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Eggshell contamination with special reference to different methods of disinfection on the isolated bacteria

Essam I. El Toukhy[1], Esraa G. Hefny[2], Naglaa F. Koraney[3], Aya E. Azam[4]

[1]*Biotechnology Department, Animal Health Research Institute (AHRI), Egypt.*

[2] *Milk and Meat Hygiene Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt.*

[3] *Bacteriology and Immunology Department, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Egypt.*

[4] *Animal Hygiene and Veterinary Management Department, Faculty of Vet. Med., Benha University, Benha, Egypt*

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ABSTRACT

The study was conducted on 198 table eggs that were collected from markets, and manual and automatic farms in EL-Fayoum governorate, to assess the existence of foodborne pathogens (*Escherichia coli* and *Staphylococcus aureus*), either on the outer eggshell or inner egg contents and to evaluate the reduction capacity of three antimicrobial agents (citric acid 3%, Sodium bicarbonate 3% and hydrogen peroxide (H2O2) 3%) against eggshells bacterial load. The obtained results found that Staph. aureus has recorded the highest prevalence (90%) in table eggs, Almost, *E. coli* and *Staph. aureus* isolates exhibited high resistance to the tested antibiotics. Also, the *E. coli* isolates were positive to virulence genes (tet A gene and dfr A gene). Finally, the effect of tested antimicrobial agents on the experimentally contaminated eggs showed that H2O2 (3%) was more effective in reducing the load of *E. coli* on eggshells than the other antimicrobial agents tested, while bicarbonate 3% expressed a significant ability to reduce *Staph. aureus*.

1. INTRODUCTION

The modern trends of poultry industry have become directed towards production of healthy and safe food in the developing countries. Eggs can provide an excellent source of high-quality protein, affordable, cheap, multivitamin, good fats, and trace elements, while offering a moderate calorie source (140 kcal/100g) and low economic costs (Carrillo et al., 2012).

Moreover, the high nutritive value of eggs makes them vulnerable to microbial contamination that threatens the eggs' shelf life (Gole *et al*., 2014). The microbial quality of table eggs also , adversely affects consumers through transmission of pathogenic microorganisms causing intoxication and public health hazards(Al Momani *et al*., 2018). Egg contents could be contaminated by a vertical transmission during laying (Gantois *et al*., 2009) or horizontally by soil, faeces, nesting particles, hands of workers, during processing, during transportation, and during storage, as the bacteria can be colonized on the shell and penetrate the egg through its pores (Messens *et* al., 2007).

The most foodborne pathogens often contaminate eggshells are *E. coli and Staph. aureus* (Maktabi *et al*., 2018).

Most of *E. coli* strains are harmless bacteria and commonly inhabit the mammals and birds intestine which easily spread through water, litter, faecal matter, dust and food (Gupta and Sharma, 2015). The strain, O157:H7 has been reported to be pathogenic to humans, causing bloody diarrhea, haemolytic uremic syndrome, urinary tracts infections, pneumonia meningitis and peritonitis in humans (Musgrove *et al*., 2008).

The thermostable enterotoxins from *Staph. aureus* causing nausea, vomiting, abdominal pain, cramps and diarrhea within 6 hours of ingestion of contaminated egg (EFSA, 2014).

Food poisoning outbreaks due to consumption of eggs contaminated with pathogenic microorganisms may be higher in developing countries where little or no control measures are applied. Keeping the eggs clean and sterile is extremely important for public health. Chemical disinfection has more appropriate action against bacterial load with the rise of microbial antibiotic resistance (Michael *et* al., 2014), while choosing the perfect disinfectant that's effective, safe, and keeps egg quality without change in color or odour could be a great challenge.

Citric acid is one of the most effective choices, especially under standard doses, because it improves the flavor of foodstuffs without causing any harmful effects (Park *et al.*, 2009). Hydrogen peroxide is another inexpensive chemical substance with strong bactericidal properties (Ukuku *et al*., 2005).

2. MATERIAL AND METHODS

2.1. Sample collection

A total number of 198 table egg samples, 150 eggs were used for microbial isolation, identification, and antibiotics

Correspondence to: Aya E. Azam.
Aya.azzam@fvtm.bu.edu.eg or Aya.azam96@gmail.com. https://orcid.org/0000-0002-1988-9933

sensitivity, while 48 eggs were used for antimicrobial efficacy assessment. The eggs were randomly collected from poultry farms, markets, supermarkets, and groceries in EL-Fayoum governorate from February to April 2020. Each sample was placed in a sterile plastic bag and carried to AHRI laboratory (Animal Health Research Institute) without delay in preparation and microbiological examination.

