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Full Length Research Paper

Salmonella Heidelberg reduces nitrergic neurons in the myenteric plexus of the duodenum of broilers

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The present study aimed to characterize the effects of *Salmonella* Heidelberg challenge, as well as its changes in the inhibitory neuron subpopulation in the myenteric plexus of broilers. At the age of 21 days, after euthanasia of birds, duodenum fragments were collected from 5 male chicken broilers Cobb 500 linage from the challenged group and the control group for the quantification from the subpopulation of myenteric plexus inhibitory neurons by the histochemical method of NADPH-diaphorase. The membrane preparations obtained after neuronal marking were used for quantifying the neurons from micrographs obtained under light microscope, corresponding to 60 microscope fields per animal. Regarding the location of the neurons, they were grouped into those forming ganglia and those isolated between the fibers connecting the neuron groupings. The average number of neurons obtained was 24.38 neurons per mm^2 in the control group, and 9.57 neurons per mm^2 in the challenged group, with such values presenting statistical variation. This reduction in the number of neurons is perhaps a sign of the deleterious effects of *Salmonella* Heidelberg on positive NADPH-diaphorase neurons. Such reduction may promote changes in the functional characteristics of the digestive tube, reducing or slowing down the processes of digestion and absorption of nutrients, thereby limiting the animals' productive performance.

Key words: *Gallus gallus*, NADPH-diaphorase, inhibitory neurons, enteric nervous system, *Salmonella* sp.

INTRODUCTION

The digestive tube has a great diversity of micro- organisms, with bacteria being found in great quantities

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(Oliveira-Sequeira et al., 2008). *Salmonella* which is part of the normal microbiota of birds is prevalent in poultry products. Such prevalence is as a result of improper handling and incorrect technology upon slaughtering, which poses serious risks to the final consumer as well as international trade (Colla et al., 2012; Muniz et al., 2017).

According to Rodrigues et al. (2009), Salmonella is the main cause of food poisoning or food-borne diseases in humans worldwide; it is associated with the consumption of poultry products. Lanzarin (2012) emphasized *Salmonella* Typhimurium and *Salmonella* Enteritidis serovars as important in poultry production. They also emphasized the increase in the indexes of *Salmonella* Heidelberg (Rodrigues, 2009; Brazil, 2014; Liu et al., 2017), *Salmonella* Agona and *Salmonella* Senftenberg serotypes in a few regions in Brazil.

According to Pulido-Landínez et al. (2013), Martins (2015), Sausen (2015), Souza (2015) and Liu et al. (2017), *S.* Heidelberg is currently the most prevalent serotype in poultry farms. Also, the prevalence of this serotype is 20 to 33% of the positive samples for *Salmonella* spp. between the years, 2012 to 2014 in Brazil (Souza, 2015).

Chittick et al. (2006) reported that *S.* Heidelberg is presented as the fourth most prevalent serovar in sick humans. The Public Health Agency of Canada (2007), Han et al. (2012), and Brazil (2014) report that *S.* Heidelberg is more invasive and determines diseases with greater intensity than other paratyphoid serovars in humans. Also, in the reports of Rodrigues et al. (2009), they have the capacity of being hydrophobic, forming a biofilm on the surfaces, which could increase their permanence in the environment and industries. This increases the chance of transmission to the food materials, and therefore posing a greater risk to the final consumer.

The enteric nervous system is situated in the wall of the digestive tube, and it is formed from embryonic cells of the neuroectoderm, being classified as the third component of the autonomous nervous system. It performs its activities without any command from the central nervous system (Langley, 1921; Costa and Brookes, 1994; Furness and Bornstein, 1995; Furness, 2006). The enteric nervous system is divided into plexus, more specifically with emphasis on sub-mucous and myenteric plexus, which act on the digestion and absorption of nutrients that control the motor functions, blood flow and secretions of the gastrointestinal tract (Furness, 2006; Phillips and Powley, 2007).

The myenteric plexus in birds extends from the esophagus to the cloaca, located between the circular and longitudinal layers of the muscular coat, except for the gizzard, which is located superficially between the serous and the circular layer of the muscular coat (Aisa et al., 1987). The neurons can be found arranged in ganglia or between fibers that connect the ganglia, depending on the species or digestive tube segment being studied (Matsuo, 1934; Gabella, 1971; Ali and Mclelland, 1979; Molinari et al., 1994; Mello et al., 1996; Germano et al., 2000; Stabille et al., 2000; Yang et al., 2012).

The myenteric plexus is not considered as totally excitatory since there are differences between the neural populations and the population of nitrergic neurons which have fundamental roles in the motility of the intestine by having inhibiting functions (Furness, 2006). In birds, reports describing myenteric neurons are still scarce; they are only descriptions of myenteric neurons in the proventriculus of ducks (Molinari et al., 1994), in young chicken of three weeks old, adult chicken of 18 weeks, in small and large intestines (Ali and Mclelland 1978, 1979) and ileum of 65 days old chicken (Yang et al., 2012).

