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Review

Efficacy and food safety considerations of poultry competitive exclusion products

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Competitive exclusion (CE) products are anaerobic cultures of bacteria that are applied to poultry hatchlings to establish a protective enteric microbiota that excludes intestinal colonization by human food-borne pathogens. For safety of the poultry flock and human consumers, the identities of bacteria in CE products need to be known. A CE product is a culture of intestinal contents from adult chickens. It may be microbiologically defined by analysis of bacteria isolated from the culture, but many bacteria are hard to reliably isolate, identify, and characterize with conventional techniques. Sequence analysis of 16S ribosomal RNA (rRNA) genes may be more reliable than conventional techniques to identify CE bacteria. Bacteria in CE products may contain antimicrobial drug resistance and virulence mechanisms that could be transferred to the enteric bacteria of the food animal and to the human consumer. Detection methods for specific antimicrobial drug resistance and virulence genes and the integrase genes of conjugative transposons, mostly utilizing PCR technology, are being developed that can be applied to assess these risks in CE bacteria. With improvements in efficacy, bacterial identification, and detection and control of the possible risks of gene transfer, CE product technology can be made a more effective food safety tool.

Keywords: Antimicrobial drug resistance / Competitive exclusion / Food safety / Microbial ecology / Poultry

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1 Introduction

Modern hatchery management practices prevent the intimate contact between hatchlings and hens, which, under natural conditions inoculates the hatchlings with the appropriate microbial species to develop a protective intestinal microbiota [1]. These management practices leave the hatchlings more susceptible to colonization by *Salmonella* and *Campylobacter* spp. [2, 3]. Competitive exclusion (CE) products are intended to replace this natural process of microbiota development. A CE product is distinguished from probiotics by the main purpose of replacing the natural route of microbiota succession. Probiotics are live microbial feed additives used to achieve a beneficial effect on the health of the host [4]. Probiotics are intended to enhance the functions of existing microbiota.

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Abbreviations: CE, competitive exclusion; **VRE**, vancomycin-resistant enterococci

Without the use of CE products in modern farming practices, human pathogens colonize poultry at a rapid rate [3]. For example, feces were sampled from 1 to 45 days after oral challenge of broiler chicks with *Campylobacter jejuni*. At 24 h after challenge, 50% were positive and at 48 h, 70% were positive for *C. jejuni*. The peak rate of colonization was 13 – 19 days with a steady decline thereafter. At market age, day 43, 37.5% were positively colonized with *C. jejuni* [3]. Therefore, the use of CE products is justified by the need to quickly establish a competitive microbiota after hatching. However, the CE products used for this purpose must be effective and safe for both the poultry and for the human consumers, who may become exposed to the intestinal microbiota of the birds.

There are several advantages and disadvantages of CE technology compared in the present review (Table 1). The most important advantage is that CE products ensure the establishment of the complex intestinal microbiota that resists colonization by human and poultry pathogens. They are produced as a consortium of bacteria that can coexist together as a stable community in the enteric ecosystem. Although the main goal of CE technology is to reduce poultry contamination by *Salmonella enterica* and *Campylobacter* spp., it also may provide a way to reduce agriculture

Table 1. Advantages and disadvantages of competitive exclusion technology

Advantages	Disadvantages
Reduce rates of *Salmonella enterica* and *Campylobacter jejuni* colonization of poultry	Uncertain compositions and quality control
Bacteria in products are adapted to poultry intestinal ecosystems	Difficult bacterial identifications
Assures establishment of complex intestinal microbiota	Antimicrobial drug resistance genes may be transferable to microbiota of human consumer
Reduced need for antimicrobial drug use in poultry	Virulence factors may be transferable to microbiota of human consumer

dependence on antimicrobial drugs. Antimicrobial drugs are used in poultry farming to reduce *S. enterica* colonization and to maintain the health of the birds. The prospect of reducing the use of antimicrobials in poultry agriculture by using CE products is a strong incentive to develop CE technology. Concerns are growing about the selective pressures of antimicrobial drug use in agriculture that promotes the movement of drug resistant bacteria into the human food supply [5]. Concerns about the use of antimicrobials that select for resistance have led to the withdrawal of poultry applications of several drugs that have human use analogs, such as avoparcin [6]. Thus, alternative technologies to antimicrobial drugs in poultry agriculture, like CE, have gained greater importance. Even though CE may be useful for reduction of pathogen colonization, protective microbiota replacement, microbiota stabilization, and reducing the use of antimicrobials in poultry, it is only a partially effective method for prevention of flock colonization by human pathogens [1]. Thus, antimicrobial drug use in poultry may not be completely replaced by current CE products.