2.2. Microbiological examination

2.2.1. Preparation of samples

Each sample was washed in a sterile plastic bag with 100 ml sterile saline. Eggshells were tested by the rinse method as described by Moats (1980). The eggs were prepared for the evacuation of their contents according to (APHA, 2004), and the contents were tested after egg evacuation according to Speck (1976).

2.2.2. Microbial Isolation and identification

Isolation and identification of *E. coli* from egg contents were carried out according to (Cheesbrough, 2006) While the isolation and identification of coagulase-positive Staphylococcus aureus were done according to (AM, 2003, ISO, 2003b). The isolates were confirmed by PCR. Oligonucleotide sequences, target gene, amplicon size, and cycling condition were showed in table (1).

Table 1 Oligonucleotide sequences, target gene, amplicon size, and cycling condition of genes of *E. coli* and staph. aureus

Genetic target	Primers	Primer sequences	Primary denaturation	Secondary denaturation	annealing temperature	Extension	Final extension	Amplicon size	References
tuf gene (E.coli)	TEcol553	5-TGGGAAGCGAAAATCCTG -3	95 °C 3 min.	95 °C 30 sec.	58 °C 30 sec.	72 °C 30 sec.	72 °C 5 min.	258 bp	(Maheux et al., 2009)
	TEcol754	5-CAGTACAGGTAGACTTCTG-3							
16s rRNA gene (Staph. aureus	-	(F) CCTATAAGACTGGGATAACTTCGGG (R) CTTTGAGTTTCAACCTTGCGGTCG	94 °C 5 min.	94 °C 30 sec.	55 °C 45 sec	72 °C 45 sec.	72 °C 10 min	791 bp	(Mason et al., 2001)

2.3. Antibiotics sensitivity test

The Antibiogram of *E. coli* and *Staph. aureus* isolates from the collected eggs were performed using the agar disc diffusion technique as described by (Bauer, 1966) using seventeen antibiotic discs (Himedia, India). The results were interpreted according to the standards of Clinical Laboratory Standards Institute (CLSI, 2016).

The presence of genes associated with resistance to streptomycin [aadA1 of 447 bp, its sequence are (F) TATCCAGCTAAGCGCGAACT, (R) ATTTGCCGACTA CCTTGGTC at 58°C annealing temperature (Van et al., 2008)], tetracycline [tet (A) at 577 bp, its sequence are (F) GGTTCACTCGAACGACGTCA, (R) CTGTCCGACAA GTTGCATGA at 57°C annealing temperature (Randall et al., 2004)], trimethoprim [dfrA1of 367 bp, its sequence are (F) GGAGTGCCAAAGGTGAACAGC, (R) GAGGCGA AGTCTTGGGTAAAAAC at 45°C annealing temperature (Toro et al., 2005)] and erythromycin [ere(A) of 419 bp, its sequence are (F) GCCGGTGCTCATGAACTTGAG, (R) CGACTCTATTCGATCAGAGGC at 52 °C annealing temperature (Van et al., 2008)] were determined by PCR.

2.4. Efficacy of the antimicrobial agents on eggshells

2.4.1. Preparation of bacterial suspension

Tubes containing 5 ml trypticase soy broth (TSB) were inoculated with the colonies previously isolated, then incubated at 37°C for 20 h on a shaker at 10 RPM. About 1.5 ml was moved to a different tube with 60 ml TSB and incubated as before, then centrifuged at 4000 RPM for 7 min. The supernatant was discarded, and the pellets were re-suspended in 10 ml phosphate-buffered saline (PBS). Eggs were inoculated by the previously prepared concentrated suspension as described below.

2.4.2. Eggs inoculation

In each series of tests, six medium-sized fresh eggs (a total of 48 eggs) were prepared according to (Rodriguez-Romo and Yousef, 2005). Aliquot 500 ml of sterile PBS was added to dilute the concentrated bacterium suspension to achieve the concentrations of 5.76 and 5.84 Log CFU\ml for *E. coli* and *Staph. aureus* respectively. Eggs were placed within every bacterial suspension for 30 min. and dried under a laminar flow for 1 h. at ambient temperature.

