The intentional early colonization of the intestinal tract with beneficial microflora, known as competitive exclusion, has been shown to successfully protect poultry from selected enteric pathogens. Although effective cultures have been produced and are available, an inexpensive, air-tolerant, and completely defined culture is needed. Presently, we developed an in vitro competition assay to select for individual facultative anaerobes of poultry enteric origin that could exclude *Salmonella*. Using this assay, 24 isolates were selected and stored individually. These 24 isolates were amplified in batch culture (tryptic soy broth, 4 h at 40°C) and administered at final dilutions of 10, 100, or 1,000 cfu to day-of-hatch poults. Forty-eight hours later, poults were challenged with 100 to 1,000 cfu antibiotic-resistance-marked *Salmonella enteritidis* PT 13A by oral gavage. Five days later, all poults were killed, and cecal tonsils were aseptically removed for tetrathionate enrichment (24 h at 37°C) followed by selective plating with marker antibiotics. Selected lactose-negative, antibiotic-resistant colonies typical of *Salmonella* were further confirmed by serogrouping. Treatment-related protection ranged from 0 to 100% in three experiments. Greatest protection was related to the lowest concentrations of the protective microflora in each experiment. These data suggest that effective combinations of competitive enteric microflora can be identified by appropriate in vitro selection methods.

(Key words: competitive exclusion, *Salmonella*, turkey poult, cecal microflora, poultry)

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INTRODUCTION

Many of the more than 200 pathogenic serovars of the genus *Salmonella* are able to colonize the gastrointestinal tract of poultry (Gast, 1997). Although most paratyphoid *Salmonella* infections of poultry are subclinical, poultry products have been reported to provide an important vehicle for human infections. An estimated 1.4 million cases of food-borne salmonellosis occur annually, with the total combined costs associated with medical care and lost productivity in the United States estimated at up to $3.5 billion annually (United States Department of Agriculture, 1995).

Competitive exclusion (CE), first described by Nurmi and Rantala (1973), has been an effective method of control for salmonellosis in commercial poultry flocks. This concept has been extensively studied by other laboratories including Snoeyenbos et al. (1979), Impey et al. (1987), Stavric et al. (1987), Bailey et al. (1988), and others (1992), Schneitz and Nuotio (1992), Ziprin et al. (1993), Nisbet et al. (1996), and Mead (2000). Cultures of normal gut microbiota often contain an undefined mixture of bacteria. Although no commercially available and documented effective CE product is completely defined, PRE-EMPT is a nearly defined culture that is continuously produced from the same seed stock, arguably reducing the risk of reintroduction of pathogens (Corrier et al., 1995; Nisbet et al., 1996). However, other existing cultures are periodically amplified or passaged in specific pathogen free birds (Snoeyenbos et al., 1979; Nurmi et al., 1987). Although these cultures are effective (Impey et al., 1987; Schneitz and Nuotio, 1992; Nisbet et al., 1996), a completely defined culture that is continuously derived from a single defined group of bacteria could be an inexpensive, safe alternative. To date, most investigators have argued that complex microflora may be required for effective CE to allow redundancy for adaptation to variable environments within the gastrointestinal tract (Corrier et al., 1995; Mead 2000). Additionally, attempts to create defined and effective CE cultures have been previously thwarted by assumptions that selective media for

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Abbreviation Key: BGA = brilliant green agar; CE = competitive exclusion; NA = naladixic acid; TSB = tryptic soy broth.
many enteric microflora are not available. Only approximately one-quarter of the intestinal microflora have been characterized, and valid in vitro selection criteria have not been demonstrated (Mead, 2000).

Presently, we have 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 pouls from *Salmonella* infection following challenge.

