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Review

Novel Approaches for *Campylobacter* Control in Poultry

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Abstract

The Gram-negative bacterium *Campylobacter* is the most common bacterial cause of human gastroenteritis in the United States and many industrialized countries. Poultry, particularly chickens, is considered a major source of human campylobacteriosis. Thus, on-farm control of *Campylobacter* in poultry would reduce the risk of human exposure to this pathogen and have a significant impact on food safety and public health. To date, three general strategies have been proposed to control *Campylobacter* in poultry at the farm level: (1) reduction of environmental exposure (biosecurity measures), (2) an increase in poultry's host resistance to reduce *Campylobacter* carriage in the gut (e.g., competitive exclusion, vaccination, and host genetics selection), and (3) the use of antimicrobial alternatives to reduce and even eliminate *Campylobacter* from colonized chickens (e.g., bacteriophage therapy and bacteriocin treatment). Except for biosecurity measures, the other intervention approaches are currently not commercially available and are still under development. This review is focused on two promising strategies—vaccination and bacteriocin treatment. In particular, we extensively review recent research aimed at discovering and characterizing potent anti-*Campylobacter* bacteriocins to reduce *Campylobacter* load at the primary production level in poultry.

Introduction

Microaerophilic *Campylobacter* spp., including *C. jejuni* and *C. coli*, are the most common bacterial causes of human gastroenteritis in the United States and many industrialized countries (Friedman *et al.*, 2000; Tauxe, 2002). Human *Campylobacter* illnesses are caused primarily by *C. jejuni* (~90%) and secondarily by *C. coli* (~10%). The estimated cases of campylobacteriosis in the United States are more than 2 millions per year (Mead *et al.*, 1999). The medical and productivity costs resulting from *C. jejuni* infection are estimated at 1.5–8.0 billion dollars each year in the United States (Buzby *et al.*, 1997; Buzby and Roberts, 1997). Poultry comprises the greatest concentration of *Campylobacter* and thus the main source of human campylobacteriosis (Friedman *et al.*, 2000). A recent study using a novel population genetics approach further indicated that chicken is the major source of *C. jejuni* that is pathogenic to humans, whereas wild animal and environmental sources are responsible for only 3% of campylobacteriosis (Wilson *et al.*, 2008). Quantitative risk assessment models have indicated that a reduction of *C. jejuni* numbers on a broiler carcass by 100-fold (or 2 log units) could result in a significant reduction (30 times less) in the incidence of campylobacteriosis (Rosenquist *et al.*, 2003). Therefore, reduction or elimination of *Campylobacter* in the poultry reservoir is an essential step to control this food safety problem. Although there are multiple levels at which *Campylobacter* contamination can be targeted and implemented, on-farm control of *Campylobacter* would have the greatest impact because the intestine of living poultry is the only amplification point for *Campylobacter* throughout the food chain (Wagenaar *et al.*, 2006, 2008).

Campylobacter is highly prevalent in poultry production systems, such as broilers, layers, turkeys, and ducks (Sahin *et al.*, 2002). This review is focused on broilers, the largest poultry market sector being the primary research addressed. *Campylobacter* is a commensal organism that establishes persistent and benign infections in broilers with colonization level up to 10^{10} colony-forming units (CFU) per gram of feces (Sahin *et al.*, 2002; Newell and Fearnley, 2003; Dhillon *et al.*, 2006). Although *Campylobacter* can be isolated from most intestinal sites of broiler chickens, it is mainly found in the cecal and cloacal crypts, where it does not adhere to epithelial cells but is found in the mucous layer (Beery *et al.*, 1988; Achen *et al.*, 1998). In commercial conditions, *Campylobacter* is most often absent in broilers less than 2–3 weeks of age although experimental inoculation of newly hatched chicks with *Campylobacter* can establish colonization successfully (Stern *et al.*, 1988; Newell and Wagenaar, 2000; Sahin *et al.*, 2002). The reasons for this lag phase are unknown but might be attributed to

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multiple factors, such as presence of maternal antibodies, antibiotic feed additives, intestinal development, and intestinal microbial flora (Newell and Wagenaar, 2000; Sahin *et al*., 2002). Once the first bird in a flock becomes colonized, infection spreads to the entire flock in just a few days. This rapid spread of *Campylobacter* throughout the flock is a result of high levels of shedding and efficient fecal–oral transmission compounded by communal water and feed (Lee and Newell, 2006). Horizontal transmission from environmental sources is the primary route of flock infections by *Campylobacter* (Sahin *et al*., 2002). In broiler chickens, *C. jejuni* colonization can persist for the lifetime of the animal (6–7 weeks), consequently leading to carcass contamination at the slaughter facility. Together, *Campylobacter* can rapidly disseminate throughout the flock, and establish persistent and high-level colonization in broilers, which greatly challenges the development of effective farm-based intervention measures to reduce *Campylobacter* in poultry.

