al., 2016).

World egg consumption is constantly increasing (Abín et al., 2018), therefore, food safety measures must be taken into account in modern egg production (Wang et al., 2017). Egg quality depends on external (egg weight and volume, shell characteristics, and specific gravity) and internal (albumen and yolk indices) properties (Bhat et al., 2021).

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Several foodborne illness outbreaks have been identified due to the consumption of contaminated eggs (Moyle et al., 2016). The presence of highly nutritious substances in eggs creates a suitable environment for bacterial growth, of which some are pathogenic (Jain et al., 2017). Freshly laid eggs are generally free of microbes and superficial contamination occurs immediately after laying. Various factors can influence bacterial contamination of the eggshell such as the type of housing system (Jones et al., 2016), the presence of food and feces in nests (Im et al., 2015) and season (Parveen et al., 2017). In hens, the digestive, urinary and reproductive tracts open in the same location, which could contribute to eggshell contamination during egg laying (De Reu et al., 2006a). Eggshells contamination can occur vertically (from infected hens) or horizontally (in the external environment) (Poleh et al., 2018; Isnawaida et al., 2021); the second can induce internal egg contamination (Lin et al., 2021).

A variety of microorganisms can contaminate eggshells, some of which are pathogenic. Fecal bacteria are the most common. The authors identified *Salmonella* spp., *Campylobacters* spp., *Escherichia coli* (Okorie-Kanu et al., 2016; Alter, 2017), *Bacillus* spp., *Serratia* spp*., Staphylococcus* spp. or *Pseudomonas* spp. (Ge et al., 2016; Ananou et al., 2018). High contamination of the eggshell led to a greater possibility of microorganism penetration and contamination of the egg contents (De Reu et al., 2006b).

In Algeria, no studies have been conducted to investigate the eggshell contamination. Therefore, this study was conducted to evaluate the bacterial contamination of the eggshells of commercial brown egg laying hybrid hens collected in a laying hen farm located in Algiers. The investigated parameters were: Total bacteria, total and fecal coliforms, fecal streptococci, *Raoultella planticola, Escherichia coli, Staphylococcus aureus* and *Salmonella* spp.

Material and methods

Egg collection

The study was conducted in a laying hen farm, located in Algiers. The hens were 56 weeks old ISA Brown, raised in battery cages, receiving 16 hours of light per day and fed *ad libitum* with a standard layer feed. The internal temperature of the farm is constant and the lighting and the extinction of the lights are automatic. The ventilation is dynamic; which allows the control of the air flow in the farm. A total of 100 eggs were randomly collected during the summer season and aseptically transported in sterile isothermal bags to the laboratory for analysis in the same day.

Microbiological analysis

Two methods were used for investigation and enumeration of bacteria: a classical method and a rapid method Rida®Count. The classical method concerned: total bacteria count (according to ISO 4833-1 standard), total and fecal coliforms (according to ISO 4832, NF V08-060 standard), fecal streptococci (according to ISO 7899-2 standard), *Staphylococcus aureus* (according to NF EN ISO 6888-1/A1 (ISO, 2004**)**) and *Salmonella* spp. (according to ISO 6579-1 standard).

The rapid method RIDA®COUNT concerned: Total bacteria count, total coliforms, *Staphylococcus aureus* and *Salmonella* spp.

Preparation of initial solutions and dilutions

In a sterile bag containing 70 ml of buffered peptone water (BPW; ref: AEB 140302), one egg was placed and rubbed carefully for 2 minutes, then removed (Loongyai et al., 2011).

The resulting smear is homogenized by a peristaltic stomacher device for 2 minutes. Then 1/10, 1/100, 1/1000 dilutions are prepared. These dilutions were used in both the classical and rapid methods. The results of counts were presented in Log of Colony Forming Units (CFU) / cm². The surface area of the eggs (S) in cm² was calculated from the average weight of the eggs (P) in grams according to the formula of Bonnet and Mongin (1965): $S = 4.68\ P^{2/3}$.

