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Effects of *Lactobacillus* Probiotic, P22 Bacteriophage and *Salmonella* Typhimurium on the Heterophilic Burst Activity of Broiler Chickens

ABSTRACT

Due to the constant evolution of industrial poultry production and the global emergence of bacterial resistance to antibiotics there has been an increasing interest in alternatives for the treatment of poultry salmonellosis, such as phage therapy and probiotics. The present study evaluated the effects of the oral administration of the bacteriophage P22 and of a probiotic, consisting of four *Lactobacillus* species, on the level of circulating heterophils containing a superoxide anion of one-day-old broilers challenged with *Salmonella* Typhimurium for seven days. It was concluded that the treatment with a probiotic with lactobacilli of broilers experimentally infected with *Salmonella* spp eliminates this pathogen by increasing the circulating levels of reactive heterophils. When chicks are treated with a probiotic and a bacteriophage, the agent is eliminated with no changes in circulating reactive heterophil counts. It is also concluded that the heterophils of day-old chicks are not capable of producing superoxide anion. However, this capacity is detected after 48 h of life, indicating that heterophils mature as birds age.

INTRODUCTION

Heterophils are polymorphonuclear leukocytes of poultry that are homologous to neutrophils in mammals. They have primary phagocytic action in the inflammatory response against infectious agents, such as bacteria and fungi (Montalli, 1988; Campbell, 1995), and reach the infected site after 30 minutes of the initial insult (Kogut *et al.*, 1994, He *et al.*, 2003).

The invasion of the intestinal mucosa by bacteria of the genus *Salmonella*, a Gram-negative bacillus commonly associated with food poisoning outbreaks in humans and animals, stimulates the massive recruitment of polymorphonuclear cells to lamina propria of inflammatory sites. Heterophils are attracted by chemokines resulting from the host-parasite interaction (Porter Jr. & Holt, 1993), and upon arriving at the infection site, rapidly phagocytose the invading agent. Heterophils are essential for fighting salmonellosis in newly-hatched poultry (Kogut *et al.*, 2001; Swaggerty *et al.*, 2005). During phagocytosis, heterophils use primarily a non-oxidative bactericidal mechanism mediated by lysozymes and cationic proteins, particularly α -defensin (Harmon, 1998). It was believed for many years that heterophils were not capable of generating oxygen metabolites; however, the studies of Desmidt *et al.* (1996), Farnell *et al.* (2003), and He *et al.* (2003; 2007) demonstrated this activity.

Due to the increase of bacterial resistance to antibiotics, several studies have been developed for the control and eradication of *Salmonella* spp., mainly focusing on methods that maintain the quality of poultry products. Among the researched natural alternatives, phage



therapy and probiotics have been acknowledged by the scientific community.

Bacteriophages or phages are viral predators of bacteria and are commonly found in the environment. Their parasitic action is well-accepted for the biocontrol of *Salmonella* (Rohwer & Edwards 2002; Atterbury *et al.*, 2007). Bacteriophages are cell-specific, i.e., each species of bacteria is attacked by specific phages that cause damage only to their own host cell (Mayr & Guerreiro, 1981). The advantages of phage therapy are that it is selective, not affecting the beneficial intestinal microbiota and eukaryotic cells, and its side effects are rare (Matsuzaki *et al.*, 2005).

Probiotics are pure or compound cultures of previously identified live microorganisms that, when administered to humans and animals, benefit the host by stimulating the properties of the natural microbiota (Fuller, 1989; Havennar & Huis In't Veld, 1992), consequently preventing the intestinal colonization by pathogenic bacteria (Sandler *et al.*, 1969; Stuart, 1984). Probiotics composed of lactobacilli are the most commonly applied in the poultry industry. These bacteria present several mechanisms that inhibit the growth and may even kill some species of pathogenic bacteria (Fuller, 1989), and the use of different strains may boost this inhibitory capacity (Sarra & Badini, 1997).

The constant intake of lactobacilli has a local immunostimulants effect on the intestinal mucosa. Lactobacilli attract lymphocytes to the intestinal lamina propria (Lillehoj & Chung, 1992) and stimulate the intestinal synthesis of IgA by the immune system, increasing the resistance to diseases (Pulverer *et al.*, 1990). In addition, there is a systemic effect, characterized by lymphomonocytic leukosis (Marietto-Gonçalves *et al.*, 2011), which may prevent possible septicemia.

