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Antimicrobial activity of acid-hydrolyzed *Citrus unshiu* peel extract in milk

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ABSTRACT

Citrus fruit (*Citrus unshiu*) peels were extracted with hot water and then acid-hydrolyzed using hydrochloric acid. Antimicrobial activities of acid-hydrolyzed *Citrus unshiu* peel extract were evaluated against pathogenic bacteria, including *Bacillus cereus*, *Staphylococcus aureus*, and *Listeria monocytogenes*. Antilisterial effect was also determined by adding extracts at 1, 2, and 4% to whole, low-fat, and skim milk. The cell numbers of *B. cereus, Staph. aureus,* and *L. monocytogenes* cultures treated with acid-hydrolyzed extract for 12 h at 35°C were reduced from about 8 log cfu/mL to <1 log cfu/mL. *Bacillus cereus* was more sensitive to acid-hydrolyzed *Citrus unshiu* peel extract than were the other bacteria. The addition of 4% acid-hydrolyzed *Citrus unshiu* extracts to all types of milk inhibited the growth of *L. monocytogenes* within 1 d of storage at 4°C. The results indicated that *Citrus unshiu* peel extracts, after acid hydrolysis, effectively inhibited the growth of pathogenic bacteria. These findings indicate that acid hydrolysis of *Citrus unshiu* peel facilitates its use as a natural antimicrobial agent for food products.
Key words: antimicrobial effect, *Citrus unshiu* peel, acid hydrolysis, flavonoid, pathogen

INTRODUCTION

Citrus fruits in the food industry are principally used for the production of fresh juice or citrus-based drinks. As a result, citrus fruit peels are generated as waste, although they contain a high amount of flavonoids (Pfaltzgraff et al., 2013). The most abundant citrus flavonoids, generally known as the flavanones, include hesperidin, naringin, narirutin, and neohesperidin, and these compounds have been found to provide health benefits such as antioxidative, anticancer, antiinflammatory, and cardiovascular protective activities. Furthermore, the consumption of naringin and hesperidin reduced cholesterol levels in hamsters by 32 to 40% (Kurowska and Manthey, 2004).

Flavonoids, a large group of plant polyphenols, are present in plant tissues in relatively high concentrations either as sugar conjugates or as aglycones (Chinapongtitiwat et al., 2013). The glycosidic isoflavones are very poorly absorbed in the small intestine, compared with their aglycones, because of their high molecular weight and hydrophilic properties (Chang and Nair, 1995; Cha and Cho, 2001). Naringin and hesperidin, glycosidic forms of flavonoids, are less bioactive than their respective aglycones, naringenin and hesperetin (Mattila and Kumpulainen, 2002; Chinapongtitiwat et al., 2013). Phenolic acids bound to sugars are typically liberated using base hydrolysis, acid hydrolysis, or both (Hertog et al., 1992). This method can be applied to glycosides of flavonoids present in *Citrus unshiu* (satsuma orange) peel to improve their bioactivities.

Pathogenic bacteria such as *Bacillus cereus, Staphylococcus aureus*, and *Listeria monocytogenes* are public health concerns. In particular, *L. monocytogenes* has been implicated in several foodborne outbreaks associated with the consumption of pasteurized milk (Fleming et al., 1985). Although *L. monocytogenes* is destroyed by pasteurization, several studies have reported its heat resistance and its ability to survive refrigeration (Doyle et al., 1987; Kotzekidou and Giannakidis 2008). Its presence in raw and processed foods may not be avoidable. Therefore, the growth of such microorganisms that cause decay and transmutation in foods during processing and storage should be inhibited.

In recent years, interest has increased in using natural antimicrobial agents in food products. *Citrus unshiu* peel, generated as a waste product of juice processing and known to contain functional compounds, can be an excellent candidate as a natural antimicrobial agent. Currently, there are no reports on the antimicrobial activity of acid-hydrolyzed *Citrus unshiu* peel extracts against commonly occurring foodborne pathogens. The objective of this study was to evaluate the antimicrobial activities of acid-hydrolyzed *Citrus unshiu* peel extract against 3 important foodborne pathogens including *B.*

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cereus, S. aureus, and *L. monocytogenes*. In addition, the antilisterial effect of acid-hydrolyzed *Citrus unshiu* peel extract in sterilized milk was determined to evaluate its potential application as a natural preservative in foods.

