Characteristics of Chinese chives (Allium tuberosum) fermented by Leuconostoc mesenteroides

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Abstract This study was conducted to investigate the characteristics of bioactive compounds in Chinese chives juice (FC) fermented with Leuconostoc mesenteroides SK1962, a lactic acid bacteria isolated from Kim-chi. L. mesenteroides SK1962 only (LO) was used as comparison. The pH of FC gradually decreased from 6.21 to 4.23 during the 48-h incubation period, which was similar to that of LO. The growth of L. mesenteroides SK1962 in FC was higher compared with that in LO for various incubation times, with the exception of the 8-h incubation. Total polyphenol and flavonoid contents in FC were higher as compared with those in LO during incubation, leading to increased antioxidant activity in FC at different incubation times. Moreover, FC was more effective in reduction of superoxide free radical production in primary bovine mammary alveolar cells. In addition, FC demonstrated antibacterial properties against pathogenic bacteria such as Listeria monocytogenes, Pantoea agglomerans, Haemophilus parasuis, Salmonella gallinarum, Escherichia coli O157, and Burkholderia. sp. Although LO also showed antibacterial effects against the above-mentioned pathogenic bacteria, its antibacterial activities were generally lower compared with those of FC. The results show that the antioxidant and antibacterial activities in Chinese chives was induced by fermentation with L. mesenteroides SK 1962. In conclusion, fermentation may lead to an increase in bioactive compounds including total polyphenol and flavonoid.

Keywords Antimicrobial activity · Antioxidative activity · Chinese chives juice · Fermentation · Leuconostoc mesenteroides

Introduction

Historically, mankind has used various plants and their extracts for the treatment of diseases (Cowan 1999). Approximately 250,000–500,000 plant species have been found on Earth, however, only 1 % of those plants have been researched for their pharmaceutical potentials (Meléndez and Capriles 2006). Plants and their extracts possess numerous health benefits, and their functions can be classified into three categories: direct antimicrobial, immunomodulating, and growth promoting (Hernández et al. 1984). These functions can be applied in the maintenance and promotion of human health. The Chinese chive (Allium tuberosum) is a monocotyledonous plant belonging to the Allium genus. The Allium genus also includes garlics and onions, which have been recognized as rich sources of secondary metabolites such as polyphenols, flavonoids, and sulfides (Havey et al. 2004; Wouters et al. 2013). In
For many years, it has been utilized as foodstuffs and traditional herbs due to its antioxidant, detoxification, and anticancer effects. It has been reported that the strong antioxidant effects of Chinese chives are due to the presence of polyphenols and antioxidant vitamins (Lundegårdh et al. 2008; Bernaert et al. 2012). Furthermore, its sulfur-containing compounds have also been shown to demonstrate antibacterial (Mostafa et al. 2000; Seo et al. 2001; Lundegårdh et al. 2008) and anticancer (Park et al. 2002; Xiao et al. 2005) activities. Fermentation is known as a promising procedure for increasing the nutritional quality of plant foods (Frias et al. 2005), and improving biological activities of plant compounds including antioxidant and antimicrobial activities (Lee et al. 2004; Wu et al. 2011). In addition, several products of microbial fermentation are incorporated into food as additives and supplements such as antioxidants, anticancer agents, sweeteners, and preservatives (Couto and Sanromán 2006). Microorganisms play an important role in the production of a wide range of primary and secondary metabolites (Wijesinghe et al. 2012). For example, Leuconostoc sp., lactic acid bacteria, is used in a large variety of dairy and food fermentations (Kleerebezem et al. 1997). It is also used in the production of several biologically useful materials such as polyphenolic compounds and ginsenosides, which also possess antioxidative properties (Park et al. 2012; Yang et al. 2012; Bernaert et al. 2013). Therefore, there is increasing interest in the physicochemical properties in the fermented vegetables. Several studies have tried to characterize the fermentation process of Allium plants. Bernaert et al. (2013) reported that spontaneous fermentation process was highly related to the enhancement of biological activities such as antioxidant activity and profiles of flavonoids and polyphenol. Another study concerning leek fermentation could be found in the fermentation or ripening of Kim-chi, Korean traditional fermented cabbage. Over ripening via rapid growth of some lactic acid bacteria in Kim-chi is a factor of low quality cause of strong acidic tastes. With the reason, Lee and Kim (1999) tried to supplement chive in Kim-chi to suppress the growth of lactic acid bacteria Leuconostoc involved in over ripening. Very recently, Yang et al. (2014) have reported the isolation of lactic acid bacteria from leek Kim-chi and suggested Weissella confusa LK4 as a starter culture for leek fermentation.