Even with certain advantages, CE technology has some potential disadvantages (Table 1). Because a CE treatment affects the health of the birds, it is regulated as an animal drug and its components, by law, must be specified [7]. The problem lies in accurately specifying the bacteria in the product. As CE products are intended to become permanently established as part of the animals' intestinal microbiota, the potential for these bacteria to reach the human consumer exists. Exposure of humans to the poultry microbiota may be minimized by careful management of production facilities, but some bacteria often remain associated with poultry carcasses [8]. In relation to the human consumer, two other problems with CE products must be considered: the presence of antimicrobial resistance and the possibility of transferable virulence genes in the component bacteria of CE products that could affect the microbiota of the consumer. These risks must be weighed against the benefits of CE product use, which are reduced poultry contamination by frank human pathogens (*e.g. S. enterica* and *C. jejuni*), improved health of the animals, and reduced dependence on antimicrobial drugs.

This review will present evidence that CE products are partially effective and beneficial and what factors need to be optimized to improve their effectiveness. The review will then discuss the issues of defining the composition of CE products and the techniques for identification and characterization of their component bacteria. The potential risks of CE products to introduce antimicrobial resistance and virulence genes into the microbiota of poultry and to the human consumer will be also discussed. The focus of the present review is the application of CE in poultry, but successful use of CE in other areas of animal agriculture should also be mentioned. The use of CE has been proven effective for protection of swine from infections of enterotoxigenic *Escherichia coli* [9], and *Salmonella* sp. [10]. In addition, protection of cattle from herd transmission of *E. coli* O157:H7 has also been described [11].

2 Evidence for efficacy of CE products

A goal for CE products would be the decontamination of poultry flocks harboring *Salmonella* or other food-borne pathogens. However, this goal is not met by existing products, which are more capable of preventing colonization than decontamination. For example, complete decontamination of *Salmonella* sp. from laying hens was not achieved with combined CE, enrofloxacin treatment, and movement of birds to a clean environment [12]. Chicken breeds vary somewhat in susceptibility to *S. enterica* Serovar Enteritidis colonization; however, once colonized, translocation of bacteria to internal organs is the same regardless of the chicken strain tested [13]. A summary of CE product efficacy reports is given in Table 2. Use of a CE product, Aviguard™, with enrofloxacin treatment on previously *Salmonella*-colonized chicks reduced, but could not eliminate, *Salmonella* sp. from the ceca of laying hens [14]. In another study, Aviguard™ was tested for elimination of *Salmonella* sp. from an egg production facility. It reduced fecal spread of *Salmonella* sp. to the laying hens and *Salmonella* sp. contamination of the egg crating plant was eliminated [15]. An undisclosed commercial CE product was used in broiler chicks against *S. enterica* Serovar Enteritidis from four broiler flocks. The results of the study were reduced mortality due to salmonellosis and modest reductions in spleen, liver, and cecal colonization [16]. A commercial CE product, Broilact™ was sprayed in the hatchery and cultures of *Salmonella* sp. were taken from broiler chick transport pads and from litter samples 2 weeks before slaughter. The researchers reported significant, but incomplete reduction of positive flocks on the farm and int the slaughterhouse

[17]. In broilers, Preempt™ reduced the horizontal transfer of *S. enterica* Serovar Gallinarum and the mortality of direct challenge of chickens [18]. These studies show that numerous critical control points exist in the hatching and rearing environments where CE can help reduce *Salmonella* sp. contamination, but pre-existing *Salmonella* sp. contamination cannot be completely eliminated from flocks.

As pointed out above, CE products are effective for prevention of poultry contamination by *Salmonella* sp., but they also reduce the level of colonization by several other important species of bacteria. *C. jejuni* is second in importance after *S. enterica* as an infectious agent acquired from poultry, and the prevalence of campylobacteriosis from poultry may be higher than salmonellosis, but the less severe disease goes underreported [19]. Broilact™ was used against *C. jejuni* with oral challenge. Protection of several different groups of Broilact™-treated chickens was reported to range from 0 to 62%, *i. e*. a 38 to 100% reduction in numbers of *C. jejuni*-colonized birds [20]. Using avian isolates of *Lactobacillus acidophilus* and *Enterococcus faecium*, as specific CE cultures against *C. jejuni*, a 70% reduction in fecal shedding could be achieved; only 27% reduction in colonization was reported [21]. A combination of one commercial CE product (Broilact™) and another preparation of mucosal bacteria (K-bacteria™) were tested for CE activity against *Campylobacter* sp. colonization. The CE product alone failed to protect chickens from *Campylobacter* spp. Combination therapy of Broilact™ and K-bacteria™ reduced, but did not eliminate *Campylobacter* colonization of the chicks [19].

Protection against other food-borne pathogens, such as *Listeria monocytogenes,* and *E. coli* by CE products has also been demonstrated. Preempt™ was used to prevent *L. monocytogenes* colonization of the cecae in Leghorn chickens by application before oral challenge with the pathogen. Efficacy was 100% in a flock of 50 chicks (25 tested and 25 used as controls) [22]. Although some strains of *E. coli* are pathogenic for humans, and poultry is a minor source, CE products do provide some protection of chickens from colonization by exogenous *E. coli* isolates, as observed in a study using fecal cultures as CE products in broilers and layers [23]. Some protection from colonization by *E. coli* was seen in both types of chickens, but the layers responded better than broilers. In another study using Aviguard™, colonization of chickens by *E. coli* O78:K80, a poultry pathogen, was reduced 14 days after challenge [24].

