Journal of Microbiological Methods 177 (2020) 106045

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

Multi fragment melting analysis system (MFMAS) for one-step identification of lactobacilli

Zülal Kesmen[a,*], Özge Kılıç[a], Yasin Gormez[b], Mete Çelik[c], Burcu Bakir-Gungor[d]

[a] *Erciyes University, Engineering Faculty, Department of Food Engineering, Kayseri, Turkey*
[b] *Cumhuriyet University, Faculty of Economic and Administrative Sciences, Sivas, Turkey*
[c] *Erciyes University, Engineering Faculty, Department of Computer Engineering, Kayseri, Turkey*
[d] *Abdullah Gul University, Department of Computer Engineering, Faculty of Engineering, Kayseri, Turkey*

ARTICLE INFO

Keywords:
Multi-fragment melting analysis system (MFMAS)
High resolution melting (HRM)
Lactobacilli
One-step identification
Machine learning
Logistic regression (LR)

ABSTRACT

The accurate identification of lactobacilli is essential for the effective management of industrial practices associated with lactobacilli strains, such as the production of fermented foods or probiotic supplements. For this reason, in this study, we proposed the Multi Fragment Melting Analysis System (MFMAS)-lactobacilli based on high resolution melting (HRM) analysis of multiple DNA regions that have high interspecies heterogeneity for fast and reliable identification and characterization of lactobacilli. The MFMAS-lactobacilli is a new and customized version of the MFMAS, which was developed by our research group. MFMAS-lactobacilli is a combined system that consists of *i)* a ready-to-use plate, which is designed for multiple HRM analysis, and *ii)* a data analysis software, which is used to characterize lactobacilli species via incorporating machine learning techniques. Simultaneous HRM analysis of multiple DNA fragments yields a fingerprint for each tested strain and the identification is performed by comparing the fingerprints of unknown strains with those of known lactobacilli species registered in the MFMAS. In this study, a total of 254 isolates, which were recovered from fermented foods and probiotic supplements, were subjected to MFMAS analysis, and the results were confirmed by a combination of different molecular techniques. All of the analyzed isolates were exactly differentiated and accurately identified by applying the single-step procedure of MFMAS, and it was determined that all of the tested isolates belonged to 18 different lactobacilli species. The individual analysis of each target DNA region provided identification with an accuracy range from 59% to 90% for all tested isolates. However, when each target DNA region was analyzed simultaneously, perfect discrimination and 100% accurate identification were obtained even in closely related species. As a result, it was concluded that MFMAS-lactobacilli is a multi-purpose method that can be used to differentiate, classify, and identify lactobacilli species. Hence, our proposed system could be a potential alternative to overcome the inconsistencies and difficulties of the current methods.

1. Introduction

Lactobacilli are an important bacterial group that contains several species widely used in the fermentation industry and animal/human nutrition, due to their technological or functional activities (Bernardeau et al., 2006; Giraffa et al., 2010; Altay et al., 2013). The term “lactobacilli” refers to all organisms that were classified in the genus *Lactobacillus* until the taxonomic re-evaluation of the genus in 2020 (Zheng et al., 2020). Several lactobacilli species have an essential role in the production of fermented foods, and some of them are used as starter cultures to improve the organoleptic properties of these products (Leroy and De Vuyst, 2004; Settanni and Moschetti, 2010; dos Santos Cruxen et al., 2019). Their antimicrobial metabolites, such as bacteriocins, can promote food safety (Omar et al., 2008; Kumariya et al., 2019). Several species in the genera *Lacticaseibacillus, Lactiplantibacillus, Ligilactobacillus*, and *Limosilactobacillus* are considered as probiotics that provide beneficial health effects by colonizing in human and animal intestines (Herbel et al., 2013; Ramos et al., 2013; Ren et al., 2014; Kumar and Kumar, 2015). Therefore, species-level identification of lactobacilli is important to understand biochemical changes associated with the fermentation process, to detect potential starter cultures in traditional fermented foods, and to monitor the viability of probiotic bacteria during the storage time of probiotic products (Turková et al., 2012; Fusco et al., 2016).

A variety of methods have been used for the identification and characterization of lactobacilli isolated from foods and other microbial

---

* Corresponding author.
E-mail address: zkesmen@erciyes.edu.tr (Z. Kesmen).

https://doi.org/10.1016/j.mimet.2020.106045
Received 2 September 2019; Received in revised form 18 August 2020; Accepted 19 August 2020

ecosystems (Wang et al., 2008; Pot and Tsakalidou, 2009). In recent decades, genotypic methods have become widely used and have increasingly replaced the phenotypic and biochemical methods that suffer from poor reliability and long processing times (Ehrmann and Vogel, 2005; Singh et al., 2009; Carafa et al., 2015). DNA fingerprinting techniques, e.g., PCR amplification of repetitive bacterial DNA elements (rep-PCR) (Gevers et al., 2001; Mohammed et al., 2009), randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) (Andrighetto et al., 2001), pulsed-field gel electrophoresis (PFGE) (Bouton et al., 2002), and amplified fragment length polymorphism (AFLP) (Torriani et al., 2001a) are reliable tools for classifying and typing a large number of isolates, but they often exhibit low reproducibility and require laborious post-PCR handling steps (Foxman et al., 2005; Adzitey et al., 2013; Tamburro and Ripabelli, 2017). Sequence analysis of complete or partial ribosomal RNA genes have been successfully used for the identification of lactobacilli but it is often difficult to identify closely related species based on sequencing a single gene (Collins et al., 1991; Huang et al., 2010). Other genotypic approaches, such as species-specific PCR, restriction fragment length polymorphism (RFLP) analysis, and ribotyping, which have varying degrees of discriminatory power, have also been employed for the identification and differentiation of lactobacilli (Tynkkynen et al., 1999; Rodas et al., 2005; Blaiotta et al., 2008). However, none of the current methods are standardized, and these are often characterized as time-consuming and laborious methods. Moreover, in most cases, a combination of these methods is required to obtain the correct results (Turková et al., 2012).

Recently, the post-PCR melting curve analysis has become a popular tool for microbial detection (Tamburro and Ripabelli, 2017). The melting curve analysis is based on the melting properties of a double-strand DNA fragment that varies with increasing temperature. This technology uses real-time PCR instrumentation and does not require laborious post-PCR procedures for the characterization of amplicons (Tong and Giffard, 2012; Lin and Gänzle, 2014; Erdem et al., 2016). HRM analysis is an advanced version of the melting curve analysis. The HRM technique provides higher discriminatory power because it employs third generation dsDNA binding fluorescent dyes that do not inhibit PCR amplification, even at saturating concentrations (Reed and Wittwer, 2004; Druml and Cichna-Markl, 2014). HRM analysis has been employed for the specific discrimination of species within different microbial communities including lactobacilli, acetic acid bacteria, and yeasts (Szén et al., 2014; Iacumin et al., 2015; Yetiman and Kesmen, 2015; Kesmen et al., 2018a).

The recently developed multi-fragment melting analysis system (MFMAS) is a novel identification technique based on HRM analysis of multiple DNA regions. MFMAS was firstly employed to identify yeast species isolated from foods, and very promising results have been obtained (Kesmen et al., 2018b). This system assumes that, as the number of target DNA regions that have high inter-species heterogeneity in HRM analysis increases, the discrimination ability of the method increases. The MFMAS is a combined system that consists of a ready-to-use plate, a systems-integrated software program, and trained models that are generated from the melting curve data of the known species for each target DNA region. In this system, multiple DNA fragments of a microbial isolate to be identified are amplified simultaneously in a separate well on a single plate, and then HRM analysis is applied to each amplification product. When the HRM data obtained for the target DNA regions of each microbial isolate are evaluated simultaneously, a fingerprint is obtained for each one of the isolates and the identification is performed by comparing the HRM fingerprints of unknown isolates with those of known species. MFMAS is a one-step identification system that has high discriminatory power and is suitable for rapid and practical analysis of large numbers of microbial isolates. MFMAS-lactobacilli is a new version of MFMAS, which was specifically customized for species-level identification of lactobacilli.

The current methods used in the identification of lactobacilli are problematic, especially for the discrimination of closely related species. It is considered that the MFMAS-lactobacilli has a potential to meet the need for a fast and reliable method to be used in species-level identification of lactobacilli. Therefore, in this study, we aimed to assess the success and applicability of MFMAS-lactobacilli for the genotypic differentiation and identification of lactobacilli isolated from food and probiotic supplements.

2. Material and methods

In this study, MFMAS was applied to identify lactobacilli species (i.e. species that were classified in the genus *Lactobacillus* until April 2020), isolated from various fermented food products, probiotic supplements, and reference strains. The MFMAS-based identification was confirmed through rep-PCR fingerprinting analysis, followed by sequencing of different DNA regions.