On-farm intervention measures to reduce *Campylobacter* in poultry have been comprehensively reviewed recently (Wagenaar *et al*., 2006, 2008; de Zoete *et al*., 2007; Connerton *et al*., 2008). Three general strategies have been proposed to control *Campylobacter* on the poultry farm: (1) reduction of environmental exposure (biosecurity measures), (2) an increase in poultry's host resistance to reduce *Campylobacter* carriage in the gut (e.g., competitive exclusion, vaccination, and host genetics selection), and (3) the use of antimicrobial alternatives to reduce and even eliminate *Campylobacter* from colonized chickens (e.g., bacteriophage therapy and bacteriocin treatment). The rationale and effectiveness of different intervention measures are briefly summarized in Table 1. Theoretically, reduction of environmental exposure of chickens to *Campylobacter* (the first general strategy in Table 1) should protect poultry against *Campylobacter*. However, effective implementation of this intervention strategy, such as biosecurity measures, relies on a better understanding of risk factors and sources of *Campylobacter* for poultry (Wagenaar *et al*., 2008). In addition, several practical limitations have impeded wide application of biosecurity measures (Table 1). To date, the remaining two general strategies (Table 1) are not commercially available and are still under development. This review will not discuss several specific measures, including competitive exclusion, host genetics selection, and bacteriophage therapy (Table 1), because of limited progress of these measures; the detailed information on these measures can be found in corresponding reviews or journal articles (Table 1). This review is primarily focused on two promising measures—vaccination and bacteriocin treatment. In particular, we will extensively review recent breakthroughs in the discovery and characterization of potent anti-*Campylobacter* bacteriocins because these bacteriocins dramatically reduced *C. jejuni* colonization in chickens and are being developed for on-farm control of *Campylobacter* in poultry.

Vaccination of Chickens Against *Campylobacter*

Campylobacter *infections and chicken host immunity*

Through oral ingestion, *C. jejuni* enters the host intestine and colonizes the distal intestine, primarily the cecum in chicken. Although *Campylobacter* was considered a commensal of the avian host, *C. jejuni* infection triggers both a systemic and mucosal immune response in chickens (de Zoete *et al*., 2007). *C. jejuni*–specific serum IgG, IgA, and IgM, and mucosal IgA and IgG increased after oral infection with *C. jejuni* (Myszewski and Stern, 1990; Cawthraw *et al*., 1994; Widders *et al*., 1996). Specifically, *Campylobacter*-specific serum IgG, IgA, and IgM levels were elevated gradually 2–3 weeks after experimental inoculation, and mucosal IgA rose 3–4 weeks after oral infection. The antibodies are directed against multiple *Campylobacter* antigens, among which flagellin is usually the first antigen to be recognized by all antibody isotypes (Cawthraw *et al*., 1994; Rice *et al*., 1997). The elevated levels of *Campylobacter*-specific antibodies are correlated with reduced colonization level of *Campylobacter*, suggesting a protective role of the antibodies in anti-*Campylobacter* infection in chickens. The *Campylobacter* maternal antibodies could also be vertically transferred from infected layer hens to newly hatched chickens (Sahin *et al*., 2001). The high-level of *Campylobacter* maternal antibodies in young chickens may partly contribute to the lack of *Campylobacter* infection in young broiler chickens in natural environments during the first 2 weeks of life, which was also supported by laboratory challenge experiments (Sahin *et al*., 2001, 2003). Together, these findings demonstrated the protective nature of *Campylobacter*-specific antibodies and supported the feasibility of development of immunization-based approaches to control *Campylobacter* infections in poultry.

Interaction between Campylobacter *and chicken immune system*

It is not surprising that *Campylobacter*-specific antibody response is slow and moderate in chickens because *Campylobacter* infection in chicken does not cause a strong inflammatory response or tissue damage in intestine. It is still largely unknown how *Campylobacter* interacts with the chicken immune system to trigger the immune response. Understanding the delicate interactions between *Campylobacter* and the chicken immune systems would greatly facilitate development of immunization-based approaches to control *Campylobacter* infections in poultry. In some studies, *Campylobacter* was also isolated from the spleen, liver, and blood in young chickens, suggesting that *Campylobacter* may invade intestinal epithelial cells and become systemic (Sanyal *et al*., 1984; Knudsen *et al*., 2006). Recent studies (Byrne *et al*., 2007; Van Deun *et al*., 2008) further demonstrated that *C. jejuni* could adhere to and invade chicken intestinal epithelial cells *in vitro* and *in vivo*. Notably, the *in vitro* invasiveness of *C. jejuni* was correlated with the magnitude of spleen colonization in *C. jejuni*–inoculated chickens. The *C. jejuni* strains that invaded chicken epithelial cells were not able to proliferate intracellularly, but quickly evaded from the cells. Therefore, Van Deun *et al.* (2008) proposed a novel colonization mechanism of *C. jejuni* by escaping rapid clearance through short-term epithelial invasion and evasion, combined with fast replication in the mucus. Interestingly, a recent report showed that *C. jejuni* also colonized the bursa of Fabricius of day-old chicks with 10^4–10^7 CFU/g of content in bursa for up to 28 days (Bingham-Ramos *et al*., 2008). Given that the bursa of Fabricius is an important immune organ in chickens, further examination of the colonization of *C. jejuni* in the bursa may provide novel information on the interaction between *Campylobacter* and the host immune system.

