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Research Note

Assessment of the Effects of Nurmi-Type Cultures and a Defined Probiotic Preparation on a *Salmonella* Typhimurium 29E Challenge In Vivo

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ABSTRACT

The effects of treatment with an undefined commercial Nurmi-type culture (NTC), cultured cecal contents, and a dual-strain probiotic, containing *Enterococcus faecalis* and *Pediococcus pentosaceus*, on *Salmonella* Typhimurium colonization were evaluated in a specific-pathogen-free bird model. Two sets of trials were performed, and each study was arranged as a randomized complete block design with three treatments. Treatments consisted of (i) control, (ii) commercial NTC, and (iii) cultured cecal contents in the first set of trials and (i) control, (ii) defined probiotic, and (iii) cultured cecal contents in the second set. On day 1, birds were administered 1.2×10^7 CFU of the appropriate treatment by oral gavage. On day 3, all birds were challenged with 1×10^6 CFU of *Salmonella* Typhimurium 29E (nalidixic acid resistant). Chicks were asphyxiated with argon gas on day 10, and ceca were aseptically removed. *Salmonella* Typhimurium counts (CFU per milliliter of cecal contents) were determined on brilliant green agar containing 30 mg of nalidixic acid per liter, and CFU counts were log transformed prior to analysis. Cecal pH and volatile fatty acid concentrations were also determined. Data were analyzed by one-way analysis of variance, and means were compared by Tukey's pairwise analysis. Commercial NTC and cultured cecal contents treatments resulted in a significant decrease ($P \leq 0.05$) in *Salmonella* Typhimurium 29E colonization, with the NTC offering a higher level of protection. In the second set of trials, the defined probiotic tended to reduce colonization by *Salmonella* Typhimurium ($P = 0.07$), while chicks treated with cultured cecal contents displayed a significant decrease ($P = 0.03$) when compared to the negative control. No significant change was observed in cecal pH or in acetate and propionate concentrations; however, a significant increase in butyrate concentrations in both the cultured cecal contents and defined probiotic treatment groups was observed when compared to the control birds. These observations suggest that defined cultures are less effective *Salmonella* control agents than are preparations generated from the complete cecal microflora.

Salmonella is not a native member of the microbiota of poultry, but it readily colonizes the intestines of young chicks and persists during rearing *(49).* Cross-infection in the flock and transfer of *Salmonella* to carcasses by fecal contamination during processing are common. Consequently, poultry products contaminated with *Salmonella* are a major source of foodborne disease in most developed countries.

Competitive exclusion (CE) is the treatment of newly hatched chicks with suspensions of cecal or fecal droppings obtained from healthy adult birds. Administration of such cultures allows the early establishment of a normal avian gut flora, thus excluding *Salmonella* from 1-day-old chicks. The protective effect of indigenous intestinal microflora against *Salmonella* colonization was first demonstrated by Nurmi and Rantala in 1973 *(35)* and has since been confirmed by numerous researchers *(2, 7, 33, 38).* Over the decades, various commercial CE cultures have been developed and demonstrated to reduce contamination of *Salmonella* in poultry *(8, 13, 39, 41).*

There are many commercial poultry CE products available on the market today. These include Broilact (Orion Corporation, Turku, Finland), PREEMPT (MS Bioscience, Madison, Wis.), and Aviguard (Microbial Developments Ltd., Spring Lane North, Malvern Link, Worcestershire, UK). One feature they all have in common is that the original inoculum was obtained from a chicken cecum. Broilact, which is derived from the entire cecal contents of an adult chicken, undergoes a selection process that avoids the presence of clostridia or other human pathogens. This product has been shown to be effective against *Salmonella (41, 42)* and *Campylobacter jejuni (16)* colonization in poultry. The efficacy of PREEMPT has been well documented *(21, 23, 27, 33, 47).* Undefined Aviguard has also been proven effective in controlling pathogen colonization in poultry *(13, 20, 31).*

In many areas of the world, it is impossible to gain approval for the use of undefined CE products from regulatory agencies because of the potential risk of transmitting human or avian pathogens. Therefore, isolated strains from

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undefined gut microflora, used singly or in mixtures, have been assessed for their ability to protect chicks against challenge with *Salmonella.* In some cases, mixtures of defined cultures have been shown to offer substantial protection in the control of *Salmonella* colonization *(33).*

