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Research article

Biotransformation of major ginsenosides in ginsenoside model culture by lactic acid bacteria

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ABSTRACT

Background: Some differences have been reported in the biotransformation of ginsenosides, probably due to the types of materials used such as ginseng, enzymes, and microorganisms. Moreover, most microorganisms used for transforming ginsenosides do not meet food-grade standards. We investigated the statistical conversion rate of major ginsenosides in ginsenosides model culture during fermentation by lactic acid bacteria (LAB) to estimate possible pathways.

Methods: Ginsenosides standard mix was used as a model culture to facilitate clear identification of the metabolic changes. Changes in eight ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg2) during fermentation with six strains of LAB were investigated.

Results: In most cases, the residual ginsenoside level decreased by 5.9–36.8% compared with the initial ginsenoside level. Ginsenosides Rb1, Rb2, Rc, and Re continuously decreased during fermentation. By contrast, Rd was maintained or slightly increased after 1 d of fermentation. Rg1 and Rg2 reached their lowest values after 1–2 d of fermentation, and then began to increase gradually. The conversion of Rd, Rg1, and Rg2 into smaller deglycosylated forms was more rapid than that of Rd from Rb1, Rb2, and Rc, as well as that of Rg1 and Rg2 from Re during the first 2 d of fermentation with LAB.

Conclusion: Ginsenosides Rb1, Rb2, Rc, and Re continuously decreased, whereas ginsenosides Rd, Rg1, and Rg2 increased after 1–2 d of fermentation. This study may provide new insights into the metabolism of ginsenosides and can clarify the metabolic changes in ginsenosides biotransformed by LAB.

1. Introduction

Ginseng (*Panax ginseng* Meyer) is a traditional herbal medicine that has been used for a long time in Asia because of its numerous medicinal functions [1] and its use continues to increase worldwide. Ginseng contains various bioactive components, including ginsenosides (ginseng saponins), acidic polysaccharides, polyphenols, and polyacetylenes [2]. Among these components, ginsenosides, which are glycosides with steroids or triterpenes as aglycons, have been highly characterized for their biological activities. To date, more than 50 ginsenosides have been identified and are divided into two major categories based on their chemical structures: (1) protopanaxadiol [PPD, aglycone (20S)-protopanaxadiol], which includes ginsenosides Rb1, Rb2, Rc, Rd, Rg3, compound K (CK), and Rh2, and (2) protopanaxatriol [PPT, aglycone (20S)-protopanaxatriol], which includes ginsenosides Re, Rf, Rg1, Rg2, and Rh1 [3]. Among those, six major ginsenosides (Rb1, Rb2, Rc, Rd, Re, and Rg1) have been determined to account for 90% of the total ginsenoside content of *P. ginseng* Meyer [4].

The sugar chains of ginsenosides were found to be closely related to their functionality. The conversion of ginsenosides into smaller deglycosylated forms may therefore markedly change the biological activity especially effective for *in vivo* physiological actions [5]. For example, CK transformed from ginsenosides Rb1, Rb2, and Rc was reported to demonstrate improved anti-inflammatory and antitumor effects [6,7]. Therefore, the transformation of these major ginsenosides (Rb1, Rb2, Rc, Rd, Re, and Rg1) into smaller deglycosylated ginsenosides is necessary. Many studies have

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focused on the conversion of major ginsenosides into more active deglycosylated forms using physicochemical transformation methods such as heating and acid treatment [8,9]. By contrast, biological approaches such as microbial or enzymatic methods have been proposed, owing to their marked selectivity, mild reaction conditions, environmental compatibility, and convenience for industrial applications [10,11]. Many studies have identified the biotransformation of ginsenosides into smaller deglycosylated forms such as Rb1 → Rd, F2, Rg3, CK [12], Re, Rb1, Rc → Rg1, Rd, CK [13], Rb1 → Rd [1], and Rb1 → Rd, Rg3 [10]. The structures and possible biotransformation pathways of PPD- and PPT-type ginsenosides by microbial or enzymatic methods are shown in Fig. 1. The specific pathways of the PPD and PPT ginsenosides were indicated based on published information (see Table S1 in Supplementary Material online). Some differences in the biotransformation of ginsenosides have been reported according to the biological methods used; however, these differences may also be due to the types of materials used such as ginseng, enzymes, and microorganisms. Moreover, most microorganisms used for biological transformation do not satisfy food-grade standards [14].

