Review

Bacteriocins to control Campylobacter spp. in poultry—A review

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ABSTRACT The unacceptably high frequency of Campylobacter jejuni transmission from poultry to humans encourages scientists to consider and create alternative intervention strategies to control the pathogen in poultry production. Extremely high numbers of Campylobacter (often >10⁸ cfu/g of poultry intestinal material) potentiate high numbers of the organism on the processed broiler carcass with increasing consequent human health risk. Many scientists believe interventions during poultry production portend the greatest opportunity for reducing risk of disease. Over the past 10 yr, we have focused our studies on nonantibiotic bacteriocin application to intervene during animal production and this is the subject of the current review. The application of therapeutic bacteriocin treatments to reduce poultry colonization diminishes Campylobacter from >10⁸ cfu/g of cecal materials to nondetectable or very low levels in treated birds. Further, the review provides scientists with a useful starting point for the further development of industry-applicable interventions leading to reduced transmission of this agent in human disease.

Key words: Campylobacter, bacteriocin, colonization, broiler, intervention

INTRODUCTION

The major reservoir for Campylobacter jejuni leading to human infections is commercial broiler chickens (Stern, 1992; Mead et al., 1999). Levels of Campylobacter in excess of 10³.⁵ cfu/processed broiler carcasses have been implicated in transmission of the organism to humans, resulting in human gastroenteritis (Callicott et al., 2008). As a corollary, levels of 10².⁷ cfu/processed broiler carcasses were not implicated in disease transmission. There is a need by the poultry industry for intervention strategies that predictably reduce Campylobacter levels in preharvest poultry and poultry carcasses. Regulatory oversight and monitoring of Campylobacter levels is the responsibility of regulatory agencies to promote a safer poultry product.

Approximately all 30- to 45-d-old broilers on Russian poultry farms are colonized by Campylobacter spp. (Stern et al., 2004). The same high level of colonization of Campylobacter spp. in broilers is observed in the United States (Stern and Robach, 2003). Carcasses and poultry products are contaminated by poultry intestinal materials contaminated with C. jejuni and provide a major source for the spread of campylobacteriosis in humans. Eliminating or dramatically reducing Campylobacter contamination during bird production is a direct approach to minimize foodborne infections.

To reduce levels of Campylobacter spp. before slaughter, various approaches have been explored. These include vaccination, treatment of birds with bacterial antagonists, bacterial phage, and antibiotics or drugs. Unfortunately, the use of vaccines and lytic phages against C. jejuni are still at an experimental phase. The prolonged application of antibiotics or drugs in human medicine and in veterinary fields to control Campylobacter spp. or other pathogens in birds is undesirable because of the resultant development of multidrug-resistant bacteria and residues. Research has focused on applying microbial antagonists as an alternative for antibiotics and chemicals. The initial focus of our cooperative studies was to identify and use nonpathogenic enteric bacteria that would inhibit or compete with C. jejuni within the intestinal tract of commercial broiler chickens. Each of our C. jejuni antagonistic enteric isolates came from healthy, commercially reared broiler chickens. Unfortunately, we were never able to identify bacterial isolates (acting as probiotics) that would successfully compete within the gastrointestinal tract and control Campylobacter. These same enteric bacteria, however, were able to produce bacteriocins (BCN) that could dramatically control the high levels of gut colonization of this target pathogen in birds (Stern et al., 2008). As predicted in his 2003 review, Joerger indicated that “purified or partially purified bacteriocins could be used...for the reduction or elimination of cer-
tain pathogens” (Joerger, 2003). This is the subject of the present summary on this line of research.

**REVIEW OF BCN**

Researchers from the State Research Center for Applied Microbiology and Biotechnology (Obolensk, Moscow, Russian Federation) and the Agricultural Research Service-Richard B. Russell Research Center (Athens, GA) have found that by culturing selected microbial antagonists isolated from broilers we could produce specific BCN that were highly effective in vitro against *Campylobacter* spp. When purified BCN were provided in feed or water, we eliminated or markedly reduced *C. jejuni* from infected chicks. Bacteriocins are low molecular weight peptides that are produced in bacterial ribosomes and possess antimicrobial properties (Klaenhammer, 1993; Nissen-Meyer and Nes, 1997; Cleveland et al., 2001; Eijsink et al., 2002; Riley and Wertz, 2002). They are mainly cationic, hydrophobic, or amphiphilic peptides, with molecular weights of 5 to 6 kDa. Mature peptides usually carry 20 to 60 amino acids (Nissen-Meyer and Nes, 1997; Riley and Wertz, 2002).

