Melting curve analysis of a *groEL* PCR fragment for the rapid genotyping of strains belonging to the *Lactobacillus casei* group of species

Short title: HRMa to distinguish *L. casei* group of species

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Abstract

L. casei group (*Lc*s) consists of three phylogenetically closely related species (*L. casei*, *L. paracasei*, and *L. rhamnosus*), which are widely used in the dairy and probiotic industrial sectors. Strategies to easily, and rapidly characterize *Lc*s are therefore of interest. To this aim, we developed a method according to a technique known as high resolution melting analysis (HRMa), which was applied to a 150 bp *groEL* gene fragment. The analysis was performed on 53 *Lc*s strains and 29 strains representatives of species that are commonly present in dairy and probiotic products and can be most probably co-isolated with *Lc*s strains. DNA amplification was obtained only from *Lc*s strains, demonstrating the specificity of the *groEL* primers designed in this study. The HRMa clustered *Lc*s strains in three groups that exactly corresponded to the species of the *L. casei* group. A following HRMa separated the 39 *L. paracasei* strains in two well distinct intraspecific groups, indicating the possible existence of at least two distinct genotypes inside the species. Nonetheless, the phenotypic characterization demonstrated that the genotypes do not correspond to the two *L. paracasei* subspecies, namely *paracasei* and *tolerans*. In conclusion, the melting curve analysis developed in this study is demonstrably a simple, labor-saving, and rapid strategy obtain the genotyping of a bacterial isolate and simultaneously potentially confirm its affiliation to the *L. casei* group of species. The application of this method to a larger collection of strains may validate the possibility to use the proposed HRMa protocol for the taxonomic discrimination of *L. casei* group of species. In general, this study suggests that HRMa can be a suitable technique for the genetic typization of *Lactobacillus* strains.

Keywords: probiotic; *Lactobacillus*; HRM analysis; molecular typing

1. Introduction

Lactobacillus casei, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* are three distinct species of phylogenetically closely related facultative heterofermentative lactic acid bacteria (LAB), collectively known as the species of the *L. casei* group (*Lc*s). *Lc*s includes typical non-starter LAB, commonly isolated from human gut (Wall et al., 2007) and vaginal mucosa, and widely found in many traditional fermented foods, where they contribute, through specific biochemical activities, to the quality and, in many cases, to the preservation of finished products. Particularly, they represent the major components of the autochthonous microbial population of many traditional cheeses and are used as subsidiary cultures (or ripening cultures) in cheese production (Colombo et al., 2009; Franciosi et al., 2008; Koirala et al., 2014).

Many studies have also shown the health-promoting potential of *Lc*s, as demonstrated by their extensive use as probiotics (Ferrario et al., 2014), or mixed cultures in dairy products and, as functional component, in a new generation of probiotic food products, including soy yoghurt (Donkor et al., 2005) and table olives (Lavermicocca et al., 2010). In fact, the two most-studied and best-known probiotic microorganisms worldwide are *Lactobacillus paracasei* Shirota (Yakult Honsha Co., Ltd.) and *Lactobacillus rhamnosus* GG (Valio Ltd.). However, the high relatedness of the members of this bacterial group makes difficult their differentiation. As a consequence, the taxonomic position and nomenclature of this group has been controversial until recently (Collins et al., 1989; Dellaglio et al., 1991; Tindall, 2008).

Correct taxonomic assignment of microorganisms deliberately used in food and feed is a mandatory requirement, according to the Joint FAO/WHO Expert Meeting reports on probiotics (FAO/WHO, 2002) and the European Food Safety Authority (EFSA) consultations on the Qualified Presumption of Safety (QPS) approach (EFSA, 2004). Manufacturers, in fact, must follow the recommendation that each labeled denomination for microbial species conforms to the International Code of Nomenclature of Bacteria (Lapage et al., 1992).

Another important aspect concerning industrial strains refers to the fact that technological or probiotic features are mostly strain dependent and, therefore, genotyping methods are needed to assure quality management (Herbel et al., 2013). Several methods of genetic typization have been used to characterize strains belonging to the *Lc*s (Diancourt et al., 2007; Dimitonova et al., 2008; Capra et al., 2011; Sato et al., 2012); however, these methods have often inherent limitations. For instance, multi-locus sequence typing protocols (Diancourt et al., 2007) need DNA sequencing steps that make the protocol quite expensive and time-intensive. Furthermore, PCR-based fingerprinting techniques such as RAPD, ERIC-PCR, BOX-PCR, and rep-PCR (Capra et al., 2011; Dimitonova et al., 2008) are poorly reproducible and

do not allow the confirmation that the isolate under investigation actually belongs to the *L. casei* group of species. Also MALDI-TOF MS of ribosomal proteins (Sato et al., 2012) have been proposed for *Lc*S characterization, but this method imply a quite complex protocol that requires equipment not easily found in the research and clinical laboratories of microbiology.

In this study, we designed a protocol to run a melting curve analysis of a small hyper-variable region that we identified inside the *Lactobacillus groEL* gene. Gene *groEL* codes for the 60-kDa group I chaperonin GrolEL that, because of its essential function, exhibits high sequence conservation across species (Goyal et al., 2006). Our results show that melting curve analysis protocol here presented is a simple and fast strategy to perform the genetic typization of bacterial strains belonging to the species of the *L. casei* group.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Eighty-two bacterial isolates originating from human intestine, vagina, oral cavity, dairy products, and commercial probiotic foods were included in this study (Table 1). These isolates were obtained from the bacterial culture collection of our Department or from international culture collections (American Type Culture Collection, ATCC; Deutsche Sammlung von. Mikroorganismen und Zellkulturen, DSMZ; Belgian Coordinated Collections of Microorganisms BCCM/LMG). Lactobacilli were grown in de Man, Rogosa and Sharpe (MRS) (Fluka, Sigma-Aldrich, St. Louis, MO, USA). Bifidobacteria were cultivated in MRS (Difco Lab., Augsburg, Germany) supplemented with 0.05 % cysteine-HCl in anaerobic conditions. Streptococci and enterococci were grown in M17 (Difco Lab.) supplemented with 2 % lactose or glucose. *Bacillus* was cultivated in nutrient broth (Difco). All strains were incubated at 30°C, with the exceptions of *Lactobacillus helveticus*, *Streptococcus thermophilus* and bifidobacteria that were grown at 37°C.

