Antimicrobial Effect of Kaempferol on Psychrotrophic *Bacillus cereus* Strains Outbreakable in Dairy Products

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Abstract

The objective of this study was to evaluate the antimicrobial effects of various natural flavonoids against growth of psychrotrophic *Bacillus cereus* strains, which cause dairy food outbreaks. Flavonoids were first screened for their ability to inhibit growth of *B. cereus* strains using the paper-disc diffusion test. Second, the growth inhibitory effect of selected flavonoids was evaluated in tryptic soy broth supplemented with 0.6% yeast extract, and the bactericidal effect of the flavonoids was measured in 0.8% (w/v) NaCl solution. Based on the paper-disc diffusion test, kaempferol was effectively active against *B. cereus* P14 and *B. cereus* KCCM 40935. Kaempferol had an antimicrobial effect at concentrations greater than 100 μ M, and the numbers of *B. cereus* P14 and *B. cereus* KCCM 40935 decreased by 3.55 and 1.5 log cycles, respectively. The cell numbers of *B. cereus* P14 and *B. cereus* KCCM 40935 treated with 50 μ M kaempferol were reduced by 4.18 and 2.84 log cycles during a 24 h incubation to test the bactericidal effect of kaempferol ($p < 0.05$). The results indicate that kaempferol had the greatest antimicrobial effect among the psychrotrophic *B. cereus* strains and the natural flavonoids tested.

Key words: psychrotrophic *Bacillus cereus*, kaempferol, flavonoid, antimicrobial effect, dairy food

Introduction

Bacillus cereus is a gram positive, aerobic, spore-forming microorganism and a common cause of food poisoning (Andersson *et al.*, 1995; Kramer *et al.*, 1989). This species, which is a common soil inhabitant, is often present in foods such as rice, spices, milk and dairy products, vegetables, meats, cakes and other desserts (Granum, 2001). In particular, *B. cereus* is a contaminant of raw milk (Lin, 1998) and also frequently isolated from a variety of dairy products. Some psychrotrophic strains of *B. cereus* are known to grow in foods at temperatures as low as 4-6°C (Dufrenne *et al.*, 1994; Griffiths *et al.*, 1990; Netten *et al.*, 1990). This growth represents a problem in chilled products such as milk and other dairy products, and in ready-to-eat or fresh chilled foods. Generally, raw milk and dairy products are contaminated by *B. cereus* from the soil and grass (Andersson *et al.*, 1995). The heat resistant spore-forming bacteria also pose a special prob-

lem for the food industry because it is not always possible to apply enough heat during food processing to kill the spores (Rowan *et al.*, 1998; Borge *et al.*, 2001).

Outbreaks of *B. cereus* are responsible for two types of food-borne illness such as the emetic and diarrheagenic syndromes (Granum *et al.*, 1997). A variety of different enterotoxins have been characterized for each syndrome including hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe) (Agata *et al.*, 1994; Agata *et al.*, 1995; Yokoyama *et al.*, 1999; Little *et al.*, 1994).

Many researchers have studied about the antimicrobial effects of natural compounds extracted from medical plants toward *B. cereus* strains (Palombo *et al.*, 2001). Flavonoids are natural organic compounds in fruits, flowers, stems, and roots. Flavonoids appear to have antimicrobial, anti-oxidative, anti-inflammatory, and anti-carcinogenic effects (Kim *et al.*, 2005; Lee *et al.*, 2006; Son *et al.*, 2003), and have played major roles in successful medical treatments in ancient times as well as the present. In addition, there are numerous studies on the functional effects of flavonoids as health foods and pharmaceuticals (Aviram and Fuhrman, 2002; Bosetti *et al.*, 2005; Joshipura *et al.*, 1999). Therefore, the objective of this study was to evaluate *in vitro* the inhibitory effects of flavonoid on the

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growth of psychrotrophic *B. cereus* strains outbreakable in dairy products.

Materials and Methods

Microorganisms

B. cereus P14 was obtained from Pulmuone, Inc. (Seoul, Korea) and *B. cereus* KCCM 40935 was purchased from the Korea Culture Center of Microorganisms (KCCM; Seoul, Korea). The stock cultures were stored at -70°C in tryptic soy broth (Difco Becton Dickinson, USA) with 15% glycerol and sub-cultured three times in 10 mL tryptic soy broth (Difco) supplemented with 0.6% yeast extract (TSB-YE) at 37°C for 12 h.

