Effects of 5-hydroxytryptophan and m-hydroxybenzylhydrazine associated to Lactobacillus spp. on the humoral response of broilers challenged with Salmonella Enteritidis


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ABSTRACT This study investigates the effects of different doses of serotonin, its precursor 5-hydroxytryptophan (5HTP), and m-hydroxybenzylhydrazine inhibitor (NSD1015), administered via intraperitoneal for 5 consecutive days, on behavior and average body weight of broilers. We also measured the humoral immune response and quantification of Salmonella Enteritidis in broilers chickens that received the drugs evaluated and a Lactobacillus pool. The study was divided into 3 experiments: Experiment 1 - administration of pharmaceuticals with choice of dosage; Experiment 2 - administration of pharmaceuticals and a Lactobacillus pool in birds that were not challenged with S. Enteritidis, and Experiment 3 - administration of pharmaceuticals and a Lactobacillus pool in birds challenged with S. Enteritidis. The ELISA was used to scan dosages of intestinal IgA and serum IgY. We used colony-forming units to quantify S. Enteritidis. The concentrations of IgA and IgY did not show significant differences (P > 0.05) in Experiment 2. In Experiment 3, NSD1015 associated with Lactobacillus determined higher IgA concentrations, promoting greater stimulus to the immune system than 5HTP. Regarding quantification of S. Enteritidis in the cecal content of birds, 5HTP associated to Lactobacillus determined the smallest number of bacteria, showing possible interaction of 5-hydroxytryptophan and Lactobacillus spp. with the immune system of broiler chickens.

Key words: serotonin, Salmonella Enteritidis, immune response, broilers

INTRODUCTION

Foodborne pathogenic microorganisms, namely Salmonella spp., are of great interest in public health, causing morbidity and mortality in humans in many countries (Shinohara et al., 2008).

Ingestion of contaminated water and food, mostly animal products, such as poultry and eggs, causes Salmonellosis in humans (Jackson et al., 1991; Revolledo et al., 2006). Therefore, preventive and control measures for Salmonella in poultry are essential to avoid contamination of the food chain by this microorganism (Berndt et al., 2007).

The use of probiotics, such as Lactobacillus spp., for the control of foodborne pathogens has been gaining prominence in recent years due to the benefits promoted to the host (Brisbin et al., 2010). Probiotics are able to form a complex and dynamic system that greatly influences microbiological, immunological physiological, and biochemical factors of the host (Debonnet et al., 2009).

The contact between the enteropathogen and the host occurs in the cells that compose the intestinal mucosa. Therefore cell integrity in this area is highly important, because it is the first line of defense against viral and bacterial infections (Muir et al., 2000; Liu et al., 2010). In the intestinal mucosa, enteroendocrine cells produce substances such as gastrin, secretin, cholecystokinin, and serotonin, among others, for the processes of digestion, absorption, epithelial proliferation, and modulation of the immune system (Uribe et al., 1994).

Serotonin modulates the innate and adaptive immune response through its receptors and can often act as mediator in inflammatory intestinal diseases (Garabal et al., 2003). Multiple serotonergic receptors are characterized in lymphocytes, monocytes, macrophages, and dendritic cells (Cloez-Tayarani and Changeux, 2007), reinforcing the hypothesis that serotonin plays an important role in pathogenesis of intestinal diseases (Manocha and Khan, 2012).

This study investigated the influence of precursor (5-hydroxytryptophan) and inhibitor
(m-hydroxybenzylhydrazine) of serotonin, associated with Lactobacillus spp., on the immune response of broilers challenged with Salmonella Enteritidis. We also analyzed the recovery of S. Enteritidis in the cecal content.

**MATERIAL AND METHODS**

**Bacterial Samples and Culturing Conditions**

The strains of Lactobacillus spp. used in this study (Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus acidophilus, Lactobacillus rhamnosus, and Lactobacillus spp.) were isolated from broilers and selected according to the adhesion capacity and immunomodulatory effect described by Rocha et al. (2012).

They were cultured separately in 15 mL of Man Rogosa Sharp liquid medium, incubated at 37°C, for 24 h in an anaerobic system. On the following day, the cultures were mixed in equal parts and administered to the birds. The number of colony-forming units (CFU) was determined through decimal dilutions in PBS (pH 7.2) and plated on Man Rogosa Sharp agar.