Some *in vitro* studies using chicken cells (e.g., primary chicken embryo intestinal cells, primary chick kidney cells, or

Table 1. Major On-Farm Strategies to Reduce *Campylobacter jejuni* in Poultry

Strategies	*Rationale and effectiveness*	*References*
I. Reduce environmental exposure		
Biosecurity measures	*Campylobacter* is widespread in the farm environment. General biosecurity measures (e.g., hygiene and physical barriers) and specific biosecurity measures (e.g., single-species farming and partial depopulation) should protect poultry against *Campylobacter*. High biosecurity levels were correlated with absence of *Campylobacter* in poultry. However, it is difficult to assess the effectiveness of improving biosecurity in reducing *Campylobacter* in poultry. In addition, biosecurity measures may not work well for free-range flocks. The cost and practicality of such measures need to be determined.	Reviewed by Newell and Wagenaar (2000); Wagenaar *et al.* (2006, 2008)
II. Increase poultry's host resistance to reduce *Campylobacter* carriage in the gut		
Competitive exclusion	Competitive exclusion (CE) products consist of defined or undefined bacterial agents from the microbiota of adult chickens. It is hypothesized that chickens fed with CE products should establish a protective enteric microbiota that prevents *C. jejuni* colonization. However, CE has only limited and inconsistent success for *Campylobacter*. For acceptance by the poultry industry and regulatory agencies, complete identification of complex species in CE products is needed but is challenging.	Reviewed by Mead (2000); Wagner (2006); Wagenaar *et al.* (2008)
Vaccination	There is a correlation between increasing levels of *Campylobacter* antibodies and reducing levels of *C. jejuni* colonization in poultry. Vaccination of chickens against *Campylobacter* has had only partial success. The challenges for an effective vaccine against *Campylobacter* in poultry are significant.	Reviewed by de Zoete *et al.* (2007)
Host genetics selection	It has been observed that different chicken lines showed different susceptibilities for *Campylobacter* colonization. Given significant difference in the susceptibility between individual chickens with same line under identical conditions, this approach requires an improved knowledge about interactions between *Campylobacter* and chicken.	Stern *et al.* (1990a); Boyd *et al.* (2005); Li *et al.* (2008)
III. Use antimicrobial alternatives to reduce/eliminate *Campylobacter* from colonized chickens		
Bacteriophage therapy	Bacteriophages are viruses that can infect and kill susceptible bacteria. Some *Campylobacter*-specific phages reduced *Campylobacter* shedding about 2–3 log units in chickens under experimental conditions. The self-replicating nature and high specificity of phage make bacteriophages a potential alternative to conventional antibiotics. However, *C. jejuni* may develop resistance quickly in response to phage therapy and gain virulence genes via phage-mediated horizontal gene transfer. In addition, production of phage using *Campylobacter* host, a potential human pathogen that requires microaerophilic growth condition, raises concerns regarding safety and bulk production.	Reviewed by Connerton *et al.* (2008)
Bacteriocin treatment	Bacteriocins are antimicrobial peptides produced by bacteria with narrow or broad host ranges. The natural bacteriocins have considerable potential to fulfill the need for more effective antibiotics. Recently, several potent anti-*Campylobacter* bacteriocins have been identified in bacteria isolated from chicken intestine. These bacteriocins dramatically reduced *Campylobacter* colonization in chickens (>5–8 log reductions) and are being directed toward on-farm control of this pathogen in poultry.	Stern *et al.* (2005, 2006, 2008); Cole *et al.* (2006); Line *et al.* (2008); Svetoch *et al.* (2008)

chicken macrophage cell HD11) also provided compelling evidence that *Campylobacter* could stimulate the expression of proinflammatory cytokines and chemokines in chickens (Smith *et al.*, 2005; Borrmann *et al.*, 2007; Li *et al.*, 2008). Recently, Smith *et al.* (2008) also reported that a significant induction of proinflammatory chemokin transcript was observed in both day-old and 2-week-old chickens upon infection with *C. jejuni*. These *in vitro* and *in vivo* studies indicated that *C. jejuni* could intimately interact with the chicken immune system to trigger an immune response although no pathological signs are observed for *Campylobacter* infection in chickens.

Antigenicity of Campylobacter *components*

Elucidation of immunogenic and protective antigens in *C. jejuni* is a primary step toward the design of effective vaccines. However, very limited studies have been done to characterize the immunological properties of *Campylobacter* components, primarily due to a lack of understanding of pathogenesis mechanisms and the antigenic complexity of this organism. Outer membrane proteins (OMPs) have been exploited and demonstrated as attractive targets of immune intervention in Gram-negative bacteria (Stern *et al.*, 1990b; Lin *et al.*, 2002a). Immunogenic OMPs identified in *Campylobacter* include flagellum (Fla) (Guerry, 1997), major outer membrane protein MOMP (Zhang *et al.*, 2000), cell-binding factor Peb1 (Pei and Blaser, 1993), multidrug efflux pump component CmeC (Lin *et al.*, 2002b, 2003, 2005b), and ferric enterobactin receptor CfrA (Zeng *et al.*, 2008). Motility-mediating Fla is the best-characterized immunogenic protein shown to be required for *Campylobacter* colonization in birds and mammals (Morooka *et al.*, 1985; Pavlovskis *et al.*, 1991; Nachamkin *et al.*, 1993; Wassenaar *et al.*, 1993; Guerry, 1997). However, as an immunodominant protein in *C. jejuni*, Fla is modified by glycosylation and undergoes both phase and antigenic variation, which complicates the use of Fla for vaccination (Caldwell *et al.*, 1985; Logan *et al.*, 1989; Doig *et al.*, 1996; Szymanski *et al.*, 1999). Regarding another immunodominant protein MOMP, the definitive role of MOMP in microbe–host interaction is still not clear, and both conserved and variable regions were observed in MOMP (Zhang *et al.*, 2000). The antigenicity of MOMP is also unique as reflected by predominant conformational epitopes in nature (Zhang *et al.*, 2000). Peb1 functions as an outer membrane adhesin to mammalian cells and as an aspartate/glutamate-binding protein of an ABC transporter. However, it seems that Peb1 is localized mainly in the periplasm (de Zoete *et al.*, 2007). CmeC is an essential OMP component of CmeABC efflux system that plays a critical role in multidrug resistance and pathogenesis (Lin *et al.*, 2002b, 2003). Recent studies have shown that (1) CmeC is broadly expressed and highly conserved in *C. jejuni*, (2) CmeC is immunogenic *in vivo*, (3) CmeC is essential for colonization of *Campylobacter* in the intestine by mediating bile resistance, (4) expression of CmeC is dramatically induced by bile salts and highly upregulated in the intestinal tract, and (5) inhibition of CmeABC efflux pump by pump inhibitor increased susceptibility of *C. jejuni* to multiple antimicrobials and reduced *in vivo* colonization of *C. jejuni* in chicken (Lin *et al.*, 2002b, 2003, 2005a, 2005b; Stintzi *et al.*, 2005; Lin and Martinez, 2006; Martinez and Lin, 2006; Fakhr and Logue, 2007; Zeng and Lin, 2008). These findings strongly suggest that CmeC is an attractive and novel vaccine candidate that may not only prevent *in vivo* colonization of *C. jejuni* but also combat antibiotic resistance in *C. jejuni*. CfrA, an OMP component of ferric enterobactin iron acquisition system, is dramatically induced by iron-restricted condition and plays an essential role in colonization of *C. jejuni* in chickens (Palyada *et al.*, 2004). Our recent work showed that CfrA is broadly distributed, expressed, and antigenically conserved among *C. jejuni* strains from various sources (Zeng *et al.*, 2008). In addition, sera from *Campylobacter*-infected chickens showed vivid reaction with CfrA, indicating CfrA is also immunogenic *in vivo* (Zeng *et al.*, 2008). Therefore, CfrA is another potential vaccine candidate against *C. jejuni*.

