



Research article

Evaluation of ginsenoside bioconversion of lactic acid bacteria isolated from kimchi



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ABSTRACT

Background: *Panax ginseng* is a physiologically active plant widely used in traditional medicine that is characterized by the presence of ginsenosides. Rb1, a major ginsenoside, is used as the starting material for producing ginsenoside derivatives with enhanced pharmaceutical potentials through chemical, enzymatic, or microbial transformation.

Methods: To investigate the bioconversion of ginsenoside Rb1, we prepared kimchi originated bacterial strains *Leuconostoc mesenteroides* WiKim19, *Pediococcus pentosaceus* WiKim20, *Lactobacillus brevis* WiKim47, *Leuconostoc lactis* WiKim48, and *Lactobacillus sakei* WiKim49 and analyzed bioconversion products using LC-MS/MS mass spectrometer.

Results: *L. mesenteroides* WiKim19 and *Pediococcus pentosaceus* WiKim20 converted ginsenoside Rb1 into the ginsenoside Rg3 approximately five times more than *Lactobacillus brevis* WiKim47, *Leuconostoc lactis* WiKim48, and *Lactobacillus sakei* WiKim49. *L. mesenteroides* WiKim19 showed positive correlation with β -glucosidase activity and higher transformation ability of ginsenoside Rb1 into Rg3 than the other strains whereas, *P. pentosaceus* WiKim20 showed an elevated production of Rb3 even with lack of β -glucosidase activity but have the highest acidity among the five lactic acid bacteria (LAB).

Conclusion: Ginsenoside Rg5 concentration of five LABs have ranged from ~ 2.6 $\mu\text{g/mL}$ to 6.5 $\mu\text{g/mL}$ and increased in accordance with the incubation periods. Our results indicate that the enzymatic activity along with acidic condition contribute to the production of minor ginsenoside from lactic acid bacteria.

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1. Introduction

Lactic acid bacteria (LAB) have been used as probiotics and are present in many fermented foods (cheese, yogurt, butter, and kimchi), where they influence the taste and preservation by producing lactic acid and/or alcohol. Some enzymes produced by LABs can efficiently utilize ingested nutrients to benefit the host, i.e., linoleic acid isomerase from *Lactobacillus acidophilus* produces conjugated linoleic acid, which has biological properties, from linoleic acid [1], and β -glucosidase from *Lactobacillus plantarum* converts isoflavone glucosides, which are not absorbed by enterocytes [2], to absorbable aglycones [3]. Recent studies reported the bioconversion of ginsenosides using *Lactobacillus*

pentosus and *Leuconostoc citreum* isolated from fermented foods due to β -glucosidase activity [4,5].

The major commercial ginsengs such as *Panax ginseng* Meyer (Korean Red Ginseng), *Panax quinquefolium* (American ginseng), and *Panax notoginseng* (Burk.) F.H. Chen (Notoginseng) have been widely used as traditional herbal medicines [6]. Ginsenosides (ginseng saponins) are the major pharmacological constituents of ginseng, and over 100 ginsenosides have been identified [5,7]. Major ginsenosides (80% of the ginsenosides) are composed of Rb1, Rb2, Rc, Rd, Re, and Rg1; minor ginsenosides are their deglycosylated forms and composed of Rg3, Rh2, Rh1, F2, C-K, Rg2, Rh1, Rg5, and F1 [8].

Minor ginsenosides are known to have a greater pharmaceutical potential than major ginsenosides [9–14]. However, naturally

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occurring minor ginsenosides are present at very low concentrations. Therefore, hydrolysis of sugar moieties from abundant major ginsenosides are needed to produce minor ginsenosides. Gut microbiota metabolize orally administered ginseng and help transport across the epithelial membrane [15] and human intestinal microbiota convert major ginsenosides to minor ginsenosides [10,16,17]. However, ginsenoside metabolism varies between individuals depending on the population of gut microbiota, such as *Ruminococcus* spp., *Bacteroides* spp., and *Bifidobacterium* spp. [17]. Acidic environments as well as intestinal microbiota have important influences on the bioconversion of ginsenoside and the low pH of gastrointestinal environment could activate the deglycosylation of ginsenoside by acidic hydrolysis response [18–20].