2.1. Sampling procedures

A total of 23 food samples including pickle ($N = 4$), regional cheese ($N = 3$), sucuk (Turkish fermented sausage) produced without starter cultures (N = 3), home-made yoghurt ($N = 2$), sourdough (N = 3), kefir (N = 3), and probiotic supplements ($N = 5$) were collected from local markets in Kayseri, Turkey. Ten g of each sample was homogenized with 90 ml of Maximum Recovery Diluent (MRD, Merck) for 2 min using a stomacher machine (Masticator Basic, IUL Instruments, Spain). Serial dilutions were prepared in MRD, and the appropriate dilutions were plated onto Rogosa agar (Merck, Germany). The plates were incubated at 30 °C for 48 h in anaerobic jars with CO_2-generating kits (Anaerocult C, GasPak Merck). About 10–15 morphologically different colonies were randomly selected from the Rogosa agar plates for isolation, purification, and subsequent characterization. The purities and cell morphologies of the isolates were examined microscopically and Gram-stain and catalase test were performed. The pure isolates were stored as stock cultures at −80 °C in MRS medium containing 20% glycerol.

2.2. Reference strains

The reference strains were obtained from the American Type Culture Collection (ATCC), the Agricultural Research Service Culture Collection (NRRL), Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ) and Erciyes University Culture Collection (EUCC). The strains, *Lactobacillus* (*L.*) *acidophilus* NRRL B-23431, *L. acidophilus* NRRL B-4495, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 12315, *L. delbrueckii* subsp. *bulgaricus* EUCC-07BL5, *Lactobacillus gasseri* EUCC-07G1, *Lactobacillus helveticus* NRRL B-734, *Lactiplantibacillus* (*Lacp.*) *plantarum* (formerly L. *plantarum*) ATCC-8014, *Lacp. plantarum* DSMZ 20174, *Levilactobacillus* (*Lev.*) *brevis* (formerly L. *brevis*) EUCC-07B7, *Lacticaseibacillus* (*Lact.*) *casei* (formerly L. *casei*) DSMZ 20011, *Lact. casei* NRRL B-1922, *Lacticaseibacillus paracasei* (formerly L. *paracasei*) EUCC-07CA9, *Lacticaseibacillus rhamnosus* (formerly L. *rhamnosus*) ATCC 11982, *Limosilactobacillus* (*Lim.*) *fermentum* (formerly L. *fermentum*) ATCC 14931, *Lim. fermentum* NRRL B-1932, *Limosilactobacillus reuteri* (formerly L. *reuteri*) NRRL B-14172, *Lentilactobacillus* (*Lent.*) *buchneri* (formerly L. *buchneri*) NRRL B-1860, *Lentilactobacillus hilgardii* (formerly L. *hilgardii*) NRRL B-1127, *Lentilactobacillus kefiri* (formerly L. *kefiri*), *Latilactobacillus* (*Lat.*) *curvatus* (formerly L. *curvatus*) NRRL B-4562, *Latilactobacillus sakei* (formerly L. *sakei*) EUCC-07S7, NRRL B-1839, *Companilactobacillus* (*C.*) *farciminis* (formerly L. *farciminis*) EUCC-07FA3, *Companilactobacillus heilongjiangensis* (formerly L. *heilongjiangensis*) EUCC-07HJ3 (Zheng et al., 2020) were used as reference strains. All reference strains were cultured in MRS broth at 30 °C under microaerophilic atmosphere conditions before DNA extraction.

2.3. DNA extraction

The genomic DNA was isolated from overnight cultures in MRS broth according to the method described by Gevers et al. (2001). The DNA extraction was replicated three times for each isolate and reference strain. The concentration of DNA was measured with a micro-volume UV–Vis spectrophotometer (Quawell 5000, CA, USA) and adjusted to 100 ng/µl with TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA) to use in subsequent steps.

2.4. MFMAS-based identification of lactobacilli strains

2.4.1. Loading of bacterial DNA into the MFMAS plate

MFMAS analysis based on simultaneous HRM analysis of 12 different DNA regions of each bacterial isolate was performed on a 12 × 8 well plate. The ready-to-use MFMAS plate contained the reaction components required for the amplification and the subsequent melting analysis steps of the target DNA fragments. The reaction mixture consisted of 10 µl commercial real-time PCR master mix with ResoLight HRM dye (Roche Applied Science, Germany), 20 pmol each of primers, 2.5 mmol l $- ^{1}$ MgCl$_2$, in a total volume of 20 µl. The first row of the plate was dedicated to negative control analysis; and hence, 1 µl DNase free water was loaded into all wells in the first row (row A). The DNA of the *Lacp. plantarum* strain ATCC-8014 was used as a positive control, and it was loaded into all wells in the second row (row B) of the plate. The 3rd-8th rows (rows C—H) were used for the analysis of bacterial isolates and 50 ng/µl genomic DNA of each isolate was loaded into all wells in an individual row of the plate. MFMAS plate assays were replicated three or more times for all isolates and reference strains.

2.4.2. MFMAS reaction conditions

After the bacterial DNAs were loaded into the wells, the plate was placed in the real-time PCR device (light cycler 480, Roche, Germany), and a common temperature program was applied for all target DNA fragments. The amplification process was performed with the following steps: an initial denaturation of DNA at 94 °C for 5 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 40 s, extension at 72 °C for 50 s, and a final extension at 72 °C for 10 min. The melting curve program was started immediately after the amplification reaction was completed. The amplification products were exposed to heating from 60 °C to 95 °C with 0.03 °C/s increments, and fluorescence accumulation was measured automatically in each step. All fluorescence accumulation data was subsequently converted to numerical data by the melting curve program and exported to process with the MFMAS software.

2.4.3. MFMAS software

MFMAS was equipped with MFMAS software that analyzes the melting curve data obtained from target DNA fragments of each analyzed strain in comparison to the melting curve data of known bacterial species. MFMAS software, which was developed in the python programming language, employs a robust machine learning algorithm, namely logistic regression (LR) to learn from the melting curves of the known bacterial species and to generate models. Then, these models were used to predict the species of the analyzed strains. As shown in Fig. 1, this system had two phases, namely the training phase and the prediction phase. In the training phase, 12 matrices, which correspond to the melting curve data of 12 target DNA regions of known species, were entered into the system as an input file. In these matrices, while the rows correspond to different species, the columns correspond to the melting curve data (fluorescence accumulation at different temperatures) of each species. Hence, the parameters of the LR were estimated using this data. At the end of the training phase, 12 models corresponding to the 12 target DNA regions were generated.

In the prediction phase, using these trained models, the species of the analyzed strains were predicted. The strength of our method stems from its ability to combine information from different target DNA regions. In order to make use of the information hidden in each target DNA region, via employing a soft voting technique from the machine learning domain, an ensemble method was developed. In this regard, firstly, the melting curve data from all of the target DNA regions for each analyzed strain was sent to the system as an input. Secondly, for each analyzed test sample, using the melting curve data of unknown strain for an individual target DNA region, the probabilities of belonging to known species were calculated. In the generated probability vector, each cell of the vector represented the probability of belonging to a known species based on the melting curve data of this particular target DNA region. This step was repeated for all target DNA regions. Thirdly, for each known species, via taking the averages of the 12 probabilities calculated for the test sample for 12 different target DNA regions, a combined probability (in terms of target DNA regions) was computed. In other words, in this step, the probabilities of belonging to each one of the known species were calculated for the analyzed strain (test sample). Lastly, by comparing these probabilities, the most probable species for the analyzed strain was predicted. Hence, at the end of the prediction phase, the tested unknown strain was assigned to a species of lactobacilli with the highest prediction probability.

It is worthwhile to note that once the training phase was completed, the system could be run in the prediction phase several times without a need to rerun of the training phase. However, once a new melting curve data of a known species was collected, the trained models could be updated by repeating the training phase. In this way, the system could adapt to new data and stay up to date.

Additionally, when the HRM data acquired for 12 target DNA regions of each microbial isolate were evaluated simultaneously, a fingerprint was obtained for each of the isolates. The numerical data, which was used in the construction of the fingerprint patterns, were subjected to cluster analysis using the unweighted pair group method with the arithmetic averages (UPGMA) algorithm to examine the phylogenetic relationships among the analyzed strains.

2.5. Confirmation of MFMAS-based identification of lactobacilli isolates

The preliminary grouping of isolates using PCR-based fingerprinting analysis, and the subsequent sequencing of representative strains, is one of the most common and reliable methods for the identification of a large number of bacterial isolates. Therefore, in this study, this approach was utilized to confirm the MFMAS-based identification of

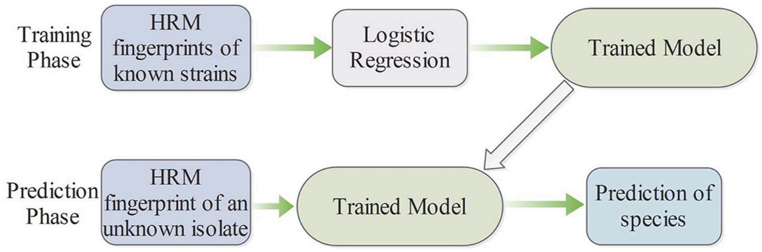

Fig. 1. Schematic representation of MFMAS software.

lactobacilli isolates.

