Bacteriocins Reduce *Campylobacter* Colonization and Alter Gut Morphology in Turkey Poults[1,2]

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ABSTRACT *Campylobacter* is a leading cause of foodborne illness in the United States. Recent evidence has demonstrated that bacteriocins produced by *Bacillus circulans* and *Paenibacillus polymyxa* reduce cecal *Campylobacter* colonization in broiler chickens infected with *Campylobacter jejuni*. As *Campylobacter coli* is the most prevalent *Campylobacter* isolate recovered in turkeys, the objectives of the present study were to evaluate the efficacy of these bacteriocins against *C. coli* colonization and their influence on the gastrointestinal architecture of young turkeys. In 3 separate trials, a total of 135 day-of-hatch poults (n = 45/trial) were orally challenged on d 3 with approximately 10^6 cfu of a mixture of 3 *C. coli* isolates. Immediately before bacteriocin treatment (d 10), cecal *Campylobacter* concentrations averaged 1.1×10^7 cfu/g of cecal contents (n = 15/trial). On d 10 to 12 posthatch, 2 bacteriocin treatment groups were given free access to feed supplemented with purified, microencapsulated bacteriocins, whereas the positive control treatment group had access to untreated feed (n = 10/treatment group per trial). At the end of the 3-d dosing period, ceca and duodenal loops were collected for analysis. In each of the 3 separate trials, treatment with bacteriocin eliminated detectable ceca *Campylobacter* concentrations (detection limit, 1×10^2 cfu/g of cecal contents) vs. controls (1.0×10^6 cfu of *Campylobacter*/g of cecal contents). Duodenum crypt depth and goblet cell numbers were also reduced in turkeys treated with either bacteriocin vs. controls ($P < 0.05$). The dynamic reduction in crypt depth and goblet cell density in turkeys dosed with bacteriocin may provide clues to how bacteriocins inhibit enteric *Campylobacter*.

Key words: *Campylobacter*, ceca, bacteriocin, turkey, gastrointestinal tract

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INTRODUCTION

Campylobacter is one of the leading bacterial causes of human foodborne illness in the United States (Centers for Disease Control and Prevention, 2005). A substantial number of poultry and retail poultry products are contaminated with *Campylobacter*, with isolation rates approaching 100% (Stern et al., 2001; Zhao et al., 2001; Newell and Fearnley, 2003). Epidemiological evidence has emphasized the importance of poultry products as a significant source of human *Campylobacter* infection (Jacobs-Reitsma, 2000; Corry and Attabay, 2001). Therefore, the reduction or elimination of this organism in commercial poultry flocks should greatly reduce the incidence of human *Campylobacter* infection (Jacobs-Reitsma, 1997; Sahin et al., 2002).

One approach to reduce *Campylobacter* colonization in preharvest poultry is the use of competitive exclusion (**CE**) cultures (Stern et al., 2001). Competitive exclusion is the administration of nonpathogenic enteric microflora that may compete with and reduce enteric pathogens. Competitive exclusion, first described by Nurmi and Rantala (1973) has been used to successfully control *Salmonella* contamination in poultry (Corrier et al., 1995; Bielke et al., 2003). Unfortunately, the use of CE cultures has not consistently reduced *Campylobacter* colonization (Stern et

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al., 2001; Mead, 2002). Through efforts to improve the effectiveness of CE cultures against *Campylobacter*, researchers have observed that certain bacteria produce metabolites that are inhibitory to *Campylobacter* growth in vitro (Schoeni and Doyle, 1992; Newell and Wagenaar, 2000; Svetoch et al., 2005). These metabolites, identified as bacteriocins, are proteins naturally produced by bacteria that kill or inhibit the growth of other bacteria (Cleveland et al., 2001). Unlike antibiotics, bacteriocins have no known toxic effects and have a narrow killing spectrum (Riley and Wertz, 2002). The use of bacteriocins as antimicrobials has already been applied in food preservation; as the bacteriocin nisin is considered a generally recognized as safe compound and is approved for use in foods (Joeger, 2003).

Recently, Svetoch et al. (2005) found that bacteriocins produced by certain strains of *Bacillus circulans* and *Paenibacillus polymyxa* were inhibitory to *Campylobacter* growth in vitro. In a follow-up study, these purified bacteriocins, microencapsulated and administered via feed, reduced cecal *Campylobacter* colonization in young broiler chickens experimentally infected with *Campylobacter jejuni* (Stern et al., 2005). However, the efficacy of these bacteriocins in turkeys has not been determined. Furthermore, the influence of bacteriocins on enteric histology has not been evaluated. Therefore, the objectives in the present study were to evaluate the efficacy of these bacteriocins against *Campylobacter coli* colonization and to evaluate the influence of bacteriocins on the gastrointestinal morphology in turkeys.