The present study evaluated the dynamics of the activation of the superoxide anion in heterophils of day-old chicks challenged with *Salmonella enterica*, subspecies *enterica*, serotype Typhimurium, and treated with the bacteriophage P22 and a probiotic composed of lactobacilli for seven days.

MATERIAL AND METHODS

Birds and facilities

One hundred and ninety two one-day-old Avian Farm chicks (*Gallus gallus domesticus*), derived from a same breeder flock not vaccinated against *Salmonella*, were used. Chicks were housed in battery cages at the Poultry Pathology Laboratory of the Clinics Department

of the School of Veterinary Medicine and Animal Science, Universidade Estadual Paulista (FMVZ-UNESP), Botucatu, Brazil. The study was previously approved by the Committee of Ethics of Animal Experimentation of FMVZ-UNESP. Birds were offered water and an antibiotic-free diet *ad libitum*, and were maintained under controlled temperature ($32^{\circ}\text{C} \pm 3^{\circ}$).

The absence of *Salmonella* spp. was confirmed by the microbiological analysis of 10% additional chicks before the beginning of the experiment. Twenty chicks were sacrificed by neck dislocation and their organs (liver, ceca, spleen, and yolk sac) were aseptically collected. Organs were evaluated as individual pools. After maceration, samples were incubated at 40°C for 24 h in test tubes containing 10 mL tetrathionate broth (TB; Merck, Darmstadt, Germany) and in selenite-cystine broth (SCB; Merck, Darmstadt, Germany). After incubation, samples were plated on brilliant-green agar (BGA; Oxoid, Basingstoke, England) and xylose-lysine deoxycholate agar (XLD; Oxoid, Basingstoke, England) and again incubated at 37°C for 24 h, after which the presence of bacterial lactose-negative H_2S -producing colonies was determined. No growth of colonies morphologically compatible with *Salmonella* spp was detected.

Table 1 – Experimental groups.

Group	Treatment	Number of birds
Cr	Control*	48
St	<i>Salmonella</i> Typhimurium	48
St+Pb	Probiotic	48
St+Pb+Ph	<i>Salmonella</i> Typhimurium, probiotic and bacteriophage P22	48

*Group not challenged and not treated.

Experimental design

Chicks were distributed into four treatments ($n=48$). On their first day of life, birds in the groups St, St+Pb, and St+Pb+Ph were inoculated with *S. Typhimurium*. Three hours after inoculation, the St+Pb+Ph group was treated with the bacteriophage and the probiotic, and group St+Pb only with probiotic. The birds of the Cr group were not inoculated with *S. Typhimurium* and did not receive any treatment. Birds were inoculated and received the treatment via gavage before feeding.

Cultures and preparation of *Salmonella* Typhimurium inoculum (ST), probiotic, and bacteriophage

The inoculum used to challenge of the groups St, St+Pb, and St+Pb+Ph consisted of a culture containing



4×10^4 CFU/mL of marked ST resistant to nalidixic acid and rifampicin.

The probiotic culture was previously tested *in vitro* for its inhibitory effect on ST using the technique "spot-on-the-lawn", according to Lima *et al.* (2007). The culture contained strains of *Lactobacillus acidophilus*, *L. fermentum*, *L. reuteri*, and *L. salivarius*, and was daily cultivated in DeMan-Rogosa-Sharpe broth (MRS; Oxoid, Basingstoke, England) under anaerobiosis (Probac do Brasil Ltda., São Paulo, Brazil) at 37°C. Each *Lactobacillus* strain was individually cultured and the probiotic was mixed only at the time treatments were applied. Birds of the groups St+Pb and St+Pb+Ph were supplied 6×10^9 CFU/mL of the fresh probiotic culture every 24 h.

The bacteriophage P22 (ATCC 19585-B1) was used. Before the experiment was carried out, its lytic activity against the *Salmonella* inoculum was also tested using the "spot-on-the-lawn" technique. It was amplified in a nutrient broth solution (Acumedia, Lansing, USA) with the addition of 0.5% NaCl, containing 2×10^{10} PFU (plaque forming unit) and 3 mL ST culture in triptone-soy broth (TSB; Merck, Darmstadt, Germany). After 4h at 40°C, the culture was purified by filtration in 0.22- μ m filter (Medical Millex-GS filter unity, Millipore, Billerica, USA). Viral load was determined by counting PFU in Petri dish with TSB2x previously cultivated with ST. Birds of the St+Pb+Ph treatment were inoculated with 0.5 mL of the culture containing 3×10^{10} PFU/mL of P22 directly in the crop via gavage.