Characterization of the biotransformation of ginsenosides during fermentation is needed to explain the pharmacological actions of fermented ginseng. However, most reports only deal with one or a few ginsenosides, ignoring the full action of ginsenosides. Moreover, the ginsenosides levels in ginseng plants are influenced by a range of factors such as the species, age, part of the plant, cultivation method, harvesting season, and preservation method [15]. It can be difficult to clearly identify the comprehensive ginsenoside transformation, probably due to the chemical complexity of the metabolites, the lack of reference standards, experimental error during the extraction of ginsenosides, or the limitations inherent in analytical methods [16]. Therefore, the investigation of targeted changes in major ginsenosides during fermentation with selected microorganisms is needed [17].

In this paper, the biotransformation in ginsenosides using food-compatible microorganisms [lactic acid bacteria (LAB)] was identified and quantified. A standard mix of ginsenosides containing eight major ginsenosides was used as a model culture to facilitate clear identification of the metabolic changes.

2. Materials and methods

2.1. Materials

Ginseng ginsenosides mix, including Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg2 (100 μg/mL of each component in methanol), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (1 mL) was evaporated under vacuum using liquid nitrogen, and diluted with 100 mL of distilled water for use as a ginsenosides model culture (GMC) for LAB.

2.2. Fermentation

Six LAB strains were selected for this study based on β-glucosidase activity as previously reported [18,19]. *Lactobacillus plantarum* KCCM11322, *Lactobacillus delbrueckii* subsp. *bulgaricus* KCCM35463, *Lactobacillus fermentum* KCCM40401, and *Bifidobacterium longum* KCCM11953 were purchased from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea). *L. delbrueckii* subsp. *lactis* KCTC 1058 and *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC 3718 were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). The strain was inoculated into Man–Rogosa–Sharpe (MRS, Difco, Sparks, MD, USA) broth and grown at 37°C for 48 h to obtain a final cell count of 10^8 CFU/mL. GMC was produced using ginseng ginsenosides mix diluted with distilled water by supplementing MRS broth (5.5%) with the LAB nutrient. GMC was inoculated with 1% (vol/vol) of LAB starter culture and fermentation was performed at 30°C for 5 d. Samples were taken on the 1st, 2nd, 3rd, and 5th days of fermentation for chemical and microbiological analyses.

2.3. Microbiological analysis

One milliliter of GMC was aseptically transferred into a conical tube prior to the preparation of 1/10 serial dilutions for microbiological analysis. LAB count was determined after growing the LAB in MRS agar and incubating at 37°C for 48 h. Tests were carried out in duplicate and the results were expressed as log CFU/g.

2.4. Physicochemical analysis

After centrifuging for 5 min at 12,000 rpm, the supernatant liquid was filtered through a 0.45-μm membrane (Whatman No. 2) and used in all of the test systems. The total soluble solids (degrees Brix) in GMC were measured using a digital refractometer (PR-32, Atago, Tokyo, Japan) with temperature compensation. pH was determined using a pH meter (pH-250L, ISTEK, Seoul, Korea) and the means of three measurements were recorded. Titratable acidity (TA) as lactic acid was determined by titrating to pH 8.3 with 0.1N NaOH. Reducing sugars were measured according to the 3,5-dinitrosalicylic acid (DNS) method [20]. Reaction mixtures contained 3 mL of the sample and 3 mL of the DNS reagent. Glucose solution was used for the standard curve. The reaction mixture was heated in a water bath for 5 min to develop a red-brown color and was then cooled to room temperature in a water-ice bath. Subsequently, 1 mL of a 40% potassium sodium tartrate solution was added to stabilize the color. The absorbance was measured at 540 nm using a spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan).

2.5. β-glucosidase activity

Beta-glucosidase activity was evaluated via the colorimetric method using *p*-nitrophenyl-β-D-glucopyranoside (pNPG) as a substrate [10]. The reaction mixture, which contained 5mM of pNPG in 1 mL of sodium phosphate buffer (pH 7.0) and 100 μL of enzyme solution, was incubated at 30°C for 10 min. The reaction was subsequently terminated by adding 1 mL of 0.5M NaOH, and the absorption of the released *p*-nitrophenol (PNP) was measured against the prepared reagent blank at 400 nm using a spectrophotometer. One unit of β-glucosidase activity was defined as the quantity of enzyme required to liberate 1 μM of pNP/min under standard conditions.