MATERIALS AND METHODS

Bacteriocins

Bacteria producing the bacteriocins were recovered from the intestinal tracts of broiler chickens. Associated bacteriocin purification and microencapsulation procedures have been previously described in detail (Stern et al., 2005; Svetoch et al., 2005). Briefly, bacteriocin B602 was secreted by the isolate *P. polymyxa* (NRRL B-30509), whereas bacteriocin OR7 was secreted by the isolate *Lactobacillus salivarius* (NRRL B-35014; N. J. Stern, E. A. Svetoch, B. V. Eruslanov, V. V. Perelygin, E. V. Mitsevich, I. P. Mitsevich, V. D. Pokhilenko, V. P. Levchuk, and O. E. Svetoch, unpublished data). Each bacteriocin was precipitated with saturated ammonium sulfate, dissolved, dialyzed, and purified by Superose 12HR 16/50 column chromatography (Pharmacia, Uppsala, Sweden), followed by passing the protein over a 300-mL SP Sepharose Fast Flow column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The purified bacteriocins were then mixed with polyvinylpyrrolidone powder to produce microencapsulated bacteriocins, which were used to produce a medicated feed. The final concentration of each bacteriocin was 250 mg/kg of feed.

Campylobacter Isolates and Growth Conditions

Poults used in this study were challenged with a solution containing an equal combination of 3 *C. coli* isolates, 2 wild-type turkey isolates, and an American Type Culture Collection isolate 43481. A frozen culture of each isolate was inoculated into 9.0 mL of *Campylobacter* enrichment broth and grown individually for 24 h at 42°C in a microaerobic environment (5% O_2, 10% CO_2, 85% N_2), as previously described (Cole et al., 2004). After 24 h, 10 uL of each culture was passed into another 9.0 mL of *Campylobacter* enrichment broth and grown for 24 h in a microaerophilic environment. After 24 h, each culture was combined in a 50-mL conical tube and used for poult inoculation (see below).

Experimental Design

A total of 135 poults were used in this study. In each of 3 separate trials, 45 day-of-hatch poults were obtained from a local commercial hatchery and randomly allocated to 1 of 3 treatment groups: positive control, bacteriocin B602, or bacteriocin OR7 (n = 15/pen). Each treatment group was housed in an individual floor pen on fresh pine litter and provided water and feed ad libitum. Three days posthatch, all poults in each treatment group were inoculated, via oral gavage, with 0.25 mL of a solution containing a mixture of 3 *C. coli* isolates (approximately 10^6 cfu/mL), as described previously (Farnell et al., 2005). Immediately before bacteriocin treatment (d 10), 5 of the 15 birds from each of the 3 treatment pens (n = 15/trial) in each trial were euthanized and ceca was collected for *Campylobacter* enumeration. On d 10 to 12 posthatch, the 2 bacteriocin treatment groups were given free access to feed supplemented with purified, microencapsulated bacteriocins, whereas the positive controls had access to untreated feed. At the end of the 3-d dosing period, ceca were collected from all remaining turkeys (n = 10/pen; 30/trial) for *Campylobacter* enumeration, and their duodenal loops were collected for morphometric analysis.

Enumeration of Campylobacter in Cecal Contents

The cecal contents of each poult were serially diluted 1:9 in buffered phosphate diluent, and 100 uL of each dilution was plated onto *Campylobacter* Line agar plates (Line, 2001). The plates were incubated for 48 h at 42°C in a microaerobic environment. After incubation, characteristic colonies were confirmed as *Campylobacter* using a commercial latex agglutination test kit (Panbio Inc., Columbia, MD). The direct counts were converted to $\log_{10}$ colony-forming units per gram of cecal contents. The detection limit for *Campylobacter* was 1×10^2 cfu/g of cecal contents.

Morphometric Analysis of the Gut

The gastrointestinal morphometric variables evaluated were villus height, villus surface area, lamina propria

Table 1. Reduction of cecal *Campylobacter* concentrations and incidence in commercial turkey poults treated with bacteriocins[1]

Trial	Positive control	Bacteriocin B602	Bacteriocin OR7
1	$2.6 \times 10^{6, a}$ (10/10)	ND[b] (0/10)	ND[b] (0/10)
2	$3.6 \times 10^{5, a}$ (10/10)	ND[b] (0/10)	ND[b] (0/10)
3	$3.4 \times 10^{5, a}$ (10/10)	ND[b] (0/10)	ND[b] (0/10)

[a,b]Means within rows with no common superscript differ significantly ($P < 0.01$).

