



D- and L-amino acid concentrations in culture broth of *Lactobacillus* are highly dependent on the phylogenetic group of *Lactobacillus*

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ABSTRACT

D-amino acids produced by *Lactobacillus* are thought to contribute to the taste quality and health functions; however, no studies have comprehensively evaluated the concentrations of the D- and L-forms of amino acids separately in individual *Lactobacillus* strains. To gain insight into amino acid concentrations in *Lactobacillus*, we evaluated amino acid concentrations in culture broth of *Lactobacillus* separately for the D- and L-forms. *Lactobacillus* strains were cultured in culture broth, and the amino acid concentrations in supernatant were assessed. The amino acid concentrations obtained by liquid chromatography-tandem mass spectrometry (LC-MS/MS) were subjected to cluster analysis based on Bray-Curtis distance with Ward's minimum variance method. In the analysis of amino acid concentrations under culture with different monosaccharides, the distances among strains cultured with the same monosaccharide were significantly greater than those among cultures of the same strain under different monosaccharides ($p < 0.01$). The cluster analysis of amino acid concentrations under culture with the same monosaccharide suggested that strains belonging to the same phylogenetic group of *Lactobacillus* exhibited similar concentrations of amino acids. Data analyses of 70 strains belonging to 17 *Lactobacillus* taxa indicated that the concentrations of amino acids were highly dependent on the phylogenetic group of *Lactobacillus* and that the group differences in amino acid concentration were strongly driven by differences in L-serine and D-alanine concentrations. Our results indicate that it is important to evaluate D- and L-amino acids separately when evaluating variations in amino acid concentrations. Because D-alanine has the potential to affect taste quality, the results of this study may provide insight into the taste quality of fermented food produced by *Lactobacillus*.

1. Introduction

Species of the genus *Lactobacillus* are among the most important taxa involved in food microbiology and human health due to their roles in food production and preservation and their beneficial effects on human health [1]. *Lactobacillus* bacteria are non-spore-forming, catalase-negative, obligate saccharolytic rod or coccobacillus bacteria; they are generally characterized by a low guanine and cytosine genome content and are able to produce lactate as the main end-product of fermentation. Other side products include acetate, ethanol, carbon dioxide, formate and succinate [2]. These bacteria require complex nutrients, such as salts, amino acids, peptides, vitamins, and fatty acids, and they reside in food (e.g., dairy products, grain products, beer, wine and fruits), water, soil, sewage and intestinal environments, including those of humans. The ability of *Lactobacillus* to metabolize several carbohydrates provides

the members of this genus competitive advantages in numerous environments and makes them unique and distinctive in terms of their fermentation potential [3].

The traditional method of fermented food production is the inoculation of food with a sample of a previous-day product that contains certain microorganisms, such as members of the genus *Lactobacillus*. This method is still used for some homemade products; however, the quality of the products produced by this method varies. For stabilization and large-scale production of fermented food, commercial starter cultures containing *Lactobacillus* are used for successful production of fermented food [4]. To date, many *Lactobacillus* strains have been used in the food industry for their specific activities, such as the ability to utilize carbohydrates.

Bacteria can produce D-amino acids [5], and fermented food produced by *Lactobacillus* contains D-amino acids. A previous study

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indicated that the umami taste in Japanese rice wine, which is a fermented alcoholic drink, was more influenced by D-alanine supplementation than by L-alanine supplementation [6]. Because several D-amino acids, such as D-alanine, D-leucine and D-phenylalanine, are sweeter than their L-amino acid counterparts, D-amino acids might contribute to the taste quality of fermented foods [7].

Lactobacillus is also used as a probiotic, which is defined as a live microorganism that confers a health benefit to the host when administered in adequate amounts, and it has been demonstrated to improve host health [8]. *Lactobacillus acidophilus* (*L. acidophilus*), *Lactobacillus crispatus* (*L. crispatus*), *Lactobacillus gasseri* (*L. gasseri*), *Lactobacillus johnsonii* (*L. johnsonii*), *Lactobacillus rhamnosus* (*L. rhamnosus*), *Lactobacillus paracasei* (*L. paracasei*), *Lactobacillus plantarum* (*L. plantarum*), *Lactobacillus reuteri* (*L. reuteri*), *Lactobacillus fermentum* (*L. fermentum*), *Lactobacillus salivarius* (*L. salivarius*) and *Lactobacillus brevis* (*L. brevis*) are widely used as probiotics [9–11], and the beneficial effects of probiotics are thought to derive from their surface components and metabolites [12]. Probiotics produce D-amino acids as metabolites, and a previous study in mice indicated that D-tryptophan derived from *Lactobacillus* ameliorated allergic airway inflammation [13]. Other reports have indicated the health functions of D-amino acids in the intestinal environment. D-amino acids produced by the gut microbiota protect the mucosal surface of the small intestine from pathogens by inducing D-amino acid oxidase production in intestinal epithelial cells [14]. Furthermore, intestinal microbiota-derived D-serine has been shown to protect against acute kidney injury [15]. Therefore, the D-amino acids produced by *Lactobacillus* as probiotic bacteria in the intestinal environment might contribute to host health.

To determine their effects on taste quality and host health condition,

the concentrations of D- and L-amino acids produced by *Lactobacillus* should be evaluated separately. However, no reports have comprehensively evaluated the concentrations of the D- and L-forms of amino acids separately in individual *Lactobacillus* strains. In this study, we evaluated the concentrations of D- and L-forms and their characteristics in individual *Lactobacillus* strains. Since the ability to assimilate carbohydrates is one of the major factors affecting the fermentation potential of *Lactobacillus*, we evaluated whether carbohydrate type or bacterial taxon had a greater impact on the concentrations of amino acids by investigating 11 type strains of *Lactobacillus* and 6 species of monosaccharides. In addition, we used 70 strains belonging to 17 *Lactobacillus* taxa to evaluate the influence of *Lactobacillus* strain.