Although CE products are mainly used to prevent contamination of chickens by human pathogens, they can be used to protect the chickens from avian pathogens. Necrotic arteritis by *Clostridium perfringens* in broiler chicks was treated with non-antibiotic feed additives and CE cultures. A CE culture containing lactic acid bacteria protected the chickens, while undefined CE and other feed additives did not [25].

Although CE has been applied mostly to chicken production, other poultry uses have been reported for CE products. Aviguard™, a commercial CE product sprayed on hatching turkey eggs prevented colonization from a *Salmonella* sp. challenge [26]. Antibiotic-resistant *E. coli* are more prevalent in poults from young turkey hens than old hens, showing that environment affects colonization. Antibiotics are fed to turkeys to reduce *E. coli* colonization, as it causes potentially lethal immunological stress in poults. This problem makes a case for CE use in turkey production for both contaminant and disease prevention [27]. Aviguard™ and a pure culture of *L. acidophilus* (Avi-Pac™) were compared with fresh and 24-h-old turkey cecal contents for CE efficacy [28]. Fresh cecal contents were superior to the commercial CE products (of chicken origin), which were superior to the commercial *L. acidophilus* culture in preventing colonization of turkeys with *Salmonella* sp. [28]. Even unconventional poultry, like pheasants, have benefited from CE applications. A commercial chicken cecal-derived CE product reduced *Salmonella* colonization and reduced mortality of pheasant chicks [29].

Throughout this section, some studies have shown promising results for complete effectiveness of CE products. Most of the studies listed in Table 2 show that CE as currently used, is only partially effective against human pathogen contaminants in poultry. The use of antimicrobials in poultry production probably cannot be replaced by CE until more effective CE products are developed.

3 Factors affecting the efficacy of CE products

To increase the use of CE as an alternative to antimicrobial drugs, optimization of efficacy must be achieved. Optimization of CE efficacy requires attention to a number of factors, such as the number and composition of bacteria present in a product and how stable it is in storage, at application, and the degree of colonization by CE bacteria in the birds after treatment. Different preparations, from direct feeding of feces to association with specific, characterized microbial isolates have been evaluated as CE products. Complex mixtures containing a diversity of bacteria are more effective, and have longer shelf lives than single species or simple defined bacterial mixtures for protection of chicks from intestinal colonization by *S. enterica* [1, 28]. Fresh cultures are considered optimal for CE products, but the source of bacteria from the gastrointestinal tract is also important. For example, lyophilized cecum contents were better than fresh fecal cultures for CE orally applied to chickens [30].

Table 2. Cumulative reports of competitive exclusion efficacy *in vivo*

Animal species	CE type (Brand name)	Target species	Reported result	Reference
Broiler chicken	Cecal culture	*Salmonella*	6 $\log_{10}$ reduced intestinal *Salmonella*	[88]
Chicken	Feces or cecal rinses	*Salmonella*	25 – 70% reduced colonization	[30]
Broiler chicken	(Preempt™)	*Salmonella*	64% reduced colonization	[36]
Chicken	Undefined	*Salmonella*	7 $\log_{10}$ reduced intestinal *Salmonella*	[45]
Broiler chicken	(MSC™), (Aviguard™), (Avifree™) comparison	*Salmonella*	39%, 28%, 0% reduced colonization	[46]
Chicken	(Broilact)	*Salmonella*	Prevented colonization	[43]
Chicken oral challenge	(Aviguard™) + enrofloxacin	*Salmonella*	40% reduced colonization	[14]
Laying chicken	(Aviguard™)	*Salmonella*	9–fold reduced colonization	[15]
Broiler chicken	Undefined	*Salmonella*	34% reduced colonization	[16]
Broiler chicken	(Broilact™)	*Salmonella*	86% reduced colonization	[17]
Broiler chicken	(Preempt™)	*Salmonella*	90% reduced mortality	[18]
Chicken	Defined mixture[a)]	*Campylobacter*	62% reduced colonization	[31]
Broiler chicken	Cultured mucosal scrapings	*Campylobacter*	Reduced colonization	[32]
Broiler chicken	(Broilact + K-Bacteria™)	*Campylobacter*	Delayed infection 1.5 wk.	[19]
Broiler chicken	(Broilact™)	*Campylobacter*	38 – 100% reduced colonization	[20]
Broiler chicken	*L. acidophilus, Enterococcus faecium*	*Campylobacter*	27% reduced colonization 70% reduced fecal shedding	[21]
Chicken	(Preempt™)	*Listeria monocytogenes*	100% reduced colonization	[22]
Broiler and layer chicken	Fecal culture	*Escherichia coli*	30 – 100% reduced colonization	[23]
Broiler chicken	(Aviguard™)	*E. coli*	3 – 4 $\log_{10}$ reduction of intestinal *E. coli*	[24]
Broiler chicken	Lactic acid bacteria	*Clostridium perfringens*	30% reduced mortality	[25]
Turkey eggs at hatch	(Aviguard™)	*Salmonella*	Reduced environmental detection	[26]
Turkey	*L. acidophilus*, (Aviguard™), fresh cecal contents compared	*Salmonella*	Fresh cecal contents and (Aviguard™) reduced intestinal *Salmonella*	[28]
Pheasant	Cecal culture	*Salmonella*	3% reduced mortality	[29]

a) Mixture of the following species: *Citrobacter diversus, Klebsiella pneumoniae, Escherichia coli.*