[1]Data represent $\log^{10}$ colony-forming units of *Campylobacter* per gram of cecal contents collected from 3 separate trials (n = 10 poults/treatment per trial; total 30 poults/trial). Incidence of *Campylobacter* is represented as the number of positive ceca out of 10 birds. In each trial, poults were orally challenged 3 d posthatch with approximately 10^6 cfu of a mixture of 3 *Campylobacter coli* isolates. On d 10 to 12 posthatch, the 2 treatment groups were fed a diet containing bacteriocins, and the positive control group was fed the same commercial diet without bacteriocins. After 72 h of treatment with bacteriocins, turkeys were euthanized, and ceca were collected for enumeration of *Campylobacter*. ND = the concentration of bacteria was below detectable levels ($<10^2$ cfu/g of cecal contents).

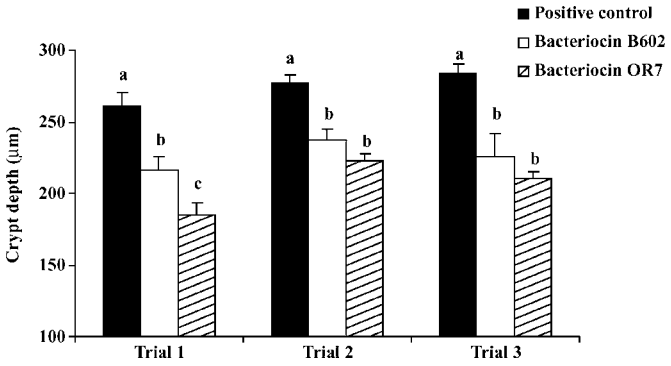

Figure 1. Effect of bacteriocins on duodenal crypt depth in turkey poults after oral challenge with *Campylobacter*. Values are means ± SEM, representing 10 birds/group and 10 measurements/parameter per bird from 3 separate trials. In each trial, poults were orally challenged 3 d posthatch with approximately 10^6 cfu of a mixture of 3 *Campylobacter coli* isolates. On d 10 to 12 posthatch, the 2 treatment groups were fed a commercial diet containing bacteriocins, and the positive control group was fed the same commercial diet without bacteriocins. On d 13 posthatch (10 d postchallenge), turkeys were euthanized, and duodenal loops were collected for morphometric analysis. Means with no common superscripts differ significantly ($P < 0.05$) between treatments within trials.

thickness, villus crypt depth, and goblet cell number per villus from the duodenum. A 1-cm segment of the midpoint of the duodenum was removed and fixed in 10% buffered formalin for 72 h. Each segment was then embedded in paraffin, and a 2-μm section of each sample was placed on a glass slide and stained with hematoxylin and eosin for examination with a light microscope (Sakamoto et al., 2000). Morphological parameters were measured using the Image-Pro Plus Version 4.5 software package (Media Cybernetics Inc., Silver Springs, MD), as previously described (Solis de Los Santos et al., 2005). Ten replicate measurements for each variable studied were taken from each poult. Briefly, the villus height was measured from the top of the villus to the top of the lamina propria. Surface area was calculated using the formula surface area = (2π) (VW/2) (VL) where VW = villus width and VL = villus length (Sakamoto et al., 2000). The lamina propria thickness was measured in the space between the base of the villus and the top of the muscularis mucosa. Crypt depth was measured from the base to the region of transition between the crypt and villus (Aptekmann et al., 2001). Goblet cell number was determined by counting the number of goblet cells in 10 individual histologically well-oriented villi (Yunus et al., 2005).

Statistical Analysis

Data were subjected to ANOVA using the GLM procedure of SAS (SAS Institute, 2003). Treatment means were partitioned by LSMEANS analysis. A probability of $P < 0.05$ was required for statistical significance.

RESULTS

Campylobacter Colonization

Immediately before bacteriocin treatment (d 10), the 5 birds collected from each of the pens used in this study (n = 15/trial) were colonized with *Campylobacter*. The average cecal *Campylobacter* numbers were 2.0×10^7, 8.2×10^4, or 1.3×10^7 cfu/g of cecal contents for the turkeys from trials 1, 2, or 3, respectively. In each of the 3 separate trials, the 3-d treatment with bacteriocins eliminated detectable ceca *Campylobacter* concentrations in all turkeys (detection limit, 1×10^2 cfu/g of cecal contents) when compared with the unmedicated positive control groups ($P < 0.01$; Table 1).

