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Bacterial Inhibition and Antioxidant Activity of Kefir Produced from Thai Jasmine Rice Milk

[1]Deeseenthum Sirirat and [2]Pejovic Jelena
[1]Department of Biotechnology, Faculty of Technology,
Mahasarakham University, Maung, Mahasarakham-44000, Thailand
[2]Department of Biochemical Engineering and Biotechnology,
Faculty of Technology and Metallurgy, University of Belgrade, Serbia

Abstract: The aim of this study was to investigate the bacterial inhibition and antioxidant activity of 24 and 48 h of rice milk-kefir and cow milk-kefir. Bacterial inhibition activity of kefir was investigated against *Staphylococcus aureus*, *Bacillus subtillis*, *Escherichia coli* and *Pseudomonas fluorescens* by using the disk diffusion method. Kefir showed some activity against all organisms tested. Antioxidant activity of kefir was measured using three different methods: DPPH radical scavenging activity of extracts, lipid peroxidation assay and hydroxyl radical scavenging activity. As a standard BHA (Butylated hydroxyanisole) was used. Rice milk-kefir was displayed significantly greater of antioxidant activity. These findings have suggested that rice milk-kefir may be considered among the more promising food components in term of preventing oxidative damage.

Key words: DPPH, lipid peroxidation, rice milk-kefir, *E. coli*, *Staphylococcus aureus*, LAB

INTRODUCTION

Kefir is a fermented milk drink produced by the actions of bacteria and yeasts contained in kefir grains and it is reported to have a unique taste and properties. Both bacteria and yeasts are surrounded by a polysaccharide matrix, namely kefiran (a water-soluble branched glucogalactan) which has been reported to have antibacterial, antimycotic and antitumour activity (Micheli *et al*., 1999). Kefir has frequently been claimed to be effective against a variety of symptoms and diseases. Kefir can be made of any type of milk: cow, goat, sheep, coconut, rice and soy, but cow milk is commonly used. Traditionally, kefir is homemade but the former product has been commercialized in many countries (Farnworth, 2005).

Since antibiotic use became widespread 50 years ago, bacteria have relentlessly developed resistance. Because of this, efforts have been made to develop and study new sources of natural compounds with antibacterial activity (Martínez and Baquero, 2002). Some of the recent studies show that many lactobacilli are capable of producing a wide range of bacterial inhibition compounds, including organic acids, carbon dioxide, hydrogen peroxide, ethanol, diacetyl and peptides (bacteriocins) that may reduce foodborne pathogens and spoilage bacteria during food production and storage (Farnworth, 2005). Garrote *et al*. (2000) tested the inhibitory activity of a supernatant of cow's milk fermented with kefir grains, against gram-negative and Gram-positive bacteria. Both lactic and acetic acids were found in the supernatants. Both gram-negative and Gram-positive bacteria were inhibited. Cevikbas *et al*. (2006) found similar results against gram-positive coccus and Gram-positive bacillus.

In present study, we compared antioxidant activity of kefir with antioxidant activity of BHA (Butylated hydroxyanisole). BHA is of the most used antioxidant. BHA preserved fats and it is used in food (butter, meats, cereals, chewing gum, baked goods, snack foods, dehydrated potatoes and beer) and cosmetic industry. BHA has undergone the additive application and review process required by the US Food and Drug Administration. However, the same chemical properties which make BHA excellent preservatives may also be implicated in health effects. The oxidative characteristics and/or metabolites of BHA may contribute to carcinogenicity or tumorigenicity. However, the same reactions may combat oxidative stress. There is evidence that certain persons may have difficulty metabolizing BHA, resulting in health and behavior changes (Lyck *et al*., 2006). These are all reasons why scientists are searching for new natural antioxidants. Nevertheless,

Corresponding Author: Deeseenthum Sirirat, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Maung, Mahasarakham-44000, Thailand

there is not sufficient scientific evidence to confirm all these hypothesis or therapeutic properties and more clinical studies are required to substantiate such claims.

