Microbiological characterization and functionality of set-type yogurt fermented with potential prebiotic substrates *Cudrania tricuspidata* and *Morus alba* L. leaf extracts

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ABSTRACT

The objective of this study was to investigate the effect of 2 plant leaf extracts on fermentation mechanisms and health-promoting activities and their potential as a nutraceutical prebiotics ingredient for application in dairy products. The individual active phenolic compounds in the plant extract-supplemented milk and yogurts were also identified. Compared with control fermentation, the plant extracts significantly increased the growth and acidification rate of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. In particular, plant extract components, including monosaccharides, formic acid, and hydroxycinnamic acid, such as neo-chlorogenic, chlorogenic, and caffeic acid, together play a stimulatory role and cause this beneficial effect on the growth of yogurt culture bacteria through fermentation. In addition, supplementation with the plant extracts enhanced antioxidant activities with increased total phenolic contents, especially the highest antioxidant activity was observed in yogurt supplemented with *Cudrania tricuspidata* leaf extract.

Key words: yogurt, fermentation, phenolic compounds, antioxidant activity

INTRODUCTION

Yogurt containing pro- and prebiotics is known to have many beneficial effects such as strengthening the immune system and intestinal system, assisting lactose digestion, and alleviating inflammatory bowel disease and allergies (Adolfsson et al., 2004). In addition to these health benefits, the physical properties,

appearance, and flavor of the yogurt are important factors for consumer acceptability. Texture, flavor, acid production from the starter cultures, bacteria during fermentation, and even storage care can be modified by altering the process conditions or by adding supplementary ingredients. The ingredients that determine the nutritional value or technical applications (or both) of yogurts include milk protein (Peng et al., 2009), prebiotics (Bruno et al., 2002), and plants (Amirdivani and Baba, 2011). Fructooligosaccharide, galactooligosaccharide, and isomaltooligosaccharide (Manning and Gibson, 2004) are widely used prebiotics that enhance the activity of gut microorganisms. Recently, research efforts have been focused on finding prebiotics from plant extracts, but only a few studies have shown the functional and prebiotic properties of plant extracts in yogurt processing. Plant extracts have been shown to possess health-promoting properties such as antimicrobial and antioxidant effects. Therefore, new prebiotics derived from plant extracts that stimulate the growth of lactic acid bacteria (**LAB**) are needed. In a search for new natural prebiotic sources for yogurt production, we manufactured yogurts supplemented with functional plant extracts of *Cudrania tricuspidata* leaves (**CT**) and *Morus alba* L. leaves (**MA**), and studied various functional properties of these yogurts.

Cudrania tricuspidata, which has beneficial properties such as antimicrobial, anti-inflammatory, and antitumor effects and α -glucosidase inhibitory activity (Park et al., 2006), is one of the most ubiquitous traditional plant remedies in Asia. *Morus alba* L. is a deciduous tree that is distributed throughout Asia, Africa, Europe, and South and North America and found in a wide range of tropical areas (Srivastava et al., 2006). Tree bark, fruits, and leaves of *M. alba* have been used as natural medicines and the leaves are used as tea infusion in Asian countries (Katsube et al., 2009). Extensive reports are available on the inhibitory effect of water-soluble *C. tricuspidata* and *M. alba* leaf extracts on lipid

Received December 23, 2015.

Accepted March 28, 2016.

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peroxidative formation (Cha et al., 2000) and on the antibacterial efficacy of *C. tricuspidata* fruit essential oil (Bajpai et al., 2013). However, the use of these 2 plant leaf extracts in food has hardly been studied.

Previously, our group has screened aqueous extracts from 100 herb species to test their prebiotic activity by determining oligosaccharides, water-soluble dietary fiber, and total phenol contents (data not shown). On the basis of the results, 2 plant extracts were selected for this study, which may serve as a good source of functional nutrients and prebiotics for yogurt starter cultures to improve microbial and chemical properties. Here, we investigated the effects of yogurt supplementation with the extracts on the acidification kinetics, growth of yogurt starter cultures, and fermentation mechanisms during fermentation. In addition, the phenolic compounds in the plant extract-supplemented yogurts were profiled, and the antioxidant properties of the final product immediately after production and during 28 d of refrigerated storage were evaluated to investigate the effects of the plant extracts on fermentation.

