Comparison of Mucosal Competitive Exclusion and Competitive Exclusion Treatment to Reduce *Salmonella* and *Campylobacter* spp. Colonization in Broiler Chickens

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ABSTRACT Control of *Salmonella* spp. during the earliest phases of broiler production may provide the best opportunity to reduce human pathogens on processed broiler carcasses. Application of the "Nurmi concept" has been demonstrated to be an effective means in reducing *Salmonella* colonization among broiler chicks. In 1989, Aho et al. developed a competitive exclusion (CE) culture for control of *Salmonella* spp., whereas a mucosal competitive exclusion culture (MCE) developed in the United States was originally created to control *Campylobacter* colonization (Stern et al., 1995). The major differences in the two patents were the higher level of anaerobic culture required, the degree of epithelial scraping and washing of the ceca, media used for subculturing, and the culture incubation temperatures (35 C vs. 42 C). The CE and MCE were compared for efficacy in reducing *Salmonella* and *Campylobacter* colonization in broiler chicks. Nine adult birds (three for each of three replicates) were slaughtered, and each of a bird's paired ceca were used to produce corresponding antagonistic microflora, which were administered to day-of-hatch chicks. The chicks (a total of 210) were challenged 24 h later with *Salmonella* and *Campylobacter* and were killed 1 wk later, and levels of the pathogens were determined. Ninety CE-treated birds were significantly more colonized by *Salmonella typhimurium* than those 90 chicks treated with the MCE microflora (3.97 log 10 cfu/g cecal contents vs. 1.25 log 10 cfu/g cecal contents). Also, *Campylobacter* spp. colonization of these birds was significantly higher for CE-treated birds when compared with MCE-treated birds (6.96 log 10 cfu/g cecal contents vs. 5.03 log 10 cfu/g cecal contents). These results can be useful in developing intervention strategies to reduce chicken colonization by *Salmonella* and *Campylobacter*.

(*Key words*: competitive exclusion, poultry, *Salmonella*, *Campylobacter* spp.)

INTRODUCTION

Campylobacter spp. and *Salmonella* spp. have caused sporadic cases of food-borne illness and are often associated with consumption of poultry (Tauxe et al., 1988). Elimination or reduction of these organisms from the food supply is a difficult task. Approaches from "farm to fork" will likely be required to impact the incidence of food-borne illness associated with these pathogens. Elimination of these organisms from broilers and other poultry before they reach the processing plant will improve the chances of producing processed carcasses free from these organisms. Even when a few birds arrive at the plant colonized or externally contaminated with pathogens such as these, other birds, and ultimately other carcasses, may become contaminated as well (The National Advisory Committee on Microbiological Criteria for Foods, 1994). One means of impacting on-farm levels of colonization that has been used world-wide is treatment of hatching eggs and newly hatched chicks with competitive exclusion (CE) cultures as described by Nurmi and Rantala (1973) and Rantala and Nurmi (1973).

Thorough reviews of CE culture methodology and application have been published (Bailey, 1987; Stavric, 1987; Corrier and Nisbet, 1999). These articles cover the different means of generating cultures (defined and undefined) and methods of preparation and their effectiveness in reducing contamination by *Salmonella* and *Campylobacter* spp.

A U.S. patent was issued for a method developed at the Agricultural Research Service for a mucosal competitive exclusion (MCE) culture, which selects microorganisms associated with the mucus layer in the intestines of birds.

Abbreviation Key: CE = competitive exclusion; CF = colonization factor; MCE = mucosal competitive exclusion; PF = protection factor; PRAS-BHI = prereduced annerobically sterile brain heart infusion broth.

The MCE was devised to maximize the populations occurring in the intestinal mucosa, the same niche as occupied by *Campylobacter* spp. Aho et al. (1989) developed a CE culture for control of *Salmonella* spp. by using a different method described in a patent issued in Great Britain. These experiments were performed to compare the effectiveness of MCE and CE cultures.