The efficacy of CE products may depend on factors such as growth and storage conditions that include exposure to oxygen, storage temperature, and composition of culture medium components. In one experiment, comparison of aerobic and anaerobic growth of a defined culture of *Citrobacter diversus*, *Klebsiella pneumoniae*, and *E. coli* gave some level of protection to chicks from *Campylobacter* spp. colonization, but there was no difference between culture conditions [31]. In this case, none of the bacteria in the product were obligate anaerobes, but a product consisting of them would be expected to lose efficacy if stored aerobically. Long-term storage of CE products may also affect their capacity to competitively exclude. Storage at -80°C for 3 months significantly reduced the efficacy of an undefined culture that protected broilers from *Campylobacter* sp. challenge [32]. Product stability was an issue for CE products from early in the development of the technology. Manufacturers learned that fresh frozen cultures were more effective than lyophilized cultures.

Bacterial composition of CE products may vary from lot to lot. If strains of bacteria that contribute significantly to CE activity are reduced in number, the efficacy of the product can be diminished. Production of volatile fatty acids by lactic acid bacteria is a putative mechanism for CE activity. When hens are molting, there are reduced volatile fatty acids in their gastrointestinal tracts, which corresponds with increased susceptibility to *Salmonella* sp. colonization, a problem that can be controlled by addition of lactose to their drinking water [33]. These facts show that production of volatile fatty acids by CE bacteria may be important for their efficacy in preventing *S. enterica* and *Campylobacter* spp. colonization. Production of volatile fatty acids must be considered in the selection of bacteria for a defined CE product, and can be measured for quality control purposes during CE product manufacturing [34].

The method of application of a CE product is an important consideration for efficacy. The most effective technique for application is spraying on chicks within the first day after hatching with an aerosol of CE bacteria [35]. Application of a CE product in drinking water has also been shown to be effective against *Salmonella* sp. colonization of chicks [36]. In an attempt to get CE treatments to chicks earlier, in the protected environment of the shell, *in ovo* injection of the commercial CE product Broilact™ caused mortality and failed to provide a CE effect in chickens [37].

It was once thought that CE treatments might help accelerate weight gain in animals due to reduced stress from *Salmonella* sp. infections. However, CE products did not increase feed conversion, a measure of weight gain per unit of feed [38, 39]. In a similar context, antimicrobial compounds are often added to animal feed to accelerate and

enhance growth of the animals. Addition of CE products to feed with or without antibiotics does not enhance weight gain. CE products included with antibiotic supplements in broiler feed failed to provide enhancement in weight gain [38]. Another study used the feed additive, bacitracin with a CE treatment, and this did not improve feed conversion performance of broilers [39].

Addition of antibiotic treatment with CE treatment does not provide complete decontamination of mature birds, but can prevent colonization of chicks, as shown in a study with enrofloxacin [40]. The drug treatment followed by CE treatment reduced *Salmonella* sp. isolation from adult breeder chickens, but hatchlings were free of *Salmonella* sp. Another point to consider with combined antimicrobial drug and CE therapy is that the antimicrobials have an effect on the efficacy of the CE product. Antimicrobial drug residues in the cecum reduce the effectiveness of Preempt™ [41]. Using an assay for enrofloxacin, gentamicin, tetracycline, ceftiofur, and tylosin, Caldwell *et al*. [41] found that these drugs in low concentrations reduced the protection provided by the CE product. Further indications of enrofloxacin susceptibility by the same CE product have also been reported [42]. As CE products appear to interfere with feed conversion enhancement provided by antimicrobial feed additives, this is another factor that accounts for the low acceptance of CE technology as an alternative to antimicrobial drug use in the poultry industry.

The use of vaccine strains of *Salmonella* sp. that are avirulent have been a hopeful means of enhancing the efficacy of CE products for preventing poultry contamination with the pathogen. A CE product, Broilact™, was compared to attenuated vaccine strains; *Salmonella*-free spleens and livers were observed in the CE treated groups, but most vaccine-treated groups had pathogenic *Salmonella*-contaminated organs [43].

The numerous factors that can affect the activity of CE products need to be evaluated *in vitro* on defined mixtures of CE bacteria to find the most effective combination. An *in vitro* CE assay, using chicken intestinal cell cultures, defined microbial mixtures, and cellular invasion by *Salmonella* sp. was developed that can help in the assessment of potential CE products [44]. The study showed that reducing the number of *S. enterica* that invaded the tissue culture cells was proportional to the ability of the CE bacteria to competitively exclude the pathogen. This procedure can be used to test mixtures of defined bacteria with different attributes, such as increased organic acid production or enhanced intestinal cell adhesion for colonization resistance before they are tested as CE products *in vivo*. In this way, more effective, safer CE products can be developed.