2. Material and methods

2.1. Bacterial strains and culture conditions

Lactobacillus strains were obtained from Japan Collection of Microorganisms (JCM) (Ibaraki, Japan) and Asahi Group Culture Collection (AGCC) (Ibaraki, Japan) (Table 1). For bacterial culture, we used the method described in a previous study [16], which allowed the cultivation of all *Lactobacillus* strains used in this study. Bacterial cultures of *Lactobacillus* were subcultured in de Man, Rogosa and Sharpe (MRS) broth (Becton, Dickinson and Company, Sparks, MD) and incubated statically at 37 °C for 16 h.

2.2. Sample collection

Precultures of *Lactobacillus* in MRS broth were centrifuged at 8000 g

Table 1

Phylogenetic groups of *Lactobacillus* according to Salvetti et al. [2] and the strain names and sources of *Lactobacillus* in this study.

Strain name	Source	Phylogenetic group	Strain name	Source	Phylogenetic group
<i>Lactobacillus acidophilus</i> JCM1132 ^T	JCM	<i>Lactobacillus delbrueckii</i> group	<i>Lactobacillus fermentum</i> JCM1173 ^T	JCM	<i>Lactobacillus reuteri</i> group
<i>Lactobacillus acidophilus</i> LAC1613	AGCC		<i>Lactobacillus fermentum</i> LFE1299	AGCC	
<i>Lactobacillus acidophilus</i> LAC1780	AGCC		<i>Lactobacillus fermentum</i> LFE1753	AGCC	
<i>Lactobacillus acidophilus</i> LAC3525	AGCC		<i>Lactobacillus fermentum</i> LFE3024	AGCC	
<i>Lactobacillus amylovorus</i> JCM1126 ^T	JCM		<i>Lactobacillus mucosae</i> LMU3014	AGCC	
<i>Lactobacillus amylovorus</i> LAM1562	AGCC		<i>Lactobacillus mucosae</i> LMU3018	AGCC	
<i>Lactobacillus amylovorus</i> LAM1587	AGCC		<i>Lactobacillus mucosae</i> LMU3021	AGCC	
<i>Lactobacillus amylovorus</i> LAM1750	AGCC		<i>Lactobacillus mucosae</i> LMU3025	AGCC	
<i>Lactobacillus crispatus</i> JCM1185 ^T	JCM		<i>Lactobacillus mucosae</i> LMU3368	AGCC	
<i>Lactobacillus crispatus</i> LCR1740	AGCC		<i>Lactobacillus mucosae</i> LMU3451	AGCC	
<i>Lactobacillus crispatus</i> LCR1744	AGCC	<i>Lactobacillus mucosae</i> LMU3470	AGCC		
<i>Lactobacillus crispatus</i> LCR1762	AGCC	<i>Lactobacillus oris</i> LOR1717	AGCC	<i>Lactobacillus casei</i> group	
<i>Lactobacillus crispatus</i> LCR1779	AGCC	<i>Lactobacillus oris</i> LOR3356	AGCC		
<i>Lactobacillus crispatus</i> LCR3453	AGCC	<i>Lactobacillus oris</i> LOR3360	AGCC		
<i>Lactobacillus delbrueckii</i> LDE688	AGCC	<i>Lactobacillus reuteri</i> JCM1112 ^T	JCM		
<i>Lactobacillus gasseri</i> JCM1131 ^T	JCM	<i>Lactobacillus reuteri</i> LRE720	AGCC		
<i>Lactobacillus gasseri</i> LGA3450	AGCC	<i>Lactobacillus reuteri</i> LRE3017	AGCC		
<i>Lactobacillus gasseri</i> LGA3465	AGCC	<i>Lactobacillus reuteri</i> LRE3062	AGCC		
<i>Lactobacillus gasseri</i> LGA3516	AGCC	<i>Lactobacillus reuteri</i> LRE3084	AGCC		
<i>Lactobacillus gasseri</i> LGA3529	AGCC	<i>Lactobacillus casei</i> LCA3518	AGCC		
<i>Lactobacillus gasseri</i> LGA3568	AGCC	<i>Lactobacillus casei</i> LCA3560	AGCC		
<i>Lactobacillus johnsonii</i> JCM2012 ^T	JCM	<i>Lactobacillus casei</i> LCA3489	AGCC		
<i>Lactobacillus johnsonii</i> LJO721	AGCC	<i>Lactobacillus casei</i> LCA3490	AGCC		
<i>Lactobacillus johnsonii</i> LJO1572	AGCC	<i>Lactobacillus casei</i> LCA3493	AGCC		
<i>Lactobacillus plantarum</i> JCM1149 ^T	JCM	<i>Lactobacillus paracasei</i> JCM8130 ^T	JCM		
<i>Lactobacillus plantarum</i> LPL3504	AGCC	<i>Lactobacillus paracasei</i> LPA3507	AGCC		
<i>Lactobacillus plantarum</i> LPL3530	AGCC	<i>Lactobacillus paracasei</i> LPA3526	AGCC		
<i>Lactobacillus plantarum</i> LPL3543	AGCC	<i>Lactobacillus paracasei</i> LPA3546	AGCC		
<i>Lactobacillus plantarum</i> LPL3551	AGCC	<i>Lactobacillus paracasei</i> LPA3562	AGCC		
<i>Lactobacillus salivarius</i> JCM1231 ^T	JCM	<i>Lactobacillus rhamnosus</i> JCM1136 ^T	JCM		
<i>Lactobacillus salivarius</i> LSA3355	AGCC	<i>Lactobacillus rhamnosus</i> LRH1616	AGCC		
<i>Lactobacillus salivarius</i> LSA3496	AGCC	<i>Lactobacillus rhamnosus</i> LRH3344	AGCC		
<i>Lactobacillus salivarius</i> LSA3510	AGCC	<i>Lactobacillus rhamnosus</i> LRH3544	AGCC		
<i>Lactobacillus brevis</i> JCM1059 ^T	JCM				
<i>Lactobacillus brevis</i> LBR3509	AGCC				
<i>Lactobacillus brevis</i> LBR3550	AGCC				
<i>Lactobacillus brevis</i> LBR3573	AGCC				

