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Bacteriological Investigation of Microorganisms (Salmonella sp. and Other Enterobacteriaceae) in Common Quails (Coturnix coturnix) Submitted to Different Forced-Molting Procedures

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■Keywords

Eggs, forced molting, zinc oxide, fasting, wheat midds diet.

ABSTRACT

The aim of this study was to investigate the presence of *Salmonella* in common quails submitted to forced molting. A total of 240 quails were divided at 40 weeks of age into four groups: CG (control, quails not submitted to molting); FM (fasting method); WM (fed wheat midds ad libitum); and ZM (zinc oxide method). From each group, 10 cloacal swabs, 10 fecal samples, and 20 egg samples were collected before molting (two weeks) and after molting (two weeks). The microbiological procedures for *Salmonella* spp. identification were performed in four steps. The agglutination test, using somatic and flagellar antigens, was used to confirm *Salmonella*-suspected colonies. According to the methodology applied, none of the samples was positive for *Salmonella* spp. The results showed that 20.0% of the egg samples from birds submitted to forced molting were contaminated with enterobacteria. It was concluded that, under the conditions of the present experiment, the stress caused by forced molting did not induce infection by *Salmonella* spp. or increased Enterobacteriaceae contamination levels in the eggs.

INTRODUCTION

Low egg quality is a common problem in the poultry industry and cause extensive losses. The main causes are related to environmental, health, and management issues (Cardoso *et al*., 2001). Among health factors, egg contamination by *Salmonella* spp. is extremely important in the poultry production chain (Hafez, 2005), because this genus is one of the main causes of food poisoning in humans (Chernaki-Leffer*et al*., 2002; Tirolli & Costa, 2006).

Microorganisms may contaminate internal egg contamination by several pathways: through the ovary, when the agent remains inside the egg during its formation; through the uterus, when the agent is present in the oviduct epithelium or in the serosa of the air sacs; through the cloaca at the time of lay, when the egg may get in contact with contaminated feces; and through the external environment after lay, which is the most frequent form of microorganism invasion (Soncini & Bittencourt, 2003).

Despite the significant role played by *Salmonella* spp. in food poisoning around the world and the economic losses caused in the poultry industry, other enterobacteria that may potentially cause foodborne diseases in humans should be considered, as several studies report their occurrence in eggs used for human consumption. The most frequent enterobacteria are *Enterobacter* sp., *Klebsilella* sp., *Citrobacter* sp., *Serratia* sp. *Proteus* sp., *Pseudomonas* sp., *Hafnia* and *Providencia* sp. (Adesiyun*et al*., 2006; Salles, 2007; Musgrove *et al*., 2008; Siqueira *et al*., 2008).

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The main source of contamination of poultry products by food pathogens are animal feces (Rasmussen *et al*., 2004), particularly in the case of eggs. One of the most frequent forms of egg contact with feces is when birds present watery feces due to pathological (e.g., enteritis) or physiological conditions (e.g., changes in feed formulation), leading to egg contamination at the time of lay (Soncini & Bittencourt, 2003).

In order to prevent bacterial contamination problems, good management practices need to be applied in egg production, providing hens proper health conditions that prevent the spread and replication of microorganisms. A common management practices applied in the egg industry is forced molting by fasting, aiming at obtaining a second laying cycle. However, this frequently affects hens' health. The stress caused by feed fasting results in an increase in blood corticosterone levels (Webster, 2003). It is known that stress hormones, such as corticosterone, have anti-inflammatory properties, thereby impairing the immune function (Golden *et al*., 2008). Therefore, molting induced by fasting increases hen susceptibility to several pathogenic microorganisms, including *Salmonella* (Holt, 2003).

Holt (2003) asserted that chickens submitted to forced molting are 100 to 1000 times more susceptible to *Salmonella* Enteritidis (SE) infection and may easily spread the bacterium to the non-infected hens in neighboring cages. This demonstrated that fasting to induce forced molting in commercial layers may eventually cause severe consumer health problems. This hypothesis was confirmed by Golden *et al*. (2008), who demonstrated that layers submitted to forced molting by feed fasting produced SE-contaminated eggs more frequently.