MATERIALS AND METHODS

Plant Extracts

The CT and MA were obtained from the local market (Sunchang, Jeollabuk-Do, South Korea). One hundred grams of leaves was washed and then soaked in distilled water (1,000 mL) in a water bath (100°C) with occasional shaking for 9 h for extraction. Then, the leaf extract was filtered through filter paper. The clear solution was concentrated by evaporation to dryness under vacuum at temperatures not higher than 50°C. The concentrated plant extracts were freeze-dried before use in yogurt production.

Preparation of Fermented Milks Supplemented With Plant Extracts

The powdered plant extracts [0.2% (g/g)] were added to prewarmed (60°C) milk, and the mixtures were pasteurized (85°C, 15 min) and cooled to 41°C. Milks with and without plant extract were inoculated with a commercial yogurt starter culture containing *Streptococcus thermophilus* (ST-BODY-1) and *Lactobacillus delbrueckii* ssp. *bulgaricus* (LB-12) direct vat set (DVS) cultures (Chr. Hansen, Hørsholm, Denmark). The inoculum was prepared using direct vat set cultures at concentration of 0.002 g of starter culture (each per liter). The mixtures were incubated at 41°C until a pH of 4.5 was achieved. Samples were collected directly

after production and after 7, 14, 21, and 28 d of refrigerated storage at 4°C. The yogurt without added plant extracts was used as control in this study. All samples were lyophilized and stored at −20°C before analysis.

Kinetic Parameters

Each fermentation, performed in 3 replicates, was monitored as described previously (Oliveira et al., 2009). The acidification rate ($V_{\max}$) was calculated as the time variation of pH (dpH/dt) and expressed as 10^{-3} pH units/min. During the fermentation period, the following kinetic parameters were calculated: (1) $t_{\max}$ (h), time at which $V_{\max}$ was reached; (2) $t_{\text{pH } 5.0}$ (h), time to reach pH 5.0; and (3) t_f (h), time to complete the fermentation.

Viability of Starter Cultures

For each run of the experimental design, samples were analyzed immediately after fermentation and after 4 wk of storage at 4°C. *Streptococcus thermophilus* was counted on M17 agar (Oxoid, Basingstoke, UK) plates after aerobic incubation at 37°C for 48 h. *Lactobacillus delbrueckii* ssp. *bulgaricus* was enumerated on MRS (Difco Laboratories, Detroit, MI) at pH 5.2 with acetic acid after anaerobic incubation at 45°C for 72 h in an anaerobic jar with anaerobe gas packs (MGC, Tokyo, Japan; Ravula and Shah, 1998; Tharmaraj and Shah, 2003).

Analysis of Sugars and Organic Acids

Sugars (fructose, glucose, galactose, and lactose) in the plant yogurts were analyzed by using HPLC (Agilent Technologies, Waldbronn, Germany) based on AOAC Official Methods 980.13 and 972.16 (AOAC International, 2012).

All organic acid standards (citric acid, lactic acid, propionic acid, pyruvic acid, and formic acid) were supplied by Sigma Chemical Co. (St. Louis, MO). Organic acids in the plant yogurts were determined following procedure reported by Donkor et al. (2005). Briefly, the plant yogurt samples (2 g) were mixed with 0.005 N H_2SO_4 (6 mL). The mixture was centrifuged at $5,000 \times g$ for 10 min and the supernatant was filtered with 0.2- μm pore size membrane filter. The separation of organic acids was achieved using HPLC fitted with Aminex HPX-87C column (Bio-Rad, Hercules, CA) maintained at 50°C. The mobile phase was 0.005 N H_2SO_4 with a flow rate 0.6 mL/min. Quantification of organic acids was determined from the standard curves of solutions of known concentrations.

Ultra-Performance Liquid Chromatography–MS/MS Analysis of Phenolic Compounds

For extraction of phenolic compounds from the plant yogurts, lyophilized yogurt samples (2 g) were homogenized with 14 mL of 50% ethanol containing 0.05 M H_3PO_4 in water. The extracts were sonicated in an ultrasonic bath at room temperature for 20 min and centrifuged at $5,180 \times g$ for 30 min. The supernatant was filtered through a 0.2- μm pore size membrane filter into HPLC vials for analysis.

