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Coliform Bacteria and Hatching Egg Disinfectants

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Abstract

(This study aimed to monitoring the microbial status of broiler breeder hatching eggs through isolation of coliform bacteria, especially Salmonella and E coli from eggs and hatched chicks and identification of isolated microorganism by PCR. Besides, the reduction of the contamination rate by using different egg disinfectants. A total of 600 broiler breeder Saso fertile eggs was purchased from a commercial broiler breeder flock aged 27 wk, which reared on a deep litter system at a private farm in Gharbiya Governorate, Egypt. The eggs were allocated randomly into four equal treatment groups (n＝150) according to the disinfectant used. The eggs in the first group were kept as a control without treatment. While, the eggs in the second group were disinfected by 1.4% H_2O_2. The eggs in the third group were disinfected by 0.5% TH4. The eggs in the fourth group were disinfected by 0.5% Virkon S. Results revealed that, the hatchability percent was increased by applying the different egg disinfectants. The highest total embryonic deaths were recorded in eggs disinfected by TH4 in comparison to other disinfectants used. The weak chicks' quality appeared only in eggs disinfected by H_2O_2 and TH4 but the highest percent of omphalities was recorded in eggs disinfected by H_2O_2 followed by the control group. Egg disinfectants were significantly reduced the eggshell total bacterial count from 27 Logs to 4.3 Logs with a 84.08% reduction by using H_2O_2, from 24 Logs to 4.7 Logs with a 80.42% reduction after using virkon S and reduced the total bacterial count from 25 Logs to 3 Logs with a 88% reduction after using TH4 in comparison to 28 Logs for the control group. PCR findings showed that salmonella and Escherichia coli were the predominant bacteria on the surface of the eggshell. The prevalence of Escherichia coli was the highest, followed by salmonella. It could be concluded that E. coli is more predominant than salmonella.

Keywords: bacterial count; hatchability; embryonic mortality; salmonella; E. coli.

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1. Introduction

The egg is formed in a sterile condition, but microbial contamination of their eggshell occurred during oviposition through the cloaca or immediately after oviposition [16]. Microbial contamination of hatching eggs causes poor hatchability and chick performance, so it is a main concern of poultry producers to minimize the bacterial numbers by practicing the highest standards of hygiene in hatcheries [4]. When cleaning and disinfection procedures are not performed properly during hatching egg incubation, the occurrence and maintenance of microorganism in the hatcheries were developed to give what is called incubator infections by transferring the microbes among the different batches of newly hatched chicks leading to spreading of poultry diseases [9, 12].

After hatching egg contamination the microorganisms may penetrate the eggshell and infect the embryo resulted in embryonic mortality, inability to hatch weak chicks, high chick mortality and poor chick or poults growth [2]. Non-coliform gram-negative bacteria like Salmonella and Escherichia coli (E. coli) are the causes of yolk sac infection. Moreover, E. coli is considered the main microorganism which is believed to do an adverse effect on chick quality and cause embryonic deaths [20].

Poor standards of hatchery hygiene not only the main causes of severe economic loss in poultry production, but also, poor egg treatment with a disinfection which used by the poultry producers. As, the highest standers procedures of hatching eggshell disinfection improve day-old chick quality, hatchability and keeping quality by reducing the microbial population on the eggshell surface [14, 17].

So, the objective of this study was planned to evaluate the role of disinfectants in reducing the Coliforms on eggshells and hatcheries surfaces.

2. Materials and Methods

2.1. Breeder Flock and eggs

A total number of 600 fertile eggs were collected from freshly laid layers at the 12th week from egg production from one farm located in Gharbia Governorate El-Santa in safety transportation. A total number of 200 samples from egg shell, hatchery surfaces, unhatched eggs and cloacal swabs divided as shown: 105 samples from three disinfectant groups before and after disinfection and the control group, 30 samples from disinfectant hatchery groups before and after disinfection, 5 from the control group, 40 cloacal swabs from day old chick and 20 from dead unhatched eggs.

Selected eggs for hatching were candled on the 7^{th} day of incubation to eliminate infertile eggs. The eggs were again candled on the 16^{th} day of incubation. The embryonated eggs that died between 7^{th} and 21^{th} day of incubation were used for this study Examination to all samples for isolation and identification of Salmonella and E. coli. Examination of day old chick revealed some had omphalitis, weak limbs, distorted peek, and weak chick.

