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Research article

Metabolite profiling of fermented ginseng extracts by gas chromatography mass spectrometry

Seong-Eun Park [1], Seung-Ho Seo [1], Kyoung In Lee [2], Chang-Su Na [1], Hong-Seok Son [1,*]

[1] School of Korean Medicine, Dongshin University, Naju, Republic of Korea
[2] Biotechnology Industrialization Center, Dongshin University, Naju, Republic of Korea

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ABSTRACT

Background: Ginseng contains many small metabolites such as amino acids, fatty acids, carbohydrates, and ginsenosides. However, little is known about the relationships between microorganisms and metabolites during the entire ginseng fermentation process. We investigated metabolic changes during ginseng fermentation according to the inoculation of food-compatible microorganisms.
Methods: Gas chromatography mass spectrometry (GC-MS) datasets coupled with the multivariate statistical method for the purpose of latent-information extraction and sample classification were used for the evaluation of ginseng fermentation. Four different starter cultures (*Saccharomyces bayanus, Bacillus subtilis, Lactobacillus plantarum,* and *Leuconostoc mesenteroide*) were used for the ginseng extract fermentation.
Results: The principal component analysis score plot and heat map showed a clear separation between ginseng extracts fermented with *S. bayanus* and other strains. The highest levels of fructose, maltose, and galactose in the ginseng extracts were found in ginseng extracts fermented with *B. subtilis.* The levels of succinic acid and malic acid in the ginseng extract fermented with *S. bayanus* as well as the levels of lactic acid, malonic acid, and hydroxypruvic acid in the ginseng extract fermented with lactic acid bacteria (*L. plantarum* and *L. mesenteroide*) were the highest. In the results of taste features analysis using an electronic tongue, the ginseng extracts fermented with lactic acid bacteria were significantly distinguished from other groups by a high index of sour taste probably due to high lactic acid contents.
Conclusion: These results suggest that a metabolomics approach based on GC-MS can be a useful tool to understand ginseng fermentation and evaluate the fermentative characteristics of starter cultures.

1. Introduction

Ginseng (*Panax ginseng* Meyer), a traditional medicinal herb in Asia, has been used for a long time due to its various medicinal functions [1], such as anticancer [2], anti-inflammatory [3], and antidepressant effects [4]. Many studies have focused on the conversion of ginsenosides, which are 30-carbon glycosides derived from the triterpenoid dammarane, into more active deglycosylated forms using microbial or enzymatic methods to change the biological activity. The conversion of ginsenosides into smaller deglycosylated forms changes the biological activities, such as the antiallergic [5] and antidiabetic effects [6].

As ginsenosides have been highly characterized for their major active components [7], most reports only deal with some ginsenosides, ignoring the full action of metabolites. However, ginseng also contains many small metabolites such as amino acids, fatty acids, and carbohydrates [8,9]. The investigation of metabolic changes in ginseng during fermentation is important to evaluate the fermentation characteristics and assess the fermentative behaviors of microorganisms. As compounds in ginseng are not always easily detected by classical analysis, many metabolites should be analyzed, and powerful analytical methods for the determination of the metabolites are needed.

Metabolomics can directly delineate the physiological and biochemical status by its “metabolome profile” as a whole and therefore can provide systems information that differs from other traditional approaches [10]. There are a few existing reports regarding the fingerprinting or metabolic profiling of ginseng using analytical methods, such as proton nuclear magnetic resonance spectroscopy [9,11], high performance liquid chromatography

* Corresponding author. School of Korean Medicine, Dongshin University, 185, Geonjae-ro, Naju-si, Jeollanam-do 58245, Republic of Korea.
E-mail address: hsson@dsu.ac.kr (H.-S. Son).

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[12,13], and fourier transform infrared spectroscopy [14]. However, little is known about the relationships between microorganisms and metabolites during the entire ginseng fermentation process. Thus, this study was performed to investigate metabolic changes during ginseng fermentation according to the inoculation of food-compatible microorganisms. Gas chromatography mass spectrometry (GC-MS) datasets coupled with multivariate statistical methods for the purpose of latent-information extraction and sample classification were used to understand ginseng fermentation.

2. Materials and methods

2.1. Strain cultures

Lactobacillus plantarum KCCM 11322 was purchased from the Korean Culture Center of Microorganisms (KCCM, Seoul, Korea). *Leuconostoc mesenteroides* subsp. *mesenteroides* KCTC 3718 and *Bacillus subtilis* KCTC 2023 were purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). *Saccharomyces bayanus* EC-1118 (Lalvin, Montreal, Canada) was also used for comparison.

Before inoculation with ginseng extracts, strains were precultured at 37°C for 48 h in de Man Rogosa Sharpe broth (Difco, Sparks, MD, USA) for *L. plantarum* and *L. mesenteroides*, nutrient broth (Difco, Sparks, MD, USA) for *B. subtilis*, and yeast malt broth (Difco, Sparks, MD, USA) for *S. bayanus* to obtain a final cell count of above 10^7 CFU/mL.

2.2. Fermentation conditions using ginseng

Six-year-old ginseng roots with a disheveled-hair shape cultivated at Geumsan (Korea) were used in this study. Ginseng was hot-air-dried for 2 d and was subsequently powdered using a blender. Next, 15 g of ginseng powder was dissolved in 300 mL of distilled water and extracted at 121°C for 15 min by autoclaving. The mixture was then air-cooled to room temperature for inoculation. The ginseng extracts were inoculated with 1% (v/v) of each starter culture, and fermentation was performed at 30°C for 4 d. Samples were taken on the 1st, 2nd, and 4th days of fermentation for analyses. Measurements were performed in triplicate for each starter culture.

2.3. Viable cell counts

First, 1mL of ginseng was aseptically transferred into a conical tube prior to the preparation of 1/10 serial dilutions for microbiological analysis. *L. plantarum* and *L. mesenteroides* counts were determined after growing the bacteria in Man Rogosa Sharpe agar and incubating at 37°C for 48 h. The counts of *S. bayanus* and *B. subtilis* were determined in yeast malt agar and potato dextrose agar by incubation at 30°C for 48 h. Measurements were performed in triplicate, and the results were expressed as log CFU/mL.

2.4. pH and titratable acidity

After centrifuging for 5 min at 10,000 rpm, the supernatant liquid was used in all of the test systems. 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.

