

Original Article

Antibacterial effects of carvacrol against *Staphylococcus aureus* and *Escherichia coli* O157:H7

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The objective of this study was to investigate the antimicrobial effects of carvacrol (CV) against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* O157:H7 (*E. coli* O157:H7) strains in milk. The minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of CV against *S. aureus* and *E. coli* O157:H7 were determined. In addition, bactericidal kinetics and antimicrobial activity of CV against the aforementioned pathogens in milk over a period of 2 weeks were investigated. CV exhibited antibacterial activity against both foodborne pathogens tested. The MIC and MBC of CV against *S. aureus* were 15.0 and 20 mg/mL, respectively, whereas those against *E. coli* O157:H7 were 16.0 and 32 mg/mL, respectively. In time-kill assays, CV at MBC reduced the number of *S. aureus* and *E. coli* O157:H7 in milk to undetectable levels within 24 hr. The antibacterial effects of CV persisted for 14 days without any loss of activity. Results of this study suggest that CV has a potential antibacterial activity against foodborne pathogens such as *S. aureus* and *E. coli* O157:H7 in milk.

Key words: foodborne disease, carvacrol, antibacterial activity, *Staphylococcus aureus*, *Escherichia coli* O157:H7

Introduction

With rapid globalization of food production and trade, many outbreaks of foodborne diseases now take place on worldwide dimensions. Foodborne disease resulting from consumption of food contaminated with pathogenic bacteria and/or their toxins is a vital concern to public health [1].

In general, it is estimated that up to one-third of the population for industrialized countries suffer a foodborne illness each year [2]. In the United States, Centers for

Disease Control and Prevention (CDC) estimates that each year roughly 1 in 6 Americans (or 48 million people) get sick, 128,000 are hospitalized, and 3,000 die of foodborne diseases. Although the vast majority of cases are mild, a significant number of deaths do occur and the high levels of acute infections and chronic sequela lead to billions of dollars in medical costs and lost productivity [3]. According to the data of Ministry of Food and Drug Safety during 2006~2012 [4], the number of foodborne disease occurrences and patients was 2,137 cases and 54,386 persons, respectively. Pathogenic *Escherichia coli* (*E. coli*), Norovirus, *Staphylococcus aureus* (*S. aureus*), nontyphoidal *Salmonella* and *Vibrio parahaemolyticus* were the top five pathogens causing domestically acquired foodborne illnesses in Korea.

S. aureus is one of the major bacteria causing foodborne disease. The bacterium is a gram positive coccus bacterium that is a member of the Firmicutes, and is frequently found in the human respiratory tract and on the skin. Foodborne disease-associated strains often promote infections by producing potent protein toxins and expressing cell-surface proteins that bind and inactivate antibodies [5]. In addition, *E. coli* O157:H7 is an enterohemorrhagic serotype of the bacterium *E. coli* and a cause of foodborne illness, typically through consumption of contaminated food [6].

With the investigation of the bacterial safety for meats, fishery and vegetables in the local market by Korean Consumer Agency [7], *S. aureus* and *E. coli* were detected at 27.8 and 62.7% of 212 cases surveyed, respectively, and among them, 94.8% of *S. aureus* and 92.9% of *E. coli* were identified as antibiotic-resistant bacteria.

Around the world, the widespread use of antibiotics in food animal production systems has resulted in the emer-

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gence of antibiotic-resistant bacteria that can be transmitted to humans through the food chain [8]. Infection with antibiotic-resistant bacteria negatively impacts on public health, due to an increased incidence of treatment failure and severity of disease [9].

In previous studies, the antimicrobial properties of several plant-derived essential oils, and a variety of active components of these oils have been identified [10-14]. Carvacrol (CV) and thymol, the two main phenols that constitute about 78-85% of oregano oil, are mainly responsible for the antibacterial activity of the oil obtained from *Origanum vulgare* L. [15].

Generally recognized as a safe food additive, CV is used as a flavoring agent in several products [16, 17]. Moreover, this natural phytochemical has a broad-spectrum antimicrobial activity and has not induced resistance in gram-positive and gram-negative bacteria after prolonged exposure [18, 19].