2.6. Ginsenosides analysis

Eight ginsenoside peaks were detected by liquid chromatography (Agilent 1200 Series) coupled with 6410A triple quadrupole mass spectroscopy (Agilent, Santa Clara, California, USA). Samples were ionized and detected by electrospray ionization–mass spectrometry with the selected ion-monitoring mode of negative ions. The nebulizer gas was set to 10 L/min at a temperature of 320°C and the capillary voltages were set to 4 kV. Separation was achieved using the XDB-C_{18} column (50 mm × 4.6 mm i.d., 1.8 μm; Agilent) with a column oven temperature of 35°C. The mobile phase was composed of (A) 5mM ammonium acetate–formic acid (0.1%, vol/vol) and (B) methanol. The mobile phase B was kept at 50% for 2 min and then gradually increased to 90% for 25 min. The flow rate was kept at 0.35 mL/min, and 5 μL of the sample solution was injected in each run.

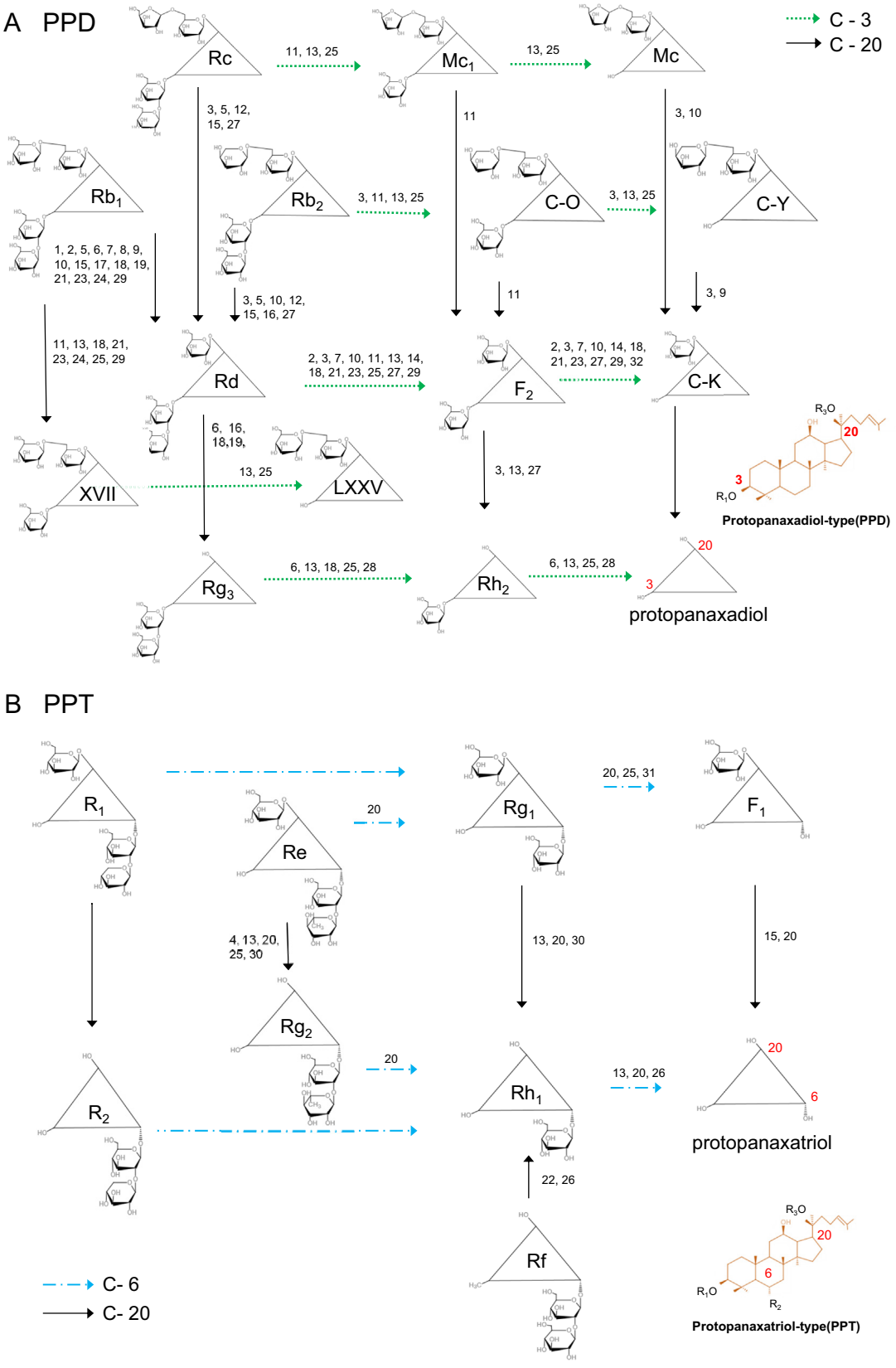

Fig. 1. The structures and possible biotransformation pathways of (A) protopanaxadiol (PPD)- and (B) protopanaxatriol (PPT)-type ginsenosides by microbial or enzymatic methods. The pathways of ginsenosides were drawn based on published information and the reference numbers are indicated in Table S1 (see Supplementary Material online). The fundamental chemical structures of ginsenosides were simply expressed to highlight their conversion into smaller deglycosylated forms.

2.7. Statistical analysis

Statistical analyses were performed using the SPSS version 14.0 statistical package for windows (SPSS Inc., Chicago, IL, USA). Analysis of variance and Duncan multiple range tests were applied to the data to determine significant differences, and a value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Microbiological analysis