2.4.3. Disinfection of eggs with the antimicrobial agents

Sterile stomacher plastic bags containing 200 ml from each citric acid, H_2O_2 and sodium bicarbonate was prepared individually at a concentration of 3% for egg treatment (Park *et al.*, 2009). The final bacterial concentrations in eggs treated with various antimicrobial agents were compared to the bacterial concentrations in eggs immersed once in controls (BPS without disinfection) (Upadhyaya *et al.*, 2013).

2.5. Statistical analysis

One way analysis of variance was used (ANOVA) and significance was done used Tukey test using SPSS (version 16; SPSS Inc., Chicago, USA).

3. RESULTS

3.1. Prevalence of *E. coli* and *Staph. aureus* in eggshell and inner contents

The microbial prevalence of eggs is summarized in Table (2). The highest prevalence rate from collected eggs was recorded by *Staph. aureus* (90%) followed by *E. coli* (70%). Only 20% of eggshells were contaminated with *E. coli* and *Staph. aureus* and the contamination did not reach to the inner content of eggs. From both eggshell and egg contents, staphylococcus aureus was the highest bacteria isolated (70%) followed by *E. coli* (50%).

Table 2 Prevalence of *E. coli*, *Staphylococcus aureus* and *S. Typhimurium* on eggshell and contents from collected samples

Samples	*E.coli*		*Staph. Aureus*	
	No of egg	Prevalence (%)	No of egg	Prevalence (%)
Positive eggshells only	30	20%	30	20%
Positive egg contents only	0	0%	0	0%
Positive both eggshells & contents	75	50%	105	70%
Contamination free	45	30%	15	10%
Total contaminated eggs	105	70%	135	90%

3.2. Effect of source of collection on microbial prevalence in table eggs.

The results showed that all eggs collected from local markets and manual farms were contaminated with *Staphylococcus aureus* against 66,6% from automatic ones. Similarly, 95% of the egg from local markets were contaminated with *E. coli* followed by manual farms (73%) and automatic farms (33.3%) (Table 3).

Table 3 Effect of source of collection on prevalence of *E. coli, Staph. aureus* and *S. Typhimurium* in table eggs

Source of collection		*Number of collected eggs*	*Positive samples*			
			E.coli		*Staph. Aureus*	
			N	%	N	%
Local markets		60	57	95%	60	100%
Layers farm	Manual (Deep litter)	45	33	73%	45	100%
	Automatic (cages)	45	15	33.3%	30	66.6%
Total		150	105	70%	135	90%

3.3. Molecular detection of isolated bacteria:

From all positive isolates, 20 samples (randomly selected) of *E.coli* and *Staph. aureus* were used for PCR amplification targeting the *tuf* and *16s rRNA* , respectively**.** All the samples (100%) were positive as showed in fig.1.

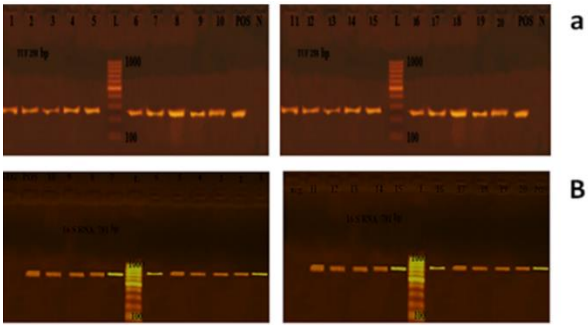


Fig. 1 Agarose gel electrophoresis 1.5 % showing PCR products Lane (L): molecular weight marker (100 bp DNA ladder, Thermo).
a: *tuf* gene of E.coli. Lanes (1) to (20) were positive DNA samples showed a band at 258 bp. Lane (POS) was positive control. Lane (N) was negative control.
b: 16s rRNA gene of *Staph. Aureus*. Lanes (1) to (20) were positive DNA samples showed a band at 791 bp. Lane (POS) was positive control. Lane (N) was negative control

