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Isolation of *Salmonella enterica* in Laying-Hen Flocks and Assessment of Eggshell Contamination in France

MARIANNE CHEMALY,[1]* ADELINE HUNEAU-SALAÜN,[2] ANNIE LABBE,[1] CATHERINE HOUDAYER,[1] ISABELLE PETETIN,[2] AND PHILIPPE FRAVALO[1]

[1]*Unité Hygiène et Qualité des Produits Avicoles et Porcins and* [2]*Unité Epidémiologie et Bien-Être en Aviculture et Cuniculture, Laboratoire d'Etude de Recherche Avicole et Porcine, AFSSA site de Ploufragan, BP 53, 22440 Ploufragan, France*

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ABSTRACT

The present investigation was conducted in conjunction with the European Union baseline study for the estimation of *Salmonella* prevalence in laying-hen flocks. It aimed at evaluating eggshell contamination in farms positive for *Salmonella,* characterizing the genetic patterns of *Salmonella* strains and identifying the factors associated with *Salmonella* contamination of eggshells. For this purpose, a total of 4,200 eggs were collected from 28 positive flocks and analyzed according to draft Annex D of International Organization for Standardization Method 6579. Molecular characterization of the *Salmonella* strains was obtained by the pulsed-field gel electrophoresis method with two restriction enzymes, *Xba*I and *Bln*I. The relationship between the presence of *Salmonella* on eggshells and rearing practices was studied by using multiple correspondence analysis. Results showed that 39.3% of the positive flocks had at least one positive eggshell, with a total of 1.05% of eggshells testing positive for *Salmonella*. We detected the same serovars on samples taken from the farm and from eggshells within a given flock, with isolates sharing the same genetic pattern in 7 of 11 flocks. Eggshells tested positive for *Salmonella* in flocks (i) located where delivery trucks pass near air entrances of the poultry house, (ii) with high holding capacity (>30,000 laying hens), and (iii) with more than five positive samples coming from the farm environment, as well as in cases of flocks with a maximum egg-laying rate of >96% and in cases where farmers worked in other animal production. This study provided valuable information that could be used for risk management and risk assessment studies.

Salmonella is a major zoonotic pathogen and the cause of numerous outbreaks worldwide each year *(3, 9, 12, 34)*. Poultry and eggs remain the major source of infection in developed countries *(29, 30)*. Between 1996 and 2005, eggs and egg-based products were responsible for 59% of the salmonellosis cases in France *(7)*.

The prevalence of *Salmonella* on eggshells has not yet been fully investigated. The microbiological quality of eggshells influences the quality of the egg products. Eggshells can become contaminated with *Salmonella* either because of an infection of the oviduct or by environmental contamination due to the shedding of the bacteria by infected animals. A survey recently performed in the United Kingdom reported a prevalence of *Salmonella* in non–United Kingdom raw shell eggs at the retail level; within the French sample, 2 (0.6%) of 348 eggs were found to be positive for *Salmonella* Mbandaka and *Salmonella* Rissen *(24)*. However, the vertical transmission of *Salmonella* Enteritidis from the shell to the egg yolk is believed scarce. In one study, only 1 of 14,040 eggs tested positive for *Salmonella (15),* while results from another epidemiological investigation after an outbreak showed that 6 (1.7%) of 355 eggs presented positive yolks for *Salmonella* Enteritidis *(13)*. In 2003, the contamination rate of eggs retailed in the United Kingdom was estimated at 0.3% *(10)*.

In 2004, European Commission Decision 2004/665/EC *(11)* prompted European Union member states to carry out an epidemiological investigation between September 2004 and October 2005 in order to estimate the prevalence of *Salmonella enterica* in laying-hen flocks at the end of the rearing period. In France, 524 flocks were sampled from 70 areas distributed throughout the country, with poultry reared either in cages or on the floor, with or without a free-range system. The estimated prevalence of *Salmonella* was found to be 17.7% overall *(4),* and major serovars recovered included *Salmonella* Typhimurium (4.2%) and *Salmonella* Enteritidis (3.8%). Risk factor analyses performed during this baseline study revealed that the prevalence of *Salmonella* was significantly higher in cage flocks than in on-floor flocks. In cage flocks ($n = 227$), the risk of *Salmonella* contamination increased with flock size. In on-floor flocks ($n = 292$), a higher risk of contamination was associated with multistage management (presence of hens of different ages on the farm), and the contamination of a previous flock by *Salmonella* Enteritidis *(17)*.