Vaccine development against Campylobacter *in chickens*

Vaccine development against *Campylobacter* in chickens has been comprehensively reviewed by de Zoete *et al.* (2007) recently. There is no vaccine available to date to control *Campylobacter* infections in poultry. A successful chicken vaccine should prevent colonization or cause a strong reduction of *Campylobacter* numbers in chickens (>2 log units) (de Zoete *et al.*, 2007).

The following three approaches have been explored for developing effective and safe vaccine against *Campylobacter* in poultry:

1. Live attenuated vaccines. Because infection with wild-type *C. jejuni* strain induced anti-*Campylobacter* antibodies (Myszewski and Stern, 1990; Cawthraw *et al.*, 1994; Widders *et al.*, 1996), it is likely that a live attenuated vaccine will have a protective effect. However, experimental colonization with a noncolonizing *C. jejuni* strain did not protect upon homologous challenge (Ziprin *et al.*, 2002). In addition, the paucity of information on the pathogenesis of the organism complicates this strategy.
2. Killed whole-cell vaccines. This type of vaccine could induce high protective immunity without the concern regarding potential pathogenesis to human. Vaccination with killed *C. jejuni* whole cells enhanced the immune responses and partly reduced colonization of *C. jejuni* in chickens (<2 log) (de Zoete *et al.*, 2007).
3. Subunit vaccine. Successful development of subunit vaccine needs improved knowledge on immunogenic and protective antigens in *C. jejuni*.

Several studies have been focused on immunodominant antigen Fla with variable success (reviewed by de Zoete *et al.*, 2007). However, Fla is modified by glycosylation and undergoes both phase and antigenic variation, which limits the application of Fla-based vaccines. The most encouraging vaccination study was published by a Polish group, in which oral vaccination of chickens with CjaA via a *Salmonella* carrier strain reduced *C. jejuni* colonization by 6 logs (Wyszynska *et al.*, 2004). However, this finding is intriguing and needs to be confirmed because of the following two reasons. First, only two treatment groups (untreated chicken vs. vaccine treatment) were used in this study, and there was no *Salmonella* carrier strain control group included (Wyszynska *et al.*, 2004). Therefore, it is likely that the protective effect observed in this vaccination trial was mediated by general boost of host im-

munity due to *Salmonella* infection instead of specific anti-CjaA antibodies. Second, a recent study (Wyszynska *et al.*, 2008) indicated that CjaA is an N-glycosylated lipoprotein localized in the inner membrane of *C. jejuni*. Thus, it is difficult for specific CjaA antibodies to pass through outer membrane and gain access to CjaA, consequently conferring protection. Regarding CmeC and CfrA, the two promising vaccine candidates, the protective efficacy of these subunit vaccines needs to be determined in chicken in the future.

Oral delivery systems would be appropriate for *Campylobacter* vaccine in poultry as far as cost and simplicity of administration are concerned (Wagenaar *et al.*, 2008). Particularly, successful identification of protective antigens as well as epitope mapping will lead to the development of inexpensive and practical oral vaccines for chickens to prevent *Campylobacter* infections using appropriate delivery systems, such as attenuated *Salmonella*-based vaccines (Curtiss *et al.*, 1989) and genetically modified *Lactobacillus* (Mota *et al.*, 2006). In conclusion, the short average life span of broiler chickens (~6 weeks) poses a significant challenge to induce a strong antibody response against *Campylobacter* in chickens. To develop an effective vaccine against *Campylobacter* in poultry, three main challenges have been identified: (1) the identification of cross-protective antigens, (2) the induction of rapid and strong immune response, and (3) the development of novel adjuvants to further stimulate immunity against *Campylobacter* (de Zoete *et al.*, 2007).

