Metabolome analysis of milk fermented by γ -aminobutyric acid-producing *Lactococcus lactis*

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ABSTRACT

γ -Aminobutyric acid (GABA) is one of the most important functional components in fermented foods because of its physiological functions, such as neurotransmission and antihypertensive activities. However, little is known about components other than GABA in GABA-rich fermented foods. A metabolomic approach offers an opportunity to discover bioactive and flavor components in fermented food. To find specific components in milk fermented with GABA-producing *Lactococcus lactis* 01-7, we compared the components found in GABA-rich fermented milk with those found in control milk fermented without GABA production using capillary electrophoresis time-of-flight mass spectrometry. A principal component analysis score plot showed a clear differentiation between the control milk fermented with *L. lactis* 01-1, which does not produce GABA, and GABA-rich milk fermented with a combination of *L. lactis* strains 01-1 and 01-7. As expected, the amount of GABA in GABA-rich fermented milk was much higher (1,216-fold) than that of the control milk. Interestingly, the amount of Orn was also much higher (27-fold) than that of the control milk. Peptide analysis showed that levels of 6 putative angiotensin-I-converting enzyme (ACE)-inhibitory peptides were also higher in the GABA-rich fermented milk. Furthermore, ACE-inhibitory activity of GABA-rich fermented milk tended to be higher than that of the control milk. These results indicate that the GABA-producing strain 01-7 provides fermented milk with other functional components in addition to GABA.

Key words: metabolome, γ -aminobutyric acid (GABA), *Lactococcus lactis*, fermented milk

INTRODUCTION

Various fermented milk products worldwide are produced by microorganisms, and lactic acid bacteria (LAB) are often used in starter cultures. Studies on the fermentation characteristics of LAB have been re-

viewed extensively (Auclair and Accolas, 1983; Kok, 1991; Heller, 2001; Smit et al., 2005; Cogan et al., 2007; Smid and Lacroix, 2013). Understanding lactic acid production and proteolytic activity is important in improving upon the fermentation process used during the manufacture of fermented milk. Sugar metabolism during the fermentation process and enzymes such as proteases or peptidases that can degrade casein have been investigated for many years (McKay and Baldwin, 1974; Thomas et al., 1974; Bosman et al., 1990; Meijer et al., 1996; Lapierre et al., 2002; Neves et al., 2010; Zhang et al., 2014). The degradation of milk by LAB can lead to the production of various metabolites such as volatiles, peptides, and organic acids. Some of these metabolites have beneficial effects on human health as well as on the texture of yogurt and cheese (Meisel and Bockelmann, 1999; St-Gelais et al., 2009). Although various elements in fermented milk have been investigated, many components that could influence texture and human health remain to be considered.

Recently, whole component analyses of cheese and yogurt have been conducted with metabolomic approaches using GC-MS, nuclear magnetic resonance, and liquid chromatography-electrospray ionization-MS (LC-ESI/MS; Ochi et al., 2012; Sforza et al., 2012; Le Boucher et al., 2013; Settachaimongkon et al., 2014). According to a recent peptidomic analysis using LC-MS/MS, novel bioactive components could be found in human skim milk (Wan et al., 2013). Metabolomic analyses that include an analysis of peptides may provide a new insight into fermented milk products because several peptides in fermented milk that contain lactotripeptides have bioactivity, such as angiotensin-converting enzyme (ACE)-inhibitory activity and immunomodulatory effects (Meisel and Bockelmann, 1999; Seppo et al., 2003). The use of capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) for metabolomics has recently attracted attention because of its high resolution and sensitive detection of unstable metabolites during the derivatization of plant and intestinal metabolites (Kusano et al., 2011; Matsumoto et al., 2012).

Components in fermented milk are considered richly diverse; a variety of fermented starters or nonstarters with various enzymatic and metabolic activities are

Received June 11, 2015.

Accepted October 1, 2015.

