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Efficacy of several vaccination programmes in commercial layer and broiler breeder hens against experimental challenge with *Salmonella enterica* serovar Enteritidis

Rafael Antonio Casarin Penha Filho[1], Jacqueline Boldrin de Paiva[1], Yuli Melisa Sierra Argüello[1], Mariana Dias da Silva[1], Yannick Gardin[2], Fernando Resende[3], Angelo Berchieri Junior[1] and Luiz Sesti[3]*

[1]*Avian Pathology Group, FCAV-Unesp, Jaboticabal-SP 14884-900, Brazil,* [2]*CEVA SANTÉ ANIMALE – Libourne Cedex 35501, France, and* [3]*CEVA SAÚDE ANIMAL – Paulínia-SP 13140-000, Brazil*

Two experiments were performed to evaluate the protective effect of various vaccination combinations given at 5 and 9 weeks of age against experimental challenge with *Salmonella enterica* serovar Enteritidis (SE) phage type 4 at 12 weeks of age. In Experiment 1, groups of commercial layers were vaccinated by one of the following programmes: Group 1, two doses of a SE bacterin (Layermune SE); Group 2, one dose of a live *Salmonella enterica* serovar Gallinarum vaccine (Cevac SG9R) followed by one dose of the SE bacterin; Group 3, one dose of each of two different multivalent inactivated vaccines containing SE cells (Corymune 4K and Corymune 7K; and Group 4, unvaccinated, challenged controls. In Experiment 2, groups of broiler breeders were vaccinated by the same programmes as Groups 1 and 2 above while Group 3 was an unvaccinated, challenged control group. All vaccination programmes and the challenge induced significant ($P < 0.05$) seroconversion as measured by enzyme-linked immunosorbent assay. Overall, in both experiments, all vaccination schemes were significantly effective in reducing organ (spleen, liver and caeca) colonization by the challenge strain as well as reducing faecal excretion for at least 3 weeks. Vaccinated layers in Groups 1 and 2 and broiler breeders in Group 2 showed the greatest reduction in organ colonization and the least faecal excretion. In Experiment 1, layers vaccinated with multivalent inactivated vaccines containing a SE component (Group 3) were only moderately protected, indicating that such a vaccination programme may be useful in farms with good husbandry and housing conditions and low environmental infectious pressure by *Salmonella*.

Introduction

Salmonella infections may be responsible for chronic and acute diseases in poultry, but in the field are usually subclinical, leading to a risk of food poisoning in consumers of contaminated poultry products. Infections occur frequently and are difficult to control, especially in countries with intensive large-scale industrial production (World Health Organization & Food and Agriculture Organization of the United Nations, 2002; Rosu *et al.*, 2007).

Salmonella enterica serovar Enteritidis (SE) is considered to be a zoonotic agent commonly found in domestic poultry and has been responsible for many outbreaks of human salmonellosis through consumption of contaminated food, especially those prepared with raw eggs or other poultry products (Rodrigue *et al.*, 1990; Duguid & North, 1991; Morse *et al.*, 1994; Santos *et al.*, 2000; Altekruse *et al.*, 2006; Humphrey, 2006; Gast, 2007). Although some strains of SE phage type 4 (PT4) may cause increased morbidity and mortality in broilers (Barrow *et al.*, 1987; Poppe, 2000), birds often have subclinical infection and the organism can be disseminated readily by vertical and horizontal routes (Guard-Petter, 2001). Therefore it is necessary to implement monitoring programmes based on bacteriological and serological testing to prevent the infection or to control dissemination of the organisms. Additional measures such as competitive exclusion, heat treatment of feed, incorporation of organic acids in feed, control of rodents, and so forth, can be taken as part of a comprehensive biosecurity programme (Iba & Berchieri Jr, 1995; Patterson & Bukholder, 2003).

Vaccination of layer and broiler breeder hens could contribute significantly to reducing *Salmonella* numbers in the table egg industry and broiler processing plants (Collard *et al.*, 2008). Vaccination might afford protection for the entire productive life of the birds (Mastroeni *et al.*, 2000), reduce the duration and severity of infection and help to prevent reinfection (Gast, 2007). Cogan &

*To whom correspondence should be addressed. Tel: +55 19 8136 0569. Fax: +55 19 3833 7722. E-mail: luiz.sesti@ceva.com
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Humphrey (2003) reported that cases of human salmonellosis due to food poisoning decreased significantly in the United Kingdom after the implementation of a widespread vaccination programme in commercial layers.

Gast *et al*. (1992) observed a rapid primary humoral response after vaccination of specific pathogen free chickens with a killed oil-emulsion SE vaccine, and there was partial protection against experimental challenge with SE. The presence of the challenge strain in organs and egg contents was reduced, but the enteric tract was still susceptible to colonization. It seems that such colonization is more difficult to prevent than that of internal organs (Hassan & Curtiss, 1996; Adriaensen *et al.*, 2007). Gast *et al.* (1993) reported that two different oil-emulsion SE bacterins significantly reduced faecal shedding 1 week after SE challenge. However, no protective effect could be measured by the second week because intestinal colonization by the challenge strain had reached such low levels in all groups.

The efficacy of the bacterins depends upon several factors such as stress conditions (Nakamura *et al.*, 1994) and the composition of the vaccine (Liu *et al*., 2001). Freitas Neto *et al*. (2008) observed variable performance among three commercial SE killed vaccines. A recent study by Davies & Breslin (2004) supports earlier observations that the maximum performance of a vaccination programme is obtained when associated with very strict on-farm hygiene measures. Several studies have shown that vaccines can reduce faecal shedding and systemic spread of SE in laying hens, and thus reduce egg contamination (Miyamoto *et al.*, 1999; Woodward *et al.*, 2002; Davies & Breslin, 2004; Gantois *et al.*, 2006; Collard *et al*., 2008).

Live vaccines against *Salmonella* are able to induce cell-mediated and humoral immune responses (Babu *et al.*, 2003; Rana & Kulshreshtha, 2006). Although killed SE bacterins do not promote a full immune response, they are considered safer because there is no risk of reversion to virulence, no spread in the environment and they are good enough, in general, to protect chickens when applied in large-scale poultry production (Barrow *et al.*, 1991; De Buck *et al*., 2004). Alternatively, it has been suggested that the utilization of an attenuated rough strain of *Salmonella enterica* serovar Gallinarum (SG9R) may be a safe and very effective way of controlling SE infections in chickens (Tan *et al*., 2008a,b; Witvliet *et al.*, 2008). This strain has been shown to be non-pathogenic for chickens (Smith, 1956; Gordon & Luke, 1959; Gordon *et al.*, 1959; Gupta & Mallick, 1976; Smith & Tucker, 1980) and has no public health significance as it cannot cause infection in humans. Lee *et al.* (2005)) demonstrated that SG9R was able to control the systemic infection by *Salmonella* Gallinarum, drastically reducing the morbidity and mortality rates among vaccinated birds. In field studies, Feberwee *et al*. (2001a,b) observed that SG9R contributed to the reduction of SE in layer flocks and, since the vaccine strain could not be detected in the faeces or eggs, it could be considered that it offers minimum risk for the environment.

The present study assessed the efficacy of commercial killed SE vaccines alone or in combination with a commercial live SG9R vaccine in controlling an experimental SE challenge.

Materials and Methods

Birds. Chickens from a commercial genetic strain of table egg layers (Dekalb white; Granja Planalto, Uberlândia-MG, Brazil) were used in Experiment 1, and female birds from a commercial genetic strain of broiler breeders (Cobb 500; Cobb-Vantress Brasil, Guapiaçú-SP, Brazil) were used in Experiment 2. They were obtained at 1 day of age and were reared and fed as recommended in the respective production manuals.

On arrival, the *Salmonella*-free status of the birds was checked by sampling the transport boxes with drag swabs for detection of *Salmonella* spp. (Zancan *et al*., 2000). All samples tested were negative.

Experiment 1 was carried out first and then a downtime period of 4 weeks was allowed for cleaning and disinfection before the start of Experiment 2. The birds were reared in the same house until challenge. Each group consisted of 36 birds, equally divided into separate sets of six cages. Empty cages were placed in between the groups so there was no contact between birds of different groups, facilitating the daily husbandry, cleaning and disinfection of the house. Two weeks before challenge, the groups were moved to separate rooms with controlled ambient conditions.

Vaccines. Commercial vaccines produced by CEVA-Phylaxia (Cevac Corymune 4K and 7K; Budapest, Hungary), CEVA Biomune (Layermune SE; Lenexa,USA) and CEVA Campinas (Cevac SG9R; Campinas, SP, Brazil) were used. Cevac Corymune 4K contains an inactivated combination of *Avibacterium paragallinarum* serotypes A, B and C, an SE strain, homogenized with aluminium hydroxide adjuvant and thiomersal as a preservative. Cevac Corymune 7K is as above but with the addition of Newcastle disease virus (La Sota strain), infectious bronchitis virus (Massachusetts strain) and egg drop syndrome virus (B8/78 strain). Layermune SE is an inactivated bacterial vaccine (bacterin) containing multiple selected strains of SE in oil adjuvant. Cevac SG9R contains live *Salmonella* Gallinarum strain 9R in freeze-dried form. The vaccine is at a concentration of at least 10^7 colony-forming units per dose and is naturally attenuated and non-pathogenic for chickens.

Vaccines were administered either intramuscularly in the breast muscle (Cevac Corymune 4K and 7K and Layermune SE) or subcutaneously in the dorsal lower part of the neck (Cevac SG9R) as recommended by the manufacturer.