Despite these health issues involving the fasting method, forced molting has still been successfully used in commercial layer production. Alternative forced molting methods have been researched and used in poultry industry during the last decades to provide animal welfare and to prevent health problems. Among the employed methods, the supply of high dietary zinc levels is the most frequently studied and applied in the field, particularly in the United States (Mesquita Filho, 2008). Forced-molting methods using wheat-based feeds have also shown good results (Biggs *et al*., 2004; Dalanezi, 2007).

Forced molting is still not commonly applied in quail egg production (Garcia *et al*., 2001), although a few published studies have reported its effects on production and physiological aspects (Zamprônio *et al*., 1996; Garcia *et al*., 2002.; Teixeira *et al*., 2007; Mesquita Filho, 2008; Faitarone *et al*., 2008). Scientific data on the effects of forced molting on quail health are lacking. Therefore, the objective of the present study was to evaluate the effect of different forced molting methods on the presence of *Salmonella* spp. in the eggs, feces, and cloacal swabs of common quails (*Coturnix coturnix*).

MATERIALS AND METHODS

Molting methods

In this experiment, 240 common quails at the end of their production cycle (40 weeks old) were used. Three forced molting methods were used: wheat method (WM), zinc oxide method (ZM), and fasting method (FM).The control group (CG) was composed of quails that were not induced to molting and were offered a commercial laying feed (Table 01) and water *ad libitum* during the entire experimental period.In

Table 1 – Ingredient composition and nutritional levels of the feed supplied to the experimental quails.

Ingredients	Percentage
Ground corn 8%	46
Soybean meal 45.5%	25
Semi-defatted soybeans 10% EE	19
Dicalcium phosphate 45%	1.8
Limestone 38%	7.3
Salt	0.3
DL-Methionine 99%	0.17
L-Lysine 78%	0.1
Mineral supplement1	0.13
Vitamin supplement2	0.2
Total	100
Composition	
Crude protein (%)	21.985
AME (kcal/kg)	2807
Calcium	3.320
Available phosphorus	0.693
Methionine + cystine (%)	0.78
Lysine (%)	1.10
Methionine (%)	0.43

1. Mineral supplement (per kg product):Cu.10,000 mg; Fe.100,000 mg; I, 1,500 mg; Mn, 150,000 mg; Zn, 100,000 mg.

2. Vitamin supplement (per kg product):Vitamin A, 12,000,000 IU; Vitamin D3, 3,600,000 IU; Vitamin K, 1,600 mg; Vitamin B1, 2,500 mg; Vitamin B12 – 12,500 mg; niacin – 3,750 mg; pantothenic acid – 12,500mg; folic acid –15,000 mg; antioxidant – 25,000mg.

total, 60 birds were designated to each experimental group (WM, ZM, FM, CG). Birds were weighed and housed in conventional pyramid-type cages (64 birds/m^2) belonging to the experimental houses of the School of Veterinary Medicine of the State University of Ceará, Brazil.

Molting was induced in WM and ZM quails by feeding wheat midds and a laying feed containing 25000 ppm zinc oxide, respectively. FM birds were submitted to complete feed fasting until the end of the treatment. Independently of treatment group, all birds were offered water *ad libitum*. Birds were submitted to the treatments until the body weight loss (BWL) specific for each method was achieved, as follows: WM (25% BWL), and ZM and FM (30% BWL).

Microbiological evaluation and sample collection

The microbiological evaluation consisted in the investigation of the presence of *Salmonella* spp. in cloacal swabs, fresh fecal samples, and eggs two weeks before and two weeks after quails were submitted to forced molting. The same procedure was applied to investigate the presence of other Enterobacteriaceae in egg samples. Ten fecal samples and ten cloacal swabs (pool of three birds) were collected from each experimental group. Twenty egg samples, consisting of two eggs each, were evaluated.