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present. Volatile profiles differ among fermented milk products produced from different starters (Gallardo-Escamilla et al., 2005). In a recent review, Settanni and Moschetti (2010) observed that nonstarter LAB could also influence cheese quality and components with health benefits. A further investigation of various fermented milk products using the metabolomic approach could lead to the discovery of new biological processes and components.

There have been many reports on γ -aminobutyric acid (GABA)-rich foods such as cheese, yogurt, soy milk, and fermented bread produced by LAB (Nomura et al., 1998; Siragusa et al., 2007; Li and Cao, 2010) because GABA has physiological functions in important processes such as neurotransmission and antihypertensive activities (Owens and Kriegstein, 2002; Inoue et al., 2003). Although GABA attracts attention for its physiological functions, little is known about components other than GABA in GABA-rich fermented foods. Other components such as peptides could influence the antihypertensive activity of the product. Chiu et al. (2013) reported that *Lactobacillus* strains harboring the glutamate decarboxylase gene could improve the ACE-inhibitory activity of fermented pepino milk. This result suggested that components other than GABA have ACE-inhibitory activity because GABA alone cannot inhibit ACE (Inoue et al., 2003). The metabolomic approach may provide insight into GABA-rich fermented milk products and their fermentation processes.

In our previous study, the GABA-producing strain *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* 01-7 and non-GABA-producing strain *L. lactis* ssp. *cremoris* 01-1 were isolated from commercial starters, and the coculture of these strains showed a high level of GABA production during cheese ripening (Nomura et al., 1998). Strain 01-7 is able to vigorously produce GABA, although the growth level of strain 01-7 in milk is low. Strain 01-1, which grows rapidly in milk, is not able to produce GABA or hydrolyze arginine and citrate (Nomura et al., 1999). The functional activities of both strains are unknown. In this study, to identify the specific components in GABA-rich fermented milk, we investigated components in milk fermented with and without the GABA-producing strain 01-7 using CE-TOFMS. In addition, putative ACE-inhibitory peptides and the ACE-inhibitory activity of fermented milk were investigated.

MATERIALS AND METHODS

Strains and Fermented Milk Products

The GABA-producing strain *L. lactis* ssp. *lactis* biovar *diacetylactis* 01-7 and the non-GABA-producing

strain *L. lactis* ssp. *cremoris* 01-1 were grown at 30°C for 24 h in M17 medium (Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose (GM17 medium). The cells were centrifuged at $13,000 \times g$ for 5 min and washed twice with saline. The harvested cells were resuspended in 8% skim milk and used to inoculate 5 mL of skim milk (1% vol/vol). The cultures were statically incubated at 30°C for 48 h. After centrifugation at $15,300 \times g$ for 10 min, the supernatants were used for metabolic analysis. Milk fermented with strain 01-1 alone and with a mixture of strains (01-1 and 01-7) are referred to as control fermented milk and GABA-rich fermented milk, respectively. Milk fermentations in each group were carried out in triplicate, and all 6 fermented milk samples were used for CE-TOFMS analysis.

CE-TOFMS

The analysis of metabolites including peptides in the fermented milk was performed using CE-TOFMS. One hundred microliters of each prepared supernatant was mixed with 900 μ L of methanol, 1,000 μ L of chloroform, and 400 μ L of Milli-Q water (Millipore, Billerica, MA), and the mixture was centrifuged at $2,300 \times g$ for 5 min at 4°C. The aqueous phase was passed through an ultrafiltration membrane (5-kDa cutoff filter; Ultrafree MC, Millipore) at $9,100 \times g$ for 2 h at 4°C. After the filtrate was completely evaporated, the residue was dissolved in 25 μ L of Milli-Q water. The prepared samples were analyzed using an Agilent CE-TOFMS system (Agilent Technologies, Waldbronn, Germany) as described previously (Matsumoto et al., 2012). Cationic and anionic metabolites were analyzed using a fused silica capillary (50 μ m, 80 cm) with a cation buffer solution (H3301-1001) and anion buffer solution (I3301-1023), respectively (Human Metabolome Technologies, Tsuruoka, Japan). To perform cation analysis, the samples were injected at a pressure of 5 kPa (50 mbar) for 10 s. The applied voltage was set at 27 kV. Electrospray ionization-mass spectrometry (ESI-MS) was conducted in the positive ion mode with the MS capillary voltage set at 4,000 V. To perform anion analysis, the samples were injected at a pressure of 5 kPa (50 mbar) for 25 s. The applied voltage was set at 30 kV. The ESI-MS was conducted in negative ion mode with the MS capillary voltage set at 3,500 V. The MS scan range was 50 to 1,000 (m/z). The CE-TOFMS data were analyzed using MasterHands ver. 2.16.0.15 software (Keio University, Tsuruoka, Japan). Metabolites including dipeptides were identified based on m/z and their migration times. Tri- or tetrapeptides were identified based on the only m/z . Peptides (2–4 amino acid residues) and other metabolites were annotated using the peptide list and metabolite library maintained by

