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PAPER

Histomorphometrical evaluation of gastrointestinal tract and performance of Ross broilers hatched from eggs inoculated with *Salmonella* Enteritidis phage type 4

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Abstract

An experiment was carried out with 128 Ross chicks to evaluate the effects of *Salmonella* Enteritidis phage type 4 inoculated in eggs (contamination site *vs inoculum*), observing the site of inoculation by injection in allantoidal cavity or by shell direct contact and the combination with presence or absence of *Salmonella* Enteritidis phage type 4. Chicks were distributed in a completely randomised 2x2 factorial design (contamination site and *inoculum*). We evaluated the frequency of infected chicks, the morphology of the small intestinal mucosa from day 1 to 21 of age, and its performance. *Salmonella* Enteritidis phage type 4 inoculated in allantoidal cavity showed high frequency of intestinal colonisation at hatch, but reduced with age. The histomorphometrical evaluation indicated reduction in villus height ($P<0.05$) and increase in crypt depth in duodenum and ileum at day 1. At day 21, villus height was similar to the control, and crypt depth was deeper ($P<0.05$). The lowest performance ($P<0.05$) was observed at days 14 and 21 and highest feed conversion at day 14. The fast-growth lineage showed increased jejunum crypt depth ($P<0.05$). At day 21, no difference was found in the intestinal mucosa parameters or in weight gain and feed conversion in chicks inoculated with *Salmonella* Enteritidis in the albumen. Changes were evident in the histomorphometric parameters, which resulted in lower weight gain at days 14 and 21.

Introduction

Paratyphoid infections have been considered of high risk in poultry production, especially *Salmonella* Enteritidis and *Salmonella* Typhimurium, due to their threat to public health. *Salmonella* Enteritidis is one of the most serious pathogens for humans and the poultry industry, and phage type 4 (PT4) shows the highest dissemination in Brazil (Nunes *et al.*, 2003; Santos *et al*., 2003; Castilla *et al.*, 2006). These data explain the persistence of bacteria in the birds and their probability of contaminating food. Andrade *et al*. (2012) observed that the contamination of the eggshell before hatching generated birds susceptible to infection at birth, and the frequency of isolation of *Salmonella* Enteritidis persisted until 28 days of age in turkeys. Desmidt *et al*. (1997) reported the penetration of *Salmonella* Enteritidis phage type 4 through the intestinal wall in the early stages of infection. A high percentage of chicks with gastrointestinal tract (GIT) colonisation in the first week may exhibit intestinal alterations and modified performance in the first days of life.

Experimental inoculations with *Salmonella* can contribute to pinpoint those factors influencing the ability to colonise the intestine, and to determine injuries and the absorption of nutrients (Andrade *et al*., 2009). The GIT presents the highest tissue regeneration rates (Friedman *et al*., 2003) and may be anatomically complete upon hatching, but it also shows post-hatching development with increased absorption surface, enterocyte proliferation and crypt differentiation and definition. Factors such as bacterial flora, intestinal motility, vascular supply, nutrition, and immune cell function may affect the GIT mucosa morphofunctionally. The scantiness of data on the invasive capacity of *Salmonella* Enteritidis phage type 4 in broilers, the effect of this virus on the histomorphometry of the GIT, and the fact that it is widely disseminated among commercial poultry flocks (Nunes *et al.* 2003; Rocha *et al*., 2003; Rezende *et al*., 2005, 2008; Andrade *et al*., 2012) are important factors that the poultry industry has to take into consideration. In view of the above, the present study aimed to evaluate the susceptibility of Ross chicks originating from embryos inoculated with *Salmonella* Enteritidis phage type 4 on eggshell surfaces or in the albumen at the moment of incubation, based on chicks' biometry, intestinal morphohistometry, colonisation and overall performance.

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Key words: *Salmonella* Enteritidis PT4, Inoculum, Crypt depth, Villus height, Intestinal health.

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Materials and methods

Experimental design

The experiment was performed in the Department of Poultry Diseases, Laboratories of Histopathology and Bacteriology of the School of Veterinary and Animal Science of the Federal University of Goiás in Goiânia, Goiás, Brazil. A total of 128 Ross lineage chicks were distributed in a completely randomised 2x2 factorial design according to the treatments of embryonated eggs (contamination site *versus* inoculum) previously made. Embryonated eggs were obtained from farm with effective control for *Salmonella*. Approximately 4 h after collection and arrival at the laboratory, eggs were sprayed with a compound derived from quaternary ammonia at a concentration of 1000 ppm. The eggs were kept at room temperature around 18 h, until they were inoculated. All procedures were carried out utilising sterile equipment in an aseptic chamber at security level 2. Eggs were exposed to *inocula* of *Salmonella* Enteritidis PT 4 by contact with hands coated with disposable gloves, simulating cross-contamination after the process of sanitising eggs. Each glove was moistened with 0.1 mL of buffered saline 0.85% and