The structures and possible biotransformation pathways of PPD- and PPT-type ginsenosides by microbial or enzymatic methods are shown in Fig. 1. The specific pathways of the PPD and PPT ginsenosides were indicated based on published information (see Table S1 in Supplementary Material online). Some differences have been reported in the biotransformation of ginsenosides according to the types of materials used such as ginseng, enzymes, and microorganisms. We investigated the statistical conversion rate of major ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg2) in GMC during fermentation by LAB to estimate the possible pathways involved.

Fig. 2 shows the growth kinetics of LAB during fermentation in GMCs inoculated with six strains of LAB. Each batch was inoculated with 1% LAB by volume, with a final cell count of 8–9 log CFU/mL. GMCs contained about 9–11 log CFU/mL on the 1st day of fermentation, which increased slightly on the 2nd day of fermentation, with the exception of one starter culture (*L. fermentum*). Afterward, the number of viable cells in GMCs began to decrease and the cell population reached 6–7.5 log CFU/mL in all tested samples after 5 d of fermentation. Although similar, the population growth pattern displayed by LAB in GMCs showed several variations in the inoculated strains.

3.2. Physicochemical analysis

The reducing sugar contents before fermentation of the GMCs was 550 mg/L. The reducing sugars were depleted to 20–25 mg/L by all strains except for *L. delbrueckii* subsp. *bulgaricus* (68 mg/L, data not shown). These results confirmed that fermentable sugar was almost exhausted within the 5 d of fermentation. The pH and the TA of GMCs fermented with the six LAB strains are presented in Fig. 3. The pH of GMCs before inoculation was 6.37 and the value decreased during fermentation. Although the viable LAB numbers

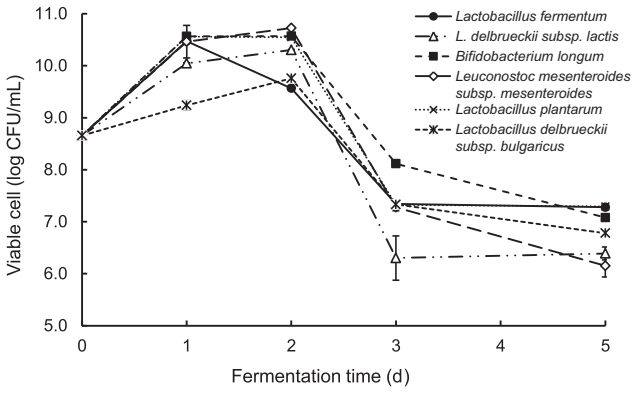

Fig. 2. Growth kinetics of lactic acid bacteria (LAB) during fermentation in ginsenosides model cultures inoculated with six LAB strains. Each batch was inoculated with 1% LAB by volume, with a final cell count of 8–9 log CFU/mL. Values are expressed as the mean ± standard deviation ($n = 3$).