The ultra-performance liquid chromatography–MS/MS analyses were carried out using an ACQUITY Ultra Performance Liquid Chromatography system (Waters, Milford, MA) equipped with a Z-spray electrospray ionization source and ZEVO TQ iontrap (MS/MS; Waters) operating in negative mode. MassLynx software (version 4.0, Waters) was used to control the instruments, and for data acquisition and processing. Sample solutions were injected into a reversed-phase column (BEH C18, 1.7 μm , 2.1×150 mm, Waters), which was maintained at 30°C. The separation was executed with a mobile phase consisting of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) with linear gradient elution performed as follows: 0 to 9.8 min, 8% B; 9.8 to 21.80 min, 15% B; 21.8 to 23.8 min, 22% B; 23.8 to 27.8 min, 40% B; 27.8 to 28.2 min, hold on 40% B; and 28.2 to 29.8 min, back to 8% B. The linear binary gradient was set to a flow rate of 0.2 mL/min and total run time was 29.8 min. Ten microliters of sample was injected into the electrospray source (source temperature 150°C, desolvation temperature 360°C, capillary voltage 2.5 kV, cone voltage 25 V). Argon was used as collision gas (collision energy 25 eV at the start).

Antioxidant Capacity

Total phenolic compounds were determined by an assay from Maksimović et al. (2005). Total phenolic contents (TPC) were expressed as micrograms of gallic acid equivalent (GAE)/mL using a regression of known concentrations of gallic acid, which was determined every time total phenolic assay was carried out. Also, we evaluated the antioxidant activities of yogurt by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, ferric-reducing antioxidant power assay, and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity using the method of Oh et al. (2013).

Statistical Analysis

All data were expressed as means $\pm$ SD. Statistical significance for the differences between the groups

was assessed using Duncan's multiple range tests. SAS software version 9.2 (SAS Institute Inc., Cary, NC) was used to perform all statistical tests. Values of $P < 0.05$ were considered to indicate a significant difference.

RESULTS AND DISCUSSION

Fermentation Kinetics and Culture Growth During Fermentation

The acidification kinetic parameters of the 2 plant yogurts are presented in Table 1. Both plant extracts significantly accelerated yogurt acidification. The addition of CT and MA increased V_{max} by 57 and 75%, respectively, whereas t_{max} of the CT-supplemented yogurt was the shortest. The fermentation was more rapidly completed in the plant extract-supplemented samples than in the control. Especially, $t_{\text{pH } 5.0}$ and t_f were slightly shorter in CT-supplemented than in MA-supplemented yogurt. These results showed that CT and MA significantly accelerated acidification and fermentation. Similarly, Amirdivani and Baba (2011) reported that fermentation with herbs enhanced the metabolic activity of yogurt bacteria and that the pH was decreased due to increased production of organic acids by LAB. According to Oliveira et al. (2009), coculture of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* was the best for yogurt preparation; however, in this study, the prebiotic effect of nondigestible oligosaccharides (such as maltodextrin, polydextrose, and oligofructose) on probiotics was evaluated. Multiple other studies have reported that various commercial prebiotics such as inulin, maltodextrin, oligofructose, and polydextrose accelerated acidification of the yogurt and reduced the fermentation time (Jaya and Das, 2004). Here, we tested the effects of 2 novel prebiotic ingredients from plant sources on yogurt fermentation kinetics. Our results indicated that CT and MA might be appropriate prebiotic ingredients for yogurt preparation in terms of acidification and fermentation time. The viability of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in the yogurts during fermentation is presented in Figure 1. In agreement with the results for acidification kinetics, both yogurts supplemented with plant extracts showed a more rapid increase in microbe counts than the control yogurt. The viabilities of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in all yogurts increased with 2.61 to 2.95 log cfu/mL and with 0.69 to 1.14 log cfu/mL, respectively, during fermentation. The *S. thermophilus* counts in the CT- and MA-supplemented yogurts were significantly higher than in the control, whereas the viability of *L. delbrueckii* ssp. *bulgaricus* at the end of fermentation was in the following order: control > MA > CT. After approximately 4 h of fermenta-

Table 1. Acidification kinetic parameters of the yogurts supplemented with the plant extracts¹

Kinetic parameter ²	Type of yogurt		
	Control	CT	MA
$V_{\max}$ (10^{-3} pH units/min)	8.33 ± 0.83^c	12.83 ± 0.50^b	14.58 ± 0.25^a
$t_{\max}$ (h)	10.67 ± 0.58^a	3.67 ± 0.58^c	4.00 ± 0.00^b
$t_{\text{pH } 5.0}$ (h)	10.71 ± 0.16^a	4.31 ± 0.05^c	4.72 ± 0.06^b
t_f (h)	11.48 ± 0.49^a	5.80 ± 0.18^b	5.95 ± 0.14^b

^{a-c}Different letters within a row denote significant differences ($P \leq 0.05$).