2.5. Ginsenoside analysis

Eight ginsenoside peaks were detected using liquid chromatography (Agilent 1200 Series) coupled with 6410A triple quadrupole mass spectroscopy (Agilent, USA). Samples were ionized and detected by electrospray ionisation mass spectrometry with the selected ion monitoring mode for negative ions. The following ions were extracted for the quantitative analysis of ginsenosides: m/z 784.5 (ginsenoside Rg_2), m/z 799.5 (ginsenoside Rf and Rg_1), m/z 945.6 (ginsenoside Re and Rd), m/z 1077.6 (ginsenoside Rb_2 and Rc), and m/z 1107.6 (ginsenoside Rb_1). The quantity of the ginsenosides was determined by comparison with the chromatogram of a standard mixture of the ginsenosides, purchased from Sigma-Aldrich (St Louis, MO, USA). The nebulizer gas was set to 10 L/min at a temperature of 320°C, and the capillary voltages were set to 4kV. Separation was achieved using a XDB-C18 column (50 mm × 4.6 mm i.d., 1.8 μm, Agilent, USA), with a column oven temperature of 35°C. The mobile phase was composed of (A) 5mM ammonium acetate-formic acid (0.1%, v/v) and (B) methanol. B was kept at 50% for

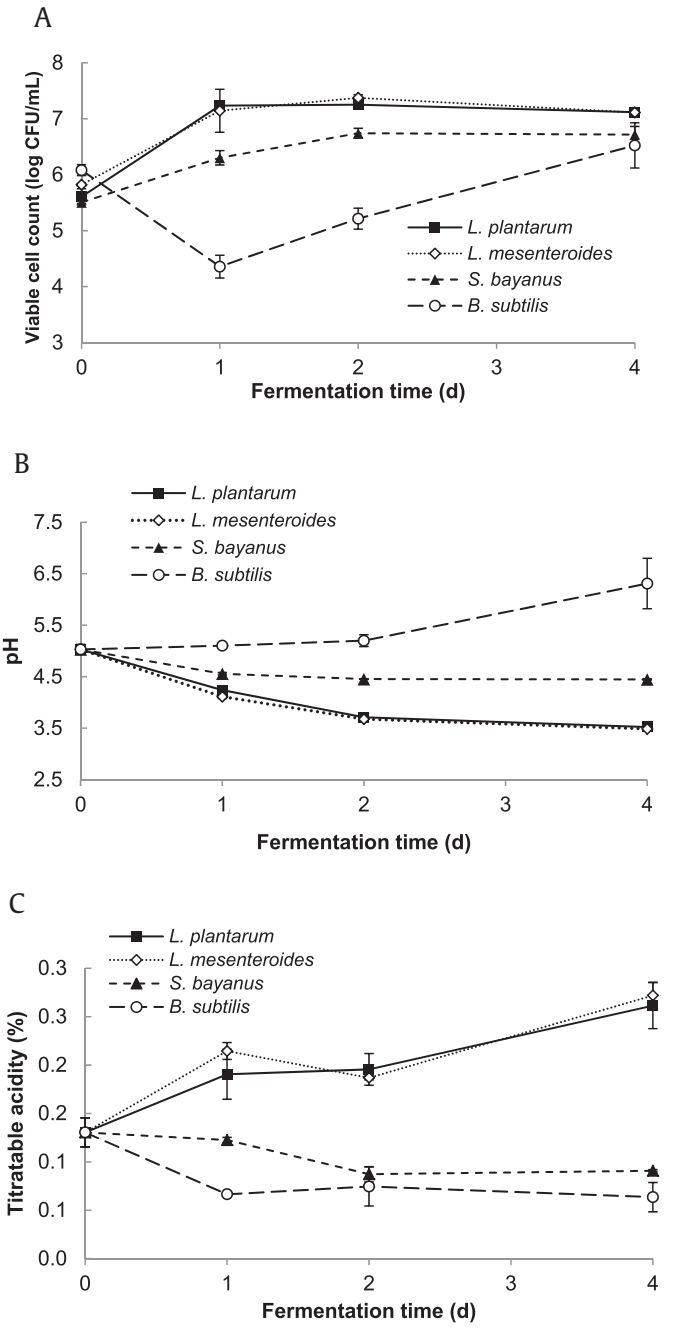

Fig. 1. (A) Changes in viable cell counts; (B) pH; and (C) titratable acidity in ginseng extract during fermentation. Values are expressed as the mean ± standard deviations ($n = 5$).