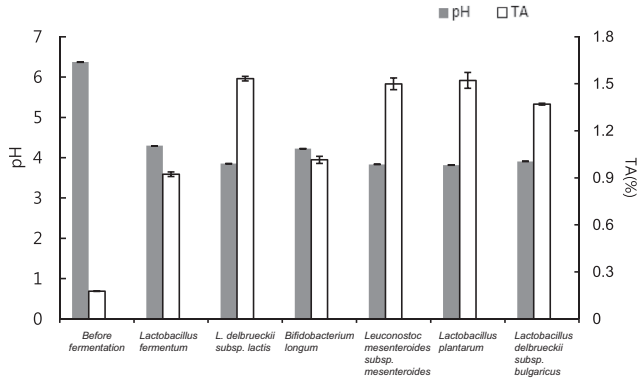

Fig. 3. pH and titratable acidity (TA) of ginsenosides model cultures fermented with six lactic acid bacteria strains. TA was expressed as percent lactic acid. Values are expressed as the mean ± SD ($n = 3$).

in the GMCs inoculated with *L. fermentum* and *B. longum* were relatively higher than those in other samples during fermentation, the pH values were significantly higher at 4.2–4.3 compared with the other GMCs (3.8–3.9). In general, the TA was inversely proportional to the pH value. As expected, the GMCs inoculated with *L. fermentum* and *B. longum* had lower TA values, with final values of 0.92–1.02%. GMCs inoculated with other strains had significantly higher TA value of 1.37–1.53%. It may be that the pH reduction was due to LAB rapidly becoming the predominant microorganism, producing lactic acid and leading to pH reduction.

3.3. β-glucosidase activity

The β-glucosidase activity of GMCs fermented with six LAB strains are listed in Fig. 4. As the fermentation progressed, β-glucosidase activity of all GMCs increased and peaked at 2 d of fermentation. Afterward, the β-glucosidase activity in the samples began to decrease and reached 74–109 U in all tested samples on the 5th day of fermentation. Although β-glucosidase activity was lower compared with the 2nd day of fermentation, its activity in the GMCs inoculated with *L. mesenteroides* and *L. plantarum* stabilized or decreased slowly by the 5th day of fermentation. This indicated that a fermentation period longer than 5 d was needed for the biotransformation of ginsenosides. By contrast, a large and rapid decrease in β-glucosidase activity was observed in the GMCs inoculated with *L. fermentum* and *B. longum*. The β-glucosidase activity pattern displayed by *L. delbrueckii* subsp. *bulgaricus* in GMC was significantly lower than other tested samples during fermentation. Because of the special structure of the dammarane skeleton,

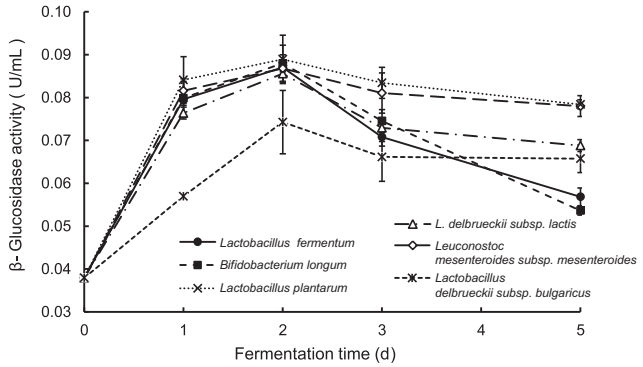

Fig. 4. β-Glucosidase activity of ginsenosides model cultures fermented with six lactic acid bacteria strains. Values are expressed as the mean ± SD ($n = 3$).

only specific β-glucosidases hydrolyze ginsenoside-β-glucoside linkages [14]. To date, several β-glucosidases producing LABs have been reported [21–23]. Yan et al [24] purified seven β-glucosidase fractions from *Paecilomyces* Bainier sp. 229, and found that the biotransformation pathway of Rb1 to CK by each fraction was different. Specific bioconversion of ginsenoside Rb1 into various ginsenosides may be possible using the appropriate combination of specific microbial enzymes.

