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High resolution melting analysis (HRM) as a new tool for the identification of species belonging to the Lactobacillus casei group and comparison with

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1 2	High resolution melting analysis (HRM) as a new tool for the identification of species
3	belonging to the Lactobacillus casei group and comparison with species-specific PCRs
4	and multiplex PCR.
5	
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24	
25	Abstract

26	The correct identification and characterisation of bacteria is essential for several reasons: the
27	classification of lactic acid bacteria (LAB) has changed significantly over the years, and it is
28	important to distinguish and define them correctly, according to the current nomenclature,
29	avoiding problems in the interpretation of literature, as well as mislabelling when probiotic
30	are used in food products. In this study, species-specific PCR and HRM (high-resolution
31	melting) analysis were developed to identify strains belonging to the Lactobacillus casei
32	group and to classify them into L. casei, L. paracasei and L. rhamnosus. HRM analysis
33	confirmed to be a potent, simple, fast and economic tool for microbial identification.
34	In particular, 201 strains, collected from International collections and attributed to the L. casei
35	group, were examined using these techniques and the results were compared with
36	consolidated molecular methods, already published. Seven of the tested strains don't belong
37	to the L. casei group. Among the remaining 194 strains, 6 showed inconsistent results, leaving
38	identification undetermined. All the applied techniques were congruent for the identification
39	of the vast majority of the tested strains (188). Notably, for 46 of the strains, the identification
40	differed from the previous attribution.
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43	Keywords: Lactobacillus casei group, High Resolution Melting Analysis, Identification
44	methods, multiplex PCR, species-specific PCR.
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51	1. Introduction

53	Lactic acid bacteria (LAB) are important for the food industry because they promote human
54	health and have therefore been the focus of recent studies (Iqbal et al., 2014). These
55	microorganisms are extremely widespread in nature and are characteristic of many habitats:
56	the gastro-intestinal tracts of various animals such as mice, rats, pigs, chickens and humans;
57	milk and dairy products; fish products; fermented products; and the surfaces of certain plants
58	and fruits. LAB are used in the production and preservation of food products such as cheese,
59	sauerkraut, meat and yogurt (Konings et al., 2000; Settanni and Moschetti, 2010; Shiby and
60	Mishra, 2013; Rubio et al., 2014; Han et al., 2014; Corbo et al., 2014; Beganović et al., 2011,
61	2014; Mani-López et al., 2014). Their important impact on fermented foods and intestinal
62	microflora is due to their antagonistic activity against potential pathogens (de Vrese and
63	Marteau, 2007; Ortolani et al., 2010; Aguilar et al., 2011).
64	The Genus Lactobacillus spp. have been extensively studied because of several factors: the
65	importance of these microorganisms in human health; their use in improving the quality or
66	health aspects of many foods; and queries by legislative bodies, industry and consumers about
67	safety, labelling, patents and strain integrity (Shu et al., 1999; Holzapfel and Schillinger,
68	2002; Singh et al., 2009; Doherty et al., 2010; Giraffa et al., 2010; Crittenden, 2012; Harrison
69	et al., 2012; Chen et al., 2014; Didari et al., 2014; El-Abbadi et al., 2014; Fijan, 2014).
70	Lactobacillus spp. includes the L. casei group, which consists of Lactobacillus casei, L.
71	paracasei and L. rhamnosus; these species are used in various commercial and traditional
72	fermented foods. These three species are closely genetically related to each other (Holzapfel
73	and Schillinger, 2002; Ong et al., 2007; Sakai et al., 2010).
74	Recently, the classification of these bacteria has changed considerably because it is difficult to
75	discriminate between L. casei, L. paracasei and L. rhamnosus. However, this distinction is
76	important to understand the relationship between strains, to monitor the genetic stability of the
77	strains and to classify them into recognisable species based on the current taxonomy of these

78 organisms. Furthermore, because of their industrial importance, accurate taxonomic

identification of these microorganisms is essential to generate accurate labels for foodproducts 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).

85 Several techniques have been used to identify and characterise *Lactobacillus* spp. isolates 86 based on their physiological characteristics; these techniques include the study of the 87 fermentative pathways, assays on carbohydrates, lactic acid configuration or peptidoglycan 88 analysis. However, because of the strong similarities, the results of such analyses are often 89 ambiguous (Richiard et al., 2001; Dubernet et al., 2002; Huang et al., 2011); therefore, other 90 studies have focused on genetic characterisation using molecular methods (Klijn et al., 1991; 91 Nuor, 1998; Baele et al., 2002; Comi et al., 2005; Huang and Lee, 2011; Turkova et al., 2012; 92 Salvetti et al., 2012). 93 This study developed and optimised two molecular techniques, high-resolution melting

94 (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

96 L. rhamnosus, were obtained from International Collections and subjected to a series of novel

- 97 trials for accurate identification using two consolidated molecular methods described
- 98 previously. These results were compared to the results obtained using the species-specific
- 99 PCR and HRM analyses developed in this study.

100

101 **2. Materials and methods**

- 102
- 103 2.1. Strains and culture conditions

105	Two hundred one (201) strains belonging to the species Lactobacillus casei, L. paracasei and
106	L. rhamnosus isolated from different sources (Table 1) were used in this study. The strains
107	were previously isolated and identified by the respective Universities or Research Institutes
108	using biochemical and morphological tests or different molecular techniques.
109	All strains were maintained as frozen stocks in reconstituted 11 % (w/v) skimmed milk
110	containing 0.1 % (w/v) ascorbic acid (RSM) in the Culture Collection of the Department of
111	Food Science, University of Udine. The isolates were routinely propagated (1 % w/v) in MRS
112	broth (pH 6.8) (Oxoid, Milan, Italy) for 16 h at 37 °C.
113	L. casei (DSM 20178), L. paracasei (DSM5622) and L. rhamnosus (DSM20021) were used
114	as reference strains for optimisation of all the molecular methods used for identification. The
115	following strains were used as negative controls: Lactobacillus fermentum (DSM 20049), L.
116	pontis (DSM 8475), L. sanfranciscensis (DSM 20451), L. brevis (DSM 20054), L. reuteri
117	(DSM 20053), L. plantarum (DSM 20174), L. sakei (DSM 6333), Lactococcus lactis (DSM
118	20481), Leuconostoc citreum (DSM 5577), Leuc. gasicomitatum (DSM 15947), Leuc.
119	mesenteroides subsp. mesenteroides (DSM 20343) and Pediococcus pentosaceus (DSM
120	20336).
121	
122	2.2. DNA extraction from pure cultures
123	
124	Two millilitres of a 48-h culture in De Man-Rogosa-Sharp (MRS) broth were centrifuged at
125	13,000g for 10 minutes at 4 °C to pellet the cells, which were then subjected to DNA
126	extraction using the MasterPureтм Complete DNA & RNA Purification Kit (Epicentre