JCM; The Japan Collection of Microorganisms, AGCC; Asahi Group Culture Collection.

for 5 min, and pellets were washed and resuspended in an equal volume of phosphate-buffered saline. The bacterial suspension was inoculated with 5% v/v into modified MRS (mMRS) broth in which the carbohydrate source had been changed from dextrose to other monosaccharides, and the bacteria were cultured at 37 °C for 16 h. The mMRS broth comprised 1% (w/v) protease peptone No. 3 (Becton, Dickinson and Company, Sparks, MD), 0.5% (w/v) yeast extract (Becton, Dickinson and Company), 1% (w/v) beef extract (Becton, Dickinson and Company), 0.2% (w/v) triammonium citrate (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 0.1% (w/v) polyoxyethylene (20) sorbitan monooleate (Fujifilm Wako Pure Chemical Corporation), 0.5% (w/v) sodium acetate (Fujifilm Wako Pure Chemical Corporation), 0.01% (w/v) magnesium sulfate (Fujifilm Wako Pure Chemical Corporation), 0.005% (w/v) manganese (II) sulfate (Fujifilm Wako Pure Chemical Corporation), 0.2% (w/v) dipotassium hydrogen phosphate (Fujifilm Wako Pure Chemical Corporation), and 2% (w/v) monosaccharide. The monosaccharide was selected from among glucose (Fujifilm Wako Pure Chemical Corporation), galactose (Fujifilm Wako Pure Chemical Corporation), rhamnose (Dextra Laboratories Ltd., Reading, UK), arabinose (Fujifilm Wako Pure Chemical Corporation), mannose (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), xylose (Nacalai Tesque, Inc., Kyoto, Japan) and fructose (Kanto Chemical Co., Inc., Tokyo, Japan).

2.3. Sample preparation

After the bacterial cultures were incubated in mMRS broth, the pH of the broth was measured. Then, the bacterial cultures were centrifuged at 8000 g for 5 min. The supernatants were frozen at -20 °C until use.

Standard and sample preparation were performed as previously described [17] with some modifications. DL-alanine (DL-Ala), DL-arginine hydrochloride (DL-Arg), DL-asparagine monohydrate (DL-Asn), DL-aspartic acid (DL-Asp), DL-cysteine hydrochloride monohydrate (DL-Cys), DL-glutamic acid (DL-Glu), DL-glutamine (DL-Gln), DL-histidine (DL-His), DL-isoleucine (DL-Ile), DL-leucine (DL-Leu), DL-lysine monohydrochloride (DL-Lys), DL-methionine (DL-Met), DL-phenylalanine (DL-Phe), DL-serine (DL-Ser), DL-threonine (DL-Thr), DL-tryptophan (DL-Trp), DL-tyrosine (DL-Tyr) and DL-valine (DL-Val) were obtained from Tokyo Chemical Co., Ltd. Glycine (Gly) and L-proline (L-Pro) were obtained from Fujifilm Wako Pure Chemical Corporation. DL-alanine-2,3,3,3-d4 (DL-Ala-d4)

was obtained from Santa Cruz Biotechnology (Dallas, TX). Each amino acid standard was dissolved in 50% methanol, 50% methanol with 0.02 mol/ml hydrochloride (for DL-Glu, DL-His and DL-Trp), or 50% methanol with 0.05 mol/ml hydrochloride (for DL-Asp and DL-Tyr). A solution of DL-Ala-d4 (1000 nmol/ml) was prepared as described above and used as an internal standard (IS) solution. After thawing a frozen sample, 50 µl of the sample was mixed with 175 µl of methanol, 10 µl of the IS solution, and 25 µl of water in a tube. The contents were then mixed and centrifuged at 10000 rpm for 10 min at 4 °C. Then, 180 µl of supernatant was transferred to a new tube and mixed with 90 µl of water and 180 µl of chloroform. After mixing and centrifugation, 50 µl of supernatant was diluted with 160 µl of acetonitrile and 40 µl of ethanol. Approximately 50 µl of diluted sample was transferred to a vial and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. A standard was prepared using the same method described above.

2.4. Amino acid quantification

LC-MS/MS analysis was performed as previously described [17] with some modifications. Briefly, we used the Nexera HPLC System (communication bus module: CBM-20A; pump: LC-30AD; autosampler: SIL-30AC; degasser: DGU-20A5R; column oven: CTO-20AC, Shimadzu, Kyoto, Japan) connected to a Sciex Triple quad 6500+ mass spectrometer (AB SCIEX, Concord, Canada). The Sciex Triple quad 6500+ (temperature: 500 °C; ion spray voltage floating: 5500 V; entrance potential: 10 V) was used with the parameters described in Table 2. Chromatographic separation for DL-Ala, DL-Arg, DL-Asn, DL-Asp, DL-Cys, DL-Glu, DL-His, DL-Ile, DL-Leu, DL-Lys, DL-Met, DL-Phe, DL-Ser, DL-Thr, DL-Trp, DL-Tyr and DL-Val was performed with a CROWNPAK CR-I (+) (3.0 mm i.d. × 150 mm, 5 µm, Daicel CPI, Osaka, Japan) enantioseparation column. Chromatographic separation for DL-Gln and DL-Lys was performed with a CROWNPAK CR-I (-) (3.0 mm i.d. × 150 mm, 5 µm, Daicel CPI) enantioseparation column. Because D- and L-Pro could not be separated from each other, total proline (a racemic mixture) was quantified using a CROWNPAK CR-I (-) column. In addition, chromatographic separation for Gly was performed with a CROWNPAK CR-I (-) column. The injection volume was 1 µl, and the oven temperature was maintained at 30 °C. The mobile phase consisted of a mixture of acetonitrile, ethanol, water and trifluoroacetic acid (80/15/5/0.5), and

Table 2
Optimized multiple reaction monitoring transition and parameters for 20 amino acids and the internal standard (IS).