The major objective of this work was to characterize the possible effects of *S.* Heidelberg on the sub-population of nitrergic neurons in the myenteric plexus of broilers. Such changes may be responsible for manifestations such as anorexia, diarrhea and weight loss, which can have a direct impact on the productive performance of these animals, as well as on their well-being.

MATERIALS AND METHODS

The experiment, which is exploratory and descriptive, was conducted at the experimental Biotherium at the Universidade Federal do Paraná, Sector Palotina – PR. There was a total of 120 male broilers (*Gallus gallus domesticus*) of the Cobb 500 lineage. They were distributed into two groups with four repetitions of 15 birds each. They were grouped into orally challenged and not challenged with *S.* Heidelberg. They were obtained from field isolates, using 1 mL of the inoculate in a 10^5 CFU/mL concentration at six days old. They remained in the house until slaughtered at 21 days old (C - negative control, not challenged group; and D - group challenged with *S.* Heidelberg).

The study was conducted using the method approved by *Comitê de Ética em Pesquisa Envolvendo Experimentação Animal* (CEPEEA), at Universidade Paranaense (protocol number 26695/2014).

Animal euthanasia and collection of duodenum fragments

After 21 days of experimental period, five birds from each group were randomly chosen and euthanized. Xylazine hydrochloride was used as the pre-anesthetic medication at 4 mg/Kg intramuscularly, and sodium thiopental was used as the anesthesia at 25 mg/Kg intravenously through ulnar vein.

The birds were then necropsied, and fragments of the proximal duodenum were collected, immediately after the duodenal loop. This was done to detect positive NADPH-diaphorase ($NADPHd^+$) myenteric neurons, which are simulated by the nitric oxide neurotransmitter, also referred to as nitrergic neurons.

Detection of positive NADPH-diaphorase myenteric neurons [($NADPHd^+$ neurons) Scherer-Singler et al. (1983)]

In order to show the nitrergic myenteric neurons ($NADPHd^+$ neurons), 10 duodenum fragments were rinsed with phosphate buffer solution (pH 7.4), one of the ends with suture wire filling inside with phosphate buffer solution (pH 7.4). Following this, the

opposite end was also tied, and the entire fragment was immersed in the buffer solution for 48 h. The fragment was then rinsed twice (10 min each) in PBS and permeabilized in PBS (pH 7.3) containing 0.3% Triton X-100® (Sigma-Aldrich Inc. Fluka – St. Louis) at 0.3%. It was diluted in sodium phosphate buffer (pH 7.3) for 10 min.

After permeabilization, the duodenum was rinsed twice (10 min each) in PBS and incubated for 120 min in the reaction medium containing 50 mg nitroblue tetrazolium® (NBT) (Sigma-Aldrich Inc. Fluka – St. Louis), 100 mg β-NADPH® (Sigma-Aldrich Inc. Fluka – St. Louis) and 0.3% Triton X-100® in Tris-HCl® buffer (Sigma-Aldrich Inc. Fluka – St. Louis) (0.1M, pH 7.6). After this treatment, the ends were released and the fragments were immersed in 4% paraformaldehyde solution for the cessation of the reaction, fixation and storage. Membrane preparations were obtained from these treated duodenum fragments.

Membrane preparations and photomicroscopy

In order to obtain the membrane preparations, the duodenum fragments were transversally sectioned to obtain a fragment of approximately 8 mm width. This was sectioned along the extension of the mesenteric border. The fragments were then micro-dissected on glass plate with the aid of pliers and stereomicroscope with transillumination to collect the mucous and sub-mucous coats. The muscular coat was preserved with its longitudinal and serous coat, where the myenteric plexus is located.

The duodenal membrane preparations obtained from the duodenum were subjected to histochemical procedures in order to detect $NADPHd^+$ myenteric neurons. These were dehydrated in alcohol at an increasing series of concentrations (90%, 95% and absolute), diaphanized with three consecutive immersions in xylene and placed between blade and glass blade with synthetic resin.

Under light microscope with 40X magnification, and high-resolution photographic camera with an image analysis system, images of 60 random microscopic fields by membrane preparation were captured. This was done contemplating the mesenteric, intermediary and anti-mesenteric areas, destined for the quantitative analysis of nitrergic myenteric neurons ($NADPHd^+$).

The duodenum circular strata circumference region corresponding to the insertion of the mesentery (0°) was used as a reference to determine the intermediary (between 60 and 120°C, and between 240 and 300°C) and anti-mesenteric regions (between 120 and 240°C) in the duodenum (Miranda-Neto et al., 2001) to guide the capture of the images. This was performed so as to sample the three areas in equivalent number of images.

