Assessment of 2 *Salmonella enterica* serovar Typhimurium-based vaccines against necrotic enteritis in reducing colonization of chickens by *Salmonella serovars* of different serogroups

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Abstract

This study assessed the protective efficacy of oral vaccination with 2 experimental attenuated *Salmonella* Typhimurium-vectored vaccines for necrotic enteritis in protecting chickens against intestinal colonization by common serovars of *Salmonella* belonging to the 4 major serogroups affecting chickens. Birds were vaccinated orally with 1×10^8 colony-forming units (CFU) of 1 of the vaccine strains χ9241 and χ9352, which express a plasmid-encoded partial recombinant hypothetical protein gene (*tHP*) of *Clostridium perfringens*, at days 1 and 7 of age, and then were challenged at 14 d of age with 10^6 CFU of *Salmonella* serovars Anatum, Enteritidis, Heidelberg, Kentucky, or Typhimurium (representative serovars of serogroups B, C, D, and E). Birds were necropsied at 4 wk of age, and samples were collected to determine reduction in tissue and intestinal colonization. The chickens vaccinated with χ9241-*tHP* showed reduced colonization by *Salmonella* Enteritidis (serogroup D) and by *Salmonella* Heidelberg and *Salmonella* Typhimurium (serogroup B) compared with the control birds. No reduction in colonization was observed in the chickens vaccinated with χ9352-*tHP*. There was an association between the efficacy of these vaccine strains in protecting against necrotic enteritis, assessed on an earlier occasion, and their efficacy in protecting against *Salmonella* colonization. Thus, the choice of an attenuated *Salmonella* Typhimurium vaccine vector for delivery of heterologous antigens to chickens should be based partly on the vaccine's value in protecting against colonization by serovars within serogroups B and D. Such vectors would have the additional benefit of reducing colonization of important *Salmonella* serovars.

Résumé

La présente étude visait à évaluer l'efficacité protectrice d'une vaccination orale avec deux vaccins atténués expérimentaux envers Salmonella *Typhimurium vectorisés pour l'entérite nécrotique à protéger les poulets contre la colonisation intestinale par les sérovars usuels de* Salmonella *appartenant aux 4 sérogroupes majeurs affectant les poulets. Les oiseaux étaient vaccinés oralement à l'âge de 1 et 7 jours avec 1×10^8 unités formant des colonies (CFU) de l'une des souches vaccinales χ9241 et χ9352, qui exprime un recombinant partiel codé sur un plasmide d'un gène d'une protéine hypothétique* (tHP) *de* Clostridium perfringens, *et par la suite soumis à une infection défi à 14 jours d'âge avec 10^6 CFU de* Salmonella *sérovars Anatum, Enteritidis, Heidelberg, Kentucky ou Typhimurium (sérovars représentatifs des sérogroupes B, C, D, et E). Une nécropsie a été pratiquée sur les oiseaux à 4 semaines d'âge et des échantillons prélevés afin de déterminer la réduction de la colonisation intestinale et tissulaire. Comparativement aux oiseaux témoins, une réduction de la colonisation par* Salmonella *Enteritidis (sérogroupe D) et par* Salmonella *Heidelberg et* Salmonella *Typhimurium (sérogroupe B) était notée chez les poulets vaccinés avec* χ9241-tHP. *Aucune réduction de la colonisation n'était notée chez les oiseaux vaccinés avec* χ9352-tHP. *Il y avait une association entre l'efficacité de ces souches vaccinales à protéger contre l'entérite nécrotique, évaluer lors d'une occasion antérieure, et leur efficacité à protéger contre la colonisation par* Salmonella. *Ainsi, le choix d'un vaccin atténué contre* Salmonella *Typhimurium comme vecteur pour la livraison d'un antigène hétérologue à des poulets devrait être basé en partie sur la capacité du vaccin à protéger contre la colonisation par les sérovars retrouvés dans les sérogroupes B et D. De tels vecteurs auraient le bénéfice additionnel de réduire la colonisation par les sérovars importants de* Salmonella.