2.5.1. Rep-PCR clustering of lactobacilli isolates

(GTG)5–PCR fingerprinting was used as a genotypic tool for the clustering of the strains before sequence analysis (Gevers et al., 2001). Amplification reactions were carried out in a total volume of 25 µl in the presence of 2 U of Taq DNA polymerase (Ampliqon, Denmark), using a thermal cycler (Techne, TC-5000, ABD). Subsequently, the PCR products were separated on a 1.5% (wt/vol) agarose gel, containing 0.5 µg/ml ethidium bromide in 1× TAE buffer at 50 V. (GTG)5- PCR fingerprints were visualized under UV light (Gel-Doc XR, Bio-Rad Laboratories, Hercules, Calf. ABD) and then analyzed using the BioNumerics software (version 7.5, Applied Maths). The similarities between the fingerprint patterns were calculated using Pearson correlation, and cluster analysis was performed using an unweighted-pair group method with arithmetic averages (UPGMA) algorithm.

2.5.2. Sequence analysis

At least 3 representative isolates were randomly selected using a random number generator (http://www.random.org) from each (GTG) 5-PCR fingerprint cluster and subjected to sequence analysis for three target DNA regions, including the full length of a 16S rRNA gene, 16S−23S rRNA intergenic spacer region (ISR), and *dnaK* gene. The amplification of the 16S rRNA gene was performed using the primers, designed by Kesmen et al. (2012). The ISR was amplified with the primers 16 (5′-GCTGGATCACCTCCTTTC-3′) (Berthier and Ehrlich, 1998) and 23-10C (5′-CCTTTCCCTCACGGTACTG-3′) (Gürtler and Stanisich, 1996). An approximately 1060 bp fragment of the *dnaK* gene was amplified with the primers DNaK1f (CCATTGGMCGTTCACCYTG) and DNaK2r (ATYAAGMGYCACATGGGTGA), designed in this study. All amplification reactions were carried out by applying the annealing temperature at 50 °C (for the *dnaK* gene), 55 °C (for a 16S rRNA gene), and 60 °C (for an ISR region). The PCR products were sequenced (MedSanTek, Turkey) and aligned with the sequences in the NCBI database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

3. Results

In this study, a new version of the MFMAS, MFMAS-lactobacilli was applied to a total of 254 isolates recovered from 23 different samples consisting of fermented food and probiotic supplements. A total of 18 different lactobacilli species were identified (Table 1). The most common species was *Lacp. plantarum,* which represented 25.6% (65 isolates) of all isolates. It was found at levels ranging from 1.9% to 10.5% in all samples except yoghurt. The second most common species was *Lev. brevis*, which represented 18.5% (47 isolates) of all isolates. *Lacp. plantarum* and *Lev. brevis* were codominant species in cheese samples and were followed by *Lact. rhamnosus, Lact. casei,* and *Lact. paracasei*. Additionally, *L. delbrueckii* subsp. *bulgaricus* (percentage in all isolates 7.9%), and *Lent. kefiri* (percentage in all isolates 3.9%) were defined in yoghurt and kefir, respectively as characteristic dominant species. Seven different lactobacilli species were identified in fermented sausage in which *Lat. sakei* (percentage in all isolates 4.3%) was the dominant species and followed by *Lev. brevis*. Among the five different species of lactobacilli identified in sourdough samples, the first most dominant species was *Lat. curvatus* and the second was *Lim. fermentum. Lacp. plantarum* was predominantly isolated in the bacterial population of pickle samples, as expected. Among all identified isolates, *Lent. hilgardii* and *Lent. buchneri* were represented by only one isolate.

A total of 10 different lactobacilli species (57 isolates) were identified from probiotic supplement products. *Lact. rhamnosus* constituted 12.2% (31 isolates) of all isolates and was followed by *Lacp. plantarum, L. acidophilus,* and *Lact. paracasei*. In addition, MFMAS was applied to a total of 23 reference strains from different culture collections and all strains were correctly identified.

In this study, 12 different DNA regions on 5 different genes, including 16S rRNA, 23S rRNA, *dnaK*, *tuf,* and *recA* genes were simultaneously amplified and the high resolution melting curves of the amplifications were constructed to obtain fingerprints. These were used to generate the models of known species. In this study, three different fragments called as 16S1, 16S2, and 16S3 on the 16S rRNA gene were targeted. These fragments produced amplicons ranging from about 176–238 bp. The first of these fragments, 16S1 produced amplicons with a relatively narrow range of Tm values that ranged from 82.835 ± 0.053 °C to 85.208 ± 0.053 °C for analyzed strains and only 53.12% of the strains were accurately identified by MFMAS analysis of this fragment. The fragment with the widest range of Tm (80.08 ± 0.070 °C -84.26 ± 0.032 °C) on the 16S rRNA gene was 16S3. The MFMAS analysis of this fragment allowed correct determination for 90.62% of the strains analyzed (Table 2). As a result of MFMAS analysis of these three fragments targeting the 16SrRNA gene, the highest level of discrimination was obtained for *Lim. fermentum,* L. *acidophilus* and *L. gasseri*. Although in the MFMAS analysis of 16S rRNA gene fragments, the *Lact.casei* group (*Lact. casei, Lact. paracasei,* and *Lact. rhamnosus*) was distinctly separated from the other species, no clear discrimination was obtained between the group members.

The four fragments (called 23S2, 23S6, 23S7, and 23S8) targeting

Table 1

Distribution of identified species according to the analyzed samples.

Species	Kefir	Pickle	Sucuk	Sourdough	Cheese	Yoghurt	PFS	Total
Lactiplantibacillus plantarum	5	27	3	6	14	0	10	65
Levilactobacillus brevis	0	25	9	0	13	0	0	47
Lacticaseibacillus rhamnosus	0	0	0	0	9	0	22	31
Lacticaseibacillus casei	3	0	0	0	4	0	4	11
Lacticaseibacillus paracasei	0	0	0	0	6	0	6	12
Latilactobacillus curvatus	0	0	2	10	4	0	0	16
Latilactobacillus sakei	0	0	11	0	0	0	0	11
Lactobacillus delbrueckii subsp. *bulgaricus*	0	0	0	0	0	20	0	20
Lactobacillus acidophilus	0	0	0	0	0	0	6	6
Lactobacillus gasseri	0	0	0	0	0	0	2	2
Lactobacillus helveticus	0	0	0	0	0	0	1	1
Lentilactobacillus kefiri	10	0	0	0	0	0	0	10
Lentilactobacillus buchneri	0	0	0	0	1	0	0	1
Lentilactobacillus hilgardii	0	1	0	0	0	0	0	1
Limosilactobacillus fermentum	0	0	0	6	0	0	3	9
Limosilactobacillus reuteri	0	0	0	0	0	0	2	2
Companilactobacillus farciminis	0	0	2	3	0	0	1	6
Companilactobacillus heilongjiangensis	0	0	1	2	0	0	0	3
Total	19	54	30	27	51	20	57	254

Table 2
The specifications of target DNA regions.

Target gene	Fragment name	Lenght (bp)	Tm range (°C)* ± SD	Tm difference (°C)	Prediction accuracy
16S rRNA	16S1	176	82.84 ± 0.053–85.21 ± 0.055	2.37	53.12%
	16S2	238	81.94 ± 0.063–84.46 ± 0.014	2.52	81.25%
	16S3	194	80.08 ± 0.070–84.26 ± 0.032	4.18	90.62%
23S rRNA	23 s2	252	81.50 ± 0.061–85.55 ± 0.037	4.05	59.37%
	23 s6	321	80.93 ± 0.038–85.96 ± 0.059	5.03	84.37%
	23 s7	238	83.65 ± 0.042–86.86 ± 0.071	3.21	71.87%
	23 s8	245	81.02 ± 0.031–84.94 ± 0.101	3.92	87.50%
dnaK	DNaK1	249	78.15 ± 0.071–86.82 ± 0.048	8.67	81.25%
	DNaK2	320	78.22 ± 0.063–85.38 ± 0.089	7.16	65.62%
tuf	Tuf1	362	77.65 ± 0.078–83.18 ± 0.050	5.53	68.75%
	Tuf2	315	79.97 ± 0.060–84.31 ± 0.093	4.34	61.29%
recA	RecA	209	78.84 ± 0.085–86.98 ± 0.050	8.16	60.60%

Tm range (°C):minimum average Tm value ± SD - maximum average Tm value ± SD.
The average Tm values were calculated using the Tm values obtained from all isolates and reference strains of each *Lactobacillus* species.

the 23S rRNA gene produced amplicons with lengths ranging from about 238 to 321 bp. Using these four fragments, 59.37% to 87.5% of the analyzed strains were correctly identified. The amplicons of the 23S6 fragments produced the Tm values changing from 80.93 ± 0.038 °C to 85.96 ± 0.059 °C. This was the widest Tm range produced by fragments targeting the 23S rRNA gene. Among the fragments on the 23S rRNA gene, the highest accurate identification rate (87.50%) was produced by the 23S8 fragment, while the lowest accurate identification rate (59.37%) was obtained from the 23S2 fragment (Table 2). These results showed that there was no relationship between the width of the Tm range and the correct detection rates of amplicons produced by the analyzed fragments. The fragments, targeted on 23S rRNA allowed discrimination with a good resolution level of all tested species except for the discrimination of the *Lact. casei* and *Lact. paracasei* strains.