**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 acid3 (NA). For these experiments, *Salmonella* was grown in tryptic soy broth4 (TSB) for approximately 8 h. The cells were washed three times with 0.9% sterile saline by centrifugation (3,000 x 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 brilliant green agar5 to determine the number of colony-forming units (cfu) of each sample was then added to the wells of a sterile 96-well microtiter plate7 (80 µL/well). Eighty microliters of TSB containing SE at 10³, 10⁴, or 10⁵ cfu/mL was added to the microtiter plate, in duplicate with supplemented medium as described above. Control plates consisted of the same arrangement of cecal isolates without SE. Paired plates were incubated in a bacteriological incubator at 37°C, or a modified anaerobic incubator flushed with eight volumes of CO₂ prior to incubation at 40°C. To ensure the selection of facultative anaerobes, the optical density (625 nm) of the control plates incubated in the modified anaerobic chamber was determined. Subsequent to incubation of the microtiter plates, each well of the microtiter plates containing cecal isolates and SE was streaked on BGA that contained antibiotics to which the SE was resistant. After overnight incubation at 37°C, the BGA plates were qualitatively analyzed for the ability of the cecal isolates to inhibit SE growth as described above. All cecal isolates (33 isolates) able to inhibit or reduce SE growth were selected for further study.

All selected cecal isolates were identified in our laboratory using API strips11 and at the Arkansas State Diagnostic Laboratory (Springdale, AR). All potential pathogens including *Escherichia, Klebsiella*, and *Proteus* were safety tested in broilers and turkeys (5 per tested isolate for each route of administration) by intraperitoneal, air sac, and subcutaneous injection. Inoculated birds were examined for morbidity, mortality, and lesions. All bacteria that caused any evidence of disease were removed as candidates from the culture. The 24 remaining individual cecal isolates included in the culture were combined, aliquoted, and stored with 30% glycerol at −80°C.

**In Vitro Selection of Cecal Microflora**

Ceca were aseptically removed from 46 apparently healthy chickens (6 to 74 wk of age) housed at the University of Arkansas poultry farm and placed in sterile sample bags. Ceca were immediately frozen in liquid nitrogen for 1 to 3 h and stored overnight at −80°C. Four grams of each sample was thawed and individually diluted 10-fold weight to volume in TSB. The final dilution of each sample was then added to the wells of a sterile 96-well microtiter plate2 (80 µL/well). Eighty microliters of TSB containing SE at 10³, 10⁴, or 10⁵ cfu/mL was added to the wells of the microtiter plates containing the cecal samples so that each well was inoculated with 3.3 x 10³, 10⁴, or 10⁵ cfu of SE. Ferric ammonium citrate6 at 2.75 g/100 mL TSB (10.1 mg/mL final concentration per well) and sodium thiosulfate9 at 0.040 g/100 mL TSB (0.15 mg/mL final concentration per well) were used as indicators of SE growth and added at 80 µL/well.

Microriter plates were incubated overnight in a bacteriological incubator at 37°C. Plates were then qualitatively examined for the presence or absence of black precipitate consistent with *Salmonella* growth (DIFCO Laboratories, 1984). Using a sterile loop, samples from the wells without black precipitate (no color change) were streaked for isolation on BGA and tryptic soy agar10 and incubated overnight in a bacteriological incubator at 37°C. All resulting non-*Salmonella* colonies were reisolated on tryptic soy agar to ensure purity. A single, isolated colony was grown in 10 mL TSB for 8 h, or until the culture was turbid. Sterile glycerol was then added to the culture in TSB, and the suspension was aliquoted and stored at −80°C.

Continued screening began with growing each cecal isolate for 8 h in TSB in a bacteriological incubator at 37°C. Twenty-five microliters of each cecal isolate was combined with 3.3 x 10³ cfu SE and 215 µL TSB in 96-well microtiter plates, in duplicate with supplemented medium as described above. Control plates consisted of the same arrangement of cecal isolates without SE. Paired plates were incubated in a bacteriological incubator at 37°C, or a modified anaerobic incubator flushed with eight volumes of CO₂ prior to incubation at 40°C. To ensure the selection of facultative anaerobes, the optical density (625 nm) of the control plates incubated in the modified anaerobic chamber was determined. Subsequent to incubation of the microtiter plates, each well of the microtiter plates containing cecal isolates and SE was streaked on BGA that contained antibiotics to which the SE was resistant. After overnight incubation at 37°C, the BGA plates were qualitatively analyzed for the ability of the cecal isolates to inhibit SE growth as described above. All cecal isolates (33 isolates) able to inhibit or reduce SE growth were selected for further study.