MATERIALS AND METHODS

Birds used to prepare the CE and MCE cultures were floor-reared broilers obtained from the loading dock of a local processing plant. These birds, approximately 6 wk old and 1.8 kg, were transported back to the laboratory alive. Immediately following euthanasia by cervical dislocation, each bird was dissected, and both ceca were removed. From each bird, one cecum was used to prepare a culture by the MCE procedure (US Patent # 5,451,400), and the other cecum was used to prepare a CE culture (GB Patent # 2,233,343). The result was paired cultures (CE and MCE) that were derived from the same bird. This procedure allowed for an accurate comparison of the efficacy of cultures generated by the two methods.

MCE Preparation

Stern et al. (1988) and Shanker et al. (1990) had reported that standard preparations of CE, even when effective against *Salmonella*, offered no obstacle to colonization by *Campylobacter* spp. because *Campylobacter* spp. is associated with the crypts in the ceca (Beery et al., 1988; Meinersmann et al., 1991). Stern (1994) proposed that deeper tissue scrapings might be useful in generating a CE culture more appropriate to preventing colonization by campylobacters. Procedures followed for the preparation of the MCE cultures used in this study were the same as described by Stern et al. (1995). After a cecum was aseptically removed from a chicken, it was placed in a sterile petri dish and moved to a large container flushed with heated N_2 (oxygen scavenged) gas. Materials were kept below the edge of the container to markedly decrease the exposure of the cecum and the bacteria inside the cecum to atmospheric oxygen. To further reduce exposure of tissues and cells to the ambient air, all procedures were performed as quickly as possible (<5 min/cecum). A sterile glass rod was used to evert the cecum without touching the inside of the organ. Next, the dull edge of a sterile scalpel blade was used to gently remove adherent cecal contents. Any remaining materials were washed off the epithelium with a syringe containing 5 to 10 mL of prereduced, anaerobically sterilized, brain heart infusion broth[3] (PRAS-BHI). At least two such washings were required to remove all visible cecal contents. Next, the epithelium of the everted cecum (about 0.25 to 0.5 inches at

the tip) was scraped with the sharp edge of a fresh, sterile scalpel blade. A syringe of the PRAS-BHI was then used to wash off both the scraped epithelium and the scalpel blade. A clean, sterile petri dish was placed under the cecum and scalpel to catch the tissue and rinse during the rinsing procedure. A sterile syringe and needle were then used to collect the rinse from the bottom of the petri dish, and the bacterial suspension was injected into a sterile tube of PRAS-BHI through a rubber septum at the top of the tube, allowing anaerobic conditions to be achieved as quickly as possible. The tubes were incubated at 35 C for 48 h. After this incubation, a sterile needle and syringe were used to obtain 1 mL of incubated culture for injection into a fresh tube of PRAS-BHI. After a second transfer and incubation, an aliquot from each culture was enriched in TT broth and then plated onto BG-Sulfa[4] and MLIA[5] (Bailey et al., 1988b) to determine whether or not these cultures contained *Salmonella*. Campy-Cefex agar (Stern et al., 1992) was used to demonstrate that *Campylobacter* spp. were absent from the cultures. After these procedures, each culture was then administered to chicks before being frozen at −80 C in a glycerol solution for future use.

CE Preparation

Procedures performed were as outlined in Aho et al. (1989). For each broiler used to generate cultures, the second cecum was aseptically removed and handled as described for the ceca used in the MCE preparation. The everted cecum was washed six times with a syringe containing 5 to 10 mL of 1% peptone. A sterile scalpel was used to scrape the cecal epithelium, which was then rinsed with 1% peptone. A sterile syringe was used to collect the washings, which were transferred to a 15-mL tube with a screw top cap containing 10 mL of 10% mucin broth. A recipe for this medium was included in the patent information. Tubes were incubated at 42 C for 48 h and subcultured twice before they were used to treat 1-d-old chicks and then frozen at −80 C in a glycerol solution for storage. These cultures were also tested for the presence of *Salmonella* and *Campylobacter* spp. The main differences between the procedures used to prepare the two types of cultures were the higher level of anaerobic culture for MCE; broth used for rinsing, incubation, and expansion of the culture; and incubation temperature (35 C vs. 42 C).