In addition to fragments targeting the 16S rRNA and 23S rRNA genes, the fragments on protein-encoding genes including *dnaK*, *tuf,* and *recA* were also analyzed in the MFMAS. In this system, 2 fragments on the *dnaK* gene called as DNaK1 and DNaK2 with an approximate length of 249 and 320 bp respectively, were targeted. The amplicons of these fragments produced the most extensive Tm ranges obtained in this study. The DNaK1 and DNaK2 fragments allowed 81.25% and 65.62% accurate identification of the analyzed strains, respectively. The other protein-encoding gene targeted in the MFMAS was the *tuf* gene. Two fragments on the *tuf* gene, called as Tuf1 and Tuf2 produced amplicons with length of approximately 362 bp and 315 bp, respectively. The rate of accurate identification of the strains analyzed for these two fragments was found to be 68.75% and 61.29%, respectively. In the MFMAS analysis, only one fragment was targeted on the *recA* gene. The RecA fragment produced amplicons with a length of about 209 bp and Tm values ranging from 78.84 ± 0.085 °C to 86.98 ± 0.050 °C. The correct identification rate of this fragment was found as 80% (Table 2). Among the analyzed strains, the closely related species including *Lim. fermentum/Lim. reuteri, Lent. buchneri/Lent. hilgardii/Lent. kefiri, L. helveticus/L. acidophilus, Lat. sakei/Lat. curvatus* could be clearly differentiated when all of the fragments on non-ribosomal genes were targeted. However, only three fragments, DNaK1, DNaK2, and Tuf1 allowed clear differentiation of species in the *Lact. casei* group.

As a result, all of the analyzed strains were exactly differentiated when the data obtained from all of the target fragments were concomitantly evaluated. In this study, when the high resolution melting curve data from all of the target fragments were overlapped in a single plane, a fingerprint was obtained for each of the strain. When the fingerprints of analyzed strains were compared with the fingerprints of known species registered in the MFMAS, all of the unknown strains were predicted with 100% accuracy (Fig. 2). In order to verify the findings of MFMAS, all of the strains were pre-grouped by rep-PCR analysis (Figure was not shown) and then a number of isolates, representing each group were identified by sequence analysis. Sequence analysis results completely confirmed the MFMAS-based identification results.

Additionally, MFMAS generated a dendrogram of the analyzed strains by processing the melting curve data of the 12 different target DNA fragments (Fig. 3). The dendrogram contained three main clusters (A, B and C). The largest cluster (cluster A) was divided into 2 sub-clusters. One of these sub-clusters contained core clusters of *Lact. casei* group, *Lim. reuteri* and *C. heilongjiangensis.* The similarity between these core clusters was below 70%, while it was < 90% among the members of the *Lact. casei* group. The other sub-cluster contained the species *Lev. brevis, Lim. fermentum, L. delbrueckii* subsp. *bulgaricus* and L. *gasseri.* They were clearly distinguished and the similarity values between these species were below 80% (except *L.gasseri/Lim. fermentum*). The second main cluster (cluster B) contained only the *Lacp. plantarum* strains obtained from various origins and the similarity among the strains ranged from 90 to 100%. The third main cluster (cluster C) contained 2 sub-clusters. The small sub-cluster consisted only of *Lat. sakei*. The large sub-cluster included 9 well-separated species, most of which were strains of *C. farciminis, L. acidophilus, Lat. curvatus* and *Lent. kefiri*.

In this study, 12 different DNA regions, of which 7 were on ribosomal genes and the remaining 5 were on non-ribosomal genes were targeted and 12 primer sets were used to amplify the target DNA regions. The nucleotide sequence of the primers were compared with genomes of a number of closely related genera using the Primer-Blast tool (https://www.ncbi.nlm. nih.gov/tools/primer-blast) and similarity rates were determined (Table 3). The primers, targeting non-ribosomal genes showed a higher specificity than the primers targeting ribosomal genes. As the sequence similarity between target DNA fragments and primers decreases, shouldered melting curves are obtained that could yield ambiguous HRM fingerprints. According to the similarity results, it might be predicted that the primers DNaK1, DNaK2, and Tuf2 could yield ambiguous HRM curves with the target DNA fragments of the closely related, non-targeted genera.

4. Discussion

In this study, MFMAS analysis was performed for the identification of a total of 254 lactobacilli isolates from various food products and probiotic supplements. A fingerprint, consisting of HRM curves of 12 target DNA fragments was produced for each isolate using a ready-to-use MFMAS plate. The fingerprints were converted into numerical data and compared with the data of all known species registered in the MFMAS to carry out species-level identification. The registered data of known species were used to create models for each target DNA fragment, and the species of the unknown isolates were predicted using these generated models.

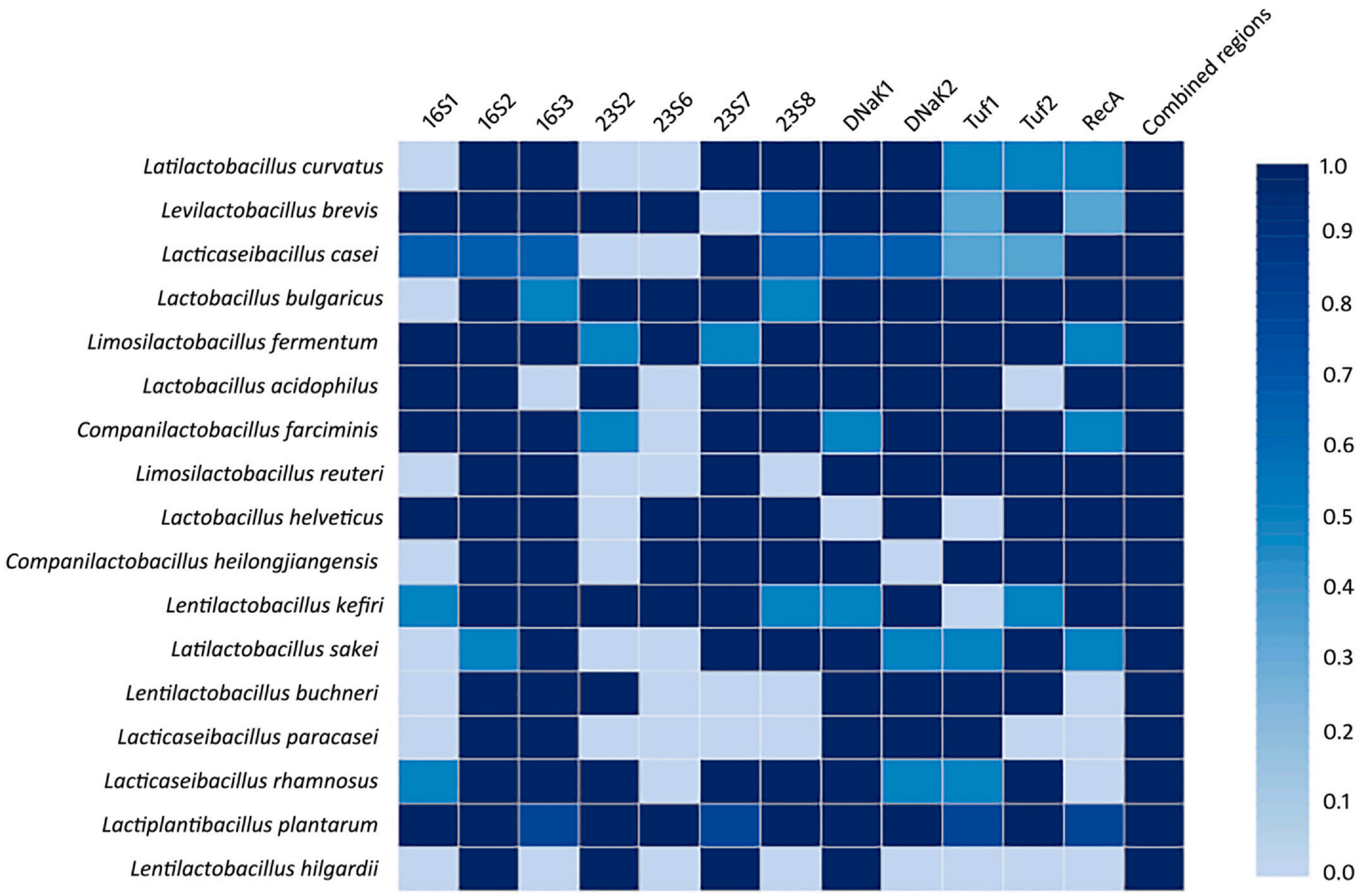

Fig. 2. Prediction performance of the MFMAS system for different lactobacilli species. The color scale refers to the prediction accuracy, the darkest color (referring to the highest score of 1) indicates that prediction accuracy = 100%, the lightest color (referring to the lowest score of 0) indicates that prediction accuracy = 0%.

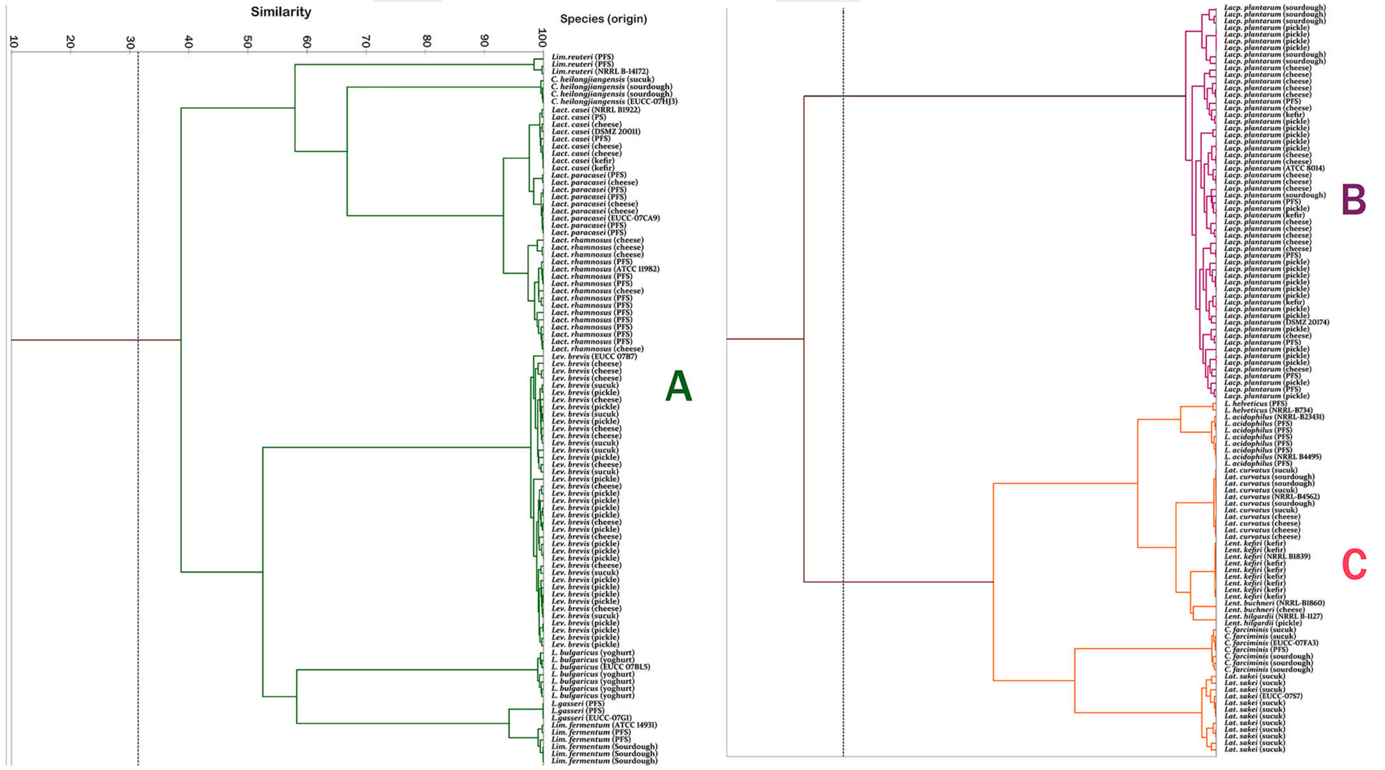

Fig. 3. The dendrogram of the cluster analysis of the lactobacilli strains constructed by using the HRM-patterns obtained for each isolate.

Table 3
The sequences of primers and their similarities with related genera.

name	Sequence (5′ → 3′)	% nucleotide identities with related genera								
		Weissella	*Leuconostoc*	*Oenococcus*	*Enterococcus*	*Tetragenococcus*	*Vagococcus*	*Lactococcus*	*Streptococcus*	*Pediococcus*
16S1	TATGACCTGGGCTACACACG	100	100	90–95	100	100	100	100	100	100
	CCCGGGAACGTATTCACC	100	100	100	100	95	100	100	100	94.4–100
16S2	GCGAACGGGTGAGTAACAC	100	100	94.7–100	100	94.7	94.7	94.7–100	94.7	94.7–100
	CGTGTCTCAGTCCCAATGTG	95–100	100	95	100	95	95	100	95	100
16S3	ACTCCTACGGGAGGCAGC	100	94.4–100	94.4	100	100	100	100	100	100
	CTACGTATTACCGCGGCTG	94.7–100	94.7–100	94.7	100	100	100	100	100	100
23S2	GTAGAATGAACCGGCGAGTTA	100	100	95.2	100	90.4–95.2	95.2–100	100	100	100
	GAGAGAACCAGCTATCTCCAA	100	95.2–100	90.4	95.2–100	95.2–100	100	95.2–100	100	100
23S6	CTAAGGTTTCCTGGGGAAGG	100	100	80	100	95–100	100	95	90–95	100
	CTGTGTCGGTTTGCGGTAC	100	100	100	94.7–100	94.7–100	100	100	100	100
23S7	TGGAGCTTTACTGTAGCTTGATATTG	100	100	100	96.15–100	92.3	92.3–96.1	88.4–96.1	92.3	96.15–100
	ACCATTCTGAGGGAACCTTTG	95.2–100	90.5	90.5–95.2	100	95.2	100	90.5–85.7	95.2–100	95.2–100
23S8	AGTACGAGAGGACCGGGATG	95.0	95–100	100	100	100	100	95–100	100	100
	GGTTAAGTCCTCGACCGATT	90.0	95–100	95	95.0–100	95	95	85–100	100	100
DNaK1	CCATTGGMCGTTCACCYTG	89.4–94.7	89.4–94.7	78.9	94.7–100	100	N	94.7–100	94.7–100	94.7–100
	TCAACCCWGAYGAAGCYGT	89.4–94.7	89.4–100	84.2	84.2	89.4	N	89.4	89.4	94.7–100
DNaK2	CCACCRCCAAGGTCRTAWAC	85–100	85.0	95	80–90	N	N	80–90	N	90–95
	ATYAAGMGYCACATGGGYGA	90–95	85–95	95	90–95	N	N	80–85	N	90–95
Tuf1	GCAACTGATGGYCCWATGCC	90–100	100	95–100	85–95.0	95	90	95–100	90–95	100
	GCAACAGTACCACGACCAGT	95–100	95–100	95	85–100	90–95	90–100	95–100	90–100	95–100
Tuf2	ACTGGTCGTGGTACTGTTGC	95–100	90–100	95	90–100	90–100	90–100	95–100	90–100	95
	TGAGAAGAATGGMGTRTGACG	81–91	81–91	90	86–91	95–100	90	86	85	100
RecA	CCCATTTCRCCTTCRATTTC	90	95–100	95	90–95	N	N	90	80–85	85–100
	GCHTATATYGATGCYGAAAA	95–100	90–100	100	80–90	N	N	95	85–90	95

M: A or C, Y: C or T, W: A or T, R: A or G, H: A or C or T (The letter "N" was used instead of the letters M, Y, R and H when the specificity of the primers was tested with primer-BLAST tool).

% nucleotide identities refer to all sequences of each target genus that are available on NCBI.

N: No primers were found in database.