The challenge was accomplished through a culture of Salmonella enterica subspecies enterica serotype Enteritidis (S. Enteritidis) phage type 4, mutant resistant to nalidixic acid (Nal) and rifampin (Rif), developed through successive cultivations in brilliant green agar containing nalidixic acid (100 μg/mL) and Rif (100 μg/mL), according to Andreatti Filho et al. (1997).

S. Enteritidis was cultivated in 20 mL of brain heart infusion broth, containing Nal and Rif (100 μg/mL) and incubated at 40°C, for 18 to 24 h. CFU The number of CFU was determined through decimal dilutions in PBS (pH 7.2) and plated in brilliant green agar containing Nal and Rif (100 μg/mL).

**Experimental Design**

The experiment was divided into 3 phases. At first, 3 doses of serotonin, its precursor, and inhibitor were administered to the birds in order to determine the best dose to use and any possible side effects. Experiments 2 and 3 were performed simultaneously after Experiment 1.

**Experiment 1.** One-day-old male broilers chickens (n = 297) were allocated into experimental cages. They received water and a nonmedicated ration ad libitum, as well as necessary warming according to age. The broiler chickens were divided into 11 groups composed of 27 animals each, as follows:

- **Treatment 1 (T1):** animals treated with 5HTP (12.5 mg/kg of live weight);
- **Treatment 2 (T2):** animals treated with 5HTP (25 mg/kg of live weight);
- **Treatment 3 (T3):** animals treated with 5HTP (50 mg/kg of live weight);
- **Treatment 4 (T4):** animals treated with serotonin (1.25 mg/kg of live weight);
- **Treatment 5 (T5):** animals treated with serotonin (2.5 mg/kg of live weight);
- **Treatment 6 (T6):** animals treated with serotonin (5 mg/kg of live weight);
- **Treatment 7 (T7):** animals treated with NSD1015 (100 mg/kg of live weight);
- **Treatment 8 (T8):** animals treated with NSD1015 (150 mg/kg of live weight);
- **Treatment 9 (T9):** animals treated with NSD1015 (300 mg/kg of live weight);
- **Treatment 10 (T10):** non-treated broilers;
- **Treatment 11 (T11):** animals treated via intraperitoneal with 0.1 mL of NaCl at 0.9% (saline solution).

The doses of serotonin (H9772, Sigma-Aldrich, St. Louis, MO), 5HTP (H9772, Sigma-Aldrich, St. Louis, MO) and NSD1015 (H9772, Sigma-Aldrich, St. Louis, MO) were diluted according to manufacturer’s recommendations. They were applied according to Reis et al. (2005), Polo et al. (2007) and Nakamura et al. (2008), via intraperitoneal from 6 to 10 d of age, at 0.1 mL/bird, with a 1 mL syringe and a 0.45 × 13 mm needle. The intraperitoneal inoculations were made in 45° angle in medium quadrant of the pelvic wall, as described by Cedraz-Mercez et al. (2007).

All birds of each treatment were weighed on a digital scale from 5 to 11 d of age.

**Experiment 2.** One-day-old male broilers chickens (n = 288) were distributed in a completely randomized experimental design. The broiler chickens were divided into 6 groups composed of 48 animals each, 8 evaluation periods and 6 repetitions per period:

- **Treatment 1 (T1):** animals treated with 5HTP;
- **Treatment 2 (T2):** animals treated with NSD1015;
- **Treatment 3 (T3):** animals treated with 5HTP and a Lactobacillus pool;
- **Treatment 4 (T4):** animals treated with NSD1015 and a Lactobacillus pool;
- **Treatment 5 (T5):** animals treated with a Lactobacillus pool;
- **Treatment 6 (T6):** animals treated with 0.1 mL of NaCl at 0.9%.

Based on the results obtained in Experiment 1, we applied 25 mg/kg of live weight of 5HTP, 150 mg/kg of live weight of m-hydroxybenzylhydrazine and NaCl once a day via intraperitoneal, for 5 consecutive days (4 to 8 d of age), at 0.1 mL/chicken with a 1 mL syringe and a 0.45 × 13 mm needle.

Once a day for 5 consecutive days (4 to 8 d of age), 500 μL from a pool of Lactobacillus spp. with 1.0 × 10⁶ CFU/mL was administered orally with the aid of a gavage needle.