Kimchi is a traditional Korean food that is fermented vegetables including cabbage and various seasonings. Kimchi has antioxidative and antidiabetic properties and bacteria isolated from kimchi produce beneficial enzymes [21–23]. Various LABs play important roles during kimchi fermentation: *Lactobacillus* and *Leuconostoc* are the predominant genera of the kimchi microbiome in the kimchi fermentation [24]. *Lactobacillus* species have neuroprotective, antifungal, and anticarcinogenic properties [25–27] and *Leuconostoc* species play key roles in decreasing foodborne pathogen growth, viral activity, and the effects of lipid profiles [28–30]. Recent studies have suggested that LABs from kimchi produce hydrolytic enzymes that catalyze ginsenoside bioconversion by removing the glycosyl group of major ginsenosides [4,5].

In this study, we isolated LABs associated with kimchi fermentation from homemade kimchi, and compared availability for ginsenoside bioconversion of five LABs such as *Leuconostoc mensesenteroides* WiKim19, *Pediococcus pentosaceus* WiKim20, *Lactobacillus brevis* WiKim47, *Leuconostoc lactis* WiKim48, and *Lactobacillus sakei* WiKim49 by quantitating transformed ginsenoside using a sensitive and reliable LC-MS/MS method.

2. Materials and methods

2.1. Materials

Leuconostoc mensesenteroides WiKim19, *Pediococcus pentosaceus* WiKim20, *Lactobacillus brevis* WiKim47, *Leuconostoc lactis* WiKim48, and *Lactobacillus sakei* WiKim49 were isolated from homemade kimchi using de Man, Rogosa, and Sharpe (MRS) media. MRS broth was purchased from Difco (Miller, Becton Dickinson, and Co., Sparks, MD, USA). Ginsenosides Rb1, Rg3, digoxin (internal standard), and β -glucosidase activity assay kit were purchased from Sigma Aldrich (St. Louis, MO, USA). Ginsenoside -F2, -Rg1, -Rf, -Ro, -Rg2, -R1, -Ra1, -Rb2, -Rb3, -F1, -Rd, -Rg5, -compound K; -Rh2, -Rh4, and gypenoside XVII were purchased from Ambo Institute (Daejeon, Korea). API 50 CH and inoculating fluid was purchased from bioMérieux (Lyon, France) [31].

2.2. Determination of 16S rRNA gene sequences and phylogenetic analysis

To identify the isolates using 16S rRNA sequencing, the isolates were sent to Macrogen Inc., Korea sequencing service (www.macrogen.com, Seoul, Korea). The obtained sequences were compared with available 16S rRNA sequences in the EzTaxon Server [32] to evaluate sequence similarity. Multiple sequence alignment of the 16S rRNA sequences from five lactic acid bacteria and these related species were performed with CLUSTAL W [33]. The phylogenetic trees were constructed using MEGA6 [34] with neighbor-joining [35] based on 1,000 random bootstrap replicates for each.

2.3. Assay of ginsenoside Rb1 bioconversion by lactic acid bacteria

L. mensesenteroides WiKim19, *P. pentosaceus* WiKim20, *L. brevis* WiKim47, *L. lactis* WiKim48, and *L. sakei* WiKim49 were inoculated in MRS broth, until absorbance reached 600 nm of 1.0. The strains were cultured at 30°C for 1 d, 3 d, and 7 d with ginsenoside Rb1 (a final concentration of 200 μ M) dissolved in MeOH. After centrifugation at 5,000g for 10 min, 2 mg/mL digoxin as an internal standard was added and purified using Sep-Pak Light C18 cartridges (Waters, Milford, MA, USA) and then dissolved in MeOH.

