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Microbiological and Functional Characterization of Kefir Grown in Different Sugar Solutions

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Kefir is an ancient beverage obtained from sugar solution fermentation of kefir grains with lactic and acetic-acid bacteria and yeasts with different probiotic properties. The aim of this work was to study the microbial composition of kefir grown in three different substrates; brown sugar, purified molasses, and high-test molasses. Antioxidant activity, angiotensin converting enzyme-inhibitory (ACE-inhibitory) activity, and antibacterial activity were also evaluated. Counts of lactic acid and acetic acid bacteria and yeasts did not change significantly in the tests; however, the microbial diversity changed depending on the media and fermentation periods used. Some of the species identified were isolated for the first time in kefir beverage (*Acetobacter indonesiensis*, *Acetobacter tropicalis*, *Gluconobacter oxydans*, *Lactobacillus farraginis*, *Oenococcus kitaharae*, and *Pichia occidentalis*). In addition, three media showed ACE-inhibitory activity, which increased after fermentation. Antioxidant and ion-chelating activities decreased after the fermentation of sugar solutions. Three fermentation solutions showed antibacterial activity against *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus*.

Keywords: sugary kefir, diversity, ACE-inhibitory activity, antioxidant, antibacterial

Introduction

Kefir is a beverage obtained from either milk or sugar solution fermentation by kefir grains. Kefir grains are a consortium of lactic and acetic-acid bacteria and yeasts (Fiorda *et al.*, 2017; Rosa *et al.*, 2017). The kefir beverage has different functional properties, including antitumor, antibacterial, antioxidant, and hypocholesterolemic properties (Rosa *et al.*, 2017; Zamberi *et al.*, 2016). There are marked differences in the current knowledge of milk kefir and sugary kefir; however, the former is better known, better studied, and more often consumed.

Nowadays, knowledge of natural products with different functional properties is increasing. Globally, cardiovascular disease accounts for approximately 17 million deaths a year, nearly one third of total deaths (World Health Organization, 2013). Of these deaths from cardiovascular disease, complications from hypertension account for 9.4 million deaths worldwide every year. Angiotensin converting enzyme (ACE) contributes indirectly to blood pressure increase. First, ACE converts angiotensin (AT)-I to AT-II, which is a potent vasoconstrictor. Second, ACE stimulates aldosterone in the kidney to retain liquid in the body, triggering an increase in

Abbreviations: ACE: Angiotensin converting enzyme, AT: Angiotensin, WHO: World Health Organization, MRS: DeMan–Rogosa–Sharpe medium, GAM: Gifu Anaerobic Medium, PDA: Potato dextrose agar, ABTS: 2,20-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid, CFU: Colony-forming unit, CFS: Cell-free supernatant

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blood pressure (Ahmed *et al.*, 2013). Identification of foods containing ACE-inhibitory compounds is therefore an important step in dealing with the increasing death rate associated with hypertension.

Antioxidant activity is another important food function. Although aerobic metabolism creates energy in the body, reactive oxygen species such as hydroxyl radicals, peroxide radicals, superoxide radicals, and hydrogen peroxide are also produced. The accumulation of such prooxidants in our bodies has been reported to be associated with health problems such as atherosclerosis, arthritis, cancer, and hypertension. Our bodies have developed different mechanisms to protect against oxidative cell damage (Halliwell and Gutteridget, 1984). However, these mechanisms are not completely effective in their preventative methods, and additional intake of foods with antioxidants is desirable.

As above-mentioned, kefir is a millenary beverage with health promotion effects. Several reports have demonstrated antioxidant, antibacterial, and ACE-inhibitory activities of milk kefir (Bourrie *et al*., 2016; Rosa *et al*., 2017). Kefir beverage produced from sugar solutions could be a beneficial product for certain consumers such as vegans, lactose intolerant people, and people who are allergic to dairy products. The aim of this work was to study the kefir microbiota during their growth in three different sugar solutions: brown sugar; purified molasses; and high-test molasses. In addition, antioxidant, ACE inhibitory, and antibacterial activities of kefir after growth in the same sugar solutions were studied.

Materials and Methods

Samples and inoculation of kefir grains Kefir grains KACMUNLP1 were obtained from the National University of La Plata (Argentina). The grains were preserved in sterilized skimmed milk (Meiji Holding Co., Ltd., Tokyo, Japan) at -80 ℃ and activated at 25 ℃ for 24 h (10 g grains with 100 mL sugar solution). The growth media were three sugar solutions: 5 % (w/v) brown sugar; 6.5 % (w/v) purified molasses; and 6.5 % (w/v) high-test molasses. Powdered brown sugar (Kanbayashi Co., Ltd.) was purchased from a local market. Purified molasses and high-test molasses were obtained from the Dai-Nippon Meiji Sugar Co., Ltd. (Tokyo, Japan). Purified molasses and high-test molasses are by-products from the manufacture of white sugar and raw sugar, respectively. The three solutions had a similar sugar concentration (4.7–4.9 °Brix). Kefir grains were grown in each solution over 60 days to ensure their ability to ferment the medium. The media were replaced by fresh media three or four times a week. Activated grains were used for the assays. The grains were incubated at 25 ℃ for 24 h. After fermentation, the grains were removed by filtration with a sieve having a 1-mm^2 mesh size. Part of each beverage obtained was used for microbial characterization, and part was centrifuged and filtrated through 0.22-μm pore-size membranes (Sartorius®, Göttingen, Germany), designated cell-free supernatants (CFS) and stored at -80 ℃ until use.