**Experiment 3.** One-day-old male broilers chickens (n = 336) were distributed in a completely randomized experimental design. The broiler chickens were divided into 7 groups composed of 48 animals each, 8 evaluation periods and 6 repetitions per period:

- **Treatment 1 (T1):** broilers treated with 5HTP and challenged with *S. Enteritidis*;
- **Treatment 2 (T2):** broilers treated with NSD1015 and challenged with *S. Enteritidis*;
- **Treatment 3 (T3):** broilers treated with 5HTP, a *Lactobacillus* pool and challenged with *S. Enteritidis*;
- **Treatment 4 (T4):** broilers treated with NSD1015, a *Lactobacillus* pool and challenged with *S. Enteritidis*;
- **Treatment 5 (T5):** broilers treated with a *Lactobacillus* pool and challenged with *S. Enteritidis*;
- **Treatment 6 (T6):** broilers challenged with *S. Enteritidis*;
- **Treatment 7 (T7):** broilers treated with 0.1mL of NaCl at 0.9%

Given the results obtained in Experiment 1, we applied 25 mg/kg of live weight of 5HTP, 150 mg/kg of live weight of m-hydroxybenzylhydrazine and NaCl once a day via intraperitoneal for 5 consecutive days (d 4 to 8 of life) 0.1 mL/chicken with a syringe 1 mL and needle 0. 45 × 13 mm.

Once a day for 5 consecutive days (4 to 8 d of age), 500 μL from a pool of *Lactobacillus* spp. with 1.0 × 10^8 CFU/mL was administered orally with the aid of a gavage needle.

At 8 d of age, 1 mL of *Salmonella Enteritidis* with 2.0 × 10^8 CFU/mL was administered orally with the aid of a gavage needle.

**Serological Monitoring** In Experiments 2 and 3, 2 mL of blood was collected from broiler chickens (n = 6 birds/group) at 4, 8, 10, 12, 14, 21, 28, and 35 d of age by puncture of an ulnar or jugular vein. The blood samples were centrifuged at 8,000 × g for 5 min at 5°C. The serum was kept at −20°C until analysis to determine IgY, as described by Rocha et al. (2012).

**Collection of Intestinal Fluid** For collection of intestinal fluid, 6 birds per group from Experiments 2 and 3 were euthanized by cervical dislocation (in compliance with the Committee of Ethics in animal experimentation of the FMVZ-UNESP - Protocol No. 191/2011-CEUA) soon after blood collection. The intestines were collected and the intestinal fluid was collected to quantify IgA, as described by Rocha et al. (2012).

**Enzyme-Linked Immunosorbent Assay – ELISA** For ELISA on intestinal secretory IgA and serum IgY, antibodies were measured using commercial kits Chicken IgA ELISA quantification and Chicken IgY ELISA quantification (Bethyl Laboratories, Montgomery, TX) following the manufacturer’s instructions. The samples of serum and intestinal fluid were diluted at 1:6,400 and 1:3,200, respectively, and tested in 4 repetitions.

**Quantification of Salmonella Enteritidis in Cecal Content** For the bacterial determinations of the cecal content, the ceca were collected from all birds and placed individually in sterile plastic collection bags. Next, they were macerated and diluted in PBS (pH 7.2) at 1:10 ratio. Subsequently, dilutions were performed in series, and plated in brilliant green agar added to 100 μg/mL of NaI and Rif. The reading of the plates was carried out 18 to 24 hours after incubation.

**Statistical Analysis** The experimental design was completely randomized, using a factorial scheme of independent groups. The data were submitted to the analysis of variance in the Tukey test at 5% significance by PROC MIXED (SAS Institute, 2009).

**RESULTS AND DISCUSSION**

In Experiment 1, the intraperitoneal administration of serotonin (T4, T5, T6) promoted behavioral change, causing a sequence of events related to a sleep-like state. The broilers showed first oral rapid movements, similar to yawns, alternating feather bristling and agitation of the wings that were followed by closing eyes and resting. These events were observed in all birds that received serotonin. In treatments T5 and T6, whose doses were 2.0 mg/Kg and 5.0 mg/Kg, respectively, birds showed a more intense and lasting effect. There was no feed intake for approximately 30 min. and the broilers remained at rest for about 10 min.

These observations corroborate Polo et al. (2007), who analyzed quails at 3 months of age that had received serotonin via parenteral, at doses 0.125, 1.25, and 2.5 mg/Kg. These authors observed sleep-like behaviors, starting with feather bristling, fast oral movements, winks, squatting, and closing of the eyes.