(Traduit par Docteur Serge Messier)

Introduction

Salmonellosis is one of the most important foodborne zoonotic diseases throughout the world, and poultry represent an important source of human infection. Among the 2500 serovars of *Salmonella*, the *Salmonella enterica* subsp. *enterica* serovars Enteritidis and Typhimurium have in the past been responsible for most of the foodborne salmonellosis in humans, but the pattern of dominant serovars varies over time and geographically. For example, in recent years serovar Heidelberg, which is associated with resistance to multiple drugs, has risen to prominence among human infections in Canada (1), and these isolates and the multidrug-resistant serovar Kentucky are now commonly isolated from retail chicken products (1). In the United States about 1.4 million cases of human

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nontyphoidal salmonellosis occur each year, with approximately 400 deaths annually (1), and in 2009 a mean of 7.2% (range 5.0% to 21.0%) of processed poultry carcasses were contaminated with *Salmonella* (2).

Effective control of foodborne pathogens including *Salmonella* is a major challenge to the poultry industry. For decades, many strategies have been applied in an effort to reduce *Salmonella* contamination on commercial poultry farms, with administration of probiotics or unusual carbohydrates among the approaches (3,4). A further approach is vaccination. Hassan and Curtiss (5–7) have described the development for chickens of attenuated *Salmonella* vaccines and vaccine vectors, including a commercially available vaccine for broiler breeders, and much is known about the basis of immunity in chickens (8–10). In recent years a number of improvements have been made in the development of attenuated *Salmonella* as a vaccine vehicle; these include regulated delayed attenuation and regulated antigen synthesis. Newer vaccine strains have been designed to express protective antigens at high levels and to stimulate strong primary and lasting memory immune responses without producing tissue damage (11–14) and to improve the immunogenicity and safety of the vaccine constructs (15,16). Experimentally, the use of apparently improved *Salmonella*-vectored vaccines has shown promise in controlling necrotic enteritis caused by *Clostridium perfringens* in poultry (17–19); such improved vaccines are not yet available commercially. If *Salmonella*-vectored vaccines are developed that can control necrotic enteritis, an additional benefit to public health might be reduced colonization by *Salmonella*. In addition, the choice of the best vaccine to control necrotic enteritis might be guided by efficacy in controlling *Salmonella* colonization.

The objective of the current study was therefore to assess the efficacy of oral vaccination with 2 recombinant attenuated *Salmonella* Typhimurium vaccine vectors, used experimentally to reduce the incidence of necrotic enteritis, in protecting broiler chickens against experimental intestinal colonization by common serovars of *Salmonella* belonging to the major serogroups.

Materials and methods

Chickens

Fertile eggs free of specific pathogens including *Salmonella* (Sunrise Farms, Catskill, New York, USA) were hatched at the Arkell Poultry Research Station, University of Guelph, Guelph, Ontario. Different experimental groups were housed in Horsfall isolators to prevent cross-contamination. All the chickens were mixed-sex White Plymouth Rock and were fed an antibiotic-free chicken starter containing 20% protein for 14 d and then a grower feed containing 28% protein (Arkell Poultry Research Station). The experiments with the chickens had been approved by the University of Guelph Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care (20).

Bacterial strains

All the *Escherichia coli* and *Salmonella* strains used are listed in Table I. *Escherichia coli* strains χ6212, a derivative of *E. coli* DH5α, and χ6097, a derivative of JM83, were used as intermediate hosts to clone the genes of interest. *Salmonella* Typhimurium χ9241 and χ9352 are avirulent strains derived from the strain UK-1 that have a chromosomal deletion of the aspartate-β-semialdehyde dehydrogenase gene (*asd*), which is complemented by an Asd$^+$ pYA-plasmid (pYA3342 or pYA3493) expressing heterologous genes, thus ensuring that the recombinant plasmid is stable (21,22). The plasmids used contained a fragment of a truncated hypothetical protein (HP) of *C. perfringens* (17,23). Wild-type representatives of *Salmonella* strains from serogroups B, C, D, and E that are commonly found in *Salmonella* isolated from Canadian poultry were used as challenge strains.

All the *Salmonella* strains used for chicken inoculation were plated on Luria–Bertani (LB) agar (Difco, Detroit, Michigan, USA) and incubated overnight at 37°C. Before challenge, stocks were grown overnight as static cultures at 37°C in LB broth (Difco). The cultures were diluted to 2% in prewarmed LB broth and grown with aeration at 37°C for approximately 4 h to an optical density at 600 nm (OD_{600nm}) of about 0.5 to 0.6. The cells were harvested by centrifugation at 8000 × *g* for 15 min at 4°C and then suspended in phosphate-buffered saline, pH 7.2, with 1% gelatin (BSG) to yield the density required for vaccination. Serial dilutions of the suspended *Salmonella* strains were plated on nalidixic acid-containing LB agar for colony-forming unit (CFU) determination.

The challenge strains were maintained in Luria–Bertani (LB) broth (Difco) containing 25% glycerol at –70°C. They were rendered resistant to nalidixic acid by growth on nalidixic acid-containing medium for subsequent ease of isolation from chickens and differentiation from the vaccine strains.

Growth of *Salmonella* for vaccination

Salmonella Typhimurium χ9352 carrying pYA3493-*tHP* (23) (hereafter called χ9352-*tHP*) was grown at 37°C overnight and then inoculated to achieve a concentration of 2% into 100 mL of prewarmed LB broth containing 0.05% L-arabinose (Sigma-Aldrich, St. Louis, Missouri, USA) and 0.1% D-mannose (ACROS Organics, Fair Lawn, New Jersey, USA) under aeration to an OD_{600nm} of about 0.8 to 1.0. The cells were recovered by centrifugation at 8000 × *g* for 15 min at 4°C, and the pellet was resuspended with 1 mL of BSG, then serially diluted and plated on MacConkey agar (Difco) supplemented with 0.5% maltose (Difco) and 0.2% arabinose to verify the phenotype and inoculum concentration. *Salmonella* Typhimurium χ9241 carrying pYA3342-*tHP* (17) (hereafter called χ9241-*tHP*) was similarly grown at 37°C in 100 mL of LB broth (unsupplemented) under aeration to an OD_{600nm} of 0.8 to 1.0 and similarly enumerated on unsupplemented MacConkey agar plates. The vaccine strains were prepared freshly on the day of vaccination.

Vaccination procedure and colonization assessment

Fifty-five 1-d-old *Salmonella*-free chickens were randomly allocated into 11 groups of 5 chickens each for 2 separate experiments. The birds were housed in individual heated Horsfall isolators equipped with high-efficiency particulate air filters. They were deprived of food and water for 4 h before oral vaccination and challenge, and food and water were returned 1 h after inoculation.

Five groups were vaccinated at 1 d of age and given a booster of the same dose at 7 d of age. The dose was 100 µL of 1×10^9

Table I. Bacterial strains used in the study

Strain (source)	Relevant genotype or phenotype	Source
Escherichia coli DH5α	F⁻ φ80*dlacZ*ΔM15 *deoR* Δ(*lacZYA-argF*)*U169 glnV44* λ⁻ *gyrA96 recA1 relA1 endA1 hsdR17*($r^- m^+$)	Stratagene, La Jolla, California, USA
Escherichia coli χ6212	DH5α Δ*asdA4* Δ*zhf-2*::Tn*10*	Dr. Roy Curtiss III
Escherichia coli χ6097	F⁻ *araD139* Δ(*proAB-lac*) φ80 d*lacZΔM15 rpsL* Δ*asdA4 thi-1 glnV44 hsdR17 recA1 endA1 relA1*	Dr. Roy Curtiss III
Salmonella Typhimurium χ9352	Δ*pmi-2426* Δ(*gmd-fcl*)*-26* ΔP_{fur77}::TT *araC* P_{BAD} *fur* ΔP_{crp527}::TT *araC* P_{BAD} *crp* Δ*asdA21*::TT *araC* P_{BAD} *c2* Δ*araE25* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT	Dr. Roy Curtiss III and Dr. Bronwyn Gunn
Salmonella Typhimurium χ9241	Δ*pabA1516* Δ*pabB232* Δ*asdA16* Δ*araBAD23* Δ*relA198*::*araC* P_{BAD} *lacI* TT	Dr. Roy Curtiss III
Salmonella Heidelberg (SA2004-4075; pooled organs)	Group B: 1,4,[5],12;r:1,2); PT-19	Laboratory for Foodborne Zoonoses, Public Health Agency of Canada, Guelph, Ontario
Salmonella Typhimurium (SA2004-4003; fluff)	Group B: 1,4,[5],12;i:1,2); PT-104	
Salmonella Kentucky (SA2004-3900; spleen)	Group C: 8,20;i:z6; PT-NA	
Salmonella Enteritidis (SA2008-0735; spleen)	Group D: 1,9,12;g,m:-; PT-8	
Salmonella Anatum (SA2007-2392; fluff)	Group E: 3,10,{15,34};e,h:1,6; PT-NA	