2.4. Assay of ginsenosides by LC-MS/MS

Ginsenosides Rb1 and minor ginsenosides in the reactions were analyzed by using UPLC (Waters), coupled to a TripleTOF 5600 plus system with electrospray ionization (ESI; AB SCIEX, Framingham, MA, USA). To investigate and separate the precursor and fragmentation ions of ginsenosides Rb1, minor ginsenosides, an Acquity UPLC BEH C₁₈ column (2.1 mm \times 100 mm, 1.7 μ m particle size) from Waters was used at a flow rate of 0.5 mL/min. UPLC conditions were as follows: solvent A, water containing 10mM ammonium acetate; solvent B, acetonitrile containing 10mM ammonium acetate; gradient, 0–0.5 min (5% B), 0.5–14.5 min (5–30% B), 14.5–15.5 min (30–32% B), 15.5–16.5 min (32–40% B), 16.5–17 min (40–55% B), 17–19 min (55% B), 19–25 min (90% B), and 25–30 min (5% B). Two microliters of each sample were injected for the UPLC analysis, and peaks were identified by comparing their retention times and fragment ion with that of reference compound.

The mass spectrometry conditions were optimized under the negative ion mode as follows: curtain gas, 30; collision energy, –30; declustering potential, –80; nebulizer gas (Gas 1), 40 at MRM mode; heater gas (Gas 2), 50. The ion spray voltage was –4,500 V. Ginsenosides in all reaction mixtures were quantified with multiple reaction monitoring (MRM) using selected transitions as follows: Rb1, m/z 1,107 \rightarrow 945; Rg3, F2, m/z 783 \rightarrow 621; Rg5, m/z 765 \rightarrow 603; digoxin, m/z 779 \rightarrow 649, Rg1, Rf m/z 799 \rightarrow 637; Ro, m/z 955 \rightarrow 793; Rg2, m/z 783 \rightarrow 637; R1, m/z 931 \rightarrow 769; Ra1, m/z 1,209 \rightarrow 1,077; Rb2, Rb3, m/z 1,077 \rightarrow 945; F1, m/z 637 \rightarrow 475; Rd, m/z 945 \rightarrow 783; XVII, m/z 945 \rightarrow 323; compound K, m/z 621 \rightarrow 459; Rh2, m/z 621 \rightarrow 459; and Rh4 m/z 619 \rightarrow 161.

Data acquisition and processing were carried out using Analyst TF 1.6 and PeakView 1.2 software (AB SCIEX), respectively. The data obtained from multiple reaction monitoring (MRM) mode were quantitated using MultiQuant software (AB SCIEX). The standard solutions containing 10–200 μ M were injected into the UPLC with 2 mg/mL digoxin. The linear calibration curve for peak area ratio (ginsenoside/digoxin) was obtained for the quantification of ginsenoside. The amounts of the ginsenosides in each sample were determined from corresponding calibration curves.

2.5. Assay of β -glucosidase activities using cell lysates

L. mensesenteroides WiKim19, *P. pentosaceus* WiKim20, *L. brevis* WiKim47, *L. lactis* WiKim48, and *L. sakei* WiKim49 were cultured for 1 d in MRS broth at 30°C. The supernatant was removed after centrifugation at 12,000g for 10 min, and cell lysates including intracellular β -glucosidase were prepared by bead beating in 50mM sodium phosphate buffer (pH 7.0). Protein concentrations of cell lysates were determined using a Pierce BCA Protein Assay Kit (Thermo, Rockford, IL, USA). The proteins were diluted to the concentration of \sim 0.5 mg/mL to assay enzyme activity. The enzyme activity was determined using β -glucosidase activity assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the

manufacturer's protocol. The release of *p*-nitrophenol was measured at 405 nm (SPECTROstar Nano, BMG Labtech, Ortenberg, Germany). Analysis was performed in duplicate for each strain. One unit of β -glucosidase is the amount of enzyme that catalyzes the hydrolysis of 1.0 μ mole substrate per min at pH 7.0. The results were reported as mean \pm standard deviation. Statistical analyses were performed using GraphPad Prism 6 (GraphPad software, La Jolla, CA, USA). One-tailed Student *t* test for unpaired samples were used to assess significance of the

differences between the three LABs. Differences with *p* values < 0.05 were considered statistically significant.

