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Aerosol Spray Treatment with Bacteriophages and Competitive Exclusion Reduces *Salmonella* Enteritidis Infection in Chickens

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SUMMARY. A combination of three different *Salmonella*-specific bacteriophages (BPs) and one competitive exclusion (CE) product were used to reduce *Salmonella* Enteritidis (SE) colonization in experimentally infected chickens. Equal numbers of 7-day-old chickens were used in each of three groups: a CE group (treated with CE), a BP group (treated with BP), and a CE-plus-BP group (treated with both products). The CE product was administered via coarse spray at 1 day of age and the cocktail of three BPs was given via spray at 6 days of age using a multiplicity of infection of 10^3 plaque-forming units. All the experimental groups, except a healthy control group, were challenged orally with 2.95×10^5 colony-forming units (CFU)/ml of an SE strain at 7 days of age. Seven days postchallenge, the chickens were euthanized for individual SE detection, quantitative bacteriology, and phage isolation from ceca and an internal organ pool. The qualitative bacteriology demonstrated that the use of the CE product diminished the incidence of SE to 75.7% and the mixture of BPs reduced it to 80%; when CE plus BP were used, the incidence dropped significantly to 38.7% ($P < 0.0001$), as compared with the infection control group (100%). A significant difference in the incidence was observed between the CE and the CE-plus-BP groups, and the BP and the CE-plus-BP groups ($P = 0.0027$ and $P = 0.0010$, respectively). The mean SE cecal count diminished with the use of CE plus BP (1.6×10^2 CFU/g, $P = 0.0003$) compared with the control group (1.56×10^5 CFU/g), the CE group (4.23×10^3 CFU/g), and the BP group (9.48×10^3 CFU/g). On the basis of the present study, it may be concluded that the use of both types of biocontrollers can be an effective method for reducing SE colonization in commercial chickens, but further basic and applied research is needed.

RESUMEN. El tratamiento por aerosol con bacteriófagos y exclusión competitiva reduce la infección con *Salmonella* Enteritidis en pollos.

Una mezcla de tres diferentes bacteriófagos (BF) específicos contra *Salmonella* y un producto de exclusión competitiva (EC) fueron utilizados para reducir la colonización de *Salmonella* Enteritidis (SE) en pollos infectados experimentalmente. Se utilizaron pollos de siete días de edad distribuidos en tres grupos con igual número de aves cada uno: el grupo EC (tratado con el producto de exclusión competitiva), el grupo BF (tratado con bacteriófagos) y el grupo EC más BF (tratado con ambos productos). El producto de EC fue administrado vía spray al día de edad, y la mezcla de tres bacteriófagos fue administrada por aerosol al día seis de edad, usando una multiplicidad de infección de 10^3 unidades formadoras de placas (UFP). Todos los grupos experimentales, excepto un grupo control sano, fueron desafiados oralmente al séptimo día de edad con 2.95×10^5 unidades formadoras de colonias (UFC)/ml de la cepa SE. Siete días post desafío (PI), se practicó la eutanasia de los pollos para realizar la detección individual de SE, la bacteriología cuantitativa y el aislamiento de fagos en ciegos y muestras de órganos internos procesadas en conjunto. La bacteriología cualitativa demostró que el uso del producto EC disminuyó la incidencia de SE a un 75.7%, la mezcla de bacteriófagos a un 80% y con la aplicación combinada de EC y BF la incidencia se redujo significativamente a un 38.7% ($P < 0.0001$), en comparación con el grupo control de infección (100%). Se observó una diferencia significativa en la incidencia entre los grupos tratados con EC y el tratamiento combinado de EC más BF ($P = 0.0027$), y entre los grupos tratados con BF y el tratamientos con la combinación de EC más BF ($P = 0.0010$). El recuento cecal promedio de SE disminuyó con el tratamiento combinado de EC más BF (1.6×10^2 UFC/g, $P = 0.0003$) comparado con el grupo control (1.56×10^5 UFC/g), con el grupo tratado con EC (4.23×10^3 UFC/g) y con el grupo tratado con BF (9.48×10^3 UFC/g). De acuerdo con el presente estudio, es posible concluir que el uso de ambos tipos de biocontroladores puede ser un método efectivo para disminuir la colonización de *Salmonella* Enteritidis en pollos comerciales, pero más investigación básica y aplicada es necesaria.