¹Values are presented as the mean $\pm$ SD ($n = 3$). CT = *Cudrania tricuspidata*-supplemented yogurt; MA = *Morus alba*-supplemented yogurt.

² $V_{\max}$ = acidification rate; $t_{\max}$ = time at which $V_{\max}$ was reached; $t_{\text{pH } 5.0}$ = time to reach pH 5.0; and (3) t_f = time to complete the fermentation.

tion, the $V_{\max}$ of the plant yogurts was higher than that of the control yogurt, consistent with the acidification kinetics. In particular, the MA-supplemented yogurt showed the highest counts of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* with 8.86 ± 0.02 log cfu/mL and 6.21 ± 0.01 log cfu/mL, respectively, among the yogurts with added plant extracts. Based on these results, we conclude that the plant extracts have potential for application in yogurt manufacture as prebiotics to shorten fermentation time, increase the viability of starter cultures, or to target beneficial LAB.

Sugar Contents

The concentrations of various sugars in the yogurts are displayed in Table 2. Before fermentation, the concentration of lactose in unfermented samples ranged from 53.02 ± 0.02 mg/g to 53.50 ± 0.19 mg/g. It considerably decreased during fermentation, ranging from 35.60 ± 0.08 mg/g to 37.42 ± 0.04 mg/g, without significant differences between the yogurt samples. The concentrations of galactose and glucose increased at the end of fermentation. Lactose is the primary carbon and energy source for many LAB, and it is metabolized to galactose and glucose during fermentation. Further, before fermentation, the concentration of fructose in the CT- and MA-supplemented samples was 0.18 ± 0.01 mg/g and 0.10 ± 0.01 mg/g, respectively, whereas fructose was not detected in the control sample. At the end of fermentation, its concentration in the CT- and MA-supplemented yogurt was decreased to 0.041 ± 0.00 mg/g and 0.037 ± 0.00 mg/g, respectively. This result indicated that fructose present in the CT and MA extracts may have served as an additional carbon source for yogurt fermentation. Because of the higher utilization rate of fructose in CT-supplemented yogurt (77%) than in MA-supplemented yogurt (61%), CT may enhance the metabolic activities of the starter cultures, resulting in dramatically shorter fermentation time than that for control yogurt. It has been reported

that metabolism of sugars by LAB varies from strain to strain and depends on the substrates and fermentation time (Amoroso et al., 1989).

Organic Acid Contents

The use of the 2 organisms *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* is beneficial for the production of fermented milk worldwide because of their synergistic interaction or symbiotic relationship (Roginski et al., 2003). The organic acid contents in the plant yogurts produced using these 2 organisms are displayed in Table 3. After fermentation, a significant change in the organic acid contents in the yogurts supplemented with the plant extracts was detected. As noted by Vedamuthu (1977), organic acids, such as lactic acid and pyruvic acid, and acetoin, diacetyl, ethanol, and CO_2 are the major end products of LAB carbohydrate metabolism. A small amount of formic acid was present in unfermented control milk. However, the amount of formic acid in unfermented milk supplemented with the plant extracts was higher than in control milk. In particular, CT-supplemented unfermented milk had the highest formic acid levels (5.03 ± 0.08 mg/g) among the samples, and 94.8% of the intrinsic formic acid in CT-supplemented unfermented milk was used during the production of plant yogurt, which enhanced the viability of the starter culture. The reason for this result may be that the formic acid in the plant extracts stimulated LAB fermentation. According to Roginski et al. (2003), *L. delbrueckii* ssp. *bulgaricus* has more proteolytic activity than *S. thermophilus*. Peptides and AA produced by the proteolytic enzymes of *L. delbrueckii* ssp. *bulgaricus* stimulate the growth of *S. thermophilus*, and the formic acid generated by *S. thermophilus* reacts with *L. delbrueckii* ssp. *bulgaricus*.

Lactic acid is a common end product of LAB fermentation, and its level increased significantly more in CT- and MA-supplemented yogurt than in control yogurt, to 6.91 ± 0.01 mg/g and 7.11 ± 0.02 mg/g, respectively.

Figure 1. Changes in (A) *Streptococcus thermophilus* counts and (B) *Lactobacillus bulgaricus* counts of the plant yogurts during fermentation. The results are presented as the mean $\pm$ SD ($n = 3$). CT = *Cudrania tricuspidata*-supplemented yogurt; MA = *Morus alba*-supplemented yogurt.

