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Performance of broilers experimentally inoculated with *Salmonella* Typhimurium and fed diets with addition of lactulosis

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ABSTRACT - The objective of this experiment was to evaluate the influence of lactulose on performance as well as its ability to prevent colonization by *Salmonella* Typhimurium in broilers orally inoculated with this pathogen. The design adopted was completely randomized, with 630 one-day-old male chicks distributed into six treatments, with seven replications and 15 birds per experimental unit. The treatments comprised the following procedures: T1 (control group) - no S. Typhimurium inoculation or supply of lactulosis; T2 - only inoculation of S. Typhimurium; T3 - only lactulosis supply; T4 - supply of lactulosis and *S.* Typhimurium inoculation on the first day of life; T5 - supply of lactulosis 48 hours before *S.* Typhimurium inoculation; and T6 - supply of lactulosis 48 hours after inoculation of *S.* Typhimurium. Performance variables were evaluated on the seventh, 14th, 21st and 28th days of age; fragments of the duodenum and jejunum were collected and sent to histomorphometric assessment at 14 days of age, and *S.* Typhimurium excretion was verified in cloacal swabs on the 10th, 24th and 35th days of age. Performance data were analyzed by ANOVA and Tukey's test (5%) and fecal excretion data were assessed by non-parametric chi-square test. Better weight gain and feed conversion were observed in groups fed lactulosis with or without challenge of *S.* Typhimurium up to 21 days of age. Reduced duodenum villous height was verified on the 14th day in groups challenged with the pathogen. Reduction of *S.* Typhimurium fecal excretion was verified in broilers fed lactulosis from the first day of life on and 48 hours before receiving *S.* Typhimurium directly into the crop. Lactulosis increases broiler performance up to one week after its inoculation, influences duodenum villous height and reduces the fecal excretion of *Salmonella* Typhimurium.

Key Words: feed conversion, prebiotic, salmonellosis, weight gain

Introduction

The use of antimicrobiobial compounds as growth promoters contributes to the sustainability of the poultry industry, improving animal performance (Fukayama et al., 2005) and controlling intestinal diseases. However, their use in animal feeding has been restricted, and Brazil, as a great exporter of poultry meat, is trying to adapt to the national and international laws and consumer demands. This restriction is a great challenge to the industry and to researchers, who seek alternative products and compounds to be incorporated to the diet which do not present any risk to animal and human health and which produce feeds with higher quality and safety.

In this group of alternatives is lactulosis, a human milk component identified in the 1950s, which acts as an inhibitor of the adhesion of pathogenic bacteria to the intestinal epithelial surface and increases the growth of Bifidobacterium and Lactobacillus, alleviating the symptoms of hepatic encephalopathy in human babies (Waitzberg, 2009).

Lactulosis is a lactose isomer that constitutes a disaccharide consisting of a galactosis and a fructose molecule (4-O-β-D-galactopryranosyl-D-frutose), which resists the gastric passage, enables growth to acidophil microorganisms and facilitates the passage of *Salmonella* through the intestine (Denipote et al., 2010).

Salmonellae belongs to the group of enteropathogens that are commercial barriers to poultry products and present extremely important serovars to public health (Marcus et al., 2007; Perdoncini et al., 2011). In these serovars, *S.* Typhimurium stands out as a source of feed toxinfections in humans (Fernandes et al., 2006), and its control is officially recognized by the Brazilian Plan of Poultry Health Control.

Salmonella can also be related to damages to the intestinal health (Assis & Santos, 2001) or determine only

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sub-clinical infections (Gast & Benson, 1995) and insidiously propagate to other animal species and result in a contaminated product and a potential problem to public health. In this context, lactulosis appears as an alternative to growth promoters, which improves animal health, modifying the balance of the intestinal microbiota and avoids colonization of pathogens, such as *S.* Typhimurium.

Therefore, this experiment was carried out with the objective of evaluating the effects of lactulosis in drinking water on performance, its ability to avoid colonization by *Salmonella* Typhimurium experimentally inoculated and the morpho-histological parameters of intestine.

