Short communication: Antioxidative and antibacterial activities on *Staphylococcus aureus* and *Escherichia coli* O157:H4 in milk with added ginseng marc extract fermented by *Lactobacillus plantarum* KCCM 11613P

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ABSTRACT

Ginseng marc, a by-product of the extraction of fresh ginseng, is known to have bioactive compounds, but is frequently discarded as agriculture waste. The objectives of our study were to assess the antioxidative activity of fermented ginseng marc extract using *Lactobacillus plantarum* KCCM 11613P and to evaluate antibacterial activity of fermented milk with added ginseng marc extract during fermentation. After 24 h of fermentation of ginseng marc extract, the viable cell number was increased to 7.7 ± 0.1 log cfu/mL, and the pH and total titratable acidity were 4.2 ± 0.4 and 0.6% lactic acid, respectively. The total phenolic and flavonoid contents of fermented ginseng marc extract increased by 32.4 and 23.3%, respectively. Higher antioxidative activity of fermented ginseng marc extract was obtained in the β -carotene bleaching, ferric-reducing ability of plasma, and ferric thiocyanate assays than the 1,1-diphenyl-2-picrylhydrazyl assay. However, the 1,1-diphenyl-2-picrylhydrazyl scavenging effect decreased due to lowered pH. During production of fermented milk with ginseng, inhibition rate of *Staphylococcus aureus* and *Escherichia coli* were 9.7 and 2.3%, respectively. The present study shows the possibilities of *Lactobacillus plantarum* KCCM 11613P used as a fermentation strain and ginseng marc used as a functional supplement in milk.

Key words: ginseng marc, *Lactobacillus plantarum*, antioxidative activity, antibacterial activity, flavonoid

Short Communication

Korean *Panax ginseng* Meyer is an herb used in Asian traditional medicine that has diverse attributes, such as antioxidant and antiaging activity, as well as

cancer-preventing properties; this herb also activates antitumor immunity, reduces memory loss, and relieves pain (Choi, 2008). Ginseng marc is a by-product of the extraction of fresh ginseng and dried ginseng, and is considered agricultural waste or is mainly used as animal feed (Choi and Hwang, 2011). However, ginseng marc is recognized to contain bioactive components, such as polyphenols and flavonoids (Attele et al., 1999).

Lipid oxidation in fatty foods and edible oils is a major problem in the food industry, exacerbated by the increasing use of polyunsaturated oils (fish, olive, and nut oils) and the evasion of synthetic antioxidants by consumers (Frankel, 2012). Oxidation of lipid occurs when lipids react with oxygen, resulting in disruption of the carbon double bond of the lipid and the production of secondary products. Lipid oxidation is associated not only with undesirable flavors, but also with decreased nutritional quality and adverse effects on human health (Spickett and Froman, 2015). For example, lipid peroxidation damages neuronal membranes leading to neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Sultana et al., 2013). Research focus has recently shifted to the application of various natural compounds rather than synthetic chemicals as antioxidants to prevent lipid oxidation.

Natural polyphenolic compounds exhibit antioxidant properties derived from their ability to scavenge peroxy radicals, superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\cdot$) with ferric-reducing antioxidant power and cupric ion (Cu^{2+})-reducing power (Gülçin, 2012). Flavonoids are widely distributed in plants and show anti-inflammatory activity, antioxidant effects, anticancer effects, and prevent the progression of chronic diseases (Park et al., 2015). Although the biofunctionality of these compounds in ginseng marc is variable and ginseng marc is a functional food material, little research has been conducted in this arena.

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Lactic acid bacteria (**LAB**) are widely used as starter cultures in many fermented foods, such as dairy, meat, vegetable and bakery products, and as natural biopreservatives that expand shelf life and improve the safety and safeguarding of typicality and sensorial property of the food (Settanni et al., 2013; Gaglio et al., 2014). *Lactobacillus plantarum* is the microorganism most widely used in the fermentation of plant-derived raw materials. Several studies have been conducted to evaluate the antimicrobial effect and probiotic effect (Burr et al., 2005) of *L. plantarum*. The antibacterial effect of fermented milk against *Escherichia coli*, *Salmonella* Choleraesuis, *Shigella dysenteriae*, and *Staphylococcus aureus* during storage period was conducted (Noël et al., 2016), whereas fewer studies of antibacterial effect during fermentation time exist. Herein, we fermented water-soluble ginseng marc extract using *L. plantarum*, investigated changes in the levels of total polyphenols, flavonoids, and the antioxidative activity during fermentation to use the active compounds in ginseng marc, and assessed the antibacterial activity of fermented ginseng marc milk for use as a milk supplement.

