Original Article

Efficacy of competitive exclusion to reduce *Salmonella* in broiler chickens

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Abstract

Competitive exclusion (CE) relies on the activity of normal flora to limit intestinal colonization by enteric pathogens. This study aims to investigate the effects of CE on *Salmonella* infection in broilers. The protective effect of CE on *Salmonella* cecal colonization was evaluated in 1 day-old chicks. In the laboratory trial, CE products were administered by oral inoculation, drinking water and whole body spray. Three days after treatment, the chickens were challenged with 10^7cfu/mL of *Salmonella* Enteritidis orally, and were evaluated for 10 days. The CE-treated chickens showed comparable protection and the number of *Salmonella* in the cecal contents significantly decreased ($P < 0.05$) compared with those of the positive control. In the farm trial, CE was administered by whole body spray at the hatchery and the second was administered by drinking water and evaluated at 32 and 42 days, respectively. The CE significantly reduced *Salmonella* contamination in the farm trial and no *Salmonella* was detected in the cecal contents compared with the control group. Average body weight gains, feed conversion ratio and the performance index of the chickens were greater than the *control group*. Thus, the use of CE had a positive effect on broiler performance. Furthermore, the CE-treated chickens displayed greater intestinal histology including villous height, submuscular layer and cell mitosis. These studies demonstrate that CE was able to protect chickens from *Salmonella* cecal colonization and it is apparent that new methodologies associated with the development of a workable CE program are needed in the poultry industry.

Keywords: broiler chickens, competitive exclusion, *Salmonella*

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Introduction

Salmonellosis is the most common and widely distributed foodborne disease and is caused by *Salmonella* spp. infection. The main sources of *Salmonella* infection for humans are poultry, meat and eggs (Antunes *et al*., 2016). Finally, *Salmonella* accesses the food chain through producing contaminated food (Thiermann *et al*., 2011). Therefore, *Salmonella* control has become a major task in poultry production to ensure poultry food safety (Chambers and Gong, 2011). One of the most effective methods for prevention and control measures for *Salmonella* is to treat young chicks with competitive exclusion (CE) culture (OIE, 2018). CE has been used to describe the protective culture of the natural or native bacterial flora of the intestine in limiting the colonization of some bacterial pathogens. Nurmi and Rantala (1973) found that the addition of CE products derived from adult intestinal contents was administered to protect newly hatched chicks against subsequent infection by *Salmonella*. This control is able to reduce the levels of *Salmonella* contaminating poultry. The mechanisms of CE products inferring resistance to pathogens include the balance of micro-ecology in the intestine of the host; and antagonizing bacteria by generating antimicrobial metabolites or volatile organic acids at pH below 6.0 that limit the growth of *Salmonella* and *Enterobacteriaceae* (Stern *et al*., 2008). CE competitively occupies attachment sites of *Salmonella* intestinal colonization and competition with microbial pathogens for essential nutrients (Milbradt *et al*., 2014; Chantziaras *et al*., 2018)

Palmu and Camelin (1997) demonstrated that the reduction of *Salmonella* intestinal colonization was effective in CE controlling *Salmonella* as has been shown in significantly reduced contamination both on the farm and at the processing plant but did not improve in broiler performance. In contrast, Schneitz (2005) and Corrier *et al*. (1995) reported that chickens treated with CE ate more feed than groups treated differently and it showed a positive effect on the performance, together with lower mortality. The protective effect of CE against *Salmonella* colonization depends on treatment systems to ensure the viability, survival and establishment of protective flora in the intestinal ecosystem of newly hatched chicks (Corrier *et al*., 1994). In addition, it also depends on *Salmonella* status in the environment, the serotype of *Salmonella* and host factors. Recent studies have demonstrated that lactic acid bacteria (LAB), *Bacillus* spp. and yeast may be successful CE agents. The effect of LAB on pathogens has been demonstrated to reduce the viability of a pathogen by producing noxious substances, such as lactic acid that is reported to suppress the growth of *Salmonella* spp., *Salmonella* Typhimurium and *Shigella flexneri* (Rinkinen, 2004). Chung *et al*. (1989) reported that Reuterin secreted by *Lactobacillus reuteri*, has broad-spectrum antimicrobial activity extending to the inhibition of at least 25 different genera of prokaryotic microbial pathogens (both gram-negative and gram-positive) and to at least 10 different eukaryotic protozoan pathogens frequently found in the intestines of most mammalian and avian species. La Ragione and Woodward (2003) showed that *Bacillus subtilis* was an effective CE agent for use in poultry pathogens such as *Salmonella* Enteritidis, *Clostridium perfringens* and *Escherichia coli* serotype 078: K80. These studies have found that *B. subtilis* spores are able to germinate and grow in an anaerobic condition also, *B. subtilis* may be a CE approach by controlling infection with enteric pathogens (Guo *et al*., 2017). For yeast, *Saccharomyces boulardii*, contains mannose in their cell wall that binds the pathogens, *S.* Typhimurium and *Campylobacter jejuni* and then, passes out of the broiler chicks (Line *et al*., 1998). Thus, bacterial colonization is diminished by mannose in the cell walls of yeast. The purpose of this study was to evaluate the efficacy of CE products against *Salmonella* cecal colonization in the intestine of chickens, when administered with CE orally through drinking water and whole body spray at 1 day old both in laboratory and farm studies.

