

Effect of immersion and inoculation in ovo of *Lactobacillus* spp. in embryonated chicken eggs in the prevention of *Salmonella* Enteritidis after hatch¹

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ABSTRACT The protection level against *Salmonella* Enteritidis was evaluated in chickens after in ovo treatment with different species of *Lactobacillus* spp. inoculated into the air cell or by immersion in broth culture. Two hundred forty embryonated eggs were distributed into 8 groups, corresponding to treatments with *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *Lactobacillus salivarius*, and control. On d 18 of incubation, 4 groups were inoculated with 0.1 mL of inoculum in the air cell and 4 groups were immersed for 3 min in culture of each treatment. Two days after hatching, 0.5

mL of *Salmonella* Enteritidis culture was inoculated by the intraesophageal route. On d 5 of life, the chicks were euthanized and the ceca were processed to obtain *Salmonella* Enteritidis counts. There was no decrease in *Salmonella* Enteritidis colonization of chick ceca, regardless of treatment or route of administration. *Lactobacillus* spp. samples used in the treatment showed no probiotic potential in chicks when inoculated in ovo, in relation to *Salmonella* Enteritidis inhibition in poultry ceca.

Key words: *Lactobacillus*, *Salmonella*, embryonated egg, in ovo

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INTRODUCTION

The complex epidemiology of *Salmonella* in the poultry production chain involves horizontal transmission with the environment and diet contamination besides the existence of different animal species that constitute reservoirs of *Salmonella* Enteritidis (Moura, 2007). The contamination of eggs by *Salmonella* spp. occurs initially and mostly through the shell. Time and temperature of storage are key factors that enable the *Salmonella* to pass from the shell surface to the internal structures of the egg.

Epidemiological studies have shown that the major source of infection of commercial poultry flocks by *Salmonella* spp. are chicks from infected hens, cross infection in the hatchery, and environmental contamination on the farm (Andreatti Filho, 2006).

Because of the complex epidemiology of *Salmonella*, it is necessary to implement control programs based on

bacteriological and serological tests, to prevent infection or control the spread of organisms, and to practice preventive hygiene measures. However, given that hygienic measures are only partially effective in controlling *Salmonella* colonization, obtaining an effective level of control requires complementary measures such as vector control, probiotics, heat treatment of feed, incorporation of organic in feeding, disinfection of hatching eggs, and vaccination, which can be implemented as part of a broad program of biosecurity (Andreatti Filho, 2006; Vandeplass et al., 2010).

Since Nurmi and Rantala (1973) suggested that pretreatment with microbiota isolated in the gastrointestinal tract of adult poultry free of *Salmonella* spp. can protect against infection by this species, many products have been developed (Ávila et al., 2006).

The use of *Lactobacillus* spp. as a probiotic for hens has been suggested as an interesting option to reduce the infections caused by *Salmonella* Enteritidis (Van Coillie et al., 2007).

Sharma and Burmester (1982) was the first study to use the concept of vaccination in ovo against Marek's disease. Andreatti Filho et al. (2006) tested the effect of administration in ovo of cecal microbiota and of *Lactobacillus salivarius* in the presence of *Salmonella* En-

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teritidis, verifying that under these conditions *L. salivarius* does not prevent the cecal infection of chicks by *Salmonella* Enteritidis.

The present study aimed to treat the embryonated eggs with different species of *Lactobacillus* spp. inoculated in the air cell or by immersion in cultivated broth, and to verify in chicks the posthatching protection level of the *Lactobacillus* spp. against oral challenge by *Salmonella* Enteritidis.

MATERIALS AND METHODS

Experimental Design

The 240 embryonated eggs used were obtained from the same broiler breeders (Cobb 500, Cobb-Vantress, Brazil), aged 35 wk, not vaccinated, from a farm in the region. They were randomly distributed into 8 groups of 30 eggs, each corresponding to a different administration route and treatment. The routes used were inoculation in the air cell or immersion in cultivated broth containing *Lactobacillus* spp. The treatments consisted of *Lactobacillus acidophilus*, *Lactobacillus fermentum*, *L. salivarius*, or PBS sterilized, separately for each route of administration.