Quantification of myenteric neurons (neuron density/mm^2 duodenum)

Photomicrographs obtained from the preparations of duodenum membranes from the broilers in groups C and D were used for the quantification of the sub-population of $NADPHd^+$ myenteric neurons. All the neurons viewed in each image were quantified and the incomplete neurons are considered in alternate fields.

The total quantified area was represented by 4x camera area, which is 1034.88 μm^2. This allowed the expression of results as neuronal density per mm^2 duodenum. In order to compare the results among the experimental groups, data were submitted to ANOVA analysis of variance and, when found to be statistically significant, they were submitted to Turkey test, using a 5% significance level ($p<0.05$).

RESULTS AND DISCUSSION

$NADPHd^+$ myenteric neurons or nitrergic neurons were arranged in ganglia and isolated between neuronal fibers establishing the communication between the ganglia (Figures 1 A and B). The area corresponding to each microscopic field was calculated as 1034.88 μm^2. The ganglion arrangement of myenteric plexus neurons is often described as evidence of evolution, presenting ganglion arrangements throughout all the segments in the poultry digestive tube (Gabella and Halasay, 1987).

Germano et al. (2000) and Stabille et al. (2000), while studying the morphology and quantification of myenteric neurons in the intestine of carps (*Ciprinus carpio*), have often isolated neurons. The authors reported the ganglia of having sparse and irregular distribution containing three or more nerve cells. Furness (2006) and Lomax et al. (2006) reported the ganglionar organization of myenteric neurons on the digestive tube wall in different species of mammals.

Yang et al. (2012) observed the ganglionic and isolated cell distribution in the ileum of 65 days old broilers. This corroborates with the results observed in this study. Studying the proventriculus of ducks (*Anas* sp.), Molinari et al. (1994) described the presence of neurons arranged in ganglia, but they did not mention the existence of isolated neurons.

However, the distribution of these cells in the intestine of these animals was not reported. Duke (1996) described that the contractions of the gizzard and duodenum depend on intrinsic connections with the gizzard since the motor pacemaker is located in the isthmus, the region between the stomach and the esophagus (Furlan and Macari, 2002). Therefore, changes in the myenteric neurons in pylorus and isthmus would terminate the contractions in these segments (Duke, 1996).

Regarding the density of $NADPHd^+$ myenteric neurons, or nitrergic neurons, there is a difference ($p<0.05$) (Table 1) between the control and challenged groups, showing that *S.* Heidelberg has significantly decreased the sub-population of $NADPHd^+$ myenteric neurons in challenged broilers.

Yang et al. (2012), studying the nitrergic neuron density in the ileum of broilers of 65 days old, found an average of 30.59 neurons per mm^2 in their ileum, while the current study found 24.38 neurons per mm^2 in group C. The number of neurons was described by different researchers from different mammal species in different intestinal segments, and, according to the authors, this fact is associated with the higher functional activity in each segment under study (Miranda-Neto et al., 2001; Furness, 2006).

The myenteric plexus responds to different types of stimuli and conditions, whether deleterious or not, changing their chemical characteristics or their functional structure to keep the digestive system homeostasis (Lomax et al., 2006). This functional characteristic is seen in the current study, where the *S.* Heidelberg represented a severe deleterious condition, reducing approximately 60% of the sub-population of nitrergic neurons. According