Bacterial strains used *Salmonella enterica* subspecies *enterica* serovar Typhimurium JCM 6977, *Escherichia coli* ATCC 11775, and *Staphylococcus aureus* ATCC 12600 were used as test microorganisms. They were activated in nutrient broth (Nissui®, Tokyo, Japan) by incubation at 37 ℃ for 24 h. Pathogens were obtained from the JCM collection (Ibaraki, Japan). A 0.5 McFarland suspension of each pathogen was prepared (corresponding to 10^8 CFU/mL) for antibacterial assays. The strains were kept at -80 ℃.

Determination of wet weight of kefir grains and pH and °Brix of fermented beverages Kefir grains grown in three sugar solutions were subcultured through seven successive passages in a suitable volume of the respective sugar solutions (10 % w/v), and incubated at 25 ℃ for 24 h. After each fermentation, kefir grains were separated from the beverages using a sieve. Grains were washed with sterile water, dried between tissue paper for 30 – 40 min at room temperature, and weighed on an analytical balance model A200S (Sartorius). According to the grain weight obtained, a suitable volume of each medium was prepared. The pH readings were made with a pH meter instrument (Docu-pH+ meter™; Sartorius). The °Brix readings were carried out with a refractometer (Pocket PAL-J™; Atago, Tokyo, Japan).

Isolation and purification of bacteria and yeast One milliliter of each fermented product was diluted in peptone water 0.1 % (w/v). Bacteria and yeasts were enumerated by the surface spread technique (Magalhães *et al*., 2010). Each diluted sample (100 μL) was spread in four different culture media. Lactic acid bacteria (LAB) were enumerated in MRS (DeMan–Rogosa–Sharpe medium) agar (Difco®, Le Pont de Claix, France) and GAM (Gifu Anaerobic Medium) agar (Nissui®, Tokyo, Japan). Acetic acid bacteria (AAB) were enumerated in GYC agar (5 % glucose [Nacalai Tesque®, Kyoto, Japan]; 1 % yeast extract [Difco]; 0.5 % calcium carbonate [Nacalai Tesque]; 0.03 % bromocresol purple [Wako, Osaka, Japan]; 2 % agar [Nacalai Tesque]) with 0.01 % (w/w) cycloheximide (Nacalai Tesque). Yeasts were enumerated in PDA (Eiken Chemical Co., Ltd., Tochigi, Japan) agar with 0.01 % (w/w) chloramphenicol (Dr. Ehrenstorfer GmbH®, Augsburg, Germany). After spreading, the GYC and PDA plates were incubated at 30 ℃ for 48 h, and the MRS and GAM plates were incubated in an anaerobic incubator at 30 ℃ for 72 h. Then, the best dilution was selected to obtain mean colony-forming units (on every plate, 30 to 300 CFU was the best dilution), and single colonies for isolation and identification. The detection limit of viable counts was 3×10^3 CFU/mL. The distinguishable colony morphology groups were independently counted on each agar plate, and randomly selected representative colonies (at least two colonies from each morphology group) were then streaked onto the same type of agar plate. The colony groups under the above lower detection limit were also isolated. Ninety-one isolates in total were used

for the 16S/26S ribosomal RNA gene (rDNA)-based species identification described below.

Identification of bacteria and yeasts by molecular methods Bacterial and yeast DNA were extracted from pure cultures. Standard genetic techniques were used, essentially as described by Sambrook and Russell (2001). Portions of the 16/26S rDNA gene were sequenced to identify genotypic characteristics of representative bacteria and yeasts. The forward primer for bacteria was 7F (5'- AGAGTTTGATYMTGGCTCAG-3'), and the reverse primer was 1510R (5'- ACGGYTACCTTGTTACGACTT-3'). The forward primer for yeasts was LSU-D2f (5'-GTGGTAACTTCCATCTAAAGC-3'), and the reverse primer was LSU-D2R (5'-GGTCCGTGTTTCAAGACGG-3'). Forward and reverse primers were provided by Thermo Fisher Scientific Inc.® (MA, USA). A total of 30 µL PCR mixture contained: 3 µL buffer solution; 2.4 µL dNTPs; 1.5 µL of each primer; 0.15 µL ExTaq polymerase (Takara, Shiga, Japan); 20.85 µL purified water; and 0.6 µL of the extracted DNA. The PCR amplification was carried out as follows: for bacteria, template DNA was denatured for 2 min at 96℃, followed by 25 cycles of denaturing at 96℃ for 15 s, annealing at 50℃ for 15 s, and primer extension at 72℃ for 1 min 30 s; for yeasts: template DNA was denatured for 2 min at 96℃, followed by 25 cycles of denaturing at 96℃ for 15 s, annealing at 50℃ for 15 s, and primer extension at 72℃ for 40 s. The amplification products were analyzed by electrophoresis on 1.0% agarose gels before being sequenced by Eurofin Genomics Co. Ltd. (Tokyo, Japan). Each sequence datum was used as a query sequence to search for similar sequences from GenBank by means of the Blast program (www.ncbi.nlm.nih.gov/blast/).

