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Full Paper

Analysis of D-amino acid in Japanese post-fermented tea, Ishizuchi-kurocha

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The D-amino acid content of Ishizuchi-kurocha, a post-fermented tea produced in Ehime, Japan, was measured. Ishizuchi-kurocha mainly contains D-glutamic acid and D-alanine, but it also contains a small amount of D-aspartic acid. Two types of lactic acid bacteria, *Lactiplantibacillus plantarum* and *Levilactobacillus brevis*, are the main species involved in lactic acid fermentation during the tea fermentation process. Therefore, the D-amino acid-producing abilities of strains of these two species isolated from Ishizuchi-kurocha were examined. Specifically, the production of D-aspartic acid, D-alanine, and D-glutamic acid by *L. brevis* and *L. plantarum* strains was observed. The amount of D-aspartic acid produced by *L. plantarum* was low. D-glutamine was detected in culture supernatant but not in bacterial cells. D-arginine was detected in bacterial cells of the *L. plantarum* strains but not in the culture supernatant. Both the *L. brevis* and *L. plantarum* strains possessed at least three kinds of putative racemase genes: alanine racemase, glutamate racemase, and aspartate racemase. However, their expression and enzyme activity remain unknown. *L. plantarum* and *L. brevis* could play an important role in the production of D-amino acids in Ishizuchi-kurocha. In fact, Ishizuchi-kurocha is expected to possess the effective physiological activities of D-amino acids.

Key words: D-amino acid, amino acid racemase, *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, Ishizuchi-kurocha

INTRODUCTION

Twenty α-amino acids are important biological molecules consisting of proteins. Nineteen of them, excluding glycine, have an optical isomer. Bacteria can synthesize a variety of D-amino acids via several racemases. In particular, D-alanine (D-Ala) and D-glutamic acid (D-Glu) are essential constituents of bacterial cell wall peptidoglycans. Although humans can only synthesize some D-amino acids, they can absorb the D-amino acids produced by intestinal bacteria [1]. D-Amino acids, first observed in the mammalian brain, have multiple physiological functions [2]. D-Serine (D-Ser) plays a key role in the physiology of the brain. Free D-Ser acts as a co-agonist against the N-methyl-D-aspartate receptor associated with mammalian brain functions such as memory and learning [3]. D-Ser has also been found to influence the clinical status of patients with Alzheimer's disease and schizophrenia [4–6]. Ser racemase has been found in the rat and mouse brain, suggesting its involvement in D-Ser synthesis. Moreover, D-Ser enhances the growth of kidney cells and improves kidney function [7]. D-Ser, produced by intestinal bacteria, also has a protective effect on the kidney [8]. The physiological functions of free D-aspartic acid (D-Asp) include a melatonin antisecretory effect, prolactin secretion activation, and testosterone synthesis promotion [9–11]. D-Amino acids, particularly D-Ala, D-Ser, and D-arginine (D-Arg), suppress lipid accumulation in hepatocytes [12]. As mentioned above, D-amino acids have various physiological functions that differ from those of L-amino acids. Therefore, D-amino acids are important for maintaining human physical functions and improving quality of life. However, because humans cannot synthesize a variety of D-amino acids, synthesis by intestinal bacteria or intake from foods is important for D-amino acid supply. Because lactic acid bacteria can synthesize D-amino acids, foods produced by lactate fermentation contain D-amino acids [13–15]. Ishizuchi-kurocha is a post-fermented tea produced in Ehime, Japan. The fermentation processes of post-fermented teas, such as

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Ishizuchi-kurocha, Goishi-cha (Kochi, Japan), Awa-bancha (Tokushima, Japan), and Miang (Northern Thailand), include lactate fermentation. Ishizuchi-kurocha is produced by a two-step fermentation process [16]. First, harvested tea leaves are steamed, and primary fermentation is performed aerobically by fungi, mainly *Aspergillus niger* and *Aspergillus luchuensis*. The secondary fermentation for Ishizuchi-kurocha is lactate fermentation, in which the tea leaves are fermented anaerobically by lactic acid bacteria. The dominant bacterial flora species in secondary fermentation are *Lactiplantibacillus plantarum* and *Levilactobacillus brevis*. The composition of lactic acid bacteria in Ishizuchi-kurocha is 80–100% L. plantarum and 20–0% *L. brevis* during secondary fermentation. Sometimes, *L. brevis* is not detected during secondary fermentation. *L. plantarum* is frequently isolated from plant-derived fermented foods. The genome sequence of *L. plantarum* strain IYO1511, which was isolated from Ishizuchi-kurocha in 2015, has been determined [17]. According to the genome sequence, *L. plantarum* IYO1511 possesses a racemase gene. However, the D-amino acid concentration of Ishizuchi-kurocha has yet to be determined. In fact, it remains unclear whether D-amino acids are present in Ishizuchi-kurocha. The D-amino acid producibility of *L. brevis* is also unknown. Therefore, in this study, the D-amino acid content of Ishizuchi-kurocha was determined, and the contributions of *L. plantarum* and *L. brevis* were examined.

MATERIALS AND METHODS

Tea samples and lactic acid bacteria

Samples of Ishizuchi-kurocha tea were provided by the Ishizuchi-kurocha producer Visee (Saijo, Ehime, Japan). The Ishizuchi-kurocha used in the present study was produced in 2020 and 2021 in Saijo, Ehime, Japan. One hundred milliliters of boiling distilled water were added to 2 g of sun-dried tea leaves and left to stand for 5 min. Then, the elution was passed through a 0.22 µm filter. This solution was filtered through an Amicon Ultra-0.5 Centrifugal Filter Unit (3 kDa NMWCO pore size; MilliporeSigma, Burlington, MA, USA) with centrifugation (14,000 × g, for 15 min at 4°C). Sample solutions were stored at −80°C until use and analyzed with an ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA). Lactic acid bacteria (*L. brevis* IYO2091, *L. plantarum* IYO2092, and *L. plantarum* IYO2179) were isolated from the tea leaves after secondary fermentation (production lots 2020-3 and 2021-1). Isolation and identification of the lactic acid bacteria were performed with the method reported by Horie *et al.* [18]. The isolated species were identified by the homology of the 16S rRNA gene sequence. Each sequence of the 16S rRNA gene has been submitted to the DNA Data Bank of Japan (DDBJ). Details of isolates and accession numbers of 16S rRNA gene sequences are shown in Table 1.