Due to the consumer risk of exposure to of *Salmonella*-contaminated eggs not having been assessed because of lack of information on this topic, an investigation was carried out in conjunction with the baseline study in order to estimate the level of contamination of eggshells collected from

* Author for correspondence. Tel: +33-2-96-01-01-52; Fax: +33-2-96-01-85-38; E-mail: m.chemaly@ploufragan.afssa.fr.

positive farms. The objectives of this work were (i) to assess the prevalence of *Salmonella* on eggshells collected from infected farms, (ii) to characterize the genetic patterns of *Salmonella* strains isolated from the farms and the eggs, and (iii) to identify factors associated with *Salmonella* contamination of eggshells by analyzing answers to questionnaires that were completed during visits made during the European baseline study and also used for this investigation.

MATERIALS AND METHODS

Egg sampling. In the European baseline study for the prevalence of *Salmonella* in French laying-hen flocks, an estimated 17.7% (93 of 525 flocks sampled; 95% confidence interval, 14.5 to 21.3) of the flocks tested positive for *Salmonella* *(17)*. A flock was considered positive when at least one of seven samples tested positive for *Salmonella*. Of the 93 positive flocks, 28 were randomly chosen for egg sampling. In order to derive the number of eggs in each sample, we considered an expected prevalence of 1% with an accuracy of 1.6 and 95% confidence limits. Thus, 150 eggs were taken from each of the 28 positive flocks as a stochastic sampling of a single day's egg production.

Microbiological analysis. For the purpose of this study, a total of 4,200 eggs were analyzed for *Salmonella*. Each egg was individually transferred aseptically in a sterile bag. Analyses were performed according to a modified version of International Organization for Standardization Method 6579 *(1)*, which required a preenrichment step in which the egg is immersed in buffered peptone water (150 ml per egg) at 37°C for 16 to 20 h. One hundred microliters of buffered peptone water was transferred to modified semisolid Rappaport-Vassiliadis agar used as the single selective enrichment medium and then incubated at 41.5°C for 24 and 48 h. Characteristic migrations were then transferred onto two selective media, xylose-lysine-deoxycholate and Rambach agars. Two typical colonies (one from each selective agar) were biochemically identified and serotyped according to the Kauffman-White scheme by using sera purchased from BioRad (Hercules, CA). All the media purchased from AES (Combourg, France) were internally prepared by following the quality control system based on International Organization for Standardization Standard 17025 *(2)*, except when specified.

Molecular characterization of *Salmonella* isolates. The strains that were isolated from positive environmental samples and positive eggshells were submitted to molecular characterization using restriction fragment length polymorphism analysis by two pulsed-field gel enzymes, *Xba*I and *Bln*I (Roche Diagnostics, Meylan, France) at 37°C for 5 to 6 h. After enzymatic restriction, the plugs were distributed into the wells of an agarose gel (1% in Tris-borate-EDTA 0.5×). The gel was then transferred to a CHEF DRIII system (Bio-Rad) for migration under a homogenized pulsed electric field (6.6 V) in a buffer (Tris-borate-EDTA 0.5×) at 14°C. Pulse time was 20 to 40 s for 12 h, and 7 to 13 s for 10 h. The *Salmonella* Braenderup H9812 strain was used as a molecular weight marker. The gels were stained with an ethidium bromide solution, and then pictures were taken under a UV lamp system (Fisher-Bioblock Scientific, Illkirch, France). Data analysis was carried out with BioNumerics software (Applied Maths, Kortrijk, Belgium) in order to establish similarities between the isolates by using the Dice coefficient *(33)* and to build the dendrograms according to the unweighted pair group method with arithmetic mean method.

Statistical analyses for factors associated with *Salmonella* contamination. The questionnaire set up for the European baseline study was used for this investigation in order to collect data regarding factors associated with the *Salmonella* contamination of eggshells. The questionnaire consisted of six main parts dealing with general characteristics of the farms and poultry houses, farm management practices, and egg production. Thus, a total of 115 questions garnered data from each sampled farm.

In a first step, we performed univariate and bivariate analyses in order to select variables related to the *Salmonella* status of each sample ($P < 0.20$) by using the FREQ procedure of SAS software (SAS Institute, Inc., Chicago, IL). The selection threshold was voluntarily raised to 20% in order to increase the number of potential variables.