4 Problems associated with CE products

4.1 Incomplete characterization of CE product bacteria

The composition of CE products appears to determine their effectiveness and presents the source of possible food safety risks associated with the technology. Defined CE products consist of a small number of strains or species of well-characterized bacteria that have been combined and grown in culture media and fed to the chicks [44]. Undefined CE products are fecal or intestinal material from adult birds that may have been cultured in growth media or taken directly from adult gastrointestinal tracts and fed to the chicks. Defined CE products are less effective and have shorter shelf lives than undefined CE products [1]. In a study comparing defined and undefined CE products, neither completely prevented *S. enterica* contamination of chicks [45]. Two different CE products [Mucosal Starter Culture™ (MSC™) and Aviguard™] were compared with a single species (*L. acidophilus*) product, Avifree™. Five days after challenge, the CE products, but not the single species product protected chickens from colonization by *S. enterica* [46]. The latter results verify the concept that complex CE products have greater efficacy than simple ones.

Undefined CE products are the most widely used, because of their demonstrated functional superiority [28]. The undefined status of the products is a problem for government regulators, who must insist on complete definition of drugs, even CE products, by virtue of their claimed health effects. In 1994, a World Health Organization Workshop on Competitive Exclusion recommended that CE products be classified as "normal gut flora," instead of as drugs (WHO/CDS/VPH/94.134). This recommendation came when the possible risks of undefined CE products were not as apparent as they are now and it was important to establish a method to prevent *Salmonella* sp. contamination of poultry. Regulatory agencies decided to treat CE products as drugs. The United States Food and Drug Administration adopted a policy of requiring CE products to be approved as new animal drugs, because of claims that CE products affect the health of the treated animals. According to the Food, Drug and Cosmetic Act [21CFR 514 Part 514.1(b)], the constituents and their concentrations in CE products must be identified and characterized as safe and effective. The Council of the European Union established Regulation (EC) Number 1831/2003, as provided in European Council Directive 70/524/EEC to regulate live bacteria feed additives in livestock [47]. The dossiers required by the regulations must include information on microorganism identifications, their numbers in the subject product, their biological origin, histories of genetic modification, compliance with genetically modified organism directives, and absence of toxins, virulence factors, and production of antibiotics or antibiotic resis-

tance factors (http://europa.eu.int/comm/food/fs/sc/scan/outcome_en.html#Guidelines).

Compliance with government regulations of CE products will require complete definition of the products. This may be achieved by rigorous microbiological analysis of the bacterial components of the CE product, however, when CE products are made by culture of cecal bacteria, many of the bacteria in the product are difficult to detect and identify. The actual determination of what bacteria are present in a CE product is even more difficult because taxonomy of anaerobic bacteria is currently changing, as molecular techniques are replacing conventional methods of identification [7]. There are many unidentified species of microorganisms in the intestinal microbiota of poultry. Before the risks of CE products can be fairly evaluated, more studies need to be done that determine whether bacteria in CE products can be fully identified. The normal intestinal microbiota of warm-blooded animals consists of at least 400 species of bacteria and other microorganisms but only about 30 species account for most of the bulk of the intestinal population [48]. It is reasonable to conclude that CE products that are effective *in vivo* consist of the major protective bacteria, even though they contain a limited number of species. This aspect of CE products makes it reasonable to expect thorough definition of the constituent microbes.

The compositions of most CE products have been characterized by conventional culture and biochemical identification techniques. These techniques have long been used to identify species of bacteria and can differentiate them to sub-species levels of taxonomy. Their drawbacks include inaccuracy for some species groups, like lactobacilli, and they require isolation of pure cultures. Molecular techniques are gaining favor as methods to detect and identify bacteria in complex mixtures like CE products. The variable region of the 16S rRNA gene of bacteria has been fortuitously exploited to differentiate anaerobic bacteria in CE products at the level of species identification [49]. In one study, 16S rRNA sequence analysis more accurately identified individual bacteria in a defined model CE mixture than phenotypic identification technologies [7]. Molecular techniques can identify bacteria that cannot be isolated in pure culture but are generally not capable of differentiating bacteria at sub-species levels of taxonomy. Using 16S rRNA gene cloning and sequence analysis techniques to identify uncultivable bacteria, a research group detected 243 sequences from 50 phylogenetic groups that were present in chicken cecal contents [49]. The *Clostridium leptum* group, *Sporomusa* group, *Clostridium coccidioides* group, and *Enterobacteriaceae* made up 89% of isolates. Since CE products are usually produced by large-scale culture techniques, and dominant species tend to overcome mixed cultures, the cultured products tend to have far fewer than 243 different kinds of bacteria in them. For example, Preempt™ consists of 29 isolates of chicken cecal bacteria, partially characterized mainly to the level of genus groups [50].