- 127 Biotechnologies, USA). The DNA concentration and purity were measured using an
- absorbance ratio of 260/280 nm and verified by agarose gel electrophoresis.
- 129

132	The L. casei group-specific PCR primer pair, LCgprpoA-F2 (5'-							
133	CACTCAARATGAAYACYGATGA-3') and -R2 (5'-CGTGGTGAGATTGAGCCAT-3')							
134	was used as described by Huang et al. (2011). The reactions were performed in a final volume							
135	of 25 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl ₂ , 0.2 mM dNTPs,							
136	0.2 mM of each primer and 1.25 U of Taq-polymerase (Applied Biosystems, Milan, Italy).							
137	The thermal cycling protocol was as follows: initial strand denaturation at 94 $^{\circ}$ C for 5 min							
138	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							
139	extension step at 72 °C for 7 min in a Thermal Cycler (DNA Engine Dyad Peltier Thermal							
140	Cycler, BioRad, Milan, Italy). The PCR products were analysed by 2 % agarose gel							
141	electrophoresis with ethidium bromide staining, and the expected amplicon size was 364 bp.							
142								
143	2.4. Species-specific PCRs							
144								
145	Three different primer pairs were used to identify strains by species-specific PCRs, as							
146	described by Ward and Timmins (1999) (Table 2). The reactions were performed in a final							
147	volume of 25 µl containing 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl ₂ , 0.2 mM							
148	dNTPs, 0.2 mM of each primer and 1.25 U of Taq-polymerase (Applied Biosystems, Milan,							
149	Italy). The amplification was performed for 30 cycles at 95 °C for 1 min, 53 °C for 1 min and							
150	72 °C for 1 min in a Thermal Cycler (DNA Engine Dyad Peltier Thermal Cycler, BioRad,							
151	Milan, Italy). An initial denaturation step (95 $^{\circ}$ C for 5 min) and a final extension step (72 $^{\circ}$ C							
152	for 5 min) were used. The PCR products were verified by electrophoresis in a 2 % agarose gel							
153	using 0.5X TBE as the running buffer. Ethidium bromide (0.5 μ g/ml) was added to the gel							
154	before solidification. After electrophoresis, the gels were examined using the BioImaging							
155	System GeneGenius (SynGene, Cambridge, United Kingdom).							

156	In this study, a second set of species-specific PCRs was developed, using a different part of							
157	the genome as a target sequence for primer annealing compared to the region used by Ward							
158	and Timmins (1999). The <i>dnaJ</i> and <i>dnaK</i> genes were targeted. All of the sequences of these							
159	genes available in GenBank for species of the L. casei group were aligned using the MultAlin							
160	software (Corpet, 1988), and the primer pairs designed were dnaKRHf/dnaKRHr,							
161	dnaKCPf/dnaKCPr, and dnaJCPf/dnaJCPr (Table 2), which were specific to the L. casei							
162	group for L. rhamnosus, L. paracasei/L. casei and L. paracasei, respectively. Before							
163	optimisation of the amplification protocol, primer specificity was tested in silico using the							
164	FastPCR 6.1 software (Kalendar et al., 2009) and in vivo using Lactobacillus fermentum (DSM							
165	20049), L. pontis (DSM 8475), L. sanfranciscensis (DSM 20451), L. brevis (DSM 20054), L.							
166	reuteri (DSM 20053), L. plantarum (DSM 20174), L. sakei (DSM 6333), Lactococcus lactis							
167	(DSM 20481), Leuconostoc citreum (DSM 5577), Leuc. gasicomitatum (DSM 15947), Leuc.							
168	mesenteroides subsp. mesenteroides (DSM 20343) and Pediococcus pentosaceus (DSM 20336) as							
169	negative controls.							
170	The reactions were performed in a final volume of 25 μ l containing 10 mM Tris HCl (pH 8.3),							
171	50 mM KCl, 1.5 mM MgCl ₂ , 0.2 mM dNTPs, 0.2 mM of each primer and 1.25 U of Taq-							
172	polymerase (Applied Biosystems, Milan, Italy). PCR was performed using the thermal							
173	cycling protocol described above, with the annealing temperatures shown in Table 2.							
174								
175	2.5. tuf multiplex PCR							
176								
177	Amplification reactions were performed with a 50 μ l (total volume) solution containing 10							
178	mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 200 µM each dNTP, 10 pmol each of primers							
179	PAR (5'-GACGGTTAAGATTGGTGAC-3'), CAS (5'-ACTGAAGGCGACAAGGA-3'),							

- 180 and RHA (5'-GCGTCAGGTTGGTGTTG-3'), 50 pmol of primer CPR (5'-
- 181 CAANTGGATNGAACCTGGCTTT-3') (Ventura et al., 2003), 25 ng of template DNA, and

182	2.5 U of Taq-DNA polymerase (Applied Biosystems, Milan, Italy). Amplification reactions						
183	were performed using a thermocycler (Perkin-Elmer Cetus 9700) with the following						
184	temperature profiles: 1 cycle at 95 °C for 5 min; 30 cycles at 95 °C for 30 s, 54 °C for 1 min,						
185	and 72 $^{\circ}$ C for 1.5 min; and 1 cycle at 72 $^{\circ}$ C for 7 min, in a Thermal Cycler (DNA Engine						
186	Dyad Peltier Thermal Cycler, BioRad, Milan, Italy). PCR amplicons were analysed by 2%						
187	(w/v) agarose gel electrophoresis in TBE 0.5X buffer at a constant voltage of 7 V/cm,						
188	visualised with ethidium bromide (0.5 μ g/ml), and photographed under UV light at 260 nm,						
189	using the BioImaging System GeneGenius (SynGene, Cambridge, United Kingdom).						
190							
191	2.6. Development and optimisation of High-Resolution Melting (HRM) analysis						
192							
193	The variable regions V1 to V3 flanked by highly conserved sequences within the 16S rRNA						
194	were selected for HRM analysis. Three consolidated primer pairs that have been used to						
195	discriminate different species by DGGE analysis were used to discriminate L. casei, L.						
196	paracasei and L. rhamnosus by HRM analysis: P1V1 and P2V1 (Klijin at al., 1991), BA-338f						
197	and UN-518r (Muyzer et al., 1993), Y1 and Y2 (Young et al., 1991). The analyses were						
198	performed in a 25-µl reaction volume containing 2X HRM PCR Master mix (Qiagen, Milan,						
199	Italy), 0.7 μ M each primer and 100 ng of DNA. The PCR amplifications were performed in a						
200	Rotor-Gene Q (Qiagen, Milan, Italy) with the following conditions: 95 °C for 1 min followed						
201	by 45 cycles at 95 °C for 10 s, 55 °C for 30 s 72 °C for 10 s. After amplification, HRM						
202	analysis was performed from 65 to 90 °C with increments of 0.1 °C/2 sec. The Rotor-Gene Q						
203	series software version 2.2.2 (Qiagen, Milan, Italy) was used to analyse the HRM data. The						
204	melting profiles were subjected to fluorescence normalisation to minimise inter- and intra-run						
205	variability. Difference plots were generated by normalising the melting profiles of strains to a						
206	negative control strain whose melting profile was converted to a horizontal line. Three						
207	difference graphs were obtained for the L. casei, L. paracasei and L. rhamnosus strains using						