bacterial suspension containing 1.5×10^2 colony-forming units (CFU)/mL. With the aid of a tuberculin syringe, 0.1 mL of the solution was placed in the glove-coated hands. Each embryonated egg was maintained for a period of 20 sec on contaminated hands, and its surface completely wet by the solution. Immediately after inoculation the eggs were transferred to incubators. For inoculation into the allantoic cavity, initially, each egg was disinfected with a solution of iodine tincture 10% at the drill site of the shell. The egg was pierced with the help of a 15-mm drill in the gauge tube. A quantity of 0.1 mL of a 0.85% saline, buffered sterile and bacterial suspension containing 1.5×10^2 CFU per mL was inoculated with the aid of a 1-mL tuberculin syringe. The needle gauge 12.7×0.33 mm was introduced into the chamber air content placed into the allantoic. After inoculation, the holes were sealed with paraffin and the eggs transferred to the incubator.

Group A_1 comprised 32 chicks hatched from eggs inoculated on the shell (direct contact) with 0.1 mL of 1.5×10^2 solution containing CFU of *Salmonella* Enteritidis phage type 4, while group A_2 was inoculated in the same way though with a negative control (0.85% sterilised buffered saline). Group B_1 consisted of 32 chicks whose albumen was inoculated with 0.1 mL of the same solution used in Group A_1, while Group B_2 was inoculated with 0.85% sterilised buffered saline (negative control). After hatching, the experimental groups were distributed in five subgroups each containing six chicks. The animals were housed in heated galvanised steel battery cages equipped with linear food troughs and drinkers and excreta collection trays. Each battery contained five tiers, each of which was equipped with a 60W incandescent light bulb that was left on until day 21 of age. Water and ration were supplied *ad libitum*. A vegetable ration without growth-promoter antibiotics was formulated based on nutritional and food composition requirements (Rostagno *et al.*, 2005). The subgroups were weighed and each chick was identified at the tarsometatarsus. Chicks were weighed at day 1, 7, 14 and 21 of life, and two chicks per group were taken to the Department of Poultry Diseases where they were kept without food for 6 to 8 h. After that, they were weighed and put to death by cervical dislocation, as required by the standard ethical treatment of the School of Veterinary Medicine and Animal Science.

Histomorphometry

Necropsy of the animals involved removing and weighing the GIT, measuring the intestinal loop (cm), and calculating the relative weight of the intestine [(organ weight/live weight)×100] (Grieves, 1991). Fragments of 3.0 cm each were removed from the three parts of the small intestine of each experimental unit: initial portion of the duodenum, jejunum (2.0 cm above Meckel's diverticulum) and ileum (next to the ileocaecal valve). The samples were sectioned longitudinally, opened, and the ends stapled onto a wooden holder. Each section was placed in a labeled flask containing 10% buffered formaldehyde solution. A total of 96 samples were treated according to the methodology proposed by Luna (1968). After 24 h of fixation, the fragments were cut into 5.0-mm-diameter pieces, placed in tissue blocks and identified. Subsequently, they were washed with tap water to remove excess formalin pigment, and dehydrated in an ascending series of ethyl alcohol concentrations from 70% to absolute alcohol. The samples were then clarified with xylol and impregnated with histological paraffin having a melting point of 56°C. The fragments were embedded in histological paraffin blocks which were cut into 5-µm slices in a rotary microtome (Spencer 820; American Optical, Buffalo, NY) using disposable blades, placed on slides, and stained with hematoxylin and eosin (HE). Morphometric dimensions were determined using Axion Vision 3.0 software. Slide images recorded in a bright field optical microscope (JENAVAL; Carl Zeiss, Oberkochen, Germany) were digitised with an analog video camera and an image capture card. The duodenum, jejunum and ileum were then analysed histometrically and quantified in micra, according to the criteria proposed by Uni *et al*. (1998). Villus height was measured from the tip of the villus to the crypt, and crypt depth was defined as the depth of the invagination between adjacent villi. Five readings per tissue fragment were performed, from right to left, totalling 60 readings of villus height and crypt depth per slide.

Salmonella research

Along with the fragments of small intestine, samples of caecal content were collected and immediately subjected to *Salmonella* research, as recommended by the Georgia Poultry Laboratory (1997). The isolates showing biochemical reactions compatible with this genus were serum-tested with somatic polyvalent antisera, placed in nutrient agar medium, and sent to the reference laboratory Oswaldo Cruz for typification of the corresponding serovar.