2.6. Measurement of pH and acidity

The LABs were cultivated in the MRS broth at a temperature of 30°C for 0 d and 3 d. The pH about 10 mL of each culture broth was measured using electrode pH meter (Mettler Toledo, Columbus,

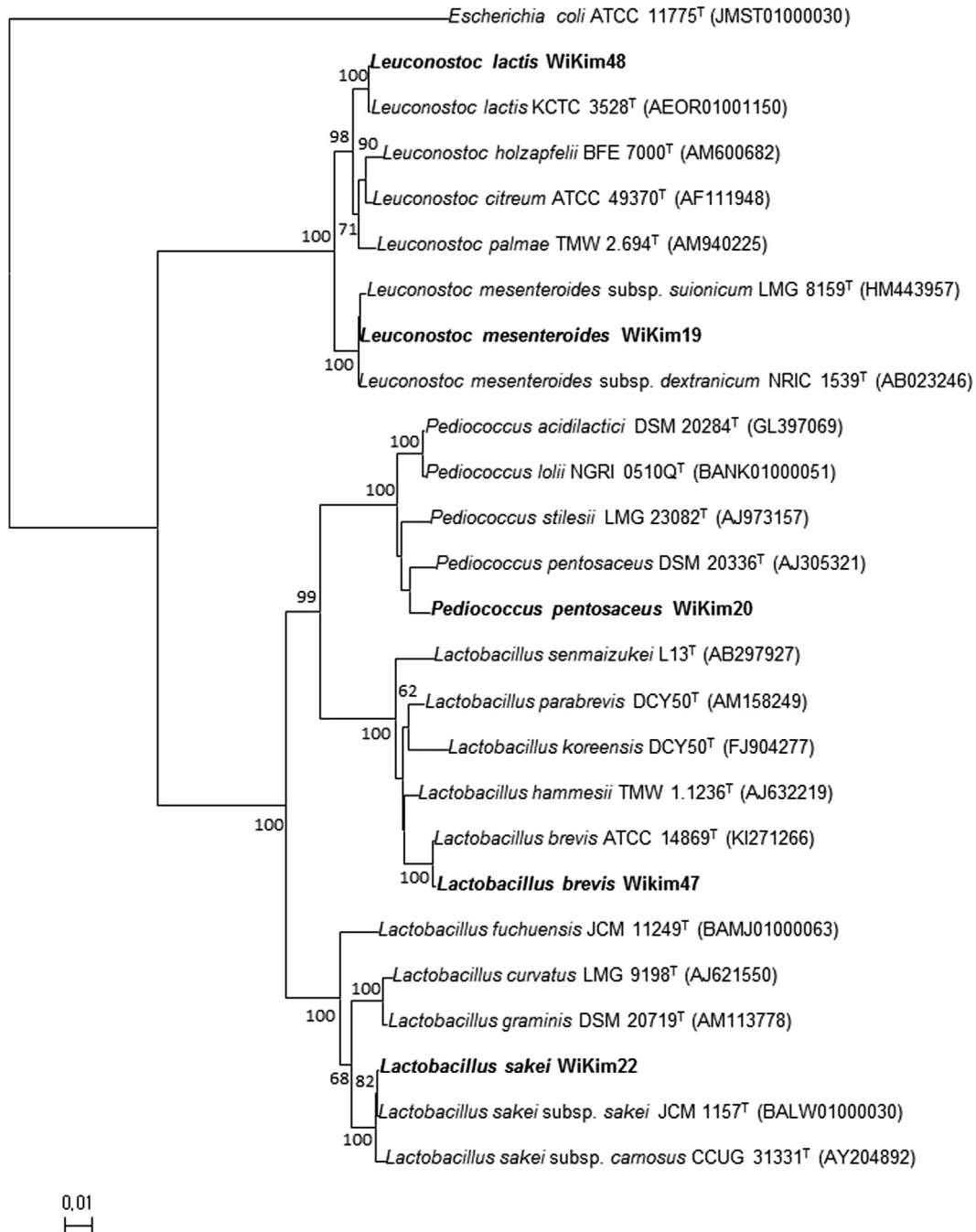


Fig. 1. Phylogenetic trees constructed from 16S rRNA gene sequences. The phylogenetic relationships of *Leuconostoc mesenteroides* WiKim19, *Pediococcus pentosaceus* WiKim20, *Lactobacillus brevis* WiKim47, *Leuconostoc lactis* WiKim48 and *Lactobacillus sakei* WiKim49 with other species are shown. Trees were constructed using the neighbor-joining method. The numbers at the nodes represent bootstrap values (> 60%) are expressed as percentages of 1,000 replicates. *Escherichia coli* ATCC 11775^T was used as an outgroup. Bar, 0.01 accumulated changes per nucleotide.