Milk is a complex medium in which lipophilic proteins reduce the bioavailability due to the potential interaction with antimicrobial compounds [20]. Therefore, milk was chosen as an *in vitro* model for studying the antimicrobial potential of CV for controlling foodborne pathogens.

The present study was to determine the efficacy of CV for killing *S. aureus* and *E. coli* O157:H7 in milk. Specifically, the antimicrobial effect of CV was investigated on each of *S. aureus* and *E. coli* O157:H7 as serious bacteria causing foodborne disease.

Materials and Methods

Materials and milk samples

CV (99%) and gentamicin sulfate were purchased from Sigma-Aldrich Korea Ltd. (Yongin, Korea). A solution was prepared by dissolving 1.0 g of CV in enough ethanol ($\geq 99.8\%$) to make 10 mL of stock solution and by dissolving 1.0 mg gentamicin sulfate in sufficient distilled water to make 10 mL of stock solution. Stock solutions were diluted with absolute ethanol prior to use. Fresh raw milk free from antibiotic residues was collected from the bulk tank at Gyeongsang National University dairy farm and autoclaved at 121°C for 15 min.

Bacterial strains and cultures

S. aureus (KVCC-BA11000330) and *E. coli* O157:H7 (KVC-BA0701446) originated from dairy cattle were obtained from the Korea Veterinary Culture Collection (KVCC, Anyang, Korea). All bacteriological media used in the study were purchased from Becton Dickinson Korea Ltd. (Seoul, Korea). The purity of each culture was ensured by characteristic morphology on mannitol salt agar (*S. aureus*) and sorbitol MacConkey agar (*E. coli* O157:H7). For the preparation of inocula, each bacterium was grown separately in 10 mL of tryptic soy broth

(TSB) for 24 hr at 37°C. The cells were then sedimented by centrifugation ($3,600 \times g$ for 15 min at 4°C), washed twice with sterile phosphate buffered saline (PBS) (pH 7.2), and resuspended in PBS. The populations of each bacterium were determined by plating 0.1-mL portions of appropriate dilutions on tryptic soy agar (TSA) plates and incubating the plates at 37°C for 24 hr. After incubation, the bacterial concentration was represented as colony forming unit (CFU)/mL.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of CV against each bacterial pathogen were determined by a broth dilution assay described by the previous study [21]. Milk tubes containing CV in the range of 0 to 50 mg/mL (2-fold dilution) were inoculated separately with each bacterial pathogen at 6.0 log CFU/mL and incubated at 37°C for 24 hr. Control samples included milk inoculated with each pathogen. Following incubation, the samples were serially diluted (1:10) in PBS and appropriate dilutions were spread on TSA plates. The plates were incubated at 37°C for 24 hr. The lowest concentration of antimicrobial treatment that inhibited visible growth of the pathogen after incubation was taken as MIC of the treatment. The lowest concentration of the treatment that prevented growth of the organism after subculture on TSA following serial dilution and plating was taken as MBC. Triplicate samples were included for each treatment, and the experiment was replicated three times.

Time-kill Assay

The bactericidal kinetics of CV were studied by inoculating sterile milk containing MIC, MBC, and $2 \times$ MBC of CV with each *S. aureus* and *E. coli* O157:H7 at 6.0 log CFU/mL. Negative and positive controls were inoculated aseptically with no added antimicrobial and supplemented with gentamicin sulfate at a dose of MBC, respectively. The samples were incubated at 37°C for 24 hr. Surviving populations of each bacterial pathogen were enumerated at 0, 6, 12, and 24 hr of incubation by plating 0.1-mL portions of the samples directly or after serial dilutions (1:10 in PBS) on duplicate TSA plates. Pathogens that were not detected by direct plating were tested for surviving bacteria by enriching 1 mL of the sample in 100 mL of TSB at 37°C for 24 hr. When growth was observed in the broth, the culture was streaked on TSA plates and observed for typical colonies of each pathogen. Each treatment was done in duplicate and the experiment was replicated three times.