3.4. Biotransformation of ginsenosides

The residual ginsenoside levels on the 5th day of fermentation are shown in Table 1. In most cases, the residual ginsenoside level decreased by 5.9–36.8% compared with the initial value. Among PPD-type ginsenosides, ginsenoside Rd was at the highest level after 5 d of fermentation. Rd is the major ginsenoside in the final fermentation product [13], and it can be converted from Rb1, Rb2, and Rc by the removal of sugar (Fig. 1). We, therefore, suggest that the relatively higher level of Rd was due to the conversion of Rb1, Rb2, and Rc during fermentation. The time courses of ginsenosides concentrations during fermentation are shown in Fig. 5. These results confirmed that Rb1, Rb2, and Rc decreased continuously during the 5 d of fermentation. By contrast, Rd was maintained or slightly increased after 1 d of fermentation. In particular, a dramatic increase in Rd was observed in the GMC inoculated with *L. delbrueckii* subsp. *bulgaricus*, which had relatively low β-glucosidase activity during fermentation. Many studies have been conducted on the biotransformation of the ginsenoside Rb1 into Rd during fermentation [1,10,24]. However, Rd can be continuously converted into CK by cell extracts from various food-grade edible microorganisms including *L. mesenteroides* [25] and *Lactobacillus paralimentarius* [26]. We confirmed that Rd decreased during the first 2 d of fermentation, suggesting that it can be converted more quickly into smaller deglycosylated forms such as Rg3, Rh2, F2, and CK [27,28]. Ye et al [1] reported that Rd yields from Rb1 were low due to the inhibition of Rd production by other ginsenosides, low substrate tolerance, requirement for pure substrates, and further transformation of Rd into other compounds. In this paper, we used the same concentration of ginsenosides standard (1 ppm) as a culture to investigate the reaction rates related to the conversion of ginsenosides into their smaller deglycosylated forms. In our model culture, it appeared that the conversion rate of Rd into its smaller deglycosylated form was more rapid than that of Rd from Rb1, Rb2 and Rc.

Among PPT-type ginsenosides, a low level of Re was observed in the GMCs after fermentation (Table 1). The decrease in Re concentration during fermentation is in agreement with other studies, but to varying degrees according to the LAB strain. The evolution of Rg1 and Rg2 contents in the GMC inoculated with LAB was very different from the other ginsenosides (Fig. 5). Unlike other ginsenosides, Rg1 and Rg2 reached their lowest levels after 1–2 d of fermentation and then increased gradually. In all cases, Rg1 and Rg2 levels in GMCs fermented with LAB were significantly higher than in other samples after fermentation, indicating that Rg1 and Rg2 were intermediate products of Re and that the bioconversion rate of Re into deglycosylated forms such as F1 and Rh1 was slower than those of other ginsenosides. Many studies have reported a decrease in Re and an increase in Rg2 during fermentation. However, the opposite was reported for Rg1 change during fermentation. Hsu et al [13] showed that after 30 d of fermentation with *Ganoderma lucidum*, ginsenoside Rg1 increased significantly, whereas ginsenoside Re decreased during fermentation. Wang et al [16] reported that Re and Rg1 were decreased slightly, whereas Rg2 and Rh1 were increased in red ginseng extract fermented by human intestinal microflora. The contrast in Rg1 changes during fermentation seems to be due to differences in bioconversion conditions such as microorganism and microbial enzymes. To avoid variability in the preparations, this research used commercially available ginsenoside mix as a model culture, and the time courses of ginsenoside concentrations during fermentation with LAB were studied. In our model system, the conversion rate of Rg1 and Rg2 during fermentation may have been similar because we added the same concentration of ginsenosides standard (1 ppm) before fermentation. Moreover, we confirmed that the conversion rate of Rg1 and Rg2 into smaller deglycosylated forms such as F1 and Rh1 was faster than the conversion rate from Re during the first 2 d of fermentation by LAB.