2.2. Preparation of Solutions

- A 1.4% H_2O_2 solution was prepared by mixing 860 ml of sterile distalled water and 140 ml of H_2O_2. Solutions were kept in a container, the top was sealed, and it was shaken well before use. Each solution was filtered (coarse filter) separately and was kept in a clean, dark bottle at 4°C until use according to [7].
- A 0.5% TH4 solution was prepared by mixing 5 ml of TH4 and 995ml of sterile distilled water. Solutions were kept in a dark container according to [13].
- A 0.5% Virkon S solution was prepared by mixing 5 g of Virkon S and 995ml of sterile distilled water. Solutions were kept in a dark container according to [13].

2.3. Experimental design and application of Solutions

- The eggs were allocated randomly into four equal treatment groups (n＝150) according to the disinfectant used. The eggs in the first group were kept as a control without treatment. While, the eggs in the second group were disinfected by 1.4% H_2O_2. The eggs in the third group were disinfected by 0.5% TH4. The eggs in the fourth group were disinfected by 0.5% Virkon S. All eggs were stored for a short period (3 days) at 15.5°C in a room with RH 75%.

2.4. Incubation Management

Eggs were marked and weighed before incubated in a commercial private incubator (Kafr El-sheikh Governorate, Egypt) with a temperature of 37.5°C and 55% RH until day 18 of incubation then incubator conditions were changed to 37.2°C and 75% RH to work as a hatchery. Eggs were turned through 90° once every 2 hrs. The time of setting eggs in incubator was recorded for each trial to obtain the exact hatch time in hours and considered from zero hour setting. Eggs were candled at 7^{th} day of incubation to detect the fertility and on day 14 of incubation to detect the livability.

2.5. Measurements

2.5.1. Hatchability

Hatchability was expressed as percentages of hatched chicks from total fertile eggs set.

2.5.2. Chick hatching weight

After 21 days, chicks that had fully emerged from eggs were removed from incubation and each hatched chicks was weighed individually to the nearest 0.1 g and recorded as chick body weight at hatch.

2.5.3. Embryonic mortality

At 14 day, the eggs which show dead embryos on candling and unhatched eggs after day 21 of incubation were

opened to establish the stage of embryonic mortality. The stages of embryonic mortality were classified as follows: day 8 to 14 early embryonic deaths (black-eye visible and embryo without feathers) and day 15 to 21 late embryonic deaths (full-grown embryo with feathers and embryo with yolk out or subtracted).

2.5.4. Chick Performance

All hatched chicks per each group were reared on a deep litter system (10 chicks/m^2) to determine their growth performance for 7 days. Chicks were weighed and identified with a leg ring number. During the 7 days of growing, a starter diet (3020 kcal of ME/kg and 23% CP) was provided adlibitum. Room temperature was set at 33°C. The photoperiod was continued light for 24hrs during the rearing period. At the end of 7 days, all chicks were individually weighed (without leg ring). For each chick, the body weight (BW) of day 1 (BW1) and the BW of day 7 (BW7) were used to calculate the growth performance (GP).

2.5.5. Microbiological examination

- A total number of 200 samples from egg shell and hatchery surfaces, embryo of unhatched eggs and cloacal swabs were taken as follows:
- 105 samples; 30 from each group (15 samples before and 15 samples after disinfection process) and 15 samples from the control group.
- 30 samples; 10 samples from each hatchery (five samples before disinfection and five samples after disinfection) and five samples from the hatchery of the control group.
- 40 cloacal swabs; five samples from (five selected day old chicks) each egg group, five samples from each hatchery and 20 samples from dead embryos of unhatched eggs representing each egg group.

Samples were examined for Total bacterial count (TBC), isolation and identification of Salmonella and E. coli.

2.5.5.1.Total bacterial count (TBC)

5 eggs per each group at laying day before and after disinfection were taken for microbiological analysis and immediately placed in sterile plastic sac containing 40 ml of sterile disinfectant. A whole-egg washing technique was used to recover the shell-associated microorganisms for estimating the total bacterial count. Serial dilutions were made from all samples and then were inoculated into sterile Petri plates containing nutrient agar [11] . The plates were packed and incubated at 37^{o}C for 48 hrs and at the end of incubation, the plates were removed and colonies were counted and multiplied by the dilution factor. Colonies were measured as log cfu/egg.