Persistence of antimicrobial activity of CV in milk

Milk samples containing each of MBC or $2 \times$ MBC of

CV were inoculated with each of *S. aureus* and *E. coli* O157:H7 at 6.0 log CFU/mL and incubated at 37°C for 14 days. Inoculated milk samples containing no CV served as controls. The surviving bacteria were determined immediately after CV addition and at 24-hr intervals until day 14. To mimic the bacterial survival after CV addition, approximate 6.0 log CFU/mL of each *S. aureus* and *E. coli* O157:H7 were inoculated into the same milk samples every 48 hr until day 6 (day 2, 4, and 6) and bacterial populations were determined on TSA after 24 hr. Duplicate samples were inoculated and the experiment was replicated three times.

Statistical Analysis

The data were expressed as the means \pm standard deviation (S.D.) for the triplicate experiments. The significance between the control group and experimental groups was determined by Student's *t*-test. A difference at the level of $P < 0.05$ was considered to be statistically significant.

Results

MIC and MBC

MIC and MBC of CV against *S. aureus* and *E. coli* O157:H7 are provided in Table 1. Against *S. aureus*, MIC and MBC of CV were 15.0 and 20.0 mg/mL, respectively, and those of CV against *E. coli* O157:H7 were 16.0 and 32.0 mg/mL, respectively. However, MIC and MBC of gentamicin were both 0.25 μ g/mL against *S. au-*

reus and *E. coli* O157:H7. The average pH of milk with no addition of CV was slightly higher than that of milk with addition of CV.

Time-kill assay

The bactericidal kinetics of CV on the foodborne pathogens in milk are depicted in Fig. 1. The average initial bacterial population in all the treatment and control samples for the two foodborne pathogens was approximate 6.0 log CFU/mL. In the control samples, the bacterial population increased during a 24-hr incubation period. In the treatment samples containing CV at MIC (15.0 mg/mL), MBC (20.0 mg/mL) and $2 \times$ MBC (40.0 mg/mL), the number of *S. aureus* was significantly reduced at 24 ($P < 0.05$), 12 ($P < 0.05$) and 6 hr ($P < 0.001$) post-incubation, respectively, compared with that of control. The presence of CV at MBC and $2 \times$ MBC reduced the population of *S. aureus* to undetectable levels in 24 and 12 hr, respectively. In addition, the population of *E. coli* O157:H7 treated with MIC (16.0 mg/mL), MBC (32.0 mg/mL) and $2 \times$ MBC (64.0 mg/mL) of CV was significantly reduced at 24 ($P < 0.05$), 6 ($P < 0.05$) and 6 hr ($P < 0.001$) post-incubation, respectively, compared with that of control. With the CV at MBC and $2 \times$ MBC, the population of *E. coli* O157:H7 reduced to undetectable levels in 24 and 12 hr, respectively. With the results of this study, the concentration of CV at MBC completely inactivated each bacterial pathogen by 24 hr of incubation. As expected, for all bacteria, CV at MIC concentra-