208	the fluorescence of each reference strain (per each graph) set as the baseline (confidence level
209	of 90 %) (Andersson et al., 2009; Gurtler et al., 2012). The ScreenClust program (Qiagen,
210	Milan, Italy) was used for Principal Component Analysis (PCA).
211	All analyses were performed in triplicate; positive/negative controls and non-template
212	controls (NTC) were included in each run. For the validation assay, 10 strains were used for
213	each species tested.
214	
215	3. Results and discussion
216	
217	3.1 Preliminary identification by L. casei group-specific PCR
218	
219	A total of 201 strains belonging to the L. casei group were collected from national and
220	international collections (Table 1). The strains were isolated from sources including raw and
221	heat-treated milk, yogurt, milking machines, green/creamy and seasoned cheeses, fermented
222	sausages, sourdoughs, wine, must and cellar equipment, beer, malt, coffee and humans; the
223	source of some strains was unknown. These strains were isolated over several years.
224	Therefore, in some cases, there was no information on the origin or method of identification
225	used. In other cases, biochemical tests or molecular analyses were performed for strain
226	identification. To uniformly identify strains, a preliminary L. casei group-specific PCR was
227	performed. The expected amplicon was obtained from 194 strains (Figure 1), confirming that
228	these strains belonged to the L. casei group. The amplicon was not obtained from 7 strains,
229	and they were excluded from subsequent analyses. None of the negative control strains
230	yielded the amplicon, confirming the specificity of the primers.
231	
232	3.2. Species identification by species-specific PCRs and tuf multiplex PCR

234 The identification methods were tested on the three reference strains and were able to 235 discriminate L. casei, L. paracasei and L. rhamnosus species (Figure 2). Species-specific 236 PCRs yielded an amplicon of the expected size (290 bp) only for the target species, and no 237 amplification product was obtained for the other two L. casei group species (Figure 2, panel 238 A). Similarly, the *tuf* multiplex PCR profiles yielded different numbers of bands for *L. casei*, 239 L. paracasei and L. rhamnosus, which enabled the discrimination of these species. The 240 amplification profile of L. casei comprised five bands of approximately 350, 450, 500, 900 241 and 1100 bp, which was not completely consistent with the profile obtained by Ventura et al. 242 (2003). The L. paracasei amplification profile comprised a strong band of approximately 200 243 bp and a thinner band of 500 bp, which was not always visible (Figure 2, panel B, lines L5 244 and L10); Ventura et al. (2003) obtained strong amplification products corresponding to these 245 sizes. The amplification profile of L. rhamnosus comprised a single amplicon of 246 approximately 500 bp, consistent with Ventura et al. (2003). Although both these techniques 247 discriminated species within the L. casei group, amplification products were also obtained for 248 specific negative control LAB strains (data not shown); these strains yielded a 290-bp 249 amplicon in the species-specific PCR analysis and profiles comparable to the L. casei group 250 species in the *tuf* multiplex PCR analysis. Therefore, a preliminary screening step comprising 251 the L. casei group-specific PCR is required for the identification of LAB isolates using these 252 techniques. 253 Inconsistent results were obtained only for 6 out of the 194-tested L. casei group strains using

the two techniques (Table 3) and, for some of them (2), the obtained results were not

unexpected. In fact, LMG6904 (synonyms ATCC393, DSM20011, CCUG21451) is a well-

256 known strain whose taxonomic classification has been repeatedly modified and is under

257 debate; the Judicial Commission of the International Committee for Systematics of

258 Prokaryotes ruled the following: i) The designation of ATCC334, a strain of *L. paracasei*, as

the neotype of *L. casei* contravenes rules 51b (1) and (2); ii) Typification of *L. casei* (Orla-

260	Jensen 1916) Hansen and Lessel 1971 is based on ATCC393; iii) The proposal to revive the
261	name L. zeae contravenes rules 51b (1) and (2); iv) The name L. paracasei has not been
262	rejected by the Judicial Commission and is legitimate, validly published and may be used as a
263	correct name. This ruling confirms the deliberations (Wayne, 1994) that followed a previous
264	Request for Opinion by Dellaglio et al. (1991) (Dellaglio et al., 1991; Waine, 1994; Dicks et
265	al., 1996; Mori et al., 1997; Chen et al., 2000; Biavati, 2001; Klein, 2001; Dellaglio et al.,
266	2002; Judicial Commission Of The International Committee On Systematics Of Prokaryotes,
267	2008). Identification of the strain DSM4905 (synonym ATCC1158) is also ambiguous based
268	on the species classification provided by the DSM and ATCC collections. In the DSM
269	collection, this strain is considered as the reference strain for the L. paracasei species,
270	whereas the ATCC considers this strain as the reference strain for the L. casei species. The
271	taxonomic classification of these two strains, as well as the remaining four strains out of the 6,
272	(DBPZ0420, DBPZ0571, DBPZ0734 and N2014) requires further studies.
273	For the other 188 strains out of the 196, the two identification methods yielded consistent
274	results, but for 46 out of the 188 strains, the results were in disagreement with the original
275	identification.
276	To confirm these results, two different methods were developed in this study: alternate
277	species-specific PCRs and HRM analysis.
278	The species-specific primer pairs designed for the <i>dnaK</i> and <i>dnaJ</i> genes were specific within
279	the L. casei group; amplicons were obtained exclusively from L. rhamnosus, L. paracasei/L.
280	casei and L. casei using the primer pairs dnaKRHf/dnaKRHr (Figure 2, panel C, a),
281	dnaKCPf/dnaKCPr (Figure 2, panel C, b) and dnaJPAf/dnaJPAr (Figure 2, panel C, c),
282	respectively. All the 194 strains, belonging to the L. casei group, were tested. The results were
283	consistent with the species-specific PCRs and <i>tuf</i> multiplex PCRs for the 188 strains. The data
284	for the 6 unidentified strains LMG6904, DSM4905, DBPZ0420, DBPZ0571, DBPZ0734, and
285	N2014 are shown in Table 3.