MATERIALS AND METHODS

Materials

Mature *Citrus unshiu* fruits, harvested in October 2009, were obtained from Jeju Island in Korea. The peels were collected and freeze-dried for 48 h and then finely ground using a high-speed blender (7012S; Waring, Torrington, CT) and a 0.5-mm sieve. The *Citrus unshiu* peel powder was stored at 4°C until use.

Preparation of Acid-Hydrolyzed Citrus Unshiu Peel Extracts

Citrus unshiu peel powder (20 g) was mixed with 200 mL of water and extracted at 80°C for 3 h in a water bath (KSB201, Sunil Co., Seoul, Korea). After repeating the extraction steps 2 more times, the extract slurries were filtered through a Whatman No. 2 filter paper. The water was removed using a rotary evaporator (Eyela N-1000V; Tokyo Rikakikai Co., Tokyo, Japan) and the concentrated extracts were then freeze-dried. The dried extract (400 mg) was added to deionized water (25 mL) in a 100-mL round-bottomed flask. Twenty-five milliliters of 50% aqueous methanol was added and 10 mL of 2 *M* HCl was slowly added over 5 min. This mixture was stirred using a magnetic stirrer at 35°C for 16 h. After cooling, the mixture was filtered through a 0.45-μm filter (25 mm i.d., GD/X 25 nylon syringe filter; Whatman Inc., Piscataway, NJ). The solvent in the filtrate was evaporated using a rotary evaporator at 50°C. The extracts (18% yield from citrus peel powder) were freeze-dried and stored at −20°C until use.

Bacterial Strains and Culture Conditions

Bacillus cereus (KCCM 40935, KCCM 40154, KCCM 11341, KCCM 40133, and KFRI 181) and *S. aureus* (KCCM 32395, KCCM 11335, KCCM 16593, KCCM 40511, and KCCM 40512) were purchased from the Korea Culture Center of Microorganisms (KCCM, Seoul, Korea). *Listeria monocytogenes* (ATCC 15313, H7969 serotype 4b, H7962 serotype 4b, H7764 serotype 1/2a, and H7762 serotype 4b) were obtained from the Department of Food Science and Human Nutrition at Iowa State University (Ames) and from the American Type Culture Collection (ATCC, Manassas, VA).

Each strain was cultured in 10 mL of tryptic soy broth (**TSB**; Difco Laboratories, Detroit, MI) supplemented with 0.6% yeast extract (**TSB-YE**) at 35°C for 12 h and stored at 4°C as stock cultures. The strain was subcultured twice in TSB-YE for activation and used for further studies. For the experiments, 100-μL aliquots of the subcultures were inoculated into 10 mL of TSB-YE and incubated at 35°C for 6 h (mid-exponential growth phase). After incubation, 1 mL of each culture broth was transferred to sterilized tubes. Cells were harvested by centrifugation (model JA-14; International Equipment Co., Needham Heights, MA) for 10 min at 10,000 × *g* at 4°C. After centrifugation, the pellets were suspended in 0.1% peptone water (Difco) for use as culture suspensions. Two- and 3-strain suspensions of each pathogen were combined in equal proportions to obtain the cocktail mixtures (Zampini and Villena, 2012).

Determination of MIC

The MIC of acid-hydrolyzed *Citrus unshiu* peel extract against 3 bacterial strains (*B. cereus*, *S. aureus*, and *L. monocytogenes*) was determined by means of the broth microdilution method (Bebear and Robertson, 1996). The extract was dissolved and diluted in TSB. Serial 2-fold dilutions ranging from 1.25 to 20 mg/mL were prepared in sterile 96-well microplates. The inocula (100 μL) of each pathogen were added to individual wells. The cell population of the inoculum was adjusted to 5.0 log cfu/mL. After incubation at 35°C for 24 h, bacterial growth was assayed by measuring absorbance at 630 nm. Bacterial growth was indicated by the presence of turbidity and a pellet at the bottom of the well. All experiments were performed in triplicate.