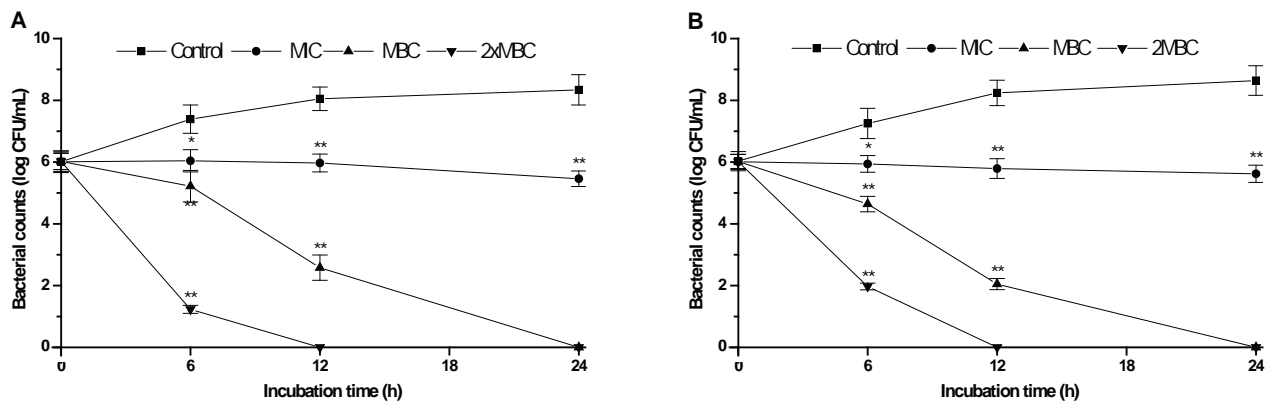


Fig. 1. Inactivation of *Staphylococcus aureus* (A) and *Escherichia coli* O157:H7 (B) in milk containing MIC, MBC, and $2 \times$ MBC of carvacrol. A: MIC=15 mg/mL, MBC=20 mg/mL, $2 \times$ MBC=40 mg/mL. B: MIC=16 mg/mL, MBC=32 mg/mL, $2 \times$ MBC=64 mg/mL.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of carvacrol against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* O157:H7 (*E. coli* O157:H7) isolated from clinical bovine mastitis cases

Compounds	MIC		MBC	
	<i>S. aureus</i>	<i>E. coli</i> O157:H7	<i>S. aureus</i>	<i>E. coli</i> O157:H7
Carvacrol (mg/mL)	15	16	20	32
Gentamicin (μ g/mL)	0.25	0.25	0.25	0.25

tion did not allow growth to occur.

Persistence of antimicrobial activity of CV in milk

The results from an experiment determining the persistence of antimicrobial activity of CV in milk are depicted in Fig. 2. *S. aureus* and *E. coli* O157:H7 (6.0 log CFU/mL) inoculated in the milk samples on day 0 were completely inactivated by each $2 \times$ MBC of CV after 24 hr. On day 3 and 6.0 log CFU/mL of *S. aureus* and *E. coli* O157:H7 reinoculated into the milk samples on day 2 were reduced to less than 1.0 log CFU/mL. On day 4, *S. aureus* and *E. coli* O157:H7 populations reinoculated at 6.0 log CFU/mL into the milk samples was reduced to less than 2.0 log CFU/mL after 24 hr. This trend in bacterial reduction was observed on the subsequent days, where 6.0 log CFU/mL of *S. aureus* and *E. coli* O157:H7 inoculated on day 6 were decreased to undetectable levels on day 12 of the experiment. A similar trend in *S. aureus* and *E. coli* O157:H7 inactivation was also observed in milk samples containing each MBC of CV, but the magnitude of killing was slightly less than that observed with each $2 \times$ MBC of CV. In control samples, all the population of *S. aureus* and *E. coli* O157:H7 increased to over than 7.0 log CFU/mL by 24 hr.

Discussion

Antibiotic administration is still widespread in many underdeveloped and developing countries and some antibiotics are accepted as global food sources. Additionally, it became more significant that the globalization of food consumption habits raises some foodborne pathogens in developed countries of which infection has not been reported before [23].

Since 1999, European Union banned antibiotic ad-

ministration for promoting the growth of farm animals. In many developed countries, antibiotic application is a question of concern. There is an awareness about the antibiotic-resistant bacteria and their effects on public health [24].

As the widespread use of antibiotics in food animal production systems has resulted in the emergence of antibiotic-resistant zoonotic bacteria that can be transmitted to humans through the food chain, the development of alternative strategies using compounds not subject to limitations associated with antibiotics is needed [9, 22]. In the present study, the data are presented for the efficacy of CV for killing foodborne pathogens *in vitro*.

In the previous studies, antibacterial activity of CV has been demonstrated against both gram-positive and gram-negative pathogens, including *Salmonella thyphimurium*, *E. coli* O157:H7, *Camphylobacter jejuni*, and *Listeria monocytogenes* [25-27]. Several studies have reported a decreased antimicrobial effect of plant extracts when used in foods or complex systems [28-30]. A higher concentration of plant-derived components is required to achieve the same antimicrobial activity in complex foods such as meat, fish, dairy products, and vegetables than in microbiological media [10, 31]. Similarly, the milk composition, especially fat level, reduces the antibacterial efficacy of components derived from plants [29]. In light of the above findings, MIC and MBC of CV in this study were determined against foodborne pathogens directly in milk rather than in any synthetic laboratory media.