Morphometric Analysis of the Gut

Administration of bacteriocins reduced the duodenum crypt depth (Figure 1) and number of goblet cells (Figure 2) in comparison with the untreated positive control groups in each of the 3 separate trials. Treatment with bacteriocin OR7 produced a greater reduction in goblet cell numbers in all 3 trials when compared with bacteriocin B602 (Figure 2). Duodenum villus height did not differ among treatment groups, except in trial 3, in which treatment with bacteriocin OR7 reduced the villus height vs. controls (Table 2). Duodenum villus surface area was lower for turkeys treated with bacteriocin OR7 in trials 1 and 2 vs. controls (Table 2). Lamina propria thickness was inconsistent among the treatment groups, with lower values for turkeys treated with bacteriocin B602 compared with controls in trials 1 and 3 and for turkeys treated with bacteriocin OR7 in trials 1 and 2 (Table 2).

DISCUSSION

In the present study, oral administration of the purified microencapsulated bacteriocins eliminated detectable cecal *Campylobacter* colonization in young turkeys in 3 separate trials. These findings are consistent with previous studies in which treatment with these same bacteriocins

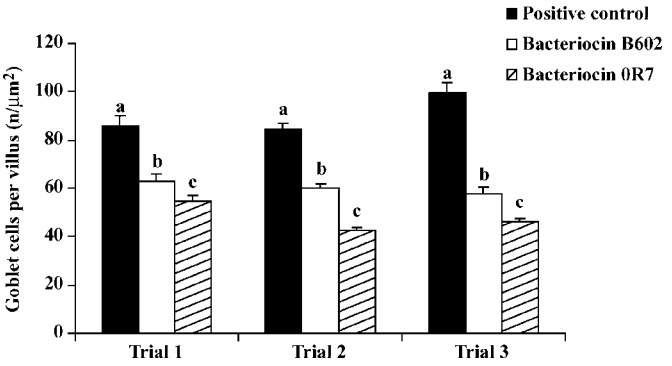

Figure 2. Effect of bacteriocins on duodenal goblet cell density in turkey poults after oral challenge with *Campylobacter*. Values are mean ± SEM, representing 10 birds/treatment group and 10 measurements/parameter per bird from 3 separate trials. In each trial, poults were orally challenged 3 d posthatch with approximately 10^6 cfu of a mixture of 3 *Campylobacter coli* isolates. On d 10 to 12 posthatch, the 2 treatment groups were fed a commercial diet containing bacteriocins, and the positive control group was fed the same commercial diet without bacteriocins. On d 13 posthatch (10 d postchallenge), turkeys were euthanized, and duodenal loops were collected for morphometric analysis. Means with no common superscript differ significantly ($P < 0.05$) between treatments within trials.

eliminated detectable *Campylobacter* in young broiler chickens infected with *C. jejuni* (Stern et al., 2005; N. J. Stern, E. A. Svetoch, B. V. Eruslanov, V. V. Perelygin, E. V. Mitsevich, I. P. Mitsevich, V. D. Pokhilenko, V. P. Levchuk, and O. E. Svetoch, unpublished data).

One of the possible mechanisms by which bacteriocins reduce *Campylobacter* colonization in poultry is by direct bactericidial or bacteriostatic activity. Bacteriocins have been demonstrated to inhibit or kill other foodborne pathogens, such as *Listeria*, *Clostridium*, and *Salmonella*, and are used in food processing and preservation (Daly et al., 1970; Tagg et al., 1976; Natrajan and Sheldon, 2000). Bacteriocin-like compounds have also been shown to have direct antimicrobial activity, in vitro, against *Campylobacter* (Schoeni and Doyle, 1992; Morency et al., 2001; Chaveerach et al., 2004), including the bacteriocins used in this study (Svetoch et al., 2005; N. J. Stern, E. A. Svetoch, B. V. Eruslanov, V. V. Perelygin, E. V. Mitsevich, I. P. Mitsevich, V. D. Pokhilenko, V. P. Levchuk, and O. E. Svetoch, unpublished data).

Another possible mechanism of action of the bacteriocins is physical or functional alteration of *Campylobacter* colonization sites. Use of either bacteriocin in this study reduced both duodenal crypt depth and goblet cell numbers. To our knowledge, this is the first study demonstrating that altering the gastrointestinal tract eliminated detectable *Campylobacter* colonization. Previous research has demonstrated that the mucus layer of intestinal crypts is an important niche for *Campylobacter* colonization in poultry (Beery et al., 1988; Meinersmann et al., 1991). The ability of *Campylobacter* to sequester itself within these crypts may be an important strategy to avoid intervention efforts, such as the use of antibiotics or CE cultures (Mead, 2002; Zhang et al., 2003; Bywater, 2004; Mead, 2004; Farnell et al., 2005). The reduction in crypt depth may have multiple effects on *Campylobacter* colonization. For example, it is possible the smaller crypt size, and subsequent greater exposure to the lumen, may change the nutrient or chemical environment (e.g., increased oxygen tension), limiting *Campylobacter* growth and colonization. It is also possible that different microflora will colonize these smaller crypts, with the ability to outcompete *Campylobacter* (CE).