Bacteriocins are produced by both gram-positive and gram-negative microorganisms that belong to different systematic groups and occupy various ecological niches (fermented milk products, cheese, meat products, fermented plant products, the gastrointestinal tract of warm-blooded animals, and soil). Strains producing BCN are commonly spread among genera of *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Carnobacterium*, *Enterococcus*, *Escherichia*, *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Pseudomonas*, *Clostridium*, and others. According to their physicochemical properties, biological activity, and specific features of their primary amino acid sequences, known BCN are divided into 3 classes (Klaenhammer, 1993; Nes et al., 1996), of which BCN in classes I and II are the best studied and have practical application. Typical peptides of class I contain from 19 to 50 and more amino acids and are characterized by availability in their compositions of unusual amino acids, such as lanthionine, β-methyl-lactonine, dehydrobutyryne, and dehydroalanine. Subclass Ia, whose most recognized representative is nisin, comprises cationic hydrophobic peptides, which form pores in bacterial membranes and have a flexible structure of molecules. Unlike peptides of subclass Ia, peptides of subclass Ib have a rigid globular structure and neutral or negative charge.

Class II comprises small thermally stable unmodified peptides, which are divided into 2 subclasses, IIA and IIB. According to the accepted classification, subclass IIA carries pediocin-like anti-*Listeria* peptides, which have a conserved N-terminus sequence of YGNGV (Tyr-Gly-Asn-Gly-Val) and 2 cysteine bridges. Bacteriocins of subclass IIB are presented by 2 differing peptides, and each is needed to provide maximal antibacterial activity. The peptides differ in primary amino acid sequences and are encoded by their neighboring genes contained on the producer chromosome and share the gene accounting for host-cell immunity to the BCN. Bacteriocins are a new generation of antimicrobials and should not be confused with antibiotics. Numerous properties of BCN distinguish them from antibiotics (Cleveland et al., 2001): i) BCN are produced on the surface of ribosomes of microbial cells, whereas antibiotics are secondary metabolites of the cell; ii) unlike producers of antibiotics, producers of BCN are tolerant to the bactericidal effect that the peptides produce; iii) BCN molecules do not have specific receptors on the cell wall of microbial cells and may attach to the cell wall anywhere; and iv) the mechanism of their injuring action is versatile for all BCN and is associated with the process of pore formation in the outer cell membrane. Bacteriocins bind with targets on cell walls of susceptible microbes, interact with the outer membrane, and generate pores. Inorganic ions leak through the pores, thereby resulting in the death of the target cell. Antibiotics, on the other hand, can inhibit synthesis of the cell walls, block intracellular protein production, and disrupt DNA and RNA replication. In addition, resistance of a bacterial target cell to a BCN develops when the target alters its cell membrane chemical composition. The development of bacterial antibiotic resistance is determined by changes in genetic determinants that are often passed on from one cell generation to the next. Last, BCN are likely produced by microorganisms in situ, which is not likely with antibiotics.

Owing to the above properties, BCN have advantages over antibiotics. There is no information suggesting that BCN could be toxic for humans and animals and accumulate in the treated subject. This is explained by the fact that BCN are highly susceptible to proteases and should degrade in the host. Bacteriocins are effective against antibiotic-resistant pathogens. This is extremely important from the standpoint of the spread of multidrug-resistant pathogens, especially those responsible for nosocomial (hospital-acquired) infections. For example, resistance to penicillin can be as high as 95% among isolates of *Staphylococcus aureus* among treated people (Breithaupt, 1999). It is known that the development of antibiotic resistance does not lead to occurrence of BCN cross-resistance and vice versa (Severina et al., 1998). Moreover, the development of resistance to one BCN does not provoke bacterial cross-resistance to the other BCN (unpublished data). In all likelihood, most BCN, especially BCN of class IIA, do not generate or have a low frequency of BCN-resistant mutants. The level of BCN resistance generated among selected mutants of *C. jejuni* is low, at just a 2- to 8-fold increase (E. A. Svetoch, unpublished data). Bacteriocin activity varies from rather narrow to an extremely wide spectra of bacterial types. This can allow selection of BCN that are active against a select pathogen but does not affect normal microflora of humans or animals. Levels required for many BCN are competitive, with present day antibiotics having a wide spectrum of activity, and BCN minimal inhibitory concentrations for some
pathogens are even superior to those antibiotics that are currently used. Bacteriocins can be considered as natural metabolites, which people and animals consume as components of food and feed throughout their lives (fermented milk products, cheese, and pickled plants). It is likely that BCN are produced in situ (in intestines of humans and animals) by representatives of normal microflora and play an important role in the kinetics of the microbial ecosystem.