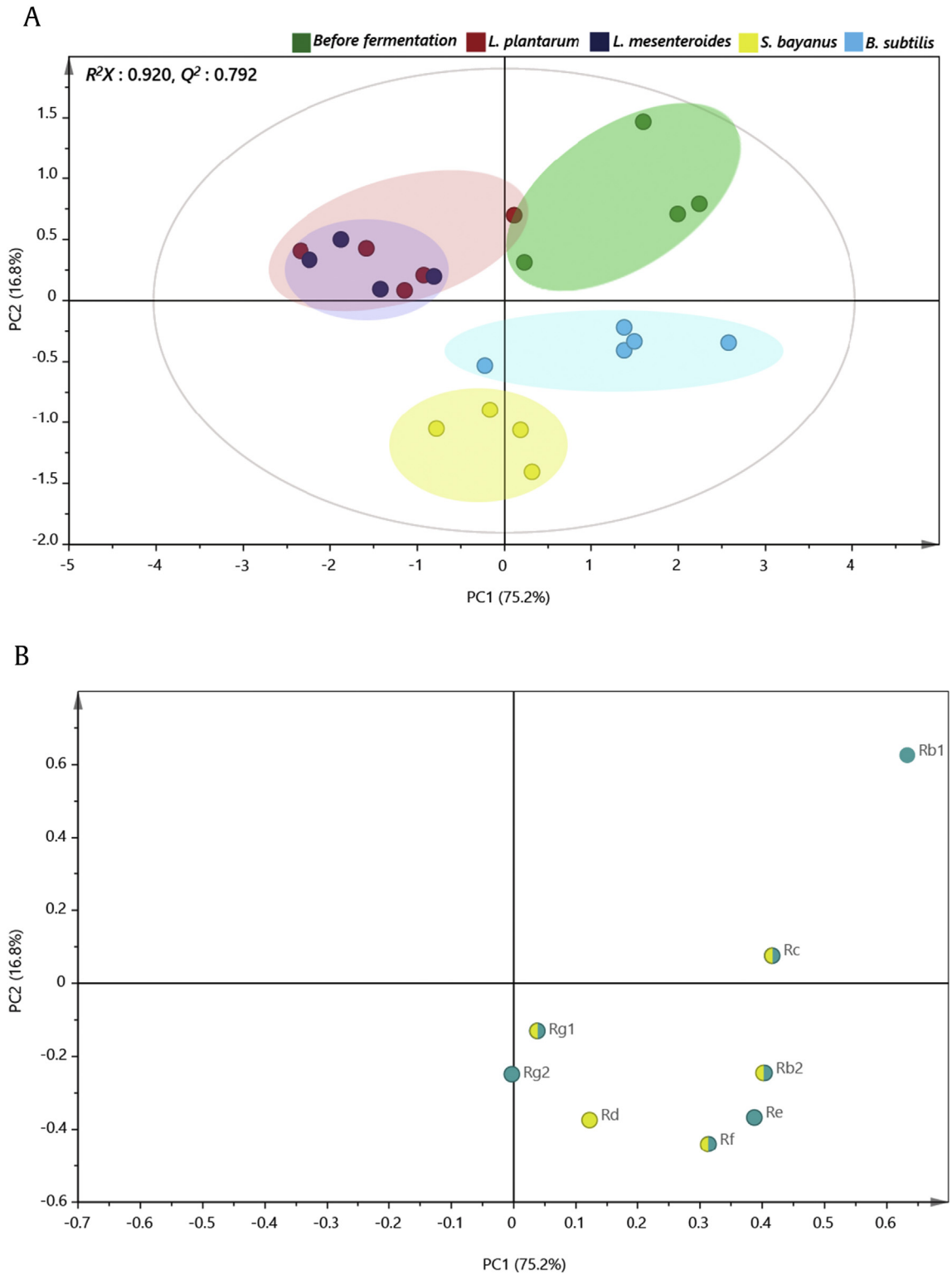

Fig. 2. (A) Principal component analysis score and (B) loading scatter plots derived from the eight ginsenosides in the ginseng extracts fermented with four different strains. Each dot in the score plot represents the replicate of samples. The more distantly the compounds are from the origin in the loading plot, the more important they are for the differentiation pattern. The blue and yellow dots on the loading plot denote the highest levels of ginsenosides in fermented ginseng extracts ($p < 0.05$) with *B. subtilis* and *S. bayanus*, respectively. The dots with two colors denote metabolites levels that were higher than those with other colors. PC = principal components.

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.

2.6. Electronic tongue

The α-ASTREE II electronic tongue system (E-tongue, Alpha M.O.S. Toulouse, France) was used to determine the taste profiles of the fermented ginseng samples. The E-tongue is composed of a 48-position autosampler, an array of chemical sensors with cross-selectivity and a chemometrics software package. The sensor set consists of seven working electrodes (SRS, GPS, STS, UMS, SPS, SWS, and BRS sensors) and a reference electrode (Ag/AgCl electrode). The integral signal during the measurement of each sample, which comprises a vector with seven individual sensor measurements, was transformed into intensity values representing the five basic tastes as follows: (1) SRS (sourness, astringency, and bitterness); (2) GPS (sourness, saltiness, and metallic); (3) STS (saltiness, spiciness, and metallic); (4) UMS (umami, saltiness, and astringency); (5) SPS (metallic, spiciness, and umami); (6) SWS (sweetness and sourness); and (7) BRS (bitterness and astringency). Test conditions included sample volume of 20 mL, analysis time of 3 min, acquisition time of 2 min, sample and room temperature, and sensor cleaning solution as 5% ethanol. After sensory measurement for each sample solution, a wash cycle was performed to ensure that there was no sample carryover to the next analysis and to ensure good reproducibility. Measurements were performed in triplicate for each sample.

2.7. GC-MS analysis

The samples were derivatized using methoxyamine hydrochloride in pyridine and N-methyl-N-(trimethylsilyl) trifluoroacetamide containing trimethychlorosilane (1%, v/v). Samples were analyzed with a 6890N gas chromatography (Agilent, Santa Clara, USA) equipped with a 5973N mass selective detector. Separation was achieved using a DB-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The GC-MS was operated as previously described [15]. Mass spectra (m/z scanning range of 50–550) were recorded at 2 scans/s with electron impact ionization at 70 eV. Ribitol served as an internal standard to monitor batch reproducibility and to correct for minor variations that occurred during sample preparation and analysis. MSD ChemStation software (Agilent, USA) was used to acquire mass spectrometric data. The mass spectra of all detected compounds were compared with spectra in the NIST and Wiley library for identification. Metabolites were identified only when the quality value of the mass spectra compared to the spectra library was 90 or higher. All metabolite identifications were manually validated to reduce deconvolution errors during automated data-processing and to eliminate false identifications. For quantitation methods, the most specific fragment ion in the spectra of each identified metabolite was determined to the quantification ion, and their summed abundance was integrated; fragment ions due to trimethylsilylation (i.e., m/z 73.1 and 147.1) were excluded from the determination of metabolite abundance.

2.8. Data processing and statistical analysis

The major GC-MS data analysis work flow was similar to our previously published work [15]. Metabolite content was calculated according to the peak area of the metabolites and the peak area of the internal standard ribitol on the same chromatograph. The generated normalized metabolite contents (variables) were imported into SIMCA-P version 14.0 (Umetrics, Umea, Sweden) for multivariate statistical analysis. The quality of the models was described by R^2 and Q^2 values. R^2 represents the variation explained by the model, whereas Q^2 indicates how well the model predicts new data [16]. The heat map was generated using the R statistical computing environment (http://www.r-project.org/). The heat map color drawn by R with ggplot2 represents the z-score transformed raw data for fermented ginseng metabolites.

Statistical analyses were performed using the SPSS version 21 (SPSS Inc., Chicago, IL, USA). Analysis of variance and Duncan's

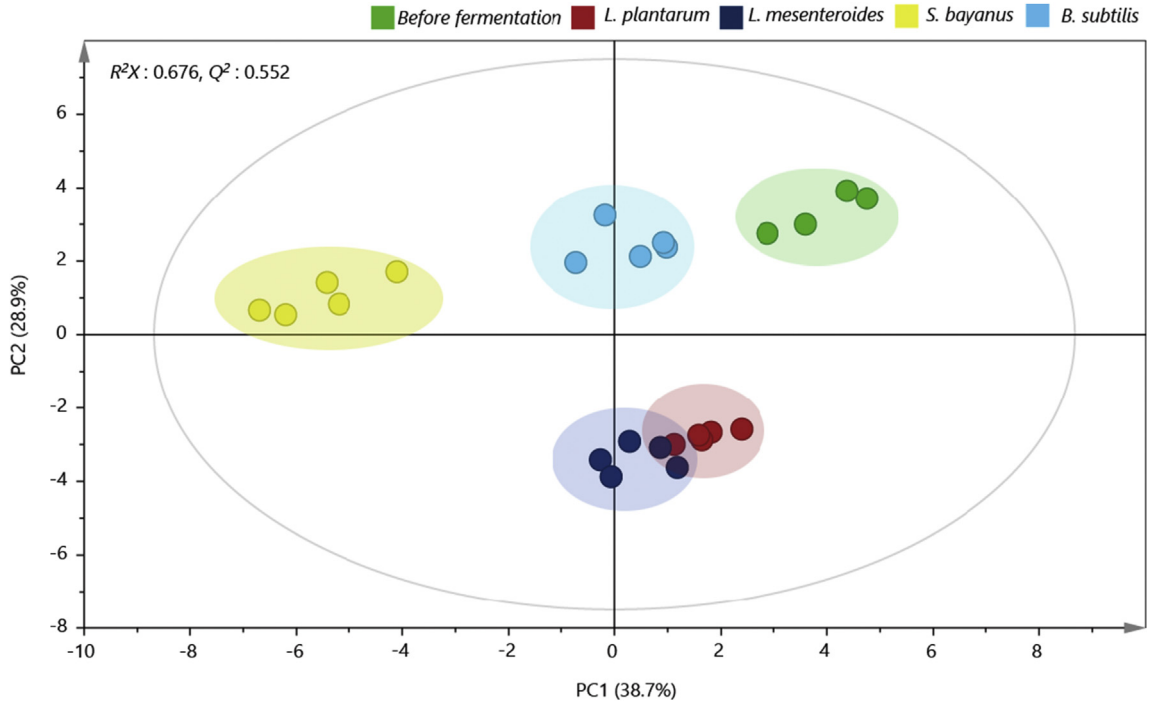

Fig. 3. Prinicpal component analysis score plot derived from GC-MS peak intensity profiles of fermented ginseng extracts, suggesting a strong strain dependence of metabolites of ginseng. Each dot in the score plot represents the replicate of samples. PC = principal components.