2.2. DNA extraction, conventional PCR and sequencing

Genomic DNA for all PCR reactions was extracted from 100 µl of an overnight culture diluted with 300 µl of TE 1 X buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, pH 8.0) as described by Arioli et al. (2007). End-point PCR was carried out with DreamTaq DNA Polymerase (Fermentas, Vilnius, Lithuania) according to manufacturer instruction. All the others PCR reagents were from Fermentas. Species-specific PCR for *L. casei* species group was performed with conserved primerY2 (5'-CCCACTGCTGCCTCCCGTAGGAGT-3') and specific primers CASEI (5'-TGCACTGAGATTCGACTTAA-3'), PARA (5'-CACCGAGATTCAACATGG-3') or RHAMN (5'-TGCATCTTGATTTAATTTTG-3'), as described by Ward and Timmins (1999). The hyper-variable *groEL* region

identified in this study was amplified with primers GroHRM-F (5'-GTTTGATCGCGGCTATCTGA-3') and GroHRM-R (5'-CCTTGTTGMACGATTTCTTG-3') through a PCR profile consisting of an initial denaturation time of 2 min at 95 °C followed by an amplification for 35 cycles of denaturation (45 s at 94 °C), annealing (45 s at 59 °C) and extension steps (30 s at 72 °C); the PCR was completed with an elongation period (5 min at 72 °C). Amplicons obtained through end-point PCR with GroHRM primers were purified from reaction master mix with UltraClean PCR Clean-Up Kit (MoBio, Cabru S.a.s., Arcore, Italy) and then sequenced on both strands (Primm srl, Milan, Italy). GenBank accession numbers of the 14 partial *groEL* gene sequences determined in this study are indicated in Table 1.

2.3. Real-time quantitative PCR and high resolution melting analysis

The concentration and purity of the DNA samples were determined spectrophotometrically with a Take3 Micro-Volume Plate in the Eon BioTek Microplate Spectrophotometer (AHSI S.p.A., Bernareggio, Italy). After quantification, DNA samples were diluted with nuclease-free water to reach a concentration of 5 ng μl^{-1} and stored at -20°C. Quantitative real-time polymerase chain reactions (qPCR) were carried out in a final volume of 15 µl containing 7.5 µl of SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories S.r.l., Segrate, Italy), 0.25 µM of each primer and 15 ng of template DNA. During the set-up of the protocol, DNA template concentrations between 50 and 0.08 ng were also tested. qPCR experiments were performed in a CFX96 thermo-cycler (Bio-Rad Laboratories) as follows: initial denaturation at 98°C for 1 min, followed by 45 cycles at 95°C for 30 s, 59°C for 20 s and 72°C for 1 s; a unique step of 65°C for 10 s was finally performed. Each DNA sample was analysed at least in duplicate. At the end of the amplification protocol, melting curves of the amplicons were immediately determined by monitoring fluorescence from 65 to 95°C, with temperature increments of 0.2°C.

For high resolution melting curve analysis (HRMa), raw fluorescence data were exported from Bio-Rad CFX-Manager software and a single text (.txt) file for each strain was prepared by indicating data from 70 to 95°C as depicted below:

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Temperature       Fluorescence
70.00             8383.74
70.20             8348.91
70.40             8314.21
70.60             8279.45
...               ...
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The .txt documents were used as input files for software uAnalyze[SM] v. 1.8 (Dwight et al., 2012); available on line at https://www.dna.utah.edu/uv/uv.php), which was employed for the HRMa. The software parameters used for the normalization of raw fluorescence melt data were as follows: horizontal sliders set at temperatures 78°C and 79.5°C for the analysis of *L. casei* species, and set at 78°C and 81°C for the analysis of *L. paracasei* intra-species genotypes.

2.4. Phenotypical tests

Carbon source fermentation was determined by using a microtitre plate assay and a basal CHL medium at pH 6.3 (Bio-Merieux., Montelieu-Vercieu, France) containing bromocresol purple as indicator, and the desired filter-sterilized carbohydrate at a final concentration of 0.5% (w/v). The plates were incubated at 30°C and were examined for color change after 24 and 48 h of incubation. Survival assay at 72°C was carried out by sinking for 40 s in a water bath a glass capillary tube containing the bacterial suspension. Growth at 40°C and survival at 72°C were determined in MRS broth. The residual viable population was determined by plate counting on MRS agar after 48–72 h of incubation at 30°C.