Challenge strain and preparation of inocula. The inocula were prepared from a strain of *Salmonella* Enteritidis PT4, kindly donated by Professor P.A. Barrow (University of Nottingham, Leicestershire, UK) and previously shown to be invasive in laying hens (Barrow & Lovell, 1991; Freitas Neto *et al*., 2008; Inoue *et al.*, 2008). For ease of enumeration in organs and faeces, a spontaneous nalidixic acid and spectinomycin-resistant mutant of this strain ($SENal^rSpec^r$) was used. The inocula consisted of a culture of $SENal^rSpec^r$ in Luria-Bertani (LB) broth (CM0395 and LP0121A; Oxoid) grown for 24 h in a shaking incubator (100 rev/min) at 37°C. This culture contained approximately 8×10^8 colony-forming units/ml. At 12 weeks of age all birds were inoculated orally, directly into the crop, with 2 ml (Experiment 1) or 3 ml (Experiment 2) of the $SENal^rSpec^r$ culture. The different volumes allowed for differences in body weight of the two types of bird and had been established by earlier pilot experiments.

Experimental design. The designs of both experiments are shown in Table 1.

Experiment 1. One hundred and forty-four commercial table egg layers were divided into four groups of 36 birds. Groups 1, 2 and 3 were immunized at 5 weeks (first dose) and 9 weeks (second dose) of age. Group 1 received two doses of Layermune SE, intramuscularly; Group 2 received one dose of CEVAC SG9R subcutaneously in the nape of the neck, and a second vaccination with Layermune SE (intramuscularly); Group 3 received one dose of Corymune 4K (intramuscularly) and a second vaccination with Corymune 7K (intramuscularly); and Group 4 was kept as non-immunized, *Salmonella*-challenged positive controls.

Experiment 2. One hundred and eight broiler breeders were divided into three different groups (36 birds per group). Groups 1 and 2 received the

Table 1. *Vaccination programmes and SE challenge*

Group[b]	Commercial table egg layer vaccination programme[a]	
	First dose (5 weeks)	Second dose (9 weeks)
Experiment 1		
1	Layermune SE	Layermune SE
2	Cevac SG9R	Layermune SE
3	Corymune 4K	Corymune 7K
4	Not vaccinated	Not vaccinated
Experiment 2	Broiler breeders	
1	Layermune SE	Layermune SE
2	Cevac SG9R	Layermune SE
3	Not vaccinated	Not vaccinated

[a]All birds challenged at 12 weeks of age with SENal^{r}Spec^r (layers challenged with 2 ml and broiler breeders with 3 ml containing $\sim 8 \times 10^8$ colony-forming units/ml). [b]Each group contained 36 birds.

same vaccination treatments as the corresponding groups in Experiment 1, and Group 3 birds were kept as non-immunized, *Salmonella*-challenged positive controls.

In both experiments all birds were challenged with a culture of SENal^{r}Spec^r at the age of 12 weeks, and in both experiments five birds from each group were killed by cervical dislocation at each of 2, 5, 7, 14 and 21 days post challenge. Approximately 1g samples of spleen, liver, and caecal contents were removed from each bird for isolation and determination of bacterial count of the challenge strain.

Twice a week, at 3, 6, 10, 13, 17 and 20 days post challenge cloacal swabs were taken from all remaining birds and assayed for faecal shedding of SE. Blood samples from six designated birds in each group were collected weekly for 5 weeks, beginning one week after the second vaccination.

Bacteriology. Bacteriological analyses were carried out as described by Barrow & Lovell (1991) with some modification. Swabs were placed in selenite broth (CM0395 and LP0121A; Oxoid) containing novobiocin (40 μg/ml) (SN) and directly plated onto Brilliant Green Agar (CM0263; Oxoid) containing nalidixic acid (100 μg/ml) and spectinomycin (100 μg/ml) (BGA Nal/Spec). Incubation was at 37°C for 24 h and, in the absence of growth, the appropriate enriched swab cultures were inoculated onto fresh plates.

After the harvesting of spleen, liver and caecal contents, the samples were diluted immediately (1:10) in phosphate-buffered saline, pH 7.4 and homogenized with a pestle and mortar. The viable count of SENal^{r}Spec^r in the samples was measured by plating aliquots of decimal dilutions on BGA Nal/Spec incubated overnight at 37°C. The first dilution of the sample was added to an equal volume of double-strength SN. This was incubated at 37°C overnight and plated on BGA Nal/Spec agar when there was no growth from the viable count assay.

Serology. Sera collected from the blood samples were stored at −20°C until the end of both experiments, when enzyme-linked immunosorbent assays (ELISAs) were carried out on all samples using a single commercial ELISA kit (FlockChek *Salmonella* Enteritidis Antibody Test Kit; IDEXX Laboratories, Westbrook, Maine, USA). Sera were diluted 1:2 and antibody titres were expressed as the sample value/negative control value (S/N) ratio of optical density (OD) values (sample OD value divided by the average OD value for the negative control samples of the ELISA kit). The S/N ratio presents a high correlation with circulating antibody titres; the higher the S/N ratio, the lower the antibody titre (a positive sample must have an S/N ratio < 0.6).