Culture Efficacy and Comparison Testing

Day-of-hatch broiler chicks were obtained from a commercial broiler hatchery and placed in isolation units designed to prevent cross-contamination from unit to unit. Each isolation unit had dimensions of 91.44 cm × 91.44 cm screened (1 cm) floors. The incubation temperature was held at 35 C, and the air was purified through HEPA filters. Each treatment (CE, MCE, or control) was assigned a separate isolation unit. There were three trials each with three pairs of cultures (CE and MCE; every pair derived

from a separate adult broiler) and a separate control for each trial in this experiment. Each of the 18 groups (9 CE and 9 MCE) contained 10 day-of-hatch chicks; the three control groups had 10 birds each. A 0.2-mL aliquot of one of the cultures was administered by oral gavage to each chick, whereas controls received 0.2 mL of sterile water. This procedure was performed before the birds were allowed access to food or water. On the following day, chicks from all groups were orally co-challenged with 0.1 mL of a suspension containing 10^7 Salmonella and 10^6 Campylobacter spp. Challenge cultures provided to chicks were Salmonella typhimurium that was resistant to 200 ppm nalidixic acid. Three separate Campylobacter spp. isolates obtained from carcass rinses were used at equal levels to generate a single cultural suspension. Stern et al. (1991) have demonstrated that Salmonella and Campylobacter do not influence one another as co-colonizers in chickens.

Chicks were provided a starter ration and water ad libitum for an additional 5 d to allow transient Salmonella and Campylobacter spp. cells to clear the intestinal tract. After euthanasia by cervical dislocation, each chick was aseptically necropsied for removal of ceca. Each chick's ceca were placed in a sterile plastic bag. For every gram of ceca and contents, 3 mL of a 1% buffered peptone solution was added. To ensure even distribution of bacteria throughout the suspension, each bag was blended in a Colworth Stomacher 80[6] for 30 s. Serial dilutions were prepared and plated onto BG-Sulfa agar with 200 ppm nalidixic acid and Campy-Cefex agar plates to determine presence and levels of Salmonella and Campylobacter spp. in the intestinal contents from the chicks.

Salmonella *Methodology*

BG-Sulfa plates with 200 ppm nalidixic acid were incubated for 18 to 24 h at 37 C and observed for presumptive Salmonella colonies. Cecal contents were also incubated at 37 C. If no Salmonella colonies were observed on the direct plates, the samples were reexamined by culturing incubated cecal contents. These plates were incubated for an additional 18 to 24 h at 37 C. Counts for each chick were converted to $\log_{10}$ cfu Salmonella/g of cecal contents.

Campylobacter *spp. Methodology*

Serial dilutions of the cecal suspensions were prepared and plated onto Campy-Cefex agar, a blood-based agar selective for Campylobacter spp. Plates were placed in gallon-sized sealable storage bags[7] and filled with microaerobic atmosphere (5% O_2, 10% CO_2, 85% N_2) before incubation at 42 C for 24 to 36 h. After incubation, plates were observed for presumptive Campylobacter spp. colonies.

Although Campy-Cefex is not a differential agar, Campylobacter spp. colonies do have a characteristic appearance on this plating medium (flat, round, translucent, pinkish colonies) that allows discrimination from contaminants that may break through the antibiotic barrier. Most contaminants are more opaque than the Campylobacter spp. colonies when the plate is held in front of a light source. Characteristic colonies were used to prepare wet mounts. Wet mount preparations were observed at 1,000x magnification by phase-contrast microscopy for characteristic cellular morphology and motility. A positive result with a latex agglutination kit specific[8] for Campylobacter jejuni, C. coli, and C. lari was confirmatory. Counts for the cecal contents of each chick were converted to $\log_{10}$ Campylobacter spp./g cecal material.