Antimicrobial Activity Assay

The antimicrobial activity of acid-hydrolyzed *Citrus unshiu* peel extract was determined by preparing 0, 0.25, 0.5, 1.0, and 2.0% extracts (wt/vol) in TSB-YE broth for assessment of the bacteriostatic effects against the 3 pathogens. *Bacillus cereus* (KCCM 40154 and KCCM 11341), *S. aureus* (KCCM 32395 and KCCM 16593), and *L. monocytogenes (*KCCM 15313, H7969 serotype 4b, and H7962 serotype 4b) were grown in TSB-YE media for 12 h at 35°C. Cocktail mixtures (Zampini and Villena, 2012) containing 2- and 3-stain suspensions were prepared and the initial cell population of the inoculum was adjusted to 5.0 log cfu/mL. After inoculation, the broths containing acid-hydrolyzed *Citrus unshiu* peel extract at different concentrations were incubated at 35°C, and aliquots were withdrawn at 0, 2, 4, 8, 12, and 24 h. At each time point, the aliquots were

serially diluted with 0.1% sterile peptone water and the appropriate dilutions were plated onto TSB-YE agar plates. After 36 h of incubation at 35°C, the viable cells were counted.

Antilisterial Activity Assay

Acid-hydrolyzed *Citrus unshiu* peel extract was added to sterilized whole milk (3.5% fat), low-fat milk (1% fat), and skim milk purchased from a local market (Seoul, Korea) at concentrations of 0, 1, 2, and 4% (wt/vol) to study its bacteriostatic effect against *L. monocytogenes*. The concentration of extract was determined after antibacterial analysis. *Listeria monocytogenes* (KCCM 15313, H7969 serotype 4b, and H7962 serotype 4b) were grown in TSB-YE medium for 12 h at 35°C. The initial inoculum population in milk was adjusted to 5.0 log cfu/mL. Inoculated milk containing acid-hydrolyzed *Citrus unshiu* peel extract was incubated at 4°C and aliquots were withdrawn at 0, 1, 2, 3, 5, 7, 9, 13, 17, and 21 d. At each time, milk aliquots were serially diluted with 0.1% sterile peptone water and the appropriate dilution was plated onto TSB-YE agar plates. After incubation at 35°C for 24 h, the viable cells were counted.

Statistical Analysis

Each experiment was independently repeated at least 2 times. The numbers of cells were converted to log cfu/mL and treatments were assigned for comparison. Analysis of variance was performed on cell numbers using SPSS software (version 8.0; SPSS Inc., Chicago, IL). The significance level of differences was defined as $P < 0.05$.

RESULTS AND DISCUSSION

MIC for Bacteria

The analysis of the MIC of acid-hydrolyzed *Citrus unshiu* peel extract against the 3 pathogens showed that the extract had a MIC of 10 mg/mL against *B. cereus* (KCCM 40154, KCCM 11341), *S. aureus* (KCCM 32395 and KCCM 16593), and *L. monocytogenes* (ATCC 15313, H7969, H7962 serotype 4b; Table 1). The MIC is the lowest concentration of an antimicrobial agent that inhibits the growth of a particular microorganism (Bebear and Robertson, 1996). Acid-hydrolyzed *Citrus unshiu* peel extract inhibited various strains of *B. cereus*, *S. aureus*, and *L. monocytogenes* at 10 to 20 mg/mL (Table 1). The antimicrobial effect of citrus essential oil has been previously shown to be effective against *Campylobacter jejuni*, *Escherichia coli* O157, *L. monocytogenes*, *B. cereus*, and *S. aureus* (Fisher and Phillips, 2006). In addition, Lin and Sheu (2010) reported that *S. aureus* was more resistant to citrus essential oil than were *L. monocytogenes* and *B. cereus*.