The DNA sequences of *Acetobacter indonesiensis* sk437, *Oenococcus kitaharae* sk452, *Acetobacter tropicalis* sk484, *Gluconobacter oxydans* sk543, *Lactobacillus farraginis* sk558, *Staphylococcus epidermidis* sk568, and *Pichia occidentalis* sk418 were deposited in the DNA Data Bank of Japan under the accession numbers LC367617-LC367623.

Determination of the ACE-inhibitory activity ACE-inhibitory activity was measured by ACE Kit-WST® (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's instructions (Hagi *et al*., 2016). As mentioned in the instructions, blank 1 (all the reagents without sample), and blank 2 (all the reagents without both sample and enzyme) were prepared and measured together with the samples. In addition, negative controls of each sample (sample + all the reagents without enzyme) were also prepared and measured.

$$ACE\ inhibition\ \% = \left[\frac{A\ blank\ 1\text{-}A\ sample}{A\ blank\ 1\text{-}A\ blank\ 2}\right] \times 100 \cdots\cdots \text{Eq. 1}$$

A blank 1 is the absorbance of positive control (without samples). *A blank 2* is the absorbance of reagent blank (without addition of enzyme mixtures). *A sample* is the absorbance in the presence of sugary kefir. Samples were tested at five concentrations to construct the standard curve for the determination of the IC_{50} value (concentration of inhibitor required to inhibit 50% of the ACE activity). The magnitude of IC_{50} was expressed as µg/mL. Samples were tested in triplicate.

Measurement of antioxidant activity 2,20-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) assay was used to determine the antioxidant activity. This activity of sugary kefir samples was determined spectrophotometrically according to the study by De Gobba *et al*. (2014), at 405 nm. The addition of antioxidant compounds reduces the ABTS acid cations, thus causing a reagent decolorization, which is measurable spectrophotometrically, depending on the antioxidant type and concentration, as well as on the reaction time. The ABTS values were expressed as mmol TE/g sample. To determine the ABTS radical scavenging activity, a standard curve was constructed with 0–0.4 mmol of Trolox. Each standard and sample was tested in triplicate.

Chelating effects upon ferrous ions' assay The ferrous ion-chelating ability was determined according to the method of Decker and Welch (1990). Fermented and unfermented samples were separately mixed with 0.1 mL of a 2 mM solution of $FeCl_2$, and 0.2 mL of 5 mM ferrozine. The mixture was then shaken and left to stand at room temperature for 10 min. The absorbance of the resulting solution was measured at 562 nm. To determine the chelating effects, a standard curve was constructed with EDTA. The standard curve was between 0–0.1 mg EDTA/mL.

Measurement of antibacterial activity The inhibitory activities of CFS of fermented and unfermented sugar solutions against *S. typhimurium* JCM 6977, *E. coli* ATCC 11775, and *S. aureus* ATCC 12600 were tested. One milliliter of each sample was inoculated with 10 µL a 0.5 McFarland standard pathogen suspension (containing 1.5×10^8 CFU/mL), uniformly mixed and incubated at 37℃ for 48 h. Pathogen growth was detected by assessing turbidity. To determine the bacteriostatic/bactericidal activity of the samples, aliquots of 100 µL of each sample were subcultured in nutrient agar (Nissui) dishes and were counted after incubating at 37℃ for 24 h. Each treatment was performed in triplicate.

Statistical analysis All growth parameters were analyzed using SIGMAPLOT 10.0® software. The results of independent assays are presented as mean values ± standard deviation. Comparisons among replications in every group were performed using analysis of variance tests by the Statgraphics Plus 5.1® software. All experiments were performed at least in triplicate.

Results and Discussion

Kefir grains can grow in various sugar solutions, including sucrose solution (10% + one fig or two dry figs and a slice of organic lemon) (Gulitz *et al*., 2013; Marsh *et al*., 2013), brown sugar (5–10%) (Magalhães *et al*., 2010; Fiorda *et al*., 2016), honey (40 °Brix) (Fiorda *et al*., 2016) and vegetable solutions

(Corona *et al*., 2016). The kefir grains studied here were able to ferment and increase their biomass in the three sugar solutions used in the study (Fig. 1). The pH of unfermented sugar solutions was between 6.01 ± 0.03 and 6.59 ± 0.16. After fermentation, the pH decreased to 4.00 ± 0.03 for purified molasses and 3.66 ± 0.02–3.78 ± 0.02 for the other two media (Fig. 1B). The pH decreased in proportion to the concentration of °Brix, indicating that sugar is used as a source of carbon for the fermentations (Fig. 1A). The biomass of kefir grains increased in the three sugar solutions, by 3.94 times, 6.30 times, and 1.37 times, when compared with their initial biomass in brown sugar, purified molasses, and high-test molasses, respectively (Fig. 1C). In addition, fermentations were confirmed by sucrose decreasing from 3298 to 995 mg/100 mL in brown sugar, from 1612 to 37 mg/100 mL in purified molasses, and from 1562 to 5 mg/100 mL in high-test molasses, as measured by ion exchange chromatography (data not published). This work followed conditions of kefir fermentation similar to those used on an industrial scale (25 ℃ for 18–24 h) (Özdestan and Üren, 2010).