In a second step, a multiple correspondence analysis *(21)* was conducted in order to analyze the relationships patterns of these dependent variables by using SPAD software, version 5.6 (Paris, France). Finally, a hierarchical classification was carried out in order to find relationships between the variables and the presence of *Salmonella* on eggshells.

RESULTS

Prevalence of *Salmonella* on eggshells. Twenty-eight flocks were selected and sampled for eggshell contamination. A flock is considered positive when at least 1 of 150 eggs sampled tests positive for *Salmonella*. In our investigation, 11 of the 28 flocks had at least 1 positive egg, equivalent to 39.3% prevalence. The prevalence of *Salmonella* on eggshells was 1.05% (95% confidence interval, 0.78 to 1.41), with 44 of 4,200 shells positive for the bacteria (Table 1). The frequency of *Salmonella* isolation varied from 0.6% (1 of 150 positive eggshells; 95% confidence interval, 0.11 to 3.7) to 8.6% (13 of 150 positive eggshells; 95% confidence interval, 5.13 to 14.3). The majority (18%) of the flocks had only 1 positive eggshell, while a single flock (4%) had 13 positive eggshells. The serotyping of *Salmonella* strains revealed five different serovars including Enteritidis, Typhimurium, Virchow, Infantis, and Montevideo. We found that within the same flock, the serovars detected on the samples taken from the farm (dust, boot swabs, or feces) and those isolated from the eggshells were the same (Table 1).

Genotyping of *Salmonella* isolates. A total of 22 isolates were genotyped according to the PFGE method. For each *Salmonella*-positive farm ($n = 11$), a *Salmonella* isolate recovered from eggshell was paired with an isolate recovered from an environmental sample on the same farm. This resulted in 10 *Salmonella* Enteritidis, 4 *Salmonella* Typhimurium, 4 *Salmonella* Virchow, 2 *Salmonella* Infantis, and 2 *Salmonella* Montevideo isolates. Isolates from the same serovar were grouped in a same cluster (Fig. 1). In 7 of 11 flocks, the isolates from eggshells and farms shared identical genetic patterns. In two of five cases with *Salmonella* Enteritidis, the isolates from eggshells and from the farms showed similarity; at any rate, all 10 isolates were genetically very close and constituted one cluster with 85% similarity. In one case, *Salmonella* Enteritidis isolates (eggshell and environmental) shared the same number of fragments, but two bands differed in their positions in the

TABLE 1. Salmonella *isolation from samples (feces, boot swabs, and dust) taken at the farm level and from eggshells from the positive farms*

Farm no.	No. of positive samples from the farm ($n = 7$)	*Salmonella* serovars found on positive samples at the farm	No. of positive eggs ($n = 150$)	*Salmonella* serovars found on positive eggs
1	6	Enteritidis	1	Enteritidis
2	4	Enteritidis	1	Enteritidis
3	2	Enteritidis	13	Enteritidis
4	3	Enteritidis	1	Enteritidis
5	7	Montevideo	2	Montevideo
6	1	Enteritidis	1	Enteritidis
7	6	Virchow	9	Virchow
8	6	Typhimurium	2	Typhimurium
9	7	Typhimurium	1	Typhimurium
10	7	Infantis	4	Infantis
11	6	Virchow	9	Virchow

*Bln*I restriction pattern (Fig. 1). In a second case, a *Salmonella* Enteritidis eggshell isolate differed from an environmental isolate by two additional fragments in the *Xba*I restriction pattern and one band position in the *Bln*I restriction pattern. For the latter case, a *Salmonella* Enteritidis eggshell isolate differed from environmental isolate by two band positions in the *Xba*I restriction pattern only. In the two cases of *Salmonella* Typhimurium, three of the four isolates had identical PFGE patterns, therefore indicating a strong similarity between the isolates from eggshells and farms in one of those cases. One *Salmonella* Typhimurium environmental isolate differed from other *Salmonella* Typhimurium isolates (eggshell and environmental) by one additional fragment in the *Xba*I restriction pattern and demonstrated a three-band difference in the *Bln*I restriction pattern. The Virchow serovar isolates were similar within a flock, although two different PFGE patterns were observed for the two different flocks. Nevertheless, all four isolates clustered in one group with 82% similarity. The PFGE patterns of the Infantis and Montevideo serovars were similar between eggshells and farms for both restriction enzymes *Xba*I and *Bln*I.