Human Metabolome Technologies (HMT), respectively. Putative ACE inhibitory di- and tripeptides were identified based on BIOPEP (<http://www.uwm.edu.pl/biochemia/index.php/pl/biopep>) and ACE-inhibitory activity reported by Foltz et al. (2009).

The relative area was calculated as follows: relative area = each peak area/(peak area of internal control $\times$ sample volume). For calculating the ratio (GABA/control) in comparative analysis, the relative area of the GABA-rich fermented milk was divided by the equivalent area of the control fermented milk. The difference between the GABA-rich fermented milk ($n = 3$) and the control fermented milk ($n = 3$) was assessed using the 2-tailed Welch's *t*-test. *P*-values of < 0.05 were considered to be significant. The data were expressed as means of the relative area $\pm$ SD.

ACE-Inhibitory Activity

The ACE-inhibitory activities of the GABA-rich fermented milk ($n = 3$) and the control fermented milk ($n = 3$) were investigated. Fermented milk samples were centrifuged at $10,000 \times g$ for 10 min. The pH of the supernatants was adjusted to 6.0 with NaOH, and the supernatant was then added to a Vivaspın 500 filter with a molecular weight cut-off of 10 kDa (Sartorius Stedim Biotech GmbH, Goettingen, Germany) for removing proteins such as caseins and BSA. After centrifugation at $13,000 \times g$ for 45 min at 4°C, the filtered samples were serially (3-fold) diluted in water. The ACE-inhibitory activities of the diluted filtrates were investigated using an ACE kit-WST (Dojindo Laboratories, Kumamoto, Japan). The assay was performed according to the manufacturer's protocol. Absorbances of the reactions were measured at 450 nm using GloMax Discover

System (Promega, Madison, WI). The ACE-inhibitory activities of the samples were calculated by the following equation: ACE-inhibitory activity (%) = $[(A_{\text{blank 1}} - A_{\text{sample}})/(A_{\text{blank 1}} - A_{\text{blank 2}})] \times 100$, where $A_{\text{blank 1}}$ is the absorbance of the positive control (without ACE inhibition), A_{sample} is the absorbance of the sample, and $A_{\text{blank 2}}$ is the absorbance of the reagent blank. The 50% inhibitory concentration (IC_{50} value) was defined as the concentration of protein (mg/mL) required for 50% reduction of the absorbance, which was determined using nonlinear regression analysis. The one-tailed Welch's *t*-test was performed to determine statistical differences. *P*-values of < 0.05 were considered significant. The data were expressed as means of $IC_{50} \pm$ SD.

RESULTS

Differences Between Metabolic Profiles of Control and GABA-Rich Fermented Milk

Capillary electrophoresis-TOFMS detected 452 peaks in the samples of fermented milk (388 cation peaks and 64 anion peaks). Of these 452 peaks, 180 were annotated by referring to the HMT library database. The other 272 peaks were annotated by referring to the HMT peptide list. A Venn diagram showed that 54 metabolites were significantly higher in the GABA-rich fermented milk compared with that of the control milk ($P < 0.05$; Figure 1). Of the 54 metabolites, 6 were detected in only the GABA-rich fermented milk. Seventy-seven metabolites were unique to the control fermented milk ($P < 0.05$). Of these, 12 were detected only in the control milk. For the 321 overlapping metabolites, there was no difference between the 2 groups.

