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ARTICLE

Antimicrobial Activity of Kefir against Various Food Pathogens and Spoilage Bacteria

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Abstract

Kefir is a unique fermented dairy product produced by a mixture of lactic acid bacteria, acetic acid bacteria, and yeast. Here, we compared the antimicrobial spectra of four types of kefirs (A, L, M, and S) fermented for 24, 36, 48, or 72 h against eight food-borne pathogens. *Bacillus cereus*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Salmonella* Enteritidis, *Pseudomonas aeruginosa*, and *Cronobacter sakazakii* were used as test strains, and antibacterial activity was investigated by the spot on lawn method. The spectra, potencies, and onsets of activity varied according to the type of kefir and the fermentation time. The broadest and strongest antimicrobial spectrum was obtained after at least 36-48 h of fermentation for all kefirs, although the traditional fermentation method of kefir is for 18-24 h at 25°C. For kefir A, *B. cereus*, *E. coli*, *S.* Enteritidis, *P. aeruginosa*, and *C. sakazakii* were inhibited, while *B. cereus*, *S. aureus*, *E. coli*, *S.* Enteritidis, *P. aeruginosa*, and *C. sakazakii* were inhibited to different extents by kefirs L, M, and S. Remarkably, *S. aureus*, *S.* Enteritidis, and *C. sakazakii* were only inhibited by kefirs L, M, and S, and *L. monocytogenes* by kefir M after fermentation for specific times, suggesting that the antimicrobial activity is attributable not only to a low pH but also to antimicrobial substances secreted during the fermentation.

Keywords: Kefir, probiotics, antimicrobial activity, food-borne pathogen, fermentation time

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Introduction

Kefir is a probiotic containing lactic acid bacteria, acetic acid bacteria, and yeast (Guzel-Seydim *et al.*, 2011). During the fermentation process, organic acids such as lactic and acetic acid and alcohol are produced and play a physiological role (Gaware *et al.*, 2011). Many studies have investigated the beneficial effects of kefir, including its antitumorigenic and antistress properties and its immunomodulatory and hypocholesterolemic functions in animal models (Gaware *et al.*, 2011; Meydani and Ha, 2000; Rodrigues *et al.*, 2005; Saloff-Coaste, 1996; Vinderola, 2005; Wheeler *et al.*, 1997). Additionally, several studies have demonstrated its inhibitory activities against gram-negative and gram-positive food-borne bacterial pathogens (Cevikbas *et al.*, 1994; Garrote *et al.*, 2000; Silva *et al.*, 2009; Ulusoy *et al.*, 2007).

In northeast Brazil, the Community Organization Pastoral da Carianca distributes kefir grains to mothers with children affected by gastrointestinal diseases (Silva *et al.*, 2009). Zacconi *et al.* (2003) reported that kefir administration is effective in preventing *Campylobacter jejuni* colonization in chicks. However, although kefir has been used in the treatment of various gastrointestinal infectious diseases in humans and animals anecdotally, there is only limited information on the relationship between fermentation time and the range, potency, and onset of antimicrobial activity of kefir. In addition, several studies showed conflicting results on the antimicrobial spectrum against various pathogens by using kefir from different origins (Anderson and Gilliland, 1999; Pintado *et al.*, 1996).

Therefore, here, we investigated the antimicrobial activities of kefirs fermented for 24, 36, 48, or 72 h against eight bacterial test strains and compare them with the activity of organic acid and ethyl alcohol to elucidate the key attributer of the antimicrobial activity. Additionally, we compared the antimicrobial spectra of four different

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kefirs to investigate the differences in the antimicrobial spectrum of kefir from different origins. This study aimed to elucidate the optimal fermentation time and conditions for achieving the broadest and most potent antimicrobial activities of kefirs against eight of food-borne pathogens and spoilage bacteria.

Materials and Methods

Kefir preparation

Four types of kefir grains, i.e., A, L, M, and S, were corrected from private households in Korea. Each kefir grain is different in the shape and size, and thus, regarded as a different kefir grain: round and 6-10 mm, Kefir grain A; oval and 4-7 mm, Kefir grain L; oval and 8-12 mm, Kefir grain M; round and 10-15 mm, Kefir grain S; round and 13-19 mm. A total of 100 g of viable kefir grains was inoculated in 1000 mL sterilized milk (10% w/v) and cultured at 25°C for 24, 36, 48, or 72 h. At the end of the fermentation process, the grains and milk were separated using a sterilized plastic filter (2-mm pore size).

Bacterial strains

Bacillus cereus ATCC14579, *Staphylococcus aureus* ATCC6538, *Listeria monocytogenes* ATCC51776, *Enterococcus faecalis* ATCC19433, *Escherichia coli* ATCC 25922, *Salmonella* Enteritidis (originally obtained from the Food and Drug Administration [FDA], College Park, USA), *Pseudomonas aeruginosa* ATCC15522, and *Cronobacter sakazakii* ATCC29544 were used in antimicrobial activity tests. Each strain was streaked onto Columbia blood agar (bioMerieux, France) for two passages and incubated in tryptic soy broth (Difco Laboratories, USA) for 24 h at 37°C for antimicrobial activity tests.

Antimicrobial activity tests with kefir

For antimicrobial activity tests, kefir milk was centrifuged at 3,134 g for 10 min, and the supernatant was sterilized by filtration using a 0.45-μm pore-size syringe filter (Millipore Co., USA). The pH of the filtered kefir supernatant was determined with a pH meter model 205 (Testo, Germany) equipped with temperature compensation, and calibrated using pH 4.00 and 7.00 buffers.

Antibacterial activity was detected by the spot on lawn method with some modifications (Cadirci and Citak, 2005). All test bacteria were cultured on Mueller-Hinton broth (MHB; Difco) and incubated at 37°C for 24 h. The culture broth was diluted using MHB to 0.5 McF and spread onto Mueller-Hinton agar (MHA; Difco) using sterilized cotton swabs. Twenty microliters of each kefir supernatant was directly dropped onto the surface of the MHA. The plates were incubated for 24 h at 37°C, and the inhibition zone was observed. The presence of a clear zone at the site of kefir supernatant inoculation was considered as total inhibition, while a decrease in cell density was considered as partial inhibition. As a control, lactic acid solution (Sigma-Aldrich, USA), acetic acid solution (Sigma-Aldrich), and ethyl alcohol (Duksan Pure Chemicals, Korea) were diluted with sterilized distilled water. The pH of the diluent was adjusted to 3.5 for both acid solutions. Ethyl alcohol was diluted to 2.0% v/v. All solutions were sterilized by filtration using a 0.45-μm pore-size syringe filter (Millipore) before use. Antibacterial activity was detected by the spot on lawn method as described above. All activity tests were performed in triplicate.

Results and Discussion

The pH values of the kefirs during the fermentation process are shown in Table 1. During the fermentation process, the pH gradually decreased in all kefir samples. Temporal changes in the antimicrobial spectra of the kefirs against the food pathogens and spoilage bacteria are also presented in Table 1. Antimicrobial activity generally increased along with fermentation time in all types of kefirs; this effect may be related to the decreased pH observed during the fermentation process. Our results conflicted with those of a study conducted by Ulusoy *et al.* (2007), in which there were no differences in the antimicrobial activities of kefirs fermented for 24 or 48 h. Silva *et al.* (2009), however, reported results that were consistent with our observations that the antimicrobial activities of kefirs generally increased with prolonged fermentation times. The traditional method for producing kefir is to ferment milk with kefir grain for 18-24 h at 20-25°C before consumption (Beshkova *et al.*, 2002; Farnworth and Mainville, 2008; Otles and Cagindi, 2003). However, here, the broadest antimicrobial spectra against eight of food pathogens and spoilage organisms were obtained after at least 36-48 h of fermentation for all types of kefirs used in this study.

In addition, kefir A showed antimicrobial activity against *B. cereus*, *E. coli*, *S.* Enteritidis, *P. aeruginosa*, and *C. sakazakii*. In contrast, kefir L, M, and S showed more broad-spectrum antimicrobial activity, inhibiting *B. cereus*, *S. aureus*, *E. coli*, *S.* Enteritidis, *P. aeruginosa*, and *C. sakazakii* growth. Those results suggest that the kefirs from dif-

Table 1. Antimicrobial spectrum of four types of kefir fermented for 24, 36, 48, or 72 h against eight food pathogens and spoilage bacteria