3.4. Antibiotic sensitivity test against *E. coli* and *Staph. aureus*:

The antibiotic susceptibly of *E. coli* isolates against different antibiotics were shown in Table (4) and histogram (1). Among all the antibiotics tested, *E. coli* was highly resistant (100%) to Ofloxacin, Ciprofloxacin, Cefuroxim, Amoxicillin, Ampcillin Sulbictam, Cefepem, Rifampin and Oxytetracyclin. Only 40% of the isolated *E. coli* was sensitive to Amikacin , followed by Kanamycin (15%) then Apramycin (10%). Staph. aureus was sensitive to Apramycin (40%), Erythromycin (35%), Vancomycin (30%), and Amikacin (25%), and sensitivity decreased gradually to Penicillin (15%), Rifampin (10%), Ciprofloxacin (10%), and Streptomycin (5%), while showed the highest resistance (100%) to Tetracycline and Sulfa/trimethoprim.

The results proved that all of the *E. coli* isolates were positive to the *tet A gene (*577bp) and *dfr A gene (*367bp). It was shown that out of 20 *E.coli* isolates, 19 isolates (95%) were positive for *aadA1 gene* at 447 bp and only 13 isolates (65%) were positive for *ere A gene* at 419bp (Fig. 2).

3.5. Effect of antimicrobial agents on *E. coli* and *Staph. aureus*:

Changes in *E. coli* count after treatments with citric acid, H2O2, and bicarbonate at concentration of 3% were displayed in Fig. 3. There was a reduction in the bacterial count by 0.36, 1.16, and 0.80 Log CFU/ml, respectively. Various disinfection treatments showed significant reductions ($P<0.05$) in bacterial concentrations compared to PBS alone (control). There was a numerical reduced bacterial concentration in *E. coli* between citric acid 3%, H_2O_2 3%, and bicarbonate 3%, but the difference wasn't significant. H_2O_2 (3%) was superior to other treatments in bacterial reduction. Similarly, there was a reduction in *Staph. aureus* count after treatment with citric acid 3%, H_2O_2 (3%), and bicarbonate 3% by 0.90, 0.83, and 1.14 Log CFU/ml, respectively. Staph. aureus appeared more sensitive to bicarbonate 3% than other antimicrobial agents tested.

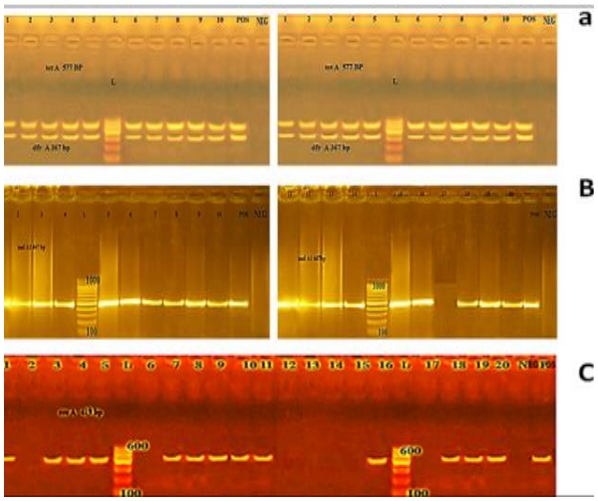
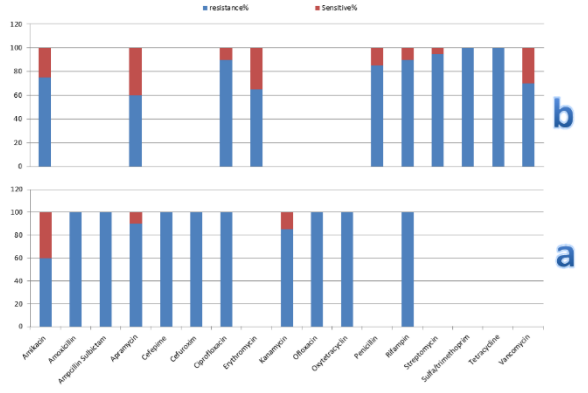