Another potentially important affect on *Campylobacter* colonization is the reduction in goblet cell numbers following bacteriocin treatment. Mucin glycoproteins are synthesized and secreted from goblet cells, which arise from stem cells at the base of the crypts and migrate toward the villus tip, in which they enter into the lumen

Table 2. Effects of bacteriocin treatment on duodenal morphology of turkey poults after oral *Campylobacter* challenge[1]

Trial	Villus height (μm)	Villus surface area (μm^2)	Lamina propria thickness (μm)
1			
Positive control	1,298.6 ± 72.8[a]	0.16 ± 0.01[a]	96.5 ± 2.9[a]
B602	1,247.5 ± 55.2[a]	0.15 ± 0.01[ab]	86.6 ± 3.1[b]
OR7	1,193.1 ± 74.3[a]	0.12 ± 0.01[b]	83.4 ± 2.1[b]
2			
Positive control	1,215.1 ± 66.1[a]	0.11 ± 0.01[a]	76.6 ± 1.4[b]
B602	1,174.4 ± 64.8[a]	0.10 ± 0.01[a]	90.1 ± 3.2[a]
OR7	1,042.8 ± 48.7[a]	0.08 ± 0.01[b]	78.5 ± 2.4[b]
3			
Positive control	1,288.2 ± 38.7[a]	0.11 ± 0.004[a]	79.7 ± 2.2[a]
B602	1,204.3 ± 26.6[a,b]	0.10 ± 0.003[a]	71.6 ± 2.0[b]
OR7	1,144.8 ± 43.3[b]	0.10 ± 0.005[a]	81.1 ± 2.4[a]

[a,b]Means within columns and trials with no common superscript differ significantly ($P < 0.05$).

[1]Mean ± SEM representing 10 birds per treatment group from 3 separate trials (n = 10 poults/treatment per trial; total 30 poults/trial). Ten separate measurements were made for each per parameter per poult. In each trial, poults were orally challenged 3 d posthatch with approximately 10^6 cfu of a mixture of 3 *Campylobacter coli* isolates. On d 10 to 12 posthatch, the 2 treatment groups were fed a diet containing bacteriocins, and the positive control group was fed a commercial diet without bacteriocins. After 72 h of treatment with bacteriocins, turkeys were euthanized, and duodenal loops were collected for morphometric analysis.

(Cheng and Leblond, 1974; Geyra et al., 2001). Previous research has demonstrated that *Campylobacter* can use mucin as a nutrient source for growth (Hugdahl et al., 1988; Schoeni and Doyle, 1992). This capability may provide a competitive advantage over other microflora. The ability of bacteriocins to reduce goblet cell number and subsequent mucin production may limit *Campylobacter* colonization. This idea is supported by previous research, reporting that colonization of *C. jejuni* in chicks can be influenced by diets that alter mucin production and viscosity (Fernandez et al., 2000).

Although bacteriocin treatment eliminated detectable *Campylobacter* colonization in this study (detection limit, 1×10^2 cfu/g of cecal contents), it is possible that undetectable numbers of *Campylobacter* may still persist in these birds. Previous research from our laboratory has demonstrated that even if *Campylobacter* is eliminated from most, but not all, enteric locations, the remaining enteric *Campylobacter* can recolonize the gut within a few days (Farnell et al., 2005). If, however, bacteriocins are dosed just before marketing, the ability of any possible *Campylobacter* to recolonize the tract would be reduced or prevented. Furthermore, even if bacteriocin treatment did not totally eliminate *Campylobacter*, the approximately 4-log reduction in *Campylobacter* concentrations obtained in the current study would provide a significant benefit to human food safety. Research by Rosenquist et al. (2003) reported that even a 2-log reduction in carcass contamination would reduce the human incidence of Campylobacteriosis in human by 30-fold.

In the present study, the administration of bacteriocins isolated from *B. circulans* and *P. polymyxa* was effective in eliminating detectable *Campylobacter* colonization in young commercial turkeys. The mechanism of bacteriocin action on *Campylobacter* colonization may be related to the ability of these compounds to reduce crypt depth and goblet cell density in young turkeys. The use of bacteriocins may be an important strategy to reduce *Campylobacter* colonization in poultry.

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