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Short communication

Characterization of yeasts isolated from kefir as a probiotic and its synergic interaction with the wine byproduct grape seed flour/extract

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ABSTRACT

Few studies have assessed the probiotic characteristics and antioxidant activity of the kefir-derived yeast, *Kluyveromyces marxianus*, and its synergistic interactions with polyphenol-rich grape seed flour (GSF)/extract (GSE). We examined several probiotic characteristics (gastric resistance, intestinal resistance, cholesterol reduction, adhesiveness to intestinal cells) of four *K. marxianus* strains. Each of the yeasts showed 5–25% greater intestinal cell adhesiveness than that of *Lactobacillus acidophilus*. Among the four yeast strains, *K. marxianus KU140723-02* (KM2) exhibited the greatest antioxidant activity. When incubated with GSF/GSE, its antioxidant activity was synergistically increased by two-fold. In conclusion, a combination of kefir-derived yeast KM2 and GSF/GSEs may be a novel functional food ingredient with antioxidant properties targeting gut health.

1. Introduction

Kefir is a traditional natural fermented probiotic milk that originated in the Caucasus Mountains. It is made with kefir grain and contains more than 50 species of lactic acid bacteria, yeast, and acetic acid bacteria. Lactic acid bacteria generally exist in a population of 10^8–10^9 CFU/g kefir grain as compared to the 10^5–10^6 CFU/g of yeast and acetic acid bacteria. These microorganisms are present in the polysaccharide and protein matrix of a kefir grain. Kefir has shown many health benefits, including anti-obesity, anti-inflammatory, cholesterol-lowering, and antioxidant effects, as well as alleviation of fatty liver and enhancement of intestinal bacterial flora (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2014; Kim, Chon, Kim, & Seo, 2015; Lim, Kale, Kim, Kim, Chon, Seo, et al., 2017; Rodrigues, Carvalho, & Schneedorf, 2005; Uchida, Ishii, Inoue, Akisato, Watanabe, Hosoyama, et al., 2010). It also reduces constipation, ameliorates gastrointestinal disorders, and enhances immune responses by facilitating intestinal activity (Ahmed et al., 2013; Gaware, Kotade, Dolas, Dhamak, Somwanshi, Nikam, et al., 2011; Lopitz, Rementeria, Elguezabal, & Garaizar, 2006).

From the kefir grains, the most extensively studied yeasts are *Kluyveromyces* spp. and *Saccharomyces* spp. (Simova et al., 2002). The potential use of *Kluyveromyces marxianus* as a probiotic has been suggested in reports of several studies showing alterations in cell adhesion, immunity, and the human gut microbiota, as well as hypocholesterolemic, anti-inflammatory, and anti-oxidative stress properties (Dellomonaco et al., 2007; Kumura, Tanoue, Tsukahara, Tanaka, & Shimazaki, 2004; Maccaferri, Klinder, Brigidi, Cavina, & Costabile, 2012; Romanin et al., 2016; Xie, Zhang, Liu, Xiong, Gao, Jia, et al., 2015). *K. marxianus* contributes to the major characteristics of kefir such as its microbial stability, chemical and organoleptic properties, and the ultrastructure of kefir grains (Gethins, Rea, Stanton, Ross, Kilcawley, O'sullivan et al., 2016).

Grape seed flour (GSF), a byproduct of grape seed in the wine-making process, contains 60–70% of the extractable flavonoids of the grape, including catechin, epicatechin, and epigallocatechin, as well as procyanidin dimers and trimers (Kim et al., 2014). Previously, our studies have shown that the antioxidant activities of GSF play major roles in the attenuation of high-fat (HF) diet-induced oxidative stress, resulting in beneficial health effects on hepatic steatosis, hypercholesterolemia, and obesity (Seo, Bartley, Tam, Kim, Kim, Chon, et al., 2016). Consistent with these findings, grape seed extracts (GSEs) showing powerful antioxidant abilities inhibited the oxidation of low-density lipoprotein (LDL) and lowered the risk of heart disease (Dai & Mumper, 2010; Shi, Yu, Pohorly, & Kakuda, 2003).