Performance

Chicks, chicken feed and leftovers were weighed at day 1, 7, 14 and 21 of life. The weight of dead chicks was recorded to correct feed intake and conversion. Weight gain, feed intake and feed conversion were calculated as well.

Statistical analysis

The averages of the completely randomised 2×2 factorial design (contamination site *vs* *inoculum*), with five repetitions, were compared by Tukey's test at the 5% level (SAS, 1998).

Results and discussion

Intestinal colonisation by bacteria in hatcheries, incubation wastes and breeding sites began before and immediately after hatching. Chicks hatched from inoculated shell and albumen showed a high rate of intestinal colonisation, based on the caecal content examined during the experimental period. According to Corrier *et al*. (1991), the composition of the microbiota in the GIT may be beneficial or harmful to the host. Beneficial gut microbiota and the development of the immune system may reduce the level of genus *Salmonella*'s intestinal colonisation in the first weeks of life (Andrade *et al.*, 2009). The relative weight of the intestine indicated that its length at days 1, 7, 14 and 21 (Table 1) was not affected by the contamination site of the Ross chicks. An analysis of the *inocula* revealed a difference ($P<0.05$) in the length of the intestine – which was longer in chicks inoculated with *Salmonella* Enteritidis – at day 7. At day 21, the weight of the intestine of chicks inoculated with the same agent was higher ($P<0.05$). Corrier *et al.* (1991) and Honjo *et al.* (1993) also reported that the development of GIT structures and functions occurs simultaneously to that of the lymphoid tissues associated with the mucosae. Infectious agents are able to overcome natural barriers in the intestinal lumen and cause disorders in the epithelial cells, thus changing the permeability of the mucosa which is in a state of perfect functional capacity (Uni *et al*., 2000, Andrade *et al*., 2012).

Similarly to the development of this tract structures and functions, the mucosa-associated lymphoid tissue develops gradually, thus coinciding with that of the villi. Despite the scantiness of studies about the participation of

the innate immune system in the first week of life of birds, Henderson *et al*. (1999) showed that phagocytosis in one-day-old birds increased in response to oral inoculation of *Salmonella* Enteritidis. At day 21 of age, duodenal villus height (Table 2) differed (P<0.05) as a function of the contamination site, showing a lower height for inoculation by allantoidal cavity. *Salmonella* Enteritidis inoculation resulted in a greater crypt depth (P<0.05). A comparison of the intestinal sections revealed lower villus height (P<0.05) and greater crypt depth (P<0.05) in the duodenum and ileum at birth. Villus height was lower (P<0.05), and crypt depth greater (P<0.05) in chicks whose albumen was inoculated with the pathogen. These changes diminished with increasing age, as did the presence of the pathogen in excreta (Andrade *et al.*, 2007), although the chicks still showed changes, mainly in crypt depth, at day 21. The contamination site and *inoculum* also affected villus height and crypt depth in the duodenum (P<0.05), with chicks inoculated in the allantoidal cavity showing lower villus height and greater crypt depth. The height of jejuna villi (Table 3) did not differ in any of the analysed situations; however, crypt depth increased (P<0.05) at day 21 in the presence of the pathogen.The villus height and crypt depth of the ileum segment were affected at birth (P<0.05), with chicks inoculated with *Salmonella* Enteritidis via the albumen showing smaller villus height and greater crypt depth (Table 4). The contamination site and the *inoculum* significantly affected crypt depth in the ileum at day 21 of age. The balance between cell loss and proliferation ensures the maintenance of the functional capacity of the intestinal epithelium. However, when the intestine responds to a stimulus that favours one of these processes, modifications such as villus height should occur in cell structures (Uni *et al*., 2000).

In this study, the hosts reacted similarly to stimuli in the GIT mucosa, resulting from the presence of *Salmonella* Enteritidis at birth. The greatest crypt depth was related to the turnover of epithelial cells during the infection, thus suggesting a compensatory response to the effects of *Salmonella* Enteritidis, as referred to by Morris *et al.* (2004). Crypt hyper-

Table 1. Relative weight and length of the intestine of Ross chicks at days 1, 7, 14 and 21 of age.