Although ginsenoside Rf is known to have higher chemical stability than other ginsenosides, it was reported that Rf transformed into ginsenoside Rg9 through a dehydration reaction under thermal and acidic conditions [29]. However, this transformation is not due to the removal of sugar from Rf leading to transformation into a smaller deglycosylated form. Rf does not have the readily hydrolytic sugar bonded to the hydroxyl at 21-C in its dammarane skeleton [30]. In this study, it may be that Rf was converted into other forms by LAB, but high variability was found among the samples.

The time courses of ginsenoside concentrations during fermentation with LAB were studied and commercially available ginsenoside mix was used as a model culture for the clear identification of the metabolic changes in ginsenosides. Among PPD-type ginsenosides, although ginsenosides Rb1, Rb2, and Rc decreased

Table 1
The residual ginsenoside contents of ginsenosides model cultures after 5 days of fermentation with six lactic acid bacteria strains

Ginsenosides (ppm)		Microorganisms						
		Lactobacillus fermentum	*Lactobacillus delbrueckii* subsp. *lactis*	*Bifidobacterium longum*	*Leuconostoc mesenteroides*	*Lactobacillus plantarum*	*L. delbrueckii* subsp. *bulgaricus*	Average
PPD	Rb1	0.772 ± 0.025^{Bcd} [1)]	0.624 ± 0.010^{Aab}	0.579 ± 0.089^{Aa}	0.797 ± 0.005^{Bab}	0.597 ± 0.041^{Abc}	0.736 ± 0.017^{Ba}	0.685
	Rb2	0.682 ± 0.064^{BCbc}	0.569 ± 0.014^{ABa}	0.607 ± 0.007^{ABCa}	0.708 ± 0.069^{BCa}	0.506 ± 0.012^{Aab}	0.719 ± 0.099^{Ca}	0.632
	Rc	0.676 ± 0.072^{bc}	0.597 ± 0.012^{a}	0.630 ± 0.007^{a}	0.683 ± 0.065^{a}	0.569 ± 0.061^{bc}	0.703 ± 0.069^{a}	0.643
	Rd	0.589 ± 0.103^{Ab}	0.741 ± 0.091^{ABabc}	0.820 ± 0.114^{BCab}	0.817 ± 0.075^{BCabc}	0.672 ± 0.053^{Ac}	1.022 ± 0.069^{Cb}	0.777
PPT	Re	0.728 ± 0.029^{ABbc}	0.723 ± 0.150^{ABabc}	0.632 ± 0.067^{Aa}	0.884 ± 0.112^{Bbc}	0.661 ± 0.053^{Ac}	0.804 ± 0.017^{ABab}	0.739
	Rg1	0.912 ± 0.070^{ABd}	0.863 ± 0.003^{Abc}	0.905 ± 0.008^{ABb}	0.959 ± 0.002^{Bc}	0.841 ± 0.027^{Ad}	0.954 ± 0.021^{Bab}	0.906
	Rg2	0.905 ± 0.065^{Ad}	0.905 ± 0.007^{Ac}	0.971 ± 0.007^{ABb}	0.947 ± 0.013^{ABc}	0.917 ± 0.026^{Ad}	0.999 ± 0.003^{Bb}	0.941
	Rf	0.423 ± 0.008^{Aa}	1.150 ± 0.214^{Bd}	0.783 ± 0.239^{ABab}	0.724 ± 0.018^{ABa}	0.386 ± 0.097^{Aa}	0.693 ± 0.260^{ABa}	0.693
Total		5.691 ± 0.029	6.176 ± 0.464	5.932 ± 0.573	6.522 ± 0.091	5.152 ± 0.103	6.634 ± 0.075	

Values with different small letters in a column and capital letters in a row are significantly different at $p < 0.05$
PPD, protopanaxadiol; PPT, protopanaxatriol
[1)] Mean ± SD ($n = 3$)

