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Effect of a Defined Competitive Exclusion Culture for Prophylaxis and Reduction of Horizontal Transmission of *Salmonella enteritidis* in Broiler Chickens

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Abstract: Effective Competitive Exclusion (CE) cultures have been shown to accelerate development of normal microflora in chicks and poults, providing increased resistance to infection by some enteric bacterial pathogens. Our objective was to develop a CE culture for prophylaxis and reduced horizontal transmission of *Salmonella enteritidis* (SE) in broiler chickens. In the present study, seven members of the family Enterobacteriaceae and 2 lactic acid bacteria isolates, each capable of *in vitro* and *in vivo* inhibition of SE, were selected and combined to form the putative CE culture. In the first experiment, day-of-hatch chicks were randomly divided into four pens. All treated chicks were orally gavaged with the CE culture and 3 pens were treated with the CE culture in the drinking water for four consecutive days. Treated and control-non treated chicks were challenged with SE 48 hr later. All 3 groups of birds that were treated with the CE culture had a significant decrease ($p<0.05$) in cecal colonization compared with non-CE-treated SE-challenged chicks. Two additional experiments were designed to measure the efficacy of the CE culture in reducing SE horizontal transmission from infected to uninfected chicks when commingled. SE was recovered in the cecal tonsils with a significantly lower incidence at days 7 and 14 in Experiment 2 and day 7 in Experiment 3 from the groups that received the CE in the drinking water as compared to controls respectively. These results suggest that a relatively simple and defined CE culture can reduce SE colonization in neonatal chicks.

Key words: Competitive exclusion, prophylaxis, horizontal transmission, *Salmonella enteritidis*, broiler chicks

Introduction

Although *Salmonella* enterica serovars are some of the best studied bacterial pathogens, the field still has a long way to go, especially when one considers that (i) they cause significant human morbidity and mortality worldwide; (ii) they have broad host ranges (iii) they are able to establish persistent colonization in some species which serve as reservoirs for transmission/shedding; and (iv) they are increasingly resistant to many antibiotics (Boyle *et al*., 2007). Poultry producers are challenged to improve production while using fewer antibiotics due to increased restriction on antimicrobial usage. Researchers worldwide are working on organic alternatives due to the ban of a wide range of drugs for animal production. Probiotics consisting of live or dead organisms and spores (Patterson and Burkholder, 2003), non-traditional chemicals (Moore *et al*., 2006), bacteriophages (Higgins *et al*., 2005), organic acids (Jarquin *et al*., 2007; Wolfenden *et al*., 2007) and others have emerged in the last decades as some of the tools that could be potentially useful in the near future for pathogen control and poultry performance improvement. Our laboratory has evaluated a simple method to select for individual enteric bacteria capable of inhibiting *Salmonella* growth *in vitro* and the ability of selected oxygen tolerant bacteria, in combination, to protect neonatal poults from *Salmonella* infection following challenge (Bielke *et al*., 2003). We have also been working toward isolation, selection and further evaluation of probiotic organisms to control food borne pathogens (Tellez *et al*., 2006). Experimental and commercial studies conducted have shown that these selected probiotic organisms are able to reduce idiopathic diarrhea in commercial turkey brooding houses (Higgins *et al*., 2005) and also to significantly reduce *Salmonella* colonization in turkeys (Vicente *et al*., 2007a) and broilers (Higgins *et al*., 2007; Vicente *et al*., 2007b). Competitive Exclusion (CE), first described by Nurmi and Rantala (1973), has been an effective method of control for salmonellosis in commercial poultry flocks. Although successful cultures have been produced and are available, an inexpensive, air-tolerant and completely defined culture is needed. The aim of this study was to extend our research and examine the effect of a competitive exclusion culture consisting of 7 Enterobacteriaceae and 2 lactic acid

bacteria into a single culture for prophylaxis and horizontal transmission of *Salmonella enteritidis* (SE) in broiler chickens.

Materials and Methods

***Salmonella* source:** A primary poultry isolate of *Salmonella enteritidis* (SE), phage type 13A, was originally obtained from the National Veterinary Services Laboratory (Ames, Iowa). This isolate was selected for resistance to nalidixic acid (NA)[1]. For these experiments, *Salmonella* was grown in tryptic soy broth (TSB)[2] for approximately 8 h. The cells were washed three times with 0.9% sterile saline by centrifugation (3,000×*g*) and the approximate concentration of the stock solution was determined spectrophotometrically. The stock solution was serially diluted and confirmed by colony counts of three replicate samples (0.1 mL/replicate) that were spread plated on xylose lactose differential agar (XLD)[3] plates containing 25 µg/mL novobiocin (NO)[4] and 20 µg/mL NA. The colony-forming units of *Salmonella* determined by spread plating were reported as the concentration of *Salmonella* (in cfu/mL) to determine total colony-forming units for the challenge experiments.