Key words: *Salmonella* Enteritidis, competitive exclusion, bacteriophage therapy, probiotics

Abbreviations: BP = bacteriophage; CE = competitive exclusion; CFU = colony-forming units; LB = Luria Bertoni; MOI = multiplicity of infection; nal^r = nalidixic acid resistant; PI = postchallenge; PFU = plaque-forming units; rif^r = rifampicin resistant; SE = *Salmonella* Enteritidis; XLD = xylose-lactose-deoxycholate

The increase of *Salmonella enterica*, together with the emergence of strains resistant to multiple antibiotics, have led to greater efforts being devoted to the search for new methods to control *Salmonella* colonization that could be used in poultry and egg production, with a consistent positive impact on public health (11).

Preventing *Salmonella* from infecting flocks requires high biosecurity standards including rodent and insect control, control of personnel and equipment movement, and *Salmonella*-free feed (11). Others measures have been studied, including vaccination (4), administration of prebiotics (27), administration of probiotics or competitive exclusion (CE) products (14,25,28), and most recently, the administration of bacteriophages (BPs) (2,7,9,16,30), but none of these alone has been shown to be 100% effective in preventing *Salmonella* infections in poultry.

The use of probiotics exerts beneficial effects in the control of intestinal bacterial pathogens given that they possess the ability to alter the composition of gut microflora by different mechanisms (1,18,28) and can be administered via coarse spray (23), by means of drinking water (6), and *in ovo* (18). The product has been

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Table 1. Experimental design.

Group	Number of birds	Treatment
1	33	SE-infected[A] (positive control)
2	33	Uninfected and nontreated (negative control)
3 (BP)	33	SE-infected[A] and BP-treated[B] by aerosol spray
4 (CE)	33	SE-infected[A] and CE-treated[C] by coarse spray
5 (BP plus CE)	33	SE-infected,[A] BP- and CE-treated[BC] by spray

[A]Infected by oral inoculation with 2.95×10^5 CFU/ml at 7 days of age.
[B]10^8 PFU/ml/doses of each of three phages (MOI 10^3) delivered at 6 days of age (two daily doses).
[C]CE product administered at 1 day of age by coarse spray.

administered as a prophylactic rather than a therapeutic measure (10,24) in those trials where its efficacy has been demonstrated. Several probiotics have been used to control *Salmonella* infection in poultry, as a colostrum probiotic (3), *Lactobacillus salivarus* CTC2197 (22), FM-B11 probiotic (different species of *Lactobacillus* and *Pediococcus parvulus*) (8), and LAB probiotic (commercial lactic acid bacteria-based probiotic culture) (14).

On the other hand, the early idea of using lytic BPs in order to kill different bacterial strains has proven to be efficient only in some cases (2,7,8,9,12,26,30). These suggest that BPs could be useful as therapeutic or prophylactic agents against *Salmonella*. The combined use of probiotics and BPs has been described against *Salmonella* Enteritidis (SE) (8) and *Salmonella* Typhimurium (30) in chickens. Both studies showed reduction in *Salmonella* colonization.

We observed promising results in a previous study (7) with mixed BPs administered via aerosol spray in specific-pathogen-free chickens infected with SE. Based in these results, this study was designed to evaluate the efficacy of combining BP and CE for reducing *Salmonella* colonization in commercial chickens.