Flavonoids

Eight different flavonoids were used in this study; the flavonols quercetin, and kaempferol; the flavones apigenin, luteolin, and 5, 4-dihydroxy-7-methoxyflavone (genkwanin); and the flavanones naringenin, hesperetin, and hesperidin. All flavonoids were purchased from Sigma Chemical Co. (USA) (Kim and Paik, 2008). All flavonoids were dissolved in dimethyl sulfoxide (DMSO) at appropriate concentrations for this study.

Screening by paper disc diffusion method

The paper disc diffusion method was used as a screening test for the antimicrobial activities of various flavonoids. Forty microliters of each flavonoid sample were applied to a paper disc (8 mm in diameter). The paper discs were placed on tryptic soy agar plates inoculated with 10^6 CFU/mL of the *B. cereus* strain being tested. Discs were impregnated with 10 μ L of distilled water. The zone of inhibition was determined after incubation at 37°C for 16 h (Osawa *et al.*, 1992).

Measurement of antimicrobial effects

The viable cell count method was used to test the effect of flavonoid on growth of *B. cereus* (Negi *et al.*, 2002). The initial density of *B. cereus* was adjusted to 2.6×10^6 CFU/mL. One hundred microliters of *B. cereus* and 100 μ L of flavonoid solution were added to 10 mL of tryptic soy broth (Difco Laboratories, USA) supplemented with 0.6% yeast extract (TSB-YE) followed by incubation at 37°C. The flavonoid concentrations were adjusted to 25, 50, and 100 μ M in the final volume of broth. As a control, 100 μ L of DMSO was added to culture broth instead of flavonoid solution. The turbidities of the cell broths after incubation for 0, 2, 4, 6, and 8 h were measured at

620 nm with a spectrophotometer (Optizen 2120 UV, Korea). The culture broths were also serially diluted in 0.1% peptone water and spread on tryptic soy agar (Difco Laboratories). After incubation at 37°C for 20 h, the colonies were counted.

Bactericidal effects of flavonoids on *B. cereus*

B. cereus was cultured in TSB-YE to a cell density of 2.6×10^6 CFU/mL. The cell broths were then centrifuged at 10,000 g for 15 min at 4°C (Micro 17TR, Inchun, Korea). The cell sediments were washed in sterile 0.8% (w/v) NaCl solution and centrifuged at 10,000 g for 10 min at 4°C. After washing twice, the cells were resuspended 5 mL of 0.8% (w/v) NaCl solution and the cell density was adjusted to 2.6×10^6 CFU/mL. The flavonoid concentrations tested were 25, and 50 μ M in the final volume, and the suspensions were incubated for 0, 2, 4, 8, 12, and 24 h at 37°C in a shaking incubator (SI-900R, JEIO-TECH, Korea) at 170 rpm (Negi *et al.*, 2002).

Results and Discussion

Screening by paper disc diffusion method

Use of the paper disc diffusion method is a very popular and useful method for screening new chemicals with antibacterial activities (Hong and Lee, 2001; Vuuren, 2008). The results of the paper disc diffusion test for 8 different flavonoids tested against several *B. cereus* strains are shown in Table 1. Kaempferol strongly inhibited the growth of *B. cereus* KCCM 40935, however, quercetin did not show antimicrobial activity toward any strains used in this study. Of the flavones tested, only apigenin had antimicrobial effects toward *B. cereus* P14 and *B. cereus* KCCM 40935, however, its antimicrobial activity was lower than that of kaempferol. Also, antimicrobial effect of apigenin was not correlated with concentration. As a results, antimicrobial effect of flavonoids differ according to the hydrophobicity, size of a molecule, and the position of their side chains on the flavonoid backbone structure (Martini *et al.*, 2004).