3. Results and discussion

Lactobacilli are one of the most important taxonomic groups of microorganisms involved in food microbiology and human nutrition, for which genetic typization and unambiguous identification must be considered a prerequisite for their correct use. In the last decade, a high number of systematic studies of lactobacilli have been carried out using molecular approaches targeting several genes, such as *16s rRNA*, *tuf*, *recA* or *rpoB* genes (Chavagnat et al., 2002; Felis et al., 2001; Piwat and Teanpaisan, 2013; Rantsiou et al., 2004; Ventura et al., 2003). A few years ago, Blaiotta et al. proposed a partial DNA sequence (499-bp) of the gene *hsp60* (*groEL*, encoding a 60-kDa heat shock protein) as a useful molecular marker for the genetic typing of *Lactobacillus* strains isolated from food samples (Blaiotta et al., 2008). Therefore, we analyzed this genetic target with the aim to identify an internal region with a potential discriminative power sufficiently high to genetically characterize strains belonging to the *L. casei* group (*Lc*s). To this aim, we performed a ClustalW alignment of the *groEL* gene sequences from the type strains of the species *L. casei*, *L. paracasei* and *L. rhamnosus*. Consequently, we identified a 110 bp region with relatively high sequence variability inside the *groEL* gene (Fig. 1). Then, we designed a pair of primers (named GroHMR) in the conserved flanking regions (Fig. 1), which allowed obtaining a 150 bp PCR amplicon from *Lc*s strains. Subsequently, we generated a dendrogram using the Neighbor Joining algorithm based on 13 *groEL* sequences available in GenBank and 14 sequences obtained during this study from *Lc*s strains available in our laboratory (Table 1; Fig. 2). The resulting dendrogram separated the bacterial strains into three clusters, which exactly corresponded to the species of the *L. casei* group. The type strain of the species *L. casei* clustered with strain *L. zeae* ATCC 15820 (Fig. 2). Accordingly, the International Committee on Systematics of Bacteria rejected the species *L. zeae* and included it in the species *L. casei* (Tindall et al., 2008). Furthermore, several strains (viz. ATCC 334, ATCC 25598, DSA-15, IMAU60056 and DSM 5622^{T}), though annotated in GenBank as *L. casei*, resulted to belong to the species *L. paracasei*. A possible explanation

for these misidentifications can be found in the fact that until quite recently the type strain of the species *L. casei* was ATCC 334, which now has been reclassified as a *L. paracasei* (Tindall et al., 2008). Therefore, our results showed that the selected 150 bp *groEL* region can be potentially useful in distinguishing among *L. casei*, *L. paracasei*, and *L. rhamnosus*.

We then tested in real-time quantitative PCR (qPCR) the specificity of GroHM primers on 53 strains belonging to the *L. casei* group (Table 1). The taxonomic identification of these strains was confirmed by PCR amplification with species-specific primers (Ward and Timmins, 1999). In addition, we also included in the analysis strains belonging to 25 different bacterial species (including 13 *Lactobacillus* species besides *Lc*s), which are commonly present in dairy and probiotic products and can be most probably co-isolated with *Lc*s strains. Among the tested bacteria, we included strains isolated from commercial probiotic products that claimed to contain a *Lc*s bacterium. Our experiments with species-specific primers (Ward and Timmins, 1999) confirmed the presence of *L. rhamnosus* in all products that labeled this species. In contrast, we found *L. paracasei* in all products asserting to include a *L. casei* strain. We never found *L. casei* in them according to PCR with species specific primers. Furthermore, in accordance with a technical opinion by EFSA (2010), our analyses confirm that the well-known bacterial strain Shirota, differently from what is claimed on the label, belongs to the species *L. paracasei* and not to *L. casei*. Though widespread use of misclassified microbial species or species with fictitious names in commercial probiotic products have been reported (Aureli et al., 2010), we believe that what we observed in this study should not be attributed to poor quality control by manufacturers but rather to the confusion that has persisted until recently about distinguishing between *L. casei* and *L. paracasei* (Dellaglio et al., 2002; Tindall, 2008).

After PCR with GroHRM primers according to the proposed protocol, we obtained an amplification curve with a Ct between 21 and 27 exclusively from strains that were previously identified as *L. casei*, *L. paracasei*, or *L. rhamnosus*, whereas the strains belonging to other taxa resulted in a Ct over 35 (Data not shown 1, only for referees). Therefore, the proposed real time PCR with GroHMR primers allowed the discrimination between *Lc*s strains and strains belonging to other taxa. However, the analysis of a larger collection of strains is needed to demonstrate the ability of GroHRM primers to amplify exclusively *Lc*s bacteria.

Since real time PCR amplification curves did not allow the discrimination among the *Lc*s strains included in our study, we developed a strategy based on melting curve analysis of the 150 bp *groEL* gene fragment amplified with GroHMR primers. We based our protocol on a technique known as high resolution melting analysis (HRMa). HRMa protocols, developed to detect small variations in DNA sequences, have found important applications in single

nucleotide polymorphism (SNP) and methylation studies (Messiaen et al., 2012; Pistek et al., 2012) and in clinical microbiology (Ruskova and Raclavsky, 2011) for identifying, detecting, and typing several pathogens, such as *Mycobacterium tuberculosis* (Lee and Ong, 2012), *Staphylococcus aureus* (Chen et al., 2012), *Listeria* spp. (Jin et al., 2012), *Clostridium difficile* (Pecavar et al., 2012), *Salmonella* serovars (Zeinzinger et al., 2012), *Enterococcus faecium* (Tong et al., 2011), and fungal pathogens (Somogyvari et al., 2012). Using HRMa to study food associated lactic acid bacteria is much more limited. To the best of our knowledge, only one study employed HRM for the characterization of lactic acid bacteria; particularly, in this study, HRM was applied to analyze denaturing gradient gel electrophoresis (DGGE) bands obtained from dairy products (Porcellato et al., 2012). In addition, recently, the use of HRMa targeting genetic regions different from *groEL* was also proposed for the taxonomic identification of species belonging to the *Lactobacillus casei* group (Iacumin et al., 2015).

According to the conventional HRMa protocols, we used in this study a PCR reaction solution including a saturating fluorescent intercalating agent and collected melt data in 0.2°C increments in a Real-Time PCR thermal cycler. Initially, we tested the effect of DNA template concentration on melting curve profiles and accordingly ran melting analyses of PCR amplicons obtained with 5 different concentrations (from 50 to 0.08 ng per reaction) of the DNA isolated from *L. paracasei* DSM 5633^{T}, which was arbitrarily chosen as reference strain. We found that DNA concentrations producing Ct values of less than 20 induced a significant shift in the melting curves, which could affect the result of the analysis. In contrast, template concentrations corresponding to Ct values of up to 28 did not affect melting curves (Fig. 3A and 3B). Similar results were obtained with DNA extracted from strains *L. casei* ATCC 339^{T} and *L. rhamnosus* GG, which were selected to represent the other two species constituting the *Lc*s group. Consequently, we used in the following experiments a DNA concentration of 5 ng μL^{-1} since it produced Ct values between 21 and 28. Specifically, we ran the assay with DNA extracted from the 53 *Lc*s strains under study (Table 1). The amplicons that we obtained from these experiments had an average melting temperature (Tm) of 79.12 ± 0.45 °C. The melting curves obtained were then analyzed in order to verify the possibility to cluster the different *Lc*s strains. To this aim, unlike other studies, we used no commercial software specifically designed for HRMa; instead, raw fluorescence melt data were normalized by means of freeware on-line software developed at the University of Utah (uAnalyze^{SM} v. 1.8; Dwight et al., 2012). In a first analysis, the 53 *Lc*s strains were clustered in 3 well distinguished groups (Fig. 4A), which had distinct average Tm values (Fig. 4). This result was obtained by setting the two horizontal sliders of the software at the temperature of 78 and 79.5°C. Notably, the resulting clusters of curves exactly divided strains according to the species (Fig. 4A).

The 39 strains belonging to *L. paracasei* were further analyzed by setting horizontal parameters at 78 and 81°C. In this way, we were able to separate *L. paracasei* strains in two well distinct genotypic groups. Strain clustering through melting curve analysis was in accordance with the dendrogram based on the 150 bp corresponding to the region amplified for melting analysis (Fig. 2). In fact, not only *L. casei, L. paracasei* and *L. rhamnsosus* strains included in the study were well distinguished by the NJ tree, but, similarly to melting analysis, the dendrogram also divided *L. paracasei* strains in two distinct genetic clusters.

Collins et al. (1989) described two subtaxa in the species *L. paracasei*, which correspond to the subspecies *paracasei* and *tolerans* (Tindall, 2008). Melting curves obtained after HRMa analysis of *L. paracasei* strains are shown in Fig. 4B. Group "a" included the type strain of the species *L. paracasei*, DSM 5622^{T}, which is also the reference strain for the subspecies *paracasei*. On the contrary, the reference strain *L. paracasei* subsp. *tolerans* LMG 9191^{T} clustered in group "*b*". We therefore hypothesized that the two genotypes observed inside *L. paracasei* could correspond to the subspecies *L. paracasei* subsp. *paracasei* (group "a") and *L. paracasei* subsp. *tolerans* (group "b"). To corroborate this hypothesis, a number of *L. paracasei* strains were characterized to verify some of the phenotypic features which have been proposed. Specifically, the subspecies *tolerans* has been described as a facultative heterofermentative bacterium that produces acid from lactose, but (differently from *L. paracasei* subsp. *paracasei*) not from amygdalin, arbutin, cellobiose, maltose, mannitol, melezitose, salicin and trehalose (Abo-Elnaga and Kandler, 1965; Collins et al., 1989). Furthermore, *L. paracasei* subsp. *tolerans* can be distinguished from *L. paracasei* subsp. *paracasei* by the fact that does not hydrolyze esculin, does not grow at 40°C, and survive heating at 72°C for 40 s (Abo-Elnaga and Kandler, 1965; Collins et al., 1989). More recent data concerning sugars fermentation abilities in *Lc*s, however, indicate that the above mentioned grouping is too restrictive. An enormous diversity of sugar utilization gene cassettes were recently identified, in fact, in the genome of several strains (Smokvina et al., 2013; Douillard et al., 2013). Accordingly, the characterization performed in our study revealed that none of the phenotypes mentioned above can univocally distinguish strains of HRM group "*a*" from strains of group "*b*" (Table 2). Therefore, we cannot conclude that the two genotypes identified inside the species *L. paracasei* by HRM correspond to the subspecies *tolerans* and *paracasei*. Nonetheless, it should be also mentioned that the phenotypic features describing *L. paracasei* subsp. *tolerans* have been based on a very limited number of strains; precisely, 6 strains isolated from pasteurized milk were considered by Abo-Elnaga and Kandler (1965) and only two strains were reported by Collins et al. (Collins et al., 1989); no other studies focusing on the phenotypic characterization of *L. paracasei* subspecies are available in

literature. As a consequence, we believe that the opportunity to maintain the two subspecies, *tolerans* and *paracasei*, inside the species *L. paracasei* should be reconsidered.

4. Conclusions

The *L. casei* group of species (*Lc*s) consists of three phylogenetically closely related species (*L. casei*, *L. paracasei*, and *L. rhamnosus*), which are widely used in the dairy and probiotic industrial sectors. Strategies to easily, unambiguously, and rapidly genetically characterize the bacteria of the *L. casei* group are therefore of interest. To this aim, we developed a method based on analysis of the melting curve of a 150 bp DNA fragment obtained by PCR from gene *groEL*. The experiments carried out during this study showed that melting curve analysis is a simple and labor-saving strategy that can be used for the initial rapid clustering of *Lactobacillus* isolates from food samples or biologic specimens. Specifically, the proposed method can represent a novel convenient molecular tool to obtain the genotyping of a bacterial isolate and simultaneously potentially confirm its affiliation to the *L. casei* group of species. In addition, the analysis of a significantly larger number of strains may also corroborate the hypothesis that the experimental protocol here presented, besides genotyping, is also suitable for the taxonomic discrimination of the species in the *L. casei* group.