Changes in Identified Metabolites

Table 1 shows the changes in the metabolites found in the GABA-rich fermented milk ($n = 3$) compared with those found in the control fermented milk ($n = 3$). Mevalolactone, *N*-acetylhistidine, and 5-methoxyindoleacetic acid were detected in 3 samples of GABA-rich fermented milk and were not detected in the control samples. On the other hand, fructose 1,6-diphosphate, citric acid, pyridoxamine, 3-aminobutyric acid, and cyclohexylamine were detected only in the 3 control fermented milk samples. Other changed metabolites were detected in all 6 samples. Focusing on the metabolites that were >1.5 times (the ratio of GABA to control) more abundant in the GABA-rich fermented milk than in the control milk, the amount of GABA present in the GABA-rich fermented milk was much higher (1,216-fold) than that in the control milk, which was expected. The decarboxylation of glutamate occurred (Figure 2A). The control fermented milk

Figure 1. Venn diagram highlighting the number of significantly different metabolites, based on the relative peak area by capillary electrophoresis time-of-flight mass spectrometry, between γ -aminobutyric acid (GABA)-rich and control fermented milk samples and the not-significantly-overlapped metabolites. The numbers in parentheses in the Venn diagram correspond to the number of metabolites observed in either GABA-rich fermented milk or control milk. Color version available online.

contained GABA because a small amount of GABA was present in the skim milk. Interestingly, the amount of Orn present in the GABA-rich fermented milk was much higher than that in the control. At the same time, levels of Cit and Arg increased and decreased, respectively, in the GABA-rich fermented milk. Arginine is thought to be converted to Orn via Cit in the arginine deiminase pathway (Figure 2B). Regarding other AA, the amounts of Gln, Thr, Asp, Ala, Gly, and Asn were increased (2.2- to 1.5-fold), and those of Ile and Met were decreased (0.5- and 0.3-fold, respectively) in the GABA-rich fermented milk. In addition to AA, purine bodies significantly changed. Levels of hypoxanthine and guanine were higher and that of adenine was lower in the GABA-rich fermented milk than in the control milk. Focusing on the metabolites in the GABA-rich fermented milk that were less abundant (<0.67-fold) in the GABA-rich fermented milk compared with those in the control milk, citric acid was not detected, whereas the level of 2-oxoglutaric acid was increased. Furthermore, the level of isocitric acid present in the GABA-rich fermented milk was lower than that in the control milk. Citric acid is thought to be converted to 2-oxoglutaric acid via both isocitric acid and diacetyl (Figure 2C).

Changes in Putative ACE-Inhibitory Peptides

Peptides present in the samples were tentatively identified as dipeptides or other peptides based on their m/z and migration time (dipeptides) or only m/z (other peptides) profile, using the HMT peptide list. Table 2 shows the putative ACE-inhibitory peptides present in the GABA-rich fermented milk at levels 2-fold higher than those present in the control sample. The dipeptide Lys-Pro was detected in the GABA-rich fermented milk but not in the control milk. A higher level of Val-Pro-Pro-containing tripeptide, which is considered a lacto-tripeptide, was detected in the GABA-rich fermented milk. In addition, levels of Pro-containing peptides and Lys-Val, known as ACE-inhibitory active peptides, were higher in the GABA-rich fermented milk than in the control milk.

With regard to ACE-inhibitory activity, the IC_{50} values of the control and GABA-rich fermented milk were 0.103 ± 0.014 and 0.073 ± 0.007 mg/mL, respectively. The level of ACE-inhibitory activity of the GABA-rich fermented milk was higher than that of the control fermented milk ($P < 0.05$).