Classical method

Determination of total bacteria counts (ISO 4833-1)

Enumeration was performed on PLAT COUNT AGAR (PCA) medium. Briefly, 1 ml of each decimal dilution was aseptically transferred to a separate sterile petri dish. Then, 15 ml of the PCA agar, previously dissolved and cooled to 47°C in a water bath, was placed in each petri dish. The mixture was shaken carefully and left to solidify. Then, a second layer of 5 ml of PCA agar was added to prevent spreading of colonies, and to obtain semi-anaerobic conditions. Afterwards, it was left to solidify. The plates were then placed in the incubator for 72h at 30°C. Then, colonies were counted.

Determination of total and fecal coliforms (ISO 4832, NF V08-060)

The enumeration of total and fecal coliforms was performed by the same method described above, but using the VRBL medium (Violet Red Bile Agar). Petri dishes were incubated for 24h at 30°C for total coliforms, and 44°C for fecal coliforms. The dark red to purplish colonies, with a diameter greater than 0.5 mm, were counted.

Determination of fecal streptococci (ISO 7899-2)

The cultivation and enumeration of fecal streptococci was performed by the same method described above, but using the Slanetz and Bartley medium.

Petri dishes were incubated for 24 h at 37°C.The colonies appeared bulging with a red-brown or pink color either in the center or on the whole colony.

A confirmation step is necessary on a Bile-Esculin-Azide Agar medium with incubation at 44°C during 2 h. After incubation, all colonies showing a brown-black color are considered positive.

Determination of Staphylococcus aureus (NF EN ISO 6888-1/A1)

0.1 to 0.5 ml of inoculum was spread on the surface of Baird-Parker agar, incubated for 24 to 48h at 37°C. Characteristic colonies of *Staphylococcus aureus* are black or gray, shiny and convex and surrounded by a halo of lightening due to hydrolysis of egg proteins. Opalescent areas (halo) due to lipolytic activity (lecithinase) may appear after 24 hours.

The biochemical confirmation concerned:

Catalase reaction: A part of the bacterial colony was put on 1 drop of H_2O_2, 20V, a positive reaction results in the appearance of air bubbles.

Coagulase reaction: A colony was cultured in a heart-brain broth. This was incubated thereafter for 20-24h at 37°C. Then, 0.1 ml of this broth was put in a tube containing 0.3 ml of rabbit plasma and incubated for 24 hours at 37°C.

The reaction is considered positive if the coagulum occupies more than ¾ of the volume of the initial liquid.

Determination of Salmonella spp. (ISO 6579-1)

A pre-enrichment was performed in buffered peptone water. The enrichment was carried out on the two selective broths: Selenite-Cystine Broth, and Rappaport-Vassiliadis Salmonella Enrichment Broth.

Isolation: It was performed on the two agar media: Hektoen and Xylose Lysine Deoxycholate (XLD agar).

Identification and confirmation of characteristic colonies was performed by:

- *Gram staining method*: It is performed on an isolated colony, the presence of small pink bacilli (Gram negative) is in favor of the bacteria sought.

- *Biochemical confirmation*: From each dish of isolation media, two (at least) suspected colonies were plated on the following media: triple sugar iron (TSI), urea indole, Lysine decarboxylase (LDC), medium for reaction medium, Methyl Red (MR), Simmons Citrate, β-Galactosidase Test and the Voges-Proskauer Simmons' Citrate.

- *Biochemical panel API (Analytical Profile Index) 20E:* Pick up a single isolated colony (from a pure culture) and make a suspension of it in sterile distilled water. Take the API 20E biochemical test strip which contains dehydrated bacterial media/biochemical reagents in 20 separate compartments. Take a Pasteur pipette and fill up (up to the brim) these compartments with the bacterial suspension. Anaerobiosis was ensured in the arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease and H_2S production tests by filling the cupules with sterile mineral oil to form a convex meniscus. The incubation boxes were closed and incubated at 37°C for 18 to 24 h. Identification of isolates was performed according to the numerical profile of API 20E observed.

Rapid method Rida®Count

This investigation was conducted using Rida®Count petrifilms, which consist of a film covered with a dry culture medium covered by a tissue, which allows a perfect absorbance of 1ml of the sample solution.

Determination of total bacteria counts

Place 1 ml of each respective dilution on Rida®Count agar film. The agar films were then placed at 35°C for 24h-48h. The colonies were then counted.

Determination of coliforms

The same protocol mentioned above has been applied here. The blue and green colonies were counted on the surface of the film after incubation.

Determination of Staphylococcus aureus

The same protocol mentioned above has been applied here. The green colonies with an approximate diameter of 1 mm were counted.

Determination of Salmonella spp.

The same protocol mentioned above has been applied here. *Salmonella* spp. form distinct, small, black colonies.

Statistical analysis

Statistical analysis was performed using the SPSS statistical package (version 17.0, SPSS Inc., USA). The Student's t test was used to study the difference in the number of bacteria between the two methods: classic and rapid. Significance is considered if $p < 0.05$.

Results

A box plot of the different bacteria enumerated from the eggshell by the classical and rapid methods was presented in Figure 1 and 2.

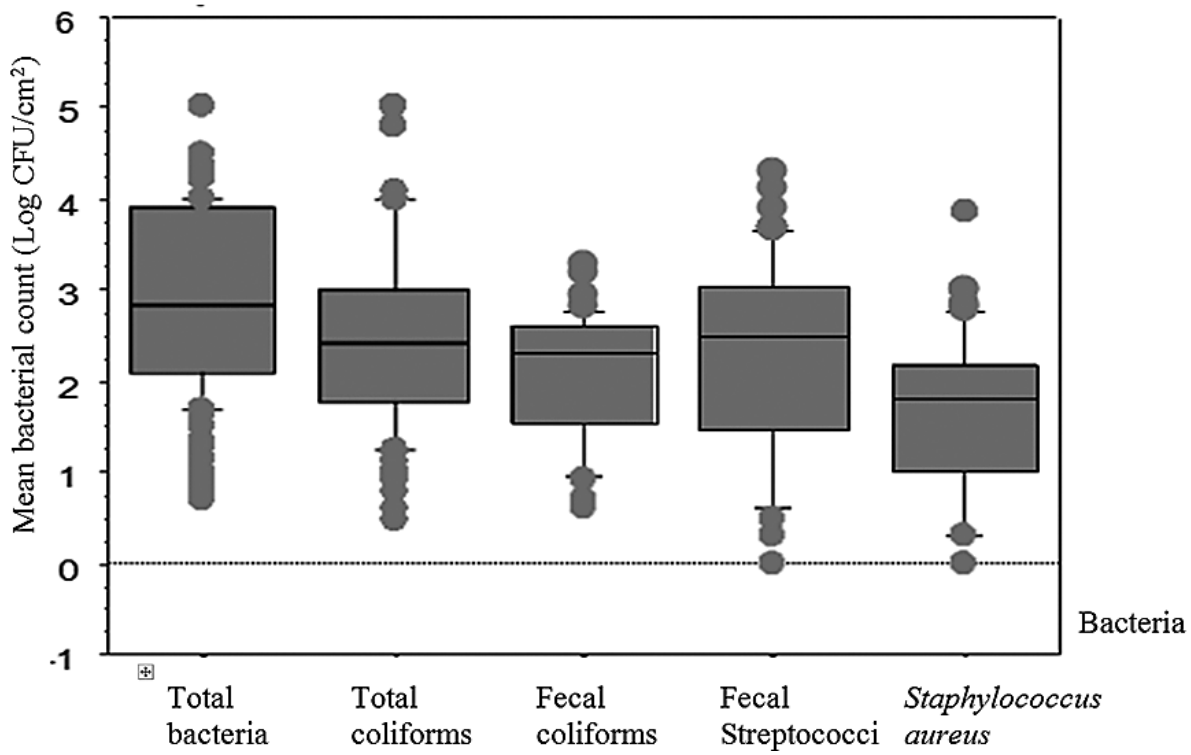

Figure 1. Box plot of the different bacteria enumerated from eggshell by classical method

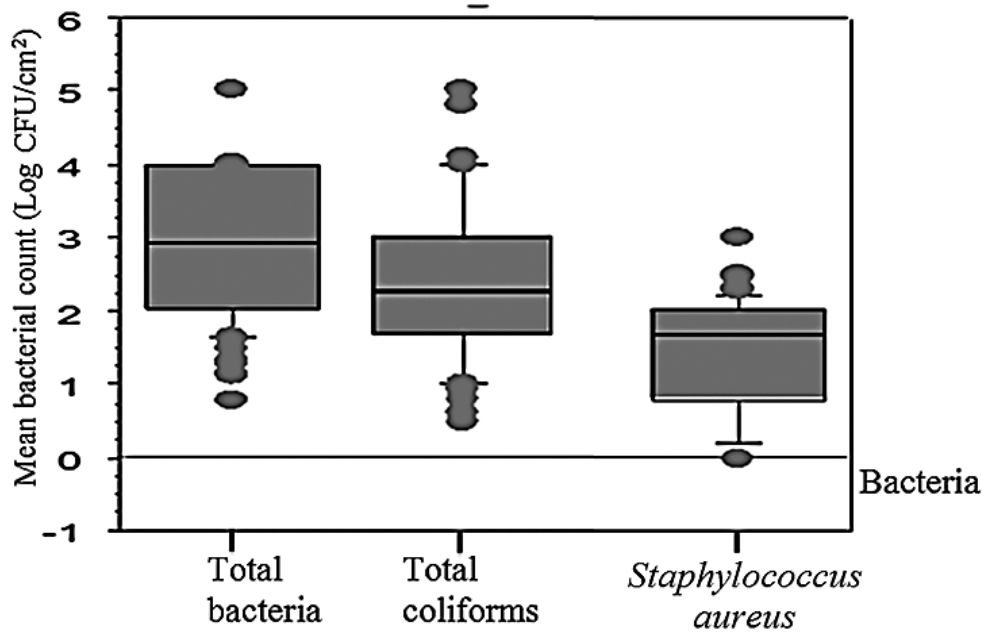

Figure 2. Box plot of the different bacteria enumerated from eggshell by the rapid method

The prevalence of contamination revealed by the classical and rapid method varies from 19% to 100% and from 43% to 100%, respectively (Table 1).

The results showed that there was no significant difference in the bacteria count revealed by the two methods.

All eggshells were found to be contaminated with aerobic bacteria. The total bacteria count revealed by the rapid method was 2.95 ± 1.06 Log CFU/cm² and by the classical method it was 2.85 ± 0.99 Log CFU/cm² (Table 1).

The rapid method revealed a higher number of eggshells infected with total coliforms (90%) and *Staphylococcus aureus* (43%) compared to the classical method (56% and 19%, respectively). The total coliforms counts (2.47 ± 0.95 Log CFU/cm²) and *Staphylococcus aureus* counts (1.67 ± 0.86 Log CFU/cm²) revealed by the classical method were close to the rapid method (2.35 ± 1.01 Log CFU/cm² and 1.43 ± 0.83 Log CFU/cm², respectively) (Table 1). The total absence of *Salmonella* spp. was confirmed by API 20E galleries. However, the presence of two eggs infected by *Raoultella planticola* (2%), and two eggs by *Escherichia coli* (2%) was found.