The application of *Enterococcus faecalis* as a probiotic has been well reviewed in the literature *(9, 22, 36).* This species has been documented to exhibit CE effects in the protection of chicks against *Salmonella* Typhimurium colonization *(43).* Indeed, it has been shown that *E. faecalis,* isolated from PREEMPT, protected epithelial cells against *Salmonella* Typhimurium invasion in vitro (47). The potential of *Pediococcus pentosaceus* as a probiotic has also been recognized *(14, 25, 30).*

The purpose of this study was to assess the efficacy of a commercial undefined Nurmi-type culture (NTC) and cecal contents, which had been cultured in-house at small scale (in a manner similar to that for the production of the commercial NTC), for their ability to decrease *Salmonella* colonization in specific pathogen-free chicks. In addition, the relative concentrations of *E. faecalis* and *P. pentosaceus* that exist in the commercial NTC served as a model for the generation of a dual-strain, defined probiotic. This culture was subsequently tested alongside cultured cecal contents for its ability to reduce *Salmonella* colonization in chicks.

MATERIALS AND METHODS

Treatments. A lyophilized sample of a commercial NTC was supplied by Alltech, Inc. (Nicholasville, Ky.).

Cecal contents (1 ml) obtained from pathogen-free adult birds were cultured anaerobically at 37°C for 3 days in 100 ml of anaerobic Viande Levure *(3)* broth, pH 6.7, with no agitation. An aliquot (400 μl) of this culture was used to inoculate fresh anaerobic Viande Levure broth (400 ml) and was incubated at 37°C, with no agitation for another 4 days. Cells were collected by centrifugation at 4,800 × *g* for 10 min using an MSE Mistral 1000 centrifuge (Shaw Scientific Ltd., Dublin, Ireland). Maltodextrin was added to obtain a final concentration of 10% (wt/wt) in cecal contents, and the pellet was frozen and lyophilized.

A defined, dual-strain probiotic preparation (containing *E. faecalis* and *P. pentosaceus* in a ratio of 98.7:1.3, respectively) was generated that was based on the concentrations at which these species occur in the commercial NTC. A minimum prerequisite for microorganisms to be accepted as probiotics is that they do not carry transferable antibiotic resistance genes *(12).* As such, *E. faecalis* and *P. pentosaceus* isolates were tested for their susceptibility to the antibiotics ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline, and vancomycin on Mueller-Hinton II agar. Agar plates contained twofold serial dilutions of antibiotics as previously described in the agar dilution method *(1, 32),* and MICs in milligram per liter were recorded for each antibiotic for both strains.

Experimental design. Ninety ($n = 90$) day-of-hatch male broiler (Cobb × Cobb) chicks were housed in chick isolator chambers (1.8 m^2; Fiberglass Unlimited, Inc., Roanoake, Ala.), which had previously been sanitized and disinfected. Each chamber contained two sterile bacterial filters to prevent the cross-contamination of bacterial populations between different treatment groups and to prevent the escape of pathogenic organisms into the environment. Chambers were equipped with a container of feed and a drinker supplying water. Feed and water, previously sterilized by autoclaving, were provided ad libitum. The composition of the antibiotic-free laboratory-mashed broiler diet is detailed in Table 1. Birds were maintained in a temperature-controlled room at 34°C for the first 2 days of the trial; the temperature was then gradually lowered to 28°C. Birds were randomly assigned to nine chambers, forming three groups of 30 chicks, two treatment groups, and a control group. Total anaerobic bacterial counts were performed on reinforced clostridial agar (Becton Dickinson, Sparks, Md.) for all treatments, and birds from each treatment group were dosed orally with 1.2×10^7 CFU of commercial NTC and cultured cecal contents in trial 1. In trial 2, birds were dosed orally with 1.2×10^7 CFU of cultured cecal contents and the defined probiotic. Treatments were administered in 0.25 ml of 0.1% (wt/vol) sterile peptone water on day 1 of the trial. Control groups were dosed with 0.25 ml of 0.1% (wt/vol) sterile peptone.