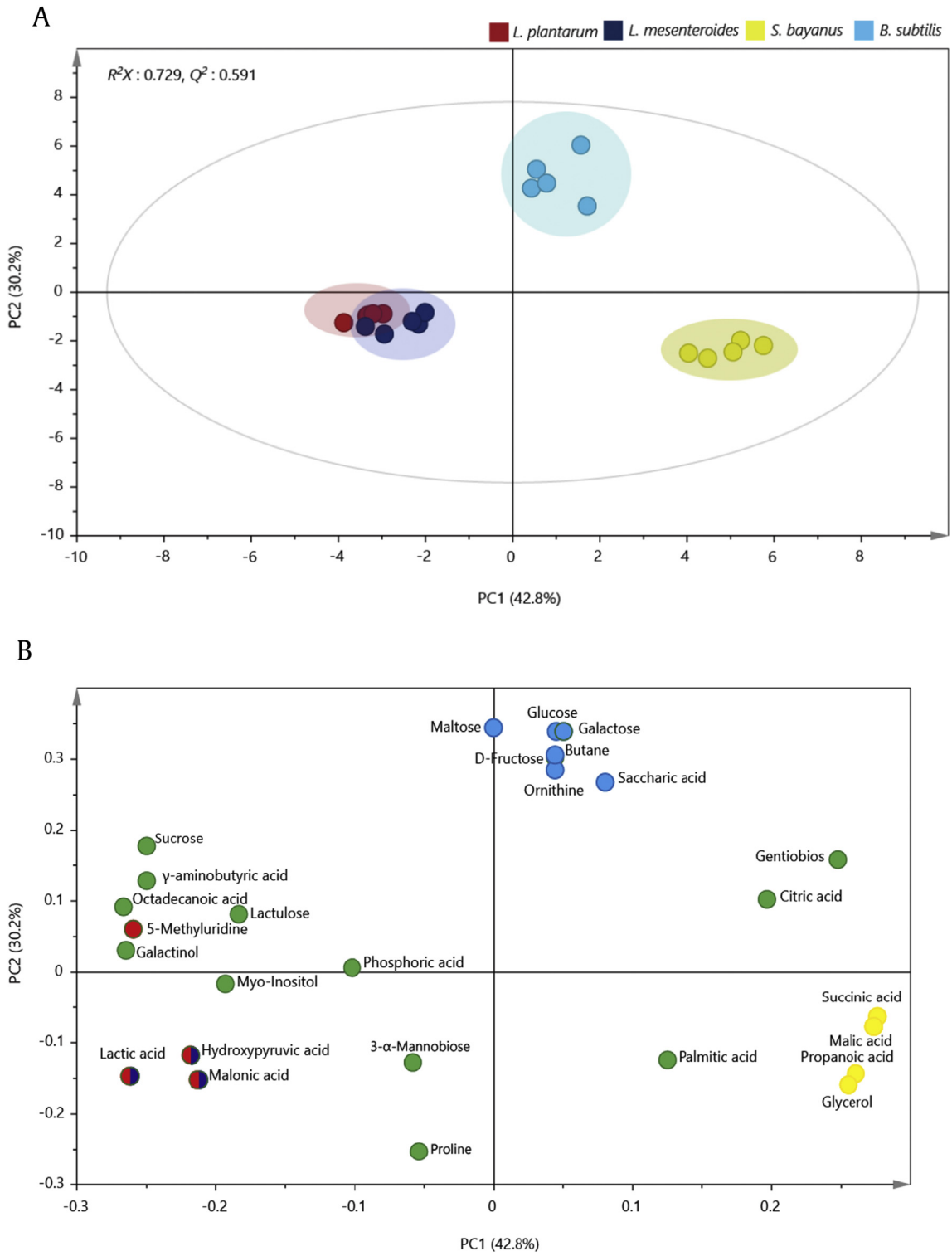

Fig. 4. (A) Principal component analysis score; (B) loading scatter plots; and (C) heat map derived from the 27 identified metabolites of fermented ginseng extracts. The red, indigo, yellow, and blue dots on the loading plot denote the highest levels of metabolites in fermented ginseng extracts ($p < 0.05$) with *L. plantarum*, *L. mesenteroides*, *S. bayanus*, and *B. subtilis*, respectively. The dots with two colors in panel B denote the metabolites levels that were higher than those with other colors. The heat map in panel C was plotted in a green-red color scale with red indicating lower metabolite levels and green indicating higher metabolite levels. PC = principal components.

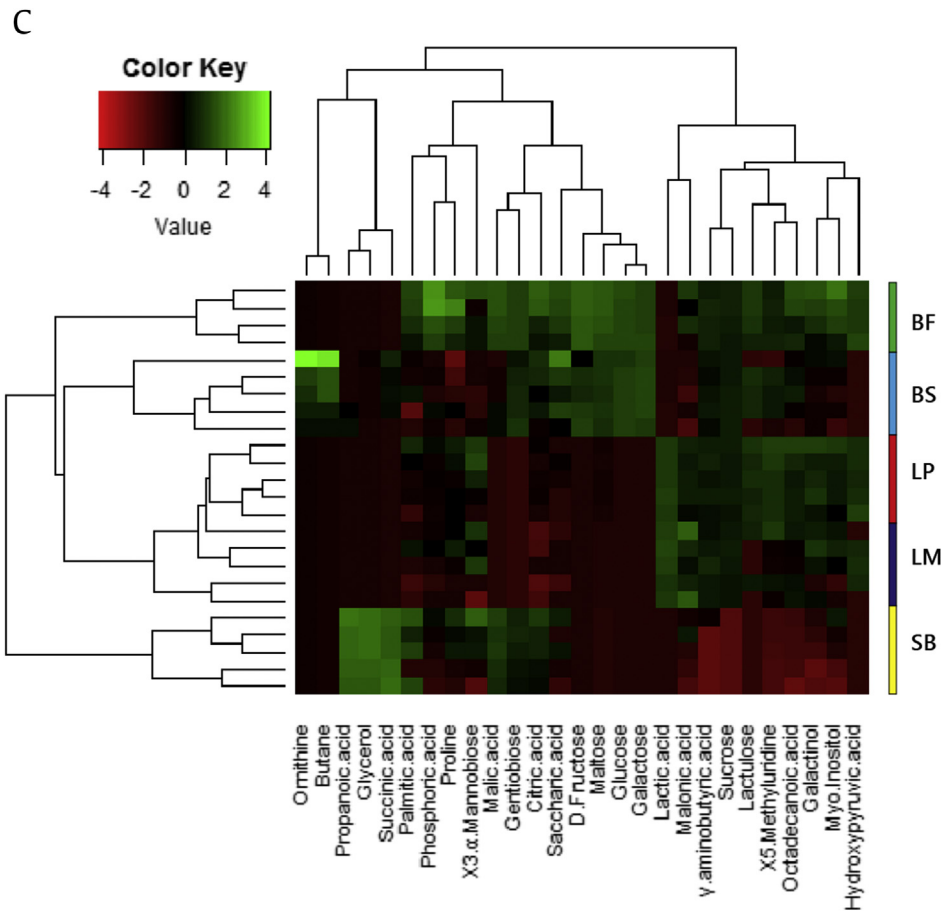

Fig. 4. (*continued*).