287 3.2. High-resolution melting (HRM) analysis

289 HRM analysis was used to resolve inconsistencies between the species-specific PCR and tuf 290 multiplex PCR analyses compared to the original identification. 291 HRM analysis is a novel technique that enables the identification of point mutations in a DNA 292 sequence. It has been previously used to characterize nonstarter lactic acid bacteria (Porcellato 293 et al., 2012a, 2012b), and the results seemed to be promising in discriminating among the L. 294 *casei* group species. This technique involves the amplification of a specific DNA sequence 295 using a primer pair that allows annealing and DNA amplification in all the three species 296 considered. The amplicons were produced using the qPCR technique and SYBR Green as an 297 intercalating fluorescent dye and then subjected to a thermal gradient with temperature 298 increments of 0.1 °C/sec using sensitive instrumentation that enables absolute precision of the 299 temperatures used. By continuously monitoring the fluorescence emitted by SYBR Green, it 300 is possible to assess the exact melting temperature of the amplicon, with a precision of 0.1 °C. 301 Base differences and/or insertions or deletions of one or more bases is revealed, and this 302 enables discrimination between amplicons and, consequently, between species. 303 Before using HRM analysis, a preliminary optimisation step was performed to determine the 304 most effective primer pair among three candidate pairs. For optimisation, six strains whose 305 original identification was confirmed by both species-specific PCRs and tuf multiplex PCRs 306 were used: Lactobacillus casei DSM20178 and LACcas7; Lactobacillus rhamnosus 307 DSM20021 and 2220; Lactobacillus paracasei DSM20258 and DSM5622. HRM analysis on 308 these strains revealed that only the primers P1V1-P2V1 were effective in discriminating 309 among the three species (Figure 3). The primer pairs BA-338f/UN518r and Y1/Y2 yielded 310 amplicons with highly similar melting curves comprising the following melting peaks: 311 DSM20178 L. casei, 85.95 °C; DSM5622 L. paracasei, 85.55 °C; DSM20258 L. paracasei,

- 312 85.38 °C; 2220 L. rhamnosus, 85.47 °C; DSM20021 L. rhamnosus, 85.40 °C; LACcas7 L.
- 313 *casei*, 84.30 °C (using BA-338f/UN518r) and DSM20178 *L. casei*, 84.90 °C; LACcas7 *L.*
- 314 *casei*, 86.40 °C; DSM5622 *L. paracasei*, 84.85 °C; 2220 *L. rhamnosus*, 84.30 °C; DSM20021
- 315 L. rhamnosus, 84.30 °C; DSM20258 L. paracasei, 84.67 °C (using Y1/Y2). Considering these
- 316 data and the melting curves (Figure 3, panel A, a; panel B, a), the normalised melting curves
- 317 (Figure 3, panel A, b; panel B, b) and the principal component analysis (PCA) graphs (Figure
- 318 3, panel A, c; panel B, c), these primer pairs could not be used to discriminate among the
- 319 three species. However, the melting profiles and the normalised fluorescence curves as well as
- 320 the PCA of the amplicons obtained using P1V1/P2V1 allowed to group the strains into 3
- 321 species-specific clusters (Figure 3, panel C, a, b, and c).
- 322 To examine the reproducibility of these data, HRM analysis was performed on five replicates
- 323 for each strain, and the curves overlapped completely. The average melting temperature of the
- 324 standard strains tested was 83.69 ± 0.03 °C for *L. casei* (DSM20178 and LACcas7, 5
- replicates per strain); 81.66 ± 0.06 °C for *L. rhamnosus* (DSM20021 and 2220, 5 replicates
- per strain), and 84.16 \pm 0.04 °C for *L. paracasei* (DSM20258 and DSM5622, 5 replicates per
- 327 strain). Therefore, HRM analysis yielded reproducible results. To highlight the differences
- among the three species, 3 difference graphs were generated using *L. casei* (DSM20171), *L.*
- 329 paracasei (DSM20258) and L. rhamnosus (DSM20021) (confidence level of 90 %) as
- 330 baselines (Figure 4, panel A, B, and C).

The blue, green and pink curves indicate ten replicates of the two *Lactobacillus rhamnosus*, *L. casei* and *L. paracasei* strains, respectively. When one species was used as the baseline, the fluorescence values for that species were almost a flat line, whereas the other two species had different performance curves. These graphs indicate the difference in the amplitudes of the curves and that this technique clearly discriminated the three species. Furthermore, the replicates yielded overlapping normalised curves, confirming the reproducibility of this

technique. The different graph amplitudes are derived from melting curves that are always

338 normalised to the same number of arbitrary fluorescence units by the Corbett 6000 software; 339 therefore, these amplitudes can be compared across different runs using *L. casei* (DSM20178), 340 L. paracasei (DSM20258) and L. rhamnosus (DSM20021) as standard controls in each run. 341 After optimisation of HRM analysis, all the 194 strains, confirmed to belong to the L. casei 342 group, were analysed using this method. Because of the large number of strains, more runs 343 were required, and standard controls were included to reveal any changes and to compare all 344 the tested strains at the end of the analysis. Therefore, after PCA, it was possible to identify 345 the strains according to the cluster in which they were grouped (Figure 5). The example 346 shown in Figure 5 demonstrates that the three species were grouped in three well-defined and 347 distant clusters (Figure 5, panel A). The normalised fluorescence curves overlapped 348 completely (Figure 5, panel B); the difference graphs showing the normalised fluorescence 349 curves vs. the control strains, also overlapped completely (Figure 5, panels C). On the basis of 350 the data obtained during the optimization, HRM confirmed to be a potent tool for microbial 351 identification, also considering their advantages: it is a simple, rapid, and inexpensive method, 352 even if depends strongly on good PCR instruments and dyes. Moreover, there is no need to 353 process the sample after the PCR reaction, and this allows to increase the sensitivity of the 354 method in respect to a traditional PCR, followed by agarose gel electrophoresis; it allows the 355 detection and, using appropriate standard curves, also the quantification of several genotypes 356 in qPCR reactions with a single primer pair, in a unique reaction, as performed by Lin and 357 Gänzle (2014). The results of the HRM analysis were consistent with the other methods used 358 in this study, confirming the identity of 188 strains; inconsistent results were obtained only for 359 the 6 strains shown in Table 3. Further studies such as whole-genome sequencing are required 360 to elucidate the taxonomic classification of these strains. For 46 of the remaining 188 strains, 361 the strain identity obtained using this method was inconsistent with the original identification 362 (Table 4). Notably, the strain DIALYac was isolated from a commercial probiotic yogurt and 363 identified as L. casei (Shirota); however, in this study, all methods classified this strain as L.

paracasei, accordingly to with Sutula et al. (2012). Therefore, there is significant ambiguity in
the use of the correct taxonomic name in industrial and scientific settings. In fact, also in
recent studies the old classification name has been used (Douillard et al., 2013).