That is the reason why scientific interest in kefir is growing due to its health benefits. With the emergence of antibiotic-resistant bacteria, it is reasonable to explore new sources of natural compounds with antibacterial compounds. Although bacterial inhibition and antioxidant activity by several isolated strains from kefir has been reported, there are no sufficient studies of bioactive properties of kefir from a mixture of pure cultures. Especially there are only few studies about activity of kefir produced by rice milk. This study, therefore, looked at the bacterial inhibition and antioxidant activities of kefir produced by rice milk.

MATERIALS AND METHODS

Preparation of bacterial solution and rice milk: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas fluorescens*, *Bacillus subtilis* were used as the test microorganisms. They were activated in nutrient broth by fermentation at 35°C for 24 h. A loop full of the bacteria that were activated in nutrient broth was transferred to sterile water and emulsified to a turbidity of McFarland 0.5 density. The absorbance at 625 nm should be from 0.08 to 0.10 (1.5×10^8 cells mL^{-1}). The organism suspension 0.1 mL was applied to agar surface of plates using spread technique.

Brown rice of Thai Jasmine Rice (Khao Dawk Mali 105) 500 g was left in 1L distillated water for 24 h. Then it was blended, filtrated with cotton sheet and cooked. The rice milk was cooked at 72°C for 15 min, after milk was cooled and ready for use.

The experiments were conducted in Department of Biotechnology, Mahasarakham University, Mahasarakham. Thailand, during June 2009 to February 2010.

Kefir culture: Freeze dried kefir cultures : Kefir DA500I (A) and Kefir DC500I (B) (Danisco, Poland) was used as the starter cultures. The composition of starter cultures, kefir A, included: *Lactococus lacti* subsp., *Leuconostoc* subsp., *Streptococcus termopfilis, Lactobacillus* subsp./kefir yeast/kefirgrains microflora; Seria 08052 A, while those of kefir B were: *Lactococus lacti* subsp., *Leuconostoc* subsp., *Streptococcus termopfilis*, *Lactobacillus* subsp./kefir yeast/kefirgrains microflora; Seria 08098 B. The starter grains were grown in MRS medium and kept at 25°C for 24 h then freezed at 4°C until used.

Fermentation process: Both commercial UHT milk and rice milk were used to produce kefir. UHT milk (total fat 9%) was used. Fermentation was done in two groups at 24-26°C for 24 and 48 h in both kefir A and B using 10% (v/v) of kefir grain inoculum.

Testing bacterial inhibition activity: The antibiotic activity of kefir was evaluated using the disk diffusion method as described by the National Committee for Clinical Laboratory Standards (NCCLS, 2000). Antibiotics, ampicillin and penicillin were used to compare bacterial inhibition activity. Paper disks (5 mm) were kept in kefir A and B and antibiotics for 2 h. The paper disks with antibiotics and kefir were applied to the agar surface previously inoculated with bacterial suspension. These plates were incubated at 35°C for 24 h. The inhabitation zones were measured at the end of the fermentation period. Experiments were performed in triplicates and mean values were used.

Physiochemical properties: The pH value was determined using a digital pH meter. The titratable acidity (Thorner degrees), °Th was determined by the following procedure: 20 mL of CO_2-free H_2O were added to 10 mL of milk; then 5 mL of alcoholic solution of phenolphthalein was added; and the mixture was titrated with 1 M NaOH to the first persistent pink color. The amount of NaOH required in milliliters was registered. This amount multiplied by 10 gave the Th per 100 mL milk. This represents the amount of alkali necessary to shifts proteins and other buffer systems in the product to the pH = 8 at which the indicator (phenolphthalein) changed color (Steffen, 1971).

Antioxidant activity: Antioxidant activity of kefir was measured using three different methods: DPPH radical scavenging activity of extracts, lipid peroxidation assay, hydroxyl radical scavenging activity. The antioxidant activity was tested at the end of 24 and 48 h fermentation for both kefir A and B produced from rice milk comparing with a standard BHA (Butylated hydroxyanisole). The experiments were performed in duplicate and mean values were used.