Table 1. Minimum inhibitory concentrations of acid-hydrolyzed *Citrus unshiu* peel extracts

Strain	*Citrus unshiu* peel extract (mg/mL)
Bacillus cereus KCCM 40935	20
B. cereus KCCM 40154	10
B. cereus KCCM 11341	10
B. cereus KFRI 181	20
B. cereus KCCM 40133	20
Staphylococcus aureus KCCM 32395	10
S. aureus KCCM 11335	20
S. aureus KCCM 16593	10
S. aureus KCCM 40511	20
S. aureus KCCM 40512	20
Listeria monocytogenes ATCC 15313	10
L. monocytogenes H7969 serotype 4b	10
L. monocytogenes H7962 serotype 4b	10
L. monocytogenes H7764 serotype 1/2a	20
L. monocytogenes H7762 serotype 4b	20

In our previous study, *Citrus unshiu* peel extract that had not been subjected to acid hydrolysis did not exhibit any antimicrobial activity (data not shown). The extract without acid hydrolysis contained flavonoids as glycosidic forms that linked with sugars (Parvathy et al., 2009). In fact, the glycosidic forms of flavonoids might have decreased the effectiveness against pathogenic bacteria compared with the corresponding aglycones. Hesperetin and naringenin, which are flavonoid glycosides and the main polyphenol compounds in *Citrus unshiu* peel, exhibited increased antimicrobial activity when converted to hesperidin and naringin, which are flavonoid aglycones (Karamanoli, 2002). Taking into account these reasons and regarding our data, acid hydrolysis of *Citrus unshiu* peel extract liberated sugars from flavonoid glycosides and improved the antibacterial activities of the *Citrus unshiu* peel extract against *B. cereus*, *S. aureus*, and *L. monocytogenes*.

Antimicrobial Activities

The cell populations of *B. cereus, S. aureus,* and *L. monocytogenes* in medium without acid-hydrolyzed *Citrus unshiu* peel extract (0%, control) gradually increased upon incubation for 8 h (Figure 1). As the concentration of acid-hydrolyzed *Citrus unshiu* peel extract was increased from 0% to 0.25, 0.5, 1, and 2%, the cell populations of all 3 strains decreased. However, the growth of the bacterial strains was not inhibited significantly ($P > 0.05$) by addition of 0.25% *Citrus unshiu* peel extract, compared with the control culture without the extract. From the data plotted in Figure

1a, no viable cells of *B. cereus* were detected in medium containing 1% or 2% *Citrus unshiu* peel extract after 2 h of incubation at 35°C. The growth of *S. aureus* was significantly inhibited by the addition of 2% *Citrus unshiu* peel extract. In medium containing 1% extract, the cell numbers of *S. aureus* were <1.0 log cfu/mL after 24 h, indicating complete abrogation of cells. Different behavior was detected in the cell population of *L. monocytogenes*, counts of which gradually decreased in medium containing 2% extract during incubation, and the growth of cells was restricted after 12 h. It is worthwhile to note that the cell populations of the 3 bacteria, *B. cereus, S. aureus,* and *L. monocytogenes*, were inhibited in a manner dependent on the concentration of acid-hydrolyzed *Citrus unshiu* peel extract.

Moreover, a clear difference in cell tolerance against *Citrus unshiu* peel extract was observed at each concentration. In the case of medium with 1% acid-hydrolyzed *Citrus unshiu* peel extract, the order of cell tolerance to *Citrus unshiu* peel extract was *L. monocytogenes* > *S. aureus* > *B. cereus*, indicating that *L. monocytogenes* was more resistant than *S. aureus* and *B. cereus*. Fisher and Phillips (2006) reported that *S. aureus* was more resistant than *L. monocytogenes* and *B. cereus* to citrus essential oil. The results of the current study are in contrast to our previous study, in which the addition of 1% acid-hydrolyzed *Citrus unshiu* peel extract did not reduce the cell populations of *Escherichia coli* and *Salmonella enteritidis* (data not shown). Generally, gram-positive bacteria seem more susceptible to phenolic compounds in citrus oils and mandarin (*Citrus reticulate*) peel extracts than gram-negative bacteria, which may be due to the relative impermeability of the outer membrane that surrounds gram-negative bacteria (Fisher and Phillips, 2006; Yi and Yu, 2008). Our results show that acid-hydrolyzed *Citrus unshiu* peel extracts exhibited better inhibitory activity against gram-positive pathogens, and that the strength of this inhibition depended on the specific bacterium.