367

368 **4. Conclusions**

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370 Accurate strain classification is critical for strains that are important for industrial purposes, 371 including strains belonging to the L. casei group, which have probiotic properties. There is 372 significant ambiguity in strain names within the L. casei group because some authors use the 373 new classification system (Dellaglio et al., 2002; Dobson et al., 2004), whereas others do not 374 (Mori et al., 1997; Ward and Timms, 1999; Vásquez et al., 2005; Desai et al., 2006). 375 Furthermore, commercial strains are often described as "L. casei", and this description is used 376 for strains of any of these species. Furthermore, these species share close genetic relationships, 377 and accurate identification is difficult (Nuor, 1998; Beale et al., 2002; Klijn et al., 1991). The 378 use of multiple coupled techniques can elucidate the taxonomic position of some strains; 379 therefore, we proposed two new molecular tools to identify species belonging to the L. casei 380 group: species-specific PCRs and HRM analysis. Both methods yielded accurate results, and 381 considering the large number of strains tested (194), these methods were effective in 382 discriminating among the three species within the L. casei group. For some strains, the results 383 obtained using these methods were inconsistent with the original identification and the results 384 obtained using other molecular methods. This discrepancy is not unexpected because in most 385 cases, the original identification was performed using phenotypical and biochemical tests. 386 These tests are often based on colour changes, which can be misinterpreted because colour 387 changes are rarely precise and sharp. Misinterpretation of these results often leads to an 388 incorrect identification. Furthermore, many strains were identified at a time when only one

389	species, L. casei, and the subsp. paracasei were classified. Therefore, the classification of
390	these strains was not consistent with the current strain taxonomy.

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644	Figure 1. Specific PCR for <i>Lactobacillus casei group</i> (amplicon size 364 bp): line 1: ladder,
645	100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2: DSM20178, L. casei; line 3:
646	DSM20021, L. rhamnosus; line 4: DSM5622, L. paracasei; line 5: DSM 20451, L.
647	sanfranciscensis; line 6: DSM 20054, L. brevis; line 7: DSM 20053, L. reuteri; line 8: DSM
648	20174, L. plantarum; line 9: DSM 6333, L. sakei; line 10: DSM 20481, Lactococcus lactis;
649	line 11: DSM 5577, Leuconostoc citreum; line 12: DSM 15947, Leuc. gasicomitatum; line 13:
650	DSM 20343, Leuc. mesenteroides subsp. mesenteroides; line 14: DSM 20336, Pediococcus
651	pentosaceous; line 15: negative control.
652	
653	Figure 2. Species identification by species-specific PCRs and <i>tuf</i> multiplex PCR. Panel A,
654	Species-specific PCRs by Ward and Timmins (1999). a) Amplification specific for L. casei; b)
655	Amplification specific for L. paracasei; c) Amplification specific for L. rhamnsosus. Line 1, Ladder,
656	100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2, DSM20178, L. casei; line 3, DSM5622, L.
657	paracasei; line 4, DSM20021, L. rhamnosus; NC, negative control. Panel B, tuf multiplex PCR by
658	Ventura et al. (2003). Lanes L1, L14: Ladder 100 bp (New England Biolabs); Lanes L2, L13: Ladder
659	50 bp (New England Biolabs); Lane L3: DSM20021, Lactobacillus rhamnosus; Lane L4: negative
660	control; Lane L5: DSM5622, Lactobacillus paracasei; Lane L6: FSG01, Lactobacillus rhamnosus;
661	Lane L7: DSM20178, Lactobacillus casei; Lane L8: N87, Lactobacillus casei; Lane L9: D44,
662	Lactobacillus rhamnosus; Lane L10: Cst7, Lactobacillus paracasei; Lane L11: N202, Lactobacillus
663	rhamnosus; Lane L12: N1110, Lactobacillus rhamnosus. Panel C, Species-specific PCRs, this study.
664	a) Amplifican specific for L. rhamnosus. Line 1, ladder, 100 bp low ladder (Sigma-Aldrich, Milan,
665	Italy); line 2, negative control; line 3, DSM20021, L. rhamnosus; line 4, N202, Lactobacillus
666	rhamnosus; line 5, DSM20178, L. casei; line 6, N87, Lactobacillus casei; line 7, DSM5622, L.
667	paracasei; line 8, Cst7, Lactobacillus paracasei. b) Amplification specific for L. paracasei/L. casei.
668	Line 1, ladder, 100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2, negative control; line 3,