Materials and Methods

Competitive exclusion culture: The CE product was isolated from the ceca of *Salmonella* free *broiler-grandparent* chickens at the age of 30 weeks. Each cecum was put into Lactobacillus MRS agar within a plastic bag to blend in the stomacher Lab Blender containing 225 mL of normal saline. Serial dilution was performed and spread onto Viande Levure agar (VL) plates and incubated at 42°C for 48 hours. The CE product was characterized. There was a mixture of 3 microbial strains in the CE product including *Bacillus* spp., *Pediococcus acidolactici* and *Kodamaea ohmeri*. All microbes could inhibit the growth of *Salmonella*. All microbes in the CE product were lacking in any important intestinal pathogens including *Salmonella* and *Escherichia coli* using selective media. *Salmonella* inhibition assay *in vitro* was performed using well diffusion assay (Sgouras *et al*., 2004).

Salmonella culture: *Salmonella enterica* serovar Enteritidis was selected for resistance to novobiocin and nalidixic acid. *Salmonella* cultures were grown in tryptic soy broth (TSB) for approximately 18 hours with shaking at 100 rpm. The viable cell concentration of the inoculums was determined by colony count on xylose lysine deoxycholate agar (XLD agar) plates. *Salmonella* was stored separately and frozen at -80 °C in enrichment broth with glycerol (40%, w/v) until used.

Chickens and experimental designs: The study consisted of 2 trials in the laboratory and farm. For the laboratory, forty, one day old mixed sex chickens of COBB500 were randomly divided into 4 groups of 10 chicks to each: group 1 was a positive control group; group 2 was orally inoculated with 0.5 mL of CE products, containing approximately $1x10^7$cfu/mL, once a day for 3 days; group 3 was provided with CE products via drinking water at 1×10^7 cfu/mL for 3 days; group 4 was sprayed with CE products by whole body spray in a confined chicken shipping box at 1.75 mL/dose with a final concentration of 1×10^7 cfu/mL

over 1-3 day-old. Feed and water were provided *ad libitum* and the chicks were observed regularly. Three days after CE application, all groups were challenged orally with 1 × 10^7 cfu/mL of *Salmonella* for 3 days. At ten days of age, 10 chickens from each group were euthanized.

For farm studies, the experiment was carried out at commercial broiler chicken farms under an evaporative cooling system to reduce *Salmonella* using CE products. Due to the limitation of chicken houses, the experiments were performed in 2 trials. In the first trial 3,600 chicks were separated into 2 groups of 1,800 chicks each. Each group was divided into 32 replicates. Group 1 was a negative control and group 2 was treated with CE products by whole body spray at the hatchery. In the second trial 3,600 chicks were separated into 2 groups of 1,800 chicks each. Each group was divided into 32 replicates. Group 1 was a negative control; group 2 was treated once for 4 hours at day 1 with CE products at 1x10^9cfu/mL in drinking water. The chickens in the first and second trials were evaluated at 32 and 42 days-old, respectively. The evaluation included body weight (BW) change (total weight/number of chickens), feed conversion ratio (FCR) (cumulative feed intake (kg)/total weight gain (kg)), performance index (PI) (average live weight (g)/average feed intake (g)×100). *Salmonella* intestinal colonization and histopathological examination were determined at 26 days of age. Animal experiments were performed according to the guidance and legislative regulations on the use of animals for scientific purposes of Chulalongkorn University, Bangkok, Thailand with permission no. 1431101.