Statistical analysis. SE antibody titres were analysed and compared within and between groups by the Students *t* test (Sigmaplot 8.0 software; http://www.sigmaplot.com). Mean viable numbers of SENal^{r}Spec^r in organ samples taken from the spleen, liver and caeca and cumulative numbers of birds SENal^{r}Spec^r positive on cloacal swabs were analysed and compared between groups by the Tukey test and the chi-square test, respectively. Statistical differences were set at the probability level of $P < 0.05$.

Results

Bacteriology. *Experiment 1.* Figure 1 shows the results of bacteriological cultures for samples of spleen, liver and caecal content. A significant ($P<0.05$) reduction in numbers of SENal^{r}Spec^r was seen in vaccinated birds in Groups 1 and 2 at the earlier time points. Birds in Group 3 showed a numerical reduction when compared with unvaccinated birds in Group 4 but the differences were significant only in caecal contents and only on two occasions.

Figure 2 shows the results for faecal excretion (cloacal swab samples) in the different treatment groups as expressed by the cumulative number of birds testing positive for SENal^{r}Spec^r isolation at different days post challenge. Birds in Groups 1 and 2 always excreted significantly less ($P<0.05$) SENal^{r}Spec^r when compared with birds in Groups 3 and 4. Vaccinated birds in Group 3 also presented a significant reduction in faecal excretion when compared with control birds in Group 4 and overall, Group 3 showed an intermediate reduction in SENal^{r}Spec^r faecal excretion.

Experiment 2. Figure 3 shows the bacteriology results of the experimental groups in Experiment 2 for samples from caecal contents and liver and spleen tissue. As in Experiment 1, vaccinated birds in Experiment 2 showed an evident protection against organ colonization by the SENal^{r}Spec^r challenge strain. The most pronounced and significant ($P<0.05$) protective effect from SE vaccination was seen in birds in Group 2, which were vaccinated with live SG9R vaccine followed by an inactivated SE bacterin.

Bacteriology results from the cloacal swab samples also showed a significant ($P<0.05$) protective effect of vaccination against faecal excretion of the SENal^{r}Spec^r challenge strain (Figure 4). Birds in both vaccinated groups excreted significantly less *Salmonella* in faeces after the heavy experimental challenge.

Serology (Experiments 1 and 2). Levels of circulating antibodies against SE in vaccinated and control birds in both experiments are shown in Figure 5. All vaccinated birds in both experiments were strongly positive for SE antibodies throughout the sampling period and the SENal^{r}Spec^r challenge did not induce any detectable change in their antibody titres. All control birds remained antibody negative for SE until the time of the experimental challenge after which all birds seroconverted within 1 week to levels similar ($P>0.05$) to those of vaccinated birds. For the ELISA assay, serum samples were diluted 1:2 as per the manufacturer's instructions and samples were simply considered either positive or negative. Due to the low sample dilution and qualitative measurement it was impossible to detect a measurable variation in SE antibody positivity among birds in the vaccinated groups. In both experiments, all vaccinated groups presented very low S/N ratios and a very low coefficient of variation (range 15 to 31) throughout. Also, no variation in SE antibody positivity was observed in vaccinated birds even after the SE