2.5.5.2. Isolation of Salmonella

Samples were pre-enriched in buffered peptone water 1/10 dilution (W/L). Incubation was carried out at 37°C ± 2 h. From the pre- enrichment culture, 0.1 ml was transferred to a tube containing 10ml of the Rappaport Vassiliadis broth and then incubated at 41. 5 °C ± 1°C for 24hrs ± 3h. The broth cultures were spread plated onto selective agar plates media to identify and observe the gross colony morphology using Xylose Lysine Desoxycholate agar (XLD), Salmonella-Shigella agar media, and MacConkeyś agar media and incubated at 37.

0 ± 1 °C for 24h ± 3h according to the **[8]** procedure**.**

Table 1: Showing the Primer used for Salmonella

Salmonella genus		Denaturation	Annealing		Primer		Cycle	Final extension		Target
OMPC-F	95ºC/2 min	95ºC /1 min	57ºC min	/1	72ºC min	/2	35	72ºC min	/5	204
OMPC-R	95ºC/2 min	95ºC /1 min	57ºC min	/1	72ºC min	/2	35	72ºC min	/5	204
Salmonella serotype Enteritidis										
ENTR-F	95ºC/2 min	95ºC /1 min	57ºC min	/1	72ºC min	/2	35	72ºC min	/5	304
ENTR-R	95ºC/2 min	95ºC /1 min	57ºC min	/1	72ºC min	/2	35	72ºC min	/5	304

2.5.5.3. Isolation of E. coli

Samples were pre-enriched in buffered peptone water and incubated 37°C for 18h± 2h under aerobic condition. A loopful from the broth of each sample was streaked onto MacConkeyś agar and Eosin Methylene Blue agar. The inoculated plates were incubated at 37°C for 24hours according to [15, 21]. The Salmonella and E. coli isolates were subjected to different biochemical tests such as sugar fermentation test, Indole production, Christenerś urea agar test, Methyl-Red and Vogues-Rescuer (MR-VP) test. Amplification of Salmonella and Escherichia coli by Polymerase Chain Reaction (PCR) TWO Primers were used for Salmonella, the first 300 bp and the second 200 bp, while for Escherichia coli was 560 bp according to [1].

Table 2: Showing the Primer used for E. coli**.**

E. coli genus		**Denaturation**	**Annealing**		**Primer extension**		**Cycle**	**Final extension**		**Target**
ECO-F	**95ºC/3 min**	**94ºC /45 sec**	**58ºC sec**	**/45**	**72ºC min**	**/1**	**35**	**72ºC min**	**/3**	**560**
ECO-R	**95ºC/3 min**	**94ºC /45 sec**	**58ºC sec**	**/45**	**72ºC min**	**/1**	**35**	**72ºC min**	**/3**	**560**

2.6. Statistical Analysis

Data were tested for distribution normality and hemogensty of variance. It reported as means and analyzed by two-way ANOVA using Graph Pad prism 5. Duncan post hoc multiple comparisons test evaluated the significance of difference among the different groups. The significance level was set at $P< 0.05$.

Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation and chi-square test by SPSS V.20.

2.6.1. Mean value $\left(\bar{X}\right)$

The sum of all observations divided by the number of observations:

$$\left(\bar{X}\right) = \frac{\Sigma\, x}{n}$$

Where Σ = sum & n = number of observations.

2.6.2. Standard Deviation [SD]

It measures the degree of scatter of individual varieties around their mean:

$$SD = \sqrt{\frac{\Sigma \left|x - x\right|^{-2}}{n-1}}$$

2.6.3. Analysis of variance [ANOVA] tests (f)

According to the computer program SPSS for Windows. ANOVA test was used for comparison among different times in the same group in quantitative data.

2.6.4. Chi-square

The hypothesis that the row and column variables are independent, without indicating strength or direction of the relationship. Pearson chi-square and likelihood-ratio chi-square. Fisher's exact test and Yates' corrected chi-square is computerized for 2x2 tables.

2.6.5. .Chi-square test

For comparison between two groups as regards qualitative data.

$$X^2 = \sum \frac{(O - E)^2}{E}$$

Where:

Σ = Summation.