The present study was conducted to isolate a specific bacterial strain that demonstrate good viability during the fermentation of the Chinese chives juice. We analyzed the fermentative characteristics and biological activities of Chinese chives juice fermented by Leuconostoc mesenteroides SK1962.

Materials and methods

Preparation of Chinese chives juice and fermentation medium

Allium tuberosum was purchased from the local market (Seoul, Korea). For the preparation of Chinese chives juice medium, the juice was prepared. Briefly, the tubers of A. tuberosum were cleaned through tap water and deskinned. The juice was prepared by grinding with juicer (Angeljuicer, Busan, Korea), and solid particles were separated by filtering using 4-layer gauzes. Then liquid fraction (filtrate) was used for medium preparation. For the fermentation of chives juice, the filtrate was added to 2× MRS (de Man, Rogosa and Sharpe) at a 1:1 (v/v) ratio, and pH was adjusted to 7.0. This media was named CMRS.

Bacterial isolation for the fermentation of Chinese chives juice

For the purpose of isolating bacteria involved in the fermentation of Chinese chives without any inoculation, the culture was incubated for 3 days as mentioned above, and was spread on MRS agar media. Among the major bacteria that overcame the antibacterial activity of Chinese chives juice, one of the isolates was selected, and identified by molecular phylogenetic analysis based on the 16S rRNA gene sequence. Polymerase chain reaction (PCR) was employed for the analysis of 16S rRNA gene sequence using universal primers (27F, 5'-AGA GTT TGA TCC TGG CTC AG-3'; 1492R, 5'-GTT TAC CTT GTT ACG ACT T-3') (Weisburg et al. 1991). The sequences of PCR products were determined by the DNA analyzer (ABI PRISM 3730XL, Applied Biosystem, Franklin Lakes, NJ, USA). Identities of the sequences from isolated strains were searched at GenBank using BLAST (Basic local alignment search tool). Multiple sequence comparisons were performed using the CLUSTAL_W program (Thompson et al. 1994). Calculation of evolutional distance and construction of phylogenetic tree were conducted to identify the isolated strain by the Maximum Composite Likelihood method using the MEGA 4 program (Tamura et al. 2004, 2007).

Determination of total polyphenols

Total polyphenol content in the extracts was determined by the modified Folin–Ciocalteu method (Wolfe et al. 2003). Aliquot of each extracted sample (0.1 mL) was added to 2 mL 2 % Na₂CO₃. Samples were vortexed for 10 min at room temperature, and 0.1 mL 50 % Folin–Ciocalteu reagent was added. The mixture was vortexed for 15 s, and
allowed to react for 30 min at 40 °C. Absorbance of each sample was measured at 700 nm using the Shimadzu UV-1601 spectrophotometer. Samples of extracts were evaluated at a final concentration of 1 mg/mL. Total polyphenol content in gallic (25–500 ppm) was calculated using the following equation based on the calibration curve: 

\[ y = 0.0261x, \quad R^2 = 0.9897, \] where \( x \) was the absorbance, and \( y \) was the gallic equivalent (mM/mL).

**Determination of total flavonoids**

Total flavonoids were estimated using previously published methods (Ordonez et al. 2006). Samples (0.5 mL) were mixed with 1.5 mL 95 % ethanol, 0.1 mL 10 % aluminum chloride, 0.1 mL 1 M potassium acetate, and 2.8 mL distilled water. After the tubes were mixed for 30 min at room temperature, the absorbance was measured at 415 nm. Sample extracts were evaluated at a final concentration of 1 mg/mL. Total flavonoid content was calculated in quercetin (5–100 ppm) using the following equation based on the calibration curve: 

\[ y = 0.0752x, \quad R^2 = 0.9996, \] where \( x \) was the absorbance, and \( y \) was quercetin equivalents in mg/mL.