Detection of Salmonella in the experimental chickens: In each experiment, chickens were euthanized. Ceca were collected aseptically and evaluated for *Salmonella* colonization using serial dilution and *pour-plate* technique using XLD agar. The plates were incubated overnight, at 37 °C. Colony morphology was used to differentiate bacterial types. The typical colonies appeared black or black- centered with a yellow periphery after 18-24 hours of incubation. Upon continued incubation, the colonies became entirely black or pink to red with black centers. *Salmonella* inhibition (%) was calculated by dividing the number of *Salmonella* negative chickens with the total number of *Salmonella* challenged chickens.

Histopathological examination: The fixed specimens of duodenum from 5 birds per group were embedded in paraffin. Transverse sections were cut into 5 micrometers. After staining with hematoxylin-eosin, the following values were measured; villous height, thickness of the submuscular layer and cellular mitosis in the intestines of chickens using a bright-field microscope EX31analyzer (Olympus). Histopathological evaluation of this experiment was performed according to Samanya and Yamauchi (2002).

Chicken embryo cross- neutralization assays: To evaluate the negative impact of the CE products with the vaccine for the Newcastle disease *virus* (NDV) strain B1 (Merial, France), the cross-neutralization assay by 50% egg infectious dose (EID_{50}) method on 9 day-old embryonated eggs and values was performed for 4 replicates according to Yen *et al.* (2011).

Statistical analysis: For the laboratory trial, the differences between the average body weight of chicks in each group were analyzed by one-way analysis of variance and Duncan's multiple range tests. For the farm trial, the differences between the average body weight, feed conversion ratio and PI of chicks in each group were analyzed by independent T test. The Chi-square test was used to evaluate the differences in inhibition (%). Differences at $P < 0.05$ were considered as statistically significant.

Results

Bacterial isolates from the cecum of normal chickens were selected based on their ability to inhibit *Salmonella* growth *in vitro* and this demonstrated that a culture consisting of LAB, *Bacillus* and yeast was efficacious *in vivo*. This finding support the hypothesis of *Salmonella* reduction using CE product as previously described (Radovcic and Grozdanic, 2003; Schneitz, 2005; Milbradt *et al.*, 2017).

Laboratory trial of the effect of CE treatment on Salmonella infection: The experimental challenge dose of 10^7cfu/mL *Salmonella* resulted in cecal colonization in 100% of the 10 day-old positive control chickens (Table 1). Additionally, cecal of the control chickens contained 4.68 x 10^{10} cfu/mL of *Salmonella* per gram of content. In contrast, all CE treated groups revealed that the *Salmonella* colony count was significantly lower than the positive control ($P < 0.05$). Specifically, the CE orally treated group was significantly lower in the *Salmonella* colony count ($P < 0.05$) compared to the others. For the percentage of *Salmonella* inhibition, the oral CE application group had the highest percentage (60%) compared to the others. On the other hand, positive control group showed 0% *Salmonella* inhibition. The number of the *Salmonella* population in the cecal contents of the body spray group was lower than in the drinking water group. This result, according to the CE products administrated by body spray, resulted in better protection against *Salmonella* colonization compared with drinking water administration. Several reports revealed that the spray application of CE products on chickens at the hatchery was advantageous over drinking water treatment because it ensured early exposure to protective CE flora before environmental *Salmonella* challenge in the rearing house (Blankenship *et al.*, 1993). The initial average BW of chickens was no different between groups (data not shown). The average BW and FCR from the laboratory trial at 10 days of age is presented in Table 2. After challenge, the chickens in the positive control group had the lowest BW compared to the other groups ($P < 0.05$).

Farm trial of the effect of CE administration on Salmonella contamination: At the 26 days of age, the control groups of the first and second trials detected *Salmonella* 10- 20% from the cecal contents. But no *Salmonella* were detected in the cecal contents of the

chickens treated with CE products (data not shown). The performance of the CE- application groups measured in terms of BW, FCR and PI was better than that of the control groups with no significant differences (Table 3).

Table 1 Effect of competitive exclusion in each group on the number of *Salmonella* count (mean ± SD) and % *Salmonella* inhibition from the cecal contents of chicks at 10 days.

Groups	*Salmonella* (log10 cfu/mL)	*Salmonella* Inhibition (%) (n/N)
Positive control	10.67 ± 10.21^{a}	0^{a} (0/10)
Oral	3.41 ± 5.50^{c}	60^{b} (6/10)
Drinking water	7.69 ± 8.90^{b}	40^{b} (4/10)
Body spray	5.10 ± 11.21^{bc}	50^{b} (5/10)

Different superscripts in each column mean a significant difference ($P < 0.05$).

Table 2 Effect of different administration of CE products from the laboratory trial on the body weight (BW) and feed conversion ratio (FCR) in chickens at 10 days of age.