In this study, 18 different lactobacilli species were identified with 100% accuracy by the MFMAS and the MFMAS-based identification was confirmed by rep-PCR grouping followed by sequence analysis. When HRM analysis was applied for a single fragment, which has a certain extent of interspecies heterogeneity, low-level discrimination was observed. However, when all the target fragments were simultaneously analyzed, excellent discrimination was obtained even between closely related species. For example, when the melting curve data obtained from only one target fragment was analyzed, any species was specifically separated or grouped with certain species. However, when the analysis was repeated for another fragment, this time, it grouped with different species, even if it was not clearly distinguished. Thus, when all target fragments were analyzed simultaneously, a species-specific discrimination pattern was obtained. The MFMAS only allows the identification of lactobacilli species of which HRM fingerprint patterns registered in the database, while unregistered species are defined as "unknown species". Species of the non-targeting Lactobacillaceae that are not included in the database likely also generate PCR amplicons but cannot be identified since HRM fingerprint patterns of reference strains are not included in MFMAS database.

In MFMAS-based identification of lactobacilli isolates a total of 7 fragments on ribosomal genes and 5 fragments on non-ribosomal genes were targeted. The 16S rRNA gene is the first universal marker and the sequence analysis of the 16S rRNA gene has been commonly used for the identification and classification of lactobacilli species for the last few decades (Collins et al., 1991; Kullen et al., 2000; Kwon et al., 2004). When the strains have more than 98.65% similarity in the 16S rRNA gene sequence, they are considered to belong to the same species (Kim et al., 2014). However, closely related species such as *Lent. buchneri* group (*Lent. buchneri, Lent. kefiri, Lent. parabuchneri,* and *Lent. parakefiri*), *Lact. casei* group (*Lact. casei, Lact. paracasei,* and *Lact. rhamnosus*), *Lacp. plantarum* group (*Lacp. fabifermentans, Lacp. plantarum, Lacp. paraplantarum,* and *Lacp. pentosus*) and L. *delbrueckii* subspecies (*L. delbrueckii* ssp. *delbrueckii, L. delbrueckii* ssp. *bulgaricus, and L. delbrueckii* ssp. *lactis*) exhibit a high level of sequence similarity and it may not always be possible to differentiate these species using only the sequence of 16S rRNA gene (Torriani et al., 1999; Watanabe et al., 2009; Huang and Lee, 2011; Yu et al., 2014). The development of high-throughput sequencing allowed the emergence of new approaches based on the whole-genome comparison. The average nucleotide identity (ANI) was the first approach, proposed to estimate the overall level of similarity between two microbial genomes (Konstantinidis and Tiedje, 2005). Since ANI is a multi-gene based approach, it provides more reliable similarity index than the methods targeted single gene, such as the 16S rRNA gene for the discrimination of bacterial species (Han et al., 2016). On the other hand, the highly conserved genes that encode a protein have emerged as alternative molecular targets for distinguishing closely related species. In previous studies, various housekeeping genes such as *tuf, recA, rpoA, hsp60, pheS, dnaK, and dnaJ/dnaK* have been used to identify species of lactobacilli through sequence analysis or species-specific PCR amplification. These genes have been shown to have higher separation ability than ribosomal RNA genes (Felis et al., 2001; Chavagnat et al., 2002; Naser et al., 2007; Blaiotta et al., 2008; Huang et al., 2011; Yu et al., 2012; Iacumin et al., 2015).

The *dnaK* gene encodes a 70-kDa heat shock protein (HSP70), which plays an important role as a chaperone in the protein folding process (Netzer and Hartl, 1998). The HSP70 is a proper candidate for advanced phylogenetic studies due to it being one of the most conserved proteins found in all biota (Gogarten, 1994). The *dnaK* sequence has been previously used to distinguish the *Lacp. plantarum* and *Lact. casei* groups, which have insufficient polymorphisms in 16S rRNA gene. In the *Lact. casei* group, the nucleotide sequence similarity of the 16S rRNA was 99.1%, while the similarity in the *dnaK* gene sequences was found to be 87.8% (Huang et al., 2010; Huang and Lee, 2011). In this study, we observed that DNaK1 and DNaK2 fragments on *dnaK* gene successfully differentiated the *Lact. casei, Lact. paracasei,* and *Lact. rhamnosus*.

In MFMAS analysis of the lactobacilli strains, two fragments on the *tuf* gene were targeted. The *tuf* gene, encoding the elongation factor Tu (EF-Tu), has been used as a target gene for phylogenetic studies (Watanabe et al., 2009; Yu et al., 2012). The EF-Tu protein catalyzes the binding of an aminoacyl-tRNA onto the ribosome during translation. The comparative analysis of nucleotide sequences revealed that the variability of the *tuf* gene was higher than the 16S rRNA gene in lactobacilli. Identities among the *tuf* genes of the lactobacilli species ranged from 79 to 98%, which allows the discrimination between very closely related species such as the *Lact. casei* group (Ventura et al., 2003).

The *recA* gene, encoding recombinase A has been suggested as a useful marker for the examination of bacterial phylogenetic relationships. The *recA* gene has been successfully used to distinguish between closely related species in lactobacilli including the *Lact. casei* (Felis et al., 2001) and *Lacp. plantarum* groups (Torriani et al., 2001b). It has also been reported that the comparison of the *recA* gene sequence allowed successful differentiation of the *Lent. hilgardii, Lent. buchneri,* and *Lev. brevis* species isolated from wine (Rodríguez et al., 2007). Similarly, in this study, a clear discrimination between these three species was obtained by the analysis of the RecA fragment.

Although 16S rRNA-based sequencing methods have been widely used for species-level identification of lactobacilli species, several closely related species cannot be distinguished precisely by these techniques. Therefore, several housekeeping genes have recently been proposed as genetic markers to identify species of lactobacilli. However, it is known that no single gene can distinguish between all lactobacilli species and the complete genomic information for some of the lactobacilli species is not available (Xie et al., 2019). Recently, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of protein profiles of microorganisms has become a popular tool for microbial identification and diagnosis. However, poor inter-laboratory reproducibility and the high initial cost of the MALDI-TOF equipment are the major limitations of this technique (Singhal et al., 2015).

In this study, we described a rapid and straightforward method for the identification of the lactobacilli species occurring in the fermented food ecosystem. MFMAS-lactobacilli is the new and customized version of the MFMAS. In MFMAS-based identification, since 12 different DNA regions with high inter-species heterogeneity are simultaneously analyzed, accurate and highly reliable identification is obtained. MFMAS does not need a specialized preparation procedure other than adding the template DNAs into the ready-to-use plates and only requires a real-time PCR device that is commonly available in molecular biology laboratories. The plate configuration of MFMAS-lactobacilli allows simultaneous analysis of six samples and the identification process of samples is completed in three hours approximately. Therefore, our proposed system, MFMAS-lactobacilli, could be a solution – to a certain degree – to the challenges and insufficiencies associated with the current methods.

5. Conclusion

In this study, the applicability of the MFMAS-lactobacilli was evaluated for fast and reliable identification of lactobacilli strains isolated from fermented food products and probiotic supplements. This new approach allowed a one-step identification of a large number of isolates (254 isolates). The MFMAS-lactobacilli provided excellent discrimination and precise identification even for closely related species such as *Lact. casei/Lact. paracasei/Lact. rhamonosus*, which produce ambiguous results with current methods. As a result, it has been concluded that the MFMAS is a promising tool that can be used for multiple purposes including differentiation, classification, and identification of lactobacilli species and it can offer an effective solution to the deficiencies and difficulties associated with the current approaches.

Declaration of Competing Interest

None.

Acknowledgments

This study was supported by The Scientific and Technological Research Council of Turkey (TÜBİTAK Project Number TOVAG-116O758) and Erciyes University Scientific and Technological Research Center/Turkey (Project Code: FDA-2017-7588). The MFMAS is a patent-protected product of Erciyes Technopark Inc.

References

Adzitey, F., Huda, N., Ali, G.R.R., 2013. Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks. 3. Biotech 3 (2), 97–107. https://doi.org/10.1007/s13205-012-0074-4.

Altay, F., Karbancıoglu-Güler, F., Daskaya-Dikmen, C., Heperkan, D. (2013). A review on traditional Turkish fermented non-alcoholic beverages: microbiota, fermentation process and quality characteristics. Int. J. Food Microbiol., 167(1), 44–56. doi:10. 1016/ j.ijfoodmicro.2013.06.016.

Andrighetto, C., Zampese, L., Lombardi, A., 2001. RAPD-PCR characterization of lactobacilli isolated from artisanal meat plants and traditional fermented sausages of Veneto region (Italy). Lett. Appl. Microbiol. 33 (1), 26–30. https://doi.org/10.1046/j.1472-765X.2001.00939.x.

Bernardeau, M., Guguen, M., Vernoux, J.P., 2006. Beneficial lactobacilli in food and feed: long-term use, biodiversity and proposals for specific and realistic safety assessments. FEMS Microbiol. Rev. 30 (4), 487–513. https://doi.org/10.1111/j.1574-6976.2006.00020.x.