Table 2. Sugar contents of the yogurts supplemented with the plant extracts¹

Sugar	Control		CT		MA	
	Before fermentation	After fermentation	Before fermentation	After fermentation	Before fermentation	After fermentation
Fructose (mg/g)	ND	ND	0.18 $\pm$ 0.01 ^a	0.041 $\pm$ 0.00 ^c	0.10 $\pm$ 0.01 ^b	0.037 $\pm$ 0.00 ^c
Glucose (mg/g)	0.11 $\pm$ 0.01 ^f	0.39 $\pm$ 0.09 ^c	0.26 $\pm$ 0.01 ^d	0.66 $\pm$ 0.007 ^a	0.17 $\pm$ 0.08 ^e	0.48 $\pm$ 0.06 ^b
Galactose (mg/g)	ND	6.48 $\pm$ 0.05 ^b	ND	5.17 $\pm$ 0.07 ^c	ND	7.96 $\pm$ 0.03 ^a
Lactose (mg/g)	53.02 $\pm$ 0.02 ^a	35.60 $\pm$ 0.08 ^b	53.50 $\pm$ 0.19 ^a	37.42 $\pm$ 0.04 ^b	53.50 $\pm$ 1.09 ^a	36.29 $\pm$ 0.28 ^b

^{a-f}Different letters within a row denote significant differences ($P \leq 0.05$).

¹Values are presented as the mean $\pm$ SD ($n = 3$). ND = not detected. CT = *Cudrania tricuspidata*-supplemented yogurt; MA = *Morus alba*-supplemented yogurt.

During the manufacturing of plant yogurts, more lactic acid is generated than other organic acids. This result is consistent with the high titratable acidity (control yogurt: 0.75%, CT-supplemented yogurt: 0.78%, and MA-supplemented yogurt: 0.88%). In addition, the level of pyruvic acid increased significantly in all samples after fermentation. Moreover, pyruvic acid showed the lowest level, ranging from 0.024 ± 0.00 mg/g to 0.036 ± 0.04 mg/g, which may be because pyruvic acid acts as an important intermediary in various metabolic pathways. Previous studies have reported that the carbohydrate source, such as lactose, is transported across the cell membrane of LAB through the mediation of a membrane-located enzyme, and β -galactosidase then hydrolyzes the sugar to glucose and galactose. The LAB use the Embden-Meyerhof pathway to produce pyruvate and lactic acid dehydrogenase and then metabolize the pyruvate to lactic acid (Roginski et al., 2003).

As shown in Table 3, citric acid was a predominant organic acid in unfermented milk, ranging from 2.64 ± 0.19 mg/g to 2.84 ± 0.01 mg/g. After fermentation, a significant decrease occurred in the citric acid contents of all yogurts: 8.8 and 12.8% of citric acid in milk was used by yogurt cultures in samples with the CT and MA extracts, respectively, compared with 3.5% in the control yogurt. The results indicated that the CT and MA extracts could aid fermentation and supply growth factors for yogurt production.

Adhikari et al. (2002) did not detect propionic acid in milk. Similarly, we did not find propionic acid in unfermented control milk. However, propionic acid was present in unfermented milk supplemented with the plant extracts, ranging from 0.096 ± 0.03 mg/g to 0.10 ± 0.00 mg/g, and its level decreased after fermentation. As mentioned previously, the addition of the plant extracts for yogurt manufacturing enhanced the fermentation kinetics and culture growth. Furthermore, the organic acid produced during plant yogurt fermentation may promote volatile compounds that determine the flavor for yogurt as well as sensory characteristics.

Phenolic Compound Contents

The phenolic compound concentrations in the yogurts were evaluated throughout fermentation. Figures 2 and 3 show ultra-performance liquid chromatography–MS total ion current chromatograms of a mixed CT- and MA-supplemented yogurt. The retention times, mass spectral characteristics, and individual multiple reaction monitoring transitions used for quantifying are specified in Table 4.