USA). Acidity was measured by titration against 0.1N NaOH to a phenolphthalein endpoint, pH 8.3.

3. Results and discussion

Although minor ginsenosides have various beneficial effects, only small percentages are found in ginseng. Therefore, many studies have investigated methods such as heating, mild acid hydrolysis, and enzymatic transformation for conversion of major ginsenosides [36–38].

Lactic acid bacteria strains were isolated from kimchi previously and the strains were identified by 16S rRNA sequencing. The identified 16S rRNA sequences were deposited on NCBI GenBank under accession No. KT759681 (*L. mesenteroides* WiKim19), KX890131 (*P. pentosaceus* WiKim20), KX886794 (*L. brevis* WiKim47), KX886799 (*L. lactis* WiKim48), and KX886806 (*L. sakei* WiKim49). The constructed phylogenetic trees clustered WiKim 19, 20, 47, 48, and WiKim 49 with the *Lactobacillus*, *Leuconostoc*, and *Pediococcus* genera and well matched with reference strains (Fig. 1).

The API 50 CH (bioMérieux) was used to determine the carbohydrate assimilation profile of five LABs. The test preparations were incubated at 30°, and readings were made after 48 h. The biochemical characteristics are listed in Table 1. *L. mesenteroides* WiKim19 showed the utilization of diverse α (1→4), β (1→4), α (1→6), α (1→2) linkage. *L. brevis* WiKim47 could not use maltose, lactose, inulin, and cellobiose which have α (1→4) and diverse β (1→4) linkage. *P. pentosaceus* WiKim20 showed the similar carbon utilizing profile with *L. mesenteroides* WiKim19. Both could use inulin and gentiobiose, which have β (1→2) and β (1→4) linkage,

respectively. However, *P. pentosaceus* WiKim20 did not use lactose, which has galactopyranosyl β (1→4) linkage.

To investigate the bacterial bioconversion of ginsenoside Rb1 into minor ginsenosides, ginsenoside bioconversion was performed by incubation of five LABs with 200 μ M of Rb1 and measured the 15 minor ginsenoside components (ginsenoside -Rg3, -F2, -Rg5, -Rg1, -Rf, -Ro, -Rg2, -R1, -Ra1, -F1, -Rd, -compound K, -Rh2, -Rh4, gypenoside XVII).

In the results, five LABs did not produce the other minor ginsenosides except ginsenoside Rg3 and Rg5 (Figs. 1A and 1B). The content of ginsenoside Rg3 increased in *L. mesenteroides* WiKim19 (5.2 ± 1.1 μ g/mL), *P. pentosaceus* WiKim20 (4.5 ± 0.9 μ g/mL) and other lactic acid bacteria produced from $\sim 0.8 \pm 0.4$ μ g/mL to 1.1 ± 0.6 μ g/mL. Interestingly, we detected the ginsenoside Rg5 from five LABs ranged from 2.6 ± 0.7 μ g/mL to 6.5 ± 3.0 μ g/mL concentration at 7 d of incubation. *L. mesenteroides* WiKim19 (6.5 ± 3.0 μ g/mL) and *P. pentosaceus* WiKim20 (4.6 ± 0.8 μ g/mL) showed the higher ginsenoside Rg5 content among the five LABs (Fig. 2B).