O = Observed value.

E = Expected value= $\frac{\text{vertical total X Horizontal total}}{\text{grand total}}$

3. Results and Discussion

3.1. Hatchability percent

An effective hatching egg sanitization program is critical to achieve a high level of hatchability and ensure the production of high quality chicks. As, the hatchability percent was increased by applying the different egg disinfectants (table 3). This significant ($P<0.05$) difference may be due to high bacterial load which penetrate the shell and infect the embryo, causing losses in hatchability beside, high egg weight loss. On the other hand, using H_2O_2 in egg disinfection lowered the hatchability percent in comparison to other disinfectants used. These findings may be explained by.

3.2. Embryonic mortality percentage

There was a significant ($P<0.05$) difference in total embryonic deaths in eggs disinfected by different egg disinfectants. As, the highest total embryonic deaths were recorded in eggs disinfected by TH4 in comparison to other disinfectants used as shown in table 3. These results may be attributed to the increase of bacterial load on the surface of the eggshell and bacterial multiplication either in the surface of the shell or inside the eggs or due to the toxicity of this disinfectant (TH4). The results of embryonic mortality are in accordance with those reported by [6] who mentioned that the microbes on egg shells of newly laid eggs can multiply rapidly when exposed to appropriate ambient conditions and penetrate the eggshell through pores, this could lead to a dramatic reduction in hatching success. In addition, rapid egg sanitization killed the microbes on the eggshell surface before penetration through the egg shell pores and affected the functional properties of the eggshell with respect to egg water loss and gas exchange during incubation. These results complicate the situation regarding application of any new egg disinfectants, therefore eggshell permeability should be taken in our concept in choosing any method of egg disinfection.

3.3. Chick weight

Concerning, the effects of disinfecting on the chick body weight after hatching, different egg disinfectants could improve the chick's weight after hatching (table 3) during the first week, and the improvement may be due to

reducing the contamination of the eggshell and reducing the events for infecting the yolk sac.

Table 3: Effect of hatching egg disinfectants on broiler performance

Variability	Control	H_2O_2	Virkon S	TH4	P-value
Hatchability (%)	78%	92%	95.2%	94%	0.001*
Mortality (%)	4%	3.3%	4%	4.6%	0.002*
Chick B.W (g)	37.08 ± 0.8	40.25 ± 0.9	40.18 ± 1.2	40.13 ± 1.0	0.001

***Means which superscript with (*) differ significantly at (P< 0.05).**

3.4. Chick quality

Data about chick quality are presented in table 4. The results showed that weak chicks' quality appeared only in eggs disinfected by H_2O_2 and TH4 but the highest percent of omphalities was recorded in eggs disinfected by H_2O_2 followed by control groups.

Physical abnormalities % as incomplete feathering and wetness didn't record in treating eggs and control groups. While, the distorted beaks were recorded only in eggs disinfected by TH4. In addition, the highest percent of weak limbs were recorded in eggs disinfected by H_2O_2 and virkon S. These findings are in agreement with that recorded by [5] who observed that optimal hatching results and chick quality can be achieved if eggs are disinfected and set after an initial adaptation period of about one to two day(s). Finally, egg disinfectants are most critical to the normal development of the embryo are those that occur before and during incubation and hatching process [11].

Table 4: Effect of different hatching egg disinfectants on optimum chick quality (%).

Optimum chick quality		Control	H_2O_2	Virkon S	TH4
Weak chick (%)		0	1.5	0	1.5
Omphalitis (%)		7.5	15	3	3
Physical abnormalities (%)	a) Incomplete feathering	0	0	0	0
	b) Weak limbs	6	7.5	7.5	6
	c) Distorted beaks	0	0	0	1.5
	d) Wetness	0	0	0	0

3.5. Total bacterial count (TBC)

The ideal environment for the embryo development is the same needed for microorganism multiplication. Therefore, contaminated eggs will disseminate microorganisms in incubators and hatchers and in turn will reduce hatchability and produce low quality chicks [3].

Data obtained concerning the effect of hatching egg disinfection on the total bacterial count of eggshell surface are shown in Table 5.