**DPPH assay**

Free radical scavenging activities were determined in vitro using the 2-diphenyl-2-picrylhydrazyl (DPPH) photometric assay, as described previously (Tadic et al. 2008) with some modification. Fermented Chinese chives (100 µL) were mixed with 900 µL 0.04 mg/mL DPPH in methanol solution. The mixture was allowed to react for 30 min with gentle agitation. Reduction of violet color was induced by free radical scavenging activity, and absorbance was measured with a spectrophotometer at 517 nm. Each solvent used in the extraction was used as a negative control, and tert-butyl-hydroxytoluene was used as a positive control. The percentage inhibition was calculated by comparing sample absorbance with negative controls, and was plotted against sample concentrations.

**Determination of superoxide radical scavenging activity in MAC-T cells**

Superoxide radical scavenging activity was determined in bovine mammary epithelial cells (MAC-T) by the method (Han et al. 2013). Samples were prepared by using only Chinese chives juice (CO), supernatants of fermented MRS broth only (LO) and Chinese chives (FC) mixing with MRS medium as 1:1 (v/v) by \( L. \) mesenteroides SK1962 at 30 °C. Aliquots (100 µL) of the Chinese chive culture were dropped onto sterilized paper disks 6 mm in diameter. The paper disk was dried for 30 min and placed on plates inoculated with pathogenic bacteria. The plate was incubated at 30 °C for 18 h. The diameter of clear zone was measured. The pathogenic bacteria used are as follows: \( Listeria \) monocytogenes KACC 10550, \( Staphylococcus \) aureus KCCM40405, \( Burkholderia \) sp., \( Pantoëa \) agglomerans, \( Haemophilus \) parasuis, \( H. \) somnus ATCC 9184, \( Salmonella \) gallinarum, and \( Escherichia \) coli O157. Some of these pathogenic bacteria were obtained from the National Veterinary Research Quarantine Service in South Korea.

**Results and discussion**

**Isolation of bacteria involved in fermentation of Chinese chives juice**

Among the major bacteria types that were able to survive or overcome the antibacterial activity of Chinese chives juice, one of isolates was selected and sequenced for the 16S RNA gene. Based on the results of the molecular phylogenetic analysis, it was determined that the isolate was closely related to \( L. \) mesenteroides LMG8159 (Accession no HM443957), and was designated as \( L. \) mesenteroides SK1962 (Fig. 1). \( L. \) mesenteroides is known as a heterofermentative lactic acid bacterium, and it is reported to be involved in the fermentation of various fruits and vegetables with other lactic acid bacteria in the \( Lactobacillus \) and \( Pediococcus \) genera (Cho et al. 2006).
Effect of Chinese chives juice on various lactic acid bacteria

Because Chinese chives juice is known to have an antibacterial activity, it is questionable whether various lactic acid bacteria including the isolated strain \(L.\) \(mesenteroides\) can grow normally on CMRS media. As shown in Table 1, CMRS did not show any antibacterial activity against \(L.\) \(mesenteroides\) SK1962, whereas Chinese chives juice only (CO) cultures demonstrated antibacterial activity (11.3 mm). Weissella sp. also displayed similar patterns as the \(L.\) \(mesenteroides\) SK1962. However, \(Lactobacillus\) \(brevis\), \(Lactobacillus\) \(plantarum\), \(Lactobacillus\) \(sakei\), and \(Lactobacillus\) \(reuteri\) showed strong resistance to both CO and CMRS. Only \(Lactobacillus\) \(lactis\) was sensitive to both CMRS and CO. \(L.\) \(lactis\) SK2083 was more sensitive to Chinese chives juice as compared with \(L.\) \(lactis\) SK2085. Therefore, CMRS was reconfirmed to be good media for the fermentation of Chinese chives juice by \(L.\) \(mesenteroides\) SK1962.

pH changes and cell growth of \(L.\) \(mesenteroides\) SK1962 at varying concentrations of Chinese chive juice