Competitive exclusion culture: Single aliquots of the combined culture containing 7 selected isolates of *Enterobacteriaceae* and 2 lactic acid bacteria growth on TSA or Mann Rogosa Sharp (MRS)[5] broth respectively were use in the present study. The 7 isolates selected from our previous study (Bielke *et al.*, 2003) included *Escherichia* (2 isolates), *Klebsiella* (2 isolates), *Enterobacter* (1 isolate), *Enterococcus* (1 isolate) and *Bacillus* (1 isolate). The 2 lactic acid bacteria included were *Pediococus parvulus* and *Lactobacillus salivarius* of poultry gastrointestinal origin, were previously selected and described (Higgins *et al.*, 2005) and contained in the commercial product (FM-B11™)[6]. Actual colony forming units administered per chick from each experiment were determined retrospectively from spread plating on TSA or MRS respectively.

Chickens: Broiler chicks from a commercial broiler cross line were obtained on the day of hatch and were orally gavaged with the appropriate culture before placement in floor pens. Each pen was approximately 1.8 m^2 in area and the floor was covered with clean softwood shavings. Chickens were provided antibiotic-free feed, formulated to meet or exceed NRC recommendations for critical nutrients for day-of-hatch chickens (NRC, 1994) and water *ad libitum*.

***Salmonella* recovery:** In each experiment, chickens were humanely killed by CO_2 asphyxiation; cecal tonsils were aseptically removed and enriched in 20 mL of tetrathionate broth[7] following incubation for 24 h at 37°C before streaking on XLD plates containing 25 µg/mL NO and 20 µg/mL NA. The plates were incubated for 24 h at 37°C and examined for the presence of lactose negative, NA-resistant *Salmonella* colonies. Selected lactose-negative, antibiotic-resistant colonies typical of *Salmonella* were further confirmed by serogrouping[8].

Experimental design

Experiment 1: Day-of-hatch chicks were randomly divided into five pens (n = 20/pen). All treated chicks were orally gavaged with the CE culture and 3 pens also received the CE culture in the drinking water for four consecutive days. Two pens of controls (Group A) were gavaged with 0.9% sterile saline solution and received no CE culture in the drinking water. Group B received the highest dose of the combined CE culture of 2.9×10^5 cfu by oral gavage and 1.20×10^7 cfu/ml in the drinking water. Group C received a dose of 2.9×10^3 cfu by oral gavage and 1.20×10^5 cfu/ml in the drinking water. Group D received a dose of 29 cfu by oral gavage and 1.20×10^3 cfu/ml in the drinking water. All chicks were challenged 48 h after placement with 4.6×10^3 cfu SE. Cecal tonsils were sampled 5 d post-challenge and cultured for presence or absence of SE.

Experiment 2: Four groups (n = 40/pen) of chicks were placed on the day-of-hatch. Ten, additional, seeder chicks per group were challenged with 1.25×10^5 cfu SE on the day-of-hatch and placed in each of the treatment groups 24 h later. Two pens were treated by inclusion of the CE culture (4.89×10^4 cfu/ml) in the drinking water for 3 consecutive days and the other two pens received no CE culture (non-treated control). On day seven, cecal tonsil samples were collected from the 10-seeder chicks and 20 contact chicks in each group. On day 14, cecal tonsil samples were collected from the remaining 20 contact chicks in each group.

Experiment 3: Four groups (n = 40/pen) of chicks were placed on the day-of-hatch. Ten, additional, seeder chicks per group were challenged with 2.13×10^5 cfu SE on day-of-hatch and placed in each of the treatment groups 24 h later. Two pens were treated by inclusion of the CE culture (1.53×10^6 cfu/ml) in the drinking water for 3 consecutive days and the other two pens received no CE culture (non-treated control). On day seven, cecal tonsil samples were collected from the 10-seeder chicks and 20 contact chicks in each group. On day 14, liver-spleen and cecal tonsil samples were collected from the remaining 20 contact chicks in each group.