Results demonstrated in this table reveal that total bacterial counts on the hatching eggshell surface were significantly ($P< 0.05$) reduced as a result of using all disinfectant treatments in comparison with the bacterial count before egg treatment. It has been demonstrated that if hatching eggs are not sanitized prior to incubation, excessive bacterial contamination and subsequent growth can lead to decreased hatchability, poor chick quality, growth and performance [18] and increased mortality.

The results of this study are in agreement with the findings of [22] who observed that turkey's egg treated with natural disinfectants represented a significant bacterial decrease of populations. Disinfectants significantly reduced the eggshell total bacterial count from 27 Logs to 4.3 Logs with a 84.08% reduction by using H_2O_2, from 24 Logs to 4.7 Logs with a 80.42% reduction after using virkon S and reduced the total bacterial count from 25 Logs to 3 Logs with a 88% reduction after using TH4 in comparison to 28 Logs for the control group. These results indicated on the best eggshell disinfectant is TH4 followed by H_2O_2 and virkon S. These findings are in coincided with that recorded by [10] who mentioned that hydrogen peroxide has been used as a satisfactory disinfectant for animate and inanimate materials and [19] who recorded that Virkon-S was essentially ineffective against the inoculated microorganisms.

Table 5: Effect of hatching egg disinfectants on TBC of broiler eggshells (cfu per egg).

	Disinfectant	H_2O_2	Virkon S	TH4
TBC	Before	27	24	25
	After	4.3	4.7	3
	Reduction %	84.08	80.42	88
	Control	28	28	28
TFC	Before	11	5	7.6
	After	2	2.3	4
	Reduction %	81.82	54	47.37
	Control	4.5	9	4.6
TCC	Before	3.5	2	4.3
	After	0.6	0.1	0.6
	Reduction %	82.86	95	86.05
	Control	9.3	9	6.6

Table 6: Effect of different hatching egg disinfectants on *E. coli* and *Salmonella*

			1st day of incubation				21th day of incubation				
Item	Disinfectant		E. coli		*Salmonella*		Cloacal swap		Unhatched eggs		
			Egg (N=15)	Hatchery (N=5)	Egg (N=15)	Hatchery (N=5)	*E. coli* (N=10)	*Salmonella* (N=10)	*E. coli* (N=5)	*Salmonella* (N=5)	(N=5)
PCR	V.S	Before	0	0	0	0	10	0	0	0	
		After	0	0	0	0					
	TH4	Before	0	0	0	0	30	0	0	0	
		After	0	0	0	0					
	H_2O_2	Before	13.33	0	13.33	0	30	0	0	0	
		After	0	0	0	0					
	Control		6.66	0	0	0	10	20	0	0	

3.6. Molecular identification of E. coli common gene PCR.

PCR findings in figures (1&2) showed that *Salmonella* and *E. coli* were the predominant bacteria on the surface of the eggshell. The prevalence of *E. coli* was the highest, followed by *Salmonella*.

