Rapid species- and subspecies-specific level classification and identification of *Lactobacillus casei* group members using MALDI Biotyper combined with ClinProTools

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ABSTRACT

Using common taxonomic methods such as 16S rDNA sequencing and physiological and biochemical analysis to identify members of the *Lactobacillus casei* group (LCG) is time-consuming, expensive and inaccurate. In this study, we applied matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to rapid discriminate LCG strains by creating an analytical in-house database (IHDB) and to develop a classification model for subspecies-level differentiation based on MS biomarkers using ClinProTools bioinformatics software (Bruker Daltonics, Billerica, MA). Genotypic methods (housekeeping gene sequencing and species-specific PCR) were also established to validate the MALDI-TOF MS platform. A total of 48 LCG reference strains were correctly identified at the species level (mean score: 2.45 ± 0.1) by using MALDI-TOF MS with an IHDB and had high score values, which was in accordance with results from *mutL* gene sequencing and specific PCR-based methods. However, one strain that was identified as *L. casei* had a relatively low score value (2.02 ± 0.02), lower sequence similarities (*mutL*: 90.4%), and failed to amplify a species-specific amplicon; it may therefore represent an undescribed novel species. In addition, after implementation of the classification model (based on 2 biomarker peaks: *m/z* 4,930 and 5,303), *L. paracasei* strains could be clearly and easily differentiated to the subspecies level. Afterward, 7 LCG-related isolates from different probiotic samples were analyzed and accurately identified. Our data demonstrate the high-resolution performance of MALDI-TOF MS for fast and accurate demarcation of LCG strains when used with an IHDB coupled to ClinProTools; this methodology can serve as an alternative for quality control of probiotic products.

Key words: MALDI-TOF MS, ClinProTools, DNA sequencing, novel candidate taxon, *Lactobacillus casei* group

INTRODUCTION

The nomenclature and taxonomic status of the *Lactobacillus casei* group (LCG) have been controversial for a long time (Skerman et al., 1980; Collins et al., 1989; Dellaglio et al., 1991, 2002; Chen et al., 2000). The Judicial Commission of the International Committee on Systematic Bacteriology in 2008 concluded that the current taxonomy of the LCG still comprises 3 closely related species: *Lactobacillus casei*, *Lactobacillus paracasei* (ssp. *paracasei* and ssp. *tolerans*), and *Lactobacillus rhamnosus* (Tindall, 2008). The LCG members contain many strains generally recognized as safe and have been largely applied in probiotic products. *Lactobacillus casei* Shirota and *L. rhamnosus* GG are well-known commercial probiotic strains in dairy supplements and foods, and they exhibit antitumor, immunostimulatory, and antimicrobial effects (Shida and Nomoto, 2013; Ashraf and Shah, 2014; Reid, 2015; Orlando et al., 2016). However, more than 28% of commercial probiotic products are mislabeled at the genus or species level because of the use of methods that have limited taxonomic resolution (Huys et al., 2006). Thus, the ability to quickly and accurately identify starter cultures is important for the management and quality control of commercial probiotic products.

Researchers have traditionally and primarily relied on carbohydrate fermentation tests to differentiate the members of the LCG. However, the phenotypic characteristics that form the basis of these tests may be affected by environmental conditions or by the possible loss or acquisition of plasmids that encode many carbohydrate fermentation traits, resulting in isolates that exhibit atypical metabolic features (Ahrne et al., 1989). Deoxyribonucleic acid fingerprinting has been used for differentiation of lactobacilli, such as amplified fragment-length polymorphisms, random amplification of polymorphic DNA, repetitive sequence-based PCR,

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Table 1. Strains used to validate the *Lactobacillus casei* group (LCG) in-house database (IHDB) by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, *mutL* gene sequencing, and species-specific PCR identification