DISCUSSION

Metabolomic analyses of milk products have recently been conducted with the goal of improving their ben-

eficial components, such as bioactive peptides and flavor compounds (Ochi et al., 2012; Sforza et al., 2012; Le Boucher et al., 2013). In this study, milk samples fermented in the presence and absence of the GABA-producing strain *L. lactis* 01-7 were analyzed using CE-TOFMS to identify unique metabolites in GABA-rich fermented milk. As expected, milk fermented with the combination of strains 01-7 and 01-1 contained a higher amount of GABA than the control milk fermented with strain 01-1 alone (Table 1). Interestingly, the level of Orn was higher in the GABA-rich fermented milk than in the control fermented milk. Ornithine is produced via arginine deiminase by metabolizing arginine (Crow and Thomas, 1982). *Lactococcus lactis* 01-7 was thought to follow the arginine deiminase pathway because the amount of arginine was lower in the GABA-rich fermented milk, and strain 01-1, which belongs to *L. lactis* ssp. *cremoris*, is not able to hydrolyze arginine (Nomura et al., 1999). In the current study, the pH of the GABA-rich milk and the control fermented milk were 4.34 and 4.27, respectively. Because both GABA and Orn are stress responses to acid (Sanders et al., 1998; Rollan et al., 2003; Su et al., 2011), conditions of low pH are thought to induce GABA and Orn production in strain 01-7. The level of citric acid, which is required for the production of diacetyl and acetoin in LAB (Drihan et al., 1976), was strongly decreased in the GABA-rich fermented milk because strain 01-7 is able to use citrate to produce diacetyl (Nomura et al., 1998). On the other hand, isocitric acid, which is produced from citrate, was decreased and 2-oxoglutaric acid, which is derived from isocitric acid, was increased in the GABA-rich fermented milk. Strain 01-7 may possess isocitrate dehydrogenase (*idh*), which converts isocitric acid to 2-oxoglutaric acid (Lapujade et al., 1998). Focusing on purine bodies, the level of adenine was decreased in the GABA-rich fermented milk, whereas guanine and hypoxanthine levels were higher. Strain 01-7 might take up adenine because it could be beneficial for growth in LAB (Torino et al., 2005). Although *L. lactis* harboring hypoxanthine guanine phosphoribosyltransferase can phosphoribosylate both hypoxanthine and guanine to inosine monophosphate and guanosine monophosphate (Nilsson and Lauridsen, 1992), the levels of these compounds were increased in the GABA-rich fermented milk. Strain 01-7 might release these 2 compounds; however, the details of this mechanism remain unclear.

The effects of GABA and Orn on human health have been thoroughly investigated; GABA is an inhibitory neurotransmitter in the central nervous system and can improve hypertension in humans (Nicoll et al., 1990; Inoue et al., 2003). Therefore, there have been many reports concerning GABA-producing LAB. Ornithine is able to improve the detoxification of ammonia in

Table 1. Metabolites at higher and lower levels detected in the γ -aminobutyric acid (GABA)-rich fermented milk compared with control milk