The antibacterial mechanism of the action of CV with a hydrophobic property is the disruption of the lipid-containing bacterial cytoplasmic membranes, which increase its permeability and depolarizes its potential [32, 33]. Besides the interaction with cytoplasmic membranes, CV has been proposed to interact with membrane proteins

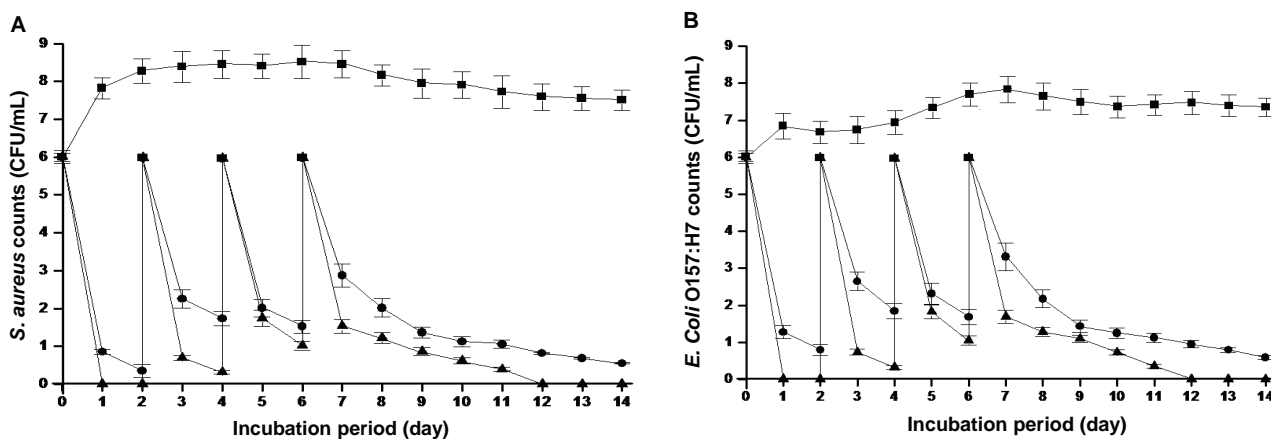


Fig. 2. Antimicrobial activity of carvacrol against *Staphylococcus aureus* (A) and *E. coli* O157:H7 in milk. A: ■=control, ●=MBC (20 mg/mL) of carvacrol, ▲= $2 \times$ MBC (40 mg/mL) of carvacrol. B: ■=control, ●=MBC (32 mg/mL) of carvacrol, ▲= $2 \times$ MBC (64 mg/mL) of carvacrol. 6.0 log CFU/mL of *Staphylococcus aureus* and *Escherichia coli* O157:H7 were reinoculated at 2, 4, and 6 hr post-incubation.

and periplasmic enzymes [34].

To be an effective antimicrobial agent as a therapy of foodborne disease under clinical conditions, CV should maintain its antimicrobial activity over an extended period. However, MIC and MBC experiments and time-kill assay determined the antimicrobial activity of CV in milk only up to 24 hr. Moreover, CV could be degraded or inactivated in milk over a period of time. Therefore, the persistence of antimicrobial activity of CV on *S. aureus* and *E. coli* O157:H7 was determined in milk for 14 days to confirm if CV could maintain antimicrobial activity over this period. *S. aureus* and *E. coli* O157:H7 were chosen for this experiment because these bacteria are a common cause of food poisoning [35].

Therefore, to investigate whether CV would maintain its effectiveness over a period of time in a milk environment, *S. aureus* and *E. coli* O157:H7 were reinoculated into milk samples on day 2, 4, and 6 of the experiment after the addition of CV on day 0. As observed in Fig. 2, the antimicrobial effect of CV persisted for the duration of the experiment without any loss of activity. It was also found that CV added to milk on day 0 was effective in killing large populations of *S. aureus* and *E. coli* O157:H7 inoculated multiple times on subsequent days.

Results from the present study suggest that CV may be useful as an alternative to antibiotics for the control of foodborne pathogens. Although CV may be applicable in controlling foodborne pathogens in milk, future experiments are needed to determine the antibacterial activity of CV in various foods.

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