LCG	BCRC ¹ strain	Other designation	MALDI-TOF MS (IHDB)	Score average ²	<i>mutL</i> sequence similarity (%)	Species-specific PCR assay ³		
						Lcas ⁴	Lparacas ⁵	Lrham ⁶
<i>Lactobacillus casei</i> (n = 3)	10697 ^T	ATCC 393	<i>L. casei</i>	2.63 ± 0.02	<i>L. casei</i> (100)	+	—	—
	17487	JCM 8129	<i>L. casei</i>	2.54 ± 0.02	<i>L. casei</i> (100)	+	—	—
	80156	CCUG 35324	<i>L. casei</i>	2.44 ± 0.01	<i>L. casei</i> (100)	+	—	—
<i>Lactobacillus paracasei</i> ssp. <i>paracasei</i> (n = 22)	12248 ^T	ATCC 25302	<i>L. paracasei</i>	2.63 ± 0.01	<i>L. paracasei</i> (100)	—	+	—
	12188	ATCC 27216	<i>L. paracasei</i>	2.40 ± 0.01	<i>L. paracasei</i> (99.2)	—	+	—
	12193	ATCC 25598	<i>L. paracasei</i>	2.46 ± 0.01	<i>L. paracasei</i> (98.9)	—	+	—
	14001	ATCC 335	<i>L. paracasei</i>	2.53 ± 0.02	<i>L. paracasei</i> (100)	—	+	—
	14023	ATCC 27092	<i>L. paracasei</i>	2.41 ± 0.01	<i>L. paracasei</i> (99.2)	—	+	—
	16093	NCIMB 8822	<i>L. paracasei</i>	2.40 ± 0.01	<i>L. paracasei</i> (99.2)	—	+	—
	16094	NCIMB 8823	<i>L. paracasei</i>	2.31 ± 0.01	<i>L. paracasei</i> (100)	—	+	—
	16100	ATCC 11582	<i>L. paracasei</i>	2.45 ± 0.02	<i>L. paracasei</i> (99.2)	—	+	—
	17001	ATCC 39392	<i>L. paracasei</i>	2.31 ± 0.02	<i>L. paracasei</i> (99.4)	—	+	—
	17002	ATCC 334	<i>L. paracasei</i>	2.36 ± 0.01	<i>L. paracasei</i> (99.4)	—	+	—
	17005	ATCC 11578	<i>L. paracasei</i>	2.41 ± 0.02	<i>L. paracasei</i> (99.8)	—	+	—
	17475	JCM 20315	<i>L. paracasei</i>	2.42 ± 0.01	<i>L. paracasei</i> (99.6)	—	+	—
	17482	JCM 1109	<i>L. paracasei</i>	2.43 ± 0.01	<i>L. paracasei</i> (100)	—	+	—
	17483	DSM 20207	<i>L. paracasei</i>	2.43 ± 0.01	<i>L. paracasei</i> (100)	—	+	—
	17484	DSM 20006	<i>L. paracasei</i>	2.31 ± 0.01	<i>L. paracasei</i> (99.6)	—	+	—
	17488	JCM 8131	<i>L. paracasei</i>	2.40 ± 0.02	<i>L. paracasei</i> (99.2)	—	+	—
	17489	JCM 8133	<i>L. paracasei</i>	2.32 ± 0.01	<i>L. paracasei</i> (99.2)	—	+	—
	18853	NRIC 1934	<i>L. paracasei</i>	2.39 ± 0.01	<i>L. paracasei</i> (99.2)	—	+	—
	18854	NRIC 1935	<i>L. paracasei</i>	2.37 ± 0.01	<i>L. paracasei</i> (99.4)	—	+	—
	18855	NRIC 1938	<i>L. paracasei</i>	2.37 ± 0.01	<i>L. paracasei</i> (99.4)	—	+	—
	80062	—	<i>L. paracasei</i>	2.38 ± 0.03	<i>L. paracasei</i> (99.2)	—	+	—
	80881	—	<i>L. paracasei</i>	2.31 ± 0.01	<i>L. paracasei</i> (99.6)	—	+	—
<i>Lactobacillus paracasei</i> ssp. <i>tolerans</i> (n = 2)	14628 ^T	ATCC 25599	<i>L. paracasei</i>	2.64 ± 0.02	<i>L. paracasei</i> (100)	—	+	—
	17485	DSM 20012	<i>L. paracasei</i>	2.53 ± 0.04	<i>L. paracasei</i> (99.2)	—	+	—
	10940 ^T	ATCC 7469	<i>L. rhamnosus</i>	2.65 ± 0.03	<i>L. rhamnosus</i> (100)	—	—	+
<i>Lactobacillus rhamnosus</i> (n = 21)	11069	NRRL B-445	<i>L. rhamnosus</i>	2.55 ± 0.02	<i>L. rhamnosus</i> (100)	—	—	+
	11197	NRRL-1445	<i>L. rhamnosus</i>	2.52 ± 0.02	<i>L. rhamnosus</i> (96.5)	—	—	+
	11673	ATCC 9595	<i>L. rhamnosus</i>	2.62 ± 0.01	<i>L. rhamnosus</i> (100)	—	—	+
	12249	NCIMB 8963	<i>L. rhamnosus</i>	2.24 ± 0.03	<i>L. rhamnosus</i> (100)	—	—	+
	12272	CSCC 2607	<i>L. rhamnosus</i>	2.50 ± 0.02	<i>L. rhamnosus</i> (100)	—	—	+
	14027	ATCC 14957	<i>L. rhamnosus</i>	2.48 ± 0.02	<i>L. rhamnosus</i> (97.0)	—	—	+
	14029	ATCC 21052	<i>L. rhamnosus</i>	2.40 ± 0.04	<i>L. rhamnosus</i> (96.3)	—	—	+
	14068	CSCC 2608	<i>L. rhamnosus</i>	2.48 ± 0.02	<i>L. rhamnosus</i> (100)	—	—	+
	16000	ATCC 53103	<i>L. rhamnosus</i>	2.36 ± 0.01	<i>L. rhamnosus</i> (97.0)	—	—	+
	16095	NCIMB 8824	<i>L. rhamnosus</i>	2.53 ± 0.02	<i>L. rhamnosus</i> (98.5)	—	—	+
	17004	ATCC 15008	<i>L. rhamnosus</i>	2.43 ± 0.01	<i>L. rhamnosus</i> (96.3)	—	—	+
	17006	ATCC 11981	<i>L. rhamnosus</i>	2.55 ± 0.03	<i>L. rhamnosus</i> (100)	—	—	+
	17007	ATCC 11982	<i>L. rhamnosus</i>	2.62 ± 0.02	<i>L. rhamnosus</i> (100)	—	—	+
	17490	JCM 8136	<i>L. rhamnosus</i>	2.42 ± 0.05	<i>L. rhamnosus</i> (97.2)	—	—	+
	18860	—	<i>L. rhamnosus</i>	2.46 ± 0.03	<i>L. rhamnosus</i> (96.3)	—	—	+
	80663	LMG 8153	<i>L. rhamnosus</i>	2.54 ± 0.02	<i>L. rhamnosus</i> (96.5)	—	—	+
	80664	LMG 10769	<i>L. rhamnosus</i>	2.57 ± 0.03	<i>L. rhamnosus</i> (100)	—	—	+
	80665	LMG 10770	<i>L. rhamnosus</i>	2.58 ± 0.01	<i>L. rhamnosus</i> (96.5)	—	—	+
	80666	LMG 10772	<i>L. rhamnosus</i>	2.48 ± 0.02	<i>L. rhamnosus</i> (98.3)	—	—	+