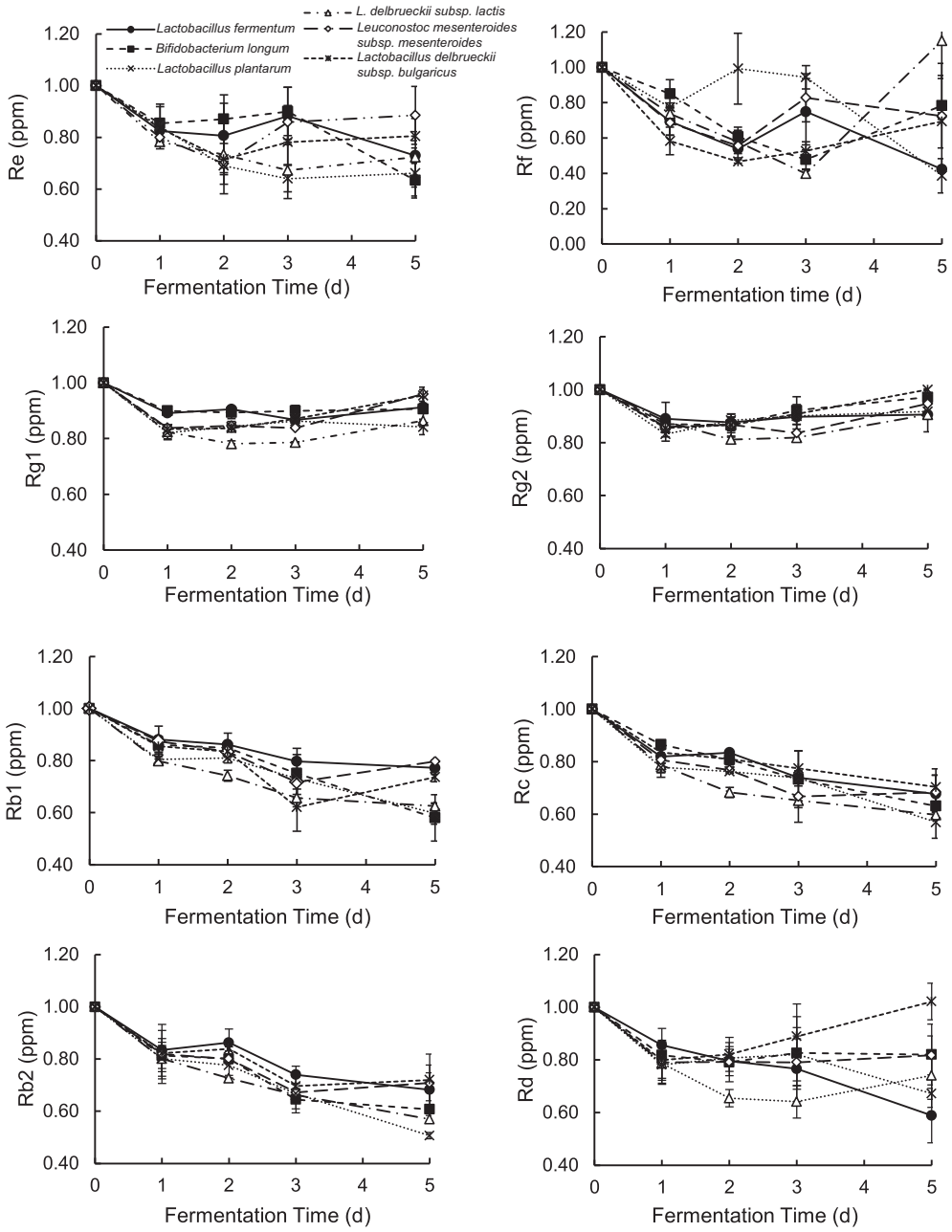

Fig. 5. The time courses of ginsenosides concentrations during fermentation with six lactic acid bacteria strains. Ginseng ginsenosides mix, including Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg2 (1 ppm of each component in distilled water), was used as a model culture to facilitate clear identification of the metabolic changes. Values are expressed as the mean ± SD ($n = 3$).

continuously, Rd was maintained or increased slightly after 1 d of fermentation, suggesting that the conversion rate of Rd into smaller deglycosylated forms was more rapid than that of Rd from Rb1, Rb2, and Rc. Among PPT-type ginsenosides, Re decreased continuously, whereas ginsenosides Rg1 and Rg2 increased after 1–2 d of fermentation, indicating that these ginsenosides were the intermediate products of Re, and that the bioconversion of Re into smaller deglycosylated forms was faster than the conversion rate from Re during the first 2 d of fermentation by LAB.

Conflicts of interest

The authors have no conflicts of interest with any parties or individuals.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgr.2015.12.008.

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