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Research Note

Paenibacillus polymyxa Purified Bacteriocin To Control *Campylobacter jejuni* in Chickens

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ABSTRACT

Campylobacter spp. cause numerous foodborne diseases. Poultry is thought to be a significant source of this zoonosis. Although many interventions designed to control this agent have been researched, none have succeeded. We evaluated a bacteriocin-based treatment to reduce *Campylobacter jejuni* colonization in poultry. A previously described purified bacteriocin (class IIa; molecular mass, 3,864 Da), secreted by *Paenibacillus polymyxa* NRRL-B-30509, was microencapsulated in polyvinylpyrrolidone, and 0.25 g of the purified bacteriocin was incorporated into 1 kg of chicken feed. One-day-old chickens were orally challenged and colonized with one of four isolates of *C. jejuni*, then reared in isolation facilities. Birds were provided ad libitum access to standard broiler starter feed and water for 7 days until 3 days before sampling, when only the treated groups of birds were provided the bacteriocin-emended feed described. In each of the eight (four by two replicates) trials, significant reductions in colonization by *C. jejuni* were observed ($P \leq 0.05$). As an example of this highly consistent data, in the first trial, 10 untreated 10-day-old chickens were colonized at a mean log 7.2 + 0.3 CFU/g of feces, whereas none of the 10 bacteriocin-treated 10-day-old chickens were colonized with detectable numbers of *C. jejuni.* Bacteriocin treatment dramatically reduced both intestinal levels and frequency of chicken colonization by *C. jejuni.* Feeding bacteriocins before poultry slaughter appears to provide control of *C. jejuni* to effectively reduce human exposure. This advance is directed toward on-farm control of pathogens, as opposed to the currently used chemical disinfection of contaminated carcasses.

For more than 30 years, poultry has been cited as an important source for the direct or indirect infection of humans by *Campylobacter jejuni (4, 6, 8, 15).* Despite numerous scientific forays into *Campylobacter* epidemiology and reports on typing methodologies, physiology, pathology, and molecular biology, no information leading to substantive intervention has been offered for the practical control of the organism in poultry. The benign commensal relationship of *Campylobacter* within poultry has been documented, and this relationship has proven obstinate to colonization intervention in the live animal *(17).*

Nurmi and Rantala *(10)* first described the potential for applying competitive exclusion as a means of controlling *Salmonella* in poultry. Stern et al. *(17)* and Shanker et al. *(13)* reported that standard preparations of competitive exclusion flora, even when effective against *Salmonella* spp., offered no obstacle to colonization by *Campylobacter* spp. *C. jejuni* preferentially colonizes within the intestinal crypts of the host ceca *(1).* With the use of a special competitive exclusion flora modification originally designed to intervene in *Campylobacter* spp. colonization, only limited and inconsistent success was reported *(16).* This modified competitive exclusion flora was based on providing intestinal bacteria derived from the same eco-niche (the intestinal mucosal lining) that *C. jejuni* occupies *(16).*

We followed these studies by identifying an important mechanism involved in *Campylobacter* spp. inhibition via antagonistic enteric bacteria *(20).* In this article, we demonstrate that microencapsulated bacteriocins (from these antagonistic bacilli) administered to colonized chickens caused dramatic reductions in poultry colonization levels of *C. jejuni.*

MATERIALS AND METHODS

Preparation of bacteriocin. The organism and its associated bacteriocin purification procedure have been described in detail *(20).* This bacteriocin-producing organism, *Paenibacillus polymyxa* NRRL B-30509, was isolated from the intestinal tract of a domestic Russian broiler chicken. This isolate producing the associated bacteriocin was selected as superior to others obtained in a similar manner, having the lowest MIC for selected strains of *Campylobacter*. The bacteriocin (no. 602) secreted by isolate NRRL B-30509 has a molecular mass (determined by amino acid sequencing) of 3,864 Da. The isoelectric point (pI; determined by isoelectric focusing) of bacteriocin 602 was 7.2. The amino acid sequence was consistent with its bacteriocin class IIa classification. With the use of these biochemical characteristics for larger scale production, the bacteriocin was precipitated with saturated

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ammonium sulfate, dissolved, dialyzed, and purified by chromatography (Superose 12HR 16/50 column, 1.6 by 50 cm; Pharmacia, Uppsala, Sweden) followed by passing the protein over an SP Sepharose Fast Flow column (300 ml; Amersham, Piscataway, N.J.).