Factors associated with the presence of *Salmonella* on eggshells. According to the univariate analysis ($P \leq 0.2$), 11 variables of a total of 115 were linked to the presence of *Salmonella* on the eggshells (Table 2). The variable number of positive samples from the farm was also included, because this factor was highly correlated to the number of positive eggshells over the 150 analyzed per flock ($P < 0.01$). Significant correlations were (i) the number of positive eggshells was higher in poultry houses with more than 30,000 laying hens (0.031), (ii) the number of positive eggshells increased with the laying rate (0.017), and (iii) the number of *Salmonella*-positive environmental samples recovered correlated with *Salmonella* status of eggshells ($P = 0.0104$) and the number of positive eggshells (0.0030). When the number of positive farm samples was fewer than five, 21% of the flocks had positive eggshells, but only 5% of the flocks had more

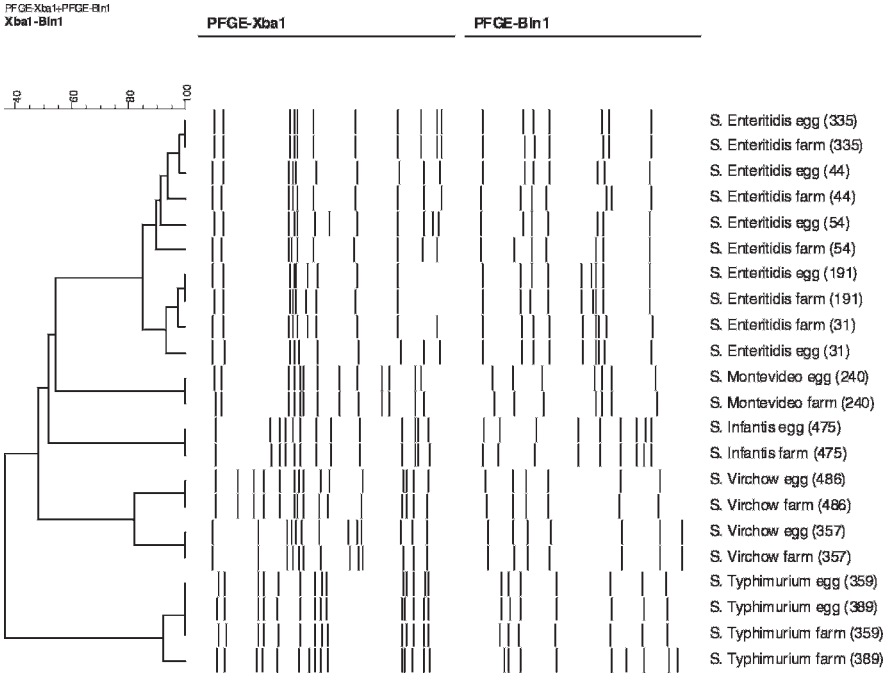

FIGURE 1. *PFGE patterns and dendrogram of* Salmonella *isolates from eggs and farms obtained with the restriction enzymes* Xba*I and* Bln*I*.

TABLE 2. χ^2 *exact test for the explanatory variables (*$P \leq 0.2$*) of* Salmonella *status of eggshells*

Code	Meaning of selected variables	No. of flocks	No. of flocks with positive eggs	χ^2 exact test
	Farm characteristics			
Explttes	Presence of future laying hens on the farm			
	Yes	6	4	
	No	22	7	0.174
Exprod	Presence of other animal production on the farm			
	Yes	8	5	
	No	20	6	0.200
	Farm practices			
Perani	Presence of farmers working in other animal production houses			
	Yes	6	4	
	No	22	7	0.178
Csnbpoul	No. of laying hens at the sampling time			
	>30,000	7	5	
	<30,000	21	6	0.076
Cspic	Maximum laying rate			
	>96%	5	4	
	<96%	23	7	0.062
	Poultry house characteristics			
Bacap	Holding capacity (no. of laying hens)			
	>30,000	8	5	
	<30,000	20	6	0.200
Bachaus	Farmer changes shoes when entering the poultry house			
	Yes	23	11	
	No	5	0	0.125
Batenu	Farmer changes clothes when entering the poultry house			
	Yes	24	11	
	No	4	0	0.023
Changement_tenue	Farmer changes completely when entering the poultry house			
	Yes	21	11	
	No	7	0	0.023
Camoeair	Egg transport trucks pass near air entrances of the poultry house			
	Yes	6	4	
	No	22	7	0.174
Circulation_air	Delivery trucks pass near air entrances of the poultry house			
	Yes	8	5	
	No	20	6	0.200

than one. When there were more than five positive farm samples, 78% of the flocks had positive eggshells, with 56% having more than one.