Material and Methods

This experiment was carried out in the Núcleo Experimental de Doenças de Aves of the Escola de Veterinária e Zootecnia of the Universidade Federal de Goiás.

A total of 630 one-day-old male Cobb chicks were allotted in heated batteries in a randomized block design with seven replications of 15 broilers each, in a total of 42 experimental units. This experiment was performed with six treatments: a control group, which did not receive the microbial inoculum or the lactulosis (Placebo); the group of birds inoculated in the crop on the first day of life with 0.5 mL of alkaline saline solution at 0.85%, with approximately 5.0×10^2 fcu/0.5 mL of *S.* Typhimurium (ST); the group that received water containing lactulosis, in a dose established in a previous assay-experiment, of 0.023 mL/g of live weight from the first to the 14th days of life (L); a group of birds that received, directly in the crop, 0.5 mL of a 0.85% buffered saline solution, containing approximately 5.0×10^2 fcu/0.5 mL of *S.* Typhimurium and water containing lactulosis at a dose of 0.023 mL/g of live weight from one to 14 days of age [(ST(1^0) +L(1^0)]; a group of birds that received water with lactulosis since the first day at the same dose as the control treatment and received 0.5 mL of a 0.85% buffered saline solution, containing approximately 5.0×10^2 fcu/0.5 mL of *S.* Typhimurium [(L(1^0) +ST(48h)] via crop after 48 hours; a group of birds inoculated directly in the crop on the first day of life, with 0.5 mL of 0.85% buffered saline solution, with approximately 5.0×10^2 fcu/0.5 mL of *S.* Typhimurium and lactulosis, after 48 hours, in the same amount of the control treatment, in the drinking water, until the 14th day of life [(ST(1^0) +L(48h)].

Birds were inoculated with *Salmonella* Typhimurium isolated from broiler samples, as recommended by Rezende et al. (2008).

The mashed corn/soybean meal diet offered to the animals was formulated according to the feed composition and nutritional requirements proposed by Rostagno (2005) and did not have anticoccidials or antibiotics as growth promoter. Birds were fed the diets *ad libitum* during all the experimental period.

Challenged and not challenged birds were allotted in separated rooms keeping the same temperature and relative air humidity conditions. Chicks were raised in galvanized steel batteries equipped with linear feeders and drinkers and trays covered with plastic protection for excreta collection. Batteries were heated with a 60 W incandescent bulb per floor until the 12th day of age.

Birds and diets were weighed on the 1st, 14th, 21st and 28th days of life for calculation of feed intake, weight gain and feed conversion. On the 14th day of life, one bird per replication was euthanized, necropsied and fragments of the initial portion of the duodenum, jejunum and ileum were collected, cut longitudinally fixed in wood plates and conserved in 10% neutral formalin. Samples were processed according to the methodology proposed by Luna (1968), stained by hematoxilin eosin (HE), and morphometrical indexes were determined with the software Axion Vision 3.0. Slide images were digitalized in a bright field microscope (Carl Zeiss model JENAVAL), captured by an analogical video camera and a capture board.

Villus and crypt dimensions determined in histological analysis were expressed in micra, according to the criteria proposed by Uni et al. (1998), with five readings for villus height and crypt depth, totaling 30 readings for each fragment collected.

At 10, 24 and 35 days of age, excreta samples were collected by cloacal swabs of 50 birds per treatment, in a total of 300 samples for each age analyzed. For the positive results for *Salmonella* Typhimurium found in excreta samples, the results were expressed in percentage and described as frequency. Samples were processed according to recommendations of Georgia Poultry Laboratory (1997) and Brasil (2003), including some changes described as follows.

After the collection by cloacal swab, samples were introduced in 9.0 mL tubes with Selenite-Cystine solution and incubated at 37 ^oC during 18 to 24 hours. After this period, aliquots were introduced in the plates and streaked in brilliant green and Hektoen agar solutions with a nickel-chrome handle and incubated at 37 ^oC during 24 hours. In each agar media, two or three colonies were selected according to the morphological characteristics of *Salmonella* and transplanted to triple sugar-iron agar media (TSI) and incubated at 37 ^oC during 24 hours.