The aim of this study was to determine the probiotic characteristics of yeasts isolated from kefir by evaluating artificial gastric and intestinal resistance, cholesterol reduction, and adherence of yeast to

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Caco-2 cells. Furthermore, this study explored the antioxidant abilities of these kefir-derived yeasts and their synergistic antioxidant activity with polyphenol-rich GSF/GSE.

2. Materials and methods

2.1. Isolation of yeasts and culture conditions

For the isolation of yeasts from kefir obtained from Sensorgen Inc. (Seoul, Korea), a loop of kefir drink was streaked on potato dextrose agar (PDA; Oxoid, Basingstoke, UK) and incubated at 30 °C for 48 h under aerobic conditions. Colonies were observed microscopically and screened by cell morphology and size. Four yeast strains were isolated from kefir and confirmed by internal transcribed spacer rRNA sequencing: *K. marxianus KU140723-01* (KM1), *K. marxianus KU140723-02* (KM2), *K. marxianus KU140723-04* (KM4), and *K. marxianus KU140723-05* (KM5). *Saccharomyces cerevisiae* ATCC6037 (SC) and *Kluyveromyces lactis* ATCC34440 (KL) were purchased from ATCC (American Type Culture Collection) and used as positive controls. Yeast strains were incubated in Man-Rogosa-Sharpe (MRS) medium at 37 °C for 24 h. Before the experiments, each yeast strains were sub-cultured three times at intervals of 24 h.

2.2. Determination of acid and bile tolerances

Acid and bile resistances were determined using the method of Hyronimus et al. (Hyronimus, Le Marrec, Sassi, & Deschamps, 2000). Briefly, to determine acid tolerance, yeasts were cultured in MRS broth at a concentration of 1.0×10^8 CFU/mL at 37 °C for 18 h. Then, 1000 U/mL of pepsin was added to 10 mL of the MRS broth at pH 2.5. Under these conditions, yeasts were inoculated and cultured at 37 °C for 0–2 h. The number of viable bacteria was counted and the survival rate determined. For bile acid resistance, 10 mL of MRS broth was adjusted to pH 8 by the addition of 0.3% (w/v) oxgall. Yeasts were grown in MRS broth at 37 °C for 18 h and then sub-cultured in the oxgall-supplemented MRS broth at 37 °C for 24 h. The survival rate was calculated by comparing the initial number of bacteria with the number of viable cells after 24 h.

2.3. Measurement of cholesterol reduction capacity

The cholesterol-lowering activity of yeasts was measured using a modification of the method of Buck and Gilliland (Buck & Gilliland, 1994). To prepare the cholesterol solution, 10 mg of cholesterol was dissolved in 1 mL of 99% ethanol at a ratio of 10:1 (w/v). Thirty microliters of the prepared cholesterol solution was added to 3 mL of MRS broth, and then the yeast was incubated for 24 h aerobically. After centrifugation for 10 min (12,000 × *g*, 4 °C), the supernatant was transferred to determine the amount of cholesterol remaining using a modification of the method of Rudel and Morris (Rudel & Morris, 1973). Briefly, 2 mL of KOH (33%, w/v) and 3 mL of 99% ethanol (Merck) were added to 2 mL of supernatant. The mixture was reacted in a water bath at 60 °C for 5 min. After cooling the solution, the cells were mixed with 5 mL of hexane. After adding 3 mL of distilled water, the solution was maintained for 15 min at room temperature for phase separation. Then, 2.5 mL of fluid in the hexane layer was transferred to a new tube, and the hexane was pressurized with nitrogas at 10 psi and 40 °C for 20 min. The remaining solution was treated with 4 mL of o-phthalaldehyde reagent (o-phthalaldehyde/glacial acetic acid, 1:2, w/v), 2 mL of sulfuric acid was added, and the reaction was allowed to proceed for 10 min. Absorbance was measured at 595 nm using a Multikan FC microplate reader (Thermo Fisher Scientific, Waltham, MA).