DPPH radical scavenging: The 0.004% (w/v) DPPH radical solution in 95% ethanol was prepared. Three milliliter of methanolic DPPH solution was mixed with 0.1 mL of sample or 95% ethanol (as a control), vortex well and incubated for 30 min in dark room at room temperature (30°C). Absorbance of each sample at 517 nm was measured. The antioxidant activity was given as percentage of DPPH scavenging, calculated as:

$$\frac{\text{(Control absorbance-extract absorbance)}}{\text{(Control absorbance)}}\times 100$$

Lipid peroxidation assay: The 0.1 mL of sample and 0.4 mL of water was mixed with 0.5 mL of egg yolk solution (10% v/v). Then this solution was vortexed well with 0.07 mL of $FeSO_4$ (10 mM) and incubated for 30 min at room temperature. After adding 1.5 mL of thiobarbituric acid solution 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulphate) samples were mixed well and heated for 60 min (95°C). After samples were cooled 5 mL of buta-1-ol was added. The samples were centrifuged for 10 min at 3000 rpm. Supernatant was used and absorbance of each sample was measured at 532 nm. 95% ethanol was used as a control. The antioxidant activity was given as an inhabitation percentage and was calculated as:

$$\frac{\text{(Control absorbance-extract absorbance)}}{\text{(Control absorbance)}}\times 100$$

Hydroxyl radical scavenging activity: The 0.075 mL of sample was mixed with 0.45 mL of sodium phosphate buffer (0.2 M, pH = 7), 0.15 mL of 2-deoxyribose (10 mM), 0.15 mL of EDTA (10 mM), 0.15 mL of $FeSO_4$ (10 mM), 0.15 mL of hydrogen peroxide (10 mM) and 0.525 mL of water. Samples were than incubated at 37°C for 4 h. After incubation 0.75 mL of trichloroactric (2.8%) acid and thiobarbituric (0.1%) acid was added. Then samples were kept in boil water for 10 min. The absorbance of each sample was measured at 520 nm and ethanol was used as a control. The antioxidant activity was given as an inhabitation percentage and was calculated as:

$$\frac{\text{(Control absorbance-extract absorbance)}}{\text{(Control absorbance)}}\times 100$$

RESULTS

Physiochemical properties: As the result of milk fermentation a foamy sparkling drink has been obtained. The viscosity of cow milk-kefir was higher than rice milk-kefir during fermentation periods of 24 and 48 h for both kefir A and B (Table 1). There was no significant difference in pH, acidity and viscosity between kefir A and kefir B growth in cow's milk. There was not much difference in the acidity, pH and viscosity of kefir A and B when they were grown in rice milk. Also, at the beginning of kefir fermentation from rice milk levels of acidity increased, which leads to pH drop. However, the viscosity of rice milk-kefir A and B was not different between the fermentation of 24 and 48 h, unlike the results obtained from cow milk-kefirs. The viscosity of both rice milk-kefir A and B was much lower than that of the same cultures when they were grown in cow's milk. Also, acidity was much lower which causes a higher value of pH.

Table 1: Psychochemical properties of kefir A and B produced from cow's milk and rice milk at 24 and 48 h

	Cow milk				Rice milk			
	Kefir A		Kefir B		Kefir A		Kefir B	
	24	48	24	48	24	48	24	48
Parameters	(h)							
pH	4.26	4.11	4.19	4.00	4.41	4.02	4.27	4.06
Viscosity (centistokes)	54.00	88.50	53.00	83.00	9.00	11.00	9.00	11.00
°Th	104.00	115.00	95.00	150.00	67.00	103.00	74.00	108.00
Total amount of lactic acid (g/100 g)	0.77	0.85	0.70	1.10	0.49	0.76	0.55	0.79

Table 2: Inhibition zones (diameter in mm) of bacterial inhibition activity of 24 and 48 h fermented kefir A and B produced from cow's milk and rice milk