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. Changes in cell counts, pH, and TA during ginseng fermentation

Fig. 1A shows the growth kinetics of strains during fermentation in ginseng extracts inoculated with four strains. Initial viable cell counts were 5.50–6.08 log CFU/mL, and the levels increased on the 1st day of fermentation, except for *B. subtilis*. Microbial populations of *L. plantarum*, *L. mesenteroides*, and *S. bayanus* showed their highest values on the 2nd day of fermentation, whereas *B. subtilis* increased gradually from the 1st day to the 4th day of fermentation. It is thought that the growth capability of *B. subtilis* is slower than those of other strains in the fermentation of ginseng extracts.

Changes in pH and TA in ginseng extracts fermented with four strains are presented in Figs. 1B and 1C. The pH value of ginseng extracts fermented with lactic acid bacteria (LAB) gradually decreased during fermentation, whereas that of ginseng extracts fermented with *B. subtilis* increased from the 2nd day of fermentation. The TAs of ginseng extracts fermented with LAB showed significantly increasing patterns compared with those of other samples, and the final TA values were 0.26–0.27%. This suggested that the decrease in pH value was due to LAB rapidly becoming the predominant microorganisms, producing lactic acid and leading to pH reduction. The decrease of TA in ginseng extracts fermented with *B. subtilis* and *S. bayanus* suggested that the fermentation characteristics of *B. subtilis* and *S. bayanus* were different from those of LAB during ginseng extracts fermentation.

3.2. Changes of ginsenosides during fermentation

Supplementary Table S1 shows the major eight ginsenosides in ginseng extracts fermented with four strains. To investigate the effects of the microbial strains on the ginsenoside differences in fermented ginseng extracts, a principal component analysis (PCA) model was generated using the eight ginsenosides (Fig. 2). As two samples (LM-3 and SB-3) were found to have very different ginsenoside contents from others, probably due to experimental errors, these two outlier samples were excluded prior to PCA to reduce the statistical power of the data analysis. A separation between microbial strains, except for LAB, was observed in the PCA model with a high-fitting ($R^2 = 0.92$) and high-predicting ($Q^2 = 792$) quality. The samples fermented with *L. plantarum* and *L. mesenteroides* were not distinguished on the left side of principal components 1 (PC1), indicating that the eight ginsenoside patterns of these two groups were more similar than those of other samples. Likewise, the samples fermented with *B. subtilis* were located close to the control samples (before fermentation), indicating that the eight ginsenosides patterns of these two groups were more similar than those of other strains. The complementary PCA loading scatter plot shows the ginsenosides that contributed to the separation (Fig. 2B). Higher levels of most ginsenosides were observed in the ginseng extracts fermented with *S. bayanus* and *B. subtilis* than in those fermented with LAB.

Biotransformation of ginsenosides into smaller deglycosylated forms has been reported, such as Rb1 → Rd, F2, Rg3, CK [17], Re,

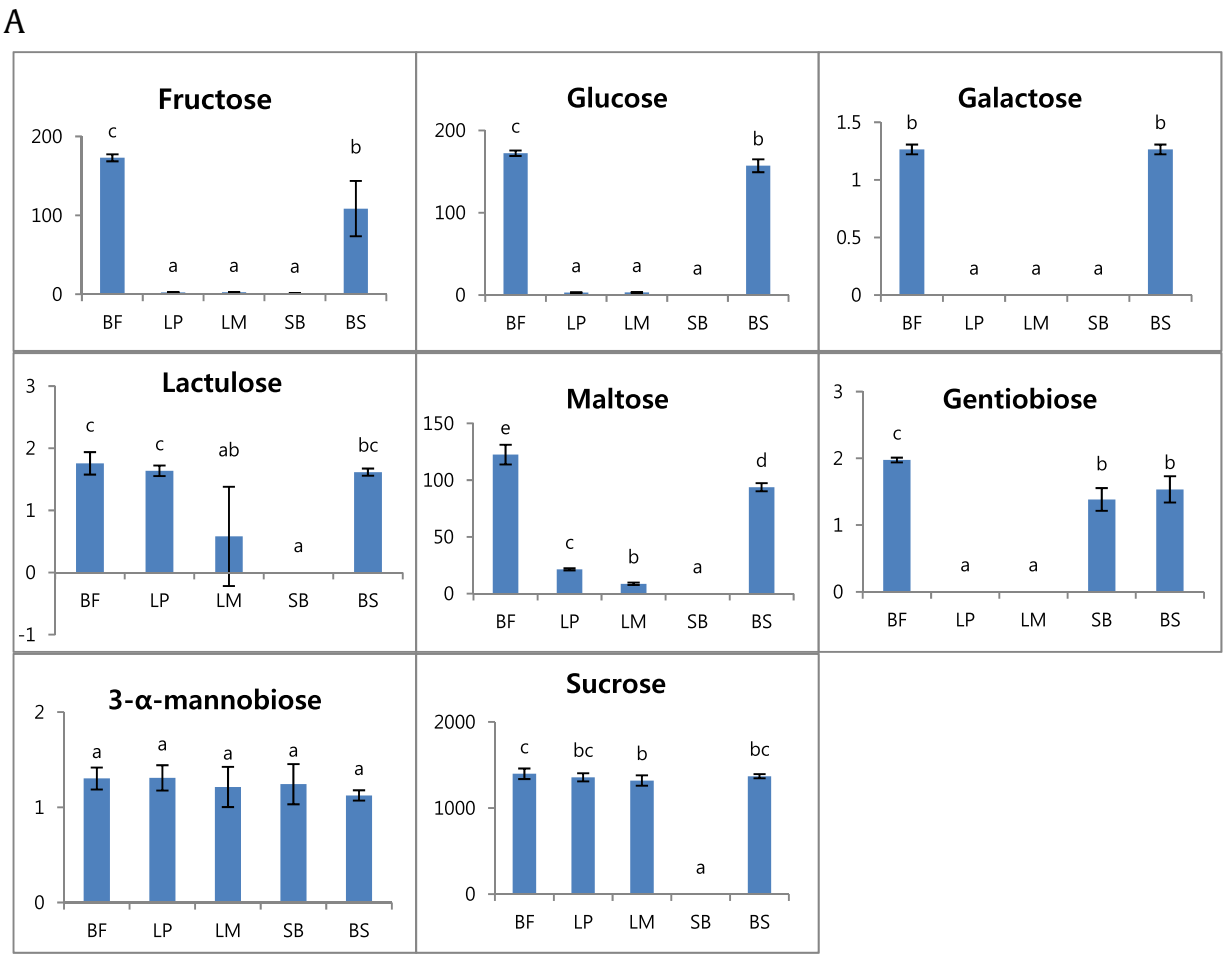

Fig. 5. Metabolite contents in (A) sugars, (B) organic acids, (C) amino acids, and (D) polyols after ginseng fermentation. Data are given as mean ± standard deviations ($n = 5$). The ginseng samples are as follows: BF = before fermentation; LP = *Lactobacillus plantarum*; LM = *Leuconostoc mesenteroides*; SB = *Saccharomyces bayanus*; BS = *Bacillus subtilis*.

