Food Microbiology 46 (2015) 357–367

Contents lists available at ScienceDirect

Food Microbiology

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

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

Lucilla Iacumin [a, *], Federica Ginaldi [a], Marisa Manzano [a], Veronica Anastasi [a], Anna Reale [b], Teresa Zotta [b], Franca Rossi [c], Raffaele Coppola [b, c], Giuseppe Comi [a]

[a] Dipartimento di Scienze degli Alimenti, Università degli Studi di Udine, Udine, Italy
[b] Istituto di Scienze dell'Alimentazione-CNR, Avellino, Italy
[c] Dipartimento di Agricoltura, Ambiente e Alimenti Università degli Studi del Molise, Campobasso, Italy

ARTICLE INFO

Article history:
Received 19 June 2014
Received in revised form
1 August 2014
Accepted 12 August 2014
Available online 9 September 2014

Keywords:
Lactobacillus casei group
High resolution melting analysis
Identification methods
Multiplex PCR
Species-specific PCR

ABSTRACT

The correct identification and characterisation of bacteria is essential for several reasons: the classification of lactic acid bacteria (LAB) has changed significantly over the years, and it is important to distinguish and define them correctly, according to the current nomenclature, avoiding problems in the interpretation of literature, as well as mislabelling when probiotic are used in food products. In this study, species-specific PCR and HRM (high-resolution melting) analysis were developed to identify strains belonging to the *Lactobacillus casei* group and to classify them into *L. casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*. HRM analysis confirmed to be a potent, simple, fast and economic tool for microbial identification.

In particular, 201 strains, collected from International collections and attributed to the *L. casei* group, were examined using these techniques and the results were compared with consolidated molecular methods, already published. Seven of the tested strains don't belong to the *L. casei* group. Among the remaining 194 strains, 6 showed inconsistent results, leaving identification undetermined. All the applied techniques were congruent for the identification of the vast majority of the tested strains (188). Notably, for 46 of the strains, the identification differed from the previous attribution.

1. Introduction

Lactic acid bacteria (LAB) are important for the food industry because they promote human health and have therefore been the focus of recent studies (Iqbal et al., 2014). These microorganisms are extremely widespread in nature and are characteristic of many habitats: the gastro-intestinal tracts of various animals such as mice, rats, pigs, chickens and humans; milk and dairy products; fish products; fermented products; and the surfaces of certain plants and fruits. LAB are used in the production and preservation of food products such as cheese, sauerkraut, meat and yogurt (Konings et al., 2000; Settanni and Moschetti, 2010; Shiby and Mishra, 2013; Rubio et al., 2014; Han et al., 2014; Corbo et al., 2014; Beganović et al., 2011, 2014; Mani-López et al., 2014). Their important impact on fermented foods and intestinal microflora is due to their antagonistic activity against potential pathogens (de Vrese and Marteau, 2007; Ortolani et al., 2010; Aguilar et al., 2011).

The Genus *Lactobacillus* spp. have been extensively studied because of several factors: the importance of these microorganisms in human health; their use in improving the quality or health aspects of many foods; and queries by legislative bodies, industry and consumers about safety, labelling, patents and strain integrity (Shu et al., 1999; Holzapfel and Schillinger, 2002; Singh et al., 2009; Doherty et al., 2010; Giraffa et al., 2010; Crittenden, 2012; Harrison et al., 2012; Chen et al., 2014; Didari et al., 2014; El-Abbadi et al., 2014; Fijan, 2014).

Lactobacillus spp. includes the *Lactobacillus casei* group, which consists of *L. casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus*; these species are used in various commercial and traditional

* Corresponding author. Tel.: +39 0432 558126; fax: +39 0432 558130.
E-mail address: lucilla.iacumin@uniud.it (L. Iacumin).

http://dx.doi.org/10.1016/j.fm.2014.08.007
0740-0020/

fermented foods. These three species are closely genetically related to each other (Holzapfel and Schillinger, 2002; Ong et al., 2007; Sakai et al., 2010).

Recently, the classification of these bacteria has changed considerably because it is difficult to discriminate between *L. casei, L. paracasei* and *L. rhamnosus*. However, this distinction is important to understand the relationship between strains, to monitor the genetic stability of the strains and to classify them into recognisable species based on the current taxonomy of these organisms. Furthermore, because of their industrial importance, accurate taxonomic identification of these microorganisms is essential to generate accurate labels for food products and probiotics (Desai et al., 2006).

Studies on the 16S rRNA genes of *L. casei, L. paracasei* and *L. rhamnosus* revealed that these microorganisms may have minor differences (polymorphisms) even within the same species, which complicates phylogenetic analyses, especially for closely related species (Vásquez et al., 2005).

Several techniques have been used to identify and characterise *Lactobacillus* spp. isolates based on their physiological characteristics; these techniques include the study of the fermentative pathways, assays on carbohydrates, lactic acid configuration or peptidoglycan analysis. However, because of the strong similarities, the results of such analyses are often ambiguous (Richiard et al., 2001; Dubernet et al., 2002; Huang et al., 2011); therefore, other studies have focused on genetic characterisation using molecular methods (Klijn et al., 1991; Nuor, 1998; Baele et al., 2002; Comi et al., 2005; Huang and Lee, 2011; Turkova et al., 2012; Salvetti et al., 2012).