 DSM20021, <i>L. rhamnosus</i>; line 4, N202, <i>Lactobacillus rhamnosus</i>; lines 5-6, DSM20178, <i>L. casei</i> line 7, N87, <i>Lactobacillus casei</i>; line 8, DSM5622, <i>Lactobacillus paracasei</i>. e) Amplification spec for <i>L. paracasei</i>. Line 1, ladder, 100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2, negative control; line 3, DSM20021, <i>L. rhamnosus</i>; line 4, N202, <i>Lactobacillus rhamnosus</i>; line 5, DSM562 <i>Lactobacillus paracasei</i>; line 6, Cst7, <i>Lactobacillus paracasei</i>; line 7, LMG13087, <i>L. paracasei</i>; li 8, DSM20178, <i>L. casei</i>. Figure 3. HRM results obtained using the three different couples of primers. Panel A, primers BA-338f / UN518r; Panel B, primers Y1 / Y2; Panel C, primers P1V1 / P2V1. a) Melting curves profiles; b) Normalized melting curves; c) Principal component analysis (PCA). Figure 4. Difference graphs obtained for the ten replicates of the three standard speci panel C) <i>L. rhamnosus</i> was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, casei; cluster 2, <i>L. paracasei</i>; cluster 3, <i>L. rhamnosus</i>. 		
 bine 7, N87, Lactobacillus casei; line 8, DSM5622, Lactobacillus paracasei. e) Amplification spec for L. paracasei. Line 1, ladder, 100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2, negative control; line 3, DSM20021, L. rhamnosus; line 4, N202, Lactobacillus rhamnosus; line 5, DSM562 Lactobacillus paracasei; line 6, Cst7, Lactobacillus paracasei; line 7, LMG13087, L. paracasei; line 6, St7, Lactobacillus paracasei; line 7, LMG13087, L. paracasei; line 7, SSM562 Figure 3. HRM results obtained using the three different couples of primers. Panel A, primers BA-338f / UN518r; Panel B, primers Y1 / Y2; Panel C, primers P1V1 / P2V1. a) Melting curves profiles; b) Normalized melting curves; c) Principal component analysis (PCA). Figure 4. Difference graphs obtained for the ten replicates of the three standard speci Panel A) L. casei was used as the baseline; panel B) L. paracasei was used as the baseline panel C) L. rhamnosus was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. 	669	DSM20021, L. rhamnosus; line 4, N202, Lactobacillus rhamnosus; lines 5-6, DSM20178, L. casei;
 for <i>L. paracasei</i>. Line 1, ladder, 100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2, negative control; line 3, DSM20021, <i>L. rhamnosus</i>; line 4, N202, <i>Lactobacillus rhamnosus</i>; line 5, DSM567 <i>Lactobacillus paracasei</i>; line 6, Cst7, <i>Lactobacillus paracasei</i>; line 7, LMG13087, <i>L. paracasei</i>; li 8, DSM20178, <i>L. casei</i>. Figure 3. HRM results obtained using the three different couples of primers. Panel A, primers BA-338f / UN518r; Panel B, primers Y1 / Y2; Panel C, primers P1V1 / P2V1. a) Melting curves profiles; b) Normalized melting curves; c) Principal component analysis (PCA). Figure 4. Difference graphs obtained for the ten replicates of the three standard speci Panel A) <i>L. casei</i> was used as the baseline; panel B) <i>L. paracasei</i> was used as the baseline panel C) <i>L. rhamnosus</i> was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, <i>casei</i>; cluster 2, <i>L. paracasei</i>; cluster 3, <i>L. rhamnosus</i>. 	670	line 7, N87, Lactobacillus casei; line 8, DSM5622, Lactobacillus paracasei. c) Amplification specific
 control; line 3, DSM20021, <i>L. rhamnosus</i>; line 4, N202, <i>Lactobacillus rhamnosus</i>; line 5, DSM56 <i>Lactobacillus paracasei</i>; line 6, Cst7, <i>Lactobacillus paracasei</i>; line 7, LMG13087, <i>L. paracasei</i>; li 8, DSM20178, <i>L. casei</i>. Figure 3. HRM results obtained using the three different couples of primers. Panel A, primers BA-338f / UN518r; Panel B, primers Y1 / Y2; Panel C, primers P1V1 / P2V1, a) Melting curves profiles; b) Normalized melting curves; c) Principal component analysis (PCA). Figure 4. Difference graphs obtained for the ten replicates of the three standard specifies panel C) <i>L. rhamnosus</i> was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, <i>casei</i>; cluster 2, <i>L. paracasei</i>; cluster 3, <i>L. rhamnosus</i>. 	671	for L. paracasei. Line 1, ladder, 100 bp low ladder (Sigma-Aldrich, Milan, Italy); line 2, negative
 <i>Lactobacillus paracasei</i>; line 6, Cst7, <i>Lactobacillus paracasei</i>; line 7, LMG13087, <i>L. paracasei</i>; line 3, L, paracasei; line 7, LMG13087, <i>L. paracasei</i>; line 7, L, paracasei; line 1, <i>casei</i>; line 4, Difference graphs obtained for the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, <i>casei</i>; cluster 2, <i>L. paracasei</i>; cluster 3, <i>L. rhamnosus</i>. 689 689 689 689 681 682 683 683 684 684 685 686 687 688 689 689 690 691 692 693 	672	control; line 3, DSM20021, L. rhamnosus; line 4, N202, Lactobacillus rhamnosus; line 5, DSM5622,
 8, DSM20178, <i>L. casei</i>. Figure 3. HRM results obtained using the three different couples of primers. Panel A, primers BA-338f / UN518r; Panel B, primers Y1 / Y2; Panel C, primers P1V1 / P2V1. a) Melting curves profiles; b) Normalized melting curves; c) Principal component analysis (PCA). Figure 4. Difference graphs obtained for the ten replicates of the three standard speci Panel A) <i>L. casei</i> was used as the baseline; panel B) <i>L. paracasei</i> was used as the baseline panel C) <i>L. rhamnosus</i> was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, <i>casei</i>; cluster 2, <i>L. paracasei</i>; cluster 3, <i>L. rhamnosus</i>. 	673	Lactobacillus paracasei; line 6, Cst7, Lactobacillus paracasei; line 7, LMG13087, L. paracasei; lines
 Figure 3. HRM results obtained using the three different couples of primers. Panel A, primers BA-338f / UN518r; Panel B, primers Y1 / Y2; Panel C, primers P1V1 / P2V1. a) Melting curves profiles; b) Normalized melting curves; c) Principal component analysis (PCA). Figure 4. Difference graphs obtained for the ten replicates of the three standard speci Panel A) L. casei was used as the baseline; panel B) L. paracasei was used as the baseline panel C) L. rhamnosus was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. 	674	8, DSM20178, <i>L. casei</i> .
 Figure 3. HRM results obtained using the three different couples of primers. Panel A, primers BA-338f / UN518r; Panel B, primers Y1 / Y2; Panel C, primers P1V1 / P2V1. a) Melting curves profiles; b) Normalized melting curves; c) Principal component analysis (PCA). Figure 4. Difference graphs obtained for the ten replicates of the three standard species Panel A) L. casei was used as the baseline; panel B) L. paracasei was used as the baseline panel C) L. rhamnosus was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. 689 690 691 692 693 	675	
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 Melting curves profiles; b) Normalized melting curves; c) Principal component analysis (PCA). Figure 4. Difference graphs obtained for the ten replicates of the three standard species Panel A) L. casei was used as the baseline; panel B) L. paracasei was used as the baseline panel C) L. rhamnosus was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. 689 690 691 692 693 	677	primers BA-338f / UN518r; Panel B, primers Y1 / Y2; Panel C, primers P1V1 / P2V1. a)
 (PCA). Figure 4. Difference graphs obtained for the ten replicates of the three standard species Panel A) L. casei was used as the baseline; panel B) L. paracasei was used as the baseline panel C) L. rhamnosus was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. 688 689 690 691 692 693 	678	Melting curves profiles; b) Normalized melting curves; c) Principal component analysis
 Figure 4. Difference graphs obtained for the ten replicates of the three standard species Panel A) L. casei was used as the baseline; panel B) L. paracasei was used as the baseline panel C) L. rhamnosus was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. 689 690 691 692 693 	679	(PCA).
 Figure 4. Difference graphs obtained for the ten replicates of the three standard species Panel A) L. casei was used as the baseline; panel B) L. paracasei was used as the baseline panel C) L. rhamnosus was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. 689 690 691 692 693 	680	
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 panel C) L. rhamnosus was then used as the baseline. Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. casei <licasei< li=""> casei casei casei<td>682</td><td>Panel A) L. casei was used as the baseline; panel B) L. paracasei was used as the baseline;</td></licasei<>	682	Panel A) L. casei was used as the baseline; panel B) L. paracasei was used as the baseline;
 Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. 688 689 690 691 693 	683	panel C) L. rhamnosus was then used as the baseline.
 Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, <i>casei</i>; cluster 2, <i>L. paracasei</i>; cluster 3, <i>L. rhamnosus</i>. 688 689 690 691 693 	684	
 686 analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, 687 casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. 688 689 690 691 692 693 	685	Figure 5. HRM analysis of 46 out of the 196 strains. Panel A, Principal component
 687 casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus. 688 689 690 691 692 693 	686	analysis; panel B, Normalised fluorescence curves; panels C, difference graphs. Cluster 1, L.
 688 689 690 691 692 693 	687	casei; cluster 2, L. paracasei; cluster 3, L. rhamnosus.
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707 Table 1. Origin and given identification of the 199 strains collected for the study.