The TSI isolates which presented characteristics of genus *Salmonella* were subjected to the following tests: indol and H_2S production, motility, urease, lysine

descarboxylation, methyl red and Simmons citrate and malonate utilization. Samples biochemically confirmed as *Salmonella* were submitted to the serological test with polyvalent anti-O serum. Samples confirmed in biochemical and serological analysis were sent to Instituto Osvaldo Cruz (FIOCRUZ) for serological typing.

Results obtained were submitted to analysis of variance and means were compared by Tukey's test, with the PROC GLM procedure of software SAS (Statistical Analysis System, version 9.2). For analysis of frequency of *Salmonella* Typhimurium excretion, chi-square test ($\alpha = 0.05$) was applied according to recommendations by Sampaio (1998).

Results and Discussion

Groups of challenged broilers fed lactulosis at different moments presented higher live weight than the placebo group and the chicks that received only lactulosis (Table 1); however, it was different from the ones inoculated only with *S.* Typhimurium ($P<0.05$). Therefore, average live weight, feed conversion and feed intake of chicks at 14 days of age were negatively affected by the bacterial inoculation ($P<0.05$).

The negative effect of the inoculated bacteria was neutralized by the lactulosis. These results suggest that this substance can act in the intestinal microbiota, delaying or avoiding bacterial colonization. According to Silva & Nörnberg (2003), prebiotic substances can block adherence loci, reduce the binding capacity of the pathogenic bacteria and, thus, reduce damages in the intestinal mucosa.

The groups that received lactulosis with or without the presence of the pathogen had higher average live weight ($P<0.05$) and better feed conversion ($P<0.05$) compared with the placebo group (Table 2).

In the placebo group, feed intake was higher and feed conversion was worse, although similar to the positive control group. These results can be explained by the reduced action of the pathogenic agent in the intestines during the experimental period, especially because of the reduced amount of feed ingested. Maiorka et al. (2001) supplemented a basal diet to broilers with additives and observed that the chicks fed a non-supplemented diet had better feed conversion results.

Similar results with incremented weight gain and feed conversion were obtained by Rostagno et al. (2003), evaluating the effect of mannan-oligosaccharides on diets containing corn grains presenting different nutritional quality.

At 28 days of age (Table 3), the productive parameters measured were not influenced by the presence of pathogen nor the addition of lactulosis. This fact can be explained by the withdrawal of lactulosis from the diets 14 days before and by the results of fecal excretion (Table 5), in which a reduction in *Salmonella* elimination was observed at older ages.

Regarding the histomorphometrical measures of duodenum and jejunum (Table 4), a statistical difference for villous height was observed in the duodenum, with reduced value for positive control group (ST), for the group fed lactulosis and inoculated with the agent on the first day [L (1st d) and ST (48h)] and for the group that received

Table 1 - Performance of broilers treated with lactulosis and challenged with *Salmonella* Typhimurium from 1 to 14 days of age

Treatments	Final weight (g)	Feed intake (g)	Feed conversion (g/g)
Placebo	441.0AB	284.4B	1.11B
Positive control (ST)	418.8B	369.1A	1.40A
Control with lactulosis (L)	456.6AB	293.7B	1.09B
ST (1st d) + L (1st d)	470.4A	313.1B	1.09B
L (1st d) + ST (48h)	454.6AB	291.8B	1.09B
ST (1st d) + L (48h)	454.7AB	287.3B	1.07B
CV (%)	6.24	8.72	6.77
P value	0.0282	0.0001	0.0001

Different letters in the same row indicate difference ($P<0.05$) by Tukey's test.
ST - *Salmonella* Typhimurium; L - lactulosis; d - day; CV - coefficient of variation.