	Molecular weight	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (volts)	Collision energy (volts)	Collision cell exit potential (volts)	Curtain gas (psi)	Collision activated dissociation gas (psi)	Ion source gas1 (psi)	Ion source gas2 (psi)
Alanine	89.1	90.0	44.0	56	17	12	40	8	30	40
Arginine	174.2	175.0	69.9	51	29	10	40	8	30	40
Asparagine	132.1	132.9	74.1	31	21	12	40	8	30	40
Aspartic acid	133.1	133.9	74.0	61	19	8	40	8	30	40
Cysteine	121.2	122.0	59.0	31	35	10	40	8	30	40
Glutamic acid	147.1	147.9	83.9	36	23	14	40	8	30	40
Glutamine	146.2	147.0	84.0	56	23	10	30	10	50	80
Glycine	75.1	75.9	30.1	56	17	14	30	10	50	80
Histidine	155.2	156.0	110.0	31	19	10	40	8	30	40
Isoleucine	131.2	132.0	86.1	51	15	12	40	8	30	40
Leucine	131.2	132.0	86.0	46	15	8	40	8	30	40
Lysine	146.2	147.0	84.0	31	25	14	30	10	50	80
Methionine	149.2	150.0	104.0	36	15	14	40	8	30	40
Phenylalanine	165.2	166.0	102.9	46	37	16	40	8	30	40
Proline	115.1	116.1	70.0	61	21	10	30	10	50	80
Serine	105.1	105.9	59.9	81	15	12	40	8	30	40
Threonine	119.1	119.8	74.0	41	15	10	40	8	30	40
Tryptophan	204.2	205.0	187.9	76	15	18	40	8	30	40
Tyrosine	181.2	182.0	136.0	36	19	16	40	8	30	40
Valine	117.2	118.0	72.0	41	15	8	40	8	30	40
Alanine-2,3,3,3-d4 (IS)	93.1	94.0	48.1	111	15	8	40	8	30	40

the flow rate was set to 0.4 ml/min under isocratic conditions. Data processing was performed using MultiQuant (AB SCIEX) and regression equations ($Y = aX + b$; with a weighting factor of $1/X$).

2.5. Statistical analysis

All statistical analyses were performed in R (ver. 4.0.0) [18]. The R packages vegan [19], RVAideMemoire [20] and randomForest [21] were used for cluster analysis, pairwise permutational multivariate analysis of variance (MANOVA) and random forest analysis, respectively. For all analyses, p values of <0.05 were considered statistically significant.

3. Results

3.1. Amino acid concentrations of *Lactobacillus* under Culture with different monosaccharides

The concentrations of amino acids in the supernatant of mMRS broth were evaluated after *Lactobacillus* culture when the pH reductions in the broth were 1.0 or greater. We measured the pH values of 77 supernatants, which were generated using 11 type strains and 7 monosaccharides. The pH reductions in the broths of 34 bacterial cultures were not large enough for evaluation; thus, the remaining 43 bacterial cultures with sufficient pH reduction and 7 uncultured broths containing certain species of monosaccharides as blanks were evaluated.

To reveal whether strain or monosaccharide type had a large impact on the concentrations of amino acids, the D- and L-amino acid concentrations in the 43 bacterial cultures and the 7 uncultured broths were used to calculate Bray-Curtis distances. Bray-Curtis distance was used to visualize the results of the hierarchical clustering analysis performed with the Ward's minimum variance method. The cluster analysis showed that the concentrations of amino acids produced by each strain were similar among the different monosaccharides (Fig. 1A). We compared the average Bray-Curtis distance among different monosaccharides within the same strain and the average Bray-Curtis distance among different strains cultured with the same monosaccharide, which were determined with a previously reported method [22]. The distance between strains within a monosaccharide type was significantly higher than the distance between monosaccharides within the same strain ($p = 1.3E-21$, Fig. 1B).

- (A) Bray-Curtis distance calculated from the D- and L-amino acid concentrations in 43 bacterial cultures and 7 uncultured broths were visualized by the hierarchical clustering analysis performed with the Ward's minimum variance method. An uncultured broth containing each monosaccharide is defined as the blank in this figure. Glc, Man, Xyl, Gal, Fru, Rha and Ara indicate glucose, mannose, xylose, galactose, fructose, rhamnose and arabinose, respectively.
- (B) Average Bray-Curtis distance (a measure of difference in amino acid concentration) between monosaccharides within a strain and between strains within a monosaccharide. Data are shown as the mean \pm SD. P-values were calculated using Welch's *t*-test. ** $p < 0.01$