Change	Compound name ¹	Relative area				Comparative analysis of GABA and control		
		Control		GABA		Ratio ²	P-value	
		Mean	SD	Mean	SD			
Increase (>1.5 fold)	Cation							
	Mevalactone	23.21	0	4.9E-04	2.7E-05	ND	0.001	**
	N-Acetylhistidine	10.58	0	7.8E-05	4.0E-06	ND	8.8E-04	***
	GABA	104.070	8.33	9.8E-05	1.0E-05	1,216	0.001	**
	Orn	133.097	7.43	3.8E-04	4.4E-05	27	0.003	**
	Hypoxanthine	137.045	11.78	4.4E-05	5.9E-06	5.2	0.010	*
	Cit	176.103	11.86	1.1E-03	7.1E-05	3.6	0.001	**
	Guanine	152.056	9.01	2.0E-04	1.6E-05	2.5	0.009	**
	Gln	147.076	11.54	1.4E-03	1.2E-04	2.2	0.002	**
	Thr	120.065	11.29	6.8E-04	2.2E-05	2.2	0.002	**
	N ⁶ -Ethylglutamine	175.108	12.19	7.4E-05	6.0E-06	1.8	4.6E-03	**
	Homocysteinethiolactone	118.032	8.06	1.7E-05	2.7E-06	1.7	0.023	*
	Asp	134.044	12.33	6.7E-03	1.2E-04	1.7	0.018	*
	Ala	90.055	9.76	5.8E-03	2.4E-04	1.6	0.017	*
	Gly	76.039	9.00	4.5E-04	4.8E-06	1.5	0.036	*
	Asn	133.060	11.23	1.6E-03	7.6E-05	1.5	0.012	*
	Anion							
	5-Methoxyindoleacetic acid	204.068	8.03	0	0	ND	0.001	**
	Mevalonic acid	147.066	8.41	9.9E-05	4.4E-06	5.9	0.001	**
	2-Oxoglutaric acid	145.013	20.50	3.0E-04	2.4E-05	4.1	2.1E-04	***
	Pyruvic acid	87.010	12.53	3.2E-03	1.8E-04	2.7	3.9E-04	***
	Dihydroorotic acid	157.025	9.19	8.1E-04	2.2E-05	2.3	0.001	**
	Cytidine-5'-monophosphate	322.044	9.31	7.9E-05	4.6E-06	2.1	0.021	*
	Homovanillic acid	181.050	8.10	5.1E-04	1.4E-05	1.8	8.5E-05	***
	N-Carbamoylaspartic acid	175.036	14.93	1.8E-04	1.5E-05	1.8	0.001	**
Decrease (<0.67)	Cation							
	Methionine sulfoxide	166.052	12.68	2.4E-04	1.8E-05	0.6	0.040	*
	Thiamine	265.111	7.24	7.1E-05	8.3E-06	0.6	0.033	*
	N-Acetyllysine	189.123	10.58	8.7E-04	7.0E-05	0.6	0.016	*
	Ile	132.101	11.02	2.8E-03	1.4E-04	0.5	3.8E-04	***
	Adenine	136.061	8.28	1.1E-04	5.4E-06	0.5	0.015	**
	Met	150.058	11.52	4.6E-03	2.7E-04	0.3	0.002	**
	Leu-Leu-Tyr	408.249	12.81	8.8E-04	1.0E-05	0.11	3.0E-05	***
	Glu	148.060	11.72	1.0E-01	1.6E-03	0.04	1.0E-04	***
	Arg	175.118	7.76	2.0E-02	8.6E-04	0.0012	6.6E-04	***
	Cyclohexylamine	100.113	8.41	5.9E-05	8.8E-06	ND	0.007	**
	3-Aminobutyric acid	104.071	8.54	5.0E-05	1.0E-05	ND	0.013	*
	Pyridoxamine	169.096	6.55	6.4E-05	4.4E-06	ND	0.002	**
	Anion							
	3-Phosphoglyceric acid	184.986	18.85	7.7E-04	4.6E-05	0.5	0.008	**
	Isocitric acid	191.020	26.92	5.9E-03	8.5E-05	0.4	1.2E-05	***
	trans-Cinnamic acid	147.045	8.57	1.2E-03	5.0E-05	0.08	6.7E-04	***
	Citric acid	191.020	24.28	4.6E-01	2.2E-02	ND	7.4E-04	***
	Fructose 1,6-diphosphate	338.990	14.09	1.1E-04	5.2E-06	ND	6.8E-04	***

¹Compound name (metabolite library maintained by Human Metabolome Technologies, Tsuruoka, Japan) based on mass-to-charge ratio (m/z) and migration time (MT).

²ND = not detected in control or GABA-rich fermented milk.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Welch t -test.

Figure 2. Pathway potentially associated with differential metabolic profiles: (A) glutamate decarboxylase, (B) arginine deiminase, and (C) citrate metabolism. *gadB* = glutamate decarboxylase; *arcA* = arginine deiminase; *arcB* = Orn carbamoyltransferase; *ak* = aconitase; *idh* = isocitrate dehydrogenase. Vertical axes show the relative area [each peak area/(peak area of internal control $\times$ sample volume)]. Black (blue) and gray (red) bars show data from control and γ -aminobutyric acid (GABA)-rich fermented milk, respectively. ** $P < 0.01$, *** $P < 0.001$ by Welch *t*-test. Color version available online.