The antimicrobial activities of plant extracts were attributed to the different phenolic compounds present in these extracts (Negi, 2012). The mechanism of the antimicrobial action of phenolic compounds is not fully understood. Likely, the activity is caused by the combined effects of adsorption of phenolic compounds to bacterial membranes that disrupts the membrane, followed by leakage of cellular contents, formation of hydroperoxide, and metal chelation by phenolics (Otake et al., 1991; Ikigai et al., 1993; Akagawa et al., 2003). Hesperidin and naringin, which are the major flavonoid compounds in citrus fruits, are effective antimicrobial agents (Yi and Yu, 2008), and acid hydrolysis of *Citrus unshiu* peel extract enhanced the antimicrobial activity by increasing the free phenolic forms of hesperidin and naringin.

Antilisterial Activity in Milk

Significant antilisterial activity of acid-hydrolyzed *Citrus unshiu* peel extract was observed against *L. monocytogenes* in different types of milk (Figure 2). The initial population of *L. monocytogenes* in whole, low-fat, and skim milk was 5.1 log cfu/mL. The cell numbers in milk without acid-hydrolyzed *Citrus unshiu* peel extract (0%, control) gradually increased during storage at 4°C and reached a maximum of 8.5 log cfu/mL after 21 d. The addition of acid-hydrolyzed *Citrus unshiu* peel extract at 1% inhibited the growth of *L. monocytogenes*. After 17 d, the cell numbers of whole, low-fat, and skim milk with 1% extract were reduced from 8.4 log cfu/mL (0%) to 1.9, 3.7, and 3.0 log cfu/mL, respectively. After 21 d, the growth of cells was totally inhibited in extract-containing milk. The addition of acid-hydrolyzed *Citrus unshiu* peel extract at 2% and 4% reduced the cell numbers to <1.0 log cfu/mL after 5 and 1 d of storage, respectively. The cell growth of *L. monocytogenes* was clearly inhibited by 2 and 4% extract in all types of milk. Yan et al. (2011) reported that green onion extract at concentrations of 2, 5, and 8% revealed a significant antilisterial effect against *L. monocytogenes* cocktail in retail full-fat milk, fat-free milk, and milk fortified with nano-calcium.

Although we observed no significant differences in the reduction of cell numbers between the types of milk containing acid-hydrolyzed *Citrus unshiu* peel extract, *L. monocytogenes* in whole milk with 1% extract tended to be less resistant than that in low-fat milk and skim milk with 1% extract. It has been reported that antibacterial efficacy varies between different carrier materials (Fisher et al., 2007). Bacteria are generally more resistant to antimicrobial agents in suspensions with heavy organic loads (Lin and Sheu, 2010). However, the high fat content of whole milk did not support bacterial growth when a higher concentration of the *Citrus unshiu* peel extract was used in this study, suggesting that the antimicrobial activity of the extract could overcome the heavier organic load in whole milk. Owen and Palombo (2007) investigated the ability of medicinal plant (*Eremophila duttonii* and *Eremophila alternifolia*) extracts to control the growth of *L. monocytogenes* in different types of foods, including whole milk, skim milk, diluted homogenates of salami, and pâté and Brie cheese. Their results showed that the extracts inhibited the growth of *L. monocytogenes* only in salami at 37°C and that the *E. duttonii* extract was effective in protecting pâté and Brie cheese stored at 4°C. Therefore, the growth of