After fermentation, the composition of the phenolic compounds in the CT- and MA-supplemented yogurts was similar to that in the unfermented samples; however, the amount of the individual compounds differed (Table 5). In total, 10 phenolic compounds were detected in the CT-supplemented yogurt. Both CT- and MA-supplemented yogurts contained a variety of phenolic compounds, and the most prominent compound was neo-chlorogenic acid followed by chlorogenic acid. The total contents of phenolic compounds content of the CT-supplemented yogurt significantly decreased when compared with nonfermented samples. The rate of phenolic compound utilization in CT-supplemented yogurt was higher than that in MA-supplemented yogurt (7.86 and 1.99%, respectively), indicating that the starter bacteria metabolized the phenolic compounds. Especially, neo-chlorogenic acid, chlorogenic acid, and caffeic acid were the most affected during fermentation in CT-supplemented yogurt, whereas 3,4-dihydroxy-hydrocinnamic acid was slightly increased after fermentation. Degradation of chlorogenic acid by cleavage of the ester bond between caffeic acid and quinic acid results in the production of 3,4-dihydroxy-hydrocinnamic acid, suggesting that caffeic acid was reduced at the double bond (Couteau et al., 2001). One study reported the detection of 3-(3-hydroxyphenyl)-propionic acid in an in vitro mixed-culture model of human colonic microflora after 24-h fermentation, resulting from dehydroxylation of 3,4-dihydroxy-hydrocinnamic acid (Rechner et al., 2004). We did not detect this compound, likely because

Table 3. Organic acids contents of the yogurts supplemented with the plant extracts¹

Organic acid	Control		CT		MA	
	Before fermentation	After fermentation	Before fermentation	After fermentation	Before fermentation	After fermentation
Citric acid (mg/g)	2.64 ± 0.19^{abc}	2.54 ± 0.01^{bc}	2.69 ± 0.02^{ab}	2.40 ± 0.15^c	2.84 ± 0.01^a	2.47 ± 0.04^{bc}
Pyruvic acid (mg/g)	0.003 ± 0.00^e	0.024 ± 0.00^c	0.003 ± 0.00^e	0.033 ± 0.00^b	0.004 ± 0.00^d	0.036 ± 0.04^a
Lactic acid (mg/g)	ND	6.48 ± 0.028^c	ND	6.91 ± 0.01^b	0.20 ± 0.00^d	7.11 ± 0.02^a
Formic acid (mg/g)	0.28 ± 0.01^d	ND	5.03 ± 0.08^a	0.41 ± 0.01^c	0.87 ± 0.02^b	ND
Propionic acid (mg/g)	ND	ND	0.096 ± 0.03^{ab}	0.083 ± 0.01^c	0.10 ± 0.00^a	0.091 ± 0.00^b

^{a–e}Different letters within a row denote significant differences ($P \leq 0.05$).

¹Values are presented as the mean $\pm$ SD ($n = 3$). ND = not detected. CT = *Cudrania tricuspidata*-supplemented yogurt; MA = *Morus alba*-supplemented yogurt.

Figure 2. (A–H) Ultra-performance liquid chromatography (UPLC)–MS/MS chromatograms of phenolic compounds in *Cudrania tricuspidata* (CT)-supplemented yogurt. 1, Neo-chlorogenic acid; 2, chlorogenic acid; 3, 3,4-dihydroxyhydrocinnamic acid; 4, caffeic acid; 5, rutin hydrate; 6, quercetin-3-galactoside; 7, quercetin-3-glucoside; 8, kaempferol-3-galactoside; 9, kaempferol-3-rutinoside; 10, kaempferol-3-glucoside. (I) UPLC-MS total ion current chromatograms of phenolic compounds of CT supplemented yogurt. TIC = total ion chromatograms.

Figure 3. (A–H) Ultra-performance liquid chromatography (UPLC)–MS/MS chromatograms of phenolic compounds in *Morus alba*-supplemented yogurt (MA). 1, neo-chlorogenic acid; 2, chlorogenic acid; 3, 3,4-dihydroxyhydrocinnamic acid; 4, caffeic acid; 5, rutin hydrate; 7, quercetin-3-glucoside; 9, kaempferol-3-rutinoside; 10, kaempferol-3-glucoside. (I) UPLC–MS total ion current chromatograms of phenolic compounds of MA-supplemented yogurt. TIC = total ion chromatograms.

Table 4. Identification of phenolic compounds by ultra-performance liquid chromatography–MS/MS

Peak	Compound	Retention time (min)	Molecular weight (g/mol)	MS (<i>m/z</i>)	MS/MS MRM (<i>m/z</i>)	Cone voltage (V)	Collision energy (V)
1	Neo-chlorogenic acid	4.81	354.31	353	191.04, ¹ 178.96	25	20
2	Chlorogenic acid	7.53	354.31	353	191.04 ¹	25	30
3	3,4-Dihydroxy-hydrocinnamic acid	8.72	182.17	180.9	59, ¹ 136.9	25	15
4	Caffeic acid	9.51	180.16	179	134.97, ¹ 107.09	25	15
5	Rutin hydrate	19.08	610.52	609.18	300, ¹ 301	25	40
6	Quercetin-3-galactoside	19.36	464.38	463.1	300, ¹ 301	25	30
7	Quercetin-3-glucoside	20.16	464.38	463.1	300, ¹ 301	25	25
8	Kaempferol-3-galactoside	22.53	448.38	447.09	284, ¹ 285	25	30
9	Kaempferol-3-rutinoside	22.99	594.52	593.18	284, ¹ 285	25	40
10	Kaempferol-3-glucoside	24.12	448.38	447.05	284, ¹ 285	25	40

¹MS/MS multiple reaction monitoring (MRM) used for the quantification of each standard.

of the different fermentation model system and short fermentation time.