The change of pH and growth of \(L.\) \(mesenteroides\) SK1962 at different concentrations of Chinese chive juice were estimated. As shown in Fig. 2A, the pH was 6.5 in LO (\(L.\) \(mesenteroides\) SK1962 only cultured on MRS broth), while it was 7.0 in FC (Chinese chives juice fermented by \(L.\) \(mesenteroides\) SK1962) at the start of the incubation period. The pH fell sharply after 8 h of incubation, and gradually decreased at different incubation times in both treatments. After 48 h of incubation, the pH was 4.16 in LO and 4.15–4.33 in FC. One of the characteristic features of fermentation by lactic acid bacteria is the resultant decrease in pH due to the production of organic acids during fermentation (Wu et al. 2011). In the presence of Chinese chives, pH was higher than the control until 24 h of incubation. After 24 h, no remarkable difference was detected between the pH of FC and LO.

Even though Chinese chive is well known for its antibacterial activity (Lee et al. 2004), \(L.\) \(mesenteroides\) SK1962 showed normal growth in 10–50 % Chinese chives juice during incubation (Fig. 2B). However, its growth in LO dropped sharply after 24 h incubation. Results indicate that addition of Chinese chives juice

| Table 1 Antibacterial activity of Chinese chives juice against various lactic acid bacteria |
|-----------------------------------------------|
| Strain | CMRS | CO |
| \(Lactobacillus\) \(brevis\) SK1304 | ND | ND |
| \(Lactobacillus\) \(sakei\) SK1958 | ND | ND |
| \(Leuconostoc\) \(mesenteroides\) SK1962 | ND | 11.3 ± 0.6 |
| \(Lactobacillus\) \(lactis\) SK2083 | 19.7 ± 0.6 | 25.3 ± 2.1 |
| \(Lactobacillus\) \(lactis\) SK2085 | 17.0 ± 1.0 | 21.3 ± 1.5 |
| \(Lactobacillus\) \(reuteri\) SK2636 | ND | ND |
| Weissella sp. SK3493 | ND | 8.0 ± 0.0 |
| \(Lactobacillus\) \(plantarum\) SK3494 | ND | ND |

CMRS Chives juice mixed with 2 X MRS broth at a 1:1 (v/v) ratio; CO Chives juice only
Hole diameter was 6 mm
ND not detected
promoted the growth of L. mesenteroides SK1962 as compared with control, suggesting that L. mesenteroides SK1962 can be used to ferment Chinese chives, and is not inhibited by its antibacterial activity. Furthermore, it was shown that Chinese chives juice could be used as a good nutrient source for the growth of L. mesenteroides SK 1962 cells. Therefore, maximum 50 % addition of Chinese chives juice was used for the fermentation in further study.

**Effect on total polyphenol content during the fermentation of Chinese chives juice**

The effect of Chinese chives juice on polyphenol content was represented in Fig. 3(A). Total polyphenol content was initially measured to be 5.67 mM prior to incubation, and this dropped following the 8-h incubation in FC. After 16- and 24-h incubations, polyphenol content transiently increased before decreasing to 3.44 mM. In LO, total polyphenol gradually decreased from 3.50 to 2.25 mM during the 48-h incubation. Total polyphenol content increased 31–87 % at various incubation times in the Chinese chives juice fermented by L. mesenteroides SK1962 (FC) as compared with Chinese chives juice only (CO, 2.63 mM). Previous studies by Bernaert et al. (2012) showed that total phenolic acid content varied between 5 and 15 mg GAE per gram of dry weight in 30 leek cultivars. In addition, fermentation of leek resulted in an increase in polyphenolic compounds as compared with that of fresh leeks. In addition, the polyphenol content of FC was 53–89 %, which was higher than that of LO. Specifically, the polyphenol content of FC was 83 and 89 % higher as compared to that of LO after 16 and 24 h of incubation, respectively. It was possible that total polyphenol content decreased due to biodegradation polyphenol compounds in Chinese chives during the process of fermentation. Similarly, Othman et al. (2009) also reported the loss of antioxidant phenolic compounds during fermentation of Chetoui olives.