	1 d		7 d		14 d		21 d	
	I, g/100 g of live weight	IL, cm	I, g/100 g of live weight	IL, cm	I, g/100 g of live weight	IL, cm	I, g/100 g I, g/100 g weight	IL, cm
Contamination site								
Shell	4.18	39.87	8.78	84.92	6.29	101.50	4.86	108.25
Internal	3.61	39.00	9.22	76.62	7.22	97.50	5.47	116.50
Inoculated agent								
SE	4.00	39.00	8.82	87.92[a]	7.38	97.75	6.17[a]	114.87
NC	3.78	39.87	9.19	76.62[b]	6.13	101.50	4.16[b]	109.87
Variation factor, %								
V	ns	ns	ns	ns	ns	ns	ns	ns
A	ns	ns	ns	ns	ns	ns	ns	ns
V × A	ns	ns	ns	ns	ns	ns	ns	ns
CV, %	19.3	4.93	6.35	3.56	11.31	4.65	48.50	45.19

I, relative weight; IL, length; V, contamination site: internal, allantoidal cavity; A, inoculated agent; SE, *Salmonella* Enteritidis; NC, negative control (0.85% sterilised buffered saline); CV, coefficient of variation; ns, not significant. [a,b]Different letters in the same column and line differ statistically in Tukey's test.

Table 2. Villus height and crypt depth of duodenum of Ross chicks at days 1 and 21 of age after inoculation.

	1 d		21 d	
	VH, µm	CD, µm	VH, µm	CD, µm
Contamination site				
Shell	369.60	69.67	1327.71[a]	106.35
Internal	226.11	70.99	1165.98[b]	99.61
Inoculated agent				
SE	280.1	76.8	1241.1	106.0[a]
NC	414.1	63.9	1352.6	84.1[b]
Variation factor, %				
V	0.01	ns	0.001	ns
A	0.01	ns	ns	0.001
V × A	0.01	0.13	ns	ns
CV, %	27.77	24.86	18.84	28.63

VH, villus height; CD, crypt depth; V, contamination site: internal, allantoidal cavity; A, inoculated agent; SE, *Salmonella* Enteritidis; NC, negative control (0.85% sterilised buffered saline); CV, coefficient of variation; ns, not significant. [a,b]Different letters in the same column and line differ statistically in Tukey's test.

plasia is a histological alteration resulting from the increase in metabolic activity to compensate for cell destruction (Rose *et al.*, 1992). In the presence of infection, this process may be augmented to prevent exposure of the cell layer and cells recover. If the stimulus leads to a higher cell extrusion rate while the cell proliferation rate is maintained or diminished, the intestine tends to respond by reducing villus height. According to Loddi (1999), if the height of the villi decreases due to augmented cell loss rate, cell production and crypt depth increase. On the other hand, villus height in the jejunum and ileum of 21-day-old chicks were not altered, despite the augmented crypt depth in the groups inoculated with *Salmonella* Enteritidis. Longer relative intestine length and higher relative weight were observed at days 7 and 21, respectively, in Ross chicks inoculated with the infectious agent. When measurements are expressed in numerical values and proportions, it should be kept in mind that the intestinal structure is elastic and susceptible to interferences such as handling and low temperatures (Madi *et al*., 2001). Nevertheless, the higher relative weight may be explained by the antigenic stimulus of *Salmonella* Enteritidis in the intestine on the first day of life. Inoculation with *Salmonella* Enteritidis by the two routes resulted in the greatest crypt depth ($P<0.05$). As for the chicks' performance, the contamination site affected their mean weight at days 14 and 21, with chicks inoculated in the albumen presenting lower mean weight gain and lower feed conversion ($P<0.05$; Table 5).

An evaluation of the chicks' performance indicated that the mean weight at days 14 and 21 of animals inoculated with *Salmonella* Enteritidis may be attributed to the presence of the infectious agent in the GIT, in view of the histomorphometric changes found in the villi and crypts upon hatching and at day 21. This injury caused by *Salmonella* Enteritidis in the mucosa affected the digestion and absorption of nutrients, as indicated by the mean weight loss of approximately 19% for the group of chicks inoculated in the albumen. These results are consistent with those of Gorhan *et al.* (1994), who reported lower weight gains in birds with inapparent *Salmonella* Enteritidis

Table 3. Villus height and crypt depth of jejunum of Ross chicks inoculated at days 1 and 21 of age.

	1 d		21 d	
	VH, µm	CD, µm	VH, µm	CD, µm
Contamination site				
Shell	166.35	67.55	863.34	162.53
Internal	164.67	64.50	941.24	166.81
Inoculated agent				
SE	168.10	80.50[a]	890.38	182.14[a]
NC	162.68	51.50[b]	914.21	147.20[b]
Variation factor, %				
V	ns	ns	ns	ns
A	ns	0.01	ns	0.001
V × A	ns	ns	ns	ns
CV, %	24.75	42.84	24.88	23.19

VH, villus height; CD, crypt depth; V, contamination site: internal, allantoidal cavity; A, inoculated agent; SE, *Salmonella* Enteritidis; NC, negative control (0.85% sterilised buffered saline); CV, coefficient of variation; ns, not significant. [a,b]Different letters in the same column and line differ statistically in Tukey's test.