Berthier, F., Ehrlich, S.D., 1998. Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region. FEMS Microbiol. Lett. 161, 97–106. https://doi.org/10.1111/j.1574-6968.1998.tb12934.x.

Blaiotta, G., Fusco, V., Ercolini, D., Aponte, M., Pepe, O., Villani, F., 2008. *Lactobacillus* strain diversity based on partial *hsp60* gene sequences and design of PCR-restriction fragment length polymorphism assays for species identification and differentiation. Appl. Environ. Microbiol. 74 (1), 208–215.

Bouton, Y., Guyot, P., Beuvier, E., Tailliez, P., Grappin, R., 2002. Use of PCR-based methods and PFGE for typing and monitoring homofermentative lactobacilli during Comté cheese ripening. Int. J. Food Microbiol. 76 (1–2), 27–38. https://doi.org/10.1016/S0168-1605(02)00006-5.

Carafa, I., Nardin, T., Larcher, R., Viola, R., Tuohy, K., Franciosi, E., 2015. Identification and characterization of wild lactobacilli and pediococci from spontaneously fermented mountain cheese. Food Microbiol. 48, 123–132. https://doi.org/10.1016/j.fm.2014.12.003.

Chavagnat, F., Haueter, M., Jimeno, J., Casey, M.G., 2002. Comparison of partial *tuf* gene sequences for the identification of lactobacilli. FEMS Microbiol. Lett. 217 (2), 177–183. https://doi.org/10.1111/j.1574-6968.2002.tb11472.x.

Collins, M.D., Rodrigues, U., Ash, C., Aguirre, M., Farrow, J.A.E., Martinez-Murcia, A., Phillips, B.A., Williams, A.M., Wallbanks, S., 1991. Phylogenetic analysis of the genus *Lactobacillus* and related lactic acid bacteria as determined by reverse transcriptase sequencing of 16S rRNA. FEMS Microbiol. Lett. 77 (1), 5–12. https://doi.org/10.1111/j.1574-6968.1991. tb04313.x.

dos Santos Cruxen, C.E., Funck, G.D., Haubert, L., da Silva Dannenberg, G., de Lima Marques, J., Chaves, F.C., … Fiorentini, Â.M., 2019. Selection of native bacterial starter culture in the production of fermented meat sausages: Application potential, safety aspects, and emerging technologies. Food Res Int. 122, 371–382. https://doi.org/10.1016/j.foodres.2019.04.018.

Druml, B., Cichna-Markl, M., 2014. High resolution melting (HRM) analysis of DNA–its role and potential in food analysis. Food Chem. 158, 245–254. https://doi.org/10.1016/j.foodchem. 2014.02.111.

Ehrmann, M.A., Vogel, R.F., 2005. Molecular taxonomy and genetics of sourdough lactic acid bacteria. Trends Food Sci. Technol. 16 (1–3), 31–42. https://doi.org/10.1016/j.tifs.2004.06.004.

Erdem, M., Kesmen, Z., Özbekar, E., Çetin, B., Yetim, H., 2016. Application of high-resolution melting analysis for differentiation of spoilage yeasts. J. Microbiol. 54 (9), 618–625. https://doi.org/10.1007/s12275-016-6017-8.

Felis, G.E., Dellaglio, F., Mizzi, L., Torriani, S., 2001. Comparative sequence analysis of a *recA* gene fragment brings new evidence for a change in the taxonomy of the *Lactobacillus casei* group. Int. J. Syst. Evol. Microbiol. 51 (6), 2113–2117. https://doi.org/10.1099/00207713-51-6-2113.

Foxman, B., Zhang, L., Koopman, J.S., Manning, S.D., Marrs, C.F., 2005. Choosing an appropriate bacterial typing technique for epidemiologic studies. Epidemiol. Perspect. Innov. 2 (1), 10. https://doi.org/10.1186/1742-5573-2-10.

Fusco, V., Quero, G.M., Chieffi, D., Franz, C.M., 2016. Identification of *Lactobacillus brevis* using a species-specific AFLP-derived marker. Int. J. Food Microbiol. 232, 90–94. https://doi.org/10.1016/j.ijfoodmicro.2016.06.002.

Gevers, D., Huys, G., Swings, J., 2001. Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. FEMS Microbiol. Lett. 205 (1), 31–36. https://doi.org/10.1111/j.1574-6968.2001.tb10921.x.

Giraffa, G., Chanishvili, N., Widyastuti, Y., 2010. Importance of lactobacilli in food and feed biotechnology. Res. Microbiol. 161 (6), 480–487. https://doi.org/10.1016/j.resmic.2010. 03.001.

Gogarten, J.P., 1994. Which is the most conserved group of proteins? Homology-orthology, paralogy, xenology, and the fusion of independent lineages. J. Mol. Evol. 39 (5), 541–543.

Gürtler, V., Stanisich, V.A. 1996. New approaches to typing and identification of bacteria using the 16S–23S rDNA spacer region. Microbiol. 142:3–16, doi:10.1099/13500872-142-1-3.

Han, N., Qiang, Y., Zhang, W., 2016. ANItools web: a web tool for fast genome comparison within multiple bacterial strains. Database (Oxford) 2016. https://doi.org/10.1093/database/baw084.

Herbel, S.R., Vahjen, W., Wieler, L.H., Guenther, S., 2013. Timely approaches to identify probiotic species of the genus *Lactobacillus*. Gut Pathogens 5 (1), 27.

Huang, C.H., Lee, F.L., 2011. The *dnaK* gene as a molecular marker for the classification and discrimination of the *Lactobacillus casei* group. Antonie Van Leeuwenhoek 99 (2), 319–327.

Huang, C.H., Lee, F.L., Liou, J.S., 2010. Rapid discrimination and classification of the *Lactobacillus plantarum* group based on a partial *dnaK* sequence and DNA fingerprinting techniques. Antonie Van Leeuwenhoek 97 (3), 289–296.

Huang, C.H., Chang, M.T., Huang, M.C., Lee, F.L., 2011. Application of the SNaPshot minisequencing assay to species identification in the *Lactobacillus casei* group. Mol. Cell. Probes 25 (4), 153–157. https://doi.org/10.1016/j.mcp.2011.03.002.

Iacumin, L., Ginaldi, F., Manzano, M., Anastasi, V., Reale, A., Zotta, T., Rossi, F., Coppola, R., Comi, G., 2015. High resolution melting analysis (HRM) as a new tool for the identification of species belonging to the *Lactobacillus casei* group and comparison with species-specific PCRs and multiplex PCR. Food Microbiol. 46, 357–367. https://doi.org/10.1016/j.fm.2014.08.007.

Kesmen, Z., Yetiman, A.E., Gulluce, A., Kacmaz, N., Sagdic, O., Cetin, B., Adiguzel, A., Şahin, F., Yetim, H., 2012. Combination of culture-dependent and culture-independent molecular methods for the determination of lactic microbiota in sucuk. Int. J. Food Microbiol. 153 (3), 428–435. https://doi.org/10.1016/j.ijfoodmicro.2011.12.008.

Kesmen, Z., Özbekar, E., Büyükkiraz, M. E. (2018a). Multifragment melting analysis of yeast species isolated from spoiled fruits. J. Appl. Microbiol. 124(2), 522–534, doi:10.1111/ jam.13645.

Kesmen, Z., Büyükkiraz, M.E., Özbekar, E., Çelik, M., Özkök, F.Ö., Kılıç, Ö., Çetin, B., Yetim, H., 2018b. Assessment of multi fragment melting analysis system (MFMAS) for the identification of food-borne yeasts. Curr. Microbiol. 1-10.

Kim, M., Oh, H.S., Park, S.C., Chun, J., 2014. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. Int. J. Syst. Evol. Microbiol. 64 (2), 346–351. https://doi.org/10.1099/ijs.0.059774-0.

Konstantinidis, K.T., Tiedje, J.M., 2005. Genomic insights that advance the species definition for prokaryotes. Proc. Natl. Acad. Sci. U. S. A. 102 (7), 2567–2572. https://doi.org/10.1073/pnas.0409727102.

Kullen, M.J., Sanozky-Dawes, R.B., Crowell, D.C., Klaenhammer, T.R., 2000. Use of the DNA sequence of variable regions of the 16S rRNA gene for rapid and accurate identification of bacteria in the *Lactobacillus acidophilus* complex. J. Appl. Microbiol. 89 (3), 511–516. https://doi.org/10.1046/j.1365-2672.2000.01146.x.

Kumar, A., Kumar, D., 2015. Characterization of *Lactobacillus* isolated from dairy samples for probiotic properties. Anaerobe 33, 117–123. https://doi.org/10.1016/j.anaerobe.2015.03.004.