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**CE Culture**

A single aliquot of the combined culture containing the 24 selected isolates was thawed and grown in 500 mL of pre-reduced TSB for 4 or 7 h in a modified anaerobic incubator, flushed with eight volumes of CO₂ at 40°C. The culture was then serially diluted with 0.9% saline 10³ to 10⁶X for administration to pouls.
Poults

Commercial cross (BUTA\textsuperscript{12}) poults were obtained on the day of hatch and were orally gavaged with the appropriate culture before placement in floor pens. Treatments consisted of 20 poults per treatment group. Each pen was approximately 1.0 m\textsuperscript{2} in area, and the floor was covered with clean softwood shavings. Poults were provided antibiotic-free feed, formulated to meet or exceed NRC recommendations for critical nutrients for day-of-hatch poults (NRC, 1994), and water ad libitum. Poults were administered SE challenges of 10\textsuperscript{2} or 10\textsuperscript{4} cfu by oral gavage 48 h after placement. At 7 d after placement, poults were humanely killed by CO\textsubscript{2} asphyxiation; cecal tonsils were aseptically removed and enriched in 20 mL of tetra-thionate broth.\textsuperscript{13} Cecal tonsils were incubated for 24 h at 37\textdegree C and streaked on BGA plates containing 25 \(\mu\text{g/mL}\) novobiacin and 20 \(\mu\text{g/mL}\) NA. The plates were incubated for 24 h at 37\textdegree C and examined for the presence of lactose-negative, NA-resistant \textit{Salmonella} colonies. Selected lactose-negative, antibiotic-resistant colonies typical of \textit{Salmonella} were further confirmed by serogrouping.

Experimental Design (In Vivo Challenge)

For administration to poults, the CE culture was grown in 500 mL of pre-reduced TSB for either 4-h or 7-h at 40\textdegree C under reduced oxygen conditions as described above. The culture was then serially diluted in 0.9% saline at 10-fold increments. Poults were gavaged with 0.25 mL of culture or vehicle (controls) on day of hatch. All poults were challenged with 10\textsuperscript{4} cfu \textit{Salmonella enteritidis} on day of hatch, and cecal tonsils were aseptically removed. Cecal tonsils were enriched for \textit{SE} recovery as described above.

Statistical Analysis

The chi-square test of independence was used to determine significant differences \((P < 0.05)\) in \textit{Salmonella} recovery between treatments within experiments testing all possible combinations as described in the figures (Zar, 1984).

RESULTS AND DISCUSSION

In experiment 1, the incidence of SE recovery was 65\% in controls, this 4-h culture reduced \((P < 0.05)\) the incidence of SE by 92\% when poults were administered CE at a lowest dilution evaluated (10\textsuperscript{5}X; Figure 1A). However, administration of 10-fold higher dose of this culture (10\textsuperscript{5}X dilution) did not affect the incidence of SE recovery, and a 100-fold higher dose of this culture actually increased \((P < 0.05)\) the incidence of SE recovery from treated poults.

Similarly, in experiment 2, treatment of SE-challenged poults with the lowest dose of 4-h culture evaluated (10\textsuperscript{5}X dilution) reduced \((P < 0.05)\) SE recovery by 88\% as compared to the untreated control group (Figure 1B). Ten to 100-fold higher doses caused smaller but significant reductions in SE recovery from culture-treated poults, but administration of a dose 1,000-fold higher (10\textsuperscript{8}X dilution) than the most effective dose (10\textsuperscript{5}X dilution) did not affect SE recovery.