It is reported that bacterial β -glucosidase activity has been particularly involved among many glycosyl hydrolases to transform ginsenoside Rb1, which removes two glucose molecules at 20-C of protopanaxadiol into ginsenoside Rg3 [39]. To determine whether β -glucosidase influences the bioconversion of ginsenoside, we tested the β -glucosidase enzyme activity. *L. mesenteroides* WiKim19, which used diverse glycosidic linkage substrates (Table 1), showed the highest β -glucosidase activity supporting the maximum bioconversion capacity. *L. lactis* WiKim48 and *L. sakei* WiKim49 exhibited the reduced productions of Rg3 along with the

Table 1

Distinctive features of the carbohydrate fermentation profiles of five LABs determined using API 50 CH (bioMérieux, Lyon, France)

Characteristic	<i>Lactobacillus brevis</i> WiKim47	<i>Pediococcus pentosaceus</i> WiKim20	<i>Leuconostoc Lactis</i> WiKim48	<i>Lactobacillus sakei</i> WiKim49	<i>Leuconostoc mesenteroides</i> WiKim19
Glycerol	–	w	–	–	–
Erythritol	–	w	–	–	–
–D-arabinose	–	w	–	–	–
L-Arabinose	+	+	+	+	+
D-Ribose	+	+	+	+	+
D-Xylose	+	+	+	–	+
D-Galactose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Mannitol	w	–	–	–	+
D-Sorbitol	–	–	–	–	+
Methyl- α -D-glucopyranoside	+	–	+	–	+
N-Acetylglucosamine	+	+	+	+	+
Amygdalin	–	+	–	–	+
Arbutin	–	+	+	–	+
Esculin ferric citrate	–	+	+	+	+
Salicin	–	+	+	+	+
D-Cellobiose	–	+	+	+	+
D-Maltose	+	+	+	–	+
D-Lactose (bovine origin)	+	–	–	+	+
D-Melibiose	+	+	+	+	+
D-Saccharose (sucrose)	+	+	+	+	+
D-Trehalose	–	+	+	+	+
Inulin	–	+	–	–	+
D-Melezitose	–	–	–	–	+
D-Raffinose	+	+	+	–	+
Amidon (starch)	–	–	w	–	–
Gentiobiose	–	+	–	–	+
D-Turanose	–	–	+	–	+
D-Tagatose	–	+	–	–	+
D-Arabitol	–	–	–	–	–
Potassium gluconate	–	–	+	w	+
Potassium 2-ketogluconate	–	w	–	–	+

–, negative; +, positive; 1, *Leuconostoc mesenteroides* WiKim19; Strain 2, *Pediococcus pentosaceus* WiKim20; Strain 3, *Lactobacillus brevis* WiKim47; Strain 4, *Leuconostoc lactis* WiKim48; Strain 5, *Lactobacillus Sakei* WiKim49; w, weak reaction