Origin	Given identification
Raw and heat treated milk,	<i>L. paracasei</i> : LMG91921, DSM56222
yoghurt, milking machines	L. Casei/paracasei: P1E53, P1E63, P2P33
	L. paracasei subsp. tolerans: LMG91911, P1E43, DSM202582
	L. rhamnosus:, HA1114, PRA1525, CI2305
Green, creamy and seasoned	L. casei: LMG69041, TMW1.14446, TMW1.12596, LACcas137, LACcas77
cheeses	L. paracasei: LMG258801, LMG258831, LMG121641, DBPZ04218, DBPZ04228,
	DBPZ04248, DBPZ04348, DBPZ04358, DBPZ04508, DBP04518, DBPZ04728,
(Italian cheeses: Scamorza,	DBPZ04758, DBPZ04768, DBPZ04778, DBPZ04788, DBPZ06358, DBPZ07338, M2668,
Parmigiano Reggiano, Grana	M2688, M2998, M3088, M3488, M3548, M3598, S18, S38, V38, W118, DSG038, DSG058,
Padano, Spressa, Asiago,	DSG078, ESG108, HSG098, PSG068, PSG098, PSG108, P719, TH12299, SP579, L249,
Montasio, Canestrato di	TH4069, FSL43610, FSL45110, DBPZ04368, DBPZ04288, M3358, M2908, M3038, H1213
Moliterno, Morlacco, Bellunese,	L. casei/paracasei: Cst711, 3LC11, DBPZ07188, M3078
Pecorino, Caciocavallo,	L. rhamnosus: M159, O148, PRA2045, PRA2325, PRA3315, DBPZ04308, DBPZ04458,
Provolone, Emmenthal, Raclette	DBPZ04468, DBPZ04488, DBPZ04498, FSG018, C1436212, CF135012, CF37/12, D4413,
de Savoie; Chinese and Tunisian	H2513, 5A919, 5D919, L99, L479, CI436812, DBPZ04208, DBPZ07348, CF14312, R6113,
Economical service ser	F1/13, N2413
Fermented sausages	L. casel/paracasel: CTC16/514
	L. casel/mamnosus: C1C16/614, 222015
Sourdoughs	L. paracasei: DBPZ05618, DBPZ05718, DBPZ05728, Q28, Q48, I14, I216
	<i>L. casei/paracasei:</i> DBPZ05638, DBPZ05648, DBPZ05798, I316
Wine, must and cellar	<i>L. paracasei</i> : LMG119611, LMG119631, LMG137171, LMG137311, B06117, B08217,
equipment's	B08317, B08517, B08717, B16117, B16917, B17117, B17417, B19517, B19617, B35017,
	B16617, B08417, B08617, B16317, B16417, B16717, B16817, B17017, B17217, B17317,
	B17517, B17917
Bier, malt	<i>L. casei</i> : LACcas257, LACcas297, TMW 1.3006
Coffee	<i>L. casei</i> : DSM201782
	L. rhamnosus: DIAL4015
Humans	<i>L. casei</i> : LMG235161
(saliva, dental caries, blood,	<i>L. zeae:</i> N8716
urethra, faeces of infants and	L. paracasei: DSM200202, LMG94381, LMG114591, LMG235111, LMG235181,
adults)	LMG235231, LMG235381, LMG235431, LMG240981, LMG241011, LMG241321,
	DBTA3418, DSM49052
	<i>L. casei/paracasei</i> : N16116, N4216, N4416, N7616, N171016
	L. rhamnosus: DBTA8618, DBTC418, N17/116, N17816, N71516, N9416, N9516, N8316,
	N20116, N20916, N201216, N13216, N2216, N2616, N81216, N17316, N111016, N13116,
	N2116, N1/216, N201016, N201316, N20216, N2516, N1/616, N201116, TMW 1.15386,
T T 1	MO216, N81116, N201416, N1/516
Unknown	L. paracaser: NKRL B-45619, DSM56222
	L. rhamnosus: NKKL B-17619, NKRL B-44219, DSM200212

1LMG: BCCM/LMG, Belgian Co-ordinated Collections of Micro-organisms (BCCMTM), Belgium.

2DSM: DSM, Deutsche Sämmlung von Mikroorganismen und Zellkülturen, Braunschweig, Germany

3Dipartimento di Agraria, Università degli Studi di Sassari, Sassari, Italy.

4Harmonium International Inc., Mirabel, Canada.

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9Università degli Studi di Verona, Dipartimento di Biotecnologie, Strada le Grazie 15, Verona, Italy

10Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy

11Istituto sperimentale Lattiero Caseario - I.L.C., Lodi, Italy.

12Dipartimento di Scienze e Tecnologie Agro-Alimentari, Unversità degli Studi di Bologna, Bologna, Italy

13 Dipartimento di Scienze delle Produzioni Agrarie e Agroalimentari, Università degli Studi di Catania, Catania, Italy.

14Institut de Recerca I Technologia Agroalimentaries (IRTA), Lleida, Spain

15Dipartimento di Scienze degli Alimenti, Università degli studi di Udine, Udine, Italy.

16Dipartimento di Agricoltura, Ambiente e Alimenti, Unversità degli Studi del Molise, Campobasso, Italy.

17 Institute for Wine Biotechnology Department of Viticulture and Oenology, Stellenbosh University, South Africa

18 Dipartimento di Biotecnologie, Università degli Studi di Verona, Verona, Italy

19 ARS Culture (NRRL) Collection, United States Department of Agriculture, USA

710 Table 2

Taget microrganism	Primer name	Sequence (5'-3')	Amplicon size (bp)	Temperature of annealing (°C)	Reference
L. casei	casei Y2	TGCACTGAGATTCGACTTAA CCCACTGCTGCCTCCCGTAGGAGT	290	53 °C	Ward and Timmins (1999)
L. paracasei	para Y2	CACCGAGATTCAACATGG CCCACTGCTGCCTCCCGTAGGAGT	290	53 °C	Ward and Timmins (1999)
L. rhamnosus	rham Y2	<i>TGCATCTTGATTTAATTTTG</i>	290	53 °C	Ward and Timmins (1999)
L. rhamnosus	dnaKRHf dnaKRHr	GAACAGCAGGGATCC GATCTTTCCGGTGTGA	235	58 °C	This study
L. paracasei/casei	dnaKCPf dnaKCPr	AAACTGTGCCCGCGT GCGACGGGGGTCTTTG	281	59 °C	This study
L. casei	dnaJPAf dnaJPAr	CGGCTGCGAACTGCATTA TTCCTGCTGGCACCCAAA	162	64 °C	This study

Table 3. Comparison of the results obtained using the different techniques on 6 out of the 194 strains: inconsistent results.