Fig. 2 Virulence genes associated with *E. coli* resistance (tet (A) gene, aad A1 gene, ere (A) gene). Lane (L): Molecular weight maker (100 bp DNA ladder, Thermo) of E.coli, Lane (POD) was positive control. Lane (NEG) was negative control.
a: tet (A) gene (577 bp) and dfr A gene (367 bp), Lane (1) to (20) were positive DNA samples for both genes.
b: aad A1 gene. Lane (L; Lanes 1-16) and lanes 18-20 were positive DNA samples showed a band at 447 bp. Lane 17 was negative sample.
c: ere (A) gene, Lane (1, 3, 4, 5, 7, 8, 9, 10, 11, 16, 18, 19, 20) were positive DNA samples showed a band at 419 bp. Lanes 2, 6, 12, 13, 14, 15 and 17 were negative DNA samples

Table 4 Antibiogram of *E. coli* and *Staph. Aureus*

	E. coli				*Staph. aureus*			
	Sensitive		Resistance		Sensitive		Resistance	
	No	%	No	%	No	%	No	%
Amikacin (30µg)	8	40	12	60	5	25	15	75
Amoxicillin (25 µg)	0	0	20	100	-	-	-	-
Ampcillin Sulbictam (20µg)	0	0	20	100	-	-	-	-
Apramycin(15µg)	2	10	18	90	8	40	12	60
Cefepime (30µg)	0	0	20	100	-	-	-	-
Cefuroxim (30µg)	0	0	20	100	-	-	-	-
*Ciprofloxacin (5*µg)	0	0	20	100	2	10	18	90
Erythromycin (15µg)	-	-	-	-	7	35	13	65
Kanamycin (30µg)	3	15	17	85				
Ofloxacin (5µg)	0	0	20	100	-	-	-	-
Oxytetracyclin (30µg)	0	0	20	100	-	-	-	-
Penicillin (5µg)	-	-	-	-	3	15	17	85
Rifampin (5µg)	0	0	20	100	2	10	18	90
Streptomycin (30µg)	-	-	-	-	1	5	19	95
Sulfa/trimethoprim(25µg)	-	-	-	-	0	0	20	100
Tetracycline (30µg)	-	-	-	-	0	0	20	100
Vancomycin (30µg)	-	-	-	-	6	30	14	70



Histogram 1 Percentage of antimicrobial sensitivity test among *E. coli* and *Staph. aureus*

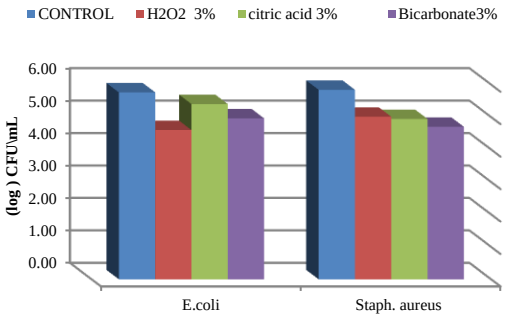


Fig.3: Effect of different treatments on reducing *E. coli* and *Staph. aureus* experimentally inoculated on eggshells. Bars with different subscripts are considered statistically different at P<0.05

4. DISCUSSION

Eggs sold in local Egyptian markets are commonly coming from different sources as deep litter farms, battery farms, automatic farms, manual farms and from backyard houses, which vary in egg handling, cleaning, disinfection, and storage. This gives different management levels for eggs ranged from very high technology in automatic farm to unwashed within the backyard houses, rather than cross-contamination and bad storage in markets.

The recorded prevalence rate of *E. coli* were in accordance with (Islam *et al*., 2018) who reported that the major egg contaminants were *E. coli* (34.64%),

Staphylococci spp. is one of the foodborne pathogens that are important for human and veterinary medicine. Our prevalence rates were nearly similar to (Islam *et al*., 2018) who reported that of *Staphylococci* isolated from egg content and shell were 41.2% and 58.8%, respectively. The lower prevalence was reported by (Eid *et al*., 2015).

Freshly laid eggs from disease-free hens generally devoid from pathogenic organism while poor storage, poor cleaning, delay in collection, and other environmental factors such as humidity, temperature, dust, litter and dirty nests causes primarily heavy contamination that could migrate to the inner contents (Evêncio-Luz *et al*., 2012).

The recorded microbial prevalence rate in the manual farms which depends on floor system and nest boxes, lacking technology and poor hygienic practice of farm workers was higher than automatic ones. While the microbial prevalence rate in markets was the highest that may be due to bad storage and collection from backyards owners as eggs have no cleaning or washing practices. Those results came in accordance with(Messens *et al*., 2007) who found that the aerobic bacterial contamination of eggs and eggshells, was higher for nest eggs collected from non-cage systems compared to nest eggs collected from furnished cages or eggs from conventional cages.

In layers farms reared on deep litter system, the presence of hen manure may pose a significant environmental load on feed, air, and water that reflected on microbial quality of the egg. Environmentally, non-caged production isn't the best choice for sustainable egg production (Gunnarsson *et al*., 2020).

Eggs in markets even supermarkets stored and offered at room temperature, not at the refrigerator, this multiply the contamination came from the farm, this may explain the highest prevalence in markets egg samples (Al Momani *et al*., 2018).

The antibiotic-resistant bacteria which isolated from table eggs can be transmitted to human by even egg handling and the ingestion of raw eggs or egg dependent products. In the present study, *E. coli* was completely resistant to ciprofloxacin, this agreed with the results of (Suleiman *et al*., 2013).

The results obtained indicated that *E. coli* was completely resistant to Rifampin, Amoxicillin, and Oxytetracyclines< These results were disagreed with those observed by (Okorie-Kanu *et al*., 2016).

About 94.09 % of *E. coli* isolates were resistant to all tested antimicrobial drugs that were agreed with (Eid *et al*., 2015).

From the obtained results, about 35%, 30%, 10%, and 5% of *Staph. aureus* isolates exhibited sensitivity against Erythromycin, vancomycin, Ciprofloxacin, and Streptomycin respectively. The higher sensitivity was reported by (Nwankwo and Nasiru, 2011) who found that the sensitivity pattern of *Staph. aureus* to Erythromycin, vancomycin, Ciprofloxacin and Streptomycin were 52.4%100%, 78.9%, and 44.2% respectively.

The resistance rate of both *E. coli* and *Staph. aureus* against Oxytetracycline was (100%), a result that was nearly similar to the result of (Eid *et al*., 2015) who reported approximately 92.9%.

In the present study, Amikacin, Kanamycin and Apramycin showed higher efficacy than other antibiotics used against *E. coli*, while Apramycin, Erythromycin and Vancomycin showed higher efficacy against *Staph. aureus*.

Eggshells contamination can reduce eggs' safety and shelf-life. Therefore, decontamination of egg surfaces should begin as soon as possible, using efficient antimicrobial agents as much as possible, this will help to achieve health goals.

Immersion of eggs in H_2O_2 solution resulted in the largest reductions in *E. coli* on eggshells compared to other tested antimicrobial agents. Those results agreed with (Maktabi et al., 2018) who reported that H_2O_2, and citric acid were similar in reducing *E .coli* contamination in eggs and different food types.

In our studies, *Staph. aureus* appeared more sensitive to bicarbonate (3%) compared with H_2O_2 (3%) and citric acid (3%). These results were nearly similar to (Sander and Wilson, 1999) who observed that H_2O_2 (3%) caused significant reductions in *Staph. aureus* counts in contaminated eggs. H_2O_2 vapor decontamination, effectively reduced *Staph. aureus* from rooms and equipment *(French et al., 2004)*.

Lemon juice (citric acid) and baking soda (bicarbonate) are readily available, natural environmentally friendly, and low in toxicity (Fong *et al*., 2014). Citric acid has higher antimicrobial efficacy than sodium bicarbonate in the treatment of hard surfaces (Bjornsdottir *et al*., 2006)

E. coli as gram-negative bacteria are more susceptible to organic acids (citric acid) than gram-positive bacteria (*Staph.* aureus) that impair the diffusion of the organic acids into the cell, preventing antimicrobial action (Rutala *et al*., 2000). Control programs and measures should apply in home-produced eggs for personal consumption or local sale, to avoid the risk of infection from their consumption.

5. CONCLUSION

Table eggs collected from different sources may harbor some natural pathogens such as *E.coli* and *Staph. aureus* infections, Table eggs may be also a vital reservoir of resistance genes with a high incidence of antibiotics resistance. This demands the limitation of antibiotic use in poultry farms, more attention, and rigorous hygienic measures in layer farms. Disinfection of table egg is beneficial to decrease microbial load on eggshell, invasion

to content and avoid potential transfer of risk to the consumer. Continuous efforts are required, strict hygienic and effective sanitary measures and proper management practices need to be adopted during egg production in poultry farms.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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