Preparation of culture media and cell-free extracts of lactic acid bacteria for amino acid analysis

The three isolates were cultured under static conditions for 24 hr at 37°C in de Man, Rogosa and Sharpe (MRS) medium (100 mL). The culture supernatant and cells were separated by centrifugation at 10,000 × g for 15 min at 4°C. For amino acid analysis of culture supernatant, aliquots (1 mL) of medium were filtered with an Amicon Ultra-0.5 Centrifugal Filter Unit (3 kDa NMWCO pore size). The prepared samples were stored at −80°C until use and analyzed with a UPLC system. The collected cells were suspended in 20 mM potassium phosphate buffer (pH 7.2) and broken using glass beads in a Multi-beads Shocker homogenizer (Yasui Kikai, Osaka, Japan). The resultant homogenate was centrifuged at 12,000 × g for 10 min at 4°C. Then, the supernatant was filtered with an Amicon Ultra-0.5 Centrifugal Filter Unit (3 kDa NMWCO pore size). The filtrate was used as a cell-free extract for amino acid analysis with a UPLC system.

Derivatization of amino acids for UPLC analysis

Derivatization of amino acids was carried out using *o*-phthaldialdehyde (OPA; Nova Biochemical, Waltham, MA, USA) plus *N*-acetyl-L-cysteine (NAC; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) or *N*-*tert*-butyloxycarbonyl-L-cysteine (NBC; Nacalai Tesque, Kyoto, Japan). For the amino acid derivatives, a methanolic solution was prepared by dissolving 8 mg of OPA and 10 mg of NAC (OPA-NAC) or 10 mg OPA and 10 mg NBC (OPA-NBC) in 1 mL of methanol. The reaction mixture (250 µL) for derivatization consisted of 25 µL of sample, 50 µL of methanolic solution, and 175 µL of 0.4 M borate-NaOH buffer (pH 10.4). After incubation for 2 min at room temperature in the dark for derivatization, aliquots (1 µL) of the reaction mixture were introduced into a UPLC system.

Analysis and quantification of amino acids using UPLC

The derivatives formed with OPA-NAC or OPA-NBC were applied to a 2.1 × 100 mm AccQ-Tag Ultra column (Waters) in an ACQUITY UPLC TUV system consisting of a Waters Binary Solvent Manager, Waters Sample Manager, and Waters FLR Detector. The excitation and emission wavelengths for fluorescent detection of the derivatized amino acids were 350 nm and 450 nm, respectively. The system was operated at a flow rate of 0.25 mL/min at 30°C. For the analysis of OPA-NAC derivatives (A=50 mM sodium acetate, pH 5.9, and B=methanol), the UPLC gradient system was 10–20% B over 3.2 min, 20% B for 1.2 min, 20–40% B over 3.6 min, 40% B for 1.2 min, 40–60%

Table 1. Properties of lactic acid bacteria used in this study

Species	Strain	Source	DDBJ Accession No.			
			16S rRNA gene	Glutamate racemase	Aspartate racemase	Alanine racemase
Levilactobacillus brevis	IYO2091	Ishizuchi-kurocha Lot 2020	LC687616	LC742524	LC742523	LC742522
Lactiplantibacillus plantarum	IYO2092	Ishizuchi-kurocha Lot 2020	LC687615	LC742518	LC742517	LC742516
Lactiplantibacillus plantarum	IYO2179	Ishizuchi-kurocha Lot 2021	LC742525	LC742521	LC742520	LC742519

DDBJ: DNA Data Bank of Japan.

B over 3.8 min, 60% B for 1 min, and 60–10% B over 0.01 min. The gradient system used for analysis of OPA-NBC derivatives (A=50 mM sodium acetate, pH 5.9, and B=acetonitrile) was 15–21% B over 7 min, 21–27.5% B over 1.5 min, 27.5% B for 2 min, 27.5–30% B over 1 min, 30–40% B over 2 min, 40% B for 0.5 min, and 40–15% B over 0.01 min.

Peak heights were used for quantification of amino acids. The standard solution consisted of 32 kinds of amino acids: D- and L- forms of Ala, Arg, Asn, Asp, Gln, Glu, His, Leu, Met, Phe, Ser, Trp, Tyr, and Val plus L-Ile, D-*allo*-Ile, L-Thr, and D-*allo*-Thr. To construct the standard calibration curves, the standard solution was diluted with 0.05 M HCl to eight concentrations (5, 10, 25, 50, 100, 250, 500 and 1,000 µM), and the eight standard solutions were analyzed on a UPLC system. Peak heights were plotted against amino acid concentrations to create a calibration curve. For all amino acid types tested, the relation was linear, with regression coefficients above 0.999. If the concentration of amino acid in a sample was over 1,000 µM, an appropriately diluted sample was derivatized, and aliquots (1 µL) of the reaction mixture were applied to the UPLC system.