In recent years, the concept of prebiotics has been developed into a more comprehensive one. Vodnar and Socaciu (2012) reported that microencapsulation of LAB with green tea extract increased the cell viability and maintained bacterial stability. Further, it has been reported that red wine polyphenol extracts and cocoa-derived flavonols modulated the human gut microbiota, indicating that these natural polyphenols have prebiotic potential (Queipo-Ortuño et al., 2012). Based on these results, phenolic compounds in plant extracts might have affected the MA- and CT-mediated enhancement of yogurt processing parameters, such as acidification kinetics and viability of starter bacteria.

Additionally, CT and MA contained a variety of phenolic compounds, and the prominent compound was neo-chlorogenic acid followed by chlorogenic acid in both yogurt samples. The concentrations of flavonoids such as quercetin-3-glucoside and rutin hydrate

($6.15 \pm 0.32 \mu\text{g/g}$ and $5.69 \pm 0.04 \mu\text{g/g}$, respectively) were significantly higher in the CT-supplemented than in the MA-supplemented yogurt. The total phenolic compounds in the CT-supplemented yogurt were higher than that in the MA-supplemented yogurt ($30.42 \pm 0.06 \mu\text{g/g}$ and $24.24 \pm 0.03 \mu\text{g/g}$, respectively). This result suggested that the CT-supplemented yogurt had higher antioxidant activity than the MA-supplemented yogurt, which was confirmed by ferric-reducing antioxidant power (**FRAP**), DPPH, and ABTS measurements (Figure 4). It has been reported that CT and MA are used in a traditional plant remedy, because of their high content of phytochemicals such as phenolic acids and flavonoids, which are beneficial for health. Moreover, extracts of leaves of these herbs exhibited higher antioxidant activities than those of other parts (e.g., stems, roots, and fruits; Jeong et al., 2009). Our results indicated that these compounds are important factors contributing to antioxidant activity of the yogurts.

Table 5. Phenolic compounds contents of the yogurts supplemented with the plant extracts¹

Compound	CT		MA	
	Before fermentation ($\mu\text{g/g}$)	After fermentation ($\mu\text{g/g}$)	Before fermentation ($\mu\text{g/g}$)	After fermentation ($\mu\text{g/g}$)
Neo-chlorogenic acid	7.01 ± 0.13^a	6.40 ± 0.11^b	10.73 ± 0.80^a	9.50 ± 0.52^a
Chlorogenic acid	5.16 ± 0.01^a	4.84 ± 0.03^b	7.21 ± 0.52^a	6.37 ± 0.47^a
3,4-Dihydroxy-hydrocinnamic acid	0.005 ± 0.00^b	0.025 ± 0.01^a	0.17 ± 0.01^b	0.26 ± 0.02^a
Caffeic acid	4.35 ± 0.10^a	3.30 ± 0.09^a	0.040 ± 0.01^a	0.035 ± 0.00^b
Rutin hydrate	5.39 ± 0.55	5.69 ± 0.04^a	1.32 ± 0.15^a	1.23 ± 0.05^a
Quercetin-3-galactoside	0.43 ± 0.21^a	0.40 ± 0.01^b	ND	ND
Quercetin-3-glucoside	6.59 ± 0.28^a	6.15 ± 0.32^b	3.49 ± 0.28^a	3.44 ± 0.33^a
Kaempferol-3-galactoside	0.10 ± 0.00^a	0.082 ± 0.01^a	ND	ND
Kaempferol-3-rutinoside	1.68 ± 0.06^a	1.55 ± 0.02^b	0.43 ± 0.05^a	0.34 ± 0.04^b
Kaempferol-3-glucoside	2.31 ± 0.04^a	1.99 ± 0.09^b	1.33 ± 0.13^a	1.36 ± 0.17^a
Total phenolic compounds	33.02 ± 0.04^a	30.42 ± 0.06^b	24.73 ± 0.33^a	24.24 ± 0.03^b

^{a,b}Different letters indicate statistically significant differences between samples taken before and after fermentation ($P \leq 0.05$).