PT — phage type; NA — not available.

CFU/mL of either *Salmonella* Typhimurium χ9352-*tHP* or *Salmonella* Typhimurium χ9241-*tHP* suspended in BSG, delivered directly into the crop with a 1-mL syringe and a 6-cm-long 18-gauge animal feeding needle (Popper and Sons, New York, New York, USA). The 5 groups of vaccinated chickens and 5 groups of nonvaccinated chickens were challenged orally at 2 wk of age with 1.2 to 2.0×10^6 CFU/mL of the wild-type strains of *Salmonella* listed in Table I. One group was neither vaccinated nor challenged and served as a control group.

All the chickens were necropsied at 4 wk of age, 2 wk after challenge. The degree of colonization protection induced by oral vaccination was assessed by comparing the ability of each challenge strain to colonize the gastrointestinal tract and invade visceral organs with the ability of the same strain to colonize and invade the organs of the control chickens (5).

Sample collection and processing

To confirm intestinal colonization by the vaccine strain, pooled 10-g fecal samples from each group were collected after vaccination at days 0, 2, 5, 8, 11, and 14 of age and on days 3, 6, 9, 12, and 14 after challenge at 2 weeks of age. The chickens were euthanized by CO_2 asphyxiation 2 wk after challenge. Necropsy procedures followed the descriptions of Hassan and Curtiss (24). The spleen, liver, and cecum were collected aseptically from each bird, and the cecal contents were milked for *Salmonella* enumeration. All samples were collected into sterile preweighed sample tubes and kept on ice.

Enumeration of vaccine strains in fecal samples

Each pooled fecal sample was added to selenite cystine broth (SCB) (1:10) and mixed thoroughly, then serially diluted in SCB. The SCB medium was used to inhibit the growth of competing non-*Salmonella* bacteria such as *E. coli* present in the intestinal tract. All dilutions were grown for 36 to 48 h at 37°C, and 100-μL aliquots were then plated onto brilliant green agar (Difco), which was incubated at 37°C for 24 h. Pink colonies were considered positive. Pink colonies isolated from fecal samples from chickens vaccinated with strain χ9352-*tHP* were subcultured on MacConkey agar plates supplemented with 0.5% maltose to verify the colorless phenotype. Pink colonies isolated from fecal samples from chickens vaccinated with strain χ9241-*tHP* were subcultured on MacConkey agar to confirm the colorless phenotype. Polymerase chain reaction was performed on representative colonies to confirm that the vaccine strains had not lost the heterologous *tHP* genes.

Enumeration of viable challenge strains in tissues

All samples were homogenized as 1:10 dilutions in SCB and then serially diluted in BSG. A 100-μL aliquot of the diluted samples was plated on *Salmonella–Shigella* (Difco) agar plates containing 30 μg/mL of nalidixic acid and incubated at 37°C for 36 to 48 h, which permitted detection of a minimum of 10^2 CFU of *Salmonella* per gram of sample. Typical colorless colonies with a black center were counted. Samples positive only by selective enrichment in SCB were recorded as containing 10 CFU; negative samples were recorded as containing 0 CFU.

Statistical analysis

Two-way analysis of variance was performed to determine whether there was a significant difference in the numbers of *Salmonella* challenge strains colonizing the gastrointestinal tract and visceral organs between the vaccinated groups and the nonvaccinated controls. The null hypothesis was rejected at $\alpha = 0.05$.