Microencapsulation of bacteriocin and dispersion into feed. Purified bacteriocin 602 (500 ml) was mixed into a 25-ml (0.8 mol K_2HPO_4 per liter) solution containing 1.25 g of completely dissolved polyvinylpyrrolidone powder; the material was termed the bacteriocin-polyvinylpyrrolidone solution. This solution was carefully and thoroughly mixed with 100 g of ground maize to produce a high-concentration medicated feed. This feed (100 g) was mixed with 1,900 g of commercial feed to produce medicated commercial feed having a bacteriocin concentration of 250 mg kg^{-1}. Bacteriocin production, its microencapsulation, and its incorporation into the high-concentration medicated feed was conducted at the State Research Center for Applied Microbiology facility and shipped to the Poultry Microbiological Safety Research Unit.

Chicken challenge with *C. jejuni*, treatments, and sampling. Chicken challenge and treatment trials were carried out at the Poultry Microbiological Safety Research Unit. Isolated U.S. strains of *C. jejuni* were used in the eight trials. The *C. jejuni* isolates were obtained as part of a previous epidemiological study *(18)* and were representatives of three distinct geographical locations in the United States. Approval for the conduct of these experiments was provided by the Institutional Animal Care and Use Committee (PMS-03-03, ''Control of *Campylobacter* in Poultry Production''). The trials were conducted over an approximately 3-month period. Day-of-hatch chicks obtained from local commercial hatcheries were placed in groups of 10 in separate isolation units. These units were equipped with feeders and water and were provided a filtered air supply along with nonmedicated feed and water ad libitum. Each group of chicks (positive control birds and treated) were challenged with individual strains of *C. jejuni* at 1 day after hatch. The challenge strains were administered individually by oral gavage. Each bird was provided a 0.2-ml suspension containing the specified *C. jejuni* isolate at 10^8 CFU per chick. Within each trial, one colonized group of chicks served as the positive control and received free access to diet without bacteriocin. Nonchallenged, nontreated groups of chickens served as negative controls. Ten *Campylobacter*-colonized positive control chickens were sacrificed at the same time as groups of the bacteriocin-treated chickens. The groups of treated chickens were provided free access to commercial chicken feed containing 250 mg of microencapsulated bacteriocin 602 per kg of feed on days 7 through 9 before sacrifice.

Groups of chickens were sacrificed by cervical disarticulation 10 days after *C. jejuni* challenge. Ceca of the individual animals were aseptically removed. Enumeration of *C. jejuni* was accomplished by diluting 1 g of cecal content suspended into 9 g of phosphate-buffered saline (pH 7.2). Tenfold serial dilutions were made in 0.1-ml portions, which were then surface plated onto Campy-Cefex *(19).* The minimum detection limit was 100 CFU g^{-1} cecal contents. Plates were incubated at 42°C for 48 h under a microaerobic atmosphere. Resulting *Campylobacter*-characteristic colonies were counted after microscopic examination and latex agglutination assay confirmation. Arithmetic estimates of the number of organisms were log transformed. Standard deviations for each experimental group were calculated and reported. Statistical analysis of *Campylobacter* numbers among the bacteriocin-treated versus nontreated chickens was made by unpaired Student's *t* test, with significance defined at the 95% level ($P \leq 0.05$).

TABLE 1. *Mean estimates of* Campylobacter jejuni *in cecal contents of 10-day-old experimental birds*

Trial	Experimental group	*Campylobacter* challenge strain	*C. jejuni* (log CFU/g of feces)[a]
1	+ Control	AL-22	7.2 ± 0.3
1	Treated	AL-22	Not detected
2	+ Control	BH-6	7.1 ± 0.4
2	Treated	BH-6	Not detected
3	+ Control	BL-1	7.8 ± 0.2
3	Treated	BL-1	Not detected
4	+ Control	CL-11	6.6 ± 0.7
4	Treated	CL-11	Not detected
1–4	Not inoculated or treated		Not detected
5	+ Control	AL-22	7.9 ± 0.7
5	Treated	AL-22	Not detected
6	+ Control	BH-6	8.3 ± 0.3
6	Treated	BH-6	Not detected
7	+ Control	BL-1	7.6 ± 0.3
7	Treated	BL-1	Not detected
8	+ Control	CL-11	7.4 ± 0.6
8	Treated	CL-11	Not detected
5–8	Not inoculated or treated		Not detected

[a] Limit of detection was 10^2 CFU/g.