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Table 1. Bacterial strains included in the study. All strains were commercially available or present in the culture collection of our Department. A, strains belonging to the *L. casei* group of species; B, other species. HMRa, High Resolution Melting analysis.

A.

Strain	Source	Species identification		HMR genotype	Partial *groEL* GenBank accession numbers[2]
		Specific primers[1]	HRMa		
LMG 23516	Belgian culture collection	*L. casei*	*L. casei*	*III*	
LMG 6904^T (= ATCC 393^T)	Belgian culture collection	*L. casei*	*L. casei*	*III*	
DH 4	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	
DH 17	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	HG422840
DH 20	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	
DH 40	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	HG422841
DH 62	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	
DH 6	Dahi traditional Nepalese dairy product	*L. rhamnosus*	*L. rhamnosus*	*I*	
GG	Commercial strain (Chr. Hansen A/S)	*L. rhamnosus*	*L. rhamnosus*	*I*	
YM 1	Commercial fermented milk	*L. rhamnosus*	*L. rhamnosus*	*I*	
LB 4	Human feces	*L. rhamnosus*	*L. rhamnosus*	*I*	
LB 11	Human feces	*L. rhamnosus*	*L. rhamnosus*	*I*	
LB 21	Human feces	*L. rhamnosus*	*L. rhamnosus*	*I*	
DSM 20021^T	German culture collection	*L. rhamnosus*	*L. rhamnosus*	*I*	
ATCC 25302^T	American culture collection	*L. paracasei*	*L. paracasei*	*IIa*	
DH 10	Dahi traditional Nepalese dairy product	*L. paracasei*	*L. paracasei*	*IIa*	HG422838
DH 65	Dahi traditional Nepalese dairy product	*L. paracasei*	*L. paracasei*	*IIa*	
DH 84	Dahi traditional Nepalese dairy product	*L. paracasei*	*L. paracasei*	*IIa*	HG422839
DH 132	Dahi traditional Nepalese dairy product	*L. paracasei*	*L. paracasei*	*IIa*	HG422837
DH 133	Dahi traditional Nepalese dairy product	*L. paracasei*	*L. paracasei*	*IIa*	
LP 3	Pecorino traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIa*	
ATCC 334	American culture collection	*L. paracasei*	*L. paracasei*	*IIb*	
DG	Human gut/commercial strain (Sofar S.p.A.)	*L. paracasei*	*L. paracasei*	*IIb*	HG422829
FMBr3	Raschera D.O.P. Traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	HG422835
I 1	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
I 2	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
I 16	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
IX 1	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	HG422830
IX 2	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 20	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 3	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 4	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 6	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 7	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
L 8	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	HG422833
L 9	Toma del Mottarone traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LB 7	Human feces	*L. paracasei*	*L. paracasei*	*IIb*	
LB 14	Human feces	*L. paracasei*	*L. paracasei*	*IIb*	
LB 22	Human feces	*L. paracasei*	*L. paracasei*	*IIb*	
LB 24	Human feces	*L. paracasei*	*L. paracasei*	*IIb*	HG422832
LMG 9191^T	Belgian culture collection	*L. paracasei*	*L. paracasei*	*IIb*	HG422831
LP 1	Pecorino traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LP 2	Pecorino traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LP 6	Bra traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LP 10	Bra tenero traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LP15	Commercial strain(GS in forma)	*L. paracasei*	*L. paracasei*	*IIb*	
LP26	Bra tenero traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	HG422834
LP28	Bra traditional Italian cheese	*L. paracasei*	*L. paracasei*	*IIb*	
LPAB	Cocoa beans fermented	*L. paracasei*	*L. paracasei*	*IIb*	
LPC-S01	Human vagina/commercial strain (Sofar S.p.A.)	*L. paracasei*	*L. paracasei*	*IIb*	HG422836
LPDT	Cocoa beans fermented	*L. paracasei*	*L. paracasei*	*IIb*	
LPT34	Fruit kefir	*L. paracasei*	*L. paracasei*	*IIb*	HG422842
Shirota	Commercial probiotic strain (Yakult Co., Ltd)	*L. paracasei*	*L. paracasei*	*IIb*	

[1], according to Ward and Timmins, 1999.
[2], GenBank accession numbers of the 14 partial *groEL* gene sequences determined in this study.