Microbial properties Kefir grains and/or their beverages show great diversity, which depends on their origin. Most kefir present LAB, AAB, and yeasts; however, the species involved may change (Gao and Li, 2016; Gulitz *et al*., 2013; Marsh *et al*., 2013). In concordance with these reports, our viable counts (in MRS, GAM, GYC, and PDA) showed variability, depending on the media type and fermentation time (Table 1). LAB constituted 82–96 %, 81–98 %, and 65–88 % of the microorganisms during fermentations in brown sugar, purified molasses, and high-test molasses, respectively. AAB constituted <0.1–16 %, <0.1–18 % and 2–24 % of the microorganisms in brown sugar, purified molasses, and high-test molasses, respectively. Yeasts constituted 1–9 %, 1–4 %, and 9–16 % of the microorganisms in brown sugar, purified molasses, and high-test molasses, respectively.

We determined the microbial species dependent on the 16/26S rDNA sequence for a representative strain of each colony morphology group (Table 1). In brown sugar, *Lactobacillus nagelii* (LAB), *Saccharomyces cerevisiae* (yeast), and *Acetobacter tropicalis* (AAB) were identified on the first day. *L. nagelii* was detected until the seventh day, and the high viable counts of the colony morphology group containing this species on the MRS and GAM plates around 10^7 CFU/mL indicated the dominance of this species in brown sugar kefir. During seven days of fermentation, *Lactobacillus farraginis*, *Lactobacillus hilgardii*, and *Lactobacillus satsumensis* were also detected, but their colony counts were lower than those of *L. nagelii* (approximately 10^6 CFU/mL). As for yeasts, only *S. cerevisiae* was isolated and its viable count increased slightly during the process, from 5.3×10^5 to 2.2×10^6 CFU/mL. *A. indonesiensis* and *Gluconobacter oxydans* were identified as AAB other than *A. tropicalis*. The AAB count was highest on the fourth day, but it decreased to 1.0×10^5 CFU/mL by the seventh day.

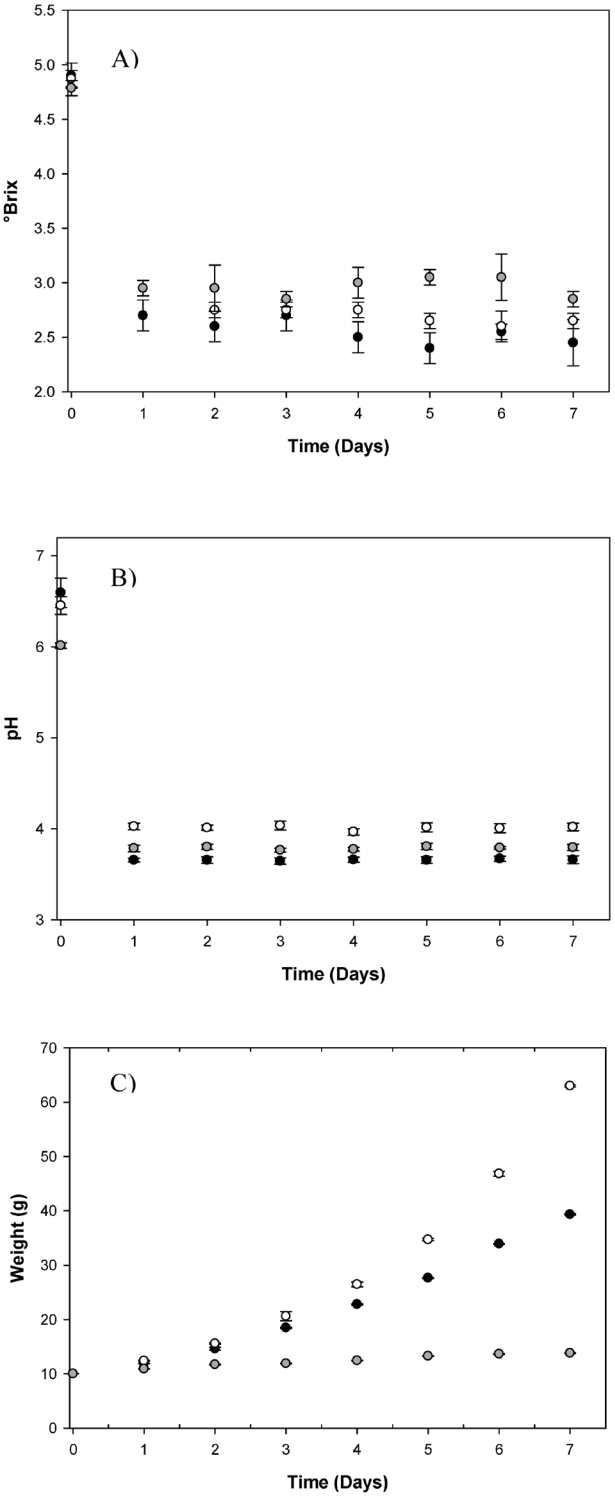

Fig. 1. pH (A), °Brix (B) and biomass increase (C) of seven consecutive fermentations in brown sugar (black circles), purified molasses (white circles), and high-test molasses (gray circles) at 25 ℃ for 24 h