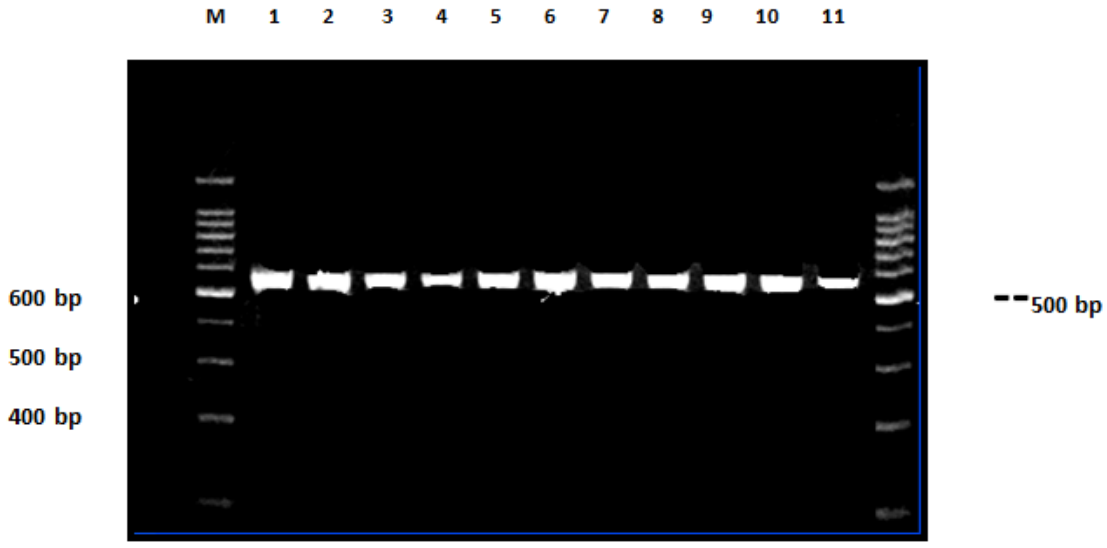

Figure 1: 2% Agarose gel Electrophoresis for PCR product of *E. coli* common gene. (lane M: 100 bp ladder, lane 1: + ve bend at 500bp, lane 2: + ve bend at 500bp, lane 3: + ve bend at 500bp, lane 4: + ve bend at 500bp, lane 5: + ve bend at 500bp, lane 6: + ve bend at 500bp, lane 7: + ve bend at 500bp, lane 8: + ve bend at 500bp, lane 9: + ve bend at 500bp, lane 10: + ve bend at 500bp and lane 11: + ve bend at 500bp).

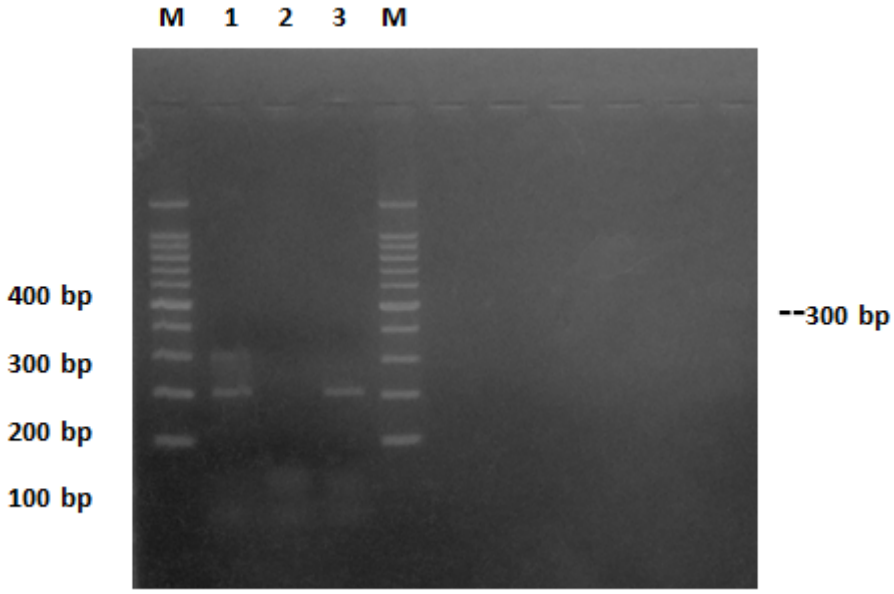

Figure 2: 2% Agarose gel Electrophoresis for PCR product *Salmonella* common gene. (lane M: 100 bp ladder, Lane 1: bend at 200 bp, bend at 300 bp, lane 2: - ve and lane 3: bend at 200 bp)

Table 7: Showing the PCR Report for E. coli.

Sample No.	Result	Target gene	Target size	Agarose gel concentration
1	**Positive**	***E. coli* genus**	**560 bp**	
2	**Positive**	***E. coli* genus**	**560 bp**	
3	**Positive**	***E. coli* genus**	**560 bp**	
4	**Positive**	***E. coli* genus**	**560 bp**	
5	**Positive**	***E. coli* genus**	**560 bp**	**2 %**
6	**Positive**	***E. coli* genus**	**560 bp**	
7	**Positive**	**E. coli genus**	**560 bp**	
8	**Positive**	***E. coli* genus**	**560 bp**	
9	**Positive**	***E. coli* genus**	**560 bp**	
10	**Positive**	***E. coli* genus**	**560 bp**	

4. Conclusion

From these results, it could be concluded that, the control of microorganisms on the shell surface of hatching eggs requires a disinfectant effective in killing the pathogens without injury to the live chick embryo. *E. coli* bacteria were the highest and predominant on eggshell.

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