MATERIALS AND METHODS

Chickens. Day-old white leghorn chickens were obtained from a *Salmonella*-free commercial flock and housed in a controlled environment under strict biosecurity. To ensure that the experimental birds remained free of natural infection, feces were obtained at 2 days of age and tested for *Salmonella* by PCR, as described by Malorny *et al.* (17), and by conventional culture methods (described below). Feed and water were supplied *ad libitum*. Feed was negative for *Salmonella* by culture and PCR. All chicken trials were performed following international animal welfare regulations approved by the Institutional Animal Care and Use Committee.

Bacterial challenge strain. An SE strain, originally obtained from a laying hen and kindly supplied by Dr. I. Acevedo (Laboratory of Bacteriology, Agriculture and Livestock Service, Ministry of Agriculture, Chile) was used. A spontaneous nalidixic acid–resistant (nal^R) and rifampicin-resistant (rif^R) mutant was used as the challenge strain.

Isolation, propagation and characterization of BPs. Three different lytic BPs were used, as previously described by us (7). Briefly, BPs were isolated from the sewage systems of commercial chicken flocks. One milliliter of a diluted sample was mixed with 10 ml of Luria Bertoni (LB), rifampicin (100 μg/ml), and 0.5 ml of an exponential growth-phase culture of SE ATCC 13076 (rifampicin-resistant mutant), and incubated for 24 hr at 37 C. After overnight shaking, 1 ml was centrifuged for 5 min at $9300 \times g$ and the resulting supernatant was treated with chloroform and then plated on a SE ATCC 13076 culture using the double-agar layered method. Plaques that formed on the plates after incubation at 37 C were stabbed with a platinum loop and replicated on the same SE ATCC strain in LB broth. This procedure yielded phage stocks in concentrations higher than 10^{10} plaque-forming units (PFU). Each of the phages was diluted in distilled water at a multiplicity of infection (MOI) of 10^3 PFU.

Competitive exclusion. Broilact (Orion Corporation, Espoo, Finland), a commercial CE product designed to introduce the protective properties of normal adult intestinal microflora into highly pathogen-susceptible chicks was used to treat newly hatched chicks. The product was reconstituted in phosphate-buffered saline and administered via coarse spray to the chickens at 1 day of age. The CE product consisted of a well-defined bacterial probiotic containing anaerobic bacteria (4.4×10^{10} colony-forming units (CFU)/g), *Enterococcus* spp. (1.0×10^9 CFU/g), *Lactobacillus* spp. (2.3×10^7 CFU/g), and nonpathogenic coliform bacteria (4.4×10^9 CFU/g).

Experimental infection. Seven-day-old chicks were used to determine if BP therapy in combination with CE reduces *Salmonella* incidence and *Salmonella* intestinal colonization (Table 1). The animals were divided into five groups, which are described in Table 1. Day 7 postchallenge (PI), chickens were euthanized by cervical dislocation (5), and ceca, spleen, and liver samples were collected aseptically during necropsy for bacterial detection (bacterial culture) and BP isolation. The spleen and the liver were analyzed as a pool. Quantitative bacteriology (CFU/g) was performed on cecal samples from 15 chickens per group. All of the birds were weighed at 1 day of age (prior to administration of probiotics) and at the end of the study (14 days of age).

Bacteriology. Samples were weighed and transferred into a sterile plastic bag containing Rappaport–Vassiliadis broth (RV; Difco, Le Pont de Claix, France; 1:100), homogenized for 3 min, and incubated at 37 C for 24, 48, and 72 hr (1 ml was separated before the incubation to quantitative bacteriology). After the initial incubation, samples were streaked onto xylose-lactose-deoxycholate (XLD) agar supplemented with nalidixic acid and rifampicin (20 μg/ml), and incubated at 37 C for 24 hr. Black colonies were serologically confirmed with *Salmonella* O antiserum, poly A-I Vi (Difco). Negative samples were frozen and processed for genome detection by PCR. A bird was considered infected when it was positive to SE isolation in either the cecal or pooled organs samples.