Areas of practical application of BCN currently exist. Today, a primary area of application of BCN is found in the food-processing industry. Research on application of nisin, the first BCN (producer Streptococcus lactis) was described in 1928 (Rogers and Whittier, 1928a,b). It has served as a biological preservative for foods and its use was initiated in the 1950s. In 1969, the World Health Organization approved it for application in the food industry as a nontoxic and harmless agent. The use of nisin as a biological preservative was approved in the United States in 1988 (Federal Register, 1988) and in Russia in 1994 (Anonymous, 1994). Now, nisin is accepted and employed in more than 50 countries (Delves-Broughton et al., 1996). According to Food and Agriculture Organization-World Health Organization estimates, a daily ratio of pure nisin for a 70-kg person could be 60 mg or 33,000 units (Hurst and Hoover, 1993).

Nisin is reported to inhibit the growth of many gram-positive bacteria, including spore-forming microorganisms accountable for food spoilage (representatives of genera Bacillus, Lactobacillus, Lactococcus, Staphylococcus, Streptococcus, and Pseudomonas), as well as of pathogens causing foodborne infections in humans (Clostridium botulinum, Clostridium perfringens, Listeria monocytogenes, and S. aureus) (Frazer et al., 1962; Hurst, 1981; Hurst and Hoover, 1993; Delves-Broughton et al., 1996; Chikindas and Montville, 2002). Due to its heat stability and panorama of pH stability, nisin is widely used as a biological preservative in producing different types of cheese, fresh, pasteurized and sterilized milk, milk deserts, cream, fruit and vegetable juices, wine, beer, caviar, liquid egg products, beans, potatoes, tomatoes, asparagus, meat, and meat products (Hurst, 1981; De Vuist and Vandamme, 1994; Cleveland et al., 2001; Papagianni, 2003). The treatment of products with nisin prolongs their storage and protects them against contamination.

In recent decades, tens of new BCN belonging mainly to class IIa were discovered and described. Because these BCN are produced by different microorganisms, they often possess a wide spectra of antibacterial activity. They inhibit the growth both of gram-positive and gram-negative bacteria, including microorganisms that cause food spoilage as well as pathogens of humans and animals. They are effective against Listeria and can be used as biological preservatives for foods in the future (Ennahar et al., 1999, 2000; Morgan et al., 1999; Cleveland et al., 2001; Eijssink et al., 2002, 2003).

### APPLICATIONS OF BCN

At present, the area of application of BCN in veterinary and human medicine remains relatively unexplored. Positive results have been reported from application of BCN to prevent mastitis in cows. Irish researchers demonstrated that the 2-component lactocin 3147 is highly effective for cows with experimentally induced mastitis as well as in field trials (Ryan et al., 1999). The disease occurred at a rate 10-fold less often in treated cows versus the control group.

In 2003, scientists from Slovakia (Strompfova et al., 2003) described the influence of a BCN-like substance produced by Enterococcus faecium EF55 on the microflora of the gastrointestinal tract of 3-d-old Japanese quails. It was shown that a single treatment of crude extract reduced the level of fecal colonization by Escherichia coli, Enterococcus, Staphylococcus, and Lactobacillus by 0.8 to 1.3 log versus control. The reduction of microbial content was observed 24 h after treatment and was followed by a decrease in selected microbial profiles of experimental versus control groups of quails. A BCN-like substance produced by strain EF55 was found to be active in vitro toward Staphylococcus spp., Lactobacillus spp., and Enterococcus spp. but not toward E. coli. Nevertheless, the content of E. coli decreased in vitro in the presence of the substance as well.

Laukova et al. (2004) investigated the effect of enterocin A produced by Enterococcus faecium EK13 on the development of Salmonella infection in gnotobiotic Japanese quail. The authors applied enterocin for therapeutic and prophylactic purposes. As a prophylactic and therapeutic agent, enterocin A was administered 8 h before challenge of quail and 8 h after their challenge, respectively. A reduced level of colonization by Salmonella enterica in the cecum (2.4 log10) and ileum (3.2 log10) was observed only when the BCN was used therapeutically. When the BCN was used prophylactically, the pathogen was reduced only in the fecal excreta but not in the cecum or ileum. Laukova et al. (2000) recommended that BCN should be used for decontaminating sewage water and manure on large cattle-breeding farms.