TABLE 1. *Composition of antibiotic-free broiler diet*

Ingredient	% content as fed (wt/wt)
Corn	53.32
Soya bean meal	38.02
Corn oil	4.37
Dical (phosphate)	2.04
Limestone	1.25
Salt	0.50
D,L-Methionine	0.25
Vitamins	0.25

Salmonella Typhimurium 29E was cultured in tryptic soy broth, and enumeration was performed on brilliant green agar containing 30 mg of nalidixic acid per liter (BG-NA). On day 3 of the trial, each bird was challenged with 1×10^6 CFU of *Salmonella* Typhimurium 29E in 0.25 ml of sterile peptone water by oral gavage. Both trials were performed in duplicate.

Sampling and sample analysis. The trial was terminated on day 10, chicks from each group were killed by asphyxiation with argon gas, and the ceca from the chicks of both groups were aseptically removed, retained, and subsequently analyzed.

***Salmonella* enumeration.** The contents of one cecum were placed in a sterile test tube, weighed, and diluted 1:10 with sterile 0.1% (wt/vol) peptone water, after which an enumeration of *Salmonella* in the cecal contents was performed on BG-NA. Nalidixic acid was added to the media to facilitate the selection of antibiotic-resistant challenge organisms *(44).* When no growth of *Salmonella* was detected on BG-NA plates, these cecal content samples were denoted as ''No Growths.'' However, it was necessary to culture the empty cecum from the corresponding No Growth in lactose broth to confirm that *Salmonella* was not present in these samples. This was determined by carrying out a series of enrichment steps and subsequent confirmatory tests *(48).*

The empty cecum was cut longitudinally, placed in lactose broth, and cultured overnight at 37°C *(44).* Aliquots of the lactose broth culture (1 ml) were further cultured in tetrathionate broth *(17)* and selenite cysteine broth *(26)* (9 ml of each) and incubated at 37°C overnight. These cultures were then streaked on BG-NA and xylose lysine deoxycholate agar and incubated at 37°C overnight *(6).* Suspect *Salmonella* colonies from these cultures were streaked on triple sugar iron agar slants *(15),* which were subsequently stabbed and incubated overnight at 37°C. Black colonies were tested by Analytab Products analysis (bioMérieux, Marcy l'Etoile, France) (API 20E) according to the manufacturer's instructions to confirm the presence of *Salmonella.*

TABLE 2. *Effects of commercial NTC and cultured cecal contents on SPF chicks*[a]

	Values by treatment:			
	Control	NTC	CCC	SEM
Log CFU g^{-1} of *Salmonella* Typhimurium 29E	6.5 A[b]	3.5 B	4.2 B	0.323
pH	5.7	5.5	5.7	0.07
VFA (mM):				
Acetate	56.4	49.6	52.3	0.349
Propionate	1.6	2.1	1.8	0.118
Butyrate	1.9	2.1	2.1	0.246
Analysis of No Growth of *Salmonella*:				
On BG-NA	1	26	21	NA
Following confirmatory analysis	1	25	21	NA

[a] Statistical analysis was performed using one-way analysis of variance and Tukey's pairwise post hoc analysis. $n = 60$ birds per treatment. NTC, commercial Nurmi-type culture; CCC, cultured cecal contents; SEM, standard error of the mean; BG-NA, brilliant green agar containing nalidixic acid; NA, not applicable; SPF, specific pathogen free.

[b] Means with no letter in common are statistically different at $P \leq 0.05$.

Cecal samples, which were found not to produce *Salmonella* colonies on BG-NA plates but resulted in *Salmonella* growth following enrichment tests, were assigned a concentration of 1.50 log CFU g^{-1} of *Salmonella*. Samples that were confirmed not to contain *Salmonella* either on BG-NA plates or following enrichment testing were assigned a concentration of 1 CFU g^{-1} of *Salmonella* (0 log CFU g^{-1}) *(34)*.

Determination of pH and VFA concentrations. Cecal contents were diluted 1:10 with distilled water, and pH was determined with a combined electrode (Orion Research Inc., Boston, Mass.). Samples were stored at −80°C for subsequent analysis of volatile fatty acids (VFAs). VFA analysis was conducted on a Hewlett-Packard model 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) fitted with a glass column (180 cm by 4 mm), containing 10% (wt/wt) SP 1000 and 1% (wt/wt) H_3PO_4 on 100/120 Chromosorb WAW (Supelco Inc., Bellofonte, Philadelphia, Pa.) with a modification described by Erwin *(11)*. The carrier gas used was nitrogen, the flow rate was 32 ml min^{-1}, and the retention times for acetate, propionate, and butyrate measurements were 2.033, 3.064, and 4.692 min, respectively. The oven temperature was set at 135°C, while the injector and detector temperatures were set at 190°C.