¹Values are presented as the mean $\pm$ SD ($n = 3$). ND = not detected. CT = *Cudrania tricuspidata*-supplemented yogurt; MA = *Morus alba*-supplemented yogurt.

Determination of Antioxidant Activity

The TPC in the yogurts with plant extracts was significantly higher than that in control yogurt both after fermentation and during cold storage (Figure 4A). The TPC gradually increased for all yogurts during storage, with the exception of the CT-supplemented yogurt. Nevertheless, the CT-supplemented yogurt still showed the highest TPC ($104.4 \pm 2.1 \mu\text{g}$ of GAE/mL), followed by MA-supplemented yogurt ($103.7 \pm 0.6 \mu\text{g}$ of GAE/mL). The fluctuations in TPC may be explained by the action of microbes and indigenous phytochemical compounds in the plant extracts during refrigerated storage

(Dalling, 1986). Microbial utilization of phenolic acids during fermentation and postacidification can lead to the production of other phenolic acids such as vanillic and *p*-hydroxybenzoic acids before the aromatic ring structure is broken down (Blum, 1998). Moreover, the decomposition of milk protein by the yogurt microbes might also contribute to the increased TPC, because the AA tyrosine, for instance, has a phenolic side chain (Shah, 2000).

The antioxidant activities of the yogurts as determined by 3 assays with different mechanisms are shown in Figures 4B to 4D. Although antioxidant activities showed slight fluctuations during the 28 d of storage,

Figure 4. Total phenolic content (A), ferric-reducing antioxidant power (FRAP) value (B), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (C), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity (D) of the yogurts supplemented with the plant extracts during refrigerated (4°C) storage. The results are presented as the mean $\pm$ SD ($n = 3$). Different letters indicate statistically significant differences among the different groups ($P < 0.05$). CT = *Cudrania tricuspidata*-supplemented yogurt; MA = *Morus alba*-supplemented yogurt.

the yogurts with plant extracts had significantly higher activities than control yogurt in all 3 antioxidant tests. At the end of storage, supplemented yogurts showed antioxidant activities approximately 2.4 to 4.4 times greater than that of control yogurt, depending on the extract. In particular, CT yogurt had the greatest antioxidant activity. Moreover, the yogurts with plant extracts maintained greater antioxidant activity during cold storage. In this study, the yogurts supplemented with plant extracts were characterized by high TPC. According to Velioglu et al. (1998) and Thompson et al. (2007), the higher antioxidant activities of yogurts by addition of plant extracts were most likely a result of the phytochemical contents of plant extracts and microbial metabolic activities. Continued microbial growth during storage may have altered some of the phenolic compounds, and hence, increased antioxidant activities (Blum, 1998). Consequently, the consumption of yogurt supplemented with plant extracts has potential health benefits associated with high antioxidant activity and live bacterial content.

CONCLUSIONS

Supplementation of plant extracts as prebiotics obviously decreased the fermentation time and increased the viability of the yogurt starter cultures when compared with control yogurt. Fructose and glucose in the plant extracts were used as energy sources by the starter cultures. In addition, fortification of yogurt with the plant extracts also led to an increase in lactic acid and pyruvic acid contents and a decrease in formic acid and propionic acid contents. Formic acid functioned to enhance the growth of LAB. Moreover, hydroxycinnamic acid, such as neo-chlorogenic, chlorogenic, and caffeic acid, in the plant extracts was degraded and metabolized during yogurt fermentation, which might be attributed to the fermentation properties of the plant extract-supplemented yogurts. Further, substantially higher antioxidant activities were observed for the yogurts with plant extracts than for nonsupplemented yogurt, concomitant with higher TPC. The improved fermentation and antioxidant properties of the yogurts were related to the phenolic compounds in CT and MA extracts; especially, CT-supplemented yogurts showed the greatest antioxidant activities. Therefore, the addition of CT extract to yogurt has the potential to be further developed for consumers as a functional yogurt with antioxidant properties.

ACKNOWLEDGMENTS

This research was supported by the Basic Science Research Program through the National Research Founda-

tion of Korea (NRF), funded by the Ministry of Education (2014R1A1A2008481) and the High Value-Added Food Technology Development Program of the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (iPET), and the Ministry for Food, Agriculture, Forestry, and Fisheries of Republic of Korea (313036-03-2-SB010).

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