Some research focused on the effect of probiotic bacteria producing BCN and BCN on Helicobacter pylori. Kim et al. (2003) assessed activities of 7 BCN toward different strains of H. pylori. They found that some lactocins (produced by Lactococcus lactis) were highly effective against the pathogen. The BCN minimal inhibitory concentrations for different strains of H. pylori varied from 0.097 to 25 mg/L. Pedocin P02 appeared to possess weak inhibitory properties. Roos and Holm (2002) have emphasized that normal flora and the generated BCN play a key role in prophylaxis of recurrent respiratory infections and acute otitis in humans. Sprules et al. (2004) considered BCN of class IIa produced by lactic acid bacteria as potential agents to cure gastrointestinal disorders in mammals.
Watanabe (1979) observed the inhibitory effect of BCN isolated from *Mycobacterium smegmatis* on tumor cells in humans and animals. Additionally, immunomodulatory properties of BCN have been described (Duc et al., 2004; Urdaci et al., 2004). This may explain the positive influence of fermented milk products and probiotic bacteria on the immunity of humans and animals.

We have focused on the application of BCN to control *C. jejuni* infection in broilers. We have screened tens of thousands of isolates of *Bacillus*, *Paenibacillus*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Escherichia*, and selected hundreds of strains, which are active in vitro against *C. jejuni*. Our first published study on anti-*C. jejuni* peptides described several class IIa BCN produced by *Bacillus circulans* and *Paenibacillus polymyxa* isolates (Svetoch et al., 2005). The complete amino acid sequences of the antimicrobial peptides were described in that publication and they contained the conserved consensus pediocin-like N-terminal YGNGV residues. The molecules ranged in weights from 3,214 to 3,864 Da and contained 30 to 39 amino acid residues in each BCN. The isoelectric point (pI) of these BCN ranged from 4.8 to 7.8. The application of BCN (B 602) to control *C. jejuni* in chickens documented use of the BCN produced by *P. polymyxa* NRRL B-30509 (Stern et al., 2005). In replicate experiments, high doses (10^8 cfu) of 4 *C. jejuni* previously isolated from US poultry production operations were orally gavaged into day-of-hatch individual chicks contained in 8 isolation units (IU), with 2 IU per *C. jejuni* isolate for each experiment. In half of the IU, the birds received BCN-amended (250 mg/kg) feed from d 7 through 9 posthatch and the corresponding second half of the IU birds served as positive controls. Untreated control groups of birds were colonized at typical levels of 10^6.6 to 10^8.3 cfu *C. jejuni*/g of cecal materials. None of the 80 BCN-treated birds were detected as colonized by *C. jejuni*. These data were highly significant in demonstrating the reduction of colonization mediated by the BCN therapeutic treatment.

Subsequently, a *Lactobacillus salivarius* (NRRL B-30514) isolate originating from the fecum of a commercial broiler was used for BCN production (Stern et al., 2006). The isolate was used to ferment, purify, and characterize the associated BCN (OR 7). The BCN comprised 54 amino acid residues, had a molecular mass of 5,123 Da, and a pI of 9.5. The BCN was susceptible to digestion by a variety of protease enzymes but was resistant to pH extremes, lysozyme, lipase, or elevated temperatures of 90°C for 15 min. Using this stable material, the BCN was microencapsulated and incorporated into chicken feed at levels of 250 mg/kg. Eight experimental bird trials consisting of 10 chickens in each IU tested 4 poultry colonizing isolates of *C. jejuni*. In these trials, the untreated control birds were colonized at expected high levels of *C. jejuni* per gram of cecal materials. The paired OR 7 BCN-treated chickens had colonization cultured at undetectable levels in 3 of the 8 treated groups or contained levels of no greater than a mean of 10^{1.3} cfu/g of cecal materials. Again, BCN treatment significantly reduced *C. jejuni* within the chicken gut.

Similar BCN treatment efficacy trials have been conducted with turkey poults (Cole et al., 2006). Using 10 poults per treatment per trial, 3 trials of birds challenged and colonized by *Campylobacter coli* were conducted in isolated floor pens. One pen per trial contained poults provided untreated feed (positive control), whereas the other 2 pens contained similarly colonized birds given feed amended with 250 mg/kg of microencapsulated BCN B 602 or BCN OR 7, respectively. In the 3 trials, the positive control birds yielded an average of 10^{7.3}, 10^{4.5}, or 10^{7.1} cfu *C. coli*/g of cecal materials, respectively. The corresponding B 602- and OR 7-treated birds had no detectable levels of *C. coli* per gram of cecal materials with the cultural detection limit of 10^2 cfu/g.