Statistical analysis. Statistical calculations were performed using the PC version of MINITAB (Minitab Inc., Philadelphia, Pa.). Bacterial CFU counts were subjected to log transformations prior to analysis. Data were analyzed by one-way analysis of variance, appropriate for the randomized complete block design, with chambers (10 birds) considered the experimental unit. Each trial was used as a blocking factor to analyze if there was any trial-to-trial variation. Trials were carried out in duplicate, and when no significant trial-by-treatment interaction was observed, the data were pooled across both trials. Post hoc analysis was carried out using Tukey's pairwise analysis.

RESULTS

Salmonella Typhimurium 29E challenge trials were carried out in duplicate, and results were pooled, as there were no significant trial-by-treatment interactions observed ($P > 0.05$). Chicks dosed with 1.2×10^7 CFU of the commercial NTC or cultured cecal contents were more resistant to colonization of the cecum than were control birds when challenged with 10^6 CFU of *Salmonella* Typhimurium 29E ($P \leq 0.05$) (Table 2). Treatment with the commercial NTC, however, significantly reduced the colonization of *Salmonella* to a larger degree than treatment with cultured cecal contents (Table 2). Confirmatory analysis of *Salmonella*-free cecal content samples indicated that a higher number of No Growths were identified in birds treated with the commercial NTC or cultured cecal contents than in controls (Table 2). No significant change in cecal pH or in acetate, propionate, and butyrate concentrations was observed when chicks were treated with commercial NTC or cultured cecal contents (Table 2).

After observing the success of the commercial NTC as a *Salmonella* control agent, a dual-strain probiotic was developed that was based on the concentrations at which *E. faecalis* and *P. pentosaceus* occur in the commercial NTC (data not shown). However, prior to the generation of the defined probiotic, *E. faecalis* and *P. pentosaceus* isolates were tested for their susceptibility to a range of antibiotics, and it was determined that both *E. faecalis* and *P. pentosaceus* were sensitive to all antibiotics tested, exhibiting MICs below the breakpoints *(12)*.

In the second set of trials, treatment of chicks with cultured cecal contents resulted in a significant decrease ($P = 0.03$) in *Salmonella* Typhimurium 29E colonization (Table 3), while treatment with the defined *E. faecalis–P. pentosaceus* combination tended to reduce colonization by *Salmonella* ($P = 0.07$) when compared to controls. Examination of the number of *Salmonella*-free cecal content samples (Table 3) demonstrated that a higher number of samples exhibiting No Growth of *Salmonella* on BG-NA plates were identified in the cultured cecal contents and defined probiotic treatment groups when compared to controls. Confirmatory tests substantiated these observations (data not shown). All cecal content samples from chicks in the control group displayed growth of *Salmonella*. However, 10 No Growths were detected in chicks treated with

TABLE 3. *Effects of the defined probiotic and cultured cecal contents on SPF chicks*[a]

	Values by treatment:			
	Control	Defined probiotic	CCC	SEM
Log CFU/g^{-1} of *Salmonella* Typhimurium 29E	7.0 A[b]	6.4 AB	5.9 B	0.04
pH	5.6	5.4	5.3	0.02
VFA (mM):				
Acetate	53.8	47.7	44.4	2.58
Propionate	1.0	1.2	1.3	0.13
Butyrate	2.2 A	3.2 B	3.2 B	0.26
Analysis of No Growth of *Salmonella*:				
On BG-NA	0	5	10	NA
Following confirmatory analysis	0	5	10	NA

[a] Statistical analysis was performed using one-way analysis of variance and Tukey's pairwise post hoc analysis. $n = 60$ birds per treatment. CCC, cultured cecal contents; BG-NA, brilliant green agar containing nalidixic acid; SEM, standard error of the mean; NA, not applicable; SPF, specific pathogen free.