B

Strain	Species	Source
LVN 4	*Bacillus coagulans*	Commercial probiotic product
BB-12	*Bifidobacterium animalis* subsp. *lactis*	Commercial probiotic strain (Chr. Hansen A/S)
MIMBb75	*Bifidobacterium bifidum*	Human feces (Guglielmetti et al., 2009)
BBR09	*Bifidobacterium breve*	Commercial probiotic strain (Sacco S.r.l.)
BNF09	*Bifidobacterium longum* subsp. *infantis*	Commercial probiotic strain (Sacco S.r.l.)
F1	*Enterococcus faecalis*	Toma del Mottarone traditional Italian cheese
YVS3	*Enterococcus faecium*	Commercial probiotic product
EIP9	*Enterococcus italicus*	Pannerone traditional Italian cheese
LA5	*Lactobacillus acidophilus*	Commercial probiotic strain (Chr. Hansen A/S)
DH 92	*Lactobacillus brevis*	Dahi traditional Nepalese dairy product
DH 94	*Lactobacillus coryniformis*	Dahi traditional Nepalese dairy product
RoCa	*Lactobacillus curvatus*	Raschera traditional Italian cheese
DH 147	*Lactobacillus delbrueckii*	Dahi traditional Nepalese dairy product
DH 98	*Lactobacillus fermentum*	Dahi traditional Nepalese dairy product
DH 95	*Lactobacillus harbinensis*	Dahi traditional Nepalese dairy product
DH 103	*Lactobacillus helveticus*	Dahi traditional Nepalese dairy product
MIMLh5	*Lactobacillus helveticus*	Grana Padano whey starter (Guglielmetti et al., 2010b)
LJHO7	*Lactobacillus johnsonii*	Human feces
DH 31	*Lactobacillus parabuchneri*	Dahi traditional Nepalese dairy product
NF1	*Lactobacillus pentosus*	Commercial probiotic product
YVS2	*Lactobacillus plantarum*	Commercial probiotic product
PRE2	*Lactobacillus salivarius*	Commercial probiotic product
V32	*Lactococcus garvieae*	Rainbow trout fillet
LGB3	*Lactococcus garvieae*	Cabbage
E1	*Lactococcus lactic* subsp. *lactis*	Toma del Mottarone traditional Italian cheese
E7	*Lactococcus lactic* subsp. *lactis*	Toma del Mottarone traditional Italian cheese
K12	*Streptococcus salivarius*	Commercial probiotic product
ST3	*Streptococcus salivarius*	Human pharyngeal mucosa (Guglielmetti et al., 2010a)
N4	*Streptoccus thermophilus*	Toma del Mottarone traditional Italian cheese

Table 2. Phenotypic characterization of *Lactobacillus paracasei* strains based on acid production from 13 carbon sources and growth at 40°C (A), and resistance to pasteurization at 72°C for 40 s (B). HRM groups *a* and *b* refer to Fig. 4B. +, positive; –, negative; +/ –, weak activity.

A

	HRM group *a*				HRM group *b*							
	ATCC25302^T	LP3	DH10	DH133	LMG9191^T	ATCC334	SHIROTA	LPC-S01	DG	LP1	LPAB	FMBr3
Amygdalin	+/–	–	+/–	+/–	–	+	+	+	+/–	+/–	+	+
Arbutin	+	–	+	+/–	–	+	+	+	+/–	+/–	+	+
Cellobiose	+	+/–	+/–	+/–	–	+	+/–	+	+	+	+	+
Esculin	+/–	–	+/–	+/–	–	+/–	+/–	+/–	+/–	+/–	+/–	+/–
Glucose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	–	+	+	+	+	+	+	+/–	+/–	+	+
Maltose	+/–	–	+	+	–	+/–	+	+/–	+	+	+	+
Mannitol	+	–	+	+	–	+	+	+	+	+	+	+
Melezitose	+	–	+	+/–	–	+	+/–	+	+	+/–	+	+
Ribose	+	+/–	+	+	–	+	+	+	+	+	+	+
Sucrose	+/–	+/–	+	+	–	+/–	+/–	+	+	+	+	+
Salicin	+	–	+	+/–	–	+	+	+	+	+	+	+
Trehalose	+	–	+	+	–	+	+	+	+	+	+	+
Growth at 40°C	+	–	+	+	–	+	+	+	+	+	+	+

B

Pasteurization resistance (CFU ml^{-1})	ATCC25302^T	LP3	LMG9191^T	ATCC334
Before treatment	3×10^7	1×10^7	2×10^7	3×10^7
After treatment	< 10	< 10	10^6	< 10

Legends

Fig. 1. ClustalW alignment of 480 bp from the sequence of gene *groEL* of strains *Lactobacillus casei* ATCC393^T (GenBank accession number AY424336), *Lactobacillus rhamnosus* ATCC 7469^T (AF429659) and *Lactobacillus paracasei* DSM 5622^T (AY424339). Grey areas indicate the target regions of primers GroHRM-F and GroHRM-R.

Fig. 2. Neighbour-joining dendrogram of aligned *groEL* gene sequences from *L. casei* group of species. Alignments of sequences were made considering the 150 bp fragment that is amplified with primers GroHRM-F and GroHRM-R (Fig. 1). Bootstrap values (1000 replicates) are shown close to the considered node. Sample indicated in bold refers to bacterial strains whose *groEL* gene sequence has been determined in this study. The *groEL* gene sequences obtained from GenBank are shown with the database accession number between brackets. Taxonomic names for database sequences are according to GenBank annotation. *Lactobacillus plantarum* ATCC 14917^T has been included as outgroup. [a], type strain of *L. paracasei* subsp. *tolerans*; [b], type strain of *L. paracasei* subsp. *paracasei*.

Fig. 3. Real time quantitative PCR (qPCR) experiment performed with the total DNA isolated from *L. paracasei* DSM 5633^T. DNA was serially 1:5 diluted from 50 ng per reaction. A, qPCR amplification curves. B, melting curves of the qPCR amplicons. Ct, real time PCR threshold cycle. RFU, relative fluorescence units. Duplicates for each condition are shown.

Fig. 4. High resolution melting curve analysis (HRMa) of the 150 bp *groEL* DNA fragment amplified by qPCR from strains belonging to species of the *Lactobacillus casei* group (*Lc*s, Table 1). Panel A, HRMa performed on 53 *Lc*s strains; the group of curves I includes 12 *L. rhamnosus* strains; group II includes 39 *L. paracasei* strains; group III includes 2 *L. casei* strains. Panel B, HRMa performed on 39 *L. paracasei* strains; the cluster of curves "a" includes *L. paracasei* subsp. *paracasei* ATCC 25302^T and other 6 strains; the cluster of curves "b" includes *L. paracasei* subsp. *tolerans* LMG 9191^T and other 31 strains. Panel C, amplicons average melting temperature (Tm) of the gentypes obtained through HRMa. Data for each bar of the histogram are reported as mean value of the Tm ± standard deviation.