The poultry farm contains numerous microbial ecosystems, including the intestinal tracts of the birds, the poultry house floor litter, and the intestinal tracts of the human farm workers. The effects of applying CE products on the microbial ecology of those ecosystems have not been thoroughly investigated. The interspecies variability of 16S rRNA sequences can be exploited using DNA molecule separation tools for population analyses of poultry-derived CE products, and how they affect the establishment of intestinal microbiota in poultry flocks [51]. When DNA is isolated from a complex bacterial mixture, like a CE product, the 16S rRNA genes from each species of bacteria present can be amplified simultaneously by PCR. Several techniques to separate the individual PCR products have been applied to mixed bacterial populations. Treatment of the PCR products with restriction enzymes take advantage of terminal restriction fragment length polymorphisms (TRFLP) that can be observed by DNA sizing using slab or capillary gel electrophoresis [52]. These techniques are limited to use with those 16S rRNA sequence variations that alter a restriction enzyme recognition site. Separation techniques that can resolve single nucleotide sequence differences are needed to differentiate some homologous DNA sequences.

A PCR product separation technique that can theoretically distinguish single nucleotide sequence differences is denaturing gradient gel electrophoresis (DGGE), which separates the mixed DNA molecules by differences in nucleotide hydrogen bonding strengths [53]. A similar gel electrophoresis separation technique, temporal temperature gradient electrophoresis (TTGE) has also been used on mixed 16S rRNA gene PCR products to study the compositions of poultry intestinal microbiota [49]. These latter techniques do not require culturing and isolation of the bacteria from the CE product, so they can reliably detect the presence of all the bacteria species. These techniques are also beneficial in assuring constant quality control of the batch culturing usually employed in the manufacture of the CE products [53].

4.2 Antimicrobial resistance

In this review, the theoretical risk of CE products as a source of antimicrobial drug resistance is considered. Antimicrobial resistance traits can be transferred from innocuous species to opportunistic or frank pathogens, possibly becoming hazardous to the food animal or human consumer [5]. Some CE products may contain bacteria with antimicrobial drug resistance determinants that are transferable from the poultry microbiota to the intestinal microbiota of humans [54], which can cause infections in susceptible individuals [55].

Although CE products reduce the risk of poultry carrying *Salmonella* sp., they would be less beneficial if they passed drug resistance traits to other bacteria, such as vancomycin resistance in *E. faecium* to related species in the intestinal microbiota of the human consumer [56]. A better understanding of how genes are transferred between bacteria and how widely genes can be dispersed between taxonomically dissimilar recipients is needed to fully assess this risk. Conjugation between competent bacterial cells appears to be the prevalent mechanism for antimicrobial drug resistance transfer. The DNA molecules that are most often transferred are plasmids that may contain the genetic elements for transpositional integration (integrons) into the recipient cell chromosome [57]. An important concern addressed in the present review is what types of antimicrobial drug resistance may be contributed to the food supply as a result of CE product use in the poultry industry; a concern that is justified because possible transfer of antibiotic-resistant bacteria from food animals to humans has been reported [58, 59].

A natural component of CE products and the human gastrointestinal microbiota, *E. coli* is a suspect in antimicrobial drug resistance transfer. In a recent epidemiological study, antibiotic-resistant *E. coli* may have been transferred from poultry to workers who handled them [60]. Antimicrobial drug-resistant bacteria have been isolated from poultry litter, suggesting that poultry microbes are an environmental reservoir for antimicrobial drug resistance [61]. Streptothricin resistance gene transfer in *E. coli* has been offered as an example of food-borne transfer of antimicrobial drug resistance to human intestinal microbiota [62]. Transferable integrons containing resistance genes for ampicillin, chloramphenicol, cephalothin, gentamicin, tetracycline, trimethoprim, sulfamethoxazole, and streptomycin have been detected in *E. coli* isolates from agricultural animals [63]. Shiga toxin-producing *E. coli* are often found with one or more such integrons. The *E. coli* present in some CE products needs to be evaluated for the presence of transferable integrons containing antimicrobial drug resistance genes.

Enterococci are also present in CE products, and in poultry and human microbiota. Much controversy has surrounded reports that enterococci can transfer glycopeptide antimicrobial drug resistance to other bacteria [64]. Vancomycin-resistant enterococci (VRE) have become a major food safety issue [65]. VRE could be a human health hazard, because of their documented ability to transfer vancomycin resistance *in vitro* to the human pathogen *Staphylococcus aureus* [66]. Although epidemiological evidence suggests that use of the vancomycin-related drug, avoparcin in poultry agriculture selected for vanocmycin resistance and may have caused food-borne transfer of the resistant bacteria to human enteric bacteria [54], other results show that human VRE appear to come from therapeutic use of vancomycin in humans and that there may not be transfer of resistance from poultry [67]. Whether the *van*A vancomycin resistance gene was transferred from the poultry enterococci to human enteric enterococci was not determined. Further research needs to be done to determine if food-borne VRE can transfer *van*A to human enteric enterococci. CE products should be tested for the presence of vancomycin-resistant *Enterococcus* sp. and their capacities to transfer vancomycin resistance genes to other species of bacteria.