Bacteriocins to Reduce *Campylobacter* in Poultry

Bacteriocins

Bacteriocins are designated as the antimicrobial peptides (AMPs) produced by bacteria with narrow or broad host ranges (Hechard and Sahl, 2002; Riley and Wertz, 2002; Cotter *et al.*, 2005). Bacteriocins are ribosomally synthesized, produced, and exported by almost every bacterial species examined to date for the apparent purpose of destroying their competitors (Riley and Wertz, 2002). Many bacteriocin-producing bacteria (e.g., lactic acid bacteria) are commensals in intestine (Riley and Wertz, 2002; Cotter *et al.*, 2005; Sit and Vederas, 2008). Therefore, the intestinal bacteriocin–producing bacteria may achieve competitive advantage and function as an innate barrier against pathogens in the gut. Bacteriocins are classified into modified bacteriocins (Class I bacteriocins, such as nisin) and unmodified bacteriocins (Class II bacteriocins, such as the anti–*C. jejuni* bacteriocins described below) (Hechard and Sahl, 2002; Riley and Wertz, 2002; Sit and Vederas, 2008). Despite the existence of a broad diversity in bacteriocin sequences and structures, it has been widely accepted that bacteriocins and other host defense peptides share a common theme in the mechanism of killing action by disruption of membrane integrity (Hechard and Sahl, 2002; Riley and Wertz, 2002; Yeaman and Yount, 2003). Generally, AMPs directly interact with target cells via initial electrostatic and hydrogen bond attraction, and then disrupt the structure or function of the bacterial membrane by permeating lipid bilayers, forming a transmembrane pore, and ultimately leading to cell death. However, transmembrane pore formation is not the only mechanism of bacterial killing by bacteriocins (Peschel and Sahl, 2006; Sahl and Bierbaum, 2008). For example, nisin, a bacteriocin widely used for food biopreservation, also has other modes of antimicrobial action, such as inhibition of cell-wall biosynthesis, inhibition of lipid bilayer function, inhibition of spore outgrowth, and activation of autolytic enzyme (Peschel and Sahl, 2006; Sahl and Bierbaum, 2008). Detailed information on bacteriocin evolution, structure–function relationships, and mode of action are available in several excellent reviews (Hechard and Sahl, 2002; Riley and Wertz, 2002; Cotter *et al.*, 2005; Peschel and Sahl, 2006).

Potential of bacteriocins as new antimicrobials

Bacterial pathogens are increasingly resistant to currently available antibiotics, and new antimicrobials are needed to combat multidrug resistance (Walsh, 2003). Bacteriocins have considerable potential for the design and production of new antimicrobials (Cleveland *et al.*, 2001; Cotter *et al.*, 2005; Kirkup, 2006; Galvez *et al.*, 2007; Rossi *et al.*, 2008; Sahl and Bierbaum, 2008; Sit and Vederas, 2008). In contrast to traditional antibiotics, bacteriocins are considered natural and nontoxic on eukaryotic cells because they are found commonly in food animal products and thus have been consumed for centuries (Cleveland *et al.*, 2001; Galvez *et al.*, 2007). In fact, two bacteriocins, nisin and pediocin PA1/AcH, have been widely used in the food industry for food biopreservation, and no toxicity due to these bacteriocins has been demonstrated (Cleveland *et al.*, 2001; Cotter *et al.*, 2005; Galvez *et al.*, 2007).

From standpoint of antimicrobial development, the emergence of bacteriocin resistance is a concern, either for food preservation or for therapeutic treatment. Because nisin is the only bacteriocin licensed as a food preservative and many potential bacteriocins are still under development, limited information is available directly addressing the development and mechanisms of bacteriocin resistance. Both Gram-positive and Gram-negative bacteria can develop resistance to bacteriocins (e.g., nisin), and the mechanism of bacteriocin resistance appears to be complex and involves various structural and physiological changes in the bacterial cell envelope (Ennahar *et al.*, 2000; Cleveland *et al.*, 2001; Breukink and de Kruijff, 2006; Peschel and Sahl, 2006; Sahl and Bierbaum, 2008). Intriguingly, it seems that bacteria have not developed highly effective mechanisms to resist natural AMPs, including bacteriocins (Peschel and Sahl, 2006; Sahl and Bierbaum, 2008). This feature is very different from many therapeutic antibiotics for which bacteria can develop high-level of resistance. Recently, it has been proposed that bacteriocins may have multiple low-affinity targets and cause pleotropic effects on various bacterial targets. Therefore, it is possible that such low-affinity interactions of bacteriocins with multiple targets are not favorable for the development of bacterial resistance. In contrast, many therapeutic antibiotics act on a single, high-affinity target, which makes it comparatively easy for bacteria to develop resistance, particularly high-level resistance (Peschel and Sahl, 2006; Sahl and Bierbaum, 2008). Together, bacteriocins have considerable potential to fulfill the need for more effective antimicrobial agents. Unlike the antibiotics that act on a single target, there is less in the way of resistance development for bacteriocin-based antimicrobials.