Blood samples

Using sterile 1-mL syringes and 0.45 x 13 mm needles, 200 μ L of blood were collected by jugular vein puncture. Samples were divided in two 100- μ L aliquots: one for leukocyte count and the other for heterophil evaluation.

Evaluation of heterophilic respiratory burst

The nitro blue tetrazolium reduction test (NBT) was used, together with leukocyte count, to evaluate heterophilic activity according to the procedure adapted from Trevelin *et al.* (2009). After blood collection, blood from each sample was smeared on a slide, which was then stained using rapid Romanovsky stain protocol. Leukocyte differential count was performed under optical microscope at 100x magnification. Immediately after blood smearing, 1 μ L sodium heparin was added to the 100- μ L blood aliquot, which was homogenized. Total leukocytes were manually counted in a

hemocytometer using macrodilution of 10 μ L of blood in 1 mL toluidine blue.

Stimulated and non-stimulated NBT were performed. For the stimulated test, 12.5 μ L Nitroblue Tetrazolium Chloride® (Sigma Aldrich, St. Louis, USA), 0.625 μ L Stimulant® (Sigma Aldrich, St. Louis, USA) and 6.25 μ L of blood were added to each microtube. After homogenization, samples were incubated in water bath at 37°C for 10 min, and then left to rest for further 10 min at room temperature. Three smears were performed, stained using rapid Romanovsky stain protocol, and read. For the non-stimulated test, the same procedure as the stimulated test was used, except that only 12.5 μ L Nitroblue Tetrazolium Chloride® and 6.25 μ L blood were added to a polypropylene microtube. The percentage of NBT-reducing cells was determined based on the count of 100 heterophils from the non-stimulated test.

Determination of the presence of *Salmonella* spp

The presence of *Salmonella* was evaluated in the ceca of birds every 48 h, starting three hours after the treatments. Evaluation times were identified as 0 h, 48 h, 96 h, and 144 h. At each evaluation time, a sample of 12 birds per treatment was sacrificed. Conventional bacteriological examination was performed and confirmation of the results was made using molecular detection.

The bacteriological examination was performed in a pool of ceca, which were weighed, macerated, and an aliquot was incubated at 40°C for 24 h in test tubes containing 10 mL tetrathionate broth (TB; Merck, Darmstadt, Germany) and in selenite-cystine broth (SCB; Merck, Darmstadt, Germany). After incubation, samples were plated on brilliant-green agar (BGA; Oxoid, Basingstoke, England) and on xylose-lysine deoxycholate agar (XLD; Oxoid, Basingstoke, England) with the addition of 100 μ g/mL nalidixic acid and 100 μ g/mL rifampicin, and again incubated at 37°C for 24 h. After screening, suspected colonies were submitted to biochemical characterization.

The presence or absence of ST in the ceca was confirmed by conventional PCR test (polymerase chain reaction), validated by the techniques commonly employed to isolate *Salmonella* spp.

DNA was extracted using Chelex-100™ (Bio-rad) with primers of Invitrogen Corporation (São Paulo, SP, Brazil). Primer sequences were suspended at the concentration of 10 pmol. The target gene *invA* (forward 5'-TTGTTACGGCTATTTGACCA-3' and



reverse 5'-CTGACTGCTACCTTGCTGATG-3') of 521 pb, with the following amplification program, was used: 5 min at 94°C, 35 amplification cycles (30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C), and final polymerization of 4 min at 72°C, according to Swamy *et al.* (1996). Amplifications were performed with 5 µL of each sample added to 1 µL of each primer and 12.5 µL of the GoTaq® Green Master Mix amplification kit (Promega) and 5.5 µL ultrapure water.

For isolation, the macerated material suspended in PBS was directly seeded on BGA plates containing 100 µg/mL nalidixic acid, and CFU were determined using serial dilution.

STATISTICAL ANALYSIS

Results were transformed into logarithm (logx+1) to analyze variance performance by the GLM procedure of SAS statistical package (version 8.0, SAS Institute, USA). Results were submitted to analysis of variance (ANOVA) and means were compared by the *post-hoc* test of Tukey. Results were considered significant when $p < 0.01$. The normality of distribution was analyzed by the Shapiro-Wilk test.