A multiple correspondence analysis was conducted in order to identify the association between rearing factors (Table 2) and *Salmonella* status of eggshells and the number of positive eggshells, introduced in the analysis as illustrative variables (Fig. 2). The first axis represents 35% of the information and separates flocks by (i) whether the delivery trucks pass near the air entrances of the poultry house, (ii) the size of the holding capacity ($\pm$30,000 laying hens), and (iii) the number of positive farm samples ($\pm$5). The second axis represents 20% of the information and distinguishes flocks based on the maximum laying rate ($\pm$96%) and whether the farmers work in other animal production.

In a second step, a hierarchical classification was created by using the independent active variables and the two illustrative variables, *Salmonella* status of eggshells and number of positive eggshells (0, 1, or >1). Because of the small number of flocks (28), the results were separated in only two classes (Table 3). The first class, characterized by a negative status for *Salmonella* on eggshells, groups the majority of the flocks (86%), which present the following characteristics: a laying rate fewer than 96%, farmers working exclusively in holding, and fewer than five positive environmental samples. The second class, characterized by a positive status for *Salmonella,* groups 14% of the flocks presenting the opposite characteristics, i.e., a laying rate higher than 96%, farmers working in other animal production, and more than five positive environmental samples (Table 3).

DISCUSSION

Although this study was not designed to estimate the overall prevalence of *Salmonella* or the specific prevalence of *Salmonella* Enteritidis on eggshells, it does provide valuable data on the incidence of *Salmonella* on

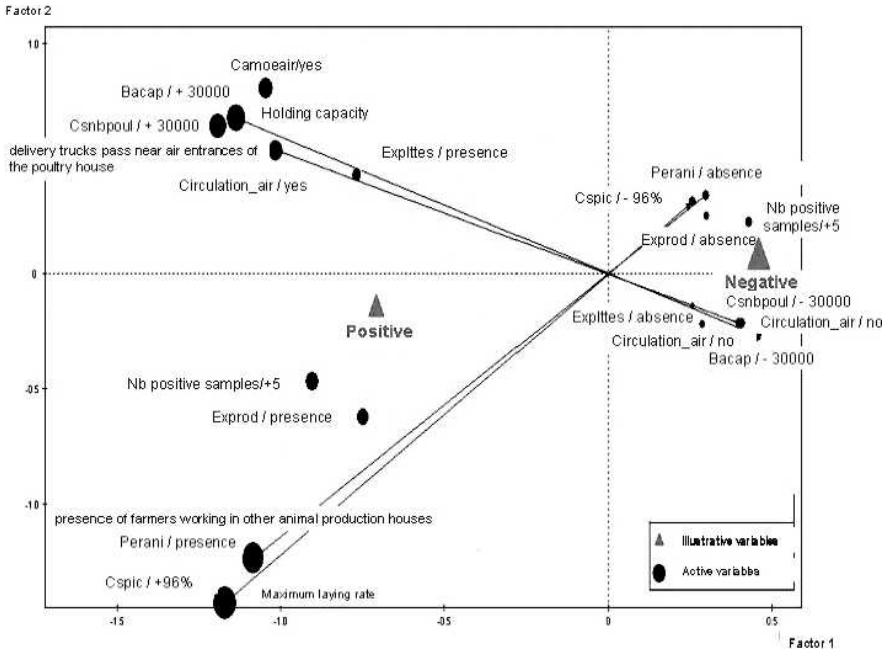

FIGURE 2. *Factorial plan built with axes 1 and 2 based on active and illustrative variables. Explttes, Presence of future laying hens on the farm; Exprod, presence of other animal production on the farm; Perani, presence of farmers working in other animal production houses; Csnbpoul, number of laying hens at the sampling time; Cspic, maximum laying rate; Bacap, holding capacity (number of laying hens); Camoeair, egg transport trucks pass near air entrances of the poultry house; Circulation_air, delivery trucks pass near air entrances of the poultry house.*