	Strain	Original ID	ID Specie- Specific PCR (Ward and Timmins, 1999)	ID Multipex (Ventura et al., 2003)	ID Specie-Specific PCR This work	ID HRM This work
Synonyms	LMG6904* DSM20011 ATCC393 CCUG 21451	L. casei L. casei L. casei L. zeae	L. paracasei	L. casei	L. paracasei	L. paracasei
Synonyms	DSM4905 ATCC 1158	L. paracasei L. casei	L. casei	L. paracasei	L. paracasei	L. casei
	DBPZ0420	L. rhamnosus	L. paracasei	L. rhamnosus	L. rhamnosus L. paracasei	L. paracasei
	DBPZ0571	L. paracasei	L. casei	L. paracasei	L. paracasei	L. casei
	DBPZ0734	L. rhamnosus	L. paracasei	L. rhamnosus	L. paracasei	L. paracasei
	N2014	L. rhamnosus	L. casei	L. rhamnosus	L. casei	L. casei

*In bold it has been underlined the original name of the tested strain, as collected from the International collections (see Table 1 and 4)

- / 20

723 **Table 4.** Final identification of the tested strains.

Origin	Identification
Raw and heat treated milk, yoghurt, milking machines	L. casei: PIE53 L. paracasei: LMG91921, DSM56222, PIE63, P2P33, DIALYac15, DIALDan15 L. paracasei subsp. tolerans: LMG91911, DSM202582 L. rhamnosus:, HA1114, PRA1525, PIE43
Green, creamy and seasoned cheeses	<i>L. casei</i> : C14368 ₁₂ <i>L. paracasei</i> : LMG258801, LMG258831, LMG121641, DBPZ04218, DBPZ04228, DBPZ04248, DBPZ04348, DBPZ04358, DBPZ04508, DBPZ04518, DBPZ04728,
(Italian cheeses: Scamorza, Parmigiano Reggiano, Grana Padano, Spressa, Asiago, Montasio, Canestrato di Moliterno, Morlacco, Bellunese, Pecorino, Caciocavallo,	DBPZ04758, DBPZ04768, DBPZ04778, DBPZ04788, DBPZ06358, DBPZ07338, M2668, M2688, M2998, M3088, M3488, M3548, M3598, S18, S38, V38, W118, DSG038, DSG058, DSG078, ESG108, HSG098, PSG068, PSG098, PSG108, P719, TH12299, SP579, L249, TH4069, FSL43610, FSL45110, DBPZ04368, M2908, M3038, TMW1.14446, TMW1.12596 LACcas77, Cst711, 3LC11, DBPZ07188, CF14312, R6113, F1713, N2413, H1213
Provolone, Emmenthal, Raclette de Savoie; Chinese and Tunisian cheeses)	<i>L. rhamnosus</i> : M159, O148, PRA2045, PRA2325, PRA3315, DBPZ04208, DBPZ04288, DBPZ04308, DBPZ04458, DBPZ04468, DBPZ04488, DBPZ04498, FSG018, CI23012, CI436212, CF135012, CF37712, D4413, H2513, 5A9T9, 5D9T9, L99, L479, LACcas137, M3358, M3078
Fermented sausages	<i>L. paracasei</i> : CTC1675 ₁₄ <i>L. rhamnosus</i> : CTC1676 ₁₄ , 2220 ₁₅
Sourdoughs	<i>L. paracasei</i> : DBPZ05618, DBPZ05728, Q28, Q48, I14, I216, DBPZ05638, DBPZ05648, DBPZ05798, I316
Wine, must and cellar equipment's	<i>L. casei</i> : B16617 <i>L. paracasei</i> : LMG119611, LMG119631, LMG137171, LMG137311, B06117, B08217, B08317, B08517, B08717, B16117, B16917, B17117, B17417, B19517, B19617, B35017 <i>L. rhamosus</i> : B08417, B08617, B16317, B16417, B16717, B16817, B17017, B17217, B17317, B17517, B17917
Bier, malt	L. paracasei: LACcas257, LACcas297, TMW 1.3005
Coffee	L. casei: DSM201782 L. rhamnosus: DIAL4015
Humans (saliva, dental caries, blood, urethra, faeces of infants and adults)	<i>L. casei</i> : LMG235161, <u>N87</u> 16, <u>N81</u> 16 <i>L. paracasei</i> : DSM200202, LMG94381, LMG114591, LMG235111, LMG235181, LMG235231, LMG235381, LMG235431, LMG240981, LMG241011, LMG241321, DBTA3418, <u>N161</u> 16, <u>N42</u> 16, <u>N44</u> 16, <u>N76</u> 16 <i>L. rhamnosus</i> : DBTA8618, DBTC418, N17116, N17816, N71516, N9416, N9516, N8316, N20116, N20916, N201216, N13216, N2216, N2616, N81216, N17316, N111016, N13116, N2116, N17216, N201016, N201316, N20216, N2516, N17616, N201116, TMW 1.15386, Mo216, <u>N1710</u> 16, N17516
Unknown	<i>L. paracasei</i> : NRRL B-45619, DSMZ 56222 <i>L. rhamnosus</i> : NRRL B-17619, NRRL B-44219, DSMZ200212

*Strains with uncertain identification: LMG 69041, DSM49052, DBPZ04208, DBPZ05718, DBPZ07348, N201416

The strains underlined in red didn't result to belong to the *L. casei* group; The identification of the strains underlined in black was in disagreement with the original identification, the new identification has been reported.

- 1LMG: BCCM/LMG, Belgian Co-ordinated Collections of Micro-organisms (BCCMTM), Belgium.
- 2DSM: DSM, Deutsche Sämmlung von Mikroorganismen und Zellkülturen, Braunschweig, Germany
- 3Dipartimento di Agraria, Università degli Studi di Sassari, Sassari, Italy.
- 4Harmonium International Inc., Mirabel, Canada.

- 5Dipartimento di Scienze Agrarie e degli Alimenti, Università delgi Studi di Modena e Reggio Emilia, Italy
- 6Lehrstuhl für Technische Mikrobiologie, Technische Universität München, Freising, Germany
- 7Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, Italy
- «Scuola di Scienze Agrarie, Alimentari e Ambientali, Università degli Studi della Basilicata, Potenza, Italy
- 9Veneto Agricoltura, Istituto per la Qualità e le Tecnologie Agroalimentari, Thiene (VI), Italy
- 10Istituto Zooprofilattico Sperimentale della Sardegna, Sassari, Italy
- 11Istituto sperimentale Lattiero Caseario I.L.C., Lodi, Italy.
- 12Dipartimento di Scienze e Tecnologie Agro-Alimentari, Unversità degli Studi di Bologna, Bologna, Italy
- 13 Dipartimento di Scienze delle Produzioni Agrarie e Agroalimentari , Università degli Studi di Catania, Catania, Italy.
- 14Institut de Recerca I Technologia Agroalimentaries (IRTA), Lleida, Spain
- 15Dipartimento di Scienze degli Alimenti, Università degli studi di Udine, Udine, Italy.
- 16Dipartimento di Agricoltura, Ambiente e Alimenti, Unversità degli Studi del Molise, Campobasso, Italy.
- 17 Institute for Wine Biotechnology Department of Viticulture and Oenology, Stellenbosh University, South Africa
- 18 Dipartimento di Biotecnologie, Università degli Studi di Verona, Verona, Italy
- 19 ARS Culture (NRRL) Collection, United States Department of Agriculture, USA