Continued

Table 1 (Continued). Strains used to validate the *Lactobacillus casei* group (LCG) in-house database (IHDB) by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, *mutL* gene sequencing, and species-specific PCR identification

LCG	BCRC ¹ strain	Other designation	MALDI-TOF MS (IHDB)	Score average ²	<i>mutL</i> sequence similarity (%)	Species-specific PCR assay ³		
						Lcas ⁴	Lparacas ⁵	Lrham ⁶

Lactobacillus sp. (n = 1)
LCG strains identified to species level, % (no.)

	80667	LMG 10773	<i>L. rhamnosus</i>	2.52 ± 0.02	<i>L. rhamnosus</i> (96.5)	—	—	+
	81062	—	<i>L. casei</i>	2.02 ± 0.02	<i>L. casei</i> (90.4)	—	—	—
				100 (48/48)	100 (48/48)	—	—	100 (48/48)

¹BCRC = Bioresource Collection and Research Center at Food Industry Research and Development Institute (Hsinchu, Taiwan).

²Score values are the mean of triplicate analysis ± SD.

³Nontarget strains testing *Lactobacillus acidophilus* BCRC 16095^T, *Lactobacillus alimentarius* BCRC 80242^T, *Lactobacillus delbrueckii* ssp. *bulgaricus* BCRC 10696^T, *Lactobacillus* *graminis* BCRC 17898^T, *Lactobacillus gasseri* BCRC 14619^T, *Lactobacillus pentosus* BCRC 11053^T, *Lactobacillus plantarum* ssp. *plantarum* BCRC 10069^T, and *Lactobacillus sakei* ssp. *sakei* BCRC 14622^T. + = PCR amplicons with each primer pair detected; — = PCR amplicons with each primer pair not detected.

⁴Species-specific PCR amplification with primer spLcas-mutIF (5'-GGCAAGTCGAGGATCAACTA-3')/-R (5'-CGACACCGTAAAGCAATGCTG-3').

⁵Species-specific PCR amplification with primer spLpara-mutIF (5'-GATATTGCCTTCACGGTTTCAC-3')/-R (5'-TTGAARTCAACCATTTGAGCG-3').