eggshells collected from positive flocks, as well as insight into the burden of salmonellosis associated with poultry products. The results show that 39% of positive flocks generated positive eggshells, and although this means that 61% of the positive flocks did not have a positive egg of 150 sampled, the ratio of positive eggs found (0.6 to 8.6%) still represents a threat to consumer health. It has been determined that *Salmonella* present on eggshells can migrate to the egg content under normal conditions of storage and moisture *(8, 16, 23),* whereas storing the eggs at a temperature below 8°C might avoid the multiplication of *Salmonella* in the yolk *(23)*. *Salmonella* can also reach the egg products during industrial preparation *(27, 30, 31),* which could be the cause of some outbreaks *(20)*.

In this study, the serovars found on the eggshells were the same as those detected in the farm environment. However, the molecular characterization of isolates by PFGE showed some discrepancies, especially among the *Salmonella* Enteritidis and *Salmonella* Typhimurium isolates. This is partly due to the high power of discrimination of this method, especially when two restriction enzymes are used. In other respects, the rather high genetic diversity of *Salmonella* Typhimurium *(5, 18, 35)* could explain the differences observed between the 2 isolates collected from the same farm, one from the environment and the second from eggshells, although their similarity with the other isolates remains high (92%). *Salmonella* Enteritidis is well known to be clonal and to have low genetic diversity; in general, many restriction enzymes or several techniques are needed to discriminate between those isolates *(19, 22, 28, 35)*. In this study, 10 isolates of *Salmonella* Enteritidis presented eight different pulsotypes grouped in one cluster, at 85%. Although different, the isolates originating from eggshells and the environment from one flock were still closely related. Our results were consistent with those

TABLE 3. *Class characteristics resulting from the hierarchical classification study*

Code[a]	Modality	% within the class	% within the samples	Class % within the modality	Test value	*P* value
		Class 1/2 (n = 24, 85.71%)				
Cspic	<96%	95.8	82.1	100.0	3.5	0.00
Perani	Absence	91.7	78.6	100.0	3.18	0.00
Ppenvi	<5	79.2	67.9	100.0	2.5	0.00
Salmonella status	Negative	70.8	60.7	100.0	2.1	0.02
		Class 2/2 (n = 4, 14.29%)				
Cspic	>96%	100.0	17.9	80.0	3.5	0.00
Perani	Presence	100.0	21.4	66.7	3.2	0.00
Ppenvi	>5	100.0	32.1	44.4	2.5	0.00
Salmonella status	Positive	100.0	39.3	36.4	2.1	0.02
Positive eggshells	>1	75.0	21.4	50.0	2.01	0.02

[a] Cspic, maximum laying rate; Perani, presence of farmers working in other animal production houses; Ppenvi, number of positive samples from the farm.

studies that reported that isolates sharing the same serotype form a single cluster *(18, 35)*.

The results of this study suggest that flocks presenting a higher level of environmental contamination (five or more contaminated samples) also present a higher level of contaminated eggs. This result could benefit quantitative risk assessments, and be used as a criterion for risk management. This confirms earlier findings by Henzler et al. *(15),* who determined that flocks with high levels of manure contamination were 10 times more likely to produce contaminated eggs than were flocks with lower levels of manure contamination. Our study also shows that egg contamination appears related to the number of hens in the poultry house (>30,000 hens), which is consistent with the results of other studies *(17, 26)*. These authors found a relationship between the number of hens housed in a caged poultry house and the risk factor for *Salmonella* contamination. In fact, large flock sizes may increase the number of susceptible birds, and contaminations within large-sized poultry houses may spread easily, especially in poultry houses connected to egg packing plants by means of a common egg conveyor *(6)*. We also noted that *Salmonella* eggshell contamination was associated with a high laying rate. Egg laying induces animal stress, and consequently, *Salmonella* shedding, which enhances the probability of eggshell contamination. The effect of production stages in laying hens apparently influences the prevalence of *Salmonella (14, 26, 32)*.

The findings from this investigation have improved our knowledge regarding the presence of *Salmonella* on eggshells. The data generated in this investigation could be used for risk assessment studies and risk management programs to ensure better, safer eggshell quality.

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