3.2. Amino acid concentrations of *Lactobacillus* under Culture with the same monosaccharide

To evaluate the effects of strain on the concentrations of amino acids produced by *Lactobacillus*, we investigated amino acid concentrations in 70 strains using mMRS broth with glucose as the sole carbon source; glucose was chosen because all the strains are capable of growing in mMRS broth with glucose. The D- and L-amino acid concentrations in 70 bacterial cultures generated from 70 strains of *Lactobacillus* were used to calculate Bray-Curtis distances. Bray-Curtis distance was used to visualize the results of the hierarchical clustering analysis performed with the Ward's minimum variance method. The concentration of each amino acid in the mMRS broth was compared using hierarchical cluster analysis with Bray-Curtis distances and Ward's clustering algorithm (Fig. 2). To validate the cluster number, we used pairwise permutational MANOVA for statistical analysis with Bonferroni correction for multiple comparisons. Significant pairwise differences between all clusters were observed when 4 clusters were selected (group 1 vs. group 2: $p = 0.006$; group 1 vs. group 3: $p = 0.006$; group 1 vs. group 4: $p = 0.012$; group 2 vs. group 3: $p = 0.006$; group 2 vs. group 4: $p = 0.006$; group 3 vs. group 4: $p = 0.012$), and when the number of clusters was greater than 4, some pairs did not show a significant difference. Group 1 consisted of 24 strains belonging to 6 species (*L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. delbrueckii*, *L. gasseri*, *L. johnsonii*), and all species belonged to the *L. delbrueckii* group. Group 2 consisted of 28 strains belonging to 6 species (*L. fermentum*, *L. mucosae*, *L. oris*, *L. plantarum*, *L. reuteri*, *L. salivarius*), and these 6 species belonged to the *L. reuteri*, *L. plantarum* or *L. salivarius*

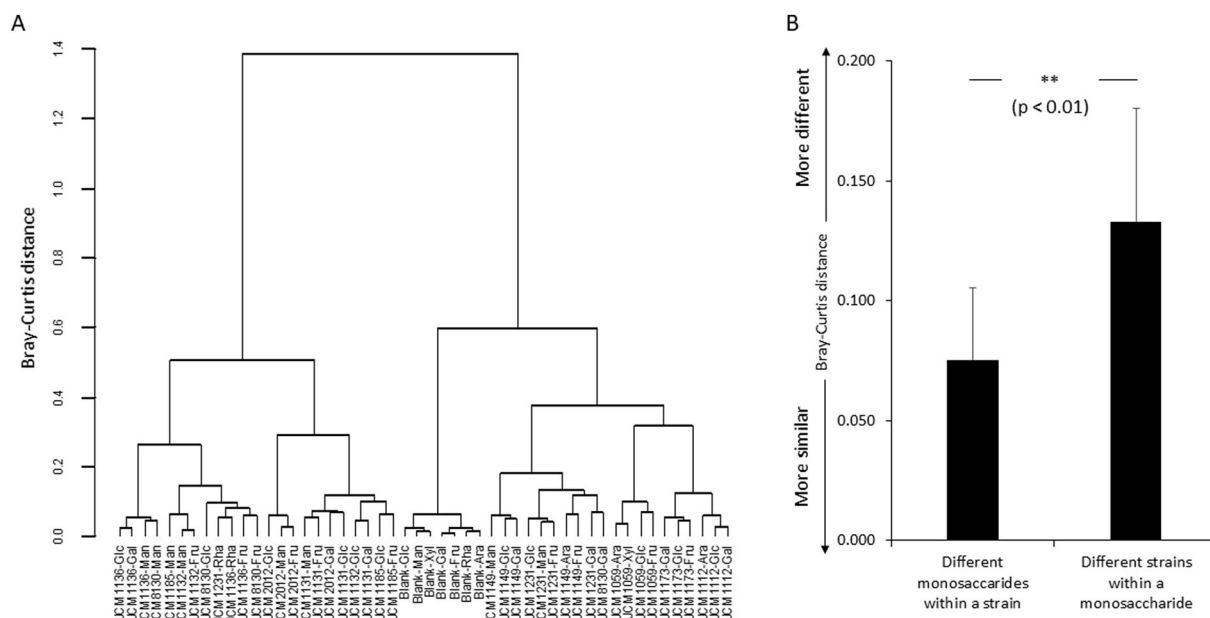


Fig. 1. Evaluation of the concentrations of amino acids in *Lactobacillus* cultured with different monosaccharides.

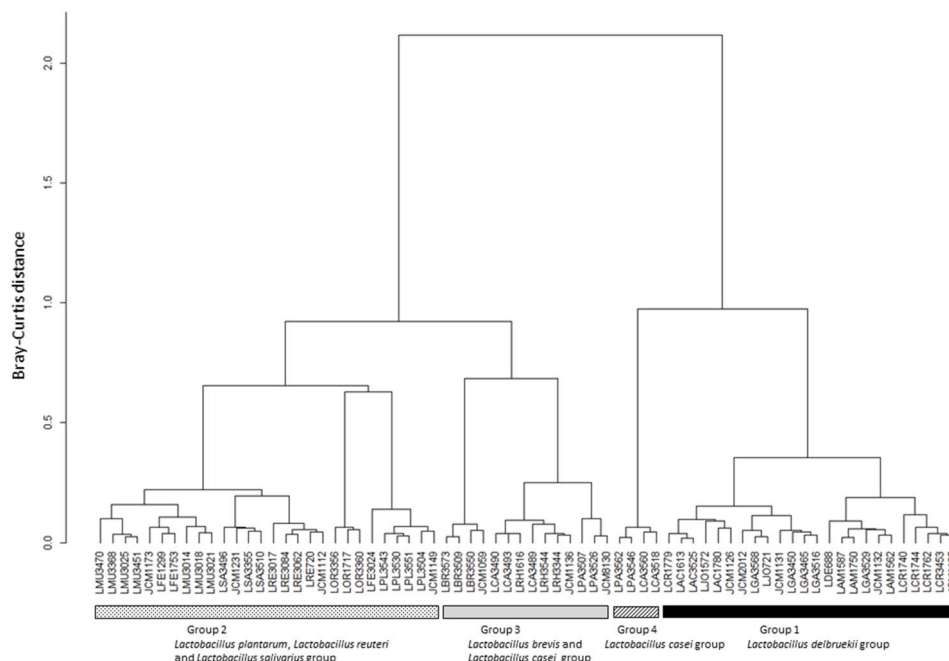


Fig. 2. Evaluation of the concentrations of amino acids in *Lactobacillus* cultured with the same monosaccharide.