RESULTS

Microbiological evaluation

During the experimental period, the presence of ST was detected by bacteriological isolation and by PCR in all samples collected from the St group. In the St+Pb group, ST was isolated and detected by PCR only in the first collection. In the St+Pb+Ph group, ST was detected only by PCR and not by bacterial isolation in

the first collection, and it was no longer detected in the subsequent samplings (Table 2).

Table 2 – Detection of *Salmonella Typhimurium* in the ceca using PCR and conventional bacterial isolation (CFU) in the different groups after three hours, according to treatment.

Treatment	Time after challenge (hours)			
	0	48	96	144
Cr	-/ND	-/ND	-/ND	-/ND
St	+/ 4×10^3	+/ 3×10^2	+/ 2×10^2	+/ 2×10^1
St+Pb	+/ 4×10^2	-/ND	-/ND	-/ND
St+Pb+Ph	+/ND	-/ND	-/ND	-/ND

*Pool of twelve birds per treatment. PCR Negative: -. Positive: +. ND, not detected.

Evaluation of heterophilic respiratory burst

White blood cells counts are shown in Table 3. In general, all groups presented a large number of leukocytes in the first collection (0h), with gradual decrease after that. The presence of circulating heterophils with superoxide anion (NBT+) was not detected in any of the groups in the first collection.

White blood cell counts were not statistically different among treatments in the first collection. However, 48h after the ST challenge, the St group presented leukopenia, as well as at the following collections. The St+Pb and St+Pb+Ph were not statistically different.

All groups challenged with ST presented significantly lower heterophil values compared with the Cr group, and the St+Pb+Ph presented intermediate values between the Cr group and the other two groups, which were not significantly different from each other. However, after 48 h, the St+Pb and St+Pb+Ph groups presented significantly higher heterophil counts compared with the Cr group. The St group presented

Table 3 – Absolute leukometric values obtained during the study.

Cell type	Treatment	Time after challenge (hours)			
		0	48	96	144
WBC	Cr	5060 ^{NSa} ± 575	3166 ^{ABb} ± 510	1962 ^{Bc} ± 387	791 ^{Bd} ± 144
	St	4476 ^{NSa} ± 452	1719 ^{Bb} ± 551	720 ^{Cc} ± 174	463 ^{Cd} ± 174
	St+Pb	4661 ^{NSa} ± 552	3520 ^{Aab} ± 646	2376 ^{Ab} ± 876	1062 ^{Ac} ± 185
	St+Pb+Ph	4908 ^{NSa} ± 512	3456 ^{Ab} ± 560	2367 ^{Ac} ± 310	983 ^{Ad} ± 201
Heterophils	Cr	4045 ^{Aa} ± 451	2678 ^{ABb} ± 290	1517 ^B ± 369	494 ^{Bd} ± 110
	St	2985 ^{Ba} ± 399	1583 ^{Bb} ± 560	486 ^{Cc} ± 138	294 ^{Cc} ± 66
	St+Pb	3036 ^{Ba} ± 371	2890 ^{Aa} ± 543	1890 ^{Ab} ± 641	858 ^{Ac} ± 115
	St+Pb+Ph	3574 ^{ABa} ± 419	2907 ^{Ab} ± 927	1914 ^{Ac} ± 243	886 ^{Ad} ± 132
NBT+	Cr	0 ^{NSns}	81 ^{Ca} ± 32	61 ^{Ba} ± 34	26 ^{Bb} ± 11
	St	0 ^{NSns}	246 ^{Aa} ± 123	117 ^{Ab} ± 25	88 ^{Abc} ± 11
	St+Pb	0 ^{NSns}	161 ^{Ba} ± 77	71 ^{Bb} ± 30	34 ^{Bb} ± 14
	St+Pb+Ph	0 ^{NSns}	81 ^{Ca} ± 30	57 ^{Ba} ± 41	29 ^{Bb} ± 9

* WBC: white blood cells; NBT+: heterophil with superoxide anion granules. Twelve birds were pooled per treatment; Small letters compare the means of the treatments against time (lines) and capital letters compare the means of the treatments within each collection time (column). Means followed by different letters are significantly different ($p < 0.01$). Means followed by at least one equal letter are not significantly different ($p < 0.05$). NS: not significant ($p > 0.05$).



low heterophil counts in all collections, which were significantly lower than that obtained in the Cr group during all evaluated periods.

In the first collection, no NBT+ heterophils were detected in none of the groups. The St group presented the highest NBT+ heterophil counts (Figure 1), with a peak at 30% of the heterophil population 144 h post-challenge (Figure 2). Groups Cr and St+Pb+Ph were not statistically different during the entire experimental period. Group St+Pb presented significantly higher NBT+ heterophil counts in the second collection (48 h) compared with Cr and St+Pb+Ph groups, but not 96 h post-challenge.