In experiment 3, batch amplification of the combined culture containing 24 bacterial isolates for either 4- or 7-h incubation was compared. Additionally, in this experiment the washed 4- or 7-h bacterial cultures were diluted to similar optical densities prior to administration, and total colony-forming units of the administered cultures were determined (less than 60\% difference), allowing

\textsuperscript{12}British United Turkeys of America.
\textsuperscript{13}210420, Becton, Dickinson, and Co., Sparks, MD.
The present culture represented a total of 24 bacterial isolates retained for in vivo testing, and these isolates were selected through screening approximately 4 to 8 × 10⁶ cecal bacteria as described above. The 24 isolates selected for in vivo testing included Escherichia (14 isolates), Citrobacter (two isolates), Klebsiella (two isolates), Enterobacter (one isolate), Staphylococcus (two isolates), Enterococcus (one isolate), and Bacillus (one isolate). 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 poult from Salmonella infection, when administered at the appropriate dose (experiments 2 and 3), also suggests that rather simple cultures can indeed provide protection.

However, the protection observed in the present experiments was clearly dose-dependent, with one- to two-log increases in dose (lower dilutions) either having no effect or actually increasing the incidence of Salmonella colonization. The reason for no or negative effects of the present CE culture at higher doses is not known. Possibilities include displacement of needed component(s) of the CE at higher doses, exclusion of environmentally derived microbiota necessary for efficacy, or inclusion of one or more organisms in the CE culture that directly reduce innate resistance to Salmonella colonization in the poult.

Because the batch culture method of the combined 24 bacterial isolates in the present experiments approached maximum turbidity by approximately 12 h, some of the 24 bacteria might out-compete others. We hypothesized that microflora diversity might be more restricted by the 7-h batch culture method. Selected dilutions of 4- or 7-h batch-amplified CE were simultaneously evaluated in experiment 3. The lowest dose (10⁵ X dilution or –10 cfu) of culture amplified 4 h and evaluated in experiment 3 caused significantly (P < 0.05) less Salmonella colonization than the 7-h culture at a similar dose. However, a much higher dose of the 7-h culture in experiment 3 did significantly reduce SE recovery as compared to controls, suggesting a difference in potency between this culture produced at different incubation times. In future studies, the issue of diversity-related efficacy may be more directly evaluated by individual propagation of individual isolates and combination of selected isolates prior to administration.

While the results of these experiments suggest that relatively simple and defined CE cultures are possible, and that in vitro selection of such microflora is possible, several issues remain to be elucidated. Although extremely high doses of other cultures have not been evaluated, negative effects of high doses of CE cultures have not been reported in previous studies with other cultures (Nurmi and Rantala, 1973; Schneitz and Nuotio, 1992; Corrier et al., 1995). The negative effects observed with the present CE combination were observed at doses at least 10- to 100-fold higher than those found to be effective (Figures 1 and 2). As many pharmaceuticals or, indeed even nutrients, have negative side effects at doses or concentrations 100-fold higher than recommended, this ob-

![FIGURE 2. Comparison of 4-h and 7-h batch culture of competitive exclusion and Salmonella enteritidis recovery in turkey poult. CE culture was grown for 4-h or 7-h in pre-reduced tryptic soy broth at 40°C in a modified anaerobic incubator, flushed with 6 volumes of CO₂. Both cultures were adjusted to approximately the same optical density (625 nm) prior to dilutions, and colony forming units determined by plating serial dilutions on tryptic soy agar. Culture was diluted to appropriate dilution in 0.9% saline and administered to day of hatch poult via oral gavage before placement in floor pens. Control birds were administered 0.9% saline. All poult were challenged with 10⁵ cfu Salmonella enteritidis two days after placement. Feed and water were provided ad libitum. Cecal tonsils were aseptically removed from poult at seven days after placement and conventionally enriched for Salmonella enteritidis. Bars with different superscripts are significantly different (P < 0.05). Bars within treatment dose with different superscripts are significantly different (P < 0.05).](https://academic.oup.com/ps/article-abstract/82/9/1378/1537442)
servation alone is not necessarily cause for concern. Never-
theless, an increased understanding of this phenomen-
on from future studies could lead to improvements in
both efficacy and safety.

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