Determination of racemase gene sequences of isolates

Racemase genes were amplified by polymerase chain reaction (PCR). The racemase gene primers were designed based on the genome of *L. plantarum* IYO1511 isolated from Ishizuchi-kurocha [17] and *L. plantarum* JCM 1149^T. The primer sequences are listed in Table 2. Each lactic acid bacterial strain was cultured in MRS broth for 18 hr. Then, bacterial cells were harvested by centrifugation at 10,000 × g for 10 min. DNA was extracted from pellets with a DNeasy Tissue & Blood Kit (Qiagen, Hilden, Germany). PCR amplification was performed in a TaKaRa PCR Thermal Cycler (Takara Bio Inc., Kusatsu, Japan). The PCRs were conducted in a total reaction volume of 50 µL containing 1 µL of template DNA, 0.5 µmol/L of each primer, Ex Taq polymerase, 5 µL of PCR buffer, and 0.2 mmol/L of deoxynucleotide triphosphate. All primers were synthesized by Eurofins Genomics K.K. (Tokyo, Japan). Thermal cycling of the Taq polymerase was conducted under the following conditions: 2 min at 93°C for initial denaturation; 30 cycles of 30 sec at 93°C, 30 sec at 55°C, and 30 sec at 72°C; and then 1 min at 72°C. PCR products were analyzed by electrophoresis in 2.0% agarose gel followed by GelRed™ (Biotium, Inc., Fremont, CA, USA) staining. Amplicons were purified by NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel GmbH & Co. KG., Düren, Germany). DNA sequencing was performed by Macrogen Japan Corp. (Tokyo, Japan). Each primer used for DNA sequencing was a forward primer. Alignment of the primary structures of the translated amino acid racemases and calculation of the phylogenetic trees were performed by ClustalW. Phylogenetic analysis was performed based on maximum likelihood with the bootstrap method using PhyML [19]. Each sequence of the racemase genes has been submitted to DDBJ. Accession numbers of the racemase gene sequences are shown in Table 1.

RESULTS

D- and L-Amino acid contents of Ishizuchi-kurocha

The D- and L-amino acid contents of Ishizuchi-kurocha produced in 2020 and 2021 were determined (Table 3). Three types of D-amino acids (D-Asp, D-Glu, and D-Ala) were identified. However, the composition of these D-amino acids differed according to the year of production. The Ishizuchi-kurocha produced in 2020 contained D-Asp, D-Glu, and D-Ala, while the tea produced in 2021 contained only D-Glu and D-Ala. The D-Ala content was higher in production lot 2021 than in production lot 2020. D-Amino acids were not detected in Batabata-cha (another post-fermented tea), which was produced without lactic fermentation (data not shown).

Table 2. Sequence of primers for amino acid racemase

Target	Species	Primer name	Sequence (5′-3′)
Glutamate racemase	*L. plantarum*	PlaGluRasF	CGTTTCCACTAATGTGTCGG
		PlaGluRasR1	GATACCGTTC TCCTTAATTT CAGG
	L. brevis	BreGluRac-3F	CGCGAAGTTCTTAATGACAGAA
		BreGluRac-3R	CACTGCTGTCGCCTTTAAAG
Aspartate racemase	*L. plantarum*	PlaAspRac-F	GCTTGTTAGGAGGTGCATGTTAG
		PlaAspRac-R	CAACGATTTGAGCGCTTACAA
	L. brevis	BreAmiRasF1	GTTCCAAGTGCAAGACGTG
		BreAmiRasR1	TTGATTGCCG CTGGCAAGTA TG
Alanine racemase	*L. plantarum*	PlaAlaRac_2-F	CCAGAATTTCTCCGGCACTCA
		PlaAlaRac_2-R	AGACAACGCCCAGGGTAAAC
		PlaAlaRac_2b-F	GGCGCAAACCTATCGTCTGA
		PlaAlaRac_2b-R	TATCCAAGTTGCTCAGGCCG
		PlaAlaRac_2c-F	TACGTGGCACCATAGCTGAC
		PlaAlaRac_2c-R	ATCGAGTTTATGGCAGGGCG
	L. brevis	BreAlaRac-F	GACATTGAGATTCGGGATAATG
		BreAlaRac-R	GCTGCTTGGTACGAGTCATG
		BreAlaRac_2-F	CGGTCGTGATGACACAGGTT
		BreAlaRac_2-R	GCCGCAACGTTACTGGTTTC

These primers were designed based on genome sequence of *L. plantarum* JCM1149^T (CP039121.1) and *L. brevis* UCCLBBS124 (CP031169.1).

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Table 3. D- and L-Amino acid content of Ishizuchi-kurocha (μM)

	2020		2021	
	NAC	NBC	NAC	NBC
L-Asp	122.6	121.6	161.6	166.0
D-Asp	4.9	6.7	0.0	0.0
d/d+l ratio	0.04	0.05	0.00	0.00
L-Glu		51.2		186.0
D-Glu		5.7		6.4
d/d+l ratio		0.10		0.03
L-Ser	25.6		34.0	
L-Gln	3.3	1.8	0.0	0.0
L-His	72.6	31.1	72.3	34.2
L-Arg	12.5		83.3	
L-Ala	85.2	83.6	123.6	123.1
D-Ala	39.3	28.1	67.9	35.4
d/d+l ratio	0.32	0.25	0.35	0.22
L-Tyr	11.1		43.5	
L-Val	76.1	74.2	85.5	86.3
L-Met	0.0	0.0	14.0	14.8
L-Trp	33.9	12.0	28.5	15.5
L-Phe	57.5	57.7	65.6	67.9
L-Ile	42.3	44.0	47.8	51.2
L-Leu	128.9	124.1	152.3	150.4

NAC: *N*-acetyl-L-cysteine; NBC: *N*-*tert*-butyloxycarbonyl-L-cysteine.

Production of D-amino acids by lactic acid bacteria isolated from Ishizuchi-kurocha