	Cow milk				Rice milk			
	Kefir A		Kefir B		Kefir A		Kefir B	
	24	48	24	48	24	48	24	48
Strains	(h)							
Staphylococcus aureus	0.0	10.0	0.0	0.0	10.0	0.0	10.0	10.0
Escherichia coli	16.7	12.5	16.7	12.5	15.2	10.0	7.5	0.0
Pseudomonas fluorescens	0.0	0.0	0.0	0.0	0.0	10.0	0.0	10.0
Bacillus subtilis	10.0	0.0	10.0	0.0	10.0	0.0	15.0	0.0

Bacterial inhibition activity: The results showed *E. coli* to be the most sensitive microorganism to both kefir A and B produced from cow milk and rice milk (Table 2). Also, bacterial inhibition effects of kefir A and B in cow's milk and rice milk were noticed against *B. subtilis* with the small diameters zones for 24 h fermented milk (10 and 15 mm, respectively). Bacterial inhibition activity against *S. aureus* was noticed only for the 48 h fermented milks with small diameter zone. There was no effect against *P. fluorescens* in kefir A and kefir B produced from cow's milk but there was a small effect of rice milk-kefir A and B at 48 h.

Antioxidant activity

DPPH radical scavenging: The results of antioxidant activity of 24 and 48 h fermented kefir A and B produced from rice milk and BHA when DPPH method was used. With the increasing concentration of sample inhibition the percentage also increased. There were no significant of the antioxidant activities during 24 and 48 h kefir A, 24 h kefir B and BHA at the high concentration (100, 200 and 400 mg mL^{-1}). In contrast, 48 h kefir B showed the lowest antioxidant activity (Fig. 1).

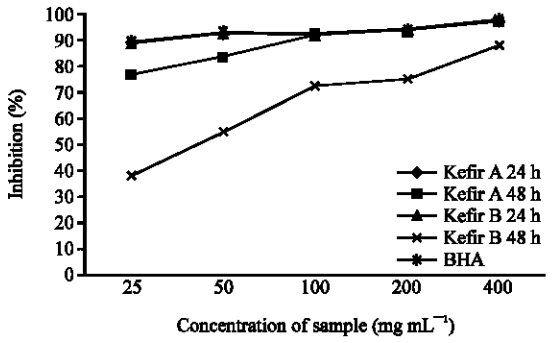

Fig. 1: Antioxidant activity of 24 and 48 h fermented kefir A and B produced from rice milk using DPPH method

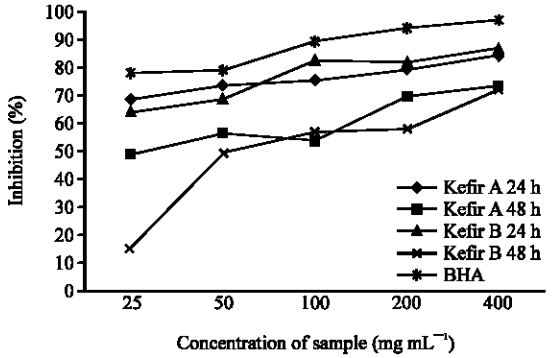

Fig. 2: Antioxidant activity of 24 and 48 h fermented kefir A and B produced from rice milk using lipid peroxidation assay

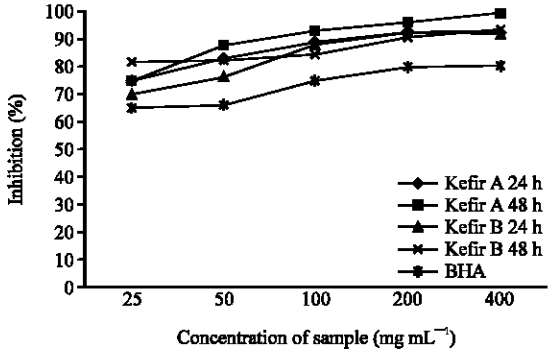

Fig. 3: Antioxidant activity of 24 and 48 h fermented kefir A and B produced from rice milk using hydroxyl radical scavenging assay

Lipid peroxidation assay: Antioxidant activity was similar for both types of kefirs and was increasing as increasing concentration. Antioxidant activity of kefirs was lower than antioxidant activity of BHA for approximately 10%. Between fermentation of 24 and 48 h of both kefirs, the 24 h kefirs showed the higher activity than 48 h kefirs (Fig. 2).