Groups	BW (Mean±SD) (g)	FCR
Positive control	135.37 ± 18.68^{c}	1.18
Oral	166.43 ± 13.47^{a}	1.06
Drinking water	150.70 ± 16.28^{b}	1.09
Body spray	156.41 ± 21.61^{b}	1.08

Different superscripts in each column mean a significant difference ($P < 0.05$).

Table 3 Effect of competitive exclusion treatment on broiler performance from the farm trial.

Groups	Age (d)	Broiler performance		
		BW(kg)	FCR	PI
		The first trial		
Control	32	1.67±0.10	1.71±0.08	282±46.17
Spray	32	1.80±0.11	1.69 ±0.08	298±31.97
		The second trial		
Control	42	2.37±0.06	1.81±0.01	308±8.50
Drinking water	42	2.45±0.09	1.76±0.02	325±8.18

Different superscripts in each column mean a significant difference ($P < 0.05$).

Intestinal villus height, submuscular layer and cell mitosis: The histological changes in the intestines of chickens is reported herein and provides new information regarding the potential for using CE-product in chickens. All parameters including intestinal villous height, thickness of the submuscular layer and cell mitosis numbers in the intestine of chickens in the CE-treated group were higher than the control group (Fig 1). The results show that cell mitosis of chickens in the CE-treated group was higher than the control group. This finding indicates that the absorptive function of the intestine may be enhanced by CE product.

Chicken embryo cross-neutralization assays: The infectiveness of NDV vaccine strain B1 was compared in embryonated chicken eggs (Table 4). The yield of virus in term of log10 EID_{50}/mL, was not significantly decreased at 0, 15, 30, 45 and 60 min after the mixing of CE products and NDV vaccine. It was found that the survival rate between the vaccine and CE group was 100% when compared with the control group. This result revealed that the CE products were compatible to ND *virus* vaccine strain B1, thus they could be applied in the hatchery by mixing them together. This is a new methodology associated with the development of a practical CE program in chicks.

Table 4 Determination of the effect of CE products on NDV strain B1 using 50% egg infectious dose (EID_{50}) method.

Time (mins)	Virus growth (Log_{10} EID_{50}/mL)	
	Virus control	*Virus* with CE
0	9.08 ± 8.80	8.96 ± 8.85
15	7.90 ± 7.87	8.55 ± 8.61
30	8.00 ± 7.91	8.98 ± 9.26
45	8.67 ± 8.40	8.48± 8.54
60	8.56 ± 0.00	8.0 ± 7.78