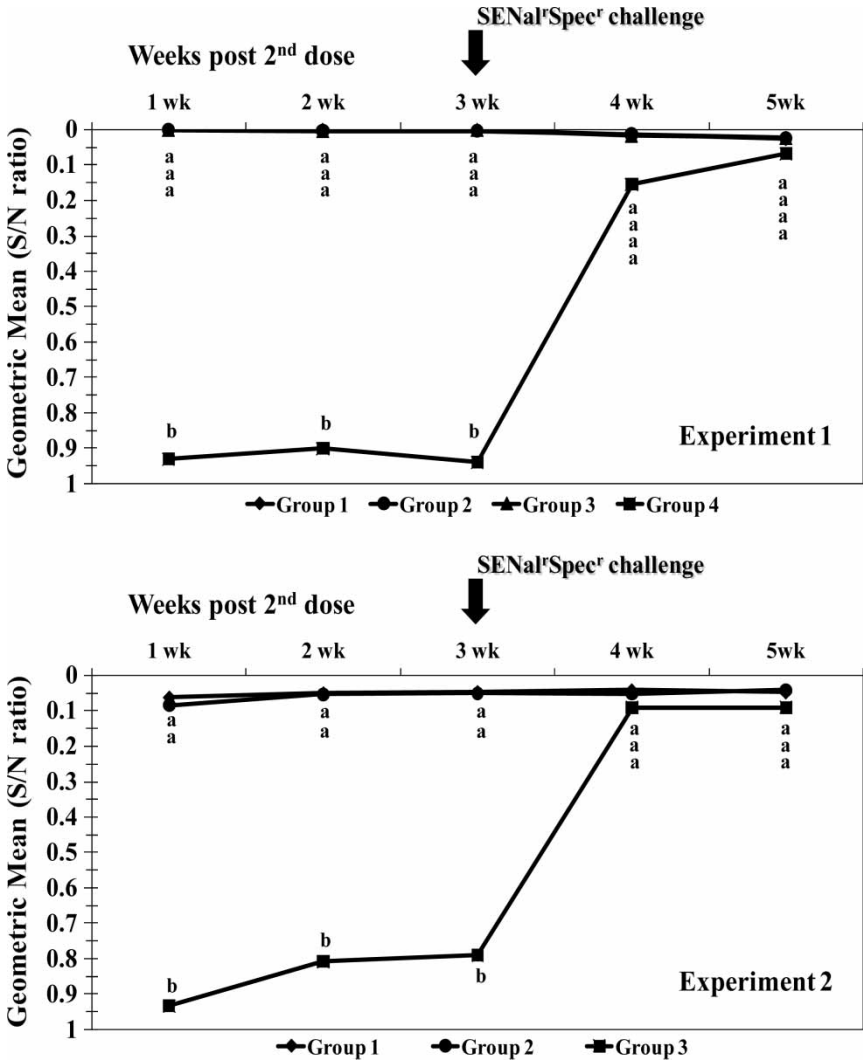

Figure 1. *Experiments 1 and 2. Circulating SE antibody titres measured by ELISA in serum taken weekly for 5 weeks after the second dose of vaccine. Titres are shown as geometric means of the S/N ratio; the higher the S/N ratio value, the lower the antibody titre. Samples presenting S/N ratio <0.6 are positive. Group means with different letters at the same sampling time are statistically different (* $P < 0.05$*).*

challenge, possibly due to already high levels of circulating antibody. A similar picture was observed in both control groups, which were negative until challenge and then showed a significant seroconversion at 1 and 2 weeks after challenge (coefficient of variation range for the S/N ratios at those 2 weeks were 60 to 78 and 51 to 89, respectively). The positive and negative control OD values for the ELISA run were 0.247/0.261 (positive controls) and 1.219/1.257 (negative controls).

Discussion

SE has been disseminated worldwide by poultry through vertical transmission (Wall & Ward, 1999; Zancan *et al.*, 2000; Gast & Holt, 2001). The emergence of this pathogen in the commercial poultry production and the risks to public health posed by *Salmonella* infection (Rodrigue *et al.*, 1990) increased interest in its control (Mead & Barrow, 1990; Zhang-Barber *et al.*, 1999). Once SE is introduced on a poultry farm it can remain in the environment for several months (Gama *et al.*, 2003; Maciorowski *et al.*, 2004), being easily disseminated by vertebrate and invertebrate vectors (Henzler & Opitz, 1992; Davies & Wray, 1996; Davies & Breslin, 2003; Hazeleger *et al.*, 2008).

In view of the complex epidemiology of SE it is economically impractical to eliminate the pathogen from farms. Establishing immunity in commercial flocks would be the most effective way to control infections, but, as with other specific control measures, vaccination alone only produces partial protection and/or control. Although Davies & Breslin (2004) found SE in vaccinated flocks, its presence can be reduced by vaccination (Gast *et al.*, 1993; Woodward *et al.*, 2002), which reduces the duration and the severity of the infection and helps to prevent re-infection (Gast, 2007). This indirectly decreases cases of human food-borne salmonellosis (Collard *et al.*, 2008).

Environmental conditions (stress, poor hygiene, etc.) and poor management practices may interfere with the success of vaccination. Kanashiro *et al.* (2008) demonstrated the extent of such interferences when they assessed the serological response of broiler breeder from commercial flocks and from experimentally vaccinated chickens. All samples from the experimentally vaccinated birds were seropositive while no antibody was