In purified molasses, *L. nagelii*, *S. cerevisiae*, and *A. tropicalis* were identified on the first day, as was the case in

brown sugar. Similar trend was observed for AAB counts, the highest value was observed on the fourth day (2.8×10^7 CFU/mL) but it decreased to 6.0×10^4 CFU/mL on the seventh day. It is noted that a different species, *Acetobacter lovaniensis*, replaced the late-stage AAB flora. As for LAB, *L. farraginis*, *Lactobacillus harbinensis*, *L. satsumensis*, and *Oenococcus kitaharae* were detected on the MRS and GAM agar plates in the course of fermentation. *Lactobacillus casei/paracasei* was also enumerated at high counts on the seventh day (1.6×10^7 CFU/mL), thus the LAB species was variable when compared to brawn sugar kefir. In contrast to brown sugar kefir, yeasts other than *S. cerevisiae*, even though remained the predominant component (10^6 CFU/mL), *Pichia occidentalis* and *Pichia membranifaciens* was detected (approximately 10^5 CFU/mL for *P. occidentalis*).

In contrast to the above two sugar kefirs, *L. casei/paracasei* was clearly the predominant LAB during fermentation in high-test molasses. It was enumerated in both MRS and GAM agar at concentrations $>1.5 \times 10^7$ CFU/mL. In addition, high counts of *L. farraginis*, *L. diovorans*, and *L. harbinensis* at 10^6-10^7 CFU/mL were observed. *L. nagelii*, which had significant population at the first fermentation day in brown sugar and purified molasses kefirs, was not detected until the fermentation reached the seventh day. The yeast inhabitants were quite similar to the case of purified molasses, with *S. cerevisiae* and two *Pichia* species (*P. membranifaciens* and *P. occidentalis*) as major populations, at 10^6–10^7 CFU/mL. The AAB detected were also similar (*A. indonesiensis*, *A. lovaniensis*, and *G. oxydans*), except that *A. tropicalis* was not detectable.

Some species identified here have been isolated by other researchers. Many diverse species of LAB have been isolated from kefir (Gao and Li, 2016; Fiorda *et al*., 2017). *L. nagelii*, *L. diolivorans*, *L. harbinensis*, *L. hilgardii*, *L. satsumensis*, and *L. paracasei* were isolated previously (Fiorda *et al*., 2016; Gulitz *et al*., 2013; Laureys and De Vuyst, 2014; Magalhães *et al*., 2010; Zanirati *et al*., 2015). *O. kitaharae* was only identified from other reported sugary kefir by culture-independent methods (Zanirati *et al*., 2015). In addition to the species mentioned before, isolation of *L. farraginis* from kefir is reported here for the first time. Four AAB species were isolated in this study. *A. lovaniensis* was isolated previously (Magalhães *et al*., 2010) and the other three species were identified by culture-independent methods (Gulitz *et al*., 2013; Walsh *et al*., 2016). These three species were isolated from kefir in this study for the first time. The yeasts *P. membranifaciens* and *S. cerevisiae* have been identified in previous articles (Stadie *et al*., 2013; Fiorda *et al*., 2016), but in this study, *P. occidentalis* was isolated from kefir for the first time. The present study thus expanded the potential species of AAB, LAB, and yeasts in kefir, providing an important information to precisely understand the microbiological nature of kefir beverages.

Functional properties There is a wide body of literature that shows the probiotic properties of kefir. Kefir and its microorganisms have a range of therapeutic effects, including antitumor, antimicrobial, antioxidant, antimutagenic, and hypocholesterolemic activities (Liu *et al.*, 2005; Rosa *et al*., 2017; Zamberi *et al*., 2016). According to WHO, hypertension complications trigger millions of deaths every year (World Health Organization, 2013), and this is becoming an important public problem for many countries around the world. The use since 1981 of ACE inhibitors has marked a great advancement in the treatment of hypertension. ACE inhibitors are now recognized as first-line agents for treating hypertension (Materson and Preston, 1994). The ACE-inhibitory activity of kefir-fermented solutions was elevated after the fermentation (Table 2). IC_{50} significantly decreased to 62.80 %, 57.19 %, and 48.17 % of the unfermented solutions in brown sugar, purified molasses, and high-test molasses, respectively. Quirós *et al*. (2005) in their study found that kefir can inhibit the activity of ACE by bioactive peptides produced after caprine milk fermentation with kefir grains, and one of the peptides showed an IC_{50} of 2 μg/mL. In the present study, the IC_{50} values were 8.92, 2.93, and 1.93 μg/mL for fermented brown sugar, purified molasses, and high-test molasses, respectively. Thus, we found that the sugary kefir has a degree of ACE-inhibitory activity comparable to that of milk-type kefir. ACE-inhibitory activity is known to be either positively or negatively affected by fermentation. Aihara *et al*. (2005) reported in their study that ACE-inhibitory activity is increased by fermenting powdered milk with *Lactobacillus helveticus* isolated from kefir. By contrast, Kwon *et al*. (2006) investigated the ACE-inhibitory activity of soymilk fermented with a commercial culture of kefir, and found that unfermented soymilk shows higher ACE-inhibitory activity, and this decreased significantly during fermentation. The present data indicate that fermentation positively influenced the ACE-inhibitory activity of sugary kefir, which is similar to the former case with caprine milk kefir. Some non-peptide ACE inhibitor have been reported such as mannitol (Hagiwara *et al*., 2005), which is known to be produced by some LAB species (Wisselink *et al*., 2002). More study is needed to clarify the production ability of ACE inhibitory compounds by LAB present in sugary kefir, but it is likely that some of these contribute to production of such components in the protein-poor environment of sugary kefir.