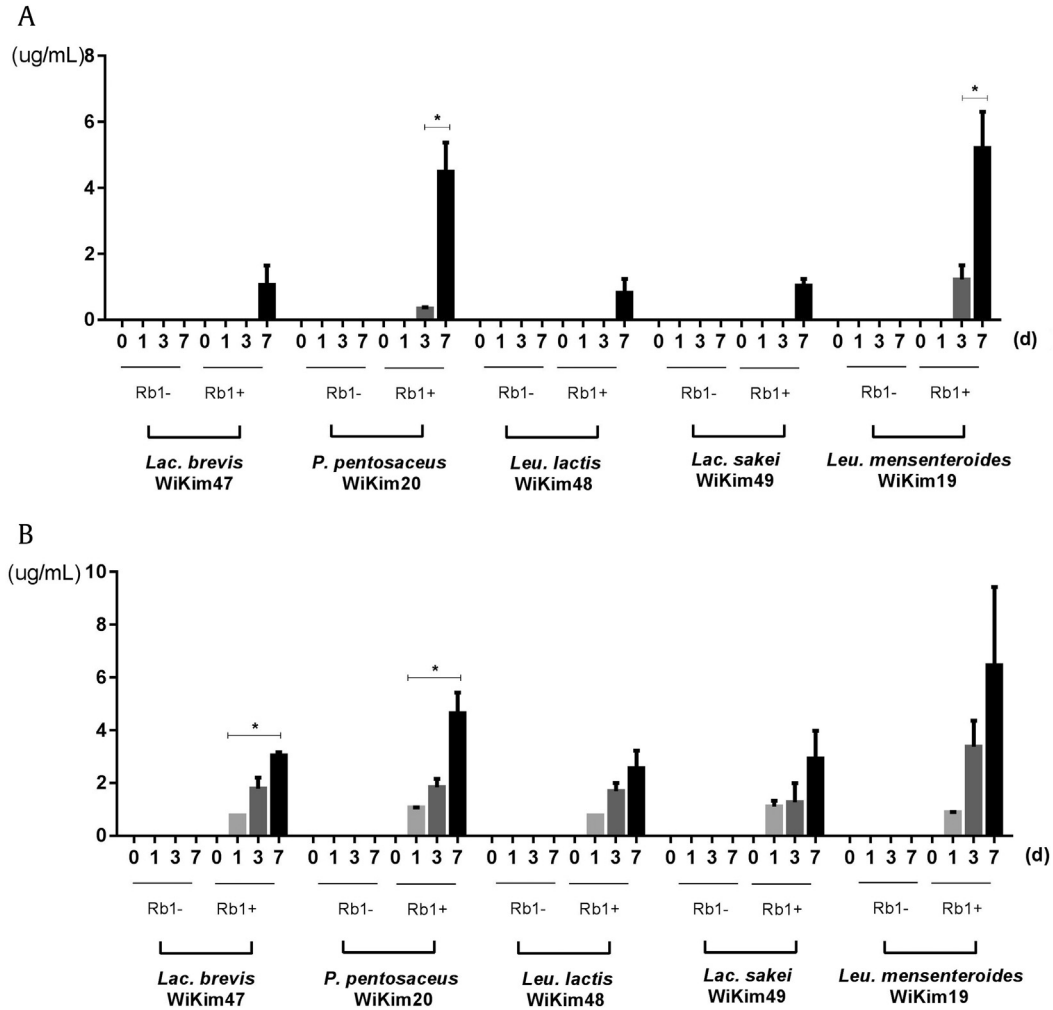


Fig. 2. Comparison of ginsenoside Rb1 bioconversion into Rg3 in cell culture supernatant of *Leu. mesenteroides* WiKim19, *P. pentosaceus* WiKim20, *Lac. brevis* WiKim47, *Leu. lactis* WiKim48 and *Lac. sakei* WiKim49. Concentrations of ginsenoside Rg3 (A) and Rg5 (B) after 1 d, 3 d, and 7 d incubation of each LAB strain with Rb1 at a concentration of 200 μ M. Data are presented as mean \pm standard deviation. * $p < 0.05$.

low β -glucosidase activities. Interestingly *P. pentosaceus* WiKim20 showed an elevated production of Rb3 in spite of lack of β -glucosidase activity (Fig. 3).

A recent study reported that the conversion of ginsenoside Rb1 into Rg3 and Rg5 with organic acid such as D-, L-tartaric acid, citric acid, and acetic acid [40]. Under acidic conditions, the low pH environment enhances the deglycosylation of ginsenoside by acidic hydrolysis response. Lactic acid bacteria are well known for their

organic acid production, which lowered the pH during the fermentation and help the long-term storage by preventing contamination. Even with varying degrees of acidity among the LABs, all culture showed the acidic property and increased accordance to the growth of bacteria.

To determine whether media acidity influences the bioconversion of ginsenoside, we measured pH and acidity of bacterial cultures (Figs. 4A and 4B). The culture supernatant of *P. pentosaceus* WiKim20 had a pH \sim 3.8, and acidity is 1.9, while *L. mesenteroides* WiKim19 had pH 4.4, and acidity is 1.1. The β -glucosidase activity and ginsenoside Rg3 production was correlated with *L. mesenteroides* WiKim19 which have higher enzyme activity and relatively low acidity among the five LABs. The elevated Rg3 production of *P. pentosaceus* WiKim20 could be explained by acidic hydrolysis instead of β -glucosidase activity. The five LABs could produce the ginsenoside Rg5 and this increase did not show the correlation with β -glucosidase enzymatic activity, supporting the theory that the organic acid could contribute to the production of Rg5.