Fig. 1

```
ATCC 393T   AAGGCAGCGGTTGATGAACTGCACAAGATCAGCCACAAGGTTAACGGCAAGAAGGAAATC
ATCC 7469T  AAGGCTGCAGTTGACGAATTGCACAAGATCAGCCACAAGGTTAACGGCAAGAAGGAAATT
DSM 5622T   AAGGCTGCCGTTGACGAATTGCACAAGATTAGCCACAAAGTTAATGGTAAGAAAGAAATC
            ***** ** ***** *** ********** ******** ***** ** ***** *****

            GCCCAGGTTGCTTCGGTTTCATCTTCCAATGAAGAAGTCGGCAATCTGATTGCGGACGCT
            GCCCAAGTTGCTTCCGTTTCCTCTTCTAATGAAGAAGTCGGCAACCTGATTGCTGACGCG
            GCGCAGGTTGCGTCCGTTTCTTCCTCAAATACAGAAGTTGGTAGTCTGATTGCCGACGCC
            ** ** ***** ** ***** ** ** ***  ****** ** *  ******** *****

            ATGGAAAAAGTTGGCCATGATGGTGTTATTACCATCGAAGAAAGCAAAGGGATCGACACT
            ATGGAAAAAGTTGGCCATGATGGTGTGATTACCATTGAAGAAAGCAAAGGGATTGATACT
            ATGGAAAAAGTTGGCCACGATGGTGTGATTACCATTGAAGAAAGCAAAGGGATTGACACT
            ***************** ******** ******** ***************** ** ***

                                        GroHRM-F →
            GAACTTTCCGTTGTTGAAGGGATGCAGTTTGATCGCGGCTATCTGAGTCAATACATGGTA
            GAACTCTCTGTTGTTGAAGGGATGCAGTTTGATCGCGGCTATCTGAGCCAGTACATGGTT
            GAACTCTCTGTTGTTGAAGGGATGCAGTTTGATCGCGGCTATCTGAGCCAGTACATGGTC
            ***** ** ***************************************** ** ********

            ACCGACAATGATAAAATGGAAGCTGATCTGGACGATCCATATATCCTGATTACCGATAAG
            ACCGACAACGACAAGATGGAAGCTGATCTGGATGATCCATATATCCTGATTACCGACAAG
            ACTGATAATGATAAGATGGAAGCTGACCTTGACGATCCTTATATCTTGATCACCGACAAA
            ** ** ** ** ** ********** ** ** ***** ****** **** ***** **

                                                    ← GroHRM-R
            AAGATTTCCAACATCCAGGACATTCTTCCACTATTACAAGAAATCGTTCAACAAGGTAAG
            AAGATCTCCAATATTCAAGATATTCTGCCACTCTTGCAAGAAATCGTGCAACAAGGCAAG
            AAGATTTCCAATATTCAGGACATTTTGCCGCTGTTACAAGAAATCGTTCAACAAGGTAAG
            ***** ***** ** ** ** *** * ** ** ** ************ ******** ***

            GCACTGTTAATCATTGCTGACGATGTTGCCGGCGAAGCATTGCCAACCCTGGTTCTTAAC
            GCACTGTTGATCATTGCTGACGATGTTGCCGGTGAAGCACTGCCGACCTTGGTTCTGAAC
            GCACTGTTGATCATTGCTGACGACGTTGCTGGTGAAGCATTGCCAACCTTAGTTCTGAAC
            ******** ************** ***** ** ****** **** *** * ***** ***
```