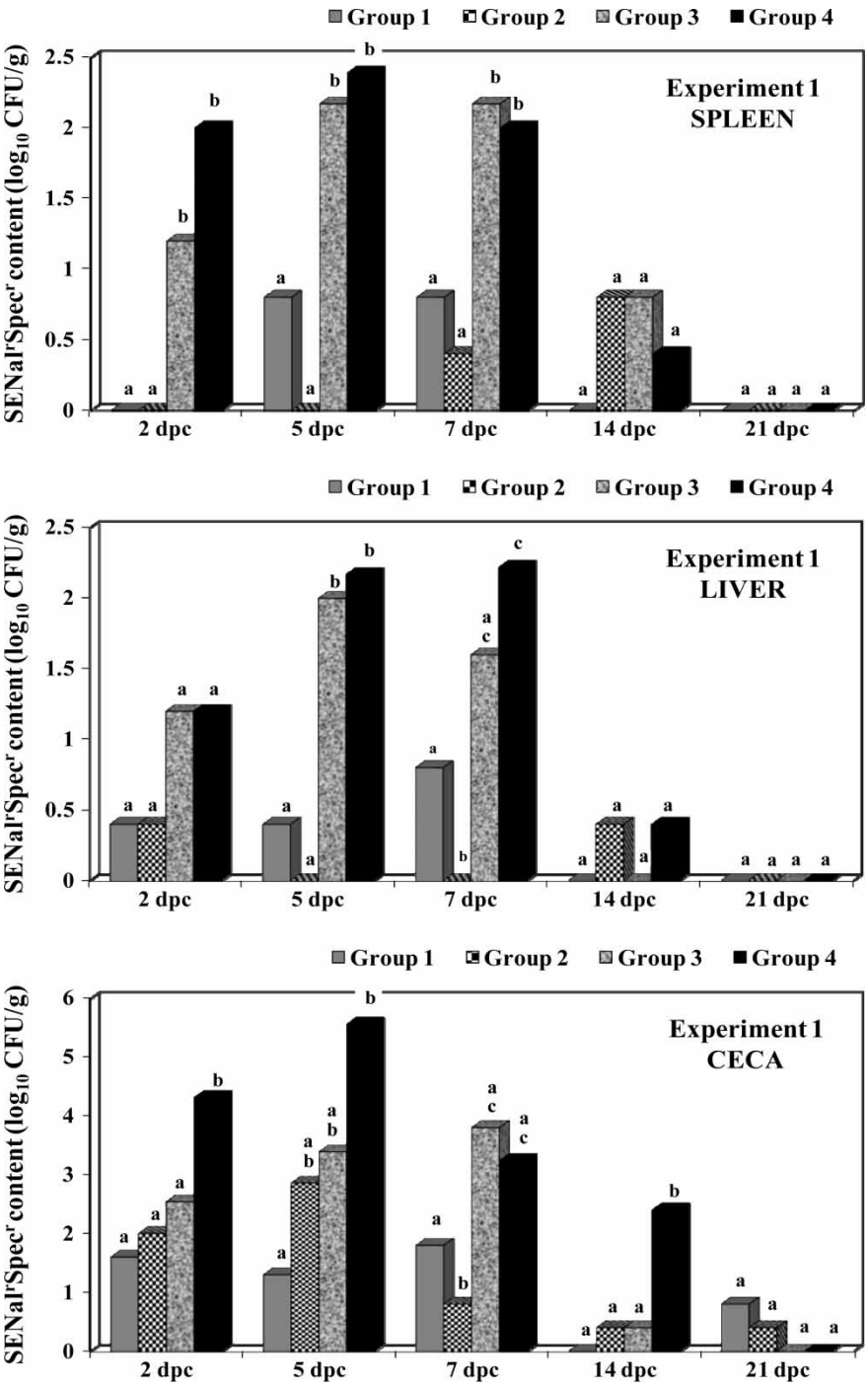

Figure 2. *Experiment 1. Isolation and quantification of SENal^{r}Spec^r challenge strain in spleen, liver and caecal contents. Bars with different letters on the same day post challenge are statistically different (* $P < 0.05$*). CFU, colony-forming units.*

detected in many broiler breeders vaccinated under field conditions, indicating vaccination failure due to poor administration procedure. The composition of the vaccine is also a matter of concern. The reduction in the systemic infection and faecal excretion are very variable (Barbour *et al.*, 1993; Freitas Neto *et al.*, 2008).

A similar situation was seen in the present study. The vaccination programmes with Layermune SE (two doses) and Cevac SG9R plus Layermune SE were superior to Corymune 4K and Corymune 7K, although the latter also reduced faecal excretion, demonstrating the beneficial effect of the vaccination with those multivalent inactivated vaccines containing a SE fraction. Previous studies (Gast *et al.*, 1992; Cooper *et al.*, 1994; Betancor *et al.*, 2005) have reported that vaccines against SE were more effective in reducing systemic infection and had only a poor action on the digestive tract. It seems likely that cell-mediated immunity is responsible for tissue clearance, but how it could be responsible for intestinal clearance remains unclear (Zhang-Barber *et al.*, 1999). Resistance to intestinal *Salmonella* infections may depend more on the humoral response, particularly IgA, and polymorphonuclear cells, although this has not been proven experimentally (Nagaraja & Rajashekara, 1999; Barrow & Wallis, 2000). In Experiment 2, Corymune 4K and 7K vaccines were not used as they were developed for use in commercial layers only. The combination of the live Cevac SG9R plus Layermune SE was more