The D-amino acid producibility of the *L. plantarum* and *L. brevis* strains isolated from Ishizuchi-kurocha was examined. The strains were isolated from the same lots of Ishizuchi-kurocha for which the D- and L-amino acids were measured in this study. They included *L. plantarum* IYO2092 and *L. brevis* IYO2091 isolated from production lot 2020 and *L. plantarum* IYO2179 isolated from product lot 2021. *L. brevis* was not detected in production lot 2021. The D- and L-amino acid contents in culture supernatants and bacterial cells after 24 hr of cultivation were determined (Tables 4 and 5). Four types of D-amino acids (D-Asp, D-Ser, D-Ala, and D-Glu) were detected in the culture supernatants of these strains. In addition, four types of D-amino acids (D-Asp, D-Glu, D-Ser, and D-Ala) were detected in the bacterial cells of *L. brevis* IYO2091. The D-amino acids detected in the culture supernatants were not detected in the MRS medium. The D-amino acid to total amino acid (D/D+L) ratios for D-Asp, D-Glu, D-Ser, and D-Ala were approximately 43–50% (Table 3). On the other hand, D-Asp was not detected in the bacterial cells of *L. plantarum* IYO2092. The intracellular D-Asp concentration of *L. plantarum* IYO2179 was lower than that of *L. brevis* IYO2091. Compared with the contents in the culture supernatants, the D/D+L ratios of amino acids in the bacterial cells were high. The production of D-Asp and D-Glu was significantly higher in *L. brevis* IYO2091 than in *L. plantarum* strains. Although *L. brevis* IYO2091 produced D-Ala and D-Ser, the production of D-Ala and D-Ser by *L. plantarum* strains was low. On the other hand, although D-Arg was not detected in the culture supernatants, the bacterial cells of *L. plantarum* strains contained D-Arg. D-Arg was not detected in either the culture supernatant or bacterial cells of *L. brevis* IYO2091. It was only detected in the cells of the *L. plantarum* strains.

Detection of amino acid racemase gene sequences in the* L. plantarum *and* L. brevis *strains

The gene sequences of the three types of amino acid racemases (glutamate racemase, alanine racemase, and aspartate racemase) were determined. The PCR primers for these racemase genes were designed based on the whole genome sequences of *L. plantarum* JCM1149^{T}, *L. plantarum* IYO1511, and *L. brevis* ATCC 14869^{T}. All the known racemase genes in *L. plantarum* IYO2092, *L. plantarum* IYO2179, and *L. brevis* IYO2091 were amplified by PCR. These strains contained at least three types of amino acid racemases: alanine racemase, aspartate racemase, and glutamate racemase. The primary structure of each racemase was determined according to its gene sequence. For glutamate racemase, the homology of the primary structures of IYO2092, IYO2179, and JCM1149^{T} was 100% (Fig. 1). The primary structure of *L. brevis* IYO2091 glutamate racemase was the same as that of *L. brevis* DSM20054^{T} (KRK19658). For aspartate racemase, the homology of the primary structures of IYO2092 and JCM1149^{T} was 100% (Fig. 2). However, the primary structure of IYO2179 aspartate racemase differed from that of IYO2092 and JCM1149^{T} by one amino acid (Glu134Asp). The primary structure of *L. brevis* IYO2091 aspartate racemase was the same as that of *L. brevis* DSM20054^{T} (KRK21458). For alanine racemase, the homology of the primary structures of IYO2092 and JCM1149^{T} was 100% (Fig. 3). However, the primary structure of IYO2179 alanine racemase differed from that of IYO2012 and JCM1149^{T} by two amino acids (Ala153Val and Gln247Leu). The primary structure of *L. brevis* IYO2091 alanine racemase was the same as that of *L. brevis* JCM1059^{T} (=DSM20054^{T}) (OQ957164). The primary structures of amino acid racemases were well conserved in *L. plantarum* species. On the other hand, the primary structure of the *L. plantarum* strains differed from that of other species in the *L. plantarum* group, i.e., *L. pentosus* and *L. paraplantarum*. The primary structures of the amino acid racemases significantly differed between *L. plantarum* group species and *L. brevis*. According to alignment by BLAST, the identities of the glutamate, aspartate, and alanine racemases between *L. plantarum* JCM1149^{T} and *L. brevis* DSM20054^{T} were 49%, 44%, and 67%, respectively.

DISCUSSION

In the present study, the D-amino acid content of Ishizuchi-kurocha was determined. Ishizuchi-kurocha from production lot 2020 contained three types of D-amino acids: D-Asp, D-Glu, and D-Ala. However, D-Asp was not detected in Ishizuchi-kurocha from production lot 2021; it contained only D-Glu and D-Ala.

All three lactic acid bacterial strains used in this study (*L. brevis* IYO2091, *L. plantarum* IYO2092, and *L. plantarum* IYO2179) possessed putative aspartate racemases. Compared with *L. brevis* IYO 2091, the D-Asp production by *L. plantarum* IYO2092 and IYO2179 was low. In particular, D-Asp was not detected in the bacterial cells of *L. plantarum* IYO2092. The intracellular D-Asp concentration of *L. brevis* IYO2091 was higher than the extracellular concentration. The homology of the primary structure of the putative aspartate racemase between *L. plantarum* and *L. brevis* was low. According to BLAST alignment, the identity between these aspartate racemases was 44%. Therefore, it is necessary to investigate the activity of these two enzymes.

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Table 4. D- and L-Amino acid contents in the culture supernatant and the bacteria cells of lactic acid bacteria (OPA-NAC derivatives)

	L.brevis IYO2091		*L. plantarum* IYO2092		*L. plantarum* IYO2179	
	Culture supernatant	Bacterial cell	Culture supernatant	Bacterial cell	Culture supernatant	Bacterial cell
	μM	nmol/g[1)]	μM	nmol/g[1)]	μM	nmol/g[1)]
L-Asp	1,553	6,061	2,542	3,241	588	596
D-Asp	217	5,063	79	0	41	36
d/d+l ratio	0	0	0	0	0	0
L-Ser	2,358	700	76	0	17	49
D-Ser	28	644	29	0	0	0
d/d+l ratio	0	0	0	0	0	0
L-Gln	48	119	43	93	66	0
D-Gln	0	0	0	0	0	0
L-His	689	585	1,119	129	546	211
D-His	0	0	0	0	0	0
L-Arg	0	164	3,835	270	1,931	429
D-Arg	0	0	0	220	0	223
d/d+l ratio	0	0	0	0	0	0
L-Ala	2,787	2,808	3,937	807	1,834	213
D-Ala	1,065	2,884	1,273	843	596	362
d/d+l ratio	0	1	0	1	0	1
D-allo-Thr	0	0	152	0	0	97
L-Tyr	682	89	950	0	439	0
D-Tyr	0	0	0	0	0	0
L-Val	3,078	799	5,446	174	2,834	196
D-Val	0	0	0	0	0	0
L-Met	1,130	0	1,810	0	861	0
D-Met	0	0	0	0	0	0
L-Trp	0	202	1,379	0	663	0
D-Trp	0	0	0	0	0	0
L-Phe	2,446	365	4,085	94	2,054	74
D-Phe	0	0	0	0	0	0
L-Ile	2,460	494	4,072	101	2,091	129
D-allo-Ile	0	0	0	0	0	0
L-Leu	5,673	1,050	9,684	308	5,332	319
D-Leu	0	0	0	0	0	0

[1)]Amount of substance (nmol) per 1 g of wet weight of cells calculated from concentration (μM).
OPA: o-phthaldialdehyde; NBC: *N-tert*-butyloxycarbonyl-L-cysteine.