Fig. 2

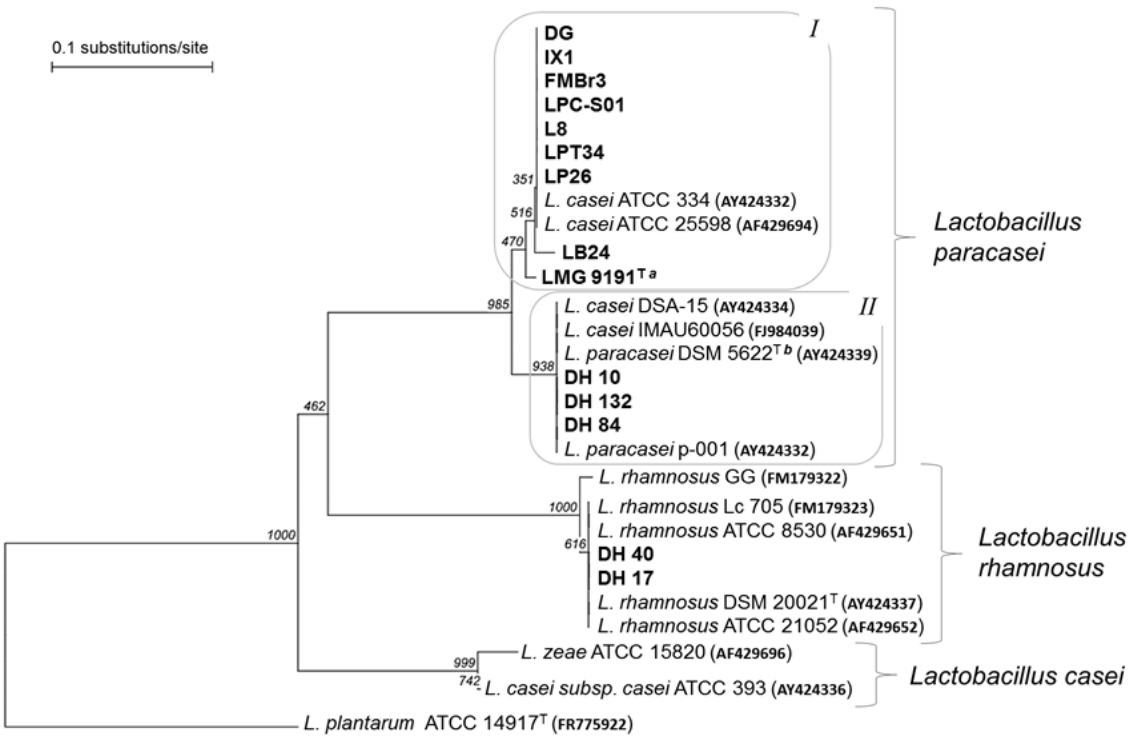
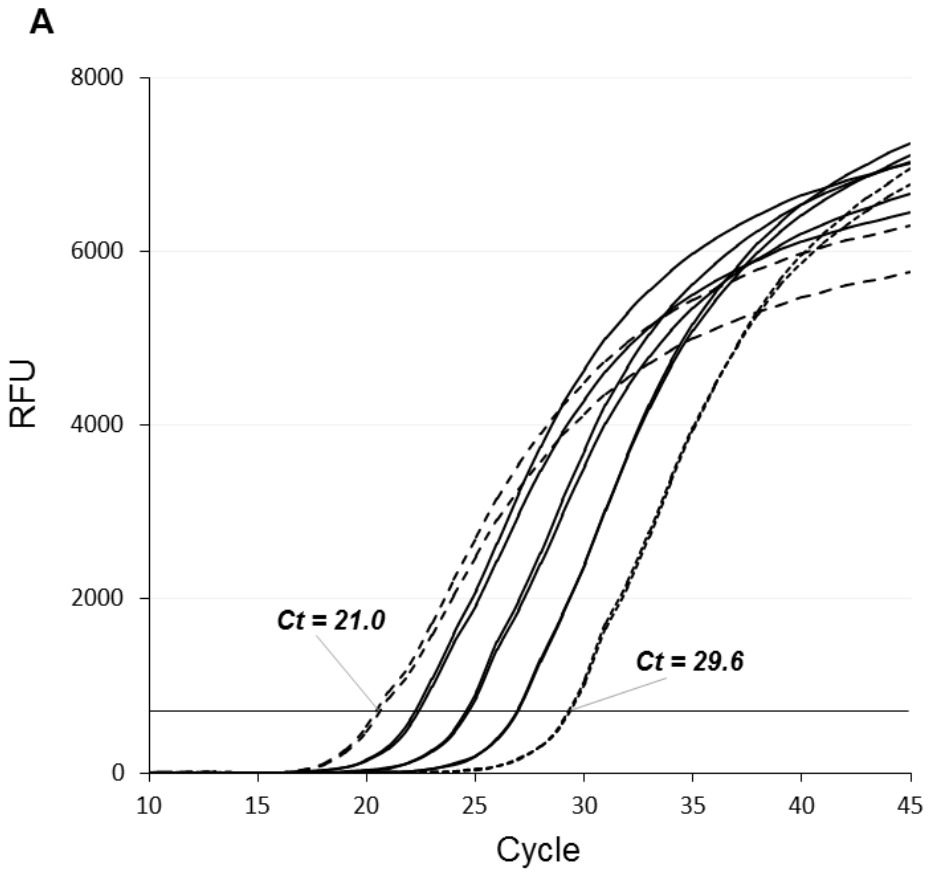


Fig. 3


A
8000
6000
4000
2000
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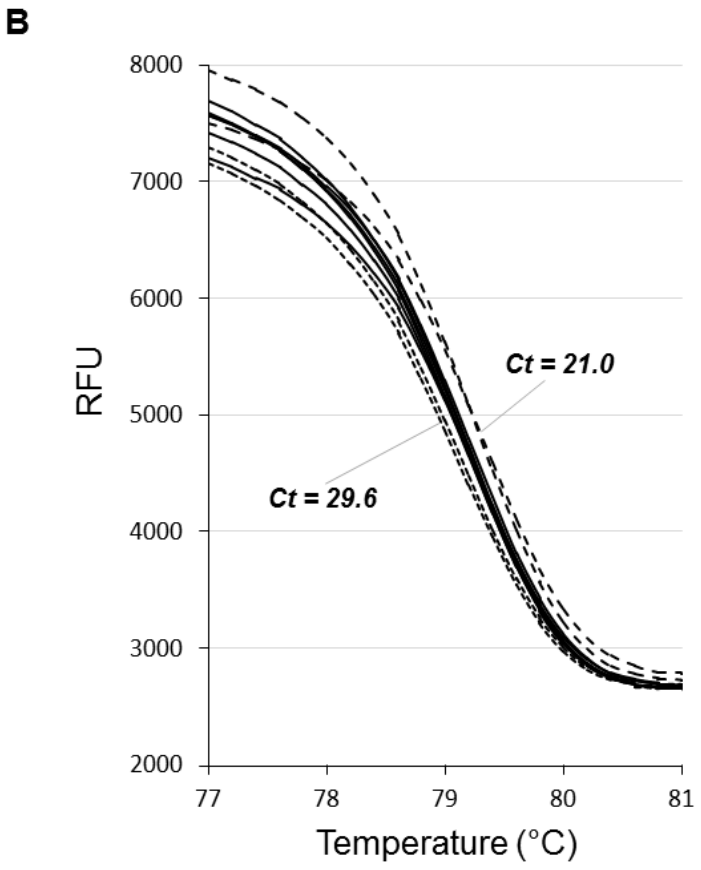
RFU
Ct = 21.0
Ct = 29.6
10
15
20
25
30
35
40
45
Cycle

Fig. 3


B
8000
7000
6000
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4000
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2000
RFU
Ct = 21.0
Ct = 29.6
77
78
79
80
81
Temperature (°C)