Results

Salmonella shedding in the feces

Oral vaccination of chickens with strain χ9241-*tHP* resulted in a higher level (10^4 to 10^7 CFU/g) and more prolonged shedding of *Salmonella* in the feces than did vaccination with χ9352-*tHP* (Figure 1). The nonvaccinated and uninfected control groups remained uninfected.

Colonization reduction induced by vaccination

Oral vaccination at 1 and 7 d of age with 10^8 CFU of strain χ9241-*tHP* significantly reduced the colonization of cecal contents and feces of vaccinated chickens challenged with the *Salmonella* serovars Heidelberg, Enteritidis, and Typhimurium (Figures 2 and 3). A significant reduction in splenic infection was also observed in chickens challenged with the Enteritidis and Typhimurium serovars.

Oral vaccination at 1 and 7 d of age with 10^8 CFU of strain χ9352-*tHP* produced no evidence of reduced colonization of the organs or cecal contents of vaccinated chickens other than a reduction of cecal colonization by *Salmonella* Anatum (Figure 2), which was, however, not supported by the results of fecal culture (Figure 3). Interestingly, there was a consistent reduction in fecal carriage in birds challenged by *Salmonella* Enteritidis (Figure 3), although no statistical analysis could be done because these were single data based on pooled samples.

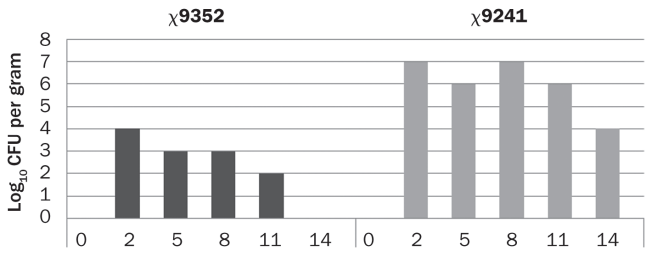

Figure 1. Isolation of recombinant attenuated *Salmonella* Typhimurium vaccine strains χ9352 and χ9241 from feces of chickens vaccinated orally at 1 d of age and given a booster at 7 d of age. Fecal samples were pooled from each group (10 g per group) at 0, 2, 5, 8, 11, and 14 d of age. Each value represents $\log_{10}$ colony-forming units (CFU) of *Salmonella* isolated per gram of feces.

Discussion

Several live attenuated *Salmonella enterica* serovar Typhimurium vaccine strains have been licensed for use against *Salmonella* infection in poultry (25–27). Recombinant attenuated *Salmonella* vaccines (RASVs) have also been developed as multivalent vaccines to deliver recombinant antigens to induce systemic and mucosal immunity against the recombinant antigens (28,29) while providing protection against salmonellosis or reducing colonization (7,29–32). One advantage of using attenuated *Salmonella* vaccine vectors to protect against necrotic enteritis is that they might have the additional human health benefit of protecting against *Salmonella* colonization. The vaccine vector used in this study, strain χ9241-*tHP*, had previously been shown to produce high antibody titers as well as significant protection against necrotic enteritis (17). It reduced colonization and invasion by the Typhimurium and Heidelberg serovars of the homologous serogroup B, as well as cross-protective immunity against the serogroup D Enteritidis serovar. Similar protection has been observed in birds vaccinated with Δ*cya*-Δ*crp*-attenuated *Salmonella* Typhimurium strain χ3985 and challenged with strains of different serovars within the homologous serogroup B, as well as with the heterologous serovar Enteritidis (5). However, in addition to reduction of colonization by serogroups B and D, the Δ*cya*-Δ*crp*-attenuated strain χ3985 provided cross-protection against several serovars in the heterologous serogroup C, as well as against the Anatum serovar in serogroup E (5). The reason for this difference in protection against these heterologous serogroups is unclear. The partial cross-protective ability against *Salmonella* Enteritidis colonization may be explained by the presence of the O12 antigen in serovars of serogroups B and D.