*Anti-*Campylobacter *bacteriocins*

In the past 3 years, significant progress has been made toward isolation of chicken commensal bacteria inhibitory to

Table 2. Overview of Bacteriocins That Reduced *Campylobacter* Colonization in Poultry

Bacteriocin-producing bacteria				
Name	*Species*	*Bacteriocin name*	*Effect*[a]	*References*
NRRL B-30509	*Paenibacillus polymyxa*	SRCAM 602	ND (4.6–6.3 log reduction in 10-day-old chickens) ND (>4 log reduction in 13-day-old turkeys)	Stern *et al.* (2005); Svetoch *et al.* (2005) Cole *et al.* (2006)
NRRL B-30514	*Lactobacillus salivarius*	OR-7	>6 log reduction in 10-day-old chickens ND (>4 log reduction in 13-day-old turkeys)	Stern *et al.* (2006) Cole *et al.* (2006)
NRRL B-30745	*Enterococcus durans/ faecium/hirae*	E-760	ND (>6.6 log reduction in 10-day-old chickens with dose as low as 31.2 mg/kg feed) ND (2.2–5.0 log reduction in 42-day-old chickens with dose of 125 mg/kg feed)	Line *et al.* (2008)
NRRL B-30746	*Enterococcus faecium*	E 50–52	ND (>6.4 log reduction in 15-day-old chickens with dose as low as 31.2 mg/kg feed) >5.3 log reduction with one day treatment of 35–41-day-old broilers (12.5 mg of E 50–52/liter of drinking water)	Svetoch *et al.* (2008)

[a]ND, no *Campylobacter* was detected in all birds after bacteriocin treatment with minimum detection of 100 CFU/g cecal contents. Unless specifically clarified, treated birds were provided specific bacteriocin at a dose of 250 mg/kg feed for 3 consecutive days. The bacteriocins were mixed with polyvinylpyrrolidone power to produce microencapsulated bacteriocins, which were used to make a medicated feed.

Campylobacter and characterization of associated bacteriocins from these bacteria (Stern *et al.*, 2005, 2006; Svetoch *et al.*, 2005, 2008; Cole *et al.*, 2006; Line *et al.*, 2008; Nazef *et al.*, 2008). Several potent anti-*Campylobacter* bacteriocins have been purified and characterized in bacteria isolated from the chicken intestinal tract, which includes SRCAM 602 from *Paenibacillus polymyxa* (Stern *et al.*, 2005; Svetoch *et al.*, 2005), OR-7 from *Lactobacillus salivarius* (Stern *et al.*, 2006), and E-760 and E 50–52 from *Enterococcus* spp. (Line *et al.*, 2008; Svetoch *et al.*, 2008). These bacteriocins also dramatically reduced *C. jejuni* colonization in poultry and are being developed for on-farm control of *Campylobacter* to protect public health. The bacteriocins that reduced *Campylobacter* colonization in poultry are summarized in Table 2.

Svetoch *et al.* (2005) first reported the identification and characterization of novel anti-*Campylobacter* bacteriocins from bacteria isolated from chicken intestine. This study involved screening 365 representative *Bacillus* and *Paenibacillus* isolates for activity against *C. jejuni*. One *B. circulans* and three *P. polymyxa* strains displayed strong antagonism to *C. jejuni*. The anti-*Campylobacter* activity was later observed to result from the secreted protein component of these antagonists. Stepwise purification using ammonium sulfate precipitation, Superose-12 gel filtration, and Mono Q anion-exchange chromatography resulted in the identification of a short peptide (~3.5 kDa) with purity up to 98.8%. The purified peptides displayed potent anti-*Campylobacter* activity and were stable at high temperature (100°C, 15 min) and a wide pH range (3.0–9.0). These peptides lost their activity after being treated with β-chymotrypsin, proteinase K, and papain but retained activity when treated with lysozyme or lipase. Amino acid sequences of these peptides indicated that these peptides are consistent with class IIa bacteriocins because of the conserved N-terminal sequence of Tyr-Gly-Asn-Gly-Val and two cysteine amino acids forming a disulfide bridge at the N-terminal portion of the peptide (Svetoch *et al.*, 2005). The biochemical and anti-*Campylobacter* activities of these bacteriocins indicate that these peptides represent a new and unreported group of bacteriocins.

To determine if above novel bacteriocins could reduce *Campylobacter* colonization in poultry, one bacteriocin, SRCAM 602 produced by *P. polymyxa* NRRL B-30509, was evaluated in chickens (Stern *et al.*, 2005) and Turkeys (Cole *et al.*, 2006). For both trials, the purified SRCAM 602 bacteriocin was microencapsulated in polyvinylpyrrolidone and then mixed with commercial feed to produce medicated feed with final bacteriocin concentration of 250 mg/kg feed. For the chicken studies, 1-day-old chickens were orally inoculated with one of four *C. jejuni* strains (Stern *et al.*, 2005). When colonization of *C. jejuni* in chickens was well established by 7 days of age, the chickens were provided with either nonmedicated feed or bacteriocin-embedded feed (10 birds per group) for 3 consecutive days. Chickens receiving nonmedicated feed displayed high levels of colonization with *C. jejuni* (6.6–8.3 log CFU/g of feces). However, none of the bacteriocin-treated chickens were colonized with *C. jejuni* (detection limit of 2 log CFU/g of feces). This finding was highly consistent in duplicated experiments for four different *C. jejuni* strains. The efficacy of this bacteriocin was also observed in turkey (Cole *et al.*, 2006). Turkey poults were orally challenged with a mixture of three *C. coli* isolates at 3 days of age. From day 10–12 posthatch, the turkeys received either nonmedicated feed or the bacteriocin-emended feed (10 birds per group). Oral administration of the purified bacteriocin

Table 3. Biochemical Characteristics of Anti-*Campylobacter* Bacteriocins

Bacteriocin[a]	*Length*[b]	*Molecular mass (Da)*	*Isoelectric point*	*Retained activity when treated with*			*Reference*
				Enzyme[c]	*Heat*	*pH*	
SRCAM 602	39	3864	7.2	Lysozyme lipase	100°C, 15 min	3.0–9.0	Stern *et al.* (2005); Svetoch *et al.* (2005)
OR-7	54	5123	9.5	Lysozyme lipase	90°C, 15 min	3.0–9.1	Stern *et al.* (2006)
E-760	62	5362	9.5	Lysozyme lipase	100°C, 5 min	5.0–8.7	Line *et al.* (2008)
E 50–52	39	3340	8.0	Lysozyme lipase	100°C, 15 min	3.0–8.4	Svetoch *et al.* (2008)

[a]All anti-*Campylobacter* bacteriocins belong to class IIa bacteriocins.
[b]Total number of amino acid residues of purified active bacteriocin.
[c]Specific bacteriocin was incubated with enzyme for 3 h of incubation at 37°C.

