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A proteomic approach for rapid identification of *Weissella* species isolated from Korean fermented foods on MALDI-TOF MS supplemented with an in-house database

Eiseul Kim [a,1], Youngjae Cho [a,1], Yoonju Lee [a], Sun-Kyung Han [a], Chang-Gyeom Kim [b], Dong-Won Choo [b], Young-Rok Kim [a], Hae-Yeong Kim [a,*]

[a] Institute of Life Sciences & Resources and Department of Food Science and Biotechnology, Kyung Hee University, Yongin 17104, Republic of Korea
[b] Department of Bioinformatics and Biosystems, Korea Polytechnics, Seongnam 13122, Republic of Korea

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ABSTRACT

Weissella are obligate heterofermentative lactic acid bacteria belonging to the *Leuconostocaceae* family. Some *Weissella* can be found in salted and fermented foods, such as kimchi and jeotgal, and plays an important role in the fermentation process. In the present study, for the first time, a rapid and accurate identification method for *Weissella* species from kimchi and jeotgal was developed based on MALDI-TOF MS, supplemented with an in-house database. Of the 135 *Weissella* spectra aligned with the MALDI bioTyper database, 56 isolates (41.5%) yielded no reliable identification results with low log scores (<1.7). After registering the spectra of six *Weissella* reference strains, all of the isolates were correctly identified, of which 113 (83.7%) and 22 (16.3%) were identified at the species and genus level, respectively. Moreover, a dendrogram generated by protein profiles of the different *Weissella* species clearly presented distinctive clusters, and PCA analysis separated the spectra of *Weissella* species into four clusters. In comparing food origins, different *Weissella* species were identified from two fermented foods. *W. soli* and *W. cibaria* were isolated from kimchi, while *W. thailandensis* and *W. halotolerans* were isolated from jeotgal. The results of our proteomic approach confirm that the MALDI bioTyper database, with our in-house *Weissella* database, is sufficient for *Weissella* identification. The MALDI-TOF MS method provides fast and reliable discrimination between different species in the genus *Weissella* and, therefore, will be useful for safety control in fish farms or in the production of fermented foods. This method can also be applied to the control of opportunistic pathogenic *Weissella* in human clinical infections.

1. Introduction

Weissella is a genus of heterofermentative bacteria that produces lactic acid (lactic acid enantiomers in some species) as the major end product of glucose fermentation, CO_2, ethanol and/or acetate, and belongs to the *Leuconostocaceae* family. This genus is comprised of 19 species at the time of this writing: *W. beninensis*, *W. ceti*, *W. cibaria*, *W. confusa*, *W. diestrammenae*, *W. fabalis*, *W. fabaria*, *W. ghanensis*, *W. halotolerans*, *W. hellinica*, *W. kandleri*, *W. koreensis*, *W. minor*, *W. oryzae*, *W. paramesenteroides*, *W. soli*, *W. thailandensis*, *W. uvarum* and *W. viridescens*. Five species were reclassified from the genus *Lactobacillus*, including *W. confusa*, *W. halotolerans*, *W. kandleri*, *W. minor* and *W. viridescens*, and one from *Leuconostoc*, *W. paramesenteroides* (Collins et al., 1993; Fusco et al., 2015).

Some *Weissella* species have health-detrimental roles in human clinical infections, while others play potentially beneficial roles in food preservation and key functions in food fermentation. Regarding its detrimental effects, two species of the genus *Weissella* were reported as opportunistic pathogens associated with human clinical infections. *W. confusa* has been isolated from many clinical specimens of human polymicrobial infection, although its clinical significance has not yet been proven (Bantar et al., 1991; Olano et al., 2001; Riebel and Washington, 1990). *W. viridescens* was isolated from the blood of a patient (Kulwichit et al., 2007) and through fecal DNA from celiac children (Sanz et al., 2007). In addition, *W. ceti* was recently reported to be a causative agent of weissellosis in rainbow trout, a disease with a high mortality rate (Snyder et al., 2015). Outbreaks of this disease have rapidly emerged among farmed (rainbow) trout in many countries (Costa et al., 2015; Liu et al., 2009; Welch and Good, 2013). Regarding its

* Corresponding author.
E-mail address: hykim@khu.ac.kr (H.-Y. Kim).
[1] Both authors contributed equally to this manuscript.

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health-beneficial effects, some *Weissella* species were reported to produce six bacteriocins, including weissellin A (*W. paramesenteroides*), weissellicin 110 (*W. cibaria*), and weissellicin D, L., M, and Y (*W. hellenica*) (Chen et al., 2014a; Leong et al., 2013; Masuda et al., 2012; Papagianni and Papamichael, 2011; Srionnual et al., 2007). Chen et al. (2014a, 2014b) reported that bacteriocinogenic *W. hellenica* strain D1501 extended the shelf-life of tofu. Previous studies demonstrated that most *Weissella* species can be isolated from various types of fermented foods (Fusco et al., 2015). Among them, *W. cibaria* and *W. koreensis* were reported as major bacteria potentially associated with kimchi fermentation (Choi et al., 2002; Kim and Chun, 2005). The *W. halotolerans* strain KNOUC4036 was previously isolated from Korean fermented jeotgal (Nam and Ahn, 2015).

A range of genomic-based identification and typing methods for *Weissella* species have been widely used to distinguish genera and species based on their characteristic differences. Most methods use DNA-DNA hybridization (De Vuyst et al., 2002; Nisiotou et al., 2014; Scheirlinck et al., 2007), pyrosequencing (Jeong et al., 2013; Roh et al., 2010; Wouters et al., 2013), polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) (Cocolin et al., 2009; Kim et al., 2009; Wouters et al., 2013), repetitive sequence-based (REP) PCR (Scheirlinck et al., 2007), restriction fragment length polymorphism (RFLP) (Chao et al., 2009; Chen et al., 2012; Lan et al., 2009) and 16S rRNA sequencing (Lee et al., 2002; Osimani et al., 2015; Urso et al., 2006). However, the above techniques are laborious and time-consuming, and require highly trained molecular biologists. As a result, the present study aimed to establish a method for MALDI-TOF MS-based identification of *Weissella* species isolated from two Korean fermented foods, kimchi and jeotgal. Kimchi is a traditional food fermented from Chinese cabbage, and the most well-known food associated with *Weissella* species. Jeotgal is a salted and fermented Korean seafood. To the best of our knowledge, no previous studies have taken a proteomic approach to identifying *Weissella* species from kimchi or jeotgal using MALDI-TOF MS, which can be available in a wide range of food industry.

This study was therefore undertaken (1) to evaluate the ability of the MALDI-TOF MS platform in a proteomic approach to identify *Weissella* species isolated from Korean salted and fermented foods based on their different patterns of TOF peaks and (2) to investigate the benefits of extending the MALDI bioTyper database with a local database for identifying *Weissella* species.

2. Materials and methods

2.1. Weissella strains

The bacteria in this study included six reference stains obtained from culture centers and 135 isolates obtained from kimchi and jeotgal. The culture centers were the Korean agricultural culture collection (KACC, Jeonju, Korea) and the Korean Collection for Type Cultures (KCTC, Daejeon, Korea). Our previous unpublished study aimed to investigate the microbial community in kimchi and jeotgal using 16S rRNA sequencing (data not shown), and 135 isolates from two fermented foods were identified as *Weissella* species. These isolates were used for establishing a reliable method of MALDI-TOF MS-based *Weissella* identification in this study.

2.2. Sampling

Kimchi and jeotgal were obtained from a Korean traditional market to access naturally occurring microflora through the fermentation process. A 10 g sample of kimchi or jeotgal was added to 90 mL of sterile water in stomacher filter bags (Seward Limited, London, UK), and the mixture was homogenized at 230 rpm for 1.5 min. The homogenized samples were serially diluted and spread onto MRS agar plates (Difco, Becton & Dickinson, Sparks, MD, USA). The plates were then incubated anaerobically at 30 °C for 48 h for DNA extraction.

2.3. 16S rRNA sequencing

Colonies were selected on the basis of different morphologies, and their total DNAs were extracted using a bacterial genomic DNA extraction kit (Intron biotechnology, Seongnam, Korea) according to the manufacturer's instructions. Briefly, the 16S rRNA gene was amplified and sequenced using the 16S rRNA-specific primers described by Hong et al., 2014. The resulting sequences were compared with the sequences from the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST).

2.4. Generation of a local database with reference strains

2.4.1. Reference strain preparation for MALDI-TOF MS

Six reference strains were incubated anaerobically at 30 °C for 48 h, and their proteins were extracted using a formic acid extraction procedure, which was provided by the company. In brief, a colony of bacteria scraped from the agar was suspended in 300 μL of sterile distilled water, followed by the addition of 900 μL ethanol. The bacterial suspension was centrifuged at 13,000 rpm for 10 min to remove the supernatant. The pellet was dried at ambient temperature and re-suspended in the mixture of 25 μL of 70% formic acid and 25 μL of acetonitrile. After another centrifugation at same condition to discard the pellet, the supernatant was carefully transferred into a clean tube and subjected to MALDI-TOF typing.

2.4.2. Typing with MALDI-TOF MS

The typing was also carried out according to the manufacturer's instructions. Briefly, the supernatant of each reference strain was deposited directly onto eight spots on a steel target plate (Bruker Daltonics, Bremen, Germany), followed by drying at ambient temperature. Dried supernatant was overlaid with 1 μL of HCCA matrix solution, followed by crystallizing at ambient temperature. The measurements were conducted using a microflex LT bench-top mass spectrometer (Bruker Daltonics) under the control of FlexControl 3.0 software at 240 laser shot steps. Mass spectra were collected within a mass range from 2000 to 20,000 Da and calibrated with the bacterial test standard (BTS; Bruker Daltonics) using FlexAnalysis 3.4 software (Bruker Daltonics). Finally, accurate spectra for each of the reference strains were uploaded into the Biotyper 3.0 software to create a single mean spectrum using the master spectra library creation method from the Biotyper software.

2.5. Sample identification for MALDI-TOF MS

Preparation of bacterial isolates for MALDI-TOF MS was carried out using an extended direct transfer extraction procedure, according to the manufacturer's instruction. In brief, a single colony of each isolate was deposited directly on a steel MSP96 target plate and subsequently overlaid with 1 μL of 70% formic acid. All spots were dried at ambient temperature before being overlaid with 1 μL of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution in acetonitrile:-water:trifluoroacetic acid (50:47.5:2.5 [v/v]). After the mixture was air-dried at ambient temperature, the plate was immediately applied to MALDI-TOF MS. The measurements were performed using a Microflex LT bench-top mass spectrometer with FlexControl 3.0 software. The procedures were as mentioned in the typing of reference strains.

2.6. MALDI-TOF MS sample data interpretation

The generated mass spectrum of each sample was compared to reference mass spectra present in the MALDI bioTyper database containing 5627 reference spectra. The software calculated integrated pattern-matching algorithms and recoded the data as logarithmic between 0 and 3.0. As specified by the manufacturer, log scores ≥ 2.0 were accepted