Lactobacillus spp.

The cultures of *Lactobacillus* spp. were obtained from the bacterial collection at the Ornithopathology Laboratory of FMVZ-UNESP-Botucatu and processed according to the "spot on the lawn" method to verify their antagonism when challenged by *Salmonella* Enteritidis.

The samples of *Lactobacillus* were inoculated in DeMan, Rogosa, Sharpe (MRS) broth and incubated at 38°C for 48 h. Subsequently, a 10- μ L volume of these cultures was added (in the form of spots) on Petri dishes containing MRS agar, and after being dried completely, the dishes were incubated again at 38°C for 18 h. The *Salmonella* Enteritidis was inoculated in tubes containing 5 mL of brain heart infusion (BHI) broth and incubated at 38°C for 12 h. Subsequently, 200 μ L of this culture was transferred to new tubes containing 20 mL of BHI broth increased to 0.75% of agar-agar, which had been previously prepared and maintained in a warm bath at 45°C. This preparation was poured carefully onto dishes previously incubated with the *Lactobacillus* samples, and after the complete solidification of the upper layer, the dishes were incubated again.

The samples of *Lactobacillus acidophilus*, *L. fermentum*, and *L. salivarius* that presented the best results in the spot on the lawn test against *Salmonella* Enteritidis were selected for the experiment. The determination of cfu of the *Lactobacillus* spp. was made by serial decimal dilution of the MRS broth in PBS and inoculated from these dilutions in Petri dishes containing MRS agar, incubated at 37°C for 24 h.

Incubation

The eggs were incubated at a temperature of 37.5°C and RH of 60%. On d 18 of incubation, after candling to determine the position of the air cell, the groups of eggs submitted to the air-cell treatments were disinfected with 70% alcohol and inoculated with 0.2 mL of the inoculum from each treatment in the center of the air cell. The groups submitted to the treatment by broth immersion containing the respective treatment were immersed for 3 min in cultures of *Lactobacillus* spp. and further dried. Next, all the eggs were returned to the incubator and maintained until hatching at a temperature of 37.5°C and RH of 60%.

Challenge Strain and Preparation of Inocula

Two days after the hatching, the chicks were challenged with *Salmonella* Enteritidis Nal/Rif phagotype 04 isolated from broiler-breeder liver and phagotyped by Instituto Adolfo Lutz-São Paulo, and kept in the bacterial collection of the Ornithopathology Laboratory at FMVZ. For use, the bacterium was inoculated in BHI and incubated at 37°C for 12 h.

On d 2 of life, all chicks were challenged by oral gavage by inoculating 0.5 mL of *Salmonella* Enteritidis inoculum containing 8.3×10^8 cfu/mL. The number of cfu in the inoculum was determined by means of decimal serial dilutions in PBS, with further plating of the respective dilution in brilliant green agar Nal/Rif, and incubated for 24 h at 37°C. On d 5 of life, the chicks were killed by cervical dislocation and the ceca removed aseptically and processed to obtain the number of cfu per gram of ceca, according to the previously described methodology.

Statistical Analysis

The technique of ANOVA was used for comparing the cfu variable in the treatments in relation to the index of cecal colonization in a scheme of 2 factors in complete randomized blocks (Zar, 2009).

RESULTS AND DISCUSSION

The cecal colonization in chicks challenged with *Salmonella* Enteritidis after inoculation in ovo or immersion of *L. acidophilus*, *L. fermentum*, *L. salivarius*, or PBS is described in Table 1. In all animals, regardless of treatment, the presence of *Salmonella* Enteritidis was observed in the ceca. The *Salmonella* Enteritidis counts in the ceca did not differ significantly between the routes and treatments.