SRCAM 602 eliminated detectable cecal *Campylobacter* colonization in all turkeys in three separate trials. Together, these findings strongly suggest that bacteriocins are effective *in vivo* and that bacteriocin treatment of colonized poultry may represent an effective intervention strategy against *Campylobacter* colonization in poultry.

Lactic acid bacteria such as *Lactobacillus* spp. are widely used probiotic organisms. Many lactic acid bacteria produce bacteriocins with different spectra ranges of inhibition (Cotter *et al.*, 2005; Galvez *et al.*, 2007). Therefore, Stern *et al.* (2006) also evaluated anti-*Campylobacter* activity among >1200 isolates of different lactic acid bacteria. One isolate, *Lactobacillus salivarius* NRRL B-30514, displayed highest anti-*Campylobacter* activity. Bacteriocin OR-7 from this strain was purified using ammonium sulfate precipitation, followed by SP Sepharose cation exchange and Octyl-Sepharose hydrophobic interaction chromatography. The purified bacteriocin OR-7 was also resistant to high temperature (90°C, 15 min) and a wide pH range (3.0–9.1). The amino acid sequence of OR-7 bacteriocin (55 aa residues) was also consistent with class IIa bacteriocins. The inhibitory effect of OR-7 on *C. jejuni* colonization in chicken was then evaluated with the same experimental design as that for SRCAM 602 described above. Bacteriocin OR-7 treatment consistently reduced *Campylobacter* colonization more than 1 million fold in chicken. Therefore, the bacteriocin OR-7 from *L. salivarius* NRRL B-30514 also has significant potential to reduce *C. jejuni* load in poultry.

Recently, the same research group identified another two novel bacteriocins, E-760 and E 50–52, which are produced by two different *Enterococcus* spp. isolated from broiler ceca (Line *et al.*, 2008; Svetoch *et al.*, 2008). The highly purified bacteriocins were obtained from culture supernatants by initial ammonium sulfate precipitation followed by ion-exchange and hydrophobic-interaction chromatography. Amino acid sequence analysis indicated that these bacteriocins also belong to Class IIa bacteriocins. These two bacteriocins also displayed tolerance to high temperature and a wide range of pH. The biochemical characteristics of all anti-*Campylobacter* bacteriocins are summarized in Table 3. Both E-760 and E 50–52 bacteriocins not only displayed potent anti-*Campylobacter* activity but also showed strong antibacterial activity against a broad spectrum of foodborne pathogens, such as *Salmonella* spp., *E. coli* O157:H7, *Listeria* spp., and *Shigella* spp. Chicken trials further showed that treatment of E-760–embedded feed eliminated detectable cecal *Campylobacter* colonization in both young chickens (10-day-old) and market-aged broiler chickens (42-day-old) (Line *et al.*, 2008; Svetoch *et al.*, 2008). With respect to bacteriocin E 50–52, young chickens received E 50–52–emended feeds at different dose (31.2, 62.5, or 125 mg/kg feed) from day 4 to day 7. All birds were sacrificed on day 15, and cecal samples were collected for enumeration of viable *C. jejuni*. Despite high levels of *C. jejuni* colonization in control chickens (8.40 $\log_{10}$ CFU/g feces), no *Campylobacter* was detected in all chickens treated with E 50–52 even at 8 days after termination of the bacteriocin treatment, suggesting that the bacteriocin treatment completely eliminated *C. jejuni* from chicken intestine (Svetoch *et al.*, 2008). E 50–52 was also very effective to reduce *C. jejuni* colonization in adult bird (35–41-day-old broilers). All the market-aged birds were environmentally colonized by *C. jejuni* at a level of about 8.00 $\log_{10}$ CFU/g feces, and 1 day treatment via drinking water (12.5 mg of E 50–52/liter of drinking water) dramatically reduced *C. jejuni* colonization in chicken intestine (>5.3 log reduction).

In summary, four anti-*Campylobacter* bacteriocins, produced by bacteria isolated from chicken intestine, have been successfully purified and characterized (Table 3). Oral administration of these bacteriocins dramatically reduced *C. jejuni* colonization in chicken intestine (Table 2). Therefore, these natural bacteriocins have been proposed as effective alternatives to therapeutic antibiotics and were being directed for on-farm control of *Campylobacter* in poultry (Casewell *et al.*, 2003; Stern *et al.*, 2005, 2006; Svetoch *et al.*, 2005, 2008; Line *et al.*, 2008).

Key issues on bacteriocins-based intervention strategy

Although the above anti-*Campylobacter* bacteriocins are very effective in reducing *C. jejuni* colonization in poultry, several important issues (e.g., production, safety, and development of resistance) need to be addressed for future regulatory approval and public acceptability of this intervention measure.