Conventional phenotypic techniques detect susceptibility of bacterial growth inhibition by antimicrobial drugs, but there are molecular techniques that detect the specific genes associated with drug resistance. Conventional phenotypic susceptibility testing can be useful to initially determine if some types of antimicrobial drug resistance that are known to be transferable are present in bacteria isolated from CE products. However, these techniques cannot directly determine if the resistance mechanisms are transferable to other bacteria. Detection of specific genes by PCR identifies the resistance mechanisms and can be used to further discern an association with transferability. These molecular detection techniques are limited in scope to characterized resistance mechanisms and require knowledge of DNA sequence information. The broad diversity of multiple antibiotic-resistant *E. coli* isolates from food animals has been evaluated by PFGE techniques [68]. These tools can be used to compare the constituents of CE products with the normal microbiota of a flock to assess whether the CE product may induce significant changes. Such assessments could be employed to provide custom CE products that would have far less potential to spread undesirable virulence and drug resistance genes into the food supply.

Conventional phenotypic detection of genetic transfer of antimicrobial drug resistance genes requires mating experiments to detect conjugative transfer of the resistance phenotype [67]. Mating experiments confirm the transferability of a resistance mechanism, but do not characterize the genes transferred or the exact transfer mechanism. Additional studies with molecular techniques that detect the resistance genes and conjugative regulatory DNA sequences are needed to fully assess transferability of resistance. An indication that transferable antimicrobial drug resistance genes may be present in bacteria is the presence of the integrase (*int*) genes that promote integration of conjugative transposons into recipient cell DNA. The class 1 and 2 *int* genes present on conjugative transposons in poultry intestinal bacteria can be detected with PCR and DNA probe hybridization assays [69, 70]. PCR techniques are also available for detection of specific antimicrobial resistance genes, such as gentamicin resistance genes [71], tetracycline resistance genes, and genes that encode drug efflux pumps [72].

4.3 Transfer of virulence genes

Like antimicrobial drug resistance, virulence gene transfer by bacteria in CE products to poultry is a theoretical risk to be considered. The challenge to agriculture from a food safety perspective is to use CE products to reduce the incidence of *S. enterica* and *Campylobacter* spp. contamination of poultry without introducing other food-borne pathogens into the human food chain in the process. New opportunities for bacteria to become pathogenic are occurring constantly and the problem of predicting where emerging pathogens will come from is far from resolved. Commensal bacteria may become pathogenic, especially for immunocompromised people, by acquiring virulence genes from other bacteria. For example, although *E. coli* is a typical member of the poultry intestinal microbiota and it is found in CE products [7], some strains can be potential human pathogens or carry transferable toxin genes that could amplify the threat from a gene-transfer recipient pathogen, like *S. enterica*.

New strains of pathogenic bacteria appear to be emerging in the environment by genetic transfer of virulence determinants. For example, RTX toxins *hly* A and *hly* B are present in some poultry isolates of *E. coli* [73]. These toxin genes could be horizontally transferred into other *E. coli* or transferred into opportunistic pathogens present in the CE product, the poultry intestinal microbiota, or the intestinal microbiota of the human poultry consumer. The *E. coli* present in CE products should not only be screened for human virulence, but also for virulence genes that could be augmented by other virulence genes transferred from other bacteria. Some CE product *E. coli* have been evaluated for the presence of virulence genes. For example, *E. coli* strains isolated from Aviguard™ were not resistant to inactivation by human sera and lacked adhesive characteristics for human cells [74]. They also lacked the *tsh* hemagglutinin gene, an essential virulence factor. The authors of the latter paper concluded that without *tsh*, the *E. coli* in Aviguard™ would not be harmful to humans if acquired from poultry. It is logical to conclude that the risk of a pathogen for virulence and transfer of virulence genes depends on the complement of virulence genes it bears. Its ability to acquire other virulence genes by horizontal transfer from other bacteria should also be considered. Further research will be needed to develop ways to assess gene transfer recipient status.

Although conjugative transposon-based mechanisms of gene transfer have attracted much recent attention, there are also other possible mechanisms of virulence gene transfer. An efficient fluoroquinolone-induced transduction (bacteriophage-mediated) mechanism for transfer of pathogenicity islands in *S. aureus* was recently described [75]. Pathogenicity islands are chromosomal DNA regions that contain clusters of virulence-related genes [76]. The pathogenicity islands of some strains of *Bacteroides fragilis* contain conjugative transposon sequences and appear to be transferable in a manner similar to antimicrobial resistance gene transfer [77]. The *B. fragilis* are already suspected of being vectors of tetracycline resistance transfer. Since some *B. fragilis* strains are components of CE products and other strains are normal intestinal bacteria in humans, their capacity to horizontally transfer both virulence and antimicrobial resistance genes is a matter of serious concern.