Quantitative bacteriology was performed from 1 ml of cecal samples that had been diluted in RV broth prior to incubation. These samples were diluted 10-fold in Oxoid buffered peptone water (Cambridge, England) and 1 ml of each serial dilution was mixed onto XLD agar containing nalidixic acid and rifampicin (20 μg/ml). The XLD plates were incubated at 37 C for 24 hr before the typical *Salmonella* colonies were counted. When accurate colony counts were not possible, appropriate dilutions were prepared from the samples incubated for 24 hr at 37 C.

PCR. Following Malorny *et al.* (17), PCR was performed using the following primers to target the *invA* gene sequence from *Salmonella* spp.: *InvA1*: 5′-GTG AAA TTA TCG CCA CGT TCG GGC AA-3′ (26 bp) and *InvA2*: 5′-TCA TCG CAC CGT CAA AGG AAC C-3′ (22 bp). Commercially available DNA extraction kits (Fermentas, Vilnius, Lithuania) were used to perform DNA extraction from frozen Rappaport-Vassiliadis–negative samples. The reaction mixture contained 5 μl of sample DNA, 5 μl of each primer, and 12.5 μl of a master mix (Fermentas) comprising dNTPs, *Taq* polymerase, buffer, and $MgCl_2$. The cycler was adjusted to the following temperature profile: initial denaturation 1 min, 95 C; denaturation 30 sec, 37 cycles of 95 C; hybridization 30 sec, 64 C; and extension 30 sec, 72 C. This was followed by a final extension cycle of 72 C for 4 min. The PCR amplicon (284-bp fragment) was detected by electrophoresis (2%

Table 2. Isolation of SE from experimental groups treated with only BPs, only CE, and BPs plus CE.

Groups[A]	Number of chickens[B]	SE-infected chickens (%)[C]	SE-positive liver/spleen (%)	SE-positive ceca (%)
Control infection	29	29 (100) A*	18 (62.0)	29 (100)
CE treated	33	25 (75.7) C*	13 (39.4)	22 (66.6)
BP treated	30	24 (80.0) C*	16 (53.3)	22 (73.3)
BP and CE treated	31	12 (38.7)B*	10 (32.2)	6 (19.3)

[A]Birds were treated by aerosol spray with 10^8 PFU/ml of each of three BPs (MOI 10^3) delivered at 6 days of age and infected by oral inoculation with 2.95×10^5 CFU/ml of SE at 7 days of age. CE product was administered at 1 day of age by coarse spray.
[B]Some animals were excluded from the experimental groups.
[C]Number of animals infected with SE, independent of the type of sample (pool of liver and spleen or ceca).
*Different letters indicate significant differences ($P < 0.05$).

agarose in Tris-acetate ethylenediaminetetraacetic acid buffer, 90 V for 90 min), stained with ethidium bromide (0.5 μg/ml) and visualized on an ultraviolet transilluminator. *Nal*^{R} *rif*^{R} SE was used as a control strain and a 50-bp DNA ladder (Fermentas) was used as molecular weight marker.

Statistical analysis. The qualitative bacteriological results were expressed as a proportions of infected animals (cecal and/or pool of organs positive), and the differences between the groups (1, 3, 4, and 5) were determined by a chi-square test. The numbers of SE recovered from cecum were transformed to logarithms (CFU $\log_{10}$) and evaluated by ANOVA. Differences between means were evaluated by a Tukey test. If ANOVA was significant, any sample that turned out to be positive to the qualitative bacteriology but negative to the quantitative bacteriology was considered as value 1; when both the qualitative and quantitative bacteriology were negative, value 0 was assigned. A P value of less than 0.05 was considered to be statistically significant (InfoStat version 2004; Argentina).

RESULTS

The infection control group had an incidence of infection that reached 100%, whereas that of the BP-plus-CE group was significantly ($P < 0.0001$) lower (38.7%); likewise, the infection incidence observed in this group (BP plus CE) was significantly lower ($P = 0.0027$) than that observed in both CE (75.7%) and BP groups (80%; $P = 0.0010$). A significant difference in SE infection incidence was observed ($P < 0.0111$) between the BP group and the infection control group, but not between CE and BP groups ($P = 0.6858$; Table 2).