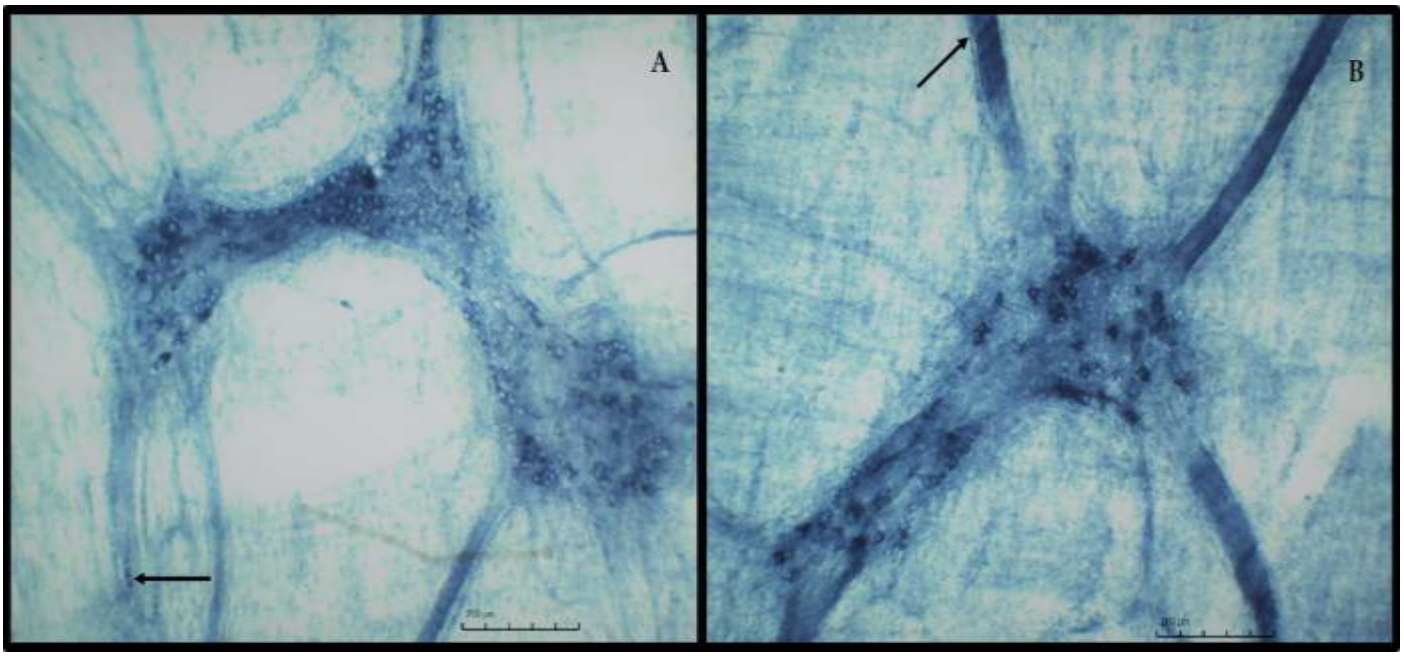

Figure 1. Photomicroscopy of *Gallus gallus domesticus* duodenum in the control group evidencing the ganglionar arrangement of the myenteric plexus in A (4X lenses); B shows the group challenged by *Salmonella* Heidelberg, where the arrangement is also shown as ganglionar with a smaller number of $NADPHd^{+}$ neurons. The arrows point to isolated neurons in the fiber region that interconnect the ganglia in the myenteric plexus.

Table 1. Mean and standard deviation of mean density of myenteric neurons by NADPH-diaphorase histochemistry corresponding to 1.00 mm^2 of the duodenum of Cobb 500 broilers (*Gallus gallus domesticus*).

Variable	Duodenum neural density in 1 mm^2	
Group	**Mean (MSE)**	**CV (%)**
C (n=5)	$24.38^{a} \pm 4.97$	40.84
D (n=5)	$9.57^{b} \pm 2.34$	48.74

Means followed by different letters in the same column differ among themselves by the Turkey test at 5% significance level (p>0.05). MSE: Mean standard error. CV: Coefficient of variation.

to Berndt et al. (2007), *S.* Heidelberg belongs to the paratyphoid *Salmonella* group. However, this is not specific to bird and can cause disease in humans through the intake of contaminated meat and eggs. It causes damage not only to the poultry industry but also to population, being a serious public health problem.

Lourenço (2016), comparing lesions of different strains of *S.* Enteritidis and *S.* Heidelberg on broilers' ileum at 16 h after inoculation, observed a greater inflammatory histological changes in the birds affected by *S.* Heidelberg. According to Rychlik et al. (2014), intestinal colonization process begins with the bacterial adaptation and replication in the intestinal lumen, followed by epithelial adhesion and transfer of the bacterial proteins to the cellular cytoplasm, recognition and triggering inflammatory response. *Salmonella* serovars may differ in their pathogenicity to possess and express genes that encode virulence factors responsible for homeostasis disturbance and causing changes that will define the severity of the disease. In the present study there was a significant reduction in the activity of neurons of the nitrergic sub-population of the myenteric plexus.

Furness (2006) describes the number of myenteric neurons as constant throughout life, and also in several situations in animals, including the growth phase. The neuronal subpopulations may be stimulated by improving the performance of the digestive system and increasing the digestion and absorption of nutrients, which allows one to infer that *S.* Heidelberg may cause performance loss due to the myenteric neuronal reduction, disturbing the normal functions of this segment.

Hitherto the literature does not mention the effects of *Salmonella* gender on the myenteric neuron populations. Moreover, there are only few reports on the density and

distribution of such neurons in broiler chickens.

Conclusion

The present study tries to assess the effects of *S.* Heidelberg on the sub-population of inhibitory neurons in the myenteric plexus of broilers. In this condition, the experiment was performed and it can be concluded that, the $NADPHd^{+}$ myenteric neurons of the duodenum of broiler chickens are presented in ganglia. They may also be found isolated between the nervous fibers of the ganglionar arrangement where it was observed that *S.* Heidelberg was able to significantly reduce this neuron subpopulation.

This reinforces the importance of further studies in the total population of neurons, as well as on the excitatory subpopulation, which have not been exploited in this phase of the research.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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