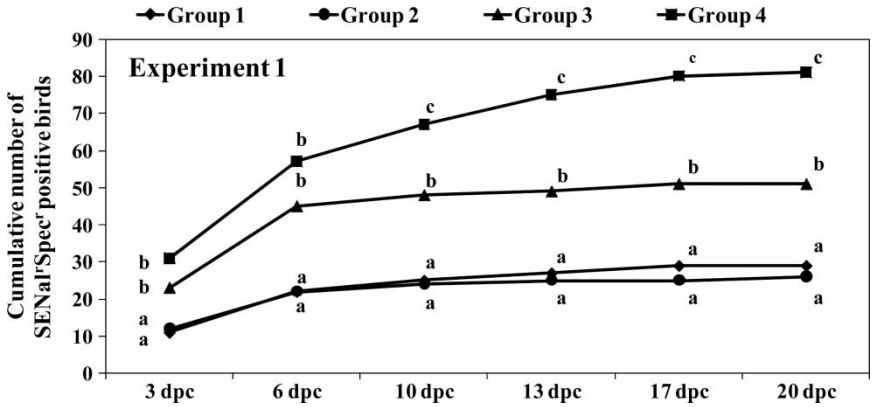

Figure 3. *Experiment 1. Cumulative numbers of birds positive for SENal^{r}Spc^r isolation at each sampling day post challenge (dpc). Different letters at each sampling dpc indicate statistically different totals (*$P < 0.05$*).*

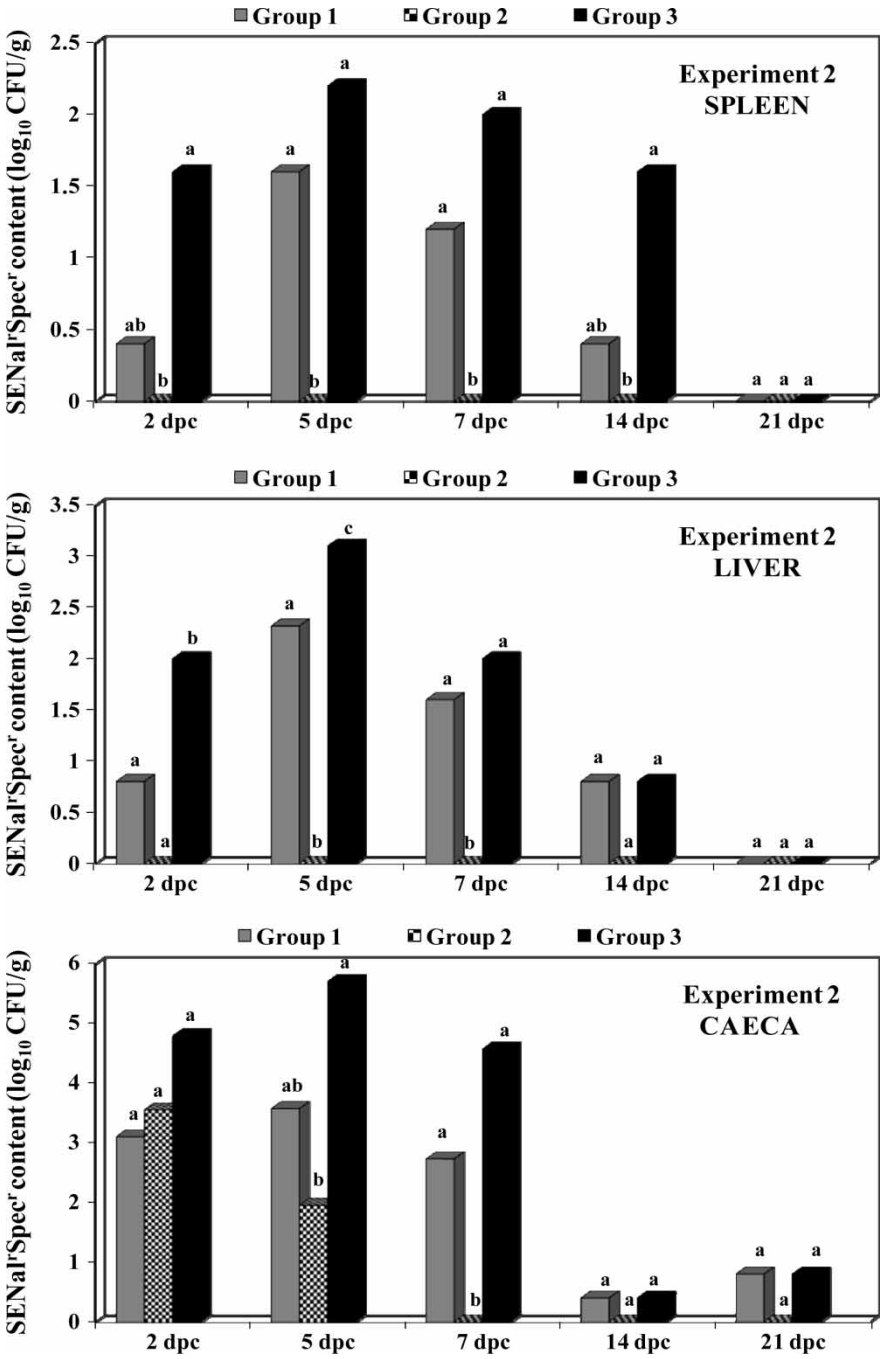

Figure 4. *Experiment 2. Isolation and quantification of SENal^{r}Spec^r challenge strain in spleen, liver and caecal contents. Bars with different letters on the same day post challenge (dpc) are statistically different (*$P < 0.05$*). CFU, colony-forming units.*