In the present study, we monitored the ginsenoside Rb1 bioconversion in LABs isolated from Kimchi with sensitive and accurate LC-MS/MS techniques. Among them, *L. mesenteroides* WiKim19 transformed ginsenoside Rb1 into ginsenoside Rg3 and showed a correlation with enzymatic activity, whereas,

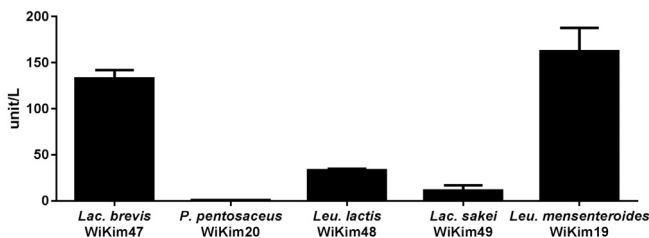


Fig. 3. Comparison of β -glucosidase activity in different LABs. The proteins were diluted to the concentration of \sim 0.5 mg/mL to assay enzyme activity and the release of *p*-nitrophenol was measured at 405 nm. One-tailed Student *t* test for unpaired samples was used to assess significance of the differences between the three LABs. One unit of β -glucosidase is the amount of enzyme that catalyzes the hydrolysis of 1.0 μ mole substrate per min at pH 7.0.

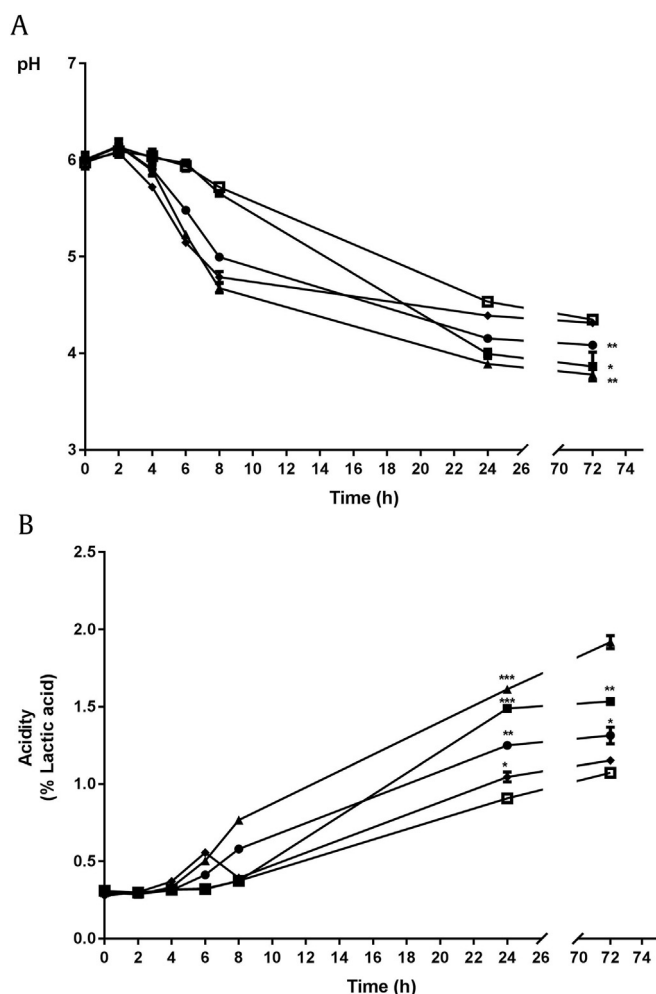


Fig. 4. pH (A) and acidity (B) during bioconversion of ginsenoside in different LABs. The pH of 10 mL of each culture broth was measured using electrode pH meter and acidity was measured by titration against 0.1N NaOH to a phenolphthalein endpoint, pH 8.3. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$. ■ *Lactobacillus brevis* WiKim47; ▲ *Pediococcus pentosaceus* WiKim20; ◆ *Leuconostoc lactis* WiKim48; ● *Lactobacillus sakei* WiKim49; □ *Leuconostoc mesenteroides* WiKim19.

P. pentosaceus WiKim20 showed an elevated production of Rb3 in spite of lack of β -glucosidase activity but have the highest acidity among the five LABs. This result suggests that ginsenoside bioconversion by microorganisms might review with the enzyme activities and the environmental conditions such as production of organic acid. Also, lactic acid bacteria are useful to convert minor ginsenoside by enzymatic conversion together with mild acidic condition.

Conflicts of interest

The authors declare that there is no conflicts of interest.

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