Rb1, Rc → Rg1, Rd, CK [18], Rb1 → Rd [1], and Rb1 → Rd, Rg3 [19]. Some differences in ginsenoside biotransformation may result from the types of materials used such as ginseng, enzymes, and microorganisms. The different ginsenoside patterns could be a characteristic of the microbial strain since the same raw materials were used for ginseng fermentation in the present study.

3.3. Analysis of the ginseng metabolome by GC-MS

In order to investigate the effect of the microbial strains used for fermentation on the metabolic profile of ginseng extracts, we analyzed the metabolites of ginseng using GC-MS with a metabolomics approach. To reduce the complexity of the datasets, whilst still retaining important information, PCA was applied. Fig. 3 shows the PCA score plot derived from the GC-MS profiles of ginseng fermentation. The PCA score plot showed a clear separation between ginseng extracts fermented with *S. bayanus* and other samples. It is interesting to note that different fermentation behaviors for each microbial strain were observed. However, the samples with *L. plantarum* and *L. mesenteroides* were not fully distinguished in the PCA score plot. The samples fermented with *B. subtilis* were located close to the control (before fermentation), indicating that the metabolites of these two groups were considered more similar than those of other strains.

3.4. Metabolite changes by microbial strains

To obtain comprehensive identification of differential metabolites in the four groups identified in the present work, PCA models were regenerated for the 27 identified metabolites from the GC-MS data (Fig. 4). The first and second principal components (PC) of the PCA score plot represented 42.8 % and 30.2 % of the total variance of the samples, respectively (Fig. 4A). As seen in Fig. 3, the variance of metabolites in ginseng extracts between *S. bayanus* and other strains was successfully captured by PC1.

To identify the metabolites responsible for the separations in the PCA scores plot, a PCA scatter loading plot was generated (Fig. 4B). The differentiations of fermented ginseng extracts between the two classes (*S. bayanus* and other strains) were due to higher levels of succinic acid, malic acid, propanoic acid, and glycerol in ginseng extracts fermented with *S. bayanus* ($p < 0.05$). Also, the *B. subtilis* group was distinguished significantly from the LAB group by PC2. The highest levels of fructose, maltose, galactose, glucose, ornithine, saccharic acid, and butane in ginseng extracts were found in ginseng extracts fermented with *B. subtilis*, whereas the levels of lactic acid, malonic acid, hydroxypyruvic acid, and 5-methyluridine were highest ($p < 0.05$) in ginseng extracts fermented with LAB. However, it was difficult to discriminate *L. plantarum* and *L. mesenteroides* using PC1 and PC2.

For quantitative analysis of metabolites, a heat map prepared using the z-score transformed raw data for fermented ginseng

B

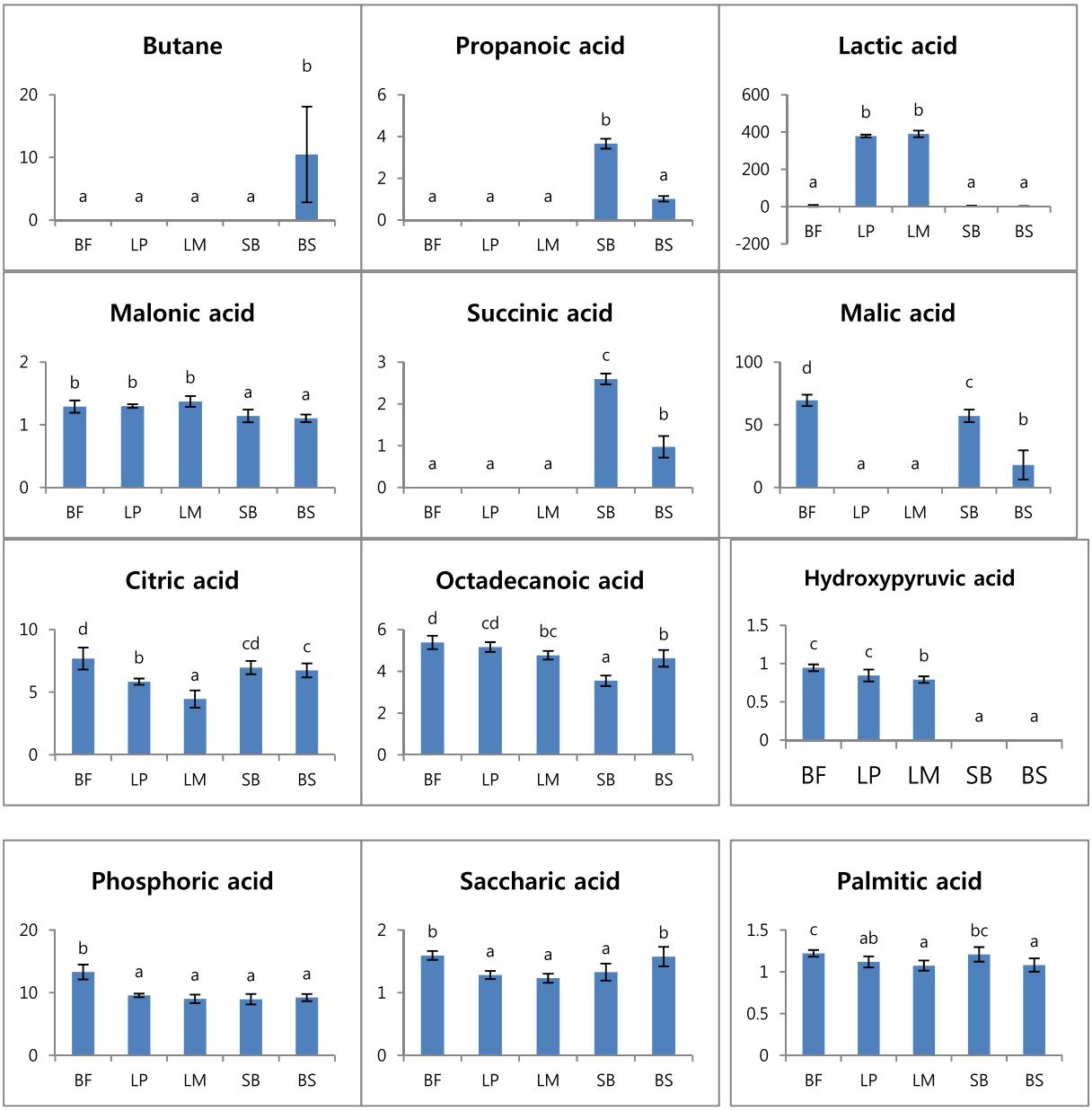

Fig. 5. (*continued*).

metabolites was plotted in a green-red color scale (Fig. 4C). Metabolic profiling for the fermented ginseng extracts showed a different pattern significantly depending on the type of inoculation strains.