2.4. Adherence to Caco-2 cells

Caco-2 cells were seeded in 24-well plates at 1×10^5/mL in Dulbecco's Modified Eagle Medium (DMEM) with 1% non-essential amino acids, 1% penicillin-streptomycin, and 10% fetal bovine serum. When Caco-2 cells were 95–99% confluent, 10^6 CFU/mL of yeast strains isolated from kefir were added to the cell cultures and incubated in 5% CO_2 at 37 °C for 1 h. Cells were then washed three times with sterile PBS to remove non-attached yeast cells and lysed with 2 mL of Triton X-100 (1% v/v). Diluted lysates were inoculated onto MRS agar. The number of colonies indicated the ability of yeasts to adhere to Caco-2 cells.

2.5. Preparation of wine GSF extracts

Chardonnay wine GSF was purchased from Sonomaceuticals, LLC/WholeVine products (Santa Rosa, CA, USA). Then, 0.3 g of GSF was mixed with 18 mL of 80% methanol or water and extracted using a sonicator (SD-350H, Seong Dong, Seoul, Korea) for 30 min. After filtering the suspension with filter paper (Whatman No. 1, Whatman International Ltd., Maidstone, England), the residue was re-extracted with 80% methanol or water for 30 min, and the filtrate was dried using a vacuum evaporator and nitrogen gas. The obtained extracts were stored at −20 °C. The (+) catechin content in GSF, sonicated grape seed water extracts (SGWE), and sonicated grape seed 80% methanol extracts (SGME) was 7, 10, and 9 mg/g, respectively.

2.6. Determination of α, α-diphenyl-β-picrylhydrazyl (DPPH) radical-scavenging activity of kefir-derived yeasts

For the preparation of intact yeast cells, each of the yeast strains was sub-cultured three times in MRS broth at 37 °C. 10^7 CFU/ml of sub-cultured yeasts were washed three times with phosphate-buffered saline (PBS) and re-suspended in PBS at the same volume as the initial sample. To prepare intracellular yeast cell-free extracts, 10^7 CFU/ml of the yeasts were washed twice with deionized water and re-suspended in deionized water, followed by ultrasonication in an ice bath at 5 s intervals every 15 s for 25 min. Yeast cell debris was removed by centrifugation at 3000 × rpm for 10 min, and the resulting supernatant was the intracellular yeast cell-free extract.

The antioxidant capacity of intact yeasts and the yeast cell-free extract was measured as described previously (Chen et al., 2010; Jung & Kim, 2015). Briefly, an aliquot of freshly prepared DPPH solution (0.2 mM in methanol) and 0.1 mL intact yeast cells or cell-free extract were mixed and allowed to react for 30 min at 37 °C. After centrifugation, 0.15 mL of supernatant from the mixture was transferred to a new plate. The scavenged DPPH was then monitored by measuring the decrease in absorbance at 517 nm. Scavenging ability was calculated using the following equation: DPPH radical-scavenging activity (%) $= \frac{(Ac-(As-Asb))}{Ac} \times 100$, where Ac is the absorbance of the DPPH solution and PBS, As is absorbance of the DPPH solution with the sample, and Asb is the absorbance of the sample blank.

To determine the antioxidant activity of kefir-derived yeasts in the presence of GSF or GSE, yeast strains were sub-cultured twice in MRS broth at 37 °C before ultrasonicated GSF or GSE was added. The amounts of GSF and GSE added were the following: 5 mg of GSF, sonicated grape seed water extract prepared from 5 mg of GSF (SGWE [L]), sonicated grape seed water extract prepared from 10 mg of GSF (SGME[H]), sonicated grape seed methanol (80%) extract prepared from 5 mg of GSF (SGME[L]), and sonicated grape seed methanol (80%) extract prepared from 10 mg of GSF (SGME[H]). After incubation at 37 °C for 24 h, the yeasts and GSF/GSE were washed three times with PBS and re-suspended in PBS at the same volume as the initial MRS broth, followed by the addition of DPPH and incubation for 30 min at 37 °C. After the GSF and GSE debris were removed by centrifugation, the supernatant of the reactant was transferred to a 96-well plate. The