No protection against *Salmonella* colonization was observed with oral vaccination by strain χ9352-*tHP*, except for a reduction in fecal carriage of serovar Enteritidis and cecal colonization by serovar Anatum. However, the latter observation is likely erroneous since the results were not supported by a reduction in fecal carriage. The general lack of colonization reduction observed with this strain is consistent with the poor efficacy of χ9352-*tHP* in protecting against necrotic enteritis (23). This strain is more attenuated than χ9241, with multiple mutations that promote both regulated delayed attenuation and regulated antigen synthesis (11). Regulated delayed RASV strains such as χ9352 display features of wild-type virulent strains of *Salmonella* at the time of vaccination that enable vaccine strains to colonize lymphoid tissue and then become attenuated in vivo. For example, the *gmd-fcl* deletion directs mannose utilization towards the synthesis of O antigen, as well as preventing the production of colonic acid. The *pmi* mutation ensures the production of wild-type O antigen in the presence of mannose, a component of the lipopolysaccharide of *Salmonella* Typhimurium. Since mannose is unavailable in the animal body, the production of wild-type O antigen eventually ceases (12). Foreign antigen expression can be controlled by the addition of arabinose to the culture medium during growth in vitro, and arabinose supplementation also results in a high level of production of iron-regulated outer membrane proteins (OMPs) (11). This strain is apparently overattenuated for chickens, as evidenced also by poor fecal colonization (Figure 1), and is not a good candidate as a vehicle for heterologous antigen delivery in poultry.

Although we did not determine the level of humoral response to *Salmonella* OMPs in this study, a previous investigation involving the use of live oral *Salmonella* vaccine strains in chickens suggested that there was no correlation between antibody response and shedding of the challenge *Salmonella* strain in eggs or cloacal swabs (33). Further work is required to choose suitable *Salmonella* vaccine strains to deliver the heterologous recombinant *C. perfringens* antigens that protect against necrotic enteritis (17–19) while reducing colonization by *Salmonella*, thus providing a simple method to reduce product contamination by *Salmonella* and enhance the safety of chicken products. The apparent association between efficacy in protecting against necrotic enteritis and efficacy in protecting against *Salmonella*