Panax ginseng marc was provided by Il Hwa Ginseng Research Institute (Guri-si, Gyeonggi-do, South Korea). Dried ginseng marc (400 g) was extracted with 2 L of 70% ethanol at 60°C and the solvent was evaporated using a rotary evaporator (EYELA N-1000V, Tokyo, Japan). Ginseng marc medium was made by mixing the aforementioned extract and distilled water at 1% (wt/vol) and sterilized by autoclaving at 121°C for 15 min.

Lactobacillus plantarum KCCM 11613 was obtained from the Korean Culture Center of Microorganisms (KCCM, Seoul, South Korea). The strain was propagated (1%, vol/vol) twice in de Man, Rogosa, Sharpe broth (Sigma Aldrich, Steinheim, Germany) and inoculated into ginseng marc medium at 1% (vol/vol, initial microbial content about 10^6 cfu/mL). Fermentation was conducted at 37°C for 24 h under stirred conditions (50 rpm). During fermentation, samples were withdrawn at 0, 4, 8, 12, 16, 20, and 24 h.

The fermented samples were serially diluted with 0.1% peptone water and pour-plated with de Man, Rogosa, Sharpe agar. The agar plates were incubated at 37°C for 24 h and the pH of each was measured with a pH meter (WTW-720, Weilheim, Germany). The total titratable acidity was determined by titration up to pH 8.2 (expressed as lactic acid %).

The total phenolic content (**TPC**) was measured using a modified Folin-Ciocalteu assay (Dordević et al., 2010). One hundred microliters of fermented ginseng marc were mixed with 2 mL of 2% sodium carbonate (Na_2CO_3) solution. After 3 min, 100 μL of Folin-Cio-

calteu reagent was added and reacted for 30 min. The absorbance was measured at 750 nm with a spectrophotometer and the TPC was calculated as gallic acid equivalents (**GAE**).

The total flavonoid content (**TFC**) was measured using the aluminum nitrate assay (Curiel et al., 2015). Five hundred microliters of fermented ginseng marc were mixed with 2.8 mL of distilled water, 1.5 mL of 99.5% ethanol, and 0.1 mL of aluminum nitrate in 1 M potassium acetate. After 30 min, the absorbance was measured at 415 nm with a spectrophotometer and the TFC was calculated as kaempferol equivalents.

Inhibition of the β -carotene and the linoleic acid oxidation were determined by the method of Park et al. (2015). A β -carotene solution was prepared by dissolving 3 mg of β -carotene, 66 μL of linoleic acid, and 300 μL of Tween 80 in chloroform. During incubation, the absorbance at 470 nm was determined at 6-h intervals. The inhibition of the β -carotene oxidation of the samples was expressed as

$$\begin{aligned} \text{Inhibition of } \beta\text{-carotene oxidation (\%)} = \\ (\text{absorbance after reaction})/(\text{initial absorbance}) \\ \times 100. \end{aligned}$$

The ferric-reducing ability of plasma (**FRAP**) assay was performed using the method of Benzie and Strain (1996), with some modification. The FRAP cocktail solution was mixed with 20 mL of 300 mM acetate buffer (pH 3.6), 2 mL of ferric 2,4,6-tripyridyl-s-triazine (**TPTZ**) in 40 mM HCl, and 2 mL of 20 mM aqueous ferric chloride and prewarmed at 37°C. The absorbance at 593 nm was measured; in this test, ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) was used as a standard reagent.

The free radical scavenging activity was measured by the method presented by Dordević et al. (2010). Briefly, 1 mL of 100 μM 2,2-diphenyl-1-picrylhydrazyl (**DPPH**) solution was added to 200 μL of the sample. After 15 min in the dark, the absorbance at 517 nm was measured. The DPPH radical scavenging activity of the samples was expressed as

$$\begin{aligned} \text{DPPH radical scavenging activity (\%)} = \\ [1 - (\text{absorbance of sample}/\text{absorbance of control})] \\ \times 100. \end{aligned}$$

Antibacterial activity of fermented milk with ginseng marc extract during fermentation was determined by viable cell number using bromocresol purple and plate count agar (Sigma Aldrich). Milk (Seoul Milk Co., Seoul, Korea) was mixed with skim milk powder (Seoul

Milk Co.), pectin, and 1% ginseng marc extract to adjust the solid content to 11%. *Lactobacillus plantarum* and pathogens were inoculated in milk and incubated at 40°C in water bath for 8 h. Tested pathogens were *Escherichia coli* FRIK125 (from Wisconsin Food Research Institute, Madison, WI) and *Staphylococcus aureus* 1573 (from Korea Veterinary Culture Collection, Kimcheon, Korea).