Table 2. Differential bioactive peptides detected in γ -aminobutyric acid (GABA)-rich fermented milk

			Relative area				Comparative analysis of GABA and control		
			Control		GABA				
Peptide name ¹	<i>m/z</i>	MT (min)	Mean	SD	Mean	SD	Ratio ²	<i>P</i> -value	
Cation									
Leu-Pro	229.154	10.94	0	0	1.2E-04	2.1E-06	ND	9.8E-05	***
Val-Pro	215.138	10.68	6.1E-05	6.2E-06	4.1E-04	5.0E-06	6.7	1.3E-05	***
Ala-Pro	187.106	10.13	9.9E-05	4.5E-06	5.7E-04	1.2E-05	5.8	0.004	**
Val-Pro-Pro	312.191	11.66	2.2E-05	2.8E-06	1.2E-04	1.4E-05	5.6	0.008	**
Val-Leu	231.170	11.28	2.0E-04	5.8E-06	4.7E-04	2.7E-05	2.4	0.002	**
Pro-Pro	213.123	10.23	1.8E-04	5.3E-06	4.0E-04	2.9E-05	2.2	0.006	**

¹Name based on mass-to-charge ratio (*m/z*) and migration time (MT).

²ND = not detected in control fermented milk.

P* < 0.01, *P* < 0.001 by Welch *t*-test.

the liver because it is used in the urea cycle (Gornall, 1942); furthermore, Orn can improve sleep quality (Miyake et al., 2014). Increases in the levels of these 2 AA in fermented milk are considered beneficial for health.

The CE-TOFMS analysis also showed differences between the GABA-rich fermented and control fermented milk in the amounts of various peptides considered to have ACE-inhibitory activity (Table 2). The level of Val-Pro-Pro-containing tripeptide, regarded as a lacto-tripeptide (Meisel and Bockelmann, 1999), was higher in the GABA-rich fermented milk; lactotripeptides improve hypertension in humans (Seppo et al., 2003). In addition, the levels of peptides containing Pro (Leu-Pro, Val-Pro, Ala-Pro, and Pro-Pro) and Val-Leu, which are known as ACE-inhibitory active peptides, were also higher in the GABA-rich fermented milk. Thus, the ACE-inhibitory activity of the GABA-rich fermented milk tended to be higher than that of the control milk. The Tyr-Pro dipeptide has been found in yogurt-like milk fermented with *Lactobacillus helveticus* (Yamamoto et al., 1999); however, Tyr-Pro peptides were not found in this study. The composition of dipeptides is considered to differ among bacterial species. The levels of other unknown peptides were also increased in the GABA-rich fermented milk (data not shown). Proteinase and peptidase are strongly related to peptide production resulting from casein degradation (Mierau et al., 1997). Further analysis of the degrading enzyme and the function of the peptides will provide a new insight into the bioactive peptides present in fermented milk products.

CONCLUSIONS

In this study, CE-TOFMS analyses provided a novel insight into GABA-rich fermented milk. The levels of GABA, Orn, and several peptides, which are considered functional components, were increased in GABA-rich

fermented milk compared with control fermented milk. Although data concerning the levels of metabolites in this study were not quantitative, the improvement in bioactive peptide and AA production may yield a more-functional fermented milk.

ACKNOWLEDGMENTS

We are grateful to Susumu Muroya (NARO Institute of Livestock and Grassland Science) for assistance with manuscript preparation. This research was supported in part by the Research Project on Development of Agricultural Products and Foods with Health-promoting benefits (NARO; Ministry of Agriculture, Forestry and Fisheries, Japan) and by the Council for Science, Technology and Innovation (CSTI), Cross-Ministerial Strategic Innovation Promotion Program (SIP), "Technologies for creating next-generation agriculture, forestry and fisheries." The authors declare no conflicts of interest.

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