The treatments that received serotonin (T4, T5, and T6) showed a slight decrease in weight gain (Table 1), most likely because the birds did not consume food soon after the administration of monoamine. This weight reduction, although statistically different from that in the control groups (T10 and T11), was small and did not influence the development of the broilers.

The weight of the birds was inversely proportional to the serotonin dose applied, that is, the higher the dose, the smaller the average body weight (ABW). In larger doses, drowsiness effects increased and, therefore, food intake decreased for a certain period influencing the daily weight gain.

The administration of 3 doses of 5HTP (T1-12.5, T2-25.0, and T3-50.0 mg/Kg) promoted effects similar to those observed in treatments with serotonin, but with a smaller intensity and only in some of the birds in the treatment. The birds showed drowsiness and feed intake was inhibited for periods shorter than 10 min.

Reis et al. (2005) reported similar results. The authors used 5HTP via intraperitoneal in quails, at one and 60 min prior to feeding, in doses of 12.5, 25.0, and...
50 mg/Kg and observed a significant reduction in food intake. However, in quails in fast for 6 hours, feed intake inhibition occurred only when the food was offered 1 min after the application of 5HTP, because in groups where feed was offered 60 min after the serotonin precursor, there was no ingestion inhibition. In this case, the fast possibly caused stress to the birds and increased neuronal uptake of serotonin precursor, anticipating its peak production (Carsia and Harvey, 2000).

The ABW of broilers treated with 5HTP (T1, T2, and T3) was similar to that in the control groups (T10 and T11) (Table 1). As observed in treatments with serotonin, higher doses promoted a reduction in ABW.

In treatments with serotonin (T4, T5, and T6), as well as in treatments with 5HTP (T1, T2, and T3), the birds remained calm and did not show aggression, similar to the observations of Shea et al. (1990).

The birds in the treatments with inhibitor NSD1015 (T7, T8, and T9) showed the same behavior of the birds that received saline (T11-NaCl) and the negative control group (T10). There was no behavioral change and feed intake remained normal. In treatments with inhibitor NSD1015 (T7, T8, and T9), the ABW of birds were similar in all doses and similar to that observed in the control groups (T10 and T11). This occurs because NSD1015 promotes serotonin reuptake inhibition, therefore, the signals of drowsiness and the feeling of satiety caused by serotonin did not occur and the birds behaved similarly to those in the control treatments, ingesting feed normally Nakamura et al. (2008).

5HTP and NSD1015 were challenged, and there was variation in the concentrations that reached 10.77 μg/mL at 14 d of age (144 h after challenge with Salmonella Enteritidis). From 21 d onwards, IgA values of all treatments again significantly increased, corroborating with Marcq et al. (2011) who reported increase in levels of secretory IgA in birds treated with Salmonella Typhimurium.

At the age of 28 d, treatment T4 (NSD1015 + Lactobacillus pH pool) showed the highest concentration of intestinal IgA (17.78 μg/mL) and differed significantly from T1 (5HTP), which presented the lowest concentration (14.69 μg/mL). The same was observed at 35 d, but T4, besides differing from T1, also differed from the negative control (T7). In experiment 1 the negative control is the T10, but in experiment 3, which is cited in this paragraph the negative control is the T7.

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Table 1. Body average weight (g) of broilers 5 to 11 d old after administration of different doses of serotonin precursor and inhibitor.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Days</th>
</tr>
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<tbody>
<tr>
<td>T1 98.7A,a</td>
<td>5 111.6A,a</td>
</tr>
<tr>
<td>T2 97.3A,a</td>
<td>111.6A,a</td>
</tr>
<tr>
<td>T3 96.6A,a</td>
<td>109.9A,a</td>
</tr>
<tr>
<td>T4 97.3A,a</td>
<td>108.6A,a</td>
</tr>
<tr>
<td>T5 99.0A,a</td>
<td>112.6A,a</td>
</tr>
<tr>
<td>T6 100.8A,a</td>
<td>111.0A,a</td>
</tr>
<tr>
<td>T7 98.7A,a</td>
<td>115.2A,a</td>
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<tr>
<td>T8 97.4A,a</td>
<td>115.6A,a</td>
</tr>
<tr>
<td>T9 96.9A,a</td>
<td>111.9A,a</td>
</tr>
<tr>
<td>T10 101.3A,a</td>
<td>117.2A,a</td>
</tr>
<tr>
<td>T11 101.7A,a</td>
<td>115.8A,a</td>
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<tr>
<td>Means 98.8A,a</td>
<td>113.2A</td>
</tr>
</tbody>
</table>

A–G Means followed by the same capital letter in the row do not differ statistically in the Tukey test (P < 0.05).