Antimicrobial effects of kaempferol on *B. cereus* P14 and *B. cereus* KCCM 40935

Kaempferol was tested to evaluate antimicrobial effects on *B. cereus* P14 and *B. cereus* KCCM 40935. As shown in Fig. 1, 1% DMSO in culture broth without kaempferol had no effect on *B. cereus* P14 and *B. cereus* KCCM 40935. For *B. cereus* P14, kaempferol had greater inhibitory effects at 100 μ M, with decreases in cell number of

Table 1. Growth inhibition of *B. cereus* P14 and *B. cereus* KCCM 40935

Flavonoids	Concentration (mM)	<i>B. cereus</i> P14	<i>B. cereus</i> KCCM 40935
Kaempferol	2.5	— ¹⁾	—
	5	+	++
	10	++	+++
	20	++	++
Quercetin	2.5	—	—
	5	—	—
	10	—	—
	20	—	—
Luteolin	2.5	—	—
	5	—	—
	10	—	—
	20	—	—
Apigenin	2.5	—	++
	5	++	++
	10	++	—
	20	+	—
Genkwanin	2.5	—	—
	5	—	—
	10	—	—
	20	—	—
Naringenin	2.5	—	—
	5	—	—
	10	—	—
	20	+	—
Hesperetin	2.5	—	—
	5	—	—
	10	—	—
	20	—	—
Hesperidin	2.5	—	—
	5	—	—
	10	—	—
	20	—	—

¹⁾No inhibition (≤ 9 mm), +: slight inhibition (10–11.9 mm), ++: moderate inhibition (12–13.9 mm), +++: strong inhibition (≥ 14 mm).

3.5 log cycles during the incubation time for 8 h ($p < 0.05$). In case of *B. cereus* KCCM 40935, the numbers of cells treated with kaempferol were decreased with about 1.5 log cycles.

Bactericidal effects of kaempferol on *B. cereus* P14 and *B. cereus* KCCM 40935

The bactericidal effects of kaempferol at 25, and 50 μM concentrations against both *B. cereus* P14 and *B. cereus*

Fig. 1. Growth inhibition of kaempferol on *B. cereus* P14 and *B. cereus* KCCM 40935. The initial number of cells was adjusted to be 2.6×10^6 CFU/mL and the concentrations of flavonoids were adjusted to 25 μM (●), 50 μM (◆), 100 μM (□). The control (■) contained DMSO only. (A), *B. cereus* P14; (B), *B. cereus* KCCM 40935.

KCCM 40935 in 0.8% (w/v) NaCl solutions were tested. As shown in Fig. 2, it appeared that the numbers of survival cells were significantly reduced at 50 μM concentration of kaempferol. *B. cereus* P14 and *B. cereus* KCCM 40935 were decreased with 4.18 log cycles and 2.84 log cycles after 24 h, respectively ($p < 0.05$).

It is known that flavonoids affect the growth of many, but not all, microorganisms and their use for inhibiting microbial growth has two purposes: i) to kill prokaryotic or eukaryotic cells, or to inhibit their growth and ii) to decrease their spread and the effects of their toxins. Although the mechanisms by which this is accomplished are not yet known, it involves the inhibition of nucleic acid synthesis, cytoplasmic membrane function, and energy metabolism (Tim and Lamb, 2005). Based on the results of this study, kaempferol has a great antimicrobial effect on psychrotrophic *B. cereus* strains, however, the detailed mechanisms and kinetics of their antimicrobial effects are as yet unknown because the effect of flavonoids on infec-

Fig. 2. Bactericidal effect of kaempferol on *B. cereus* P14 and *B. cereus* KCCM 40935 in 0.8% NaCl solution. Initial cell density was adjusted to 2.6×10^6 CFU/mL. Flavonoid concentrations were adjusted to 25 μ M (●), 50 μ M (◆). The control (■) contained DMSO only. (A), *B. cereus* P14; (B), *B. cereus* KCCM 40935.

tious agents involves many potential targets in cells and is very complicated as mentioned above. Kaempferol exist in various plants such as tea, broccoli, propolis, and other plants source. It has an effect on anticancer and cytotoxic effects

Therefore, kaempferol shows as a potential natural preservative for the raw milk and dairy products. In the further work, the antimicrobial effect of kaempferol in food system and activity of flavonoids on the sporulation or germination of *B. cereus* strains in raw milk or dairy products should be studied.

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