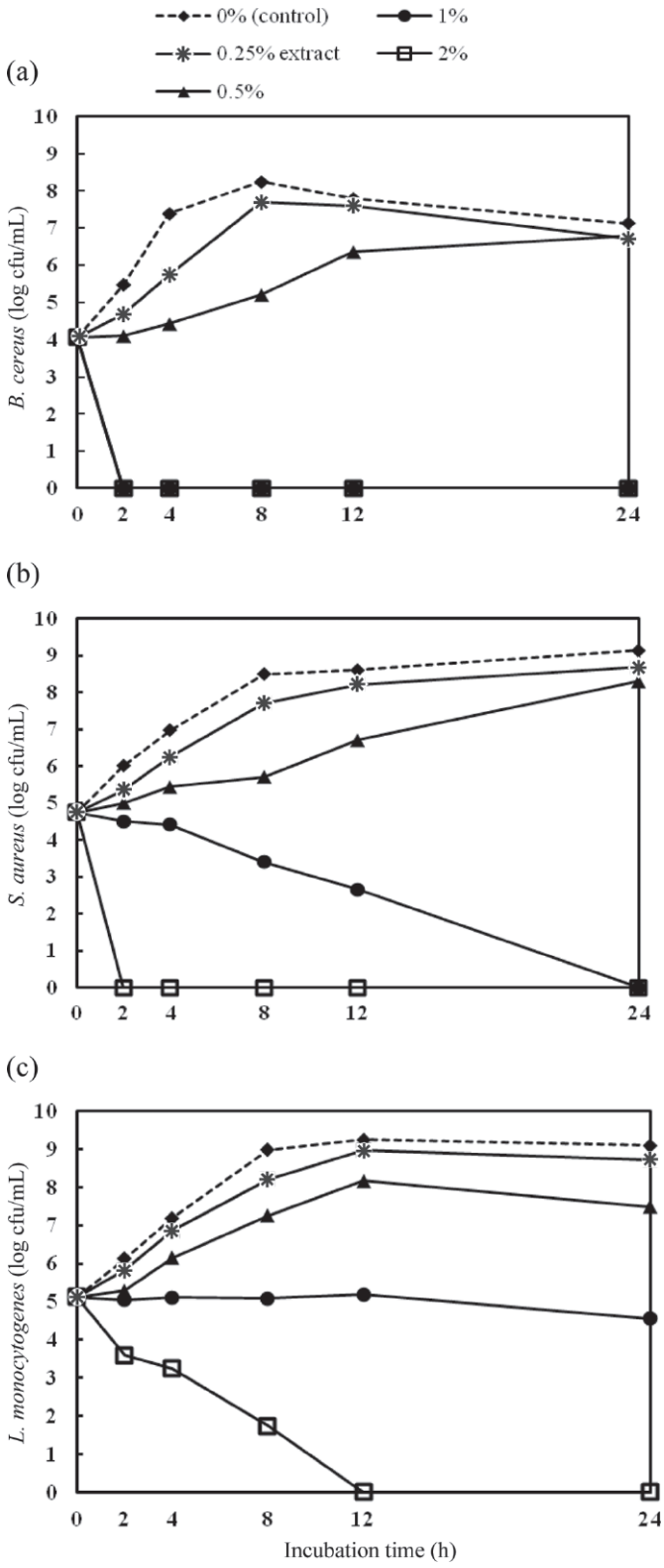

Figure 1. Inhibitory effects of acid-hydrolyzed *Citrus unshiu* peel extract on growth of (a) *Bacillus cereus*, (b) *Staphylococcus aureus*, and (c) *Listeria monocytogenes* in tryptic soy broth with yeast extract medium at 35°C for 24 h. Counts of *B. cereus* (a) with 1% (●) and 2% (□) extract were the same, thus the symbols were overlapped (■).