RESULTS

In total, we conducted eight experimental bird trials. In each of the trials, the bacteriocin treatment significantly reduced numbers of *C. jejuni* when compared with those found in the untreated control groups of birds ($P \leq 0.05$; Table 1). These reductions ranged from $10^{4.6}$ to $10^{6.3}$ CFU g^{-1}. Relatively younger birds were used in this phase of our study to conserve the amount of microencapsulated bacteriocin needed and to verify the unproven efficacy of this approach. Nonchallenged, nontreated birds were used as a negative control for the trials, and none of these birds yielded detectable levels of *Campylobacter*.

DISCUSSION

In each of the reported trials, application of bacteriocin treatment resulted in significant ($P \leq 0.05$) reductions of *Campylobacter* colonization. The most effective duration of treatment or the required amount of treatment has yet to be determined. Dose-response experiments remain to be completed. Although not tested, perhaps a single day of treatment before slaughter could be proven to be an effective approach in gaining control. Lower quantities of bacteriocin must still be tested to determine the dose-response required for effective control of *Campylobacter*. We have gathered unpublished data to verify that birds of commercial processing age also manifest dramatic reductions of *Campylobacter*. Among the control groups, as is often the case with commercial chickens, very high numbers (10^7 CFU g^{-1}) of *C. jejuni* were observed. Further study is ongoing to optimize the treatment regimen.

Metabolites secreted by competing organisms could contribute to the control of pathogens such as *C. jejuni* and *Salmonella*. Microorganisms produce a variety of com-

pounds (fatty acids, peroxides) that demonstrate antibacterial properties *(2).* Among these antibacterial compounds, bacteriocins consist of bactericidal proteins with a mechanism of action similar to ionophore antibiotics *(21).* As described in this study, the occurrence of bacteriocin production among bacterial species isolated from complex microbial communities, such as the avian intestinal tract, suggests that such bacteriocins might have a regulatory role for population dynamics within bacterial ecosystems. Bacteriocins are defined as compounds produced by bacteria that have a biologically active protein moiety and exhibit bactericidal activity *(21).* Other characteristics can include: (i) a narrow inhibitory spectrum of activity centered about closely related species, (ii) attachment to specific cell receptors, and (iii) plasmidborne genetic determinants of bacteriocin production and of host cell bacteriocin immunity *(3).* We suggest that the bacterial-derived compound applied in this study meets the criteria described by Tagg et al. *(21)* for the term bacteriocin.

Diverse biological activities are common among *Bacillus* spp. Data from this study suggest that *Bacillus* spp. and the very closely related genus of *Paenibacillus* spp. might produce pronounced antagonism to selected pathogenic microorganisms *(7). Bacillus* spp. can generate biologically active substances *(22).* Except for *Bacillus anthracis* and *Bacillus cereus,* members of the genus *Bacillus* are harmless to warm-blooded host animals and have phylogenetic relatedness to lactobacilli *(5).* Owing to these desirable characteristics, bacteria within the genus *Bacillus* spp. have found wide application as probiotics and are widely used in medicine and veterinary practice *(14).*

Piuri et al. *(11)* reported a novel antimicrobial compound secreted by a strain of *P. polymyxa* isolated from fermented sausages. Similar to the bacteriocin used in this study, their bacteriocin-like properties included a proteinaceous nature (sensitivity to proteases), insensitivity to organic solvents and chelators, and stability in heat (up to 10 min at 90°C) and acidic pH but instability in alkaline conditions. Unlike the bacteriocin used in this study, the bacteriocin-like compound Piuri et al. *(11)* described had a molecular mass of 10 kDa. Inhibitory activity to *Bacillus, Paenibacillus, Lactobacillus, Micrococcus luteus, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris,* and *Serratia marcescens* has been shown for the bacteriocin reported by Piuri et al. *(11).*

Schoeni and Wong *(12)* reported a significant reduction in broiler colonization by *C. jejuni* through the application of carbohydrate supplements with three identified antagonists: *Citrobacter diversus* 22, *Klebsiella pneumoniae* 23, and *E. coli* 25. Morishita et al. *(9)* reported a significant decrease of *C. jejuni* in intestinal samples from infected broilers after treatment with poultry-isolated cultures of *Lactobacillus acidophilus* and *Streptococcus faecium.* To our knowledge, neither of these approaches have been widely accepted by the poultry industry. In this study, we focused on the bacteriocin produced by the *P. polymyxa* NRRL B-30509 and incorporated it as a feed emendation. This treatment proved to be successful.

Our goal for this treatment is to increase our scale of operation for bacteriocin production through a willing commercial cooperator. We hope to determine the dose-response for commercial applications and, with the use of that information, to conduct large-scale commercial treatments among fully grown broilers. The application of this treatment for various pathogens in other veterinary and medical scenarios has not escaped our attention.

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