Calculation of Colonization and Protection Factor

Colonization factor (CF) was calculated as described by Bailey et al. (1988a). A mean $\log_{10}$ cfu/g of cecal material was determined for all samples within a treatment group for each challenge organism. Essentially, this calculation was the same as that described by (Pivnick et al., 1985) for infection factor, but because the colonization studied in this study was commensal and not pathogenic, the term colonization was favored over infection. Rate of colonization was calculated by dividing the number of birds colonized by the organism by the total number of birds challenged with the organism. Protection factor (PF), as described by (Pivnick et al., 1985), was the ratio of the CF for an untreated group of chicks to the CF for a treated group within the same experiment. A larger PF indicated that a treatment was more effective against colonization by the challenge organism. This ratio is widely accepted as a means for calculating CE culture efficacy. For the two treatment groups in which colonization was completely prohibited, a value of 0.01 log cfu/g cecal material was used to generate a PF.

Statistical Analysis

Data were analyzed by analysis of variance.[9] Non-continuous incidence data were compared with a chi-square test for independence; significance was assigned at $P < 0.01$. Mean CF values were compared using a Student's t test; significance was assigned at $P < 0.01$.

RESULTS

Campylobacter *spp.*

Campylobacter spp. colonization results are summarized in Table 1. Protection factors generated by the CE cultures in this experiment ranged from 0.95 to 1.29. A 1.66 log cfu/g cecal material was the greatest reduction observed with an average reduction of 0.38 log cfu/g cecal materials. Incidence of colonization was reduced by 0 to 10% with an average reduction of 2.2%. The MCE cultures

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[6]Tekmar Co., Cincinnati, OH 45222.
[7]Reynolds Qwik Seal, Richmond, VA 23261.
[8]Integrated Diagnostics, Inc., Baltimore, MD 21227.
[9]Statistica, StatSoft, Inc., Tulsa, OK 74104.

TABLE 1. Mean colonization factor (CF)[1] and rate of colonization [% (+)][2] for three replicate experiments, each replicate done with three matched pairs of competitive exclusion (CE) cultures and mucosal CE (MCE) cultures: *Campylobacter* spp. results (log cfu *Campylobacter* spp./g cecal material)

	CE[4]		MCE[5]		Control	
Replicate	CF	Number +/ total	CF	Number +/ total	CF	Number +/ total
1	6.9 ± 0.6[3]	29/30	5.0 ± 1.0	3/30	7.5 ± 0.03	10/10
2	6.9 ± 0.6	29/30	5.0 ± 1.1	10/30	7.4 ± 0.4	10/10
3	7.0 ± 0.4	30/30	5.9 ± 0.9	27/30	7.1 ± 0.8	10/10
Total	7.0[a] ± 0.3	88/90[x]	5.3[b] ± 0.6	40/90[y]	7.3[a] ± 0.3	30/30

[a,b]Colonization factor means within rows with no common superscript differ significantly ($P \leq 0.01$) by Student's *t* test.

[x,y]Incidence of positive values within rows with no common superscript differ significantly ($P \leq 0.01$) by chi-square test for independence.

[1]CF = mean log *Campylobacter* spp. count per gram of cecal material in all samples within a treatment group.

[2]Number of chicks from which *Campylobacter* spp. were isolated per number challenged.

[3]95% confidence interval.

[4]Competitive exclusion.

[5]Mucosal competitive exclusion.

resulted in PF of 1.05 to 2.22. A reduction of 4.1 log cfu/g of cecal materials was the most effective treatment; the average for all nine treated groups was 2.01 log cfu/g cecal materials. Colonization incidence reduction ranged from 0 to 60% for MCE-treated groups; a 15.6% reduction was the average for all 90 birds compared with the untreated controls.

Salmonella *spp.*

Results for *Salmonella* colonization are summarized in Table 2. A greater reduction in incidence was noted in terms of the *Salmonella* populations of the chick ceca compared to the *Campylobacter* spp. data. The PF values of CE-treated chicks ranged from 0.6 to 28.5 (average = 4.07). A reduction greater than 4 log was noted for the most effective CE culture used. On average, a reduction of 0.25 log cfu/g cecal materials was noted. Considerable variation in the log reduction was observed for this variable, thus this average value may not have much significance. Incidence was decreased from 0 to 90% with a 12.2% reduction noted on average. The PF values for MCE

treatment ranged from 0.75 to 428 (average = 114.8). The greatest reduction noted was 5.5 log with a 2.97 log reduction being observed for all nine treatment groups in terms of colonization. Application of MCE cultures resulted in decreases of *Salmonella* spp. colonization from 0 to 100% with an average reduction of 55.6% noted.