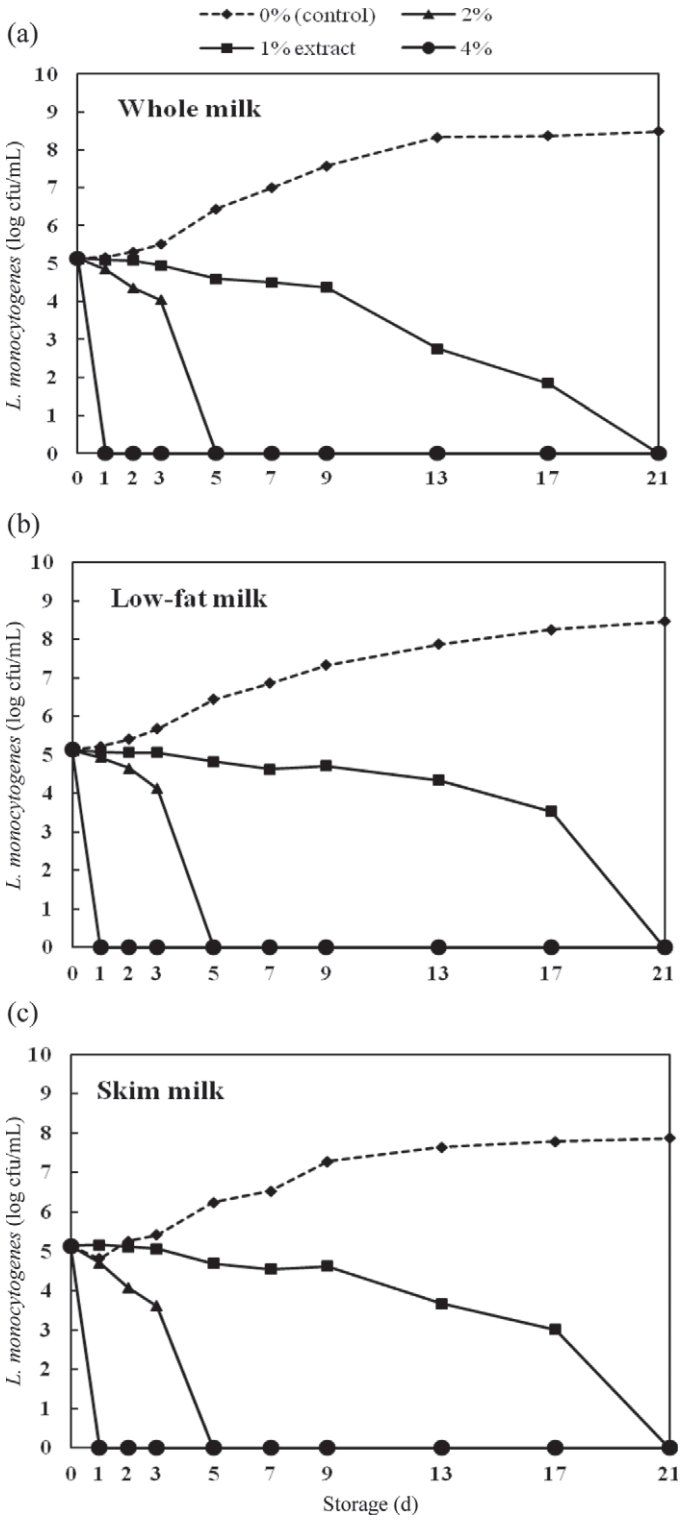

Figure 2. Inhibitory effects of acid-hydrolyzed *Citrus unshiu* peel extract against *Listeria monocytogenes* in (a) whole milk, (b) low-fat milk, and (c) skim milk over 21 d of storage at 4°C.

L. monocytogenes is probably affected not only by the specific plant extract but also by the type of foods.

Our results showed that acid-hydrolyzed *Citrus unshiu* peel extract had a substantial antilisterial effect, through direct inhibition of *L. monocytogenes* growth in whole, low-fat, and skim milk. Therefore, acid hydrolysis of *Citrus unshiu* peel extracts also improved the antilisterial activity in milk. These results indicate that acid hydrolysis of *Citrus unshiu* flavonoids could be used as a method for antimicrobial protection of food products.

CONCLUSIONS

Acid-hydrolyzed *Citrus unshiu* peel extract at 1% was effective in inhibiting the growth of 3 pathogenic bacteria, *B. cereus*, *S. aureus*, and *L. monocytogenes*. Moreover, the addition of acid-hydrolyzed *Citrus unshiu* peel extracts to whole, low-fat, and skim milk reduced the growth of *L. monocytogenes* during storage at 4°C. Acid hydrolysis enhanced the antimicrobial activity of *Citrus unshiu* peel, a waste product of juice processing, facilitating its potential use as a natural antimicrobial additive and preservative in food products.

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