Challenge strain isolation (percentage) in internal organ pool (spleen and liver) did not present significant differences ($P = 0.0863$) between experimental groups, whereas the recovery of SE from ceca differed between experimental groups and the control infection group ($P < 0.0001$; Table 2).

The quantitative bacteriology results (SE counts in ceca) are shown in Table 3. The ANOVA demonstrated that there was only a significant difference between the CE-plus-BP group and the infection control group ($P = 0.0003$). Ranges of SE counts (CFU/g) from cecal samples of different experimental groups are shown in Table 3.

BP isolation was achieved in all groups that received BP therapy (Table 4). The infection control and health groups were negative for BP isolation, demonstrating that no cross contamination between BP and control groups had occurred. In the BP group, the percentage of BP isolation observed (76.6%) was very similar to that of SE isolation (80%). In the BP-plus-CE group, a similar percentage was obtained between BP isolation (29%) and SE isolation (38.7%). It should be pointed out that none of the BPs used in this study were active against any of the bacterial strains contained in the probiotic product (data not shown).

During the study, no animals of the experimental groups presented macroscopic pathological lesions or clinical symptomatology associated with SE. Birds in the experimental groups showed no differences in weight (data not shown).

DISCUSSION

The commercial preparation Broilact was chosen for this study because of its availability on the Chilean market. By using a preventive probiotic regime (CE group) on birds experimentally infected with SE, incidence was reduced. This result is lower than those described in previous international studies conducted on birds infected with *Salmonella* and analyzed at different days PI (19,21,22). Avila *et al.* (3) observed a 61.2% reduction of SE isolation in ceca on day 3 PI, but this reduction was significantly lower on day 7 PI.

The percentage of SE isolation from internal organ pool was poor in the control group; this fact is associated with the low invasiveness of our strain.

Table 3. SE counts (CFU/g) in the ceca of 14-day-old chicks treated with only BPs, only CE, and BPs plus CE.[A]

Groups	CFU/g means ± SD	Number of chickens	Minimum and maximum counts
Control of infection	$1.56 \pm 6.01 \times 10^5$ A*	15	$10–2.33 \times 10^5$
CE treated	$4.23 \pm 14.33 \times 10^3$ AB*	15	$0–5.6 \times 10^4$
BP treated	$9.48 \pm 23.8 \times 10^3$ AB*	15	$0–10.1 \times 10^3$
BP and CE treated	$1.6 \pm 4.3 \times 10^2$ B*	15	$0–1.6 \times 10^2$

[A]Birds were treated with 10^8 PFU/ml/doses of each of three BPs (MOI 10^3) delivered at 6 days of age (two daily doses) and infected by oral inoculation with 2.95×10^5 CFU/ml SE at 7 days of age. CE product was administered at 1 day of age by coarse spray. For statistical analysis, the numbers of SE recovered from each birds were transformed to logarithms (CFU $\log_{10}$). Any sample that turned out to be positive to the qualitative bacteriology but negative to the quantitative bacteriology was considered as value 1; if both the qualitative and quantitative bacteriology were negative, a value of 0 was assigned.
*Different letters indicate significant differences ($P < 0.05$).

Table 4. Recovery of BPs from chickens infected with SE and treated with only BPs, only CE, and BPs plus CE.[A]

Groups	Number of chickens[B]	Number of BP-positive chickens (%)	Number of of BP-positive liver/spleen (%)	Number of BP-positive ceca (%)
Control Infection	29	0	0	0
CE treated	33	0	0	0
BP treated	30	23 (76.6)	14 (46.6)	22 (73.3)
BP and CE treated	31	9 (29.0)	9 (29.0)	3 (9.6)

[A]Birds were treated by aerosol spray with 10^8 PFU/ml of each of three BPs (MOI 10^3) delivered at 6 days of age and infected by oral inoculation with 2.95×10^5 CFU/ml of SE at 7 day of age. CE product was administered at 1 day of age by coarse spray.
[B]Some animals were excluded from the experimental groups.