Hydroxyl radical scavenging activity: Antioxidant activity of kefirs was higher than antioxidant activity of BHA for approximately 10%. Between fermentation of 24 and 48 h antioxidant activity of kefir A and B, the 48 h kefir A showed the highest antioxidant activity (Fig. 3).

DISCUSSION

From physiochemical properties of rice milk-kefir and cow milk-kefir, lactic acid bacteria population increased at the beginning of fermentation, which resulted in the kefir becoming more acidic and caused pH drop. Some researchers have shown that the lactic acid bacteria from kefir grow more slowly in grain milks compared to cow's milk. This may be due, in part, to the slower production of growth factors at the beginning of fermentation (Liu and Lin, 2000).

In this study, kefir supernatant was used and the highest activity of kefir was against *E. coli*. The bacterial inhibition effect against *E. coli* was also reported in the study by Garrotel *et al.* (2000). Cevikbas *et al.* (2006) found similar results against Gram positive bacillus and gram-positive coccus (*S. aureus*). They also tested lab-produced kefir for its inhibitory effect against several intestinal pathogenic microorganisms, included *E. coli*. In the study performed by Zacconi *et al.* (1995) kefir showed good inhibition against wide variety of gram-positive and gram-negative bacteria.

The target microorganisms (*S. aureus*, *E. coli*, *P. fluorescens* and *B. subtilis*) are known to promote gastrointestinal diseases such as diarrhea. Such diseases are still considered as the most important public health problems in developing countries despite advances in medical knowledge and public understanding that have been developed during recent years (Fagundes-Neto and Scaletsky, 2000). Usually, the problem is associated with the ingestion of food contaminated with microorganisms (Bremer *et al.*, 2004; Alcoba-Florez *et al.*, 2005). Zubillaga *et al.* (2001) suggested that after fermentation, the kefir product contained some inhibitory compounds which proved to be bioactive. Such as bacteriocin, hydrogen peroxide and organic acids might be responsible for killing pathogenic microbes. In addition, kefir also promote competitive adhesion to gastrointestinal epithelium surface. In this study, kefir showed the best bacterial inhibition activity against *E. coli* and *B. subtilis*. The data in this study suggested that cow milk-kefir and rice milk-kefir can be used as

effective and safe bacterial inhibition agents for treating a variety of infection.

Liu *et al.* (2005) reported the effect of milk-kefir and soy milk-kefir on the scavenging activity of DPPH radical displayed significant activity than milk and soy milk. They suggested that some components of antioxidant presented in the kefir grains were transferred to milk and soy milk during fermentation. Also Chen *et al.* (2003), Nishino *et al.* (2000) and Suetsuna *et al.* (2000) reported the increased scavenging activity of fermented milk may also be related to milk protein and soybean protein-derived peptide. McCue and Shetty (2005) suggested that the increasing of antioxidant activity during soy milk yogurt production using kefir cultures may be due to the mobilization of phenolic compounds. Phenolic compounds are plant-based materials, phytochemicals. In this study, the increasing of antioxidant activity of the rice milk-kefir may be correlated to these compounds. The high inhibition percentage of antioxidant activity of rice milk-kefirs similar to inhibition percentage of BHA activity. BHA is known to be effective antioxidants (Madhavi *et al.*, 1996). Due to their scavenging abilities on free radicals, rice milk-kefir A and B might possess good antioxidant properties. These findings have suggested that rice milk-kefir A and B are potential candidates for the role of useful natural antioxidant supplements for the human food.

CONCLUSION

Rice milk-kefir promoted higher bioactivity than cow milk-kefir. Rice milk-kefir showed the bacterial inhibition activity against *E. coli*, *B. subtilis*, *P. fluorescens* and *S. aureus* . In addition, the rice milk-kefir showed greater antioxidant activity. It is reasonable to expect that rice milk contains more antioxidant compounds and bacteriocin content. These findings have suggested that rice milk-kefir may be considered among the more promising food components in terms of preventing oxidative damage and providing safe bacterial inhibition agents.

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