Kefir	Fermentation time (h)	pH	Inhibition profile[1),2)]							
			BC	SA	LM	EF	EC	SE	PA	CS
A	24	4.05	+	−	−	−	−	−	+	−
	36	3.86	++	−	−	−	−	−	+	−
	48	3.81	++	−	−	−	+	−	++	−
	72	3.70	++	−	−	−	+	+	++	+
L	24	3.99	++	−	−	−	−	−	+	−
	36	3.77	++	−	−	−	−	−	+	++
	48	3.71	++	+	−	−	−	−	++	+
	72	3.64	++	−	−	−	+	++	++	++
M	24	3.97	++	−	+	−	−	−	+	−
	36	3.81	++	−	++	−	−	−	+	−
	48	3.77	++	+	++	−	+	−	++	+
	72	3.74	++	−	++	−	+	+	++	++
S	24	3.94	++	−	−	−	−	−	+	−
	36	3.77	++	−	−	−	+	+	+	−
	48	3.75	++	+	−	−	+	−	++	−
	72	3.65	++	−	−	−	+	++	++	++
Lactic acid solution (pH 3.5)			+	−	−	−	−	−	−	−
Acetic acid solution (pH 3.5)			+	−	−	−	−	−	−	−
Ethyl alcohol solution (2% w/w)			−	−	−	−	−	−	−	−

[1)]++, total inhibition; +, partial inhibition; −, no inhibition. [2)]BC, *Bacillus cereus* ATCC14579; SA, *Staphylococcus aureus* ATCC6538; LM, *Listeria monocytogenes* ATCC51776; EF, *Enterococcus faecalis* ATCC19433; EC, *Escherichia coli* ATCC25922; SE, *Salmonella* Enteritidis obtained from the FDA; PA, *Pseudomonas aeruginosa* ATCC15522; CS, *Cronobacter sakazakii* ATCC29544.

ferent origins have different antimicrobial spectra, which is consistent with previous studies (Anderson and Gilliland, 1999; Pintado *et al.*, 1996). Chifiriuc *et al.* (2011) reported that kefir inhibited *B. subtilis*, *S. aureus*, *E. coli*, *E. faecalis*, and *S.* Enteritidis, but did not inhibit *P. aeruginosa* or *Candida albicans*. Santos *et al.* (2003) and Ulusoy *et al.* (2007) reported that kefir could inhibit *L. monocytogenes*.

Remarkably, antimicrobial activity against *S. aureus* was only observed in kefirs L, M, and S after fermentation for 48 h. *C. sakazakii* was completely inhibited by kefir fermented for 36 or 72 h, and the activity decreased in kefir L fermented for 48 h. In kefir S, inhibitory activity against *S.* Enteritidis was present at 36 and 72 h, but not at 48 h. Considering that kefir supernatant contains various metabolites and inhibitory compounds such as organic acids, hydrogen peroxides, ethyl alcohol, diacetyl, peptides, and possibly bacteriocins, it could be postulated that these compounds interact each other to enhance or antagonize their antimicrobial effects (Kim *et al.*, 2015). For instances, the antimicrobial activities of some bacteriocins could be inactivated by organic acids or enzymatic degradation (Joshi *et al.*, 2006). It is thus inferred that the antimicrobial activity of kefir could be derived from different key compounds at each fermentation stage resulting in inconsistent antimicrobial pattern over time. Moreover, the growth of *L. monocytogenes* was only inhibited by kefir M fermented for 24 h, suggesting kefir M could contain a microorganism which produces an anti-*L. monocytogenes* molecule (i.e., bacteriocins). These data suggested that the antimicrobial activity of kefirs could be attributable to specific antimicrobial substances and not simply due to low pH values (Witthuhn *et al.*, 2005).

To demonstrate this, antimicrobial activity of lactic and acetic acid against the test strains were also investigated (Table 1). We found that only *B. cereus* growth was partially inhibited by the lactic acid and acetic acid solutions at pH 3.5 (Table 1). All other strains were resistant to both organic acid solutions. The growth of test strains was inhibited by kefir, although the pH values of kefirs in our study ranged from 4.05 to 3.64. Additionally, we conducted antimicrobial activity tests using ethyl alcohol solution because ethyl alcohol is an antimicrobial substance produced by yeast in kefir (Gaware *et al.*, 2011). The growth of all test strains, however, was not affected by the ethyl alcohol solution (Table 1). Therefore, we concluded that the antimicrobial activity of kefir was attributable to the hurdle effect of antimicrobial substances tested above, or to the single unknown bioactive compounds such as antimicrobial peptides (bacteriocins) or polysaccharides

(exopolysaccharides) (Moraes *et al.*, 2010). It is well known that the presence of bacteriocins could be screened by pH neutralization, heat and enzyme treatment of the cell-free supernatants (Harris *et al.*, 1989). Thus, future studies should be followed to elucidate the key antimicrobial compounds against each pathogenic bacterium and its mechanism.

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