DISCUSSION

In this study, a comparison in the efficacy of CE cultures generated by two methods was made. Ceca from a single bird were used to create paired cultures (one by each method) so that the differences in culture preparation would be accurately evaluated. Efficacy for the cultures was evaluated in terms of rate and level of colonization for *Campylobacter* spp. and *Salmonella*.

The *Campylobacter* spp. rate of colonization was only decreased by 2.2 and 15.6% in CE- and MCE-treated birds, respectively. Although there was a statistically significant ($P \leq 0.01$) difference between the degree of colonization in birds treated with the two culture types by chi-square analysis, it is unlikely that this degree of reduction offers

TABLE 2. Mean colonization factor (CF)[1] and rate of colonization [% (+)][2] for three replicate experiments, each replicate done with three matched pairs of competitive exclusion (CE) cultures and mucosal CE (MCE) cultures: *Salmonella* spp. results (log cfu *Salmonella* spp./g cecal material)

	CE		MCE		Control	
Replicate	CF	Number +/ total	CF	Number +/ total	CF	Number +/ total
1	3.0 ± 1.0[3]	19/30	0.1 ± 0.2	3/30	4.3 ± 1.1	10/10
2	4.5 ± 0.5	30/30	0.9 ± 0.5	10/30	5.5 ± 0.6	10/10
3	4.4 ± 0.5	30/30	2.8 ± 0.6	27/30	2.9 ± 1.1	7/10
Total	4.0[a] ± 0.4	79/90[x]	1.3[b] ± 0.3	40/90[y]	4.2[a] ± 0.6	27/30

[a,b]Colonization factor means within rows with no common superscript differ significantly ($P \leq 0.01$) by Student's *t* test.

[x,y]Incidence of positive values within rows with no common superscript differ significantly ($P \leq 0.01$) by chi-square test for independence.

[1]CF = mean log *Salmonella* spp. count per gram of cecal material in all samples within a treatment group.

[2]Number of chicks from which *Salmonella* spp. were isolated per number challenged.

[3]95% confidence interval.

much in practical significance in terms of food safety risks. The average decrease of 1.63 log cfu *Campylobacter*/g cecal material in the MCE-treated vs. the CE-treated birds was also statistically significant ($P \leq 0.01$). However, for both treatments, the PF calculated for each treatment was 1.06 and 1.47 for CE and MCE, respectively. This low level of protection would not, on its own merit, warrant the energy and expense incurred in application of either culture type. Even though MCE cultures tend to prevent *Campylobacter* spp. colonization more effectively than CE cultures, a practical level of effectiveness was only infrequently achieved. Because *Campylobacter* is known to inhabit the niche of the intestinal tract, which contains mucin, it was thought that a CE culture using bacteria harvested from this region would make a more effective treatment (Stern, 1994).

Generally, CE cultures have been proven more effective in terms of limiting *Salmonella* colonization than in preventing *Campylobacter* spp. colonization. Rate of colonization by *Salmonella* spp. decreased by almost half in MCE- vs. CE-treated birds, on average, which was a significant decrease as determined by chi-square analysis ($P \leq 0.01$). In addition, a reduction in colonization level of 525 cells of *Salmonella* spp. per gram of cecal material was achieved when the MCE-treated birds were compared with those that received the CE treatment. There was a significant difference in the average levels of colonization by analysis of variance ($P \leq 0.01$). In addition, the PF value for MCE treatment was 28 times greater than that for CE-treated birds.

A reliable method for preventing colonization by *Campylobacter* spp. is not available. In this study, effective decreases in rate and level of *Salmonella* spp. colonization were observed. Hatchery sanitation, litter treatments, and use of CE cultures may eventually be incorporated into a multifaceted intervention program that could limit colonization by *Campylobacter* spp. and *Salmonella* spp. during production. Combined with transport modifications, chill tank water amendments, and good hygiene during preparation of foods, we hope to significantly diminish the consumer exposure to *Salmonella* spp. and *Campylobacter* spp. associated with poultry.

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