Table 1. Mean bacterial count and prevalence of contamination revealed by the two methods: classical and rapid, expressed in Log CFU/cm²

Bacteria	Classical method		Rapid method	
	Mean	Prevalence %	Mean	Prevalence %
Total bacteria	2.85 ± 0.99	100	2.95 ± 1.06	100
Total coliforms	2.47 ± 0.95	56	2.35 ± 1.01	90
Fecal coliforms	2.08 ± 0.70	45	/	/
Fecal Streptococci	2.23 ± 1.13	72	/	/
Staphylococcus aureus	1.67 ± 0.86	19	1.65±1.46	43
Salmonella spp.	0	0	0	0

Discussion

Eggshell contamination is widely studied worldwide. However, no studies have been carried out in Algeria. Therefore, in this study, we have tried to identify the main bacteria contaminating eggshells.

Hen age is one of the most important factors affecting the microbiological quality of the eggshell (Tumova et al., 2014). However, some authors have found that hen age has no significant effect on eggshell bacterial contamination (De Reu et al., 2005b; 2006c). Also, the microbial quality of eggshell differed significantly due to the season and the type of production housing system (Fahim et al., 2021). Therefore, and in order to exclude the influence of age, season and type of production, our study was carried out in summer, on hens of a fixed age (56 weeks), and on ISA Brown laying hens raised in cages.

In general, eggs are contaminated after laying by dust, feces, and handling. Microbes can contaminate the shell in a short period of time and penetrate the egg interior (Adegbenro et al., 2020) and bacterial charge on the eggshell depends on several factors, including the diet of the laying hens, the environmental conditions of the egg during and after laying, and the bacterial charge of the air (De Reu et al., 2005). The longer the contact of eggs with hens on litter, the higher the bacterial load on shells compared to eggs produced from cage systems (Parisi et al. 2015; Soliman et al. 2020 ; Fahim et al. 2021), which is the case in our study.

In our survey, all eggshells were contaminated with total bacteria, which is in agreement with Fahim et al. (2021). The bacterial count on the eggshell varies widely from one study to another. The total bacteria counts revealed by the rapid and classical methods, in our investigation were 2.95 ± 1.06 Log CFU/cm² and 2.85 ± 0.99 Log CFU/cm², respectively. A bacterial charge of eggshells below 5 Log CFU considers the hygienic quality of eggs to be acceptable (De Reu et al., 2008), which is the case in our study. Our results are consistent with Clímaco et al. (2018).

The presence of coliforms on the eggshell is an indicator of poor egg handling (Poleh et al., 2018). The classical indicators commonly used in assessing the fecal contamination are the coliform group (total coliforms, fecal coliforms, *Escherichia coli*); streptococci (enterococci, fecal streptococci) (Rodrigues and Cunha, 2017). Coliform counts are also the traditional indicator of microbial quality, safety and reflect the hygiene standards adopted in the food operation (Musgrove et al., 2008). Excessive coliform counts in farm eggs indicate unhygienic conditions on poultry farms (Mo'ataz et al., 2021). Several factors contributing to the selective transfer and survival of specific bacterial species on eggshells probably include the different ability of certain bacteria to adhere to or survive on the eggshell surface (Trudeau et al., 2020).

In our study, the coliform count was in accordance with SNI-3926-2008 (2008) regarding microbiological quality requirements for fresh eggs. The prevalence of contamination revealed in our study concerning total coliforms is in concordance with Ahmed et al. (2016) and higher in comparison with Fahim et al. (2021) (38%).

Our results for fecal coliforms (2.08 ± 0.70 Log CFU/cm² representing a prevalence of 45%) are higher than these of Fahim et al. (2021) who reported a prevalence level of 38%.

In our data, 72% of eggs were contaminated with fecal streptococci which are higher than those of Al Momani et al. (2018).