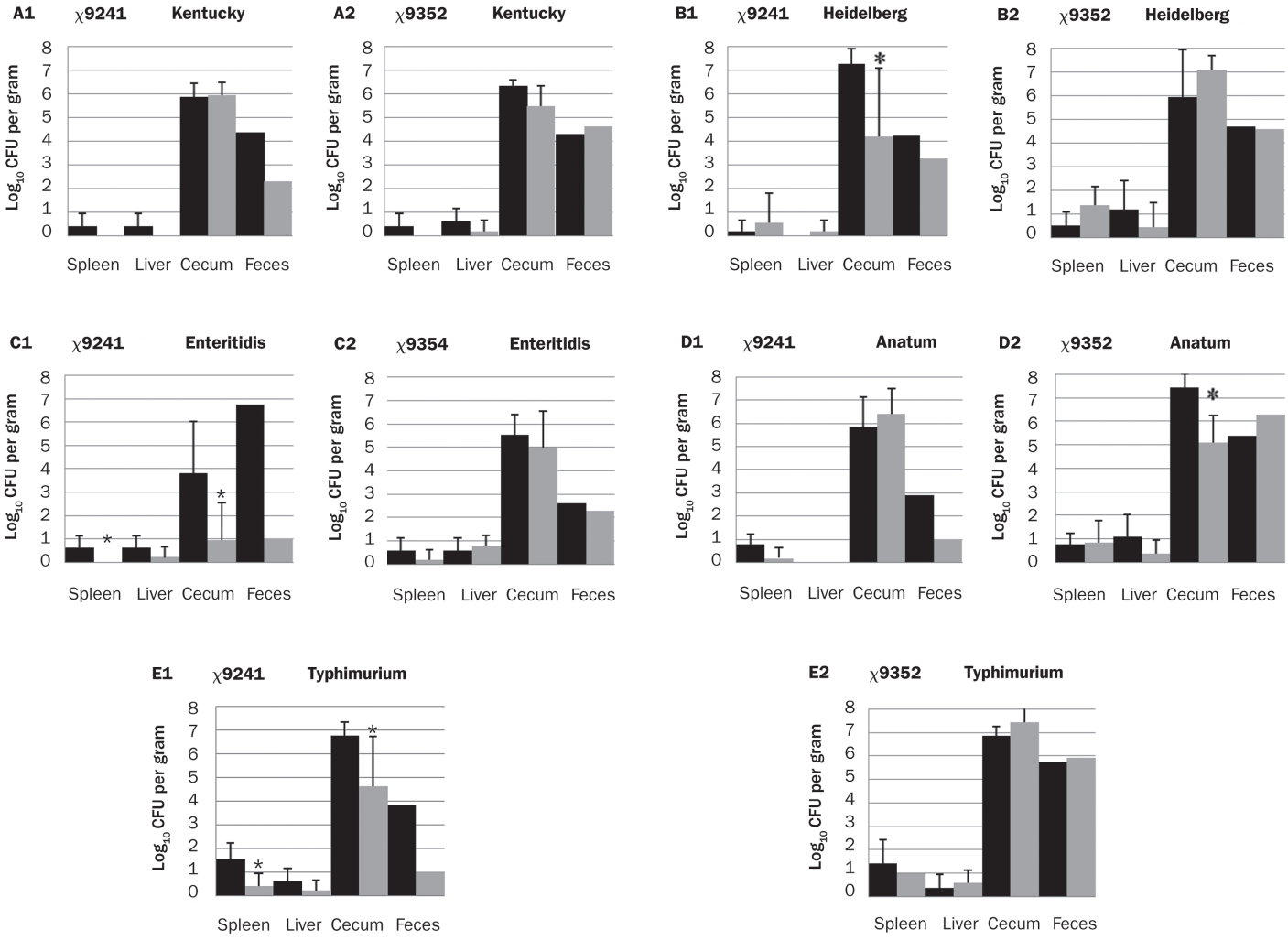

Figure 2. Colonization of chickens vaccinated (grey bars) with 10^8 CFU of *Salmonella* Typhimurium χ9241-pYA3342-*tHP* or χ9352-pYA3493-*tHP* or unvaccinated (black bars) when challenged 14 d later with 10^6 CFU of other *Salmonella* serovars. Samples of spleen, liver, and cecum were collected from individual chickens at necropsy; values presented are means ± standard error of the mean of 5 samples. Fecal samples were pooled from the group. Each value represents $\log_{10}$ CFU of *Salmonella* isolated per gram of samples. The asterisks indicate a significant difference at $P < 0.05$.

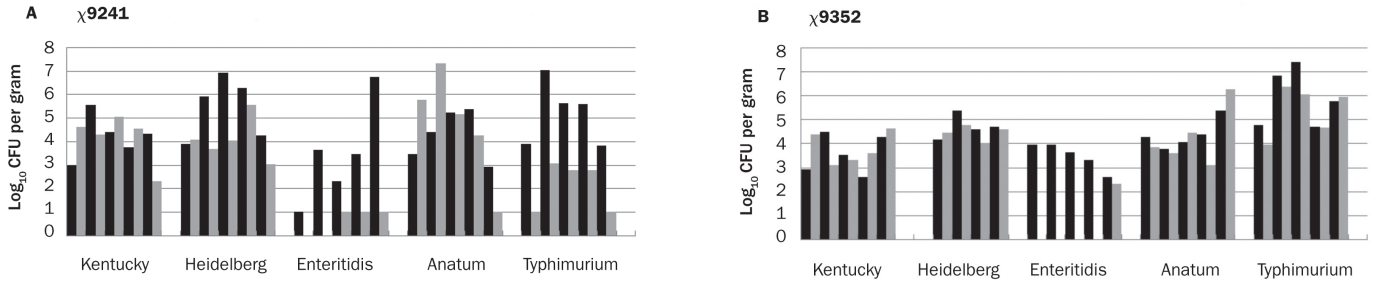

Figure 3. Colonization of pooled fecal samples (10 g per group) collected 3, 6, 9, 12, and 14 d after challenge. Vaccinated (grey bars); unvaccinated (black bars).

colonization suggests that an attenuated strain chosen as a vaccine vector should be one that gives excellent protection against colonization with serovars belonging to the homologous serogroup and to important heterologous serogroups such as that of *Salmonella* Enteritidis. The work reported here is important since it defines an approach to vector choice that avoids the current empiric approach.

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