The next BCN-producing isolate we studied was identified as *Enterococcus durans/faecium/hiraev* (NRRL B-30745), which produced BCN E 760 (Line et al., 2008). This BCN was susceptible to a variety of protease treatments, resistant to lysozyme and lipase, and was stable after exposure to a heat treatment of 5 min at 100°C and to pH extremes of 3.0 to 9.5. The polypeptide consisted of 62 amino acid residues and had a molecular weight of 5,362 Da with an pI of 9.5. Oral BCN E 760 treatment of chicks infected with 2 isolates of *C. jejuni* significantly reduced the colonization by more than 8 log_{10} cfu/g. The same BCN E 760 also controlled colonization among commercially acquired *Campylobacter* in market-aged broilers from a mean of 10^{6.2} cfu/g of cecal materials in the untreated birds to nondetectable levels (<10^2 cfu/g) when administered in feed 4 d before analysis.

The final BCN-producing isolate on which we have reported was identified as *Enterococcus faecium* (NRRL B-30746), which produced BCN E 50–52 (Svetoch et al., 2008). The biochemical traits of polypeptide E 50–52 were consistent with its classification as a class IIa BCN containing a consensus N-terminal sequence of YGNGV. The BCN had a molecular weight of 3,340 Da, was 39 amino acid residues in length, and had a pI of ~8.1. A portion of the molecule was predicted to be highly hydrophilic and the remaining portion was predicted to be hydrophobic. These characteristics are consistent with the hypothesized mechanism by which BCN are thought to perturb bacterial cell walls, penetrating both the hydrophobic and hydrophilic portions of the target cell wall. In comparative in vivo studies, young birds were challenged and colonized with 2 *C. jejuni* isolates. From d 4 to 7, the chicks were given 31 mg of BCN/kg of feed. The cecal content of the birds from the untreated IU was measured at 15 d of age and contained 10^{8.5} + 0.5 cfu/g of *C. jejuni*, whereas the birds within the treated group did not contain detectable levels of the target organism. Similarly, commercially *C. jejuni*-colonized market-aged birds (41 d old; ~2 kg) were provided 10.8 mg of BCN E 50–52 per bird.
in the drinking water over a 3-d treatment, whereas the control group was not given therapeutic treatment. The control group of broilers contained 10^8.0 ± 0.9 cfu/g of *C. jejuni*, whereas the ceca of the birds within the treated group contained 10^7.9 ± 0.2 cfu/g of *C. jejuni*. These results were statistically significant.

The BCN have effectively been used to treat about 600 naturally infected birds aged 35 to 42 d. The most effective treatment was achieved by providing experimentally and naturally infected broilers with BCN 760 in supplemented water. The most effective treatment dose of the BCN (3-d treatment) ranged from 3.5 to 25 mg per bird. Providing the birds with the BCN resulted in complete elimination of the pathogen in 90% of cases or reduced levels of *Campylobacter* spp. by 1,000,000-fold or more. The safety of our BCN has been confirmed by conducting experiments on Vero and Hela cell cultures as well as in treated mice and chickens (E. A. Svetoch, unpublished data).

**CONCLUSIONS**

Poultry worldwide have been associated with human campylobacteriosis and farmersducers have not had the tools necessary to effectively control these infections among commercial broiler flocks. Numerous epidemiological studies have pointed to a large number of factors that are associated with flock colonization. Manipulating these factors to obtain an intervention has not resulted in a marked or consistent decrease in *Campylobacter* colonization frequency among commercial broiler flocks. Dramatically enhanced biosecurity, enhanced fly screen control, competitive exclusion, immunization, and phage therapies may hold suggestions to experimentally control flock infections but have yet to be demonstrated as commercially plausible or effective. Alternatively, BCN, which are nontoxic ribosomal-produced antimicrobial peptides secreted by bacteria, have been identified, cultured, and purified for delivery through broiler drinking water. Using this farm-friendly applied approach has resulted in at least 4 different BCN being demonstrated as highly efficacious in therapeutic oral treatment of both chicken and turkeys. Initially, microencapsulated BCN were incorporated into feeds provided to *Campylobacter*-colonized poultry and resulted in dramatic colonization control. Subsequently, we determined that BCN provided in the bird drinking waters was equivalently effective. Mature *Campylobacter*-colonized broilers (>5 wk old and potentially exposed to exogenous sources) treated with BCN over the last 3 d before slaughter consistently had reduced levels of cecal colonization (5 to 6 log reductions). The simplicity of adding BCN to flock medicators over the last days of poultry production makes such interventions very easy for farmers and delivers of flocks of poultry to the processing plants with greatly reduced potentials for transmitting *Campylobacter* and human disease. The goal of producing raw chicken carcasses consistently containing <10^2.7 cfu *C. jejuni*/carcass is at hand. Large-scale commercial studies to demonstrate efficacy are planned in the near future. Reduced human campylobacteriosis is anticipated through BCN treatment of poultry on the farm.

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