Table 4. Villus height and crypt depth of ileum of Ross chicks at 1 and 21 days of age after inoculation.

	Ileum			
	1 d		21 d	
	VH, µm	CD, µm	VH, µm	CD, µm
Contamination site				
Shell	158.11	71.58	550.70	141.64
Internal	162.01	64.18	577.46	176.11
Inoculated agent				
SE	153.00[b]	76.70	559.20	193.77
NC	166.43[a]	59.18	568.95	123.99
Variation factor, %				
V	ns	ns	ns	0.001
A	3.60	0.03	ns	0.001
V x A	ns	0.02	ns	0.12
CV, %	32.62	38.43	18.35	23.67

VH, villus height; CD, crypt depth; V, contamination site: internal, allantoidal cavity; A, inoculated agent; SE, *Salmonella* Enteritidis; NC, negative control (0.85% sterilised buffered saline); CV, coefficient of variation; ns, not significant. [a,b]Different letters in the same column and line differ statistically in Tukey's test.

Table 5. Mean weight and feed conversion of Ross chicks at days 1, 7, 14 and 21 of age.

	MW1	MW7	MW14	MW21	FC7	FC14	FC21
Contamination site							
Shell	47.04	144.83	322.53[a]	586.37	1.04	1.16[a]	1.55[a]
Internal	46.65	145.43	304.24[b]	512.23	1.12	1.79[b]	1.83[b]
Inoculated agent							
SE	46.74	145.25	274.70[b]	490.51	1.07	2.21[b]	1.84[b]
NC	46.95	145.01	352.06[a]	608.09	1.08	1.29[a]	1.55[a]
Variation factor, %							
V	ns	ns	2.60	0.22	ns	0.63	0.001
A	ns	ns	0.001	0.01	ns	0.01	0.01
V × A	ns	ns	ns	3.98	ns	ns	ns
CV, %	2.08	6.22	5.31	11.08	4.21	10.16	12.01

MW1, mean weight at one day of age; MW7, mean weight at seven days of age; MW14, mean weight at 14 days of age; MW21, mean weight at 21 days of age; FC7, feed conversion at seven days of age; FC14, feed conversion at 14 days of age; FC21, feed conversion at 21 days of age; SE, *Salmonella* Enteritidis; NC, negative control (0.85% sterilised buffered saline); A, inoculated agent; V, contamination site: internal, allantoidal cavity; CV, coefficient of variation; ns, not significant. [a,b]Different letters in the same column and line differ statistically in Tukey's test.

Table 6. Bacteriological results of 16 caecal samples from 1-, 7-, 14- and 21-day-old Ross chicks hatched from eggs inoculated with *Salmonella* Enteritidis.

	1 day	7 days	14 days	21 days	Total positive
Contamination site[#]					
Shell	2/2	1/2	2/2	2/2	7/8
Internal	2/2	2/2	2/2	1/2	7/8
Total positive	4/4	3/4	4/4	3/4	14/16 (87.50%)

[#]Internal, allantoidal cavity.

infection in the first weeks of life. Dhillon *et al.* (1999) also found lower weight gains in Hubbard birds inoculated with *Salmonella* Enteritidis PT4, which presented a weight loss of 13 to 19%. In addition to the presence of the pathogen, this difference in weight gain can be attributed to the quality of the chicken feed, since no growth promoter was used. *Salmonella* Enteritidis caused lesions in the intestinal epithelial cells of chicks up to 21 days old, which affected their food conversion rate. The ability of the intestinal epithelium to renew itself may explain why changes in intestinal absorption are undetectable when the injury is not deep or persistent; however, these changes may worsen food conversion rate (Ito *et al*., 1998). Food conversion is 10 to 15% higher in the alternative rearing system than conventional breeding methods involving the use of growth promoters. An analysis was also made of the caecal content at day 1, 7, 14 and 21 of the chicks hatched from eggs inoculated experimentally with *Salmonella* Enteritidis on the shell or in the albumen (Table 6). Of the 16 samples, 87.50% (14/16) tested positive for the pathogen. An equal number of samples from the control groups was analysed and the bacterium was not detected.

Conclusions

In conclusion, eggs inoculated experimentally with *Salmonella* Enteritidis produced a high frequency of contaminated chicks, and intestinal integrity was recovered over time, although the chicks' performance was affected.

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