Antioxidant activity was measured by two methods (Table 3). An ABTS assay showed a significant decrease of antioxidant activity after fermentation in brown sugar (from 6.51 to 5.28 mmol TE/g, 19 % decrease) and purified molasses (from 21.32 to 17.93 mmol TE/g, 16 % decrease), but showed a non-significant decrease in high-test molasses (from 16.10 to 15.65 mmol TE/g). Fiorda *et al*. (2016) found that a soybean-based kefir beverage and a honey-based kefir beverage increased their radical scavenging activity after fermentation with kefir grains. The fermented beverages used in the present study showed more radical scavenging activity than did those mentioned above, even though the antioxidant activity

Table 1. Viable counts of kefir grown in brown sugar, purified molasses, and high-test molasses

Kefir	Fermentation time (d)[1]	Medium	Viable counts of respective colony morphology groups (CFU/mL)	Species of representative strains identified dependent on 16S rDNA[2]
Brown sugar	1	GYC	$1.5 \pm 0.1 \times 10^6$	*Acetobacter tropicalis*
		MRS	$2.4 \pm 0.0 \times 10^7$	*Lactobacillus nagelii*
		GAM	$2.3 \pm 0.3 \times 10^7$	*L. nagelii*
		PDA	$5.3 \pm 0.5 \times 10^5$	***Saccharomyces cerevisiae***
	4	GYC	$6.5 \pm 1.4 \times 10^6$	*Acetobacter indonesiensis*
		GYC	$7.0 \pm 4.2 \times 10^5$	*Gluconobacter oxydans*
		GYC	$1.9 \pm 1.0 \times 10^6$	*A. tropicalis*
		MRS	$1.5 \pm 0.1 \times 10^7$	*L. nagelii*
		MRS	$4.1 \pm 0.4 \times 10^6$	*Lactobacillus farraginis*
		GAM	$2.7 \pm 0.1 \times 10^7$	*L. nagelii*
		GAM	$2.2 \pm 0.9 \times 10^6$	*Lactobacillus satsumensis*
		PDA	$1.2 \pm 0.2 \times 10^6$	***S. cerevisiae***
	7	GYC	$1.0 \pm 0.1 \times 10^5$	*A. tropicalis*
		MRS	$7.2 \pm 1.0 \times 10^6$	*L. nagelii*
		MRS	$4.5 \pm 0.8 \times 10^6$	*Lactobacillus hilgardii*
		GAM	$1.2 \pm 0.3 \times 10^7$	*L. nagelii*
		GAM	$5.0 \pm 1.4 \times 10^5$	*L. farraginis*
		PDA	$2.2 \pm 0.7 \times 10^6$	***S. cerevisiae***
Purified molasses	1	GYC	$2.6 \pm 0.7 \times 10^6$	*A. tropicalis*
		MRS	$2.5 \pm 0.2 \times 10^7$	*L. farraginis*
		MRS	$9.4 \pm 1.0 \times 10^6$	*L. satsumensis*
		MRS	$6.5 \pm 0.7 \times 10^6$	*Oenococcus kitaharae*
		MRS	-[3]	*Lactobacillus casei/paracasei*
		MRS	-	*L. nagelii*
		GAM	$1.1 \pm 0.0 \times 10^7$	*L. nagelii*
		GAM	$1.0 \pm 0.1 \times 10^6$	*Lactobacillus harbinensis*
		PDA	$1.3 \pm 0.1 \times 10^6$	***S. cerevisiae***
		PDA	$7.9 \pm 2.2 \times 10^5$	***Pichia occidentalis***
	4	GYC	$2.8 \pm 0.4 \times 10^7$	*A. indonesiensis*
		MRS	$8.4 \pm 0.1 \times 10^7$	*L. farraginis*
		MRS	$9.5 \pm 4.9 \times 10^6$	*L. nagelii*
		MRS	-	*L. satsumensis*
		GAM	$3.2 \pm 0.6 \times 10^7$	*L. nagelii*
		GAM	$4.5 \pm 3.5 \times 10^6$	*L. farraginis*
		GAM	-	*L. harbinensis*
		GAM	-	*L. satsumensis*
		PDA	$2.1 \pm 0.1 \times 10^6$	***S. cerevisiae***
		PDA	$1.9 \pm 1.2 \times 10^5$	***P. occidentalis***
	7	GYC	$6.0 \pm 1.4 \times 10^4$	*Acetobacter lovaniensis*
		MRS	$2.1 \pm 0.5 \times 10^6$	*L. farraginis*
		MRS	$2.6 \pm 0.1 \times 10^6$	*L. nagelii*
		MRS	$1.9 \pm 0.7 \times 10^7$	*L. hilgardii*
		GAM	$1.5 \pm 0.0 \times 10^7$	*L. farraginis*
		GAM	$3.2 \pm 0.2 \times 10^6$	*L. nagelii*
		GAM	$1.6 \pm 0.2 \times 10^7$	*L. casei/paracasei*