This study developed and optimised two molecular techniques, high-resolution melting (HRM) analysis and species-specific PCRs, to identify species belonging to the *L. casei* group. A large number of strains (201), taxonomically indicated as *L. casei*, *L. paracasei* and *L. rhamnosus*, were obtained from International Collections and subjected to a series of novel trials for accurate identification using two consolidated molecular methods described previously. These results were compared to the results obtained using the species-specific PCR and HRM analyses developed in this study.

2. Materials and methods

2.1. Strains and culture conditions

Two hundred one (201) strains belonging to the species *L. casei*, *L. paracasei* and *L. rhamnosus* isolated from different sources (Table 1) were used in this study. The strains were previously isolated and identified by the respective Universities or Research Institutes using biochemical and morphological tests or different molecular techniques.

All strains were maintained as frozen stocks in reconstituted 11% (w/v) skimmed milk containing 0.1% (w/v) ascorbic acid (RSM) in the Culture Collection of the Department of Food Science, University of Udine. The isolates were routinely propagated (1% w/v) in MRS broth (pH 6.8) (Oxoid, Milan, Italy) for 16 h at 37 °C.

L. casei (DSM 20178), *L. paracasei* (DSM5622) and *L. rhamnosus* (DSM20021) were used as reference strains for optimisation of all the molecular methods used for identification. The following strains were used as negative controls: *Lactobacillus fermentum* (DSM 20049), *L. pontis* (DSM 8475), *L. sanfranciscensis* (DSM 20451), *L. brevis* (DSM 20054), *L. reuteri* (DSM 20053), *Lactobacillus plantarum* (DSM 20174), *L. sakei* (DSM 6333), *Lactococcus lactis* (DSM 20481), *Leuconostoc citreum* (DSM 5577), *Leuc. gasicomitatum* (DSM 15947), *Leuc. mesenteroides* subsp. *mesenteroides* (DSM 20343) and *Pediococcus pentosaceus* (DSM 20336).

2.2. DNA extraction from pure cultures

Two millilitres of a 48-h culture in De Man-Rogosa-Sharp (MRS) broth were centrifuged at 13,000 *g* for 10 min at 4 °C to pellet the cells, which were then subjected to DNA extraction using the MasterPure™ Complete DNA & RNA Purification Kit (Epicentre Biotechnologies, USA). The DNA concentration and purity were measured using an absorbance ratio of 260/280 nm and verified by agarose gel electrophoresis.

2.3. L. casei group-specific PCR

The *L. casei* group-specific PCR primer pair, LCgprpoA-F2 (5′-CACTCAARATGAAYACYGATGA-3′) and -R2 (5′-CGTGGTGA-GATTGAGCCAT-3′) was used as described by Huang et al. (2011). The reactions were performed in a final volume of 25 μl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25 U of *Taq*-polymerase (Applied Biosystems, Milan, Italy). The thermal cycling protocol was as follows: initial strand denaturation at 94 °C for 5 min followed by 25 cycles of 94 °C for 1 min, 61 °C for 1 min and 72 °C for 1.5 min, and a final extension step at 72 °C for 7 min in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad, Milan, Italy). The PCR products were analysed by 2% agarose gel electrophoresis with ethidium bromide staining, and the expected amplicon size was 364 bp.

2.4. Species-specific PCRs

Three different primer pairs were used to identify strains by species-specific PCRs, as described by Ward and Timmins (1999) (Table 2). The reactions were performed in a final volume of 25 μl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25 U of *Taq*-polymerase (Applied Biosystems, Milan, Italy). The amplification was performed for 30 cycles at 95 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad, Milan, Italy). An initial denaturation step (95 °C for 5 min) and a final extension step (72 °C for 5 min) were used. The PCR products were verified by electrophoresis in a 2% agarose gel using 0.5× TBE as the running buffer. Ethidium bromide (0.5 μg/ml) was added to the gel before solidification. After electrophoresis, the gels were examined using the BioImaging System GeneGenius (*SynGene*, Cambridge, United Kingdom).

In this study, a second set of species-specific PCRs was developed, using a different part of the genome as a target sequence for primer annealing compared to the region used by Ward and Timmins (1999). The *dnaJ* and *dnaK* genes were targeted. All of the sequences of these genes available in GenBank for species of the *L. casei* group were aligned using the MultAlin software (Corpet, 1988), and the primer pairs designed were dnaKRHf/dnaKRHr, dnaKCPf/dnaKCPr, and dnaJCPf/dnaJCPr (Table 2), which were specific to the *L. casei* group for *L. rhamnosus*, *L. paracasei*/*L. casei* and *L. paracasei*, respectively. Before optimisation of the amplification protocol, primer specificity was tested *in silico* using the FastPCR 6.1 software (Kalendar et al., 2009) and *in vivo* using *L. fermentum* (DSM 20049), *L. pontis* (DSM 8475), *L. sanfranciscensis* (DSM 20451), *L. brevis* (DSM 20054), *L. reuteri* (DSM 20053), *L. plantarum* (DSM 20174), *L. sakei* (DSM 6333), *Lactococcus lactis* (DSM 20481), *Leuconostoc citreum* (DSM 5577), *Leuc. gasicomitatum* (DSM 15947), *Leuc. mesenteroides* subsp. *mesenteroides* (DSM 20343) and *Pediococcus pentosaceus* (DSM 20336) as negative controls.

The reactions were performed in a final volume of 25 μl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM