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ORIGINAL ARTICLE

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Evaluation of the effectiveness and safety of a genetically modified live vaccine in broilers challenged with *Salmonella* Heidelberg

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ABSTRACT
Salmonellosis ranks among the major diseases of commercial poultry, and its presence in poultry flocks is responsible for economic losses and risks related to public health. Vaccines are an important tool within integrated programmes to control salmonellosis. The purpose of this study was to assess cross-protection provided by the Poulvac® ST vaccine in the control of *Salmonella* Heidelberg in experimentally challenged 3- and 21-day-old birds. Eighty birds were identified and separated into four treatments (T1: vaccinated and challenged at 3 days of age, T2: unvaccinated and challenged at 3 days of age, T3: vaccinated and challenged at 21 days of age, and T4: unvaccinated and challenged at 21 days of age). The inoculum was produced from a Brazilian field strain of SH. At the end of the experiment, caecum and liver/spleen samples were collected for quantitative and qualitative analysis of SH, respectively. Analysis of the liver/spleen showed that Poulvac® ST significantly ($P \leq 0.05$) reduced the percentage of SH positivity in the group challenged at 3 days of age, while in the group challenged at 21 days this difference was almost considered significant ($P = 0.1818$). On the other hand, there was no statistically significant difference in SH count in the caecum (CFU/g) in the group challenged at 3 days, but for the group challenged at 21 days the SH counts were significantly ($P \leq 0.05$) lower in the vaccinated group when compared to the positive control.

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Introduction

Salmonellosis ranks among the most critical food-borne diseases affecting international trade in poultry, and is used as a criterion for product quality (OIE, 2015). Bacteria of this genus have been identified as a major cause of food poisoning in humans (BRASIL, 2003; Mead *et al.*, 2010).

Salmonella Heidelberg (SH) is a significant source of non-typhoidal *Salmonella* infection and has been found in several Brazilian states where industrial poultry production is widespread, causing concerns about potential risks to public health (Dickel, 2004; Borsoi *et al.*, 2006). According to the latest results of the Pathogen Reduction Plan and European Union Rapid Alert System for Food and Feed, SH is often present in positive samples taken from Brazilian poultry products (Freitas, 2011; RASFF, 2014).

Studies examining experimental inoculation of birds have demonstrated that the behaviour of SH resembles that of *Salmonella* Enteritidis, which can generate bacteria counts in both the intestine (caeca) and the viscera (liver); additionally, the curve to quantify the positivity of the two analysed serotypes was almost identical 72 h after inoculation (Borsoi *et al.*, 2011). For all analysis times, SH counts in the intestines exceeded those in the viscera. These results demonstrate the challenge faced by the poultry sector in attempting to control SH, and the risks related to positive testing in poultry products, since this microorganism is present in considerable quantities in both the intestine and the viscera (Piao *et al.*, 2007; Wollin, 2007; Gantois *et al.*, 2008). The SH in Brazil has asymptomatic manifestation in positive birds and its presence offers a risk just for humans.

Poulvac® ST is a genetically modified live vaccine developed from a strain of *Salmonella enterica* subsp. *enterica* serovar Typhimurium (STM-1) (Alderton *et al.*, 1991; Coloe *et al.*, 1994). This serovar produces antigens in the configuration of lipopolysaccharide and flagella, which are the same as those seen in other group B salmonellas (such as SH). This similarity in the somatic and flagellar antigens indicates a possibility of good heterologous immune response. The vaccine aims to protect chickens to reduce transmission to humans.

The objective of this study was to assess the effectiveness of Poulvac® ST in controlling *S. enterica*

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subsp. *enterica* serovar Heidelberg in broiler chickens, in both the intestines and in the viscera.

Materials and methods

Poultry and treatments

Eighty one-day-old broiler chicks of both genders were separated into four treatments (T1: vaccinated and challenged at 3 days of age; T2: unvaccinated and challenged at 3 days of age; T3: vaccinated and challenged at 21 days of age; T4: unvaccinated and challenged at 21 days of age); these chicks from a Cobb Slow line breeder lineage, commonly used in Brazilian broiler companies, came from a flock certified to be free from *Salmonella* spp. When the chicks arrived from the supplier, the cardboard floor of the box was analysed to assess the presence or absence of *Salmonella* spp., using the standard methodology indicated by the Brazilian Ministry of Agriculture, Livestock, and Food Supply (Directive 126 of November 6, 1995) (BRASIL, 1995).

The birds in all groups were identified on the day they were assigned into groups, and were fed and watered using tubular feeders and bell-type watering stations (chick food and watering stations were used up to seven days of age). Throughout the experiment, birds were given free access to food and water.

This protocol was submitted to the Zoetis Ethics and Animal Welfare Committee for approval prior to the experiment, and was approved in process 07-15-70AQO.

Vaccination and placement

The study was done in the Experimental Laboratory of Mercolab, located in Cascavel, Paraná State, Brazil. Immediately after the birds arrived, they were weighed and vaccinated (Table 1). Vaccination was conducted via spraying with Poulvac® ST (batch 002/14 manufactured 08/14, expiration 02/16). A total volume of 40 ml was sprayed on the 40 chicks (T1 and T3) on the first day of life. The number of doses was one dose/bird with a minimum amount of 3×10^7 colony-forming units (CFU)/dose. The vaccine was diluted with a free chlorine inactivator containing non-toxic blue dye (batch 045/14 manufactured May/14, expiration May/16).

The vaccinated birds were placed together with their respective control birds in two pens (T1 + T2 and T3 + T4) and were marked with paint to allow identification. Just in the second dose of the vaccine, at 14 days of age, the birds were separated in the same room by a movable fence to revaccinate only the tagged birds. One day after vaccination, the fence was removed. The purpose of allowing the birds to co-mingle was to validate the independence assumption and the analytical methods used.

The birds remained in 2.6 m^2 pens on new wood shavings (floor pens) in closed rooms during the experimental period. Plant pelleted feed without any *Salmonella* inhibitory ingredients (e.g. organic acids, prebiotics, or probiotics) was provided. Care was taken to prevent cross contamination by wearing personal protective equipment: shoe covers, gowns, hair nets, and gloves. Both rooms used were identical and located side by side with independent entrance and exit. Personal protective equipment was changed when moving between rooms following the order from T3 + T4 room to T1 + T2 room. Both the wood shavings and the rooms were sampled for the presence of *Salmonella* spp. before the start of the experiment.

Vaccine

Poulvac® ST is a commercial vaccine produced by Zoetis (Madison, NJ, USA). It contains a live non-virulent strain of AWC 591 *Salmonella* Typhimurium in lyophilized form with a titre of $\geq 3 \times 10^7$ CFU/dose. This variant of *S.* Typhimurium was altered by the deletion of two genes, *aro*A and *ser*C. The deletion of these genes results in an organism that retains the structure of the cell wall and flagellum, which keeps immunizing antigens intact.

Challenge

The challenge was performed with a SH strain isolated from the field. This sample was rendered resistant to antibiotics (nalidixic acid and novobiocin), which were incorporated into brilliant green agar to inhibit gut microbiota and facilitate counting. SH was grown in BHI broth for 18–24 h at 37°C until reaching a concentration of 1.0×10^9 CFU/ml; serial dilution was then performed until a concentration of 1.0×10^6 CFU/ml was achieved (Pickler *et al.*, 2012). For birds in groups 1 and 2, 0.5 ml of this culture was inoculated orally at 3

Table 1. Parameters assessed and age at collection in different treatments.

Activity/Parameter assessed	Day 0	Day 2	Day 3	Day 7	Day 12	Day 14	Day 21	Day 28
Body weight	T1, T2, T3, T4							T1, T2, T3, T4
Vaccination (1st dose)	T1, T3							
Cloacal Swab for Poulvac ST detection		T1, T3		T1, T3	T1, T3			
Challenge (SH)			T1, T2				T3, T4	
Vaccination (2nd dose)						T1, T3		
SH count (caecum)								T1, T2, T3, T4
SH isolation (liver/spleen)								T1, T2, T3, T4

Note: T1 (vaccinated and challenged at 3 days of age), T2 (unvaccinated and challenged at 3 days of age), T3 (vaccinated and challenged at 21 days of age), T4 (unvaccinated and challenged at 21 days of age).

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days of age, and at 21 days of age for birds in groups 3 and 4 (Table 1). This dose of challenge was administered based on previous work done with Brazilian strain of SH (pilot test). The birds and environmental conditions were monitored daily.

Laboratory analyses

In order to count the *Salmonella* spp, at 28 days of age, the samples collected from the caecum and liver were macerated, weighed, and serially diluted in saline solution at a proportion of 1:10 until a dilution of 10^{-5} was achieved. Next, 0.1 ml of each dilution was seeded into brilliant green agar (which also contained 12.5 μg/ml of nalidixic acid and 20 μg/ml of novobiocin) with a Drigalski inoculation spatula and incubated at 36 ± 1°C for 18–24 h. After incubation, the dishes were read and colonies were counted. Those that were suspected to be *Salmonella* were selected for biochemical and serological antigenic confirmation.

To detect SH in the organ samples (liver and spleen) at 28 days of age, the conventional procedure for isolating *Salmonella* (pre-enrichment) was carried out according to Brazilian directive 126 (BRASIL, 1995).

To detect the Poulvac® ST vaccine strain, eight individual samples collected from cloacal swabs, in group 1 and 3, at days 2, 7, and 12, were subjected to real-time polymerase chain reaction analysis (RT-PCR) using a Rotor Gene SYBR Green PCR kit (Qiagen). Based on the sequence of the *aro*A gene region of the genome of the strain used in Poulvac® ST, a pair of oligonucleotides (primers) was designed that exclusively amplify a 280 bp fragment of the vaccine's DNA and do not detect other field isolates of *Salmonella* spp. One of the oligonucleotides is based on the IS10 element that is only present in the vaccine strain.

The oligonucleotides used for detection of the vaccine were:

Oligonucleotide 1–5′ CGG CAT TAC CGA GAA ACA GT 3′

Oligonucleotide 2–5′ AATAA CTGCA GTGAT CATAT GACAA GATGT GT 3′

The model for each challenge day (T1 and T3) was a completely random design with bird as the experimental unit. So each challenge day was separately analysed as a completely random design with only two treatments. So there was only one possible contrast for each day. All hypotheses were conducted with significance of $P \leq 0.05$ using a two-tailed test. For positive liver/spleen testing, Fisher's exact test was used. The counts for SH colonies in the caecum (CFU/g) were analysed using a generalized linear model (negative binomial distribution). Statistical analysis of body weights was done by F test at $P \leq 0.05$ using software SAS version 9.4, SAS Institute Inc., Cary, NC, USA.

Results

Samples taken from the environment, from the birds on the first day, and from the feed used in the

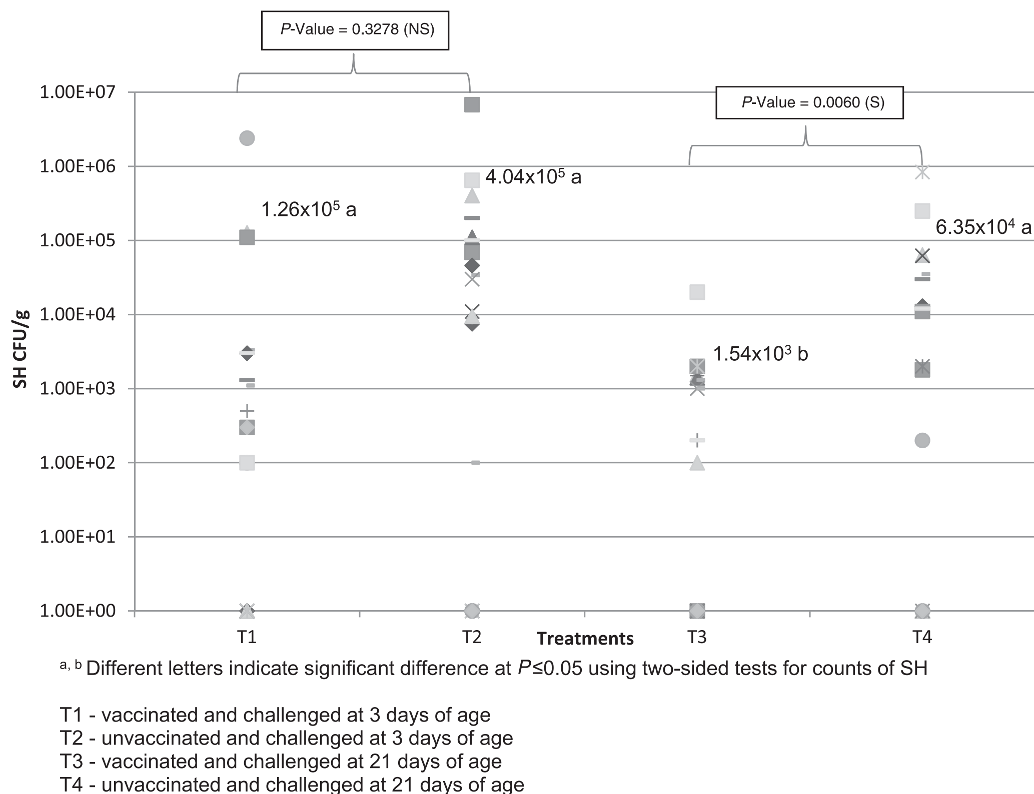

Figure 1. Average and distribution of SH counts in samples collected from broiler caeca at 28 days of age in the different groups (CFU/g caecum).

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Table 2. Average of the quantitative counts (CFU) and prevalence (positive %) of SH in the caecum on day 28.

Treatments	Average of the quantitative counts (CFU/g)	Prevalence (positive %)
1 – Vaccinated and challenged at 3 days of age	1.26×10^5	12/20 (60.0%)
2 – Unvaccinated and challenged at 3 days of age	4.04×10^5	14/20 (70.0%)
3 – Vaccinated and challenged at 21 days of age	1.54×10^3	11/20 (55.0%)
4 – Unvaccinated and challenged at 21 days of age	6.35×10^4	12/20 (60.0%)

experiment were all negative. The counts in the caecum on day 28 are shown in Figure 1. The count values for these assessments are presented in CFU per gram of caecum. The count results $<1.0 \times 10^2$ in caecum are expressed as zero in Figure 1 because of the detection limits of the technique used (count below 100/CFU/g). Samples with a negative culture result were assigned a value of 0 CFU/g in the statistical analysis. The average of quantitative counts and qualitative results in the caeca in different treatments are presented in Table 2. Table 3 presents the results of the qPCR used to detect the vaccine strain in groups 1 and 3 in order to investigate shed bacteria. Faecal swabs taken at 2, 7, and 12 days after vaccination revealed that orally vaccinated chickens were excreting the vaccine only at the first two sampling times. Table 4 presents the statistical assessments for isolation in spleen/liver and for body weight on day 28. SH isolation in spleen/liver is presented as positive/negative and not as bacterial burden because of difficulties in counting the low levels of contamination in viscera compared with caecum. The qualitative method was preferred to the quantitative method for spleen/liver analysis because many samples with low SH load would be considered negative as a function of the limit of the technique used (quantification only from 100 CFU/g). Normally for SH, the level of contamination in caecum is higher than in spleen/liver (Borsoi *et al.*, 2011).

Table 3. Detection of Poulvac® ST vaccine strain using real-time PCR of cloacal swab samples collected from broilers at different times after vaccination.

Time	Treatment 1	Treatment 3
3 days after (1st dose) vaccination (D03)	6/8 (75%)	6/8 (75%)
7 days after (1st dose) vaccination (D07)	6/8 (75%)	8/8 (100%)
12 days after (1st dose) vaccination (D12)	0/8 (0%)	0/8 (0%)

Discussion

Vaccination is the prominent means of controlling non-typhoidal *Salmonella* sorovars; both inactivated and live vaccines are widely adopted by the poultry industry according to regional challenges and governing legislation (Van Immerseel *et al.*, 2005; de Freitas Neto *et al.*, 2008; Majowicz *et al.*, 2010; Berghaus *et al.*, 2011). However, the limited ability of vaccines to act on the different serotypes that exist in the field is well known, especially in the case of inactivated vaccines, which have a more specific action (Penha Filho *et al.*, 2009). Furthermore, live vaccines offer broader protection that in turn grants more comprehensive control, since cross-immunity may occur, depending on the similarities between the involved serovars (Hassan & Curtiss, 1994). The results presented in Table 4 demonstrate the existence of a cross-immunity conferred by Poulvac® ST, which significantly reduced positive results in the liver/spleen of birds that were experimentally inoculated at 3 days of age with a Brazilian strain of SH when compared to the control group. Studies with birds and other species have demonstrated cross-immunity of live vaccines among different serovars and the highest levels of protection are found when the serovars involved have similarity between their immunizing antigens (Heithoff *et al.*, 2001; Mohler *et al.*, 2008). Cross-protection of live vaccines is an important benefit for poultry farmers, particularly in this case, where it eliminates the need for another SH-specific vaccine.

However, when we analysed the SH count in the caeca of birds challenged at 3 days of age (Figure 1), we found that the existing numerical reduction was not statistically significant, when compared to the control. This result agrees with studies that demonstrated a direct correlation between earlier challenge and greater difficulty in controlling infection through vaccination (Poppe, 1999). As the vaccination occurred very shortly before challenge, it is possible that the protection noted was due also to a non-specific increase in innate immunity in response to the vaccination. In this case as the challenge was very early, competitive exclusion effect seems to have more importance (Van Immerseel *et al.*, 2005). This short window is not sufficient for the birds to produce a complete adaptive immune response. There will be no specific immunity (such secretory IgA) at this time point. This reinforces

Table 4. Average body weight and SH presence (%) (conventional isolation methodology) in broilers liver and spleen at 28 days of age (groups 1, 2, 3, and 4).

Treatments	Average weight (g) at placement	Average weight (g) at 28 days	*P*-value/CV (%)	Liver/Spleen positive (%)	*P*-value
1 – Vaccinated and challenged at 3 days of age	48.63	1435.6 a	0.7885/10,24	0/20 (0.00) b	0.0033
2 – Unvaccinated and challenged at 3 days of age	48.54	1423.1 a		8/20 (40.00) a	
3 – Vaccinated and challenged at 21 days of age	48.90	1449.0 a	0.9321/12,67	1/20 (5.00) a	0.1818
4 – Unvaccinated and challenged at 21 days of age	48.77	1441.1 a		5/20 (25.00) a	

Notes: Different letters indicate significant difference at $P \leq 0.05$ using *F* test for average body weight or differ statistically by Fisher's exact test for liver/spleen positive presence of SH in two-sided alternative hypothesis.

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the fact that birds must be immunized as soon as possible before challenge, during the first days of life, so that they have time to develop immunity. This is often difficult, since production environments are often contaminated and flocks can be challenged as soon as they are housed (personal communication of Manfio, 2012).

Moreover, the statistically insignificant results of the samples taken from the caeca of the birds challenged at 3 days (Figure 1) also corroborate that control of *Salmonella* infection within the intestine is harder to achieve than in the case of infection in the viscera, since the reach of the immune response depends on local mucosal immunity. Secretory IgA participates directly in local immunity, since this antibody is secreted into the mucosal surface of the intestine and helps reduce bacterial colonization in the intestinal lumen (Pasetti *et al.*, 2011). In addition, cell-based immune responses where CD8+ T cells are activated as the result of live vaccines are more effective when compared to high levels of antibodies in the serum of birds that were inoculated with inactivated vaccines (Penha Filho *et al.*, 2012). Experiments with inactivated vaccines have shown significant reduction of colonization by *Salmonella* in organs, but the intestinal tract may still be susceptible to colonization. Therefore, it is more difficult to prevent colonization in this organ than in others (Adriaensen *et al.*, 2007).

The SH count in the vaccinated group, which was challenged at 21 days of age, was significantly less ($P \leq 0.05$) than in the control group (Figure 1), showing the vaccine's ability to also reduce intestinal colonization, since two doses were given before the challenge. Both the innate immune system and the specific immunity conferred by vaccination with live vaccine help prevent intestinal colonization, and the later the challenge takes place, the greater the effectiveness of the vaccination programme (McSorley, 2014). Although the differences in prevalences between the vaccinated and control groups were not statistically significant, there was a reduction in the percentage of positive liver/spleen results in birds inoculated at 21 days of age, indicating a beneficial trend from the use of the vaccine to reduce visceral contamination (Table 4). Insufficient colonization of the unvaccinated group (only 25%) was a limitation of this study, which, if higher, could have brought the significance values below the 0.05 threshold.

The mutation of the *aro*A gene of the Poulvac® ST vaccine resulted in functional loss of phosphoenolpyruvyl-shikimate-5-phosphate synthase, an enzyme required for biosynthesis of aromatic amino acids. This auxotrophic mutation can be compensated *in vitro* by supplementation of phenylalanine, tryptophan, and tyrosine. However, since these amino acids are not available for the *Salmonella* vaccine in the host tissue, this results in attenuation during growth *in vivo* (Coloe *et al.*, 1994). Consequently, the Poulvac® ST vaccine shows self-limiting behaviour where it remains viable in the bird for a restricted time, but long enough to trigger a cellular immune response. Analysis using specific qPCR in this experiment showed the vaccine's ability to survive for a limited time in the intestines of the birds (Table 3). These data agree with research conducted on the original STM-1 strain of the vaccine (Alderton *et al.*, 1991).

After a period of 12 days post-vaccination of first dose, the birds were not detected as bearers of the agent from the Poulvac® ST vaccine (Table 3). It was possible to detect the "vaccine uptake" up to the seventh day of life in the birds when the specific PCR was conducted on Poulvac® ST vaccine. This length of time would be sufficient to stimulate local and systemic immunity, preferably prior to the field challenge. In addition, studies show that during the time that live vaccines remain in the intestine, there is a potential mechanism of protection by bacteriological competitive exclusion (Van Immerseel *et al.*, 2005). Future studies should investigate if live vaccines mimic field infection, invading the host organism and reaching internal organs in the same way the field strain does. For some time, great efforts have been made towards developing this type of vaccine with gene deletion because even though these are live salmonellas and therefore retain the field challenging behaviour during initial infection, they are also extremely safe as they do not persist in poultry or even in the environment for long periods of time (Hoiseth & Stocker, 1981; Barrow *et al.*, 1990; Hormaeche *et al.*, 1991; Cooper *et al.*, 1994; Tan *et al.*, 1997; Van Immerseel *et al.*, 2002). Thus, the great advantage of genetically modified live vaccines is the low risk of reversion to virulence in comparison to vaccines made from attenuated rough strains, for example (Frey, 2007; Matsuda *et al.*, 2010; Penha Filho *et al.*, 2010; Matsuda *et al.*, 2011a, b; Shehata *et al.*, 2013; Van Immerseel *et al.*, 2013).

In the assessed experimental conditions, there was no statistical difference ($P > 0.05$) for the variable weight in the different treatments, even in birds that were only challenged with SH (Table 4). These results are in agreement with recent studies indicating that various non-typhoidal *Salmonella* serovars are highly adapted to the host and do not cause a reduction in performance parameters (Setta *et al.*, 2012; Muniz *et al.*, 2015). This asymptomatic behaviour makes the control of these serovars more difficult because contaminated flocks generally are infected without demonstrating clinical signs that are visible to producers.

The results of this experimental study demonstrate that genetically modified live vaccines can be a useful and safe tool for controlling SH in poultry. Vaccination with Poulvac® ST was able to partially decrease the bacterial load of SH in both the caecum and liver/spleen

after oral challenge. However, it is important to know their characteristics and limitations, in order to use them effectively. According to the results of this trial, the efficacy of cross-protection to SH depends on at least two applications of the vaccine. Future studies are recommended to indicate the duration of this immunity, as the test was only conducted 7 days following vaccination.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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