		GAM	-	*Lactobacillus diolivorans*
		PDA	$1.0 \pm 0.2 \times 10^6$	***S. cerevisiae***
		PDA	$1.6 \pm 1.3 \times 10^5$	***P. occidentalis***
		PDA	-	***Pichia membranifaciens***
High-test molasses	1	GYC	$1.5 \pm 0.3 \times 10^6$	*A. indonesiensis*
		MRS	$3.0 \pm 0.5 \times 10^7$	*L. casei/paracasei*
		MRS	$1.6 \pm 0.4 \times 10^7$	*L. diolivorans*
		MRS	$1.1 \pm 0.2 \times 10^7$	*L. farraginis*
		GAM	$1.6 \pm 0.0 \times 10^7$	*L. casei/paracasei*
		GAM	$2.6 \pm 0.5 \times 10^6$	*L. harbinensis*
		PDA	$2.8 \pm 1.3 \times 10^6$	***S. cerevisiae***
		PDA	$5.6 \pm 0.4 \times 10^6$	***P. membranifaciens***
	4	GYC	$1.9 \pm 0.5 \times 10^6$	*G. oxydans*
		MRS	$4.8 \pm 0.5 \times 10^7$	*L. casei/paracasei*
		GAM	$3.6 \pm 0.4 \times 10^7$	*L. casei/paracasei*
		GAM	$3.5 \pm 0.7 \times 10^6$	*L. harbinensis*
		PDA	$1.2 \pm 0.1 \times 10^7$	***S. cerevisiae***
		PDA	$5.7 \pm 0.5 \times 10^6$	***P. occidentalis***
		PDA	-	***P. membranifaciens***
	7	GYC	$2.5 \pm 0.7 \times 10^5$	*A. indonesiensis*
		GYC	$3.0 \pm 0.5 \times 10^7$	*A. lovaniensis*
		MRS	$4.5 \pm 0.3 \times 10^6$	*L. nagelii*
		MRS	$3.4 \pm 0.4 \times 10^7$	*L. casei/paracasei*
		MRS	-	*L. hilgardii*
		GAM	$3.9 \pm 0.4 \times 10^7$	*L. casei/paracasei*
		GAM	$3.1 \pm 1.0 \times 10^6$	*L. harbinensis*
		GAM	$2.8 \pm 0.3 \times 10^6$	*Staphylococcus epidermidis*
		GAM	-	*L. diolivorans*
		PDA	$4.3 \pm 0.4 \times 10^6$	***S. cerevisiae***
		PDA	$6.2 \pm 1.1 \times 10^6$	***P. membranifaciens***

[1]Seven-days of sequential fermentation (each step was conducted at 25℃ for 24 h, in total seven times) in different sugar solutions were carried out.
[2]lactic acid bacteria are underlined and yeasts are indicated in bold.
[3]-, microorganisms that were isolated and identified but viable counts of them were under the detection limit (< 3×10^3 CFU/mL).

Table 2. ACE-inhibitory activity of kefir grown in brown sugar, purified molasses, and high-test molasses

Samples	IC_{50} (μg/mL)
Unfermented brown sugar	14.21 ± 0.36^a
Fermented brown sugar	8.92 ± 0.49^b
Unfermented purified molasses	5.12 ± 0.27^c
Fermented purified molasses	2.93 ± 0.68^{de}
Unfermented high-test molasses	4.02 ± 0.39^{cd}
Fermented high-test molasses	1.93 ± 0.63^e

decreased slightly after fermentation. Purified molasses showed the strongest ABTS activity of 17.93 mmol TE/g after fermentation (Table 3). The ion-chelating assay showed a large decrease of ferrous ion availability in all kefir samples. The decrease of metal-ion availability may decrease oxidative damage, as some damaging conditions may be caused by the influence of metal-catalyzed radical formation. Liu *et al*. (2005) reported that the ferrous ion level was the same both before and after the fermentation of cow and goat milk kefirs, which contradicts our results obtained with sugary kefir. Thus, the antioxidant activities of sugary kefir exhibited different trends from other types of kefir, but the above results of the ABTS and ion-chelating assays clearly indicate the high antioxidant capacity of this beverage. To date, milk kefir has been the main focus of the investigation of antioxidant activity (Liu *et al*., 2005; Rosa *et al*., 2017; Sabokbar *et al*., 2015); therefore, the present results with sugary kefir reveal new aspects of the antioxidant capacity of kefir beverage.