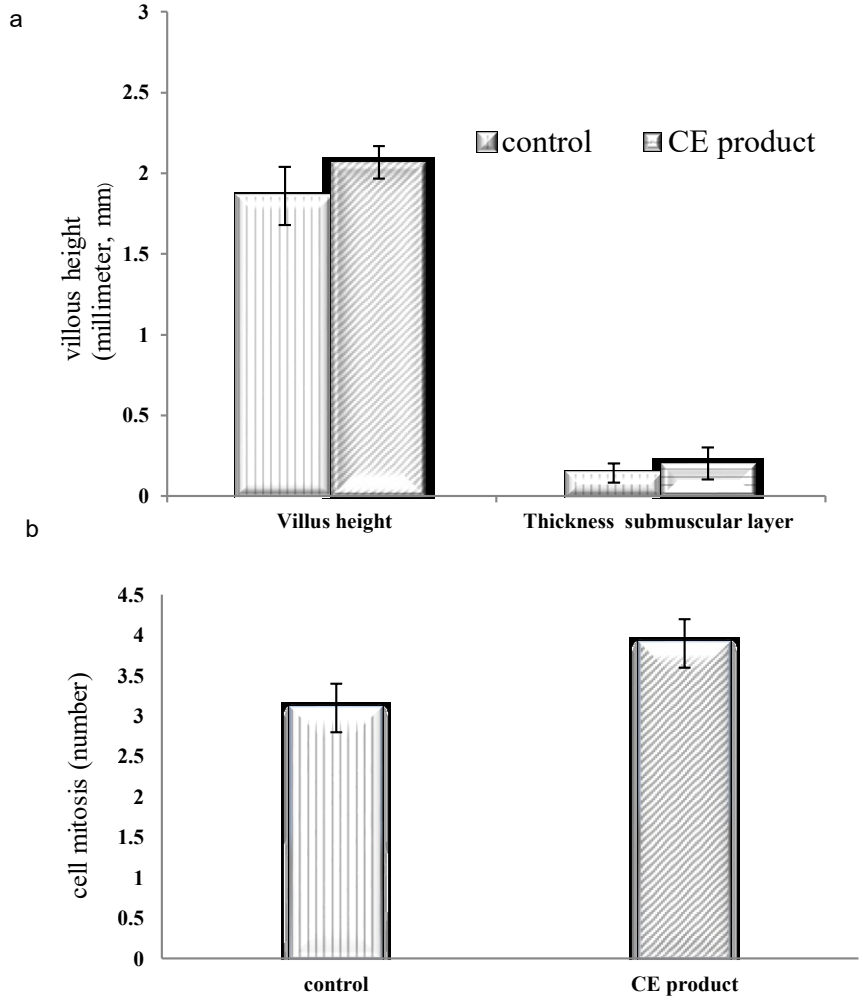

Figure 1 Intestinal villus height, thickness of submuscular layer and cell mitosis numbers in the intestines of chickens ($P > 0.05$) observed between the control and CE group.

Discussion

Salmonellosis is a major foodborne pathogen in human causing morbidity and mortality throughout the world (Kurtz *et al.*, 2017). *Salmonella* infections are mainly asymptomatic diseases in animals but are associated with infectious diseases of humans (OIE, 2016). Continuing interest in finding a way of preventing flocks with *Salmonella* infection and the contamination of poultry products is needed. In this study, the use of CE-products as a means of controlling infection or colonization of the gastrointestinal tract by *Salmonella* was performed. For the laboratory trial, statistical analysis revealed that at 10 days-old, the proportion of chickens with *Salmonella* infection in the CE-treated groups was lower than that of the control group ($P < 0.05$). This means that newly hatched chicks were protected against the establishment of *Salmonella* in the ceca when the CE-product was provided. The LAB species of CE-product can produce volatile fatty acids and lactate. Lactate will be converted in propionic acid, which has been shown to inhibit *Salmonella* colonization of the ceca and the crop of chicks (Nisbet *et al.*, 1994). According to Schneitz *et al.* (2016) revealed that an increase in the volatile fatty acid, especially that of propionic acid indicates colonization of strictly anaerobic bacteria in the ceca of CE product treated chicks. The effectiveness of CE-product has been shown to accelerate the development of normal microflora in chicks and poultry, providing increased resistance to infection by *Salmonella* and some enteric bacterial pathogens (Higgins *et al.*, 2007). In addition, CE products produce propionic acid when the level of cecal propionic acid increases and the level of *Salmonella* found in the digestive tract of the chicken decreases or is eliminated (Kubena *et al.*, 2001). In the current study, chickens were challenged with a pathogenic strain of *S.* Enteritidis and the result revealed that all chickens had been successfully infected. The infection was confirmed by determining the average bacterial count of 4.68×10^{10} *Salmonella* in 1 mL of cecum contents and also all 10 chicks showed positive infection of *Salmonella* in the control group. In this experiment, the CE-product was administered by oral inoculation, spraying and drinking water. Administration of CE-product via oral inoculation showed that *Salmonella* recovery from the cecum was significantly lowest. However, the administration of CE-product orally is impractical compared to spray application. The CE spraying group showed that *Salmonella* recovery in the cecum was

lower than the group that received the CE-product in the drinking water ($P > 0.05$). The administration of CE-product by spraying is more practical for using in the hatchery. For the field trial, both of the control groups revealed *Salmonella*-positive around 10-20% but in the CE-treated group, no *Salmonella* was found. According to previous reports the application of CE product or *Lactobacillus* and probiotics could induce gut epithelial cell proliferation and longer villi associated with activated cell mitosis (Ichikawa *et al.*, 1999). In addition, the longer villus may be induced by amylase secreted from *B. subtilis* (Dash *et al.*, 2015; Yuan *et al.*, 2017). However, amylase concentrations were not determined in the present study, and further experiments are needed to verify this effect. The efficacy of CE-product against *Salmonella* challenge in laboratory studies and against natural *Salmonella* infection during field studies has been demonstrated. Also, the CE-product in these studies has substantially protected young chicks from *Salmonella* colonization in both laboratory and field trials.

In conclusion, the CE product can protect broiler chickens from *Salmonella* infection and improve their overall performance. Greater histological analysis of the CE-treated intestine may be used to explain these results. In addition, simultaneous application of CE product and ND virus vaccine has no negative effect on each other. This new methodology will be a practical tool for application of CE product in the hatchery at the earliest age of life.

Acknowledgements

The authors would like to thank the staff of Department of Veterinary Medicine, Faculty of Veterinary Science, Chulalongkorn University for academic supports.

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