All data were analyzed via one-way ANOVA and Duncan's multiple range tests using SPSS 19 software (IBM Corp., Armonk, NY). The results were considered significant at $P < 0.05$. Each set of experiments was performed in triplicate.

To improve the growth rate of *L. plantarum*, the pH of the ginseng marc extract medium (pH 4.5) was adjusted by addition of 1 N NaOH to achieve a pH of 6.5 ± 0.2 . During fermentation at 37°C, the viable cell number, pH, and total titrate acidity were determined at 4-h intervals. After 16 h of fermentation, the number of viable cells increased from 6.2 ± 0.0 to 7.7 ± 0.1 log cfu/mL. The incubation time before logarithmic growth of the cells depends on the fermentation medium. The pH of the medium inoculated with viable cells at 6.2 ± 0.0 log cfu/mL decreased from 6.5 ± 0.2 to 4.2 ± 0.4 ; the total titrate acidity increased to 0.65% for the respective medium (data not shown).

Table 1 shows the changes in the TPC and TFC of fermented ginseng marc during fermentation. The solid content decreased from 7.73 ± 0.15 to 5.62 ± 0.07 mg/mL during fermentation; the TPC of fermented ginseng marc increased from 31.5 ± 0.4 to 41.7 ± 1.7 mg of GAE/g of solid. Dordević et al. (2010) showed that the TPC of unfermented buckwheat extracts (50.7 GAE/g of dry extract) increased to 53.2 mg of GAE/g of dry extract in the extract fermented with *Saccharomyces cerevisiae* and 59.4 mg of GAE/g of dry extract in the extract fermented with *Lactobacillus rhamnosus*. Joung et al. (2010) affirmed that during fermentation of ginseng extract, the microorganisms degrade high-molecular weight phenolic compounds into low-molecular

weight phenolic compounds or produce new phenolic compounds.

The TFC of fermented ginseng marc increased to 111.1% (as kaempferol equivalents), where kaempferol was derived from rutin (quercetin-3-O-rutinoside; Lin et al., 2014). That is, rutin can be converted to kaempferol via the kaempferol-3-rutinoside intermediate or to quercetin through dihydroxylation and deglycosylation; thus, *L. plantarum* converted rutin to kaempferol. The highest total kaempferol value was detected after 16 h of fermentation (Table 1). Yang et al. (2014) showed that, during fermentation with *L. plantarum*, the TFC increased at 6 h of fermentation, followed by a decline up to 12 h of fermentation. Sotomayor et al. (1999) reported that natural fermentation using LAB decreased the pH of the medium, which could activate hydrolysis and induce decomposition of flavonoid glycoside.

The antioxidative activity was determined by bleaching of β -carotene; β -carotene emulsion changes from orange to white due to linoleic acid free radical attack on the double bond of β -carotene (Jayaprakasha et al., 2001). In the presence of an antioxidant (which removes free radical intermediates), the orange color of the β -carotene emulsion is preserved. During fermentation of ginseng marc, the ratio of inhibition of β -carotene oxidation increased, and the highest inhibition ratio was a $67.3 \pm 1.5\%$, on average, at 16 h of fermentation. Marazza et al. (2012) reported that soymilk fermented using *L. rhamnosus* exhibited a β -carotene bleaching effect due to release of aglycones by the β -glucosidase enzyme. The β -glucosidase enzyme is known to be present in *L. plantarum*; thus, fermented ginseng marc prevented β -carotene oxidation.

The FRAP assay was used to measure the antioxidative activity of fermented ginseng marc based upon its reducing capability, where ferric iron (TPTZ- Fe^{3+}) is reduced to ferrous iron (TPTZ- Fe^{2+} ; Gülçin, 2012). According to previous study (Yang et al., 2014), the antioxidant capacity is related to the amount of *L. plantarum*, and a greater number of viable cells enhances

Table 1. Solid, total phenolic, total flavonoid contents, and antioxidative activity of ginseng marc extract fermented by *Lactobacillus plantarum* KCCM 11613P during fermentation time¹

Fermentation time (h)	Solid content (mg/mL)	Total phenol ² (mg/g of solid)	Total flavonoid ³ (mg/g of solid)	β -Carotene assay (%)	Ferric-reducing ability of plasma assay (mM FeSO_4 equivalents)	2,2-diphenyl-1-picrylhydrazyl assay (%)
0	7.73 ± 0.15	31.5 ± 0.4	9.9 ± 0.4	60.1 ± 2.7^a	268.5 ± 9.3^a	59.6 ± 0.9^d
8	7.09 ± 0.02	33.4 ± 0.4	10.6 ± 0.5	65.3 ± 1.5^{bc}	324.0 ± 20.2^b	58.0 ± 1.6^c
16	6.40 ± 0.13	37.9 ± 1.9	12.0 ± 0.8	67.3 ± 1.5^d	432.9 ± 8.8^c	53.2 ± 1.2^b
24	5.62 ± 0.07	41.7 ± 1.7	11.0 ± 0.7	65.2 ± 1.8^{bcd}	457.3 ± 17.3^d	51.8 ± 0.9^a

^{a-d}Different letters in the same column indicate significant differences determined by Duncan's multiple range tests ($P < 0.05$).