⁶Species-specific PCR amplification with primer spLrham-mutIF (5'-GGRCAGGTAGAAAGTCAACGA-3')/-R (5'-GCTGACCGTAAACGCAATCTTAG-3').

amplified rDNA restriction analysis, and intergenic spacer region-PCR (Mattarelli et al., 2014). At present, 16S rRNA gene sequencing is the genotypic method that is most commonly used to assign bacterial taxonomy and phylogeny (Petti et al., 2005). The cut-off value of 16S rRNA gene sequence similarity to identify an organism at the species level is approximately 98.65% (Kim et al., 2014). Unfortunately, members of the LCG are sometimes poorly resolved, leading to misidentification at the species level due to the high genetic similarity (reaching 98.7–99.9%) of their 16S rRNA genes (Huang et al., 2015). By contrast, protein-coding genes provide greater discrimination power and can serve as an alternative to 16S rRNA gene sequencing to identify members of the LCG (Huang and Lee, 2011; Huang et al., 2014, 2015). The traditional gold standard for bacterial species delineation is DNA-DNA hybridization (Stackebrandt et al., 2002), and the whole-genome sequence comparison method is becoming a new trend for determining microbial taxonomy. The average nucleotide identity cut-off value to establish a species boundary between 2 genomic data sets is approximately 95% (Varghese et al., 2015). However, these technologies are time consuming, labor intensive, costly, and difficult to use routinely in laboratories.

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS is a phenotype-based method that can be used for the identification, classification, and dereplication of microorganisms based on their specific protein profiles (Moore and Rossello-Mora, 2011). The advantages of MALDI-TOF MS include that it is easy to perform, fast, accurate, economical (despite the initial cost of the instrument), and high throughput (Neville et al., 2011; Sandrin et al., 2013; Tran et al., 2015). It has been successfully applied in routine clinical diagnosis, and its use has expanded to the research areas of environmental monitoring, food safety, biodiversity, and gut microbiotas (Pavlovic et al., 2013; Samb-Ba et al., 2014; Santos et al., 2016). In general, MALDI-TOF MS can discriminate at both the genus and species levels using proteomics-based identification, and the taxonomic resolution can reach the sub-species and strain levels when the method is combined with specific biomarkers (Spinali et al., 2015). However, reference databases are mainly designed for routine clinical practice (Bizzini and Greub, 2010), and adding additional entries to the database is quite important to increase identification rates via matching. In this study, we determined the performance of MALDI-TOF MS when used with an in-house database (IHDB) coupled with ClinProTools (Bruker Daltonics, Billerica, MA) to discriminate between members of the LCG; DNA-based analysis such as sequencing and species-specific PCR assays were also used as reference methods.

MATERIALS AND METHODS

Lactobacillus Strains and Culture Conditions

All reference strains used in this study were obtained from the Bioresource Collection and Research Center (BCRC; Food Industry Research and Development Institute, Hsinchu, Taiwan) and have been authenticated by 16S rRNA gene sequencing (Table 1). The *Lactobacillus* strains were incubated on lactobacilli de Man, Rogosa and Sharpe agar (Difco, Becton, Dickinson and Co., Franklin Lakes, NJ) anaerobically for 20 h at 37°C, and fresh cultures were used for further DNA-based and proteomics-based analysis.

DNA Preparation and Design of LCG Degenerate Primers

Genomic DNA was extracted using the DNeasy kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The DNA concentration and quality were calculated from the 260/280 nm absorbance ratio and checked by agarose gel electrophoresis. The degenerate primers Lcagp-mutIF3 (5'-ATTGAYCARCTATCK-GCMAC-3') and Lcagp-mutIR4 (5'-GAGCCRTAAC-CGGCAAMA-3') were designed and targeted to the most conserved region of the *mutL* gene, which was determined by comparing the *mutL* gene from the whole-genome sequences of several LCG species (accession no. AP012544, CP000423, CP003094).

DNA Sequencing of *mutL* Gene

Partial fragments of the *mutL* gene were amplified and sequenced using consensus degenerate primers. The thermal cycler protocol consisted of the following steps: initial strand denaturation at 94°C for 5 min; followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1.5 min; and a final extension step at 72°C for 7 min. The resulting amplicons were purified using a QIA quick PCR purification kit (Qiagen) and sequenced using a BigDye Terminator version 3.1 cycle-sequencing kit and a 3730 DNA sequencer (Applied Biosystems, Foster City, CA).

Phylogenetic Analysis

All sequences were aligned using ClustalX version 1.8 (Thompson et al., 1997), and the sequence identities were calculated using MatGAT version 2.02 (Campanella et al., 2003). A phylogenetic tree was constructed by using the PHYLIP computer program package and the neighbor-joining method, with genetic distances

computed using Kimura's 2-parameter model (Felsenstein, 1993). Bootstrap analyses with 1,000 replicates were used to estimate the confidence of each tree node (Kimura, 1980).