Table 2 - Performance of broilers treated with lactulosis and challenged with *Salmonella* Typhimurium from 1 to 21 days of age

Treatments	Final weight (g)	Feed intake (g)	Feed conversion (g/g)
Placebo	732.2B	433.5C	1.34B
Positive control (ST)	812.8A	464.3BC	1.34B
Control with lactulosis (L)	828.2A	471.4ABC	1.25A
ST (1st d) + L (1st d)	847.9A	490.0AB	1.26A
L (1st d) + ST (48h)	861.6A	517.0A	1.25A
ST (1st d) + L (48h)	853.8A	494.5AB	1.22A
CV (%)	5.88	6.49	3.66
P value	0.0001	0.0004	<0.0001

Different letters in the same row indicate difference ($P<0.05$) by Tukey's test.
ST - *Salmonella* Typhimurium; L - lactulosis; d - day; CV - coefficient of variation.

Table 3 - Performance of broilers treated with lactulosis and challenged with *Salmonella* Typhimurium from 1 to 28 days of age

Treatments	Final weight (g)	Feed intake (g)	Feed conversion (g/g)
Placebo	1105.78	1537.70	1.43
Positive control (ST)	1115.67	1689.17	1.56
Control with lactulosis (L)	1210.68	1621.92	1.42
ST (1st d) + L (1st d)	1118.91	1652.92	1.54
L (1st d) + ST (48h)	1129.44	1673.59	1.55
ST (1st d) + L (48h)	1162.17	1681.33	1.53
CV (%)	8.64	6.20	5.75
P value	0.3653	0.0775	0.0079

Different letters in the same row indicate difference ($P<0.05$) by Tukey's test.
ST - *Salmonella* Typhimurium; L - lactulosis; d - day; CV - coefficient of variation.

Table 4 - Performance of broilers treated with lactulosis and challenged with *Salmonella* Typhimurium from 1 to 28 days of age

Treatments	Duodenum		Jejunum	
	Villous height	Crypt depth	Villous height	Crypt depth
Placebo	1131.7A	213.0	611.0	162.6
Positive control (ST)	899.0B	219.6	629.0	168.1
Control with lactulosis (L)	1121.3A	185.8	566.1	174.4
ST (1st d) + L (1st d)	974.2AB	181.6	679.4	155.4
L (1st d) + ST (48h)	1142.5A	188.6	721.5	163.7
ST (1st d) + L (48h)	958.9AB	162.8	724.3	164.3
CV (%)	11.09	18.95	16.16	22.70
P value	0.0097	0.2819	0.1171	0.9809

Different letters in the same row indicate difference (P<0.05) by Tukey's test.
ST - *Salmonella* Typhimurium; L - lactulosis; d - day; CV - coefficient of variation.

Salmonella and then lactulosis, 48h after, [ST (1st d) and L (1st d)]. Villous height was influenced by the presence of *Salmonella* and lactulosis did not show any effect on the average values, considering that villous measures were similar for both placebo and lactulosis groups, confirming the absence of action of the oligosaccharide in the intestinal mucosa. This fact can be explained by the chemical properties of lactulosis. According to Konstantinov et al. (2004), the oligosaccharide resists the gastric digestion and cannot be hydrolyzed by the disaccharidases present in the small intestinal mucosa. It is worth stressing that *Salmonella* promoted some kind of injury or stress in the intestinal mucosa, which compromised digestion and absorption, considering the lower weight gain and worse feed conversion in the period of 1-14 days (Table 2).

On the other hand, the reduced averages of duodenum villous height were similar to the ones obtained by Okamoto et al. (2009), who challenged broilers with *S.* Enteritidis.

Despite the reduction in the average villous height in the duodenum, no statistical differences were observed for crypt depths. It is possible that the injuries caused to the epithelial cells by the pathogen were discrete or the cell recovery was really well-developed when measurements were carried out. According to Kim et al. (2011), villous height and crypt depth are important indicators for digestive tract health because they are directly connected to the capacity of absorption of the mucosa.

In the evaluation of the averages of villous height and crypt depths on the 14th day of age (Table 4), no differences were observed (P>0.05) between treatments. Considering that lactulosis is a non-digestible disaccharide with prebiotic action, the results were different from the results obtained by Oliveira et al. (2008), who utilized a mannan-oligosaccharide in diet for broilers and observed increment in jejunum villous height.