groups. Group 3 consisted of 14 strains belonging to 4 species (*L. brevis*, *L. casei*, *L. paracasei*, *L. rhamnosus*), and these 4 species belonged to the *L. brevis* or *L. casei* group. Group 4 consisted of 4 strains belonging to the species *L. paracasei*, which belonged to the *L. casei* group.

Bray-Curtis distance calculated from the D- and L-amino acid concentrations in 70 bacterial cultures were visualized by the hierarchical clustering analysis performed with the Ward's minimum variance method. The lines in the figure indicate the phylogenetic groups [2] of *Lactobacillus* belonging to four clusters. Group 1 is indicated by the black line. Group 2 is indicated by the dotted line. Group 3 is indicated by the gray line. Group 4 is indicated by the striped line.

3.3. Contribution of each amino acid to the cluster separation

To reveal the contributions of amino acids to the cluster separation, we used 4 clusters generated by hierarchical cluster analysis and pairwise permutational MANOVA as group labels.

Random forest analysis is an analytical method using a machine

learning algorithm [23]. Because a previous study [24] indicated that random forest analysis was useful for biomarker selection in metabolomic data, we used random forest analysis to calculate the contribution of each amino acid to cluster separation. In the random forest analysis, the parameters $n_{tree} = 2000$ and $m_{try} = 3$ were used for the least out-of-bag (OOB) error, and the OOB error was 1.43%. Because a previous study [25] indicated that an index based on the mean decrease in Gini generated by random forest analysis provided a more robust result than one based on the mean decrease in accuracy, the mean decrease in Gini was used as a measure of the importance of each amino acid for cluster separation.

The index indicated that L-serine and D-alanine had high impacts on cluster separation (Table 3). In the statistical analysis of L-serine differences among the 4 groups, no significant difference was observed between group 1 and group 4; however, significant differences were observed between pairs of all the other groups (group 1 vs. group 2: $p = 7.3E-14$; group 1 vs. group 3: $p = 3.1E-7$; group 1 vs. group 4: $p = 1.0$; group 2 vs. group 3: $p = 2.9E-4$; group 2 vs. group 4: $p = 1.3E-12$; group

Table 3
Mean decrease in Gini calculated by random forest analysis.

	Mean decrease in Gini		Mean decrease in Gini
L-serine	3.34	DL-proline	1.05
D-alanine	3.26	L-arginine	1.02
L-histidine	2.91	D-methionine	0.91
D-serine	2.53	L-lysine	0.91
L-leucine	2.39	D-isoleucine	0.80
D-threonine	2.16	D-valine	0.74
L-aspartic acid	2.16	Glycine	0.70
L-phenylalanine	1.99	D-arginine	0.65
L-threonine	1.78	L-glutamic acid	0.60
D-lysine	1.72	L-alanine	0.57
L-tryptophan	1.67	L-methionine	0.52
L-isoleucine	1.58	D-phenylalanine	0.50
L-glutamine	1.47	D-tyrosine	0.45
L-valine	1.44	D-aspartic acid	0.39
D-asparagine	1.41	L-asparagine	0.33
D-leucine	1.16	L-cysteine	0.30
D-glutamic acid	1.14	D-tryptophan	0.19
D-glutamine	1.11	D-cysteine	0.01
L-tyrosine	1.08	D-histidine	0.00

3 vs. group 4: $p = 2.5E-4$) (Fig. 3A). In the statistical analysis of D-alanine differences among the 4 groups, no significant difference was observed between group 2 and group 3; however, significant differences were observed between pairs of all other groups (group 1 vs. group 2: $p = 9.1E-16$; group 1 vs. group 3: $p = 1.1E-11$; group 1 vs. group 4: $p = 8.4E-10$; group 2 vs. group 3: $p = 1.0$; group 2 vs. group 4: $p = 3.4E-6$; group 3 vs. group 4: $p = 6.1E-4$) (Fig. 3B).

Data are shown as the mean \pm SD. The groups in the figure consisted of the strains in the phylogenetic groups [2] of *Lactobacillus*. Group 1 consisted of the strains belonging to the *L. delbrueckii* group and is indicated by the black bar. Group 2 consisted of the strains in the *L. reuteri*, *L. plantarum* or *L. salivarius* groups and is indicated by the dotted bar. Group 3 consisted of the strains in the *L. brevis* or *L. casei* group and is indicated by the gray bar. Group 4 consisted of 4 strains belonging to the *L. casei* group and is indicated by the striped bar. P-values were calculated using Welch's *t*-test with Bonferroni correction for multiple comparisons. $**p < 0.01$.

3.4. Characteristics of amino acid metabolism in each group

To evaluate the differences from the initial values, amino acid concentrations in the blank (i.e., uncultured mMRS broth with glucose) were measured in triplicate. The amino acid concentrations in the blank and each cluster group are summarized in Fig. 4.