Design of Species-Specific Primers and PCR-Based Identification

The PCR oligonucleotide primer sets specific for *L. casei*, *L. paracasei*, and *L. rhamnosus* were designed using VectorNTI version 9.0 (Invitrogen, Carlsbad, CA). All LCG strains and 8 nontarget strains were selected for species-specific PCR testing (Table 1). The thermal cycler protocol consisted of the following steps: initial strand denaturation at 94°C for 5 min; followed by 30 cycles of 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 7 min.

MALDI-TOF MS Analysis

A standard ethanol/formic acid extraction protocol was performed by following the method described by Huang et al. (2016). One microliter of protein extract was spotted on a 96-spot polished steel MALDI target plate. After the spots were dried at room temperature, they were overlaid with 1 µL of α-cyano-4-hydroxycinnamic acid matrix for sample/matrix cocrystallization. Mass data acquisition was performed using a MALDI-Microflex LT instrument spectrometer (Bruker Daltonics GmbH, Bremen, Germany) in positive linear mode (mass range: 2,000–20,000 Da). The bacterial test standard was used for instrument calibration. Moreover, the positive and negative controls were conducted using *Escherichia coli* BCRC 11634 and noninoculated matrix solution, respectively.

LCG IHDB Creation, Validation, and Dendrogram Construction

The raw spectra were acquired for each type strain, and the quality and relationships of each spectra were checked, screened, and estimated using FlexAnalysis software version 3.4 (Bruker Daltonics GmbH, Bremen, Germany) and a composite correlation index (CCI). The best quality spectra that remained were chosen and transferred to create a specific single main spectrum profile (MSP) for database expansion. The identities of all LCG reference strains and isolates were validated based on the newly created database. Results were analyzed following the scoring system according to the manufacturer's instructions. Samples with score values between 2.30 and 3.00 were considered to be reliably identified at the species level; samples with values

Figure 1. Phylogenetic tree of the *Lactobacillus casei* group strains based on their *mutL* gene sequences. The tree was constructed using the neighbor-joining method. Only bootstrap percentages above 80% are indicated (based on 1,000 replications). The scale bar represents 0.01% sequence divergence. BCRC = Bioresource Collection and Research Center at Food Industry Research and Development Institute (Hsinchu, Taiwan).

L.casei BCRC 10697	ACGTTAACGACGGCGACAGATTCTGGCATCGGCGCCAAGATTCACGTGAAGGGT GGCAAA spLcasei-mutlF→
L.rhamnosus BCRC 10940	ACGCTAACGACCGCTACCGATGCCGGGATTGGCGCTAAAATTCATGTCAAAGGC GGACAG spLrham-mutlF→
L.paracasei BCRC 12248	AAGTTGACAACGGCAACGGATTGAGGATTGGCACCCAGATTCACGTCAAAGGCGGTGAG
L.casei BCRC 10697	GTCGAGGATCAACTA CGGCGGCGCATCGCCGGGGCACGGATGTTGAAGTGCTGATCTG
L.rhamnosus BCRC 10940	GTAGAAAGTCAACGA CAGCGGCGCATCGTCGCGGAACCGATGTCGAAGTGAGCGATCTG
L.paracasei BCRC 12248	GTTGAAGCGCAGTCGACAGCGGCTACCGAGCGGTACGGATGTTGAAGTGAATGATTG
L.casei BCRC 10697	TTCTTCAATACACCAGCGCGCTGAAGTACATGAAGTCACAGCAGACCGAGCTTGGGAAA
L.rhamnosus BCRC 10940	TTTTTTAACTCCAGCAGCGCTGAAGTACATGAAGTCGCAACAGACCGAAGCTTGGCAAG
L.paracasei BCRC 12248	TTCTTCAACACACCGGCGCGCTGAAGTATATGAAGTCGACAGCAAACTGAACTGGGAAAA
L.casei BCRC 10697	ATTGTCGATATCGTTAGTCGGCTGGCCTTGGCTAATCC CAAGCATTGCTTTACGGTGTGG ←spLcasei-mutlR
L.rhamnosus BCRC 10940	ATTGTTGATATTGTCAGTCGGCTGGCATTAGCTAACC CTAAGATTGCGTTACGGTCAAG ←spLrham-mutlR
L.paracasei BCRC 12248	ATTGTGGATATCGTCAGCCGATTGGCGATCGCAATCCT GATATTGCTTACGGTTTCA spLpara-mutlF→
L.casei BCRC 10697	CACGATGGCAATATGGTCGTTGCGACTGCGGGTCAAGGCATCTGCGGCAGACGCTTGCG
L.rhamnosus BCRC 10940	CATGACGGCAATATGATGGTGGGACAGCCGGTCAAGGTGATCTACGCCAGACATTGGCG
L.paracasei BCRC 12248	CATGATGGCAATATGGTGGTGGCAGCAGCTGGTCAAGGTGATTGCGCCAGACGCTGGCC
L.casei BCRC 10697	GGAATCTATGGCTTGCCAGTAGCGCGGTCGATGGTTGACTTTCAGGCAGAGATCTTGAT
L.rhamnosus BCRC 10940	GGCATTACGGTTTGCCGGTTGCTCGGTCAATGGTCGACTTTCAGCCGAGGATCTTGAT
L.paracasei BCRC 12248	GGAATTTATGGCTTGCTGTGGCG CGCTCAATGGTTGATTTCAA AGCACAAAGATCTTGAT ←spLpara-mutlR