At the same time, cloacal swabs were performed for monitoring *S.* Typhimurium excretion. A statistical difference was observed (Table 5) in the frequency of pathogen elimination between treatments at the 10 days of age. Lower frequency was observed, as follows: 3/50 (6%); 4/50 (8%) and 5/50 (10%), for *Salmonella* excretion for the groups that received lactulosis on the first day or 48 hours after housing the neonatal chicks, compared with the treatments without lactulosis and inoculated with *Salmonella* Typhimurium, which presented frequency of 15/50 (30%).

Lactulosis influenced the excretion of *S.* Typhimurium. According to Martín-Peláez et al. (2008), this substance is decomposed in the colon in short-chain fatty acids, mainly lactic and acetic acids, which stimulate the peristaltic movements of the intestine. In addition, the decrease in pH benefits the proliferation of acidophil microorganisms. This change in the intestinal lumen promotes dislocation of *Salmonella*. It is important to add that this principle has been used in human medicine for years for patients infected by *Salmonella.* Yan et al. (2011) suggested that the mannan-oligosaccharydes can act in the gastrointestinal tract, blocking the binding sites of the pathogenic bacteria.

On the other hand, there was reduction in the intestinal infection from 30% (15/50) at 10 days of age, to 8% (4/50), at 24 days of age, and 2% (1/50) at 35 days of age, for positive control (ST), considering that all the broilers in the group were infected when the bacterium was inoculated. This fact

Table 5 - Frequency of excretion of *Salmonella* Typhimurium in broilers inoculated in the crop and challenged with *Salmonella* Typhimurium and treated with lactulosis at 10, 24 and 35 days of age

Age	10 days	24 days	35 days
Treatments	n (%)	n (%)	n (%)
Placebo	0/50 (0%)B*	0/50 (0%)	0/50 (0%)
Positive control (ST)	15/50 (30%)A	4/50 (8%)	1/50 (2%)
Control with lactulosis (L)	0/50 (0%)B	0/50 (0%)	0/50 (0%)
ST (1st d) + L (1st d)	4/50 (8%)B	1/50 (2%)	0/50 (0%)
L (1st d) + ST (48 hours)	3/50 (6%)B	0/50 (0%)	0/50 (0%)
ST (1st d) + L (48 hours)	5/50 (10%)B	1/50 (2%)	0/50 (0%)

* Different letters in the same row differ by the chi-square test (0.05%)
ST - *Salmonella* Typhimurium; L - lactulosis; d - day.

can be related to a natural age-based immune resistance to enteropathogens, which are related to the development of the gut-associated lymphoid tissue (GALT), and the establishment of the beneficial intestinal microbiota, in the first weeks of age (Bohórquez et al., 2011).

At 24 days of age, no statistical difference was observed in the frequency of *Salmonella* Typhimurium excretion between the treatments (Table 5). *Salmonella* was almost totally eliminated from the bird organism, although it was detected in 8% (4/50) of the samples analyzed in the positive control. This fact probably affected bird intestinal health, expressed by the worse feed conversion observed at 28 days of age. Furthermore, these birds can be considered asymptomatic carriers because of their epidemiological importance, considering that the bacteria can be eliminated in the excreta and contaminate other birds, the environment, the soil, and determine the cross-contamination in the abattoir plant during the procedures of carcass and meat processing.

At the 35 days of age, *Salmonella* was detected only in the positive control group (ST), 2% (1/50) (Table 5). This result confirms that, with ageing, the development and maturity of the bird lymphoid tissue associated to the intestine and the establishment of the normal microbiota was possible (Beal et al., 2004; Andrade et al., 2007).

Conclusions

There is incresase in weight gain in birds treated with lactulosis, and this effect can be prolonged for one week after the diet withdrawal. The use of lactulosis reduced the fecal excretion of *Salmonella* Typhimurium by the evaluated birds.

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