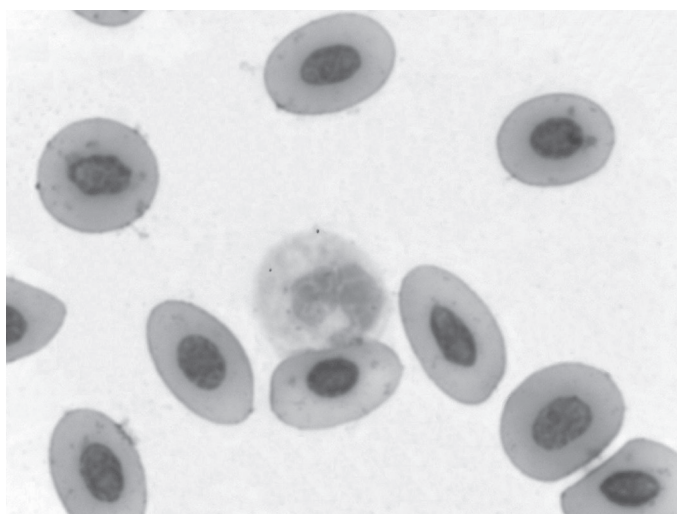


Figure 1. Reactive heterophils with superoxide anion granules (refractional black granules in optic microscopy) marked in the NBT reduction cytochemical test (Romanovsky stain, 100x).

DISCUSSION

The use of bacteriophage for the control of *Salmonella* was studied by Fiorentin *et al.* (2005), Atterbury *et al.* (2007), and Andreatti Filho *et al.* (2007), who obtained reduction of up to 2 CFU of *Salmonella* with the oral administration of bacteriophages. The inhibition of the permanence of that agent in the intestine with the use of probiotics modulated with *Lactobacillus* strains was evaluated by Gusils *et al.* (1998), Pascual *et al.* (1999), Higgins *et al.* (2008), and Vicente *et al.* (2008). Toro *et al.* (2005) reduced the load of *Salmonella* with the oral administration of competitive exclusion probiotics.

In the present study, both treatments (St+Pb and St+Pb+Ph) were effective for the control of ST. The agent was not detected in neither of the groups 48 h after the treatments. The combination of the probiotic with the bacteriophage worked faster than the treatment only with probiotic, as shown by the detection of 4×10^2 CFU of ST/g of organ in the St+Pb

group, whereas in the St+Pb+Ph group, ST was only detected by PCR. This means that after only 3 h of treatment, ST was reduced to non-detectable levels by the isolation technique or that ST was eliminated during this period, and only traces of its passage in the ceca were detectable. The success of both treatments is attributed to the use of gavage and fasting before the treatment, while in the previous studies, treatments were orally administered during the rearing phase with no fasting period applied. The absence of feed possibly allowed better action of the biological agents employed in the present study.

Leukopenia and the reduced heterophil circulating levels detected in the St group in the present study are consistent with the findings of the study of Anderson & Stephen (1970). This is due to the migration of heterophils to the infected sites because heterophils, together with macrophages, are responsible for fighting bacterial infections (Campbell, 1995). The high heterophil counts obtained here were also reported by Marietto-Gonçalves *et al.* (2011).

The present study detected the presence of circulating heterophils containing superoxide anion in all treatments after 48 hours. This was also verified in adult chickens by Desmidt *et al.* (1996), Farnell *et al.* (2003), and He *et al.* (2003; 2007). The stain NBT is yellow and it is reduced in the presence of the superoxide anion, becoming dark blue. It is a fast, cheap, and simple method to evaluate the oxidative metabolism of phagocytes to detect the presence of superoxide anion, as it does not require cell isolation (Trevelin *et al.*, 2009).

The absence of reactive heterophils in the NBT test in day-old chicks in the present study indicates initial incompetence of the oxidative response of heterophils. This supports the arguments of Ziprin *et al.* (1989), Kogut *et al.* (1994), and Beal *et al.* (2004), who associated the high susceptibility to *Salmonella* infection of newly-hatched chicks to the immaturity of their immune system during the first week of life, particularly during the first four days. As previously observed by Trevelin *et al.* (2009) in ostriches, NBT+ heterophil counts increase as birds age.