Staphylococci are the most common bacteria contaminating eggshells. The egg can be contaminated during formation and laying process. *Staphylococcus aureus* is one of the major natural egg shell contaminators of table eggs (De Reu et al., 2006a; 2006b). *Staphylococcus aureus* food intoxication is caused by ingestion of food containing staphylococcal enterotoxins, which are emetic, pyogenic, mitogenic, suppress immunoglobulin secretion, and increase toxic shock (Stewart et al., 2002).

In our investigation, *Staphylococcus aureus* was detected in 19% and 43% of the samples tested by the classical and rapid methods, respectively. This result is lower than that of Fahim et al. (2021) (86%) and higher than Parveen et al. (2017) (5.55%) and Youssef and Afifi (2017) (11%) results.

Staphylococcus aureus count was 1.67 ± 0.86 Log CFU/cm² in the classical method and 1.65±1.46 Log CFU/

cm² in the rapid method. Ahmed et al. (2016) revealed a count of 5.8 x 10^5 ± 4.8 x 10^4 CFU/ml. The presence of staphylococci in high numbers is probably the result of human contamination (Nwagu and Amadi, 2010).

Salmonella spp is one of the major causes of serious foodborne public health illness worldwide (Whiley and Ross, 2015) and the contaminated eggshell is a critical public health risk (De Reu et al., 2008; Pandeet al., 2016; Fahim et al., 2021). *Salmonella enteritidis* is the main serovar associated with human salmonellosis (Ferrari et al., 2019). Contamination of the eggs with *Salmonella* spp. can occur in the reproductive tract of hens or externally through contact with contaminated feces or surfaces. Penetration of bacteria from the shell into the egg has been demonstrated (Ferreira et al., 2020; Lin et al., 2021).

Similar to our findings, *Salmonella* spp. was not detected in eggshells (Chousalkar et al., 2010; Mahdavi et al., 2012), while some studies have revealed the presence of salmonella on eggshells (Musgrove et al., 2008; Parveen et al., 2017; Youssef and Afifi, 2017; Kapena et al., 2020; Ferreira et al., 2020; Fahim et al., 2021).

The absence of *Salmonella spp.* in isolates is due to several factors. *Salmonella* spp. are known to be much more sensitive to external conditions than most other fecal organisms (Garcia et al., 2010).

The genus *Raoultella* belongs to the family Enterobacteriaceae and is considered a normal intestinal flora of poultry (Younis et al., 2016). In our study *Raoultella planticola* was isolated from 2% of eggshells which is in agreement with Mahdavi et al. (2012) where they reported prevalence of 2.44% but of *Klebsiella pneumoniae* species. Our finding is lower than that of Al Momani et al. (2018) (15%) and Youssef and Afifi (2017) (9.6%).

Escherichia coli is one of the normal intestinal flora of poultry and humans. However, some strains are pathogenic such as enteropathogenic, enteroinvasive, enterotoxigenic, and Shiga toxin producing *Escherichia coli* (Rasheed et al., 2014). Furthermore, it was also proved that *Escherichia coli* is capable of facilitating the penetration of *Staphylococcus aureus* into the egg content (Al-Natour et al., 2011), another reason for the increased interest in controlling fecal contamination, from the surface of egg shells. *Escherichia coli* population can be used as a measure of quality and sanitary processing conditions. In fact, contamination of eggs by these bacteria is due to poor manure decomposition, water contamination and poor hygiene practices of the farmers (Johnson et al., 2006).

In our investigation, *Escherichia coli* was detected in 2% of eggshells which is lower than the findings of Mahdavi et al. (2012), Al Momani et al. (2018) and Kapena et al. (2020), who reported a prevalence of 34.26%, 15% and 6.1%, respectively.

Conclusion

This study provided partial description of the eggshell microbiota of laying hens using two methods at acceptable levels and that a good level of hygiene and management can avoid contamination by dangerous bacteria represented mainly by *Salmonella* spp.. It is therefore important that all parties involved, including producers, breeders, veterinarians, inspectors and researchers, know the microorganisms present upstream of the production chain in order to better manage the risks for poultry and public health.

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