**Effect on total flavonoid content during the fermentation of Chinese chives juice**

Total flavonoid content in FC was maintained at 1.04–1.14 mg/mL during fermentation (Fig. 3B); flavonoid content in FC was the highest at 24 h, at 1.14 mg/mL. However, flavonoid content of FC was less than that of Chinese chives juice only (CO), which was measured to be 1.17 mg/mL. This suggests that the total flavonoid content was slightly decreased by fermentation. On the other hand, the total flavonoid content of FC was two times that of LO, which slightly increased from 0.38 mg/mL to 0.44 mg/mL during the fermentation.

No agreements have been reached in previous studies regarding the total flavonoid content in fermented and non-fermented plants. The flavonoid content was increased in Lactobacillus-fermented Graptopetalum paraguayense E. walther, as observed by Wu et al. (2011). It is possible that the variability in flavonoid content may be due to specific features of biodegradation of plant compounds during fermentation.

**DPPH-radical scavenging activity during fermentation of Chinese chives juice**

Figure 3C shows antioxidant activities in LO and FC during the fermentation. DPPH-radical scavenging activity of CO was 2.74 mM, while that of FC was a little higher than CO at different fermentation times. Antioxidant activity of FC were 2.78–2.97 mM during 24 h of incubation. Antioxidant activities in FC and LO were maintained for the first 24 h, and then decreased to 2.25 mg/mL in FC and 1.93 mg/mL in LO at 48 h. This is because polyphenol and flavonoid contents also decreased at 48 h. It was also observed that antioxidant activities of FC were higher as compared to those of LO at different fermentation times. This may account for the higher total polyphenol and flavonoid contents in FC compared to those of LO.
Fig. 3 Changes in biological activities of Chinese chives juice during fermentation by \textit{L. mesenteroides} SK1962.

(A) Total polyphenol content.
(B) Total flavonoid content.
(C) DPPH-radical scavenging activity. \textit{Filled and empty circles} mean \textit{L. mesenteroides} only in medium and fermented Chinese chives juice media, respectively. \textit{FC} Chinese chives juice fermented by \textit{L. mesenteroides} SK1962.
Kusznierewicz et al. (2004) reported an increase in the antioxidant activity of cabbage following a 2-week fermentation process. Bernaert et al. (2013) also reported that fermentation of green leaves of leek for 21 days resulted in a 62% increase in antioxidant capacity as compared with activity of fresh leek. The antioxidant activity increased with increasing phenolic acid content. Nazzaro et al. (2008) observed increased DPPH-radical scavenging activity in carrot juice after 2 days of fermentation with Lactobacillus delbrueckii subsp. bulgaricus.

It is known that polyphenol acts as an antioxidant by scavenging free radicals and preventing ROS damage (Perron and Brumaghim 2009). The present study showed that enhanced antioxidant effect of fermented Chinese

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**Fig. 4** Superoxide free radical scavenging activity in MAC-T cells during fermentation of Chinese chives juice by *Leuconostoc mesenteroides* SK1962. (A) MAC-T cells without treatment of DHE. (B) Addition of DW into treated MAC-T cells with DHE. (C) Addition of MRS medium only into treated MAC-T cells with DHE. (D) Addition of chives only into treated MAC-T cells with DHE. (E) Addition of low concentration of fermented chives into treated MAC-T cells with DHE. (F) Addition of high concentration of fermented chives into treated MAC-T cells with DHE. (G) Addition of *L. mesenteroides* culture supernatant into treated MAC-T cells with DHE.
chives juice originated from the increased polyphenol and flavonoid contents.