Figure 2. Multiple sequence alignment was performed on *mutL* sequences of *Lactobacillus casei* group members. Regions representing the sequences of species-specific primers are shaded. L. = *Lactobacillus*; BCRC = Bioresource Collection and Research Center at Food Industry Research and Development Institute (Hsinchu, Taiwan).

between 2.00 and 2.29 could be reliably identified at the genus and probably the species levels. In addition, cluster analysis was performed to construct the MSP dendrogram by using the standard settings of MALDI Biotyper 3.1 (Bruker Daltronics GmbH).

ClinProTools Statistical Analysis

The *L. paracasei* strains (including 2 subspecies) were further analyzed using ClinProTools software version 3.0. The imported raw spectra of each investigated strain were automatically recalibrated and normalized so that statistical analyses such as principal component analysis and the application of classification models could be performed. A 3-dimensional principal component analysis score plot was displayed, and a supervised neural network model was generated based on the discriminating peaks. In addition, the recognition capability and cross-validation of the model were calculated to demonstrate its reliability and accuracy.

Isolation and Identification of LCG-Related Strains from Probiotic Products

Several probiotic samples (yogurt drinks, probiotic powders and capsules) of different brands were collected. The LCG-related strains were isolated using serial dilution and plating methods and were identified by MALDI-TOF MS and species-specific PCR assays.

RESULTS AND DISCUSSION

Sequencing technology has continued to advance; genome-wide comparisons have been enabled by the use of next-generation sequencing and have been used for taxonomic assignments and applied phylogeny estimation within the LCG (Toh et al., 2013). However, whole-genome sequencing-based methods are still difficult to routinely use in general laboratories. In contrast, comparing the sequences of protein-encoding genes is an alternative method to whole-genome sequencing, and the use of these sequences as molecular markers in microbial classification and identification has increased. The *mutL* gene encodes a DNA mismatch repair protein that plays a critical role in maintaining genomic stability by correcting DNA replication errors (Li, 2008). The *mutL* gene has been used as a multilocus sequence typing target for the differentiation of *L. casei*, *L. paracasei*, and *L. rhamnosus* strains (Parolo et al., 2011; Bao et al., 2016; Poluektova et al., 2017). In this study, a degenerate primer pair (Lcagp-mutLF3/R4) was used to successfully amplify a PCR amplicon consisting of approximately 800 bp of the *mutL* gene

from LCG strains; this product was then used for sequencing. The average sequence identity of the *mutL* gene among the LCG type strains was 78.8%, which was significantly lower than that of the 16S rRNA gene (98.9%) and other common housekeeping genes such as *dnaK* (85.2%), *dnaJ* (83.2%), *hsp60* (88.4%), *rpoA* (95.1%), *tuf* (93.6%), and *pheS* (82.6%) and was comparable with that of other high-resolution DNA targets such as *yycH* (78.5%) and *recA* (74.0%; Felis et al., 2001; Naser et al., 2007; Huang and Lee, 2011; Yu et al., 2012; Huang et al., 2014, 2015). The *mutL* gene could therefore serve as a valuable taxonomic marker and contribute to the current multilocus sequence analysis scheme used to characterize the LCG. Nevertheless, *L. paracasei* subspecies strains could not be discriminated by these housekeeping genes but could be differentiated based on their phenotypic characteristics (Collins et al., 1989).

We found one strain (*Lactobacillus* sp. BCRC 81062) in which the *mutL* sequence had low similarity with those of the members of LCG (78.8–90.4%) but in which 16S rRNA gene similarity was very high (99.0–99.3%). The topology of the neighbor-joined *mutL* tree was clearly separated into clusters of different species, and the nodes were supported by high bootstrap values (>80%; Figure 1). The strain BCRC 81062 was found in a separate cluster close to *L. casei*. Furthermore,

Figure 3. Gel electrophoresis of PCR products amplified using species-specific primers. M: 100-bp DNA ladder markers. Lanes: (1) *Lactobacillus casei* BCRC 10697^T, (2) *Lactobacillus paracasei* ssp. *paracasei* 12248^T, (3) *Lactobacillus rhamnosus* BCRC 10940^T, and (4) negative control. BCRC = Bioresource Collection and Research Center at Food Industry Research and Development Institute (Hsinchu, Taiwan).