The dominant bacterial species in Ishizuchi-kurocha during secondary fermentation is *L. plantarum*, followed by *L. brevis* [20]. These two species are present in the tea leaves of Ishizuchi-kurocha after secondary fermentation each year. However, *L. brevis* is not always detected in individual production lots of Ishizuchi-kurocha [20]. *L. brevis* was not detected in production lot 2021-1, which was analyzed in this study. In contrast, both *L. plantarum* and *L. brevis* were isolated from production lot 2020-3. The reason why some production lots did not contain *L. brevis* is unknown. The composition of D-amino acids in Ishizuchi-kurocha is dependent on the composition of lactic acid bacteria during fermentation. During secondary fermentation, the composition of lactic acid bacteria in Ishizuchi-kurocha is 80–100% *L. plantarum* and 20–0% *L. brevis*. When *L. brevis* is involved in the fermentation of Ishizuchi-kurocha, D-Asp may be produced. Compared with *L. brevis* IYO2091, the production of D-Asp by *L. plantarum* IYO2092 and IYO2179 was very low. If *L. brevis* is absent during the fermentation of Ishizuchi-kurocha, little to no D-Asp may be detected in the Ishizuchi-kurocha lot. *L. brevis* is an important species for D-Asp production in Ishizuchi-kurocha. Although D-Arg was not detected outside cells, it was detected in the cells of *L. plantarum* IYO2092 and IYO2179. The D/D+L ratios of Arg in *L. plantarum* IYO2092 and IYO2179 were 44.9 and 34.1, respectively. To date, arginine racemase has only been reported in *Pseudomonas* sp. [21]. The arginine racemase activity of *Pseudomonas taetrolens* was almost exclusively present in the periplasm [22]. MalY from *Latilactobacillus sakei* [23] and Lys racemase from *Oenococcus oeni* are broad-spectrum racemases that may be present in *L. plantarum* IYO2092 and IYO217. D-Ser was detected in the bacterial cells of *L. brevis* IYO 2091 but not in the culture supernatant. *L. plantarum* did not produce D-Ser in either bacterial cells or culture supernatant.

In contrast, D-Arg was detected in the bacterial cells of *L. plantarum* IYO 2092 and IYO2179 but not in their culture supernatants. *L. brevis* did not produce D-Arg in either bacterial cells or culture supernatant. Although D-Ser, D-Arg, and D-Glu were detected in the culture supernatants or bacterial cells of isolated lactic acid bacteria, they were not found in the tea leaves of Ishizuchi-kurocha. These D-amino acids were detected when lactic acid bacteria were cultured in MRS broth. MRS broth is suitable for the growth of lactic acid bacillus, whereas fermented tea leaves provide a more severe environment because

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Table 5. D- and L-Amino acid contents in the culture supernatant and the bacteria cells of lactic acid bacteria (OPA-NBC derivatives)

	L.brevis IYO2091		*L. plantarum* IYO2092		*L. plantarum* IYO2179	
	Culture supernatant	Bacterial cell	Culture supernatant	Bacterial cell	Culture supernatant	Bacterial cell
	µM	nmol/g[1)]	µM	nmol/g[1)]	µM	nmol/g[1)]
L-Asp	1,419	5,227	1,069	2,855	553	526
D-Asp	162	3,985	0	0	21	31
d/d+l ratio	0	0	0	0	0	0
L-Glu	2,447	12,958	3,357	2,805	3,206	265
D-Glu	270	12,589	131	2,658	79	1,715
d/d+l ratio	0	0	0	0	0	1
L-Asn	1,002	0	689	1,425	661	1,329
D-Asn	0	0	0	0	0	0
L-Ser	2,517	652	0	111	0	75
D-Ser	0	610	0	0	0	0
d/d+l ratio	0	0	0	0	0	0
L-Gln	0	241	0	51	56	0
D-Gln	220	0	192	0	216	0
d/d+l ratio	1	0	1	0	1	0
L-His	613	454	470	111	445	200
D-Arg	0	0	0	211	0	218
L-Ala	2,891	2,744	2,070	840	1,838	228
D-Ala	1,004	2,636	573	748	540	317
d/d+l ratio	0	0	0	0	0	1
D-Tyr	0	0	0	0	0	0
L-Val	3,445	839	3,161	183	3,054	210
D-Val	0	0	0	0	0	0
L-Met	1,085	0	947	0	874	0
D-Met	0	0	0	0	0	0
L-Ile	2,719	487	2,277	104	2,198	136
L-Trp	673	0	621	0	597	0
L-Phe	2,505	341	2,142	83	2,032	67
D-Phe	0	0	0	0	0	0
L-Leu	5,132	951	4,843	293	4,708	297
D-Leu	0	0	0	0	0	0

[1)] Amount of substance (nmol) per 1 g of wet weight of cells calculated from concentration (µM).
OPA: o-phthaldialdehyde; NBC: *N-tert*-butyloxycarbonyl-L-cysteine.

they contain antimicrobial molecules, such as catechins [24]. The nutritional conditions for bacterial growth are significantly different between MRS medium and tea leaves. The amount of substrate amino acids in tea leaves would be much lower than in MRS medium. These differences may be major factors involved in the difference in the production of D-amino acids. Furthermore, other microorganisms and their metabolites may affect the growth of lactic acid bacteria. For example, the *L. plantarum* strain isolated from Ishizuchi-kurocha exhibited stronger antibiotic resistance than *L. plantarum* strains isolated from Awa-bancha, which does not undergo aerobic fermentation by molds [18]. Thus, the metabolism and gene expression of *L. plantarum* and *L. brevis* in MRS broth and in fermented tea leaves could be significantly different. The interaction between the gene expression of amino acid racemases and D-amino acid production and secretion by *L. plantarum* and *L. brevis* during the fermentation of Ishizuchi-kurocha is an interesting process.

In conclusion, Ishizuchi-kurocha contains at least three kinds of D-amino acids (D-Asp, D-Glu, and D-Ala) with various physiological benefits, such as improved brain function and protection against kidney injury. Drinking Ishizuchi-kurocha is expected to affect the physiological activities of D-amino acids. *L. plantarum* and *L. brevis* isolated from Ishizuchi-kurocha have practical food industry applications. The co-culture of *L. plantarum* and *L. brevis* could improve the production of various D-amino acids.

CONFLICTS OF INTEREST

There is no potential conflict of interest to declare.

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(A) Alignment of putative glutamate racemase of *L. plantarum*

```
IYO2092 MANEHAIGFMDSGVGGLTVVKQALKQLPRETVYFIGDQARLPYGPRPAEQVRTFSFQMADFLMAKQIKML 70
JCM1149 MANEHAIGFMDSGVGGLTVVKQALKQLPRETVYFIGDQARLPYGPRPAEQVRTFSFQMADFLMAKQIKML 70
IYO2179 MANEHAIGFMDSGVGGLTVVKQALKQLPRETVYFIGDQARLPYGPRPAEQVRTFSFQMADFLMAKQIKML 70
        **********************************************************************
IYO2092 VIACNTATAAALPALRQQLSIPVIGVIAPGSRAALKASHRNRIGVIATEGTIRSNAYRDAILTKDPTATV 140
JCM1149 VIACNTATAAALPALRQQLSIPVIGVIAPGSRAALKASHRNRIGVIATEGTIRSNAYRDAILTKDPTATV 140
IYO2179 VIACNTATAAALPALRQQLSIPVIGVIAPGSRAALKASHRNRIGVIATEGTIRSNAYRDAILTKDPTATV 140
        **********************************************************************
IYO2092 VSQACPKFVPLVESNEYQSTVAKRVVAETLKQLKKQDVDTLVLGCTHYPLLRPLIQNVMGPGVTLIDSGA 210
JCM1149 VSQACPKFVPLVESNEYQSTVAKRVVAETLKQLKKQDVDTLVLGCTHYPLLRPLIQNVMGPGVTLIDSGA 210
IYO2179 VSQACPKFVPLVESNEYQSTVAKRVVAETLKQLKKQDVDTLVLGCTHYPLLRPLIQNVMGPGVTLIDSGA 210
        **********************************************************************
IYO2092 ETVNDVSAVLDYLDIANDRSTKRYPDEYYTTGAADQFEAIARNWLGQPDFHAQHIDLGSEAND 273
JCM1149 ETVNDVSAVLDYLDIANDRSTKRYPDEYYTTGAADQFEAIARNWLGQPDFHAQHIDLGSEAND 273
IYO2179 ETVNDVSAVLDYLDIANDRSTKRYPDEYYTTGAADQFEAIARNWLGQPDFHAQHIDLGSEAND 273
        ***************************************************************
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(B) Phylogram based on putative glutamate racemase of *L. plantarum*

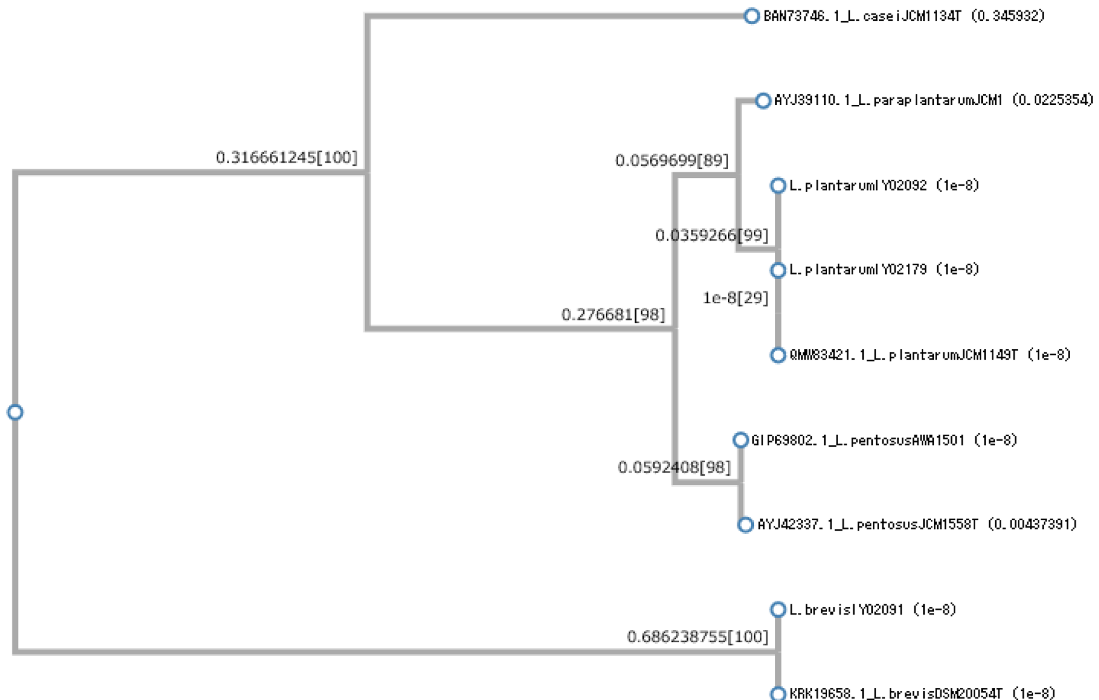

Fig. 1. Comparison of the primary structures of glutamate racemases in the *Lactiplantibacillus plantarum* group and *Levilactobacillus brevis*.

(A) Alignment of the primary structures of the glutamate racemases of *L. plantarum* IYO2092, IYO2179, and JCM1149^{T}. (B) Glutamate racemase phylogenic tree. Sequences of different types of strains were employed as a reference. The reference sequences of *L. plantarum*, *L. paraplantarum*, *L. pentosus*, and *L. brevis* were obtained from GenBank. *L. casei* was employed as the outgroup species. The accession numbers of the reference species are indicated in the figure.

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(A) Alignment of putative aspartate racemase of *L. plantarum*

```
IYO2092 MKNCFGIIGGMGTMATEDFVHTVNRLTVTHRDQDYLNYVILNDAEIPDRTAYILDHNCANPLPSLEADVQ 70
JCM1149 MKNCFGIIGGMGTMATEDFVHTVNRLTVTHRDQDYLNYVILNDAEIPDRTAYILDHNCANPLPSLEADVQ 70
IYO2179 MKNCFGIIGGMGTMATEDFVHTVNRLTVTHRDQDYLNYVILNDAEIPDRTAYILDHNCANPLPSLEADVQ 70
        **********************************************************************
IYO2092 LLNQLGVMFIVMTCNTAHYFIPALTALSNVPILNMPALAVTAACRSQQPRPLRIGILATTGTLESQLYQQ 140
JCM1149 LLNQLGVMFIVMTCNTAHYFIPALTALSNVPILNMPALAVTAACRSQQPRPLRIGILATTGTLESQLYQQ 140
IYO2179 LLNQLGVMFIVMTCNTAHYFIPALTALSNVPILNMPALAVTAACRSQQPRPLRIGILATTGTLDSQLYQQ 140
        ***************************************************************:******
IYO2092 LIRSNGHIPVIPNKQQQAQVMTLIYDDIKQRDFVDRVKFHGLIDQLLNAADCDAVILGCTELSVAQDAAP 210
JCM1149 LIRSNGHIPVIPNKQQQAQVMTLIYDDIKQRDFVDRVKFHGLIDQLLNAADCDAVILGCTELSVAQDAAP 210
IYO2179 LIRSNGHIPVIPNKQQQAQVMTLIYDDIKQRDFVDRVKFHGLIDQLLNAADCDAVILGCTELSVAQDAAP 210
        **********************************************************************
IYO2092 YDSERIIDAQYELARATVQRAQATKPALILRRRPSIRR 248
JCM1149 YDSERIIDAQYELARATVQRAQATKPALILRRRPSIRR 248
IYO2179 YDSERIIDAQYELARATVQRAQATKPALILRRRPSIRR 248
        **************************************
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(B) Phylogram based on putative aspartate racemase of *L. plantarum*

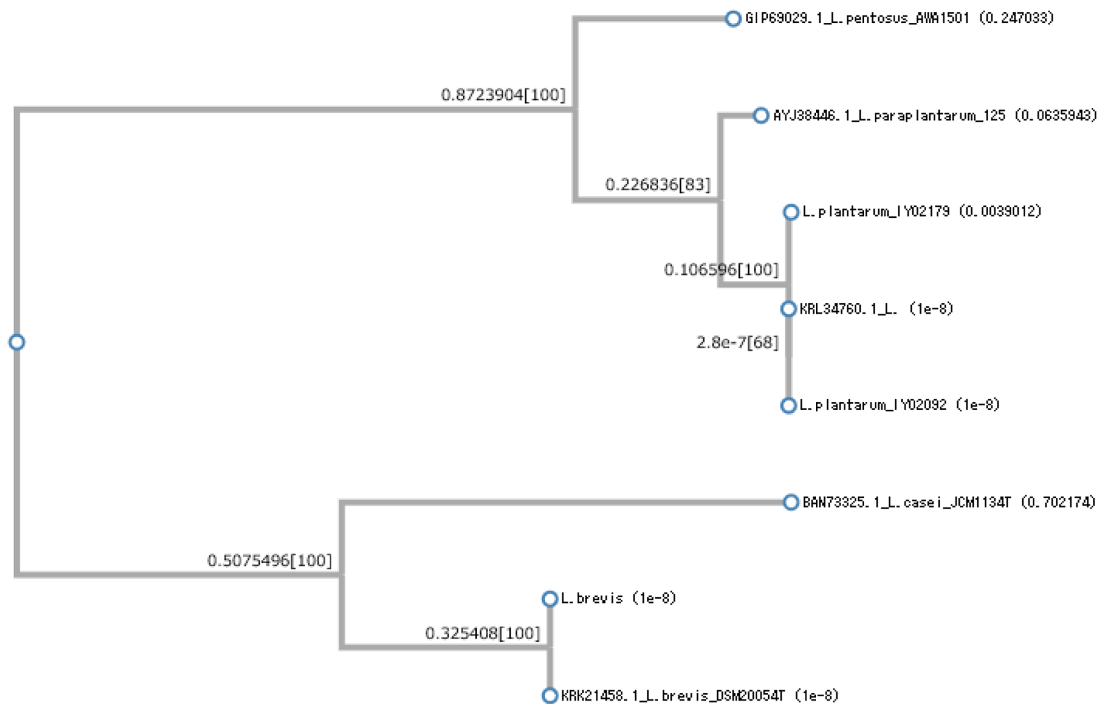

Fig. 2. Comparison of the primary structures of aspartate racemases in the *Lactiplantibacillus plantarum* group and *Levilactobacillus brevis*.
(A) Alignment of the primary structures of the amino acid racemases of *L. plantarum* IYO2092, IYO2179, and JCM1149^{T}. (B) Aspartate racemase phylogenic tree. Sequences of different types of strains were employed as a reference. The reference sequences of *L. plantarum*, *L. paraplantarum*, *L. pentosus*, and *L. brevis* were obtained from GenBank. *L. casei* was employed as the outgroup species. The accession numbers of the reference species are indicated in the figure.

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(A) Alignment of putative alanine racemase of *L. plantarum*

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IY02092 MVVIGEHRHTQVTVDLQAIKTNISNEMAQKDELTELWAVVKANGYGHGIIQVAQAAKEAGATGFCVAILDEALALRAAGF 80
JCM1149 MVVIGEHRHTQVTVDLQAIKTNISNEMAQKDELTELWAVVKANGYGHGIIQVAQAAKEAGATGFCVAILDEALALRAAGF 80
IY02179 MVVIGEHRHTQVTVDLQAIKTNISNEMAQKDELTELWAVVKANGYGHGIIQVAQAAKEAGATGFCVAILDEALALRAAGF 80
        ********************************************************************************
IY02092 AEPILVLGITEPEYAPLVAEKDISLAVGTQDWLTTAAAILAANQVTTPLHVHLALDTGMGRIGFQTPEELATAVTTLRQP 140
JCM1149 AEPILVLGITEPEYAPLVAEKDISLAVGTQDWLTTAAAILAANQVTTPLHVHLALDTGMGRIGFQTPEELATAVTTLRQP 160
IY02179 AEPILVLGITEPEYAPLVAEKDISLAVGTQDWLTTAAAILAANQVTTPLHVHLALDTGMGRIGFQTPEELATVVTTLRQP 160
        ************************************************************************.*******
IY02092 QSPFDFEGIFTHFATADQADDTYFTHQLNNWKHLIAVVDELPRYVHVSNSATSLWHQACNGNMVRFGVALYGLNPSGREL 240
JCM1149 QSPFDFEGIFTHFATADQADDTYFTHQLNNWKHLIAVVDELPRYVHVSNSATSLWHQACNGNMVRFGVALYGLNPSGREL 240
IY02179 QSPFDFEGIFTHFATADQADDTYFTHQLNNWKHLIAVVDELPRYVHVSNSATSLWHQACNGNMVRFGVALYGLNPSGREL 240
        ********************************************************************************
IY02092 SAPYPLQPALSLTARLTFVKRLARGKSVSYGATYTAAQDEWIGTVPIGYADGYERRLQGFHVLVDGEFCEIVGRVCMDQL 320
JCM1149 SAPYPLQPALSLTARLTFVKRLARGKSVSYGATYTAAQDEWIGTVPIGYADGYERRLQGFHVLVDGEFCEIVGRVCMDQL 320
IY02179 SAPYPLLPALSLTARLTFVKRLARGKSVSYGATYTAAQDEWIGTVPIGYADGYERRLQGFHVLVDGEFCEIVGRVCMDQL 320
        ****** *************************************************************************
IY02092 MVRLPHEVPVGAKVTLVGTDGARTISLQDIADYCGTIHYEIACGLAPRVPRVYID 375
JCM1149 MVRLPHEVPVGAKVTLVGTDGARTISLQDIADYCGTIHYEIACGLAPRVPRVYID 375
IY02179 MVRLPHEVPVGAKVTLVGTDGARTISLQDIADYCGTIHYEIACGLAPRVPRVYID 375
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(B) Phylogram based on putative alanine racemase of *L. plantarum*

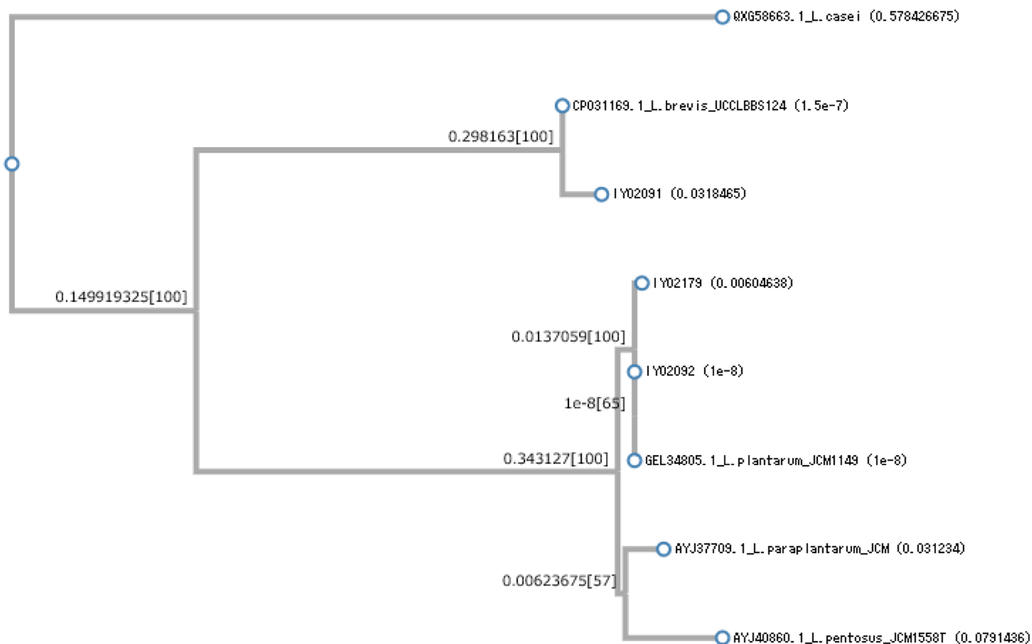

Fig. 3. Comparison of the primary structures of alanine racemases in the *Lactiplantibacillus plantarum* group and *Levilactobacillus brevis*.

(A) Alignment of the primary structures of the amino acid racemases of *L. plantarum* IYO2092, IYO2179, and JCM1149^T. (B) Alanine racemase phylogenic tree. Sequences of different types of strains were employed as a reference. The reference sequences of *L. plantarum*, *L. paraplantarum*, *L. pentosus*, and *L. brevis* were obtained from GenBank. *L. casei* was employed as the outgroup species. The accession numbers of the reference species are indicated in the figure.

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