**Superoxide radical scavenging activity in MAC-T cells during the fermentation of Chinese chives juice**

Superoxide free radical scavenging activity was estimated by the intensity of red fluorescence in MAC-T cells. The fluorescent dye DHE is sensitive to reactive oxygen species such as superoxide through oxidation of the dye which results in staining cells with a bright fluorescent red. The red areas in the cells represent oxidized DHE representing the production of superoxide. The nuclei were stained with DAPI presenting blue color. As shown in Fig. 4, both the culture supernatant of *L. mesenteroides* (LO) and non-fermented chives (CO) decreased superoxide production in MAC-T cells at 12-h post-treatment. However, fermented chives juice by *L. mesenteroides* was more effective in reducing superoxide production whether using low (25 μL/mL) or high (50 μL/mL) concentration. Thus far, lactic acid bacteria is widely known for its antioxidant effect. Wu et al. (2014) reported a strong antioxidant property on scavenging superoxide anion free radicals of different fractions of *Lactobacillus*, which could protect the Caco-2 cell from oxidative stress. Chinese chives have traditionally been used as nutraceutical foods containing abundant antioxidant compounds. In the study, the enhancing scavenging activity properly involved with the mechanism of fermentation. This may be caused by an increase in the amount of phenolic compounds and flavonoids from the degradation of Chinese chives during microbial fermentation. It may also result from the liberation or synthesis of a variety of antioxidant compounds inducing by the structural breakdown of cell walls of Chinese chives as suggested in Hur et al. (2014).

**Antibacterial activities during the fermentation of Chinese chives juice**

Antibacterial activities were determined by measuring the size of clear zone against the pathogenic bacteria: *L. monocytogenes*, *P. agglomerans*, *H. parasuis*, *S. gallinarum*, *E. coli* O157, and *Burkholderia* sp. As shown in Table 2, antibacterial activities of CO showed the strongest inhibitory effect against *L. monocytogenes* and very mild inhibitory effect against *Burkholderia* sp. However, it had no inhibitory effect on *P. agglomerans*, *H. parasuis*, *S. gallinarum*, and *E. coli* O157. FC demonstrated antibacterial effects against all tested bacteria, exerted the strongest inhibitory effects against *L. monocytogenes* and *E. coli* O157. In addition, LO also showed antibacterial effects against all pathogenic bacteria. LO demonstrated strong inhibition effect against *L. monocytogenes* and moderate inhibitory effects against *H. parasuis*. Overall, antibacterial activities of FC were higher than those of LO.

Alzoreky and Nakahara (2003) reported weak antibacterial activity in extracts from some edible plants commonly consumed in Asia. The antibacterial activity of ginger extract exhibited no inhibitory effects on *E. coli* (Chand 2013). Similarly, antibacterial activity of fresh Chinese chives had no inhibitory effect on *E. coli* O157 in this study. The diameter of the clear zone on *E. coli* for the garlic extract was 9.67 mm in the study by Chand (2013), while this study showed that the clear zone in fermented Chinese chives was 13.00 mm. Therefore, it is possible that fermented Chinese chives have increased antibacterial activity against *E. coli* as compared to fresh plants belonging to the Allium genus such as ginger, garlic, and leek. Therefore, our results showed that fermented Chinese chives with *L. mesenteroides* SK1962 could strongly inhibit bacterial growth.

According to the above results, it could be concluded that fermentation increases various bioactive compounds including polyphenols and flavonoids, which increases antioxidant and antibacterial properties in *L. mesenteroides* SK1962-fermented Chinese chives.

**Table 2 Antibacterial activities against pathogenic bacteria during fermentation by *L. mesenteroides* SK1962**

| Pathogenic bacteria                  | Clear zone diameter, mm |
|--------------------------------------|-------------------------|
|                                      | Cultured media | CO                |
|                                      | MRS         | CMRS             |
| *Pantoea agglomerans*                | 10.0 ± 0.0     | 9.7 ± 1.2        | ND                  |
| *Haemophilus parasuis*               | 11.7 ± 0.6     | 9.7 ± 0.6        | ND                  |
| *Salmonella gallinarum*              | 10.7 ± 0.6     | 10.7 ± 1.5       | ND                  |
| *Escherichia coli* O157              | 11.0 ± 1.7     | 13.0 ± 1.0       | ND                  |
| *Burkholderia* sp.                   | 10.7 ± 1.2     | 12.3 ± 0.6       | 10.3 ± 2.1          |
| *Listeria monocytogenes*             | 18.0 ± 1.0     | 18.3 ± 2.1       | 18.3 ± 1.5          |

Antibacterial activities: 8 mm = no inhibition; 8 mm ≤ very mild inhibition < 12 mm); 12 mm ≤ moderate inhibition < 15 mm; 15 mm ≤ strong inhibition.

*L. mesenteroides* SK1962 was cultured for 24 h.

ND Not detected

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