Kumariya, R., Garsa, A.K., Rajput, Y.S., Sood, S.K., Akhtar, N., Patel, S., 2019. Bacteriocins: classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. Microb. Pathog. 128, 171–177. https://doi.org/10.1016/j.micpath.2019.01. 002.

Kwon, H.S., Yang, E.H., Yeon, S.W., Kang, B.H., Kim, T.Y., 2004. Rapid identification of probiotic *Lactobacillus* species by multiplex PCR using species-specific primers based on the region extending from 16S rRNA through 23S rRNA. FEMS Microbiol. Lett. 239 (2), 267–275. https://doi.org/10.1016/j.femsle.2004.08.049.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures for the food fermentation industry. Trends Food Sci. Technol. 15 (2), 67–78 doi:10. 1016/j.tifs.2003. 09.004.

Lin, X.B., Gänzle, M.G., 2014. Quantitative high-resolution melting PCR analysis for monitoring of fermentation microbiota in sourdough. Int. J. Food Microbiol. 186, 42–48. https://doi.org/10.1016/j.ijfoodmicro.2014.06.010.

Mohammed, M., El-Aziz, H.A., Omran, N., Anwar, S., Awad, S., El-Soda, M., 2009. Rep-PCR characterization and biochemical selection of lactic acid bacteria isolated from the Delta area of Egypt. Int. J. Food Microbiol. 128 (3), 417–423. https://doi.org/10.1016/j.ijfoodmicro.2008.09.022.

Naser, S.M., Dawyndt, P., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Vancanneyt, M., Swings, J., 2007. Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. Int. J. Syst. Evol. Microbiol. 57 (12), 2777–2789. https://doi.org/10.1099/ijs.0. 64711-0.

Netzer, W. J., Hartl, F. U. (1998). Protein folding in the cytosol: chaperonin-dependent and-independent mechanisms. Trends Biochem. Sci. 23(2), 68–73, doi:10.1016/S0968-0004(97)01171-7.

Omar, N.B., Abriouel, H., Keleke, S., Valenzuela, A.S., Martínez-Cañamero, M., López, R.L., Ortega, E., Gálvez, A., 2008. Bacteriocin-producing *Lactobacillus* strains isolated from poto poto, a Congolese fermented maize product, and genetic fingerprinting of their plantaricin operons. Int. J. Food Microbiol. 127 (1–2), 18–25. https://doi.org/10.1016/j.ijfoodmicro.2008.05.037.

Pot, B., and Tsakalidou, E. (2009). Taxonomy and metabolism of *Lactobacillus*. A. Ljungh, T. Wadström (Eds.), *Lactobacillus* Molecular Biology: From Genomics to Probiotics, Caister Academic Press, Norfolk (2009), pp. 1–56.

Ramos, C. L., Thorsen, L., Schwan, R. F., Jespersen, L. (2013). Strain-specific probiotics properties of *Lactobacillus fermentum, Lactobacillus plantarum* and *Lactobacillus brevis* isolates from Brazilian food products. Food Microbiol. 36(1), 22–29. doi:10.1016/ j.fm.2013.03.010.

Reed, G.H., Wittwer, C.T., 2004. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. Clin. Chem. 50 (10), 1748–1754.

Ren, D., Li, C., Qin, Y., Yin, R., Du, S., Ye, F., ... Jin, N., 2014. In vitro evaluation of the probiotic and functional potential of *Lactobacillus* strains isolated from fermented food and human intestine. Anaerobe 30, 1–10. https://doi.org/10.1016/j.anaerobe.2014.07.004.

Rodas, A.M., Ferrer, S., Pardo, I., 2005. Polyphasic study of wine Lactobacillus strains: taxonomic implications. Int. J. Syst. Evol. Microbiol. 55 (1), 197–207. https://doi.org/10.1099/ijs.0. 63249-0.

Rodríguez, H., de las Rivas, B., Muñoz, R., 2007. Efficacy of *recA* gene sequence analysis in the identification and discrimination of *Lactobacillus hilgardii* strains isolated from stuck wine fermentations. Int. J. Food Microbiol. 115 (1), 70–78. https://doi.org/10.1016/j.ijfoodmicro.2006. 10.032.

Settanni, L., Moschetti, G. (2010). Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiol. 27(6), 691–697. doi:10.1016/j.fm.2010.05.023.

Singh, S., Goswami, P., Singh, R., Heller, K.J., 2009. Application of molecular identification tools for *Lactobacillus*, with a focus on discrimination between closely related species: a review. LWT Food Sci. Technol. 42 (2), 448–457. https://doi.org/10.1016/j.lwt.2008.05.019.

Singhal, N., Kumar, M., Kanaujia, P.K., Virdi, J.S., 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front. Microbiol. 6, 791. https://doi.org/10.3389/fmicb.2015.00791.

Szén, O.P., Kiss, A., Naár, Z., Pál, K., 2014. Evaluation of high resolution melting and other molecular methods in discrimination of *Lactobacillus* isolates. J. Appl. Microbiol. 117 (4), 1113–1121. https://doi.org/10.1111/jam.12599.

Tamburro, M., Ripabelli, G., 2017. High resolution melting as a rapid, reliable, accurate and cost-effective emerging tool for genotyping pathogenic bacteria and enhancing molecular epidemiological surveillance: a comprehensive review of the literature. Ann. Ig. 29 (4), 293–316. https://doi.org/10.7416/ai.2017.2153.

Tong, S.Y., and Giffard, P. M. (2012). Microbiological applications of high-resolution melting analysis. J. Clin. Microbiol. 50(11), 3418–3421. https://doi:10.1128/JCM. 01709-12.

Torriani, S., Zapparoli, G., Dellaglio, F., 1999. Use of PCR-based methods for rapid differentiation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*. Appl. Environ. Microbiol. 65 (10), 4351–4356.

Torriani, S., Clementi, F., Vancanneyt, M., Hoste, B., Dellaglio, F., Kersters, K., 2001a. Differentiation of *Lactobacillus plantarum*, *L. pentosus* and *L. paraplantarum* species by RAPD-PCR and AFLP. Syst. Appl. Microbiol. 24 (4), 554–560. https://doi.org/10.1078/0723-2020-00071.

Torriani, S., Felis, G.E., Dellaglio, F., 2001b. Differentiation of *Lactobacillus plantarum*, *L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. Appl. Environ. Microbiol. 67 (8), 3450–3454.

Turková, K., Rittich, B., Španová, A., 2012. Identification and determination of relatedness of lactobacilli using different DNA amplification methods. Chem. Pap. 66 (9), 842–851.

Tynkkynen, S., Satokari, R., Saarela, M., Mattila-Sandholm, T., Saxelin, M., 1999. Comparison of Ribotyping, randomly amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains. Appl. Environ. Microbiol. 65 (9), 3908–3914.

Ventura, M., Canchaya, C., Meylan, V., Klaenhammer, T.R., Zink, R., 2003. Analysis, characterization, and loci of the *tuf* genes in *Lactobacillus* and *Bifidobacterium* species and their direct application for species identification. Appl. Environ. Microbiol. 69 (11), 6908–6922.

Wang, J., Chen, X., Liu, W., Yang, M., Zhang, H., 2008. Identification of Lactobacillus from koumiss by conventional and molecular methods. Eur. Food Res. Technol. 227 (5), 1555–1561.

Watanabe, K., Fujimoto, J., Tomii, Y., Sasamoto, M., Makino, H., Kudo, Y., Okada, S., 2009. *Lactobacillus kisonensis* sp. nov., *Lactobacillus otakiensis* sp. nov., *Lactobacillus rapi* sp. nov. and *Lactobacillus sunkii* sp. nov., heterofermentative species isolated from sunki, a traditional Japanese pickle. Int. J. Syst. Evol. Microbiol. 59 (4), 754–760. https://doi.org/10.1099/ijs.0.004689-0.

Xie, M., Pan, M., Jiang, Y., Liu, X., Lu, W., Zhao, J., Zhang, H., Chen, W., 2019. groEL gene-based phylogenetic analysis of *Lactobacillus* species by high-throughput sequencing. Genes 10 (7), 530. https://doi.org/10.3390/genes10070530.

Yetiman, A.E., Kesmen, Z., 2015. Identification of acetic acid bacteria in traditionally produced vinegar and mother of vinegar by using different molecular techniques. Int. J. Food Microbiol. 204, 9–16 doi:10.1016/j.ijfoodmicro.2015.03.013.

Yu, J., Sun, Z., Liu, W., Bao, Q., Zhang, J., Zhang, H., 2012. Phylogenetic study of *Lactobacillus acidophilus* group, *L. casei* group and *L. plantarum* group based on partial *hsp60, pheS* and *tuf* gene sequences. Eur. Food Res. Technol. 234 (6), 927–934.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M., Harris, H.M., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S., 2020. A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. Int. J. Syst. Evol. Microbiol. 70 (4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107.