Purification of the four bacteriocins has been well established and standardized (Svetoch *et al.*, 2005, 2008; Stern *et al.*, 2006; Line *et al.*, 2008). The purification generally involved two steps: (1) crude bacteriocin (~9% purity) preparation from the supernatant using ammonium sulfate precipitation, and (2) bacteriocin purification from the crude preparation using two different chromatography columns, which finally results in bacteriocin purity up to 98.8%. To make bacteriocin usage in poultry economically feasible, it is important to improve the yield during production. Recently, Stern *et al.* (2008) observed that bacteriocin yield was dramatically increased when bacteriocin-producing strains were cultured together

with a novel inducer strain and inducer peptide. For example, approximate 214–225 mg of high-purity bacteriocin OR-7 could be purified from 1 L of culture liquid under modified culture condition using the novel inducer strain and peptide. Therefore, this modified procedure provides an economic method for producing large quantities of bacteriocins for commercial use.

Bacteriocins are widely considered natural and safe to animals and humans (Cleveland *et al.*, 2001; Galvez *et al.*, 2007). Some bacteriocins have been used as food preservatives for a long time (Cleveland *et al.*, 2001; Cotter *et al.*, 2005; Galvez *et al.*, 2007). All identified anti-*Campylobacter* bacteriocins are produced by commensal bacteria from chickens that have been consumed by humans for centuries. Therefore, these bacteriocins are likely nontoxic to both poultry and humans. However, to address this issue definitively, more research is needed to determine toxicity of the bacteriocins using cell culture and/or animal model systems. In addition, to date it is still not clear if oral administration of the bacteriocins could cause significant absorption of bacteriocin in intestine, consequently leading to the presence of the bacteriocin in chicken blood and tissues. Given the molecular mass of the bacteriocins (3.3–5.4 kDa; Table 3), absorption and diffusion of intact bacteriocins through the epithelial barrier may not be efficient. Svetoch *et al.* (2008) observed that oral administration of bacteriocin E 50–52 in chicken resulted in the significant reductions of *Salmonella enteritidis* in the liver and spleen, which suggests that the bacteriocin can enter the systemic system by intestinal absorption. However, it is also possible that bacteriocins, like many other AMPs, could function as a potent immune modulator and directly enhance the innate immune response to fight *Salmonella* infections (Finlay and Hancock, 2004). This hypothesis remains to be studied in the future.

The development of antibiotic resistance is inevitable in bacteria, and every antibiotic that is introduced into market to date has a limited shelf life (Walsh, 2003). However, unlike high-affinity small-molecule antibiotics, bacteria have not developed highly effective mechanisms to resist natural AMPs, including bacteriocins, which is likely because of the multiple modes of action of AMPs (Peschel and Sahl, 2006; Sahl and Bierbaum, 2008). Therefore, compared to conventional antibiotics that act on a single target, there is less in the way of resistance development for peptide antibiotics, such as bacteriocin-based antimicrobials (Peschel and Sahl, 2006; Sahl and Bierbaum, 2008). Our recent study (Hoang *et al.*, 2008) also supported this hypothesis. Examination of 146 *Campylobacter* isolates of various origins revealed only one isolate displaying resistance to bacteriocin OR-7 (MIC = 64 μg/mL), while all other isolates showed low MICs ranging from 0.25 to 1.0 μg/mL. We also observed that *Campylobacter* could develop resistance to bacteriocin OR-7 at low frequency *in vitro*. All *in vitro*–selected mutants only displayed low-level resistance (4–16-fold increase in MIC) to OR-7. The multidrug efflux pump CmeABC contributes to the intrinsic and acquired resistance of *C. jejuni* to bacteriocin OR-7 (Hoang *et al.*, 2008). Despite this recent progress in understanding the development and mechanism of bacteriocin resistance in *Campylobacter*, it is unknown if therapeutic usage of bacteriocins in poultry would promote the emergence of bacteriocin-resistant *Campylobacter* mutants *in vivo*. If so, can *Campylobacter* develop high-level bacteriocin resistance in response to therapeutic treatment with bacteriocins? It is also unclear if the bacteriocin-resistant *Campylobacter* could persist in the absence of antimicrobial selection pressure *in vitro* and *in vivo*. The specific targets of anti-*Campylobacter* bacteriocins and the molecular basis of bacteriocin resistance in *Campylobacter* are still largely unknown. Answering these questions should greatly improve our understanding on the modes of action of bacteriocins, provide helpful information for risk assessment, and facilitate the development of more sustainable bacteriocins for on-farm control of *Campylobacter* in poultry.

Conclusion

It is widely accepted that contamination of poultry by *Campylobacter* is a significant risk factor of human campylobacteriosis. Thus, the prevention and control of *C. jejuni* in poultry would reduce the risk of human exposure to *Campylobacter* and is an important food safety issue. However, there is no effective, reliable, and practical intervention measure available to reduce *C. jejuni* in poultry to date. Biosecurity measures are practical, but hygiene barriers could still be broken through. All other potential measures are still under development. Vaccination of chickens against *C. jejuni* is a feasible strategy but requires a good deal of basic research to reveal protective antigens, to examine delicate interaction between *C. jejuni* and chicken immune system, and to optimize vaccination regimen (e.g., mucosal adjuvant and delivery systems). Anti-*Campylobacter* bacteriocin treatment is clearly an effective and feasible strategy to reduce *C. jejuni* load in market-aged chickens. More research is needed to address several key issues (e.g., production, safety, and development of resistance) of bacteriocin application for future regulatory approval and public acceptability of this promising intervention measure.

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Disclosure Statement

No competing financial interests exist.

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