All fermented solutions studied showed antibacterial

Table 3. Antioxidant activity of kefir grown in brown sugar, purified molasses, and high-test molasses

	ABTS assay (mmol TE/g)	Ion-chelating assay (mg EDTA/mL)
Unfermented brown sugar	6.51 ± 0.04^{a}	5.42 ± 0.16^{a}
Fermented brown sugar	5.28 ± 0.32^{b}	0.59 ± 0.19^{b}
Unfermented purified molasses	21.32 ± 0.19^{c}	7.30 ± 0.20^{c}
Fermented purified molasses	17.93 ± 0.49^{d}	0.48 ± 0.18^{b}
Unfermented high-test molasses	16.10 ± 0.17^{e}	7.14 ± 0.22^{c}
Fermented high-test molasses	15.65 ± 0.29^{e}	0.10 ± 0.14^{d}

Table 4. Determination of antibacterial activity of kefir grown in brown sugar, purified molasses, and high-test molasses against *E. coli*, *S. enterica* subspecies *enterica* serovar Typhimurium, and *S. aureus*

	E. coli	*S. aureus*	*S. typhimurium*
Unfermented brown sugar	G ($>1.5\times10^{5}$)	NG (-)	G ($>1.5\times10^{5}$)
Fermented brown sugar	NG (-)	NG (-)	NG (-)
Unfermented purified molasses	G ($>1.5\times10^{5}$)	NG ($5.0 \pm 1.3\times10^{1}$)	G ($>1.5\times10^{5}$)
Fermented purified molasses	NG (-)	NG (-)	NG (-)
Unfermented high-test molasses	G ($>1.5\times10^{5}$)	NG ($1.8 \pm 0.5\times10^{2}$)	G ($>1.5\times10^{5}$)
Fermented high-test molasses	NG (-)	NG (-)	NG (-)
Nutrient broth	G ($>1.5\times10^{5}$)	G ($>1.5\times10^{5}$)	G ($>1.5\times10^{5}$)

G: growth observed by turbidity. NG: no growth observed by turbidity. (-): no growth observed by plate counts. The plate counts observed are shown in parentheses. CFSs were inoculated with 2.0×10^{5} CFU of *S. aureus*, 3.1×10^{5} CFU of *S. typhimurium*., and 3.5×10^{5} CFU of *E. coli*.

activity against *E. coli*, *S. typhimurium*, and *S. aureus* (Table 4). *E. coli* and *S. typhimurium* were able to grow in unfermented sugar solutions and nutrient broth ($>1.5 \times 10^{5}$ CFU/mL), but their growths were completely inhibited when inoculated in fermented solutions. *S. aureus* exhibited a weak ability to grow in unfermented purified molasses and high-test molasses, and bacterial growth was also completely inhibited in fermented solutions. Consequently, no growth was observed in samples from fermented solutions, thus confirming the bactericidal activity of kefir.

There are many reports about the antibacterial activity of milk kefir and its microorganisms (Garrote *et al*., 2000; Bourrie *et al*., 2016; Rosa *et al*., 2017). Rodrigues *et al*. (2005) demonstrated that kefir obtained from molasses fermentation exhibited antibacterial activity against *Streptococcus pyogenes*, *Streptococcus salivarius*, *S. aureus*, *Pseudomonas aeruginosa*, *S. typhimurium*, *E. coli*, *Listeria monocytogenes*, and *Candida albicans*. Our results partially agree with the results of these reports. This activity could be derived from the action of lactic and acetic acids. We found lactic and acetic acids at concentrations of not less than 70 and 40 mg/100 mL, respectively, in fermented sugary kefirs (data not published). The antibacterial activity of milk kefir is due to production of lactic and acetic acid (Garrote *et al*., 2000), which is consistent with our results. Apart from the activity of organic acids, other compounds, such as polyphenols and bacteriocins (Miao *et al*., 2016; Lee and Paik, 2017), may contribute to antibacterial activity.

Conclusion

Kefir is an ancient beverage that has been consumed over a long period of time. Its beneficial characteristics have been demonstrated in the literature. However, there is a marked difference in the current knowledge pertaining to milk-type kefir and to sugary kefir, with milk kefir being better known and more thoroughly studied than sugary kefir. Sugary kefir could be an interesting beverage for consumers who are either vegan, lactose intolerant, or allergic to dairy products. We have demonstrated that kefir grown in different sugar solutions exhibits considerable microbial diversity. Moreover, in this study, some microorganisms, such as *A. indonesiensis*, *A. tropicalis*, *G. oxydans*, *L. farraginis*, *O. kitaharae*, and *P. occidentalis*, were isolated from kefir for the first time. In addition, kefir obtained from brown sugar, purified molasses, and high-test molasses showed angiotensin converting enzyme-inhibitory, antioxidant, and antibacterial properties. Further research is necessary to study the compounds responsible for these functional properties.

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