Similar results were found by Andreatti Filho et al. (2006), who, after inoculating via air cell in *L. salivarius* and challenging with *Salmonella* Enteritidis, observed cecal colonization by *Salmonella* Enteritidis in all of the birds 5 d after challenge.

Table 1. Logarithm mean of the concentration of *Salmonella* Enteritidis in the poultry ceca according to route and treatment¹

Item	Treatment				
	<i>Lactobacillus acidophilus</i> (3.8×10^{11} cfu/mL)	<i>Lactobacillus fermentum</i> (3.4×10^{11} cfu/mL)	<i>Lactobacillus salivarius</i> (3.1×10^{11} cfu/mL)	PBS	<i>P</i> (<i>Lactobacillus</i>)
Air cell	10.09 (0.060)	9.26 (1.54)	7.82 (0.76)	9.10 (1.36)	<i>P</i> > 0.05
Immersion	9.21 (0.42)	10.01 (1.38)	8.37 (0.91)	10.00 (1.06)	<i>P</i> > 0.05
<i>P</i> (environment)	<i>P</i> > 0.05	<i>P</i> > 0.05	<i>P</i> > 0.05	<i>P</i> > 0.05	

¹SD in parentheses.

Pascual et al. (1999) reported that orally administered *L. salivarius* significantly reduced the number of positive birds after challenge with *Salmonella* Enteritidis. However, the present study promoted challenge with a larger amount of *Salmonella* Enteritidis, indicating that such *Lactobacillus* spp. treatments are not effective against a higher level of *Salmonella* Enteritidis.

A study by Leandro et al. (2010) verified the decline of birds receiving a diet of probiotics, after having been challenged with *Salmonella* Enteritidis at the age of 1 d, by collecting cecal material at different moments (7, 14, and 21 d). Thus, the trial period of up to 5 d in the present experiment may have been insufficient for *Lactobacillus* spp. to act in the gastrointestinal tract of birds. Rocha (2001) verified that 1-d-old chicks infected with *Salmonella* spp. also did not present the bacterial agent at the end of the raising period.

The hatchability ranged from 46 to 83%, including the control. An 83% hatchability rate was reported in eggs treated with *L. fermentum* route air cell, in contrast to the results reported by Cox et al. (1992), who, by administering cecal microbiota via the air cell, obtained 56% hatchability. Andreatti Filho et al. (2006), after inoculating *L. salivarius*, observed 60% hatchability.

The best hatching percentage of eggs treated with *L. fermentum* was produced by air-cell inoculation, differing significantly from eggs treated with *L. acidophilus*. The eggs that had undergone treatments with *L. salivarius* or PBS did not present hatching differences compared with the other treatments. Eggs treated by immersion in PBS showed the best hatching, whereas the *L. salivarius* treatment produced the lowest hatching rate. The treatments with *L. acidophilus* and *L. fermentum* showed no difference compared with the other treatments. In relation to the hatching rate, only the eggs that received *L. acidophilus* treatment by air cell showed a significant difference. In treatments via immersion in cultivated broth containing *Lactobacillus* spp., only the control treatment produced a significant variation in the percentages of hatched and unhatched eggs.

The present study demonstrated that there was no significant variance of hatchability of eggs before and after receiving treatment by air-cell inoculation or immersion in *Lactobacillus*, in contrast to previous results (Cox et al., 1992; Andreatti Filho, 2006).

Currently, to counteract expected contamination by *Salmonella* spp., specific alternatives to the administration of these products soon after hatching, such as in ovo use, and development of new techniques, such as dipping in broth with competitive exclusion products, are being studied (Cox et al., 1992; Leandro et al., 2010; Katayama et al., 2010). However, further elucidation is required to establish their scientific validity and economic feasibility. In the present study, the in ovo inoculation and dipping in broth cultured with *Lactobacillus acidophilus*, *L. fermentum*, or *L. salivarius* did not prevent colonization of *Salmonella* Enteritidis in poultry ceca.

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