Microbiological procedures

Cloacal swabs and fresh fecal samples (1g) were collected, transported to the Ornithological Studies Lab and immediately processed. Eggshells were disinfected by immersion in ethanol at 70%, and then broken. Egg content was homogenized by agitation in glass flasks, and 1-mL aliquots were used as sample unit. Enterobacteria isolation and identification were performed according to the following steps: Pre-enrichment, selective enrichment, plating and presumptive identification, biochemical tests, serology, and typification. During the pre-enrichment phase, 10 mL buffered peptone water at 1% were added to the collected clocal swab, fecal, and egg samples. In selective enrichment, 0.1 mL and 1 mL aliquots of the pre-enriched culture were transferred to tubes containing 10 mL Rappaport-Vassiliadis broth and 10 mL selenite cystine broth, respectively. Samples were then transferred to selective liquid media, using a platinum loop, to plates containing the selective-indicator media brilliant green agar and MacConkey agar to perform the presumptive identification. Colonies with morphological characteristics suggesting *Salmonella* spp. were inoculated in tubes with TSI (triple sugar iron) agar slant, LIA (lysine iron agar) slant, and SIM (sulfide-indole-motility) agar. Colonies were biochemically characterized by glucose and sucrose fermentation properties, gas production, H_2S production, indole production, and motility. All tests were carried out at 37 °C incubation temperature for 24 h. Colonies biochemically identified as *Salmonella* spp. were submitted to serology, using rapid plate seroagglutination tests with the use of flagellar (H) and somatic (O) antisera.

The absolute frequency of Enterobacteriaceae was determined using the plating results of isolates suspected of *Salmonella*. The isolation of bacteria not belonging to the family Enterobacteriaceae were not considered in the study. The Chi-square test at 5% significance level was used to compare the numbers of egg samples contaminated with Enterobacteriaceae among treatments before and after forced molting was implemented.

RESULTS AND DISCUSSION

The microbiological procedures did not detect the presence of *Salmonella* spp. in none of the egg, fecal, or cloacal swab samples collected from all evaluated experimental groups. Despite the absence of *Salmonella* determined in the present study, several other authors demonstrated that laying hens submitted to forced molting by the method of feed fasting are more susceptible to this microorganism (Holt & Porter, 1992; Macri*et al*., 1997; Berchieri Jr., 2000; Holt, 2003; Berry, 2003).This was clearly shown in the study of Holt *et al*. (1995). The authors induced molting in commercial layers using fasting and orally infected these birds after four days with SE (5-10 x 10^6). They observed 24 and 96 hours after the experimental infection significantly higher SE counts in the colon, ceca, and feces in the molted birds compared with the hens not submitted to forced molting. In the ileum, SE counts were higher only 48h after the experimental infection. Nakamura *et al*. (2004) observed that layers submitted to forced molting by fasting shed more *Salmonella* in the feces until day 14 post-infection than the control birds. As to *Salmonella* shedding in the eggs, Golden *et al*. (2008) observed that layers submitted to fasting may produce SE-contaminated eggs more frequently than fed layers; however, shedding declines with time.

Salmonella isolation has also been reported in layers submitted to molting using alternative methods. McReynolds et al. (2006) found that alternative molting methods usually result in low *Salmonella* incidence and significantly reduce total bacterial counts in the ceca and other internal organs in layers submitted to forced molting and challenged with *Salmonella* Enteritidis. Therefore, the authors stress that alternative forced molting methods do not completely eliminate the possible risk of *Salmonella* infection.

Most studies involving *Salmonella* isolation in laying chickens submitted to forced molting used an inoculation procedure to evaluate bird sensitivity to that microorganism. In the present study, quails were not inoculated with *Salmonella*, which may explain why its presence was not detected. However, some studies in literature show the isolation of *Salmonella* spp in layers submitted to forced molting using epidemiological investigation. Garber *et al*. (2003) surveyed farms in 15 US states using fecal matter swabs collected in layers houses with different environments. The researchers observed that layers submitted to forced molting had more probability of being positive for *Salmonella enterica* serotype Enteritidis than contemporary layers not submitted to molting. Souza *et al*. (2002) performed an epidemiological survey on the incidence of *Salmonella* in layers submitted to forced molting and isolated *Salmonella* Give from cloacal and fecal samples before and 30 days after fasting. The authors detected the presence of that microorganism in samples of meat meal and feed supplied to the layers, and argued that the finding of the same serotypes in the birds indicated the intake of contaminated feed. Therefore, the presence or absence of *Salmonella* spp. in the environment where molting is induced may determine the spread of these bacteria. According to Hinton *et al*. (2000), fasting causes physical, chemical and microbiological changes in the bird's crop, and these changes may impair the natural capacity to inhibit the local colonization by Enterobacteriacea.