all *mutL* gene sequences were deposited in GenBank (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-13642>), and this accumulated sequence data could be used to develop specific PCR primers and directly identify particular LCG species (Huang et al., 2014). Three pairs of primers, each targeting a different species in the LCG, were designed based on multiple sequence alignments of the *mutL* gene (Figure 2), and these primers successfully generated a single species-specific band (186, 125, and 186 bp) when used in PCR reactions with DNA from reference strains of *L. casei*, *L. paracasei*, and *L. rhamnosus* DNA, respectively (Figure 3; Table 1). Only BCRC 81062 and other nontarget *Lactobacillus* strains failed to amplify a species-specific amplicon.

Commercial MALDI-TOF MS databases are currently largely developed for clinical diagnosis (Croxatto et al., 2012; Murray, 2012; Starostin et al., 2015; Angeletti, 2017). A case in which an LCG-related probiotic product was misidentified by interpreting MALDI-TOF MS results using a Bruker database has been reported (Angelakis et al., 2011). Database and spectrum quality are the key factors in determining the accuracy of MALDI-TOF MS identification. Hence, it is essential to use entries representing most phylogenetic taxa within a closely related species group. In this study, all type strains of the LCG were used to build an IHDB under optimized culture conditions (cultivated anaerobically on De Man, Rogosa and Sharpe agar for 20 h at 37°C). The proteomic patterns of each type strain

Figure 4. Representative spectra from 4 *Lactobacillus casei* group type strains in the in-house database supplemented by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry data. AU = arbitrary units; m/z = mass-to-charge ratio. BCRC = Bioresource Collection and Research Center at Food Industry Research and Development Institute (Hsinchu, Taiwan). Color version available online.

contained unique peaks (Figure 4) and might serve as potential biomarkers for further differentiation. A total of 48 replicates for each strain were performed, and the obtained spectra were analyzed and screened using FlexAnalysis software. To determine the relationships among replicates, the CCI indicators between spectra were calculated. High CCI values (near 1) indicated almost identical spectra (data not shown). At least 20 good-quality spectra of each strain were loaded into a database and used for MSP creation with MALDI Bio-typer software. We attempted to blindly reidentify each strain to determine the reproducibility of the IHDB. All LCG type strains that were already present in the IHDB were correctly identified, with scores ranging from 2.63 ± 0.02 to 2.65 ± 0.03 . For subsequent validation of the IHDB, 44 LCG isolates whose identities had been confirmed on the basis of their DNA sequences were used for evaluation. The results showed that most isolates were correctly identified to the species level with high score values (2.45 ± 0.1 ; Table 1) except for strain BCRC 81062, which was identified as *L. casei* with a relatively low score (2.02 ± 0.02).

Biomarkers in bacteria are mainly ribosomal proteins and include a few housekeeping proteins, which is comparable with the multilocus sequence analysis of

the housekeeping genes used to determine higher level taxonomy and phylogeny (Welker and Moore, 2011). In recent years, a ribosomal proteins typing-based method using MALDI-TOF MS has been proposed and successfully differentiated *Bacillus* spp., *Bifidobacterium animalis*, *Bifidobacterium longum*, *Lactococcus lactis*, and LCG (Tanigawa et al., 2010; Hotta et al., 2011; Sato et al., 2011, 2012; Ruiz-Moyano et al., 2012). The MSP spectra of all reference strains were used in the construction of a dendrogram and revealed unambiguous clustering of 5 main groups, each defined by species, subspecies, and a candidate novel taxon (Figure 5), which showed good concordance with the phylogenetic tree based on *mutL* gene sequences, except for the *L. paracasei* subspecies. To identify the potential biomarkers of *L. paracasei* subspecies, we performed a statistical analysis using ClinProTools software. The resulting distribution map clearly displayed that *L. paracasei* strains could be differentiated into 2 categories (Figure 6a), and a supervised neural network model was constructed based on 2 peaks (mass-to-charge ratio 4,930 and 5,303; Figure 6b); the model resulted in recognition capability and cross-validation values of 100%. The specificity of this model was tested ($n = 24$), and it demonstrated 100% accuracy (Table 2). However,

Figure 5. Dendrogram showing the clustering of the 49 *Lactobacillus casei* group strains based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. BCRC = Bioresource Collection and Research Center at Food Industry Research and Development Institute (Hsinchu, Taiwan). MSP = main spectrum profile. Color version available online.