3.5. Quantitative changes of metabolites in ginseng fermentation

Fig. 5 shows the quantitative differences in identified metabolites after fermentation. The levels of sugars, such as fructose, glucose, and maltose, had a tendency to decrease throughout the fermentation process except for *B. subtilis*, although the remaining levels of the sugars after fermentation were different depending on the strain. As shown in Fig 5A, most of sugar (except for gentiobiose and α-mannobiose) in ginseng extracts was exhausted by *S. bayanus*. This is consistent with the findings of Jang et al [20] that almost all of the sugar in the ginseng was consumed by *Saccharomyces*. Conversely, fructose and glucose in the ginseng extracts fermented with *B. subtilis* decreased a little after fermentation. These results are in accordance with Fig. 1A, which showed the slow growth capability of *B. subtilis*. It is also interesting to note that the levels of sucrose in ginseng extracts fermented with LAB and *B. subtilis* changed little compared with the levels of glucose and fructose. This suggests that sucrose is not an optimal fermentable sugar for LAB and *B. subtilis*. Sucrose fermentation is initiated by the cleavage of sucrose hydrolase to glucose and fructose. The ability of LAB to ferment sucrose differs between different species and only a few LAB possess the ability to ferment sucrose [7,21–23]. On the other hand, gentiobiose was completely consumed by LAB after 5 d of fermentation.

The organic acids in fermented ginseng extracts can be produced or consumed by microorganisms during fermentation. The levels of propanoic acid and succinic acid in ginseng extracts

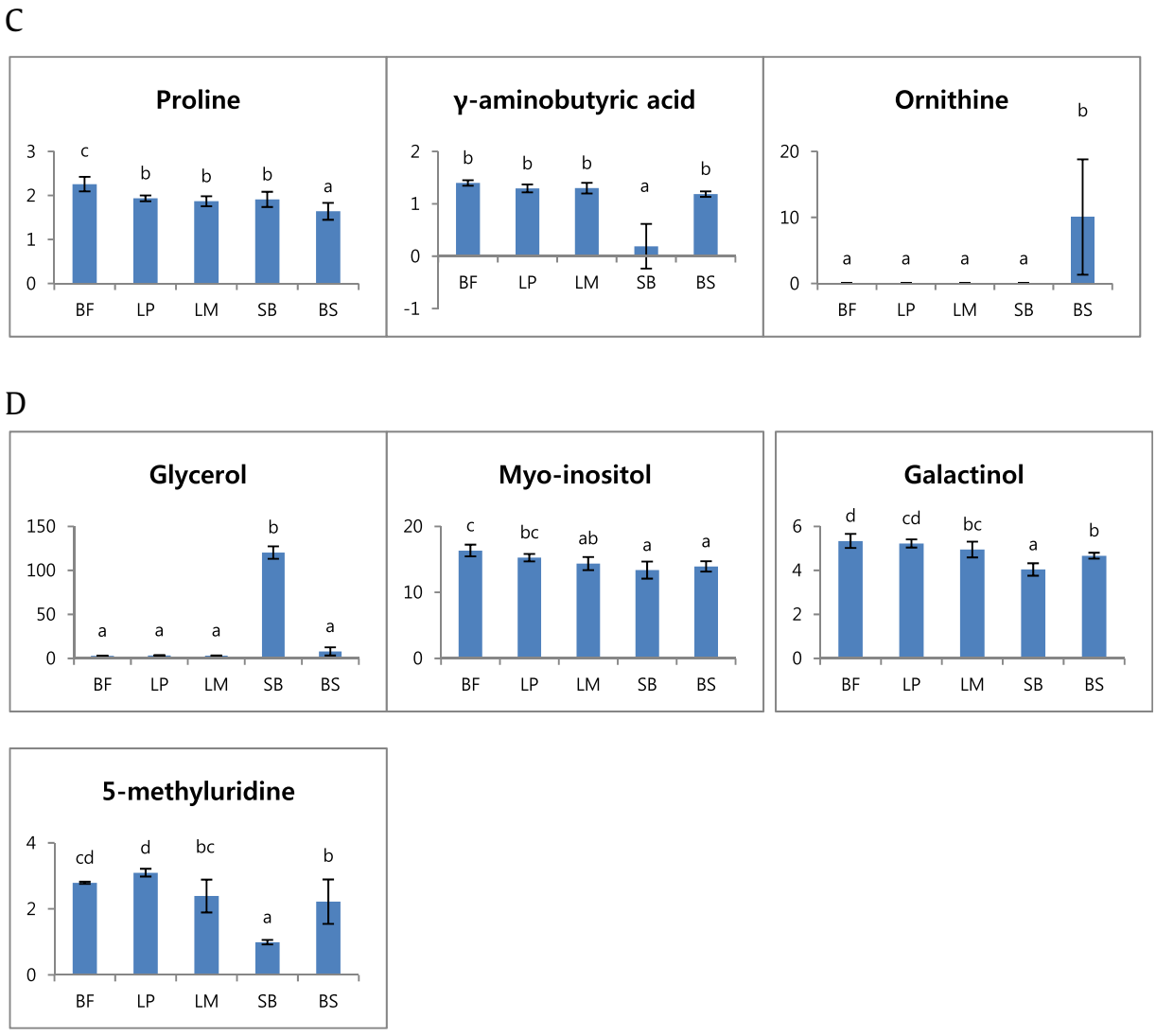

Fig. 5. (*continued*).

fermented with *B. subtilis* and *S. bayanus* increased considerably during fermentation. The drastic increase in the levels of organic acids indicated that these were mainly produced from microbial metabolism rather than released from ginseng. These results are in accordance with the increase in propanoic acid and succinic acid levels in Chinese liquor "Daqu" by *B. subtilis* [24] and the production of succinic acid in yeast fermentation [25].

As expected, lactic acid levels were the highest in the ginseng extracts fermented with LAB after fermentation. The high level of lactic acid (378.0–389.6 μg/μL) was consistent with the low pH and high TA, as shown in Figs. 1B and 1C. Increased lactic acid levels with decreased malic acid and citric acid levels indicate that malolactic fermentation occurred as LAB convert malic acid and citric acid into lactic acid during malolactic fermentation [26,27]. Higher levels of lactic acid together with lower levels of succinic and malic acid in fermented ginseng extracts could be biomarkers to characterize LAB fermentation. Also, these substances are important causative agents affecting acidity and related factors of flavor and taste [28].