[b] Means in the same row with no letter in common are significantly different at $P \leq 0.05$.

cultured cecal contents, while only five birds from the defined probiotic treatment group tested negative for *Salmonella* (Table 3). No significant change was observed in cecal pH or in the VFAs, acetate and propionate levels. However, significant increases ($P = 0.05$) in butyrate concentrations were noted in both treatment groups when compared to controls (Table 3).

DISCUSSION

Data presented in this study confirm previous reports that complex cecal preparations are very effective *Salmonella* control agents, while defined preparations are less protective *(4, 28, 45).* The first set of trials was carried out to assess the efficacy of two NTCs, one commercially sourced and the other derived in-house, as *Salmonella* control agents in chicks. Subsequently, in the second set, a dual-strain probiotic was tested alongside cultured cecal contents for its ability to reduce *Salmonella* colonization in chicks.

A minimum prerequisite for microorganisms to be accepted as probiotics is that they do not carry transferable antibiotic resistance genes *(12).* As such, *E. faecalis* and *P. pentosaceus* isolates were tested for susceptibility to a range of antibiotics, and it was determined that both species exhibited MIC values below the breakpoints *(12)* for all antibiotics tested.

Results from the first set of trials indicated that treatments with the commercial NTC and cultured cecal contents are in close agreement with previously documented reports, particularly that by Hinton et al. *(18),* who found that when chicks treated with 10^7 CFU of cultured cecal contents were challenged with 10^6 CFU of *Salmonella* Typhimurium, there was a decrease in the colonization of the challenge organism ($P < 0.05$).

In the first set of *Salmonella* challenge trials, a greater number of cecal content samples tested negative for *Salmonella* following treatment with the commercial NTC and cultured cecal contents, i.e., 25 and 21, respectively, when compared to controls (Table 2). This emphasizes that these preparations were superior CE products. Even though the same master stock of cultured cecal contents was used in this trial, following storage at −70°C for 18 months, a decrease in its effectiveness was noted in the second set of trials. Prior to the trial, this preparation was subjected to a total anaerobic count using reinforced clostridial agar *(19),* and a decrease in the viability of the culture was observed (data not shown). Sensitive protective strains and possibly nonculturable but viable bacteria may therefore have been lost during the long period of storage, resulting in a reduction in its effectiveness as a CE product. Pivnick and Nurmi *(37)* showed that the storage of undefined CE cultures results in a loss in the viability of the culture, especially if stored for more than 1 year. In addition, a more recent study has indicated that lyophilized cultures, more so than fresh cecal material, lose their initial effectiveness after some time in storage *(2).*

Cecal pH values recorded in this study were similar to the range (5.5 to 6.2) reported by Nisbet et al. *(34).* It has been demonstrated that the normal anaerobic flora of the ceca of adult chickens produce short-chain VFAs that inhibit the growth of *Salmonella (5, 40).* Indeed, another study concluded that VFAs are responsible for the reduction in numbers of members of the family *Enterobacteriaceae* in the ceca of broiler chickens during growth *(46).*

In the second set of trials, significant increases in butyrate concentrations were noted ($P = 0.05$) in both treatment groups when compared to controls (Table 3), which was not observed in the first set of trials. The reason for this is unknown. Perhaps the component strains, *E. faecalis* and *P. pentosaceus,* of the defined probiotic and the cultured cecal contents, which had been stored for 18 months, stimulated the production of this VFA more than in the initial trial. Butyrate has been documented to have a high degree of anti-*Salmonella* activity *(29),* which is thus in agreement with the reduction noted in *Salmonella* colonization in this trial. In the intestines of mice, increasing concentrations of butyrate were related to decreasing numbers

of *Enterobacteriaceae (24).* In addition, differences in cell association and invasion of epithelial cells by *Salmonella* Typhimurium were noted when this organism was grown in Luria-Bertani broth supplemented with 100 mM butyrate *(10),* suggesting a possible mechanism by which butyrate induces the exclusion of *Salmonella.*

In summary, a defined probiotic composed of two strains offered a limited degree of protection to chicks against *Salmonella* colonization but was not as effective as the more complex multistrain treatments, i.e., the commercial NTC or cultured cecal contents. These findings tend to support the view that only complex undefined CE products give adequate and consistent protection to chicks against *Salmonella* colonization.

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