The higher reactive heterophil count obtained in the St group is related to the fact that *Salmonella* stimulated the biological activity of heterophils in the peripheral blood of poultry (Kogut *et al.*, 1995). However, the intensity of this stimulus depends on the pathogenicity of the invading strain and of the time of contact with the host (Anderson & Stephens, 1970; Ziprin, 1997).



Farnnel *et al.* (2006) reported that lactobacilli systemically stimulate heterophils in health poultry. In the present study, birds contaminated with *Salmonella* and daily treated with lactobacilli did not present expressive NBT+ heterophil counts, suggesting that the stimulus caused by the pathogen was attenuated. This may be due to the fact that lactobacilli directly compete for nutrients and also produce potentially lethal substances (bacteriocins, peroxides, and organic acid) to the pathogen. Therefore, possibly there was low cytochemical expression due to tissue insult and/or little interaction of the pathogen with the intestinal mucosa, consequently reducing the stimulation of the phagocytic activity of the heterophils. This is evident in group St+Pb (Figure 2), where the presence of ST was detected in the first collection (0 h after the beginning of the treatment), with consequent increase in NBT+ heterophil values in the second collection (48 h later), when ST was no longer detected. After 96 h, St+Pb NBT+ heterophil counts were similar to those found in non-challenged and non-treated birds (group Cr). This indicates that after ST elimination, anion production was reduced, even in birds that received lactobacilli.

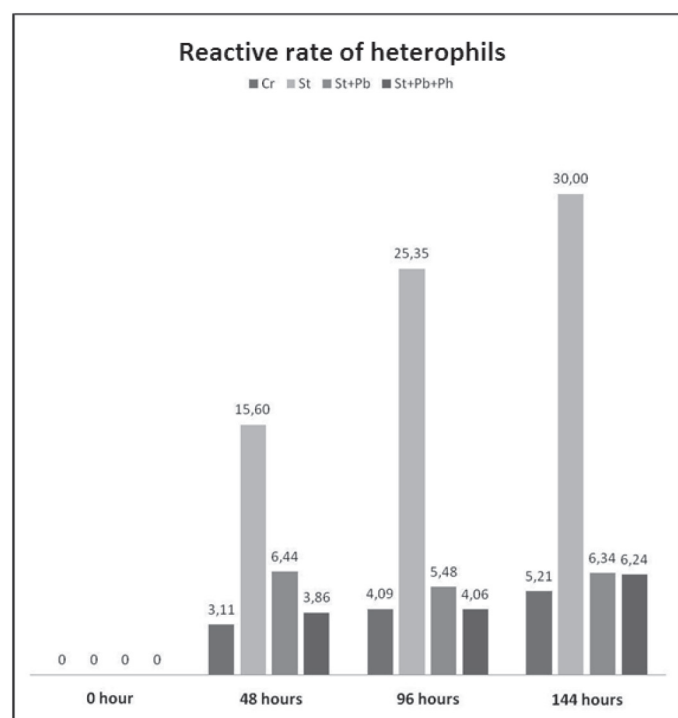


Figure 2. Heterophil NBT+ percentage rate between treatments along the experimental period; Twelve birds were pooled per treatment. ($p < 0.01$).

The group St+Pb+Ph maintained the same NBT+ heterophil counts as the Cr group. Considering that the innate immune response stimulation is influenced by the duration of the exposure of the host to the agent, it is possible that both the lytic action of the bacteriophage and the inhibitory action of the

lactobacilli allowed *Salmonella* to remain a relatively short time in the intestine of the birds of this group. This is suggested by the failure in isolating ST three hours post-treatment and only its detection by PCR.

As observed in group St+Pb+Ph, the administration of the bacteriophage did not affect NBT+ heterophil count or that of the other leukocytes. This is explained by the fact that P22 does not interact with eukaryotic cells, binding only to specific receptors of the cell wall of the host, which is *Salmonella* in this case (Baxa *et al.*, 1996; Matsuzaki *et al.*, 2005).

The obtained results showed that day-old chicks experimentally infected with *Salmonella* and treated, before being fed, daily with a probiotic composed of lactobacilli by gavage, eliminate the agent by a short-term increasing in circulating reactive heterophils. When chicks are treated with a probiotic and a bacteriophage, the agent is eliminated with no changes in circulating reactive heterophil counts. It is also concluded that the heterophils of day-old chicks are not capable of producing superoxide anion. However, this capacity is detected after 48 h of life, indicating that heterophils mature as birds age.

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