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Experimental Infection of Salmonella Enteritidis in Quails Submitted to Forced Molting by Feed Fasting

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ABSTRACT

This study aimed at evaluating bacterial shedding, as detected in swabs, feces, and eggs of quails submitted to forced molting by feed fasting and experimentally infected with a *Salmonella* Enteritidis (SE) strain. In the experiment, 84 40-week-old Italian female quails were distributed in the following groups: FI (quails induced to molt by fasting and inoculated with *Salmonella* Enteritidis – SE); CI (quails fed with a laying diet and inoculated with SE); FNI (quails induced to molt by fasting and not inoculated with SE); and CNI (quails fed with a laying feed and not inoculated with SE). Feces, cloacal swabs, and eggs were collected on day 1, 3, 7 and 14 post-inoculation (dpi) and submitted to bacteriological analyses. All samples obtained from cloacal swabs were negative for SE. None of the quails of the non-inoculated groups (FNI and CNI) were positive for SE in the fecal samples. Among the inoculated quails, the FI group presented significantly higher ($p < 0.05$) SE shedding in the feces on 1 dpi than the CI group. On 4 dpi, no significant difference was observed ($p < 0.05$) in SE shedding between the inoculated quail groups. On 7 dpi, only the FI group shed SE in the feces, whereas on 14 dpi, none of the groups shed SE. According to the results, we concluded that quails submitted to molting by fasting have higher possibility of shedding SE in the feces.

INTRODUCTION

The practice of forced molting to extend the longevity of laying hens has been widely studied and several methods have showed effective results in term of egg quality and quantity (Berry, 2003; Molino *et al.*, 2009; Aygun, 2013). However, there a few studies with quails. Some recent papers have been published evaluated the effect of forced molting on production, reproduction, and health parameters (Garcia *et al.*, 2001, Teixeira *et al.*, 2007, Faitarone *et al.*, 2008, Arora & Vatsalyia, 2011; Aljumaily *et al.*, 2012, Teixeira *et al.*, 2013). Interestingly, despite the close relation between molting and salmonellosis, this issue has not been explored yet.

The traditional forced-molting method involves submitting laying hens to several days of feed fasting, which debilitates birds and makes them susceptible to the infection by pathogens. The stress caused by fasting increases corticosterone plasma levels (Webster, 2003), and stress hormones are known to have anti-inflammatory properties, which reduce the effectiveness of the immune system in fighting diseases (Golden *et al.*, 2008). Thus, birds with compromised immune system become susceptible to microorganisms, such as Salmonella Enteritidis (Holt, 2003), and may produce contaminated eggs (Gama *et al.*, 2003), compromising public health.

Public health problems are still a concern in many countries that apply feed fasting as a forced-molting method. Brazil is a major egg producer, and there are few studies on the microbiological status of eggs produced by hens submitted to forced molting (Teixeira & Cardoso, 2011), particularly of quail eggs. Therefore, this study aimed at evaluating bacterial counts in cloacal swabs, feces, and eggs of quails submitted to forced molting by fasting and experimentally infected with Salmonella Enteritidis strains.

MATERIAL AND METHODS

Birds

In total, 82 quails at the end of egg-production cycle (40 weeks of age) were used in this experiment. Quails were individually weighed, identified, and housed in battery cages (25 x 25 x 20 cm; three birds per cage) located at the experimental facilities of the Laboratory of Ornithological Studies, State University of Ceará, Fortaleza, Brazil.

Bacteriological monitoring before inoculation

Three days before the trial started, all birds were examined to ensure they were free of *Salmonella* spp. Birds were analyzed according to methodology of Zancan *et al.* (2000) with modifications. Fresh feces and cloacal swabs were collected per cage (pool of three birds) using sterile material. Samples were placed in tubes containing 2 mL selenite-cystine broth with novobiocin addition (40µg/mL) and incubated at 37° C in a bacteriological incubator for 24h. Samples were then plated on brilliant green agar and incubated under the conditions described above, after which the growth of colonies with morphological characteristics of *Salmonella* was observed in the plates.

Experimental groups

Each experimental unit consisted of a single cage, housing three quails. Quails were distributed into four treatments with seven replicates each, as follows: FI (group of quails induced to molt by fasting and inoculated with *Salmonella* Enteritidis – SE); CI (control group: quails fed layer feed and inoculated with SE); FNI (group of quails induced to molt by fasting and not inoculated with SE); CNI (quails fed layer feed and not inoculated with SE).

Induced molting method

Molting was induced by total deprivation of feed. Water was supplied *ad libitum*. The first day of feed deprivation was considered day zero (D0). Each experimental unit was daily weighed and when mean body weight loss 30% was obtained, birds were fed a layer commercial feed *ad libitum*.

Inoculum preparation

A strain of *Salmonella* Enteritidis (SE) resistant to nalidixic acid (SENal^r) isolated from chickens (*Gallus gallus*) was used in this study. The inoculum was prepared according to Berchieri *et al.* (2001) with modifications as follows: the bacterial culture was prepared in 5 mL buffered peptone water at 0.1% and incubated in a bacteriological incubator at 37°C for 24 h. After incubation, serial dilutions were performed to determine the number of colony forming units (CFU) (Miles *et al.*, 1938). The inoculum contained 10^7 CFU of *Salmonella* Enteritidis Nal^r/mL.

Inoculation

One day after the beginning of forced molting, FI and CI quails received 0.1 mL of the inoculum directly in the crop by gavage, using a probe coupled to a 1cc syringe.

Cloacal swab samples

On 1, 4, 7, and 14 days post-inoculation (dpi), cloacal swabs were collected (pool of three birds) per experimental unit, comprising 28 samples. Processing was carried out according to methodology of Zancan *et al.* (2000) with modifications, as follows: the cloacal material was collected from all birds with the aid of sterile swabs moistened in selenite-cystine broth with novobiocin addition (40µg/mL). Immediately after collection, samples were plated on brilliant green agar containing nalidixic acid (100µg/mL). Then, both broth and plates were incubated at 37°C in a bacteriological incubator for 24h, after which the growth of colonies with morphological characteristics of *Salmonella* was observed. An aliquot from each selenite-cystine tube from samples which plates were negative was seeded on brilliant green agar plates containing nalidixic acid (100µg/mL) and placed in a bacteriological incubator for 24h at 37°C. Samples were considered negative for *Salmonella* spp only after the second plating.

Feces samples

Aluminum trays were placed under all cages to collect fresh feces on 1, 4, 7, and 14 dpi. Fecal samples (0.5 g) were placed in tubes containing 5 mL peptone water, and serially diluted for subsequent CFU counting on plates containing brilliant green agar

with nalidixic acid addition (100µg/mL) (Miles *et al.*, 1938). Plates and fecal samples cultured in peptone water were incubated in a bacteriological incubator at 37°C for 24h. Negative samples after incubation were transferred to tubes containing 2 mL of selenite-cystine broth with novobiocin (40 uL) and, after 24 h at 37°C , were plated on brilliant green agar containing nalidixic acid (100µg/mL).

Egg samples

Eggs from birds submitted to forced molting (FI and FNI) were collected for microbiological analysis during the first six days of the week and of the week they returned to lay (two weeks post-molting). The eggs produced by quails that were not submitted to molting were also analyzed according to the same selection procedure and sampling period.

Eggs were first immersed in a 70% alcohol solution for shell disinfection and processed according to Jahantigh & Nili (2010), with modifications, as follows: eggs were macerated and homogenized using spatula and sterile beaker. Samples were subsequently incubated in a bacteriological incubator at 37°C for 24 h, after which they were transferred using sterile swabs to tubes containing 2 mL selenite-cystine broth supplemented with novobiocin (40µg/mL) and incubated at 37°C for 24 h. Samples were then plated on brilliant green agar containing nalidixic acid (100µg/mL) and placed in a bacteriological incubator at the same aforementioned temperature and for the same time, and then observed for the presence of *Salmonella* Enteritidis.

Fecal microorganism count data were tested for normality by Shapiro-Wilk and Kolmogorov-Smirnov tests. Treatment means were compared by the nonparametric Mann-Whitney test (5%). Statistical analyzes were performed using the program ASSISTAT, beta version 7.6 (Silva *et al.*, 2010).

RESULTS

All cloacal swab samples were negative for SE. However, 57.10% of the fecal samples of the quails submitted to forced molting were positive on 1 dpi. The CI group showed a higher number of positive birds (37.5%) on day 4 of observation (Figure 01). On day 7, none of the CI group fecal samples was tested positive, while a sample of the FI group (14.3%) still shed SE in the feces. After 14 days, SE shedding was not observed in none of the groups.

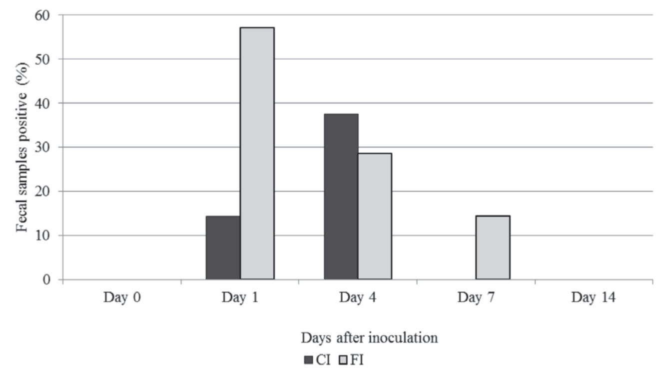

Figure 1 – Percentage of fecal samples positive for Salmonella Enteritidis obtained from quails induced to molting and experimentally infected.

None of fecal samples of the quails of the non-inoculated groups (FNI and CNI) were positive for SE and therefore, no statistical comparison was performed. On the other hand, FI birds presented significantly higher ($p<0.05$) number of bacterial cells (SE) in the feces than CI group birds on 1 dpi. On 4 dpi, no significant difference was observed ($p<0.05$) in bacterial shedding between the FI and the CI groups. On 7 dpi, the FI group was the only one that shed the pathogen in the feces, and on 14 dpi, no group shed *Salmonella* (Figure 02).

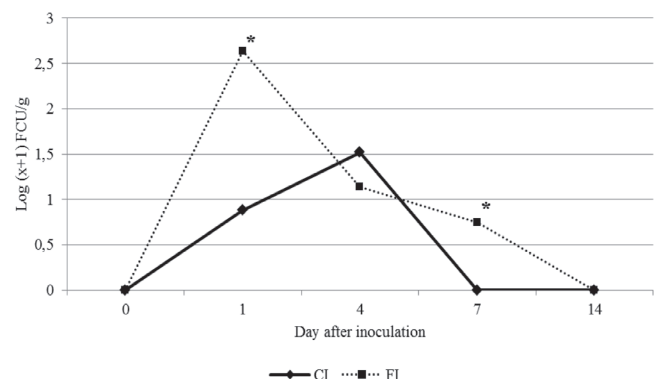

Figure 2 – Fecal shedding of Salmonella Enteritidis in quails induced to molt and experimentally infected.

*Significant differences among means on different days post-inoculation ($p<0.05$)

Quails submitted to forced molting completely ceased egg laying, which resumed only two weeks after feed supply. Microbiological analysis of all collected egg samples were negative for SE.

DISCUSSION

The negative SE results obtained in the microbiological examination of the cloacal swabs of infected quails are different from the findings of Barrow & Lovell (1991) obtained with laying hens orally infected with

a higher concentration of SE (3x10^8 CFU / mL). Those authors observed that 100% of the cloacal swabs were positive in the first week post-inoculation, and that 12.5% of the evaluated birds remained positive in the fifth week. However, García *et al.* (2011) suggested that cloacal swabs are less efficient to detect SE infection in laying hens compared with fecal samples because in that study, while fecal samples were 92% positive for SE, only 4% of cloacal swabs were positive.

The high number of SE-negative cloacal swab samples may be associated with the sensitivity of this procedure for the identification of *Salmonella* sp. Some researchers have questioned the effectiveness of swabs for the detection of *Salmonella* in animals (Aho, 1992; Weiss *et al.,* 2002). The intermittent shedding of *Salmonella*, associated with the fact that it can be shed in small amounts in the feces may impair detection, and therefore, cloacal swabbing presents limitations as a method to monitor *Salmonella* shedding (Andrade *et al.*, 2007). Pereira *et al.* (2007) studied the presence of *Salmonella* in Greater Rhea (*Rhea americana*) and concluded that the use of cloacal swabs for the detection of birds infected with *Salmonella* has low sensitivity and therefore, the risk of indicating false negative results is high. This may explain why there were no positive cloacal swab samples obtained from inoculated quails in the present study, differently from the fecal samples. Higgins (1982), working with broiler chickens, asserted that the sensitivity of *Salmonella* detection in fresh feces is greater when compared with cloacal swabs.

Concerning the microbiological analyses of the feces, detected the presence of the inoculated pathogen was detected. A significantly higher ($p<0.05$) shedding of SE in the FI group on 1 and 7 dpi was recorded. However, on 4 dpi, no significant difference ($p>0.05$) in bacterial shedding was observed between the inoculated groups considering the period in which bacterial shedding in feces was observed. This shows that fasting affects bacterial shedding in birds submitted to induced molting. The results of the present study are consistent with the findings of Nakamura *et al.* (2004), who worked with laying chickens submitted to induce molting by fasting (Body weight loss of 30% body weight loss) and orally inoculated with *Salmonella* Enteritidis (2.4 x 10^9 of the SE). These authors verified that the pathogen was eliminated in the feces during nine observation moments post-inoculation until 42 dpi, after which bacterial shedding was no longer observed. In four of these moments, the group submitted to fasting presented significantly higher bacterial shedding when compared to the group of hens not inoculated. In the other five moments, no significant difference was observed between the groups. The longer period of SE shedding in hens in that study when compared towith the shedding period observed in the quails in this study may be explained by the higher bacterial concentration used to in the inoculate.

Molting induced by fasting may compromise the immune system of laying birds, making them more vulnerable to infections by various microorganisms (Holt, 2003). Onbaşılar & Erol (2007) asserted that fasting, because it causes severe physiological stress, is the least suitable method of molting in terms of bird welfare. Those authors compared fasting with other molting methods, such as the supply of zinc oxide or of barley grains in the feed, and found that the hens submitted to fasting presented higher corticosterone levels as well as impaired humoral immunity compared with other methods. The stress caused by fasting rises plasma corticosterone levels (Webster, 2003), which results in a decrease of circulating lymphocytes (Maerz & Davis, 2010), as well as of other leukocytes, and suppresses humoral immunity, weakening the resistance of birds to diseases (Mench *et al.*, 1986).

As expected, non-inoculated quails submitted to molting by fasting were negative for *Salmonella* Enteritidis. This result shows that, although several studies in the scientific literature demonstrate that molting induced by fasting is closely associated with SE, the presence of this microorganism in the environment during the period of stress is essential for pathogen dissemination (Teixeira *et al.*, 2013).

The finding of negative SE shedding in the eggs in the present study is different from the results of Golden *et al.* (2008), who reported that laying chickens submitted to the induced molting by fasting and orally inoculated with SE laid contaminated eggs during the first weeks post-molting. However, other studies show that hens orally inoculated with SE strains may present a low or absent prevalence of this bacterium in the eggs. Yang (1992) orally inoculated Japanese quails with SE (10^{10} CFU) and found that out of 164 analyzed eggs, SE was detected in the albumen of seven eggs, in the yolk of 15 eggs, in the eggshell of 13 eggs, and in the shell membranes of 15 eggs, representing bacterial contamination of 10.9% of the eggs. In this case, the inoculum concentration was higher than that used in the quails of the present study, which may explain the absence of contaminated eggs. These results are

consistent with the findings of Barrow & Lovell (1991), who orally infected laying chickens with 3 x 10^8 CFU SE provided orally and obtained only two positive samples (0.32%) out of 633 eggs analyzed during 48 days, with the positive samples recorded during the first 4 dpi.

CONCLUSION

In conclusion, the obtained results suggest forced molting by fasting reduce the immunity of laying quails, since the group of quails inoculated with *Salmonella* Enteritidis and induced to molting by fasting shed the bacterium longer than the group inoculated with SE and not submitted to fasting.

Further studies with larger numbers of birds and during a shorter time are needed to investigate more precisely SE shedding in infected quails, as well as to observe the relationship between infection and impaired immunity caused by fasting as a method of forced molting.

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