The analysis of the recovery of SE in the CE-plus-BP–treated group of birds showed a synergic effect between both biocontrollers; that is, using only CE or only BP the results were inferior to those using CE plus BP. Likewise, according to the type of sample, the reduction of infection in cecal samples was significantly lower when using only one biocontrol product. The higher values of reduction obtained in cecal samples may be explained by 1) the combined action of both biocontrollers in the intestines or 2) the administration of two doses of BP via aerosol spray given prior to the challenge. The early administration of the probiotic could give time to the colonization and multiplication in the intestine before the challenge of SE. On the other hand, the administration of BPs via coarse spray, using two doses before the challenge, would allow a longer penetration of BPs via air, conjunctival, and oral routes. In this way, it would reach the blood stream (phagemia) reaching to the intestine. Once bacteria enter via the oral route, they search for specific receptors in the intestine that could be occupied with the probiotic; alternately, these bacteria could be destroyed by the BPs present in the intestine.

Our results differ from those described by Filho *et al.* (8), who administered BPs via the cloaca and a probiotic agent post–SE infection, but achieved a significant reduction ($P < 0.05$) in cecal isolation inferior to that observed by us. Toro *et al.* (30), who employed *Salmonella* Typhimurium as model and administered probiotics and BPs in multiple doses for 21 days, before and after the infection, failed to isolate the challenge strain by traditional culture methods.

The quantitative bacteriological (CFU/g) study conducted on ceca samples showed a slight decrease in SE count among the CE group, the BP group, and the CE-plus-BP group as compared with the control group (Table 3). Although a sizable difference was not detected between cecal counts, a significant ($P = 0.0003$) difference was observed between the CE-plus-BP group and the infection control group. Better results have been obtained by Toro *et al.* (30), who administered BP plus CE and observed a sixfold reduction in *Salmonella* Typhimurium cecal count as compared with the control group.

The administration of only BPs has had varied results in terms of *Salmonella* count as demonstrated by the results of Atterbury *et al.* (2), Higgins *et al.* (13), Fiorentin *et al.*, (9) and Higgins *et al.* (15).

The mean SE cecal count in the CE-treated group (4.23×10^3 CFU/g) was similar to that of the BP-treated group (9.48×10^3 CFU/g), and higher than the CE-plus-BP group (1.6×10^2 CFU/g), showing little synergic effect between the two biocontrollers. Toro *et al.* (30) observed significant results in the bacterial cecal count ($P < 0.05$), but did not observe a clear synergism between these two methods. Likewise, Filho *et al.* (8) detected no clear synergism using both biocontrollers.

The employment of BPs (30) and probiotics in avian models generally produces an increase in weight gain (20,29). Nevertheless, no significant differences between the different experimental groups were observed (data not shown) in this study.

The isolation of BPs in the experimental groups at 7 days PI demonstrates the phage's extended presence in the organism. As is to be expected under a ''predator–prey'' model, a reduction in bacterial isolation had the concomitant result of reducing the BP isolation (Table 4), though probably in lower numbers than that of the target bacteria.

The rapid selection of phage-resistant bacteria by phage populations has always been perceived as a great problem for their use in therapy. The use of two or more BPs reduces the possibility of selection for resistance against a specific BP (9,26,30); for this reason, the present study used a phage cocktail of three BPs. The phages used were highly specific to the challenge strain, with no activity against bacteria isolated from healthy poultry feces (7) or the probiotic bacteria (data not shown).

The results obtained from the use of CE in combination with BPs in commercial birds experimentally infected with SE are promising and might be of great value to reduce the presence of *Salmonella* in poultry, although further work is need before these treatments can be established.

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