Means followed by the same small letter in the column do not differ statistically in the Tukey test (P < 0.05).

1 T1 = 5-hydroxytryptophan 12.5mg/Kg; T2 = 5-hydroxytryptophan 25.0mg/Kg; T3 = 5-hydroxytryptophan 50.0mg/Kg; T4 = serotonin 1.25mg/Kg; T5 = serotonin 2.5mg/Kg; T6 = serotonin 5.0mg/Kg; T7 = NSD1015 100mg/Kg; T8 = NSD1015 150mg/Kg; T9 = NSD1015 300mg/Kg; T10 = negative control; T11 = NaCl 0.9%.

Ford Leibowitz and Shor-Posner (1986).
In this case, we can infer that the serotonin inhibitor (NSD1015) associated with *Lactobacillus* spp. promoted greater stimulus to the immune system than the serotonin precursor (5HTP), since IgA values of the treatment with 5HTP associated with *Lactobacillus* spp. (T3) were rather similar to those of the negative control (T7).

The studies show the presence of enterochromaffin cells, immunostaining for serotonin, and serotoninergic receptors in immune cells such as lymphocytes, monocytes, macrophages, and dendritic cells (Cloez-Tayarani and Changeux, 2007), as well as the large number of enterochromaffin cells containing serotonin in the gastrointestinal tract (Kitazawa et al., 2006). However, little is known about the effects of serotonin on the immunity system and production of secretory IgA.

In acute pathological conditions, some authors suggest widespread serotonin action in an attempt to eliminate enterotoxins produced by bacteria such as *Escherichia coli* and *Salmonella* spp. (Hansen and Witte, 2008). However, the way that serotonin and its precursor (5HTP) act remains unknown.

For serum IgY concentrations in Experiment 2 (Figure 3) and Experiment 3 (Figure 4), we observed that the treatments did not show significant differences ($P > 0.05$). In both experiments, the average IgY levels in blood serum ranged between increase and decrease over the period measured, attaining higher concentration at 35 d.

Haghighi et al. (2005) described an increase of serum IgY in birds receiving food supplemented with different samples of *Lactobacillus*. The treatments with *Lactobacillus* spp. (T3, T4, and T5), even showing higher concentrations of IgY, did not differ significantly from the negative control in both experiments.

The treatments in the 2 experiments without *Lactobacillus* spp. (T1 and T2) showed serum IgY concentrations similar to those in the negative control.

Little is known about the role of the precursor (5HTP) and inhibitor (NSD1015) of serotonin on the immune response of broilers, however, the results obtained in this study suggested that this activity might involve other components of the immune system, besides IgA and IgY.

The quantification of *S. Enteritidis* in cecal contents (Table 2) showed a reduction of *S. Enteritidis* after challenge. Treatment T3 (5HTP plus *Lactobacillus* pool) showed the highest reduction capacity of *S. Enteritidis,*
with a significant difference (P<0.05) compared to treatments T6 (positive control) and T2 (NSD1015). This suggests a reduction of S. Enteritidis in cecal contents caused by the serotonin precursor (5HTP) and Lactobacillus spp. Treatment T2 (NSD1015) introduced quantities of S. Enteritidis similar to those in treatment T6, and therefore ineffective in reducing the pathogen.

These results corroborate Hansen and Witte (2008), who suggested a widespread role of serotonin to eliminate enterotoxins produced by bacteria such as Escherichia coli and Salmonella spp. in acute
pathological conditions. However, the results observed in these experiments clearly point to the need for further studies to evaluate how this action occurs, since there was little stimulus of humoral immune response.

We can conclude that there was little influence of the treatments with precursor (5-hydroxytryptophan) and inhibitor (NSD1015) of serotonin on the induction of humoral immune response mediated by IgY and IgA. It is inferred that the availability or inhibition of serotonin synthesis can influence the immune system differently with immunoglobulins that were not evaluated in this study or by cellular immune response.

Although the treatments with 5-hydroxytryptophan (T1 and T3) did not show high IgA levels in intestinal fluid and serum IgY, we observed reduction of S. Enteritidis, mainly in treatment T3, indicating interaction of 5-hydroxytryptophan and Lactobacillus spp. with the immune system of broilers through mechanisms that need to be further elucidated.

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REFERENCES