An uncultured broth with glucose was defined as the blank and is indicated in the figure by the white bar. The groups in the figure consisted of the strains in the phylogenetic groups [2] of *Lactobacillus*. Group 1 consisted of the strains belonging to the *L. delbrueckii* group and is indicated by the black bar. Group 2 consisted of the strains in the *L. reuteri*, *L. plantarum* or *L. salivarius* groups and is indicated by the dotted bar. Group 3 consisted of the strains in the *L. brevis* or *L. casei* group and is indicated by the gray bar. Group 4 consisted of 4 strains belonging to the *L. casei* group and is indicated by the striped bar. (A) The 18 D-amino acid concentrations in the broths. (B) The 18 L-amino acid, DL-proline and glycine concentrations in the broths. Data are shown as the mean \pm SD.

4. Discussion

In the present study, it was considered important to evaluate the concentrations of D- and L-amino acids produced by *Lactobacillus* separately because the D-amino acids produced by *Lactobacillus* influence the taste of fermented food and its health benefits to the host [7,14]. Therefore, we quantified D- and L-amino acid concentrations separately by enantioseparation and LC-MS/MS methods, and we found that the concentrations of amino acids in *Lactobacillus* were highly dependent on phylogenetic group and that the group differences in amino acid concentration were strongly affected by differences in L-serine and D-alanine concentrations.

The patterns of D- and L-amino acid concentrations in this study were visualized by hierarchical cluster analysis using 11 type strains and 7 monosaccharides. Although the concentrations of a few amino acids,

especially in *L. paracasei* JCM8130, did not show obvious cluster separation, the cluster separation was highly dependent on strain (Fig. 1A). The comparison of the average Bray-Curtis distance among different monosaccharides within the same strain and that among different strains under the same monosaccharide supported the finding of strain dependence (Fig. 1B). These results suggested that compared with monosaccharide type, strain more strongly impacted the concentrations of amino acids produced by *Lactobacillus*.

We also investigated strain differences in the concentrations of amino acids in 70 strains cultured with the same monosaccharide. Glucose was selected as the best monosaccharide for this evaluation because all strains were capable of growing in mMRS broth containing glucose as the sole carbon source. Based on the hierarchical cluster analysis of amino acid concentration and the pairwise permutational MANOVA with Bonferroni correction for multiple comparisons, the *Lactobacillus* strains were separated into 4 groups (Fig. 2). The cluster analysis showed that different strains of the same bacterial species often produced similar concentrations of amino acids, although in some cases, strains produced concentrations similar to those of another bacterial species. The concentrations of amino acids in *Lactobacillus* strains were divided into the phylogenetic groups of *Lactobacillus*; this result indicated that the concentrations of amino acids produced by *Lactobacillus* were highly dependent on phylogenetic group. In previous studies [2,26,27], phylogenetic analyses of the 16S rRNA gene, selected genes, and whole genomes have been performed. All of these phylogenetic analyses have shown that the *L. delbrueckii* group clusters separately from other *Lactobacillus* groups. The cluster separation between the *L. delbrueckii* group and the other *Lactobacillus* groups in this study was consistent with previous phylogenetic analyses, which suggested that genomic differences might be partially reflected in the concentrations of amino acids. In addition, in a recent study, some *Lactobacillus* species were reclassified into new genera [28]: *L. plantarum* was reclassified into *Lactiplantibacillus*; *L. fermentum*, *L. mucosae*, *L. oris* and *L. reuteri* were reclassified into *Limosilactobacillus*; *L. salivarius* was reclassified into *Ligilactobacillus*; *L. brevis* was reclassified into *Levilactobacillus*; and *L. casei*, *L. paracasei* and *L. rhamnosus* were reclassified into *Lacticaeibacillus*. Although *L. casei* and *L. paracasei* were divided into two groups in this study, the difference in genera proposed by Zheng et al. [28] might be reflected in the concentrations of amino acids.

To reveal the contribution of each amino acid to cluster separation, we performed random forest analysis. The analysis generated the mean decrease in Gini, which indicated that L-serine and D-alanine had high impacts on the cluster separation (Table 3). In addition, significant differences between all groups were observed in either or both concentration of L-serine or D-alanine (Fig. 3A and B). These results suggested that the characteristics of each group could be partially revealed by evaluating L-serine and D-alanine concentrations. In the evaluation of D-amino acids, D-alanine was found to be especially important for understanding the variation in amino acid concentrations in *Lactobacillus*.

In *Lactobacillus*, L-serine is preferentially converted into pyruvate by serine deaminase (EC 4.3.1.17) and into glycine and acetaldehyde by serine hydroxymethyl transferase (EC 2.1.2.1) [29]. Tetrahydrofolate is

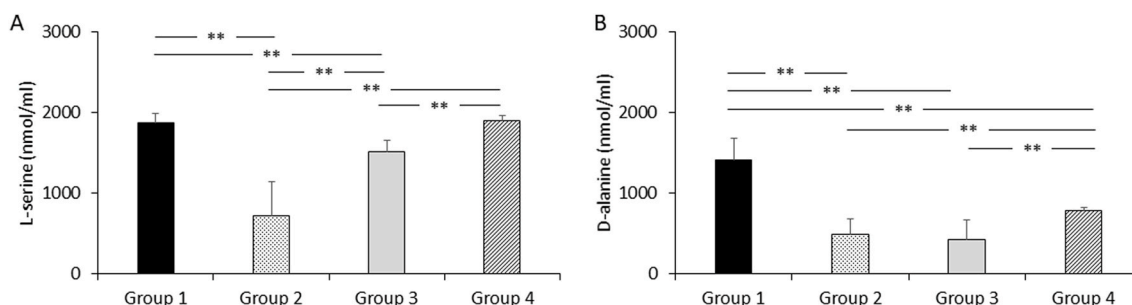


Fig. 3. Statistical analysis of L-serine and D-alanine concentrations in the 4 groups.