Table 2. Classification results of the subspecies of *Lactobacillus paracasei* strains using statistical analysis in ClinProTools software (Bruker Daltonics, Billerica, MA)

<i>L. paracasei</i> subspecies	Class	Discriminatory peaks (<i>m/z</i>) ¹	
		4,930	5,303
<i>paracasei</i> (no. = 22)	1	—	+
<i>tolerans</i> (no. = 2)	2	+	—

¹Biomarker peaks were screened by the supervised neural network model. *m/z* = mass-to-charge ratio. + = peak present; — = no peak found.

these 2 peaks could not be identified due to the low abundance and instrumental limitations. Subsequently, 7 strains isolated from different probiotic samples that

claimed to contain viable LCG-related species were analyzed using MALDI-TOF MS plus ClinProTools and species-specific PCR. These lactic acid bacteria were identified as *L. casei*, *L. paracasei* ssp. *paracasei*, and *L. rhamnosus*, respectively (Table 3). However, we found a discrepancy between the labeled species and the actual identified result in probiotic capsule products.

The current method for the confirmation of closely related prokaryotic taxa involves 2 steps: 16S rRNA sequencing is first used to assign an unknown strain at the species or species-group level, which then defines the genes and primers to be used to assign the strain to the species or subspecies level (Gevers et al., 2005). A 2-tier strategy has been successfully applied to distinguish many phylogenetically closely related bacteria,

Figure 6. ClinProTools (Billerica, MA) statistical analysis of *Lactobacillus paracasei* subspecies strains. (a) Two-dimensional peak distribution diagrams. Red and green denote class 1 and class 2 *L. paracasei*, respectively. (b) The mass peaks at mass-to-charge ratio (*m/z*) 4930 and 5303 in *L. paracasei* ssp. *tolerans* and *L. paracasei* ssp. *paracasei* strains, respectively. Color version available online.

Table 3. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and species-specific PCR identification of *Lactobacillus casei* group-related strains in commercial probiotic products

Probiotic product	Label species	MALDI-TOF MS	Species-specific PCR ¹		
			Lcas ²	Lparacas ³	Lrham ⁴
Yogurt drink 1	<i>Lactobacillus paracasei</i>	<i>L. paracasei</i> ssp. <i>paracasei</i>	—	+	—
Yogurt drink 2	<i>L. paracasei</i>	<i>L. paracasei</i> ssp. <i>paracasei</i>	—	+	—
Probiotic powder 1	<i>Lactobacillus casei</i>	<i>L. casei</i>	+	—	—
Probiotic powder 2	<i>L. casei</i>	<i>L. casei</i>	+	—	—
Probiotic powder 3	<i>Lactobacillus rhamnosus</i>	<i>L. rhamnosus</i>	—	—	+
Probiotic powder 4	<i>L. rhamnosus</i>	<i>L. rhamnosus</i>	—	—	+
Probiotic capsule	<i>L. casei</i> ⁵	<i>L. rhamnosus</i>	—	—	+

¹+ = PCR amplicons with each primer pair detected; — = PCR amplicons with each primer pair not detected.

²Species-specific PCR amplification with primer spLcasei-mutIF (5'-GGCAAAGTCGAGGATCAAACCTA-3')/-R (5'-CGACACCGTAAAAGCAATGCTG-3').

³Species-specific PCR amplification with primer spLpara-mutIF (5'-GATATTGCCTTCACGGTTTCAC-3')/-R (5'-TTGAARTCAACCATTGAGCG-3').

⁴Species-specific PCR amplification with primer spLrham-mutIF (5'-GGRCAGGTAGAAAGTCAAACGA-3')/-R (5'-GCTGACCGTAAACGCAATCTTAG-3').

⁵Discordance between the labeled and identified strains.

such as members of the *Lactobacillus acidophilus* group, the *L. casei* group, the *Lactobacillus plantarum* group, and the *Lactobacillus sakei* group (Naser et al., 2007; Huang et al., 2010, 2012, 2014). The MALDI-TOF MS ClinProTools method provides higher resolution (down to the subspecies level), faster analysis (2 h after the culture), and lower reagent costs (around \$0.20/sample) than *mutL* gene sequencing and species-specific PCR assays. Consequently, the MALDI-TOF MS ClinProTools method developed in this study is preferable.

CONCLUSIONS

Our data confirm that proteomic identification within the LCG, based on combining the use of an IHDB and the application of ClinProTools to MALDI-TOF MS data, showed superior discrimination power and required less analysis time than DNA sequencing and PCR-based methods did and can be applied to achieve efficient quality control of probiotic products. According to the preliminary sequence- and proteomics-based data, we deduced that BCRC 81062 may represent an undescribed new taxon, and we will integrate a polyphasic taxonomic strategy to describe novel species in the future.

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