¹Values are means $\pm$ SD of triplicate experiments.

²Gallic acid equivalents.

³Kaempferol equivalents.

Table 2. Viable cell number (log cfu/mL) and inhibition rate of fermented milk added with ginseng marc extract against *Staphylococcus aureus* and *Escherichia coli* during fermentation time

Fermentation time (h)	<i>Staphylococcus aureus</i> 1573			<i>Escherichia coli</i> O157:H4 FRIK125		
	Control ¹	FGM ²	Inhibition rate ³ (%)	Control	FGM	Inhibition rate (%)
0		4.9 ± 0.1	—		4.2 ± 0.2	—
4	8.1 ± 0.1	7.3 ± 0.1	9.7	7.9 ± 0.2	7.9 ± 0.1	0.1
8	8.4 ± 0.3	7.7 ± 0.2	9.0	8.7 ± 0.2	8.5 ± 0.2	2.3

¹Fermented milk using *Lactobacillus plantarum* KCCM 11613P.²Control added with ginseng marc extract.³Inhibition rate (%) = [(viable cell number of control – viable cell number of FGM)/viable cell number of control] × 100.

the ferric-reducing power. Yang et al. (2014) showed that the ferric-reducing (antioxidant) power of leek fermented with *L. plantarum* was higher than that of nonfermented leek, and the viable cell number increased from 10⁶ to 10⁸ cfu/mL after 48 h of fermentation. In the present study, after 24 h of fermentation, the ferric-reducing power of ginseng marc increased significantly from 268.5 ± 9.3 to 457.3 ± 17 mM FeSO₄ equivalents (Table 1). The increased phenolic components (GAE) of fermented ginseng marc extract act as stronger reductants in oxidation reaction and inactivate the free radical chain reaction (Du et al., 2014).

The DPPH assay is commonly used for antioxidative activity evaluation. The mechanism of this assay involves reduction of the DPPH free radical (DPPH•) to the nonradical form (DPPH-H) in the presence of an antioxidant material (Du et al., 2014). Many studies have reported that fruits and vegetables containing polyphenols exhibit antioxidant activity (Landete et al., 2008). Hence, in our study, after fermentation, the DPPH free radical scavenging activity of fermented ginseng marc was found to be lower than that of nonfermented ginseng marc, given that the DPPH radical scavenging activity is related to the pH. That is, Pękal and Pyrzynska (2015) reported that the DPPH inhibition of a sample changes based on the pH range. As the pH of fermented ginseng decreased, the DPPH scavenging effect decreased.

During manufacturing of fermented ginseng marc milk, viable cell number of *L. plantarum* increased from 6.3 to 7.5 log cfu/mL (data not shown). As shown in Table 2, it appeared that the growth inhibition rate of fermented ginseng marc milk on *S. aureus* and *E. coli* were 9.7 and 2.5%, respectively. In particular, *S. aureus* is known as a serious food-borne pathogen in many food products (Mpofu et al., 2016). Furthermore, these strains easily contaminate raw milk and dairy product during manufacturing, and their toxins can induce gastrointestinal illness (Jamali et al., 2015). Randhawa et al. (2016) reported that phenolic extracts, such as caffeic acid, coumarin, quercetin, and kaempferol, had

an inhibition effect on *S. aureus* through determination of the minimum inhibition concentration. Among phenolic components, kaempferol has been known to inhibit *S. aureus* PriA, which is an essential helicase for DNA replication (Huang et al., 2015). From our results, it appeared that the inhibition rate of *S. aureus* was enhanced by increasing content of polyphenolics due to fermented ginseng marc in milk.

Fermentation using LAB enhances the functional effects of raw material. This is the first report documenting the antioxidative and antibacterial activity of fermented ginseng marc. An increase in the content of polyphenol and flavonoid was positively correlated with the antioxidative activity of fermented ginseng marc based on β-carotene, FRAP, and ferric thiocyanate assay. These results indicate that fermented ginseng marc using *L. plantarum* is capable of inhibiting lipid oxidation and exerting a reducing effect. In addition, fermented milk with ginseng marc extract has reduced antibacterial ability against *S. aureus*. The present study shows that milk with ginseng marc extract fermented by *Lactobacillus plantarum* KCCM 11613P can be used in dairy products and functional foods.

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