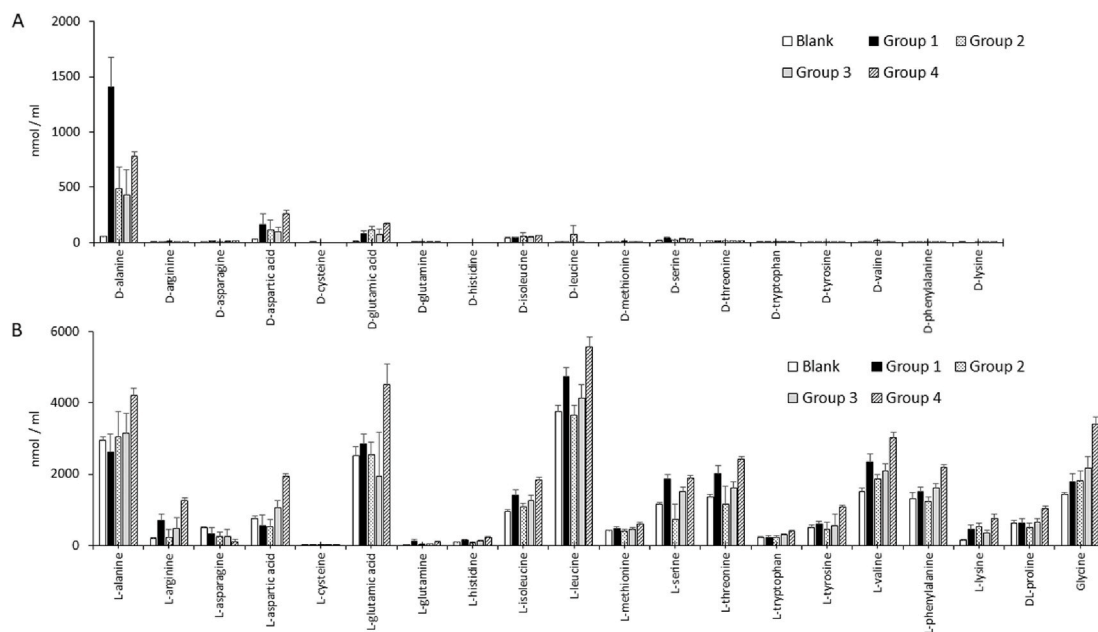


Fig. 4. D- and L-amino acid concentrations in the broths.

a cofactor of serine hydroxymethyl transferase [29], and tetrahydrofolate is produced from dihydrofolate by dihydrofolate reductase (EC 1.5.1.3) [30]. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis has indicated that the genes encoding these enzymes exist in many *Lactobacillus* species [31], suggesting that the possession of these genes does not differ among *Lactobacillus* taxa. L-alanine is converted to D-alanine by alanine racemase (EC:5.1.1.1), and its enzymatic activity has been characterized in *Lactobacillus* species [29]. In *Lactobacillus*, D-alanine is a predominant component of newly synthesized peptidoglycan [32], and the alanine racemase gene is present in all *Lactobacillus* taxa represented in the KEGG database. Regarding other enzymes for producing D-alanine in *Lactobacillus* species, amino acid racemase activity has been reported for *MalY* (EC 4.4.1.13) and isoleucine 2-epimerase (EC 5.1.1.21); these enzymes have racemase activity for many kinds of amino acids, including alanine [33,34]. These observations indicate that the differences in L-serine and D-alanine concentrations among members of *Lactobacillus* are difficult to interpret without evaluating the metabolic pathways involved and differences in metabolic activity. In addition, some *Lactobacillus* species have proteinases, such as *PrfB* and *PrfH* [35]. The concentrations of L-amino acids in *Lactobacillus* might be affected by proteolytic ability. Therefore, further analysis is needed to identify the differences in L-serine and D-alanine metabolism, which contribute to the differences in amino acid concentrations among members of *Lactobacillus*.

According to the changes from the initial values of D-amino acids in the culture broth, *Lactobacillus* mainly produced D-alanine and produced less D-aspartic acid and D-glutamic acid than D-alanine (Fig. 4A). This result was consistent with the results of a previous study [7]. The *L. delbrueckii* group produced high levels of D-alanine (Fig. 3B), which influenced taste quality in fermented food [6]; thus, the taste quality of fermented dairy foods such as yogurt and acidophilus milk, which are fermented by members of the *L. delbrueckii* group, might be affected by high D-alanine concentrations. In addition, because D-amino acids affect host health, differences in the ability to produce these acids among members of a *Lactobacillus* group might influence the health function supported by different *Lactobacillus* strains. In group 4, the levels of most L-amino acids appeared to increase above their initial values (Fig. 4B). *Lactobacillus* strains belonging to group 4 might have high proteolytic ability and thus a strong ability to increase total amino acid levels.

5. Conclusions

In this study, we found that the concentrations of amino acids in *Lactobacillus* were highly dependent on phylogenetic group and that differences in L-serine and D-alanine concentrations strongly contributed to the differences in amino acid concentrations in *Lactobacillus*. Our results suggest that revealing the variation in the concentration of D-alanine, which has the potential to affect the taste quality of fermented foods produced by *Lactobacillus*, was important for understanding amino acid metabolism in *Lactobacillus*. To our knowledge, this report is the first comprehensive evaluation of the concentrations of D- and L-amino acids in *Lactobacillus*. In future study, evaluation for the variation in the concentration of amino acid with *Lactobacillus* growth and the effects of amino acid concentrations produced by *Lactobacillus* on the taste quality and health function of *Lactobacillus*-fermented food will be expected.

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Author contributions

H. S., T. H., and Y. N. conceived and designed the experiments. H. S., K. N. and S. I. performed the experiments. H. S. analyzed the data. H. S., T. H., and Y. N. wrote the paper.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: All authors are employees of Asahi Quality & Innovations, Ltd.

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