Interestingly, markedly reduced levels of γ–aminobutyrate (GABA) and elevated levels of glycerol were detected in the ginseng extracts fermented with *S. bayanus*. In general, *Sacchromyces* can utilize GABA through GABA transaminase, which catalyzes the conversion of GABA to succinate semialdehyde in the GABA shunt pathway [29]. Glycerol is the major trialcohol produced as a byproduct of yeast fermentation in side-chain reactions from sugar to ethanol [30]. These metabolites could be characteristic of the yeast strain since the same raw materials were used for fermentation in the present study. The different amounts of metabolites observed after fermentation suggest that metabolites can be consumed or produced differently during ginseng fermentation according to the microbial strains used.

3.6. Analysis of the taste features by E-tongue

To investigate the taste features of fermented ginseng extracts, the E-tongue was used to evaluate the indices of seven tastes (Supplementary Table S2). To identify the taste features responsible for the differentiation according to the starter culture, PCA score and loading scatter plots were generated for fermented ginseng extracts using E-tongue data (Fig. 6). A clear separation with a high-fitting ($R^2X = 0.948$) and high-predicting ($Q^2 = 0.878$) quality was observed in the PCA model. Similar to the metabolomics results, the samples fermented with *B. subtilis* were located close to the control (before fermentation). These results suggested that the slow growth capability of *B. subtilis* may lead to a similar pattern of

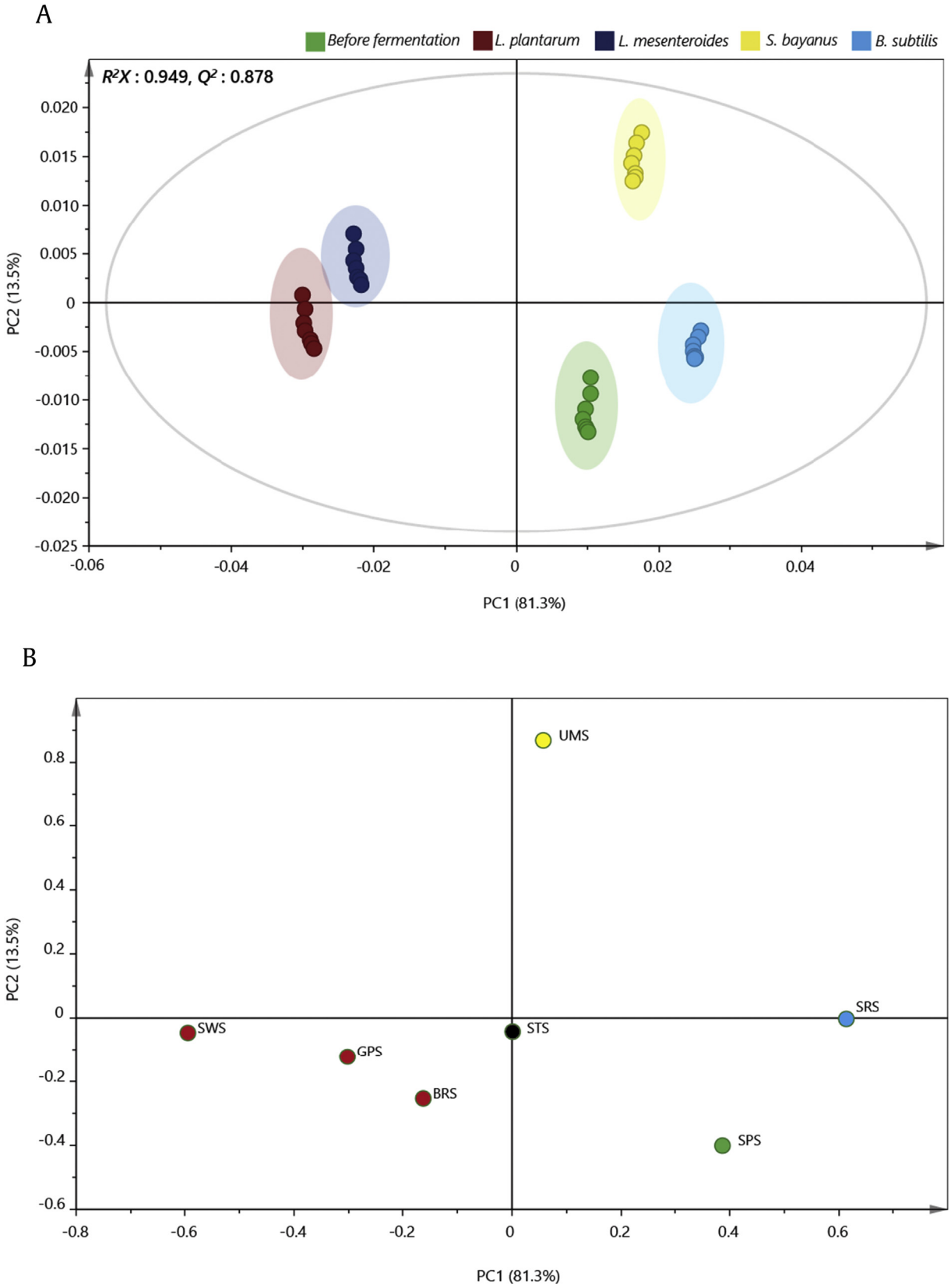

Fig. 6. (A) The principal component analysis score and (B) loading scatter plots derived from the result of E-tongue analysis on ginseng fermentation. The green, red, indigo, yellow, and blue dots on the loading plot denote the highest levels of metabolites in fermented ginseng extracts ($p < 0.05$) without inoculation (Control, before fermentation), and with *L. plantarum*, *L. mesenteroides*, *S. bayanus*, and *B. subtilis*, respectively. BRS = bitterness and astringency; GPS = sourness, saltiness, and metallic; PC = principal component; SPS = metallic, spiciness, and umami; SRS = sourness, astringency, and bitterness; STS = saltiness, spiciness, and metallic; SWS = sweetness and sourness; UMS = umami, saltiness, and astringency.

metabolites and taste index. LAB groups were significantly distinguished from other groups by a high index of SWS (sweetness and sourness) and GPS (sourness and saltiness). The high levels of lactic acid in fermented ginseng extracts with LAB are associated with the sour taste. The highest UMS (umami and saltiness) was detected in the ginseng extracts fermented with *S. bayanus*, which was significantly different from the other strains. These results suggest that the taste indices of fermented ginseng extracts were different depending on the strain used.

E-tongue, for objective evaluation such as the discrimination and quantification of tastes, is known as a sensing technology that contributes to quality management [31]. E-tongue has recently been used to evaluate the tastes of various fermented foods, such as beer [32], rice wine [33], and kimchi [34]. The different taste indices among ginseng samples may be related to the metabolite profiles for different strains. Further studies are needed to confirm the correlation between metabolites and the taste index of ginseng.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgr.2016.12.010.

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