Statistical analysis: The chi-square test of independence was used to determine significant differences (P = 0.05) in *Salmonella* recovery between treatments within experiments testing all possible combinations as described in the tables and figures (Zar, 1984).

Table 1: Experiment 1, *S. enteritidis* recovery from cecal tonsils of broiler chicks 5 days post-treatment

Treatments[1]	5 days
Control A (non treated)	88% (35/40)[a]
Group B	55% (11/20)[b]
Group C	20% (4/20)[c]
Group D	35% (7/20)[bc]

[a-c]Significantly different than control ($p<0.05$)

Table 2: *S. enteritidis* recovery from cecal tonsils of broiler chicks at 7 and 14 days post-treatment (contact birds only)

		7 days	14 days
Experiment	Treatment[1]	Cecal tonsils	Cecal tonsils
2	Control	76% (29/38)[a]	79% (31/39)[a]
	Probiotic	48% (19/40)[b]	43% (17/40)[b]
3	Control	83% (53/60)[a]	58% (23/40)
	Probiotic	59% (33/59)[b]	58% (23/40)

[a-b] Indicates significant ($P<0.05$) differences was observed between control and treated within a single experiment in each column

Results and Discussion

Many of the more than 200 pathogenic serovars of the genus *Salmonella* are able to colonize the gastrointestinal tract of poultry. Horizontal transmission under normal and fasting conditions has been reported (Holt, 1995). Heat stress and short-term withdrawal of feed and water have been associated with an increase in *Salmonella* isolation from flocks (Moore *et al.*, 2006). Therefore, practical solutions to reduce SE transmission are important for commercial poultry production. Table 1 shows the results of SE recovery from cecal tonsils of broiler chicks of Experiment 1. SE was recovered from 88% of SE challenged non-CE culture treated control chicks. In all treated groups that received the CE culture, a significantly lower incidence of SE was observed (B = 55%, C = 20%, D = 35%) compared to control (Table 1). In this experiment the CE culture was administered in the drinking water and by oral gavage. However, the administration of the CE culture by oral gavage is impractical and would prove to be very time consuming and expensive if implemented for commercial poultry production. Table 2 shows the results of CE culture efficacy against SE in Experiments 2 and 3. Both experiments showed that *Salmonella* recovery could be reduced by administration the CE culture in the drinking water. In Experiment 2, samples taken on day seven showed SE recovery from the cecal with significantly lower in the groups that received the CE culture in the drinking water (48%) as compared to controls (76%). Similar results were observed on day 14 of the experiment where SE recovery from the cecae was significantly lower in the CE culture treated group (43%) as compared to controls (79%). In Experiment 3, samples taken on day seven showed SE recovery from the cecae had a lower incidence in the group receiving the CE culture in the drinking water (59%) as compared to controls (83%). However, samples taken on day 14 of the experiment showed no significant difference between birds given the CE culture and the controls. One possible explanation for the reduced efficacy of the treatment in Experiment 3 is the dose of CE administered. In Experiment 2, the dose of CE culture was 4.89×10^4 cfu/ml of water and the dose in Experiment 3 was 1.53×10^6 cfu/ml water. It may be possible that the dose administered in Experiment 3 was too high. In support of this hypothesis reduced efficacy of high dose CE culture as has been previously described (Bielke *et al.*, 2003). Effective probiotics or competitive exclusion cultures have been shown to accelerate development of normal microflora in chicks and poults, providing increased resistance to infection by some enteric bacterial pathogens (Higgins *et al.*, 2007; Vicente *et al.*, 2007a; Vicente *et al.*, 2007b). Our objective was to create a simple, safe, defined, air tolerant and effective CE culture. We have previously selected bacteria isolated from the intestinal tract of normal chickens based on their ability to inhibit SE growth *in vitro* and demonstrated that a culture consisting of 24 organisms was efficacious *in vivo* (Bielke *et al.*, 2003). In the present experiments, seven members of the family *Enterobacteriaceae* and 2 lactic acid bacteria isolates, each capable of *in vitro* inhibition of SE, were selected and combined to form the putative CE culture. In contrast to some previous speculations regarding the necessity of complex CE cultures for prophylactic efficacy (Corrier *et al.*, 1995; Mead, 2000), the efficacy of this CE culture in protecting chicks from *Salmonella* infection, when administered at the appropriate dose (Experiments 2), also suggests that rather simple cultures can indeed provide protection. These data suggest that relatively simple defined cultures consisting of air-tolerant bacteria can inhibit *Salmonella* colonization in broiler chickens.

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