The enterococci are not only capable of transferring antimicrobial drug resistance; they also appear to be able to transfer virulence genes. The enterococcal surface protein gene (*esp*), which is found on a pathogenicity island, is readily transferred between *E. faecalis* and *E. faecium* chromosomes conjugatively and without a detectable plasmid intermediate [78]. This capacity to transfer virulence factors between species of emerging human pathogens is a matter of concern because multiple strains of these kinds of bacteria are constituents of CE products [7]. A recent report shows that antimicrobial resistance for vancomycin and β-lactam antibiotics have been disseminated along with pathogenicity islands between *E. faecalis* isolates worldwide [79].

Transferable DNA elements containing virulence genes and antimicrobial resistance genes have recently been found in plasmids isolated from *S. enterica* [80]. Transfer of virulence genes and antimicrobial drug resistance genes are not necessarily separate events, nor do they require separate genetic transfer DNA elements. Integrative conjugative elements (ICE) are DNA molecules that putatively carry virulence and antimicrobial drug resistance genes between bacteria during conjugation. An *E. coli* ICE from the ECOR31 strain has integrative functionality *in vitro* [76]. The authors of the latter paper suggest that the ECOR31 ICE may have been a progenitor for the high pathogenicity island of *Yersinia* spp. that carries virulence determinants.

The scope of genetic transfer of virulence and antimicrobial drug resistance genes between bacteria associated with animal food products is still not well appreciated or understood. *Lactococcus lactis*, a component of some CE products [7], is widely used in the dairy products industry and its genetics have been extensively studied. Plasmids in *L. lactis* containing antimicrobial resistance genes and genes for transposon mobilization bear sequences homologous to those found in *S. aureus*, *L. monocytogenes*, and *E. faecium* [81]. The *ycd* B gene (function unknown) of *L. lactis* appears to have been shared with strains of *E. coli*, *S. enterica* and *Shigella* spp. according to genomic DNA sequence analyses [82]. These latter reports show that intestinal bacteria can transfer virulence and antibiotic resistance genes to a variety of species, not just those that are closely related taxonomically.

The range of gene transfer donors and recipients appears to be large, but it is important to discern whether the likelihood of gene transfer occurring in the intestinal tract environment is great or small. A study of transfer kinetics in the intestinal tracts of mice showed that conjugative plasmid gene transfer occurs readily between bacterial cells that adhere closely together as a biofilm on mucous, but only initially upon contact [83]. Subsequent contact is obstructed by the lack of further contact of cells to others on the biofilm. This would suggest that the rate of transfer in the intestines is limited and may not be a serious problem. However, in another *in vivo* study, the rate of transfer of conjugative plasmids between *E. faecium* cells in chicken intestines was comparable to *in vitro* experiments [84]. In gnotobiotic rat intestines, antibiotic selection pressure increased tetracycline resistance gene transfer rates between *E. faecalis* strains [85]. There is sufficient evidence to be concerned about gene transfer occurring between bacteria in the intestinal tracts of poultry and people to justify investigations of whether CE products can be involved in this process.

Virulent bacteria present in CE products can be tested for directly in test animals with bioassays or serological tests, but such assays do not determine if the virulence genes can be transferred to other bacteria. Virulence assays will not detect genes in non-virulent bacteria that could enhance virulence in another bacterium that receives them. Transferable resistance genes are better detected with molecular techniques that detect the gene sequence and regulatory sequences associated with genetic transfer, usually by PCR. A real-time PCR (TaqMan) technique is available that detects a specific virulence gene in the *Salmonella* sp. pathogenicity island 2 [86]. Very sensitive DNA hybridization microarrays that detect inv A and spv B virulence genes from *Salmonella* sp. are also available [87]. As more virulence-related genes are recognized on transferable DNA elements in bacteria, they too will need to be detected in CE products.

5 Concluding remarks

There is adequate evidence that CE can be beneficial to poultry agriculture by reducing the carriage of human enteric pathogens. The most effective incompletely defined CE products, however, have drawbacks of unknown constituents that may bear transferable antimicrobial drug resistance and virulence genes to the microbiota of the human consumer. Technology now exists for thorough characterization of the bacteria present in CE products, allowing for their more complete definition. Completely defined CE products will provide assurance of safety and allow research into optimization of their efficacy. Knowledge of potential problems, like the presence of opportunistic pathogens, movable virulence factors and antibiotic resistance determinants, in the CE product component bacteria can be used to eliminate or monitor them during and after use of the products. The goal of CE product manufacture and use is a safer food supply, which must not be corrupted by the CE products themselves. With new insights emerging about the promiscuity of horizontal gene transfer between bacteria under culture conditions and in the poultry intestinal microbiota, the ecological impact of CE technology will need to be addressed. The ecological impact of administering a bacterial consortium taken from one poultry farm to animals on many poultry farms has not been adequately investigated. As these safety issues are addressed, and the efficacy of the products can be improved, a more useful food safety technology can result.

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