Table 2 shows the presumptive results relative to Enterobacteriacea isolated in quails submitted to forced molting by different methods.

There was no significant difference (p> 0.05) among treatments as to absolute frequencies of samples positive for Enterobacteriaceae before and after forced molting. The highest absolute frequency in contaminated egg samples was observed in the WM group post-molting (25%), whereas the lowest frequency was 10% (n=2) and it was observed in the CG and WM group before molting.

Table 2 – Frequency of Enterobacteriaceae isolated from the eggs of common quails submitted to forced molting by different methods

Isolated Enterobacteriaceae	Enterobacteriaceae frequency per treatment							
	Before molting				After molting			
	CG	FM	WM	ZM	CG	FM	WM	ZM
K + S	1	1	-	-	-	-	-	-
S	1	1	-	-	-	-	-	-
E	-	1	-	1	2	2		2
E + K + Sh	-	-	1	-	-	-	-	-
Sh	-	-	1	-	-	-	-	1
K	-	-	-	1	-	1	-	-
E + K + S + Sh	-	-	-	1	-	-	-	-
E + K	-	-	-	1	-	-	1	-
P	-	-	-	-	1	-	-	-
C	-	-	-	-	-	1	3	1
C + E + H + S	-	-	-	-	-	-	1	-
Total	2(10%)	3(15%)	2(10%)	4(20%)	3(15%)	4(20%)	5(25%)	4(20%)

C = Citrobacter sp.; E = Enterobacter; P = Proteus sp.; K = Klebsiella sp.; S = Serratia sp.; Sh = Shigella sp.

H = Hafnia sp.

The presumptive results found in the present study relative to absolute numbers and percentages of eggs contaminated with enterobacteria are similar or much lower than those reported in literature. This is demonstrated in the study of Stepien-Pyśniak (2010). The author qualitatively analyzed Gram-negative bacteria, particularly those belonging to Enterobacteriaceae family in the eggshell, yolk, and albumen of fresh eggs produced in large and small farms, and found that *Escherichia coli, Enterobacter, Citrobacter*, and *Klebsiella* were isolated more often in the eggshell, whereas in the albumen and in the yolk, *Escherichia coli* was more common. The least frequently isolated bacteria were *Proteus* and *Serratia.* Considering the total percentage of enterobacteria, Adesiyun *et al*. (2006) evaluated fresh eggs from Trinidad farms and found that 28.3% of the eggshells and 15.2% of the egg content were contaminated. Therefore, total egg contamination was higher than those found in quail eggs before or after forced molting. There are few studies on Enterobacteriaceae in quail eggs. However, in the metropolitan area of Fortaleza, CE, Brazil, Siqueira *et al*. (2008) carried out a study on the contamination of quail eggs by Enterobacteriaceae. The researchers found a significant contamination rate (45.59%) in quail eggs collected in supermarkets – *Enterobacter* and *Shigella* were the most frequent agents – and attributed these results to possible hygiene issues along the quail egg production chain. Therefore, the lowest contamination rate found in quail eggs before or after forced molting may be explained by the fact that the eggs were evaluated immediately after lay, and not after processing and preservation steps in a supermarket shelf.

CONCLUSIONS

Under the conditions of the present experiment, no *Salmonella* spp. was detected in cloacal swab, fecal or egg samples neither before nor after molting induced by different methods (feed fasting, feed based on wheat midds, or feed with high zinc oxide level).Therefore, it was not possible to determine if forced molting had any influence on the presence of that pathogen. Further studies are required involving previously infected quails to understand this relationship better.

The results demonstrated that the amount of quail eggs contaminated by bacteria belonging to the family Enterobacteriaceae was low before and after molting, and that this was not influenced by the evaluated forced molting methods.

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