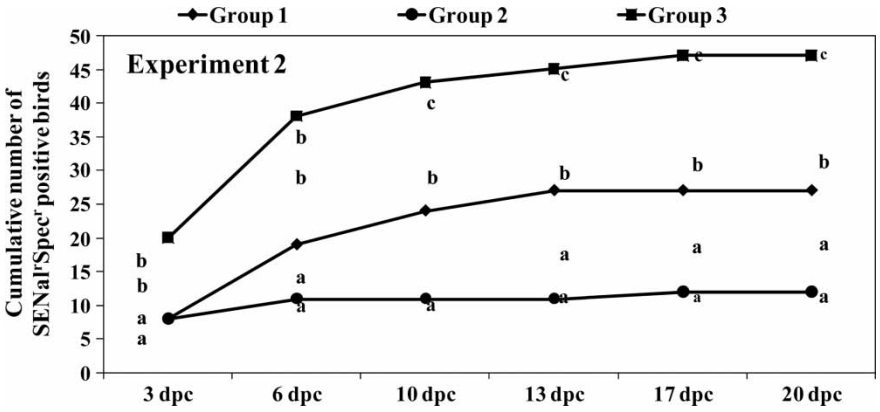

Figure 5. *Experiment 2. Cumulative numbers of birds positive for SENal^{r}Spc^r isolation at each sampling day post challenge (dpc). Different letters at each sampling dpc indicate statistically different totals (*$P < 0.05$*).*

effective than Layermune SE (two doses) in broiler breeders and equally effective in commercial layers. It appears that the use of a live SG9R vaccine in the present study induced a stronger immune response than use of inactivated vaccines only. It is widely accepted that cell-mediated immunity is more important than humoral responses in protection against *Salmonella* infections (Collins, 1974; Mastroeni *et al.*, 1993); therefore, vaccinating the birds with the live SG9R vaccine may have been responsible for the more effective protection against colonization and faecal excretion seen in birds from Group 2 in both our experiments.

The live vaccine (Cevac SG9R) used here contains an apathogenic rough strain of *Salmonella* Gallinarum. Both *Salmonella* Gallinarum and SE belong to the *Salmonella* serogroup Dl, and share the same immunodominant (Ochoa-Repáraz *et al.*, 2004) somatic "O" antigen formula (1,9,12; Ewing, 1986), and therefore reasonable cross-protection between the two serotypes is expected. This theory was confirmed in a large field trial in the Netherlands in which 80 commercial flocks were vaccinated with the SG9R vaccine strain and the flock level of occurrence of SE infections was 2.5% (2/80 flocks), which was significantly less than that in unvaccinated flocks (214 out of 1854 flocks, 11.5%; Feberwee *et al.*, 2001a). No vaccine strain bacteria were detected in 4500 eggs derived from five SG9R vaccinated flocks, while in another study no evidence was found for the faecal spread of the vaccine strain (Feberwee *et al.*, 2000, 2001b).

In the present study, birds with two very different genetic backgrounds were used in Experiments 1 and 2. Although the vaccination programme consisting of the live Cevac SG9R plus the inactivated Layermune SE vaccines was effective in both experiments and the most effective as far as faecal excretion was concerned, the results from organ colonization indicate that a more consistent protection against systemic infection occurred in the broiler breeders. This suggests that besides environmental factors, vaccine composition and bird management, the genetic background of the birds might be influencing the immune response. It is well known that genetic resistance to *Salmonella* infections varies among different lines of domestic chicken (Kaiser *et al.*, 2002).

In general, white lines of layers are much more resistant than heavy broiler breeder lines (Bumstead & Barrow, 1993; Girard-Santosuosso *et al.*, 1998). In addition, there is some evidence indicating that white layer lines may excrete SE for longer than semi-heavy and heavy lines after experimental challenge (Berchieri Jr *et al.*, 2001; A. Berchieri Jr, unpublished data). Genetic selection work carried out on commercial birds does not yet include resistance to *Salmonella* infections. Thus, the best way to prevent or control *Salmonella* infection in poultry flocks is still through the use of sound biosecurity measures. The present study clearly showed that vaccines can also be a useful tool to help decrease SE infection.

Finally, commercial layers in Group 3 in Experiment 1 (vaccinated with Corymune 4K and Corymune 7K vaccines) showed intermediate protection against the experimental SENal^{r}Spec^r challenge. This was most evident in the reduction of faecal excretion of the challenge strain. Such results could be expected because the vaccine is a multivalent inactivated formulation that contains lower SE-antigen concentration. However, such a product could be a very good choice for use in farms with good biosecurity and low environmental infectious pressure by SE.

In conclusion, results from the current experiments indicate that vaccination against *Salmonella* can be an important and effective tool within a comprehensive biosecurity programme designed for successful *Salmonella* control in industrial poultry farms.

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