Effective identification of Lactobacillus casei group species: genome-based selection of the gene mutL as the target of a novel multiplex PCR assay

Benedetta Bottari,† Giovanna E. Felis,‡ Elisa Salvetti,4 Anna Castioni,† Ilenia Campedelli, Sandra Torriani,† Valentina Bernini1 and Monica Gatti1,2

Abstract

Lactobacillus casei, Lactobacillus paracasei and Lactobacillus rhamnosus form a closely related taxonomic group (the L. casei group) within the facultatively heterofermentative lactobacilli. Strains of these species have been used for a long time as probiotics in a wide range of products, and they represent the dominant species of nonstarter lactic acid bacteria in ripened cheeses, where they contribute to flavour development. The close genetic relationship among those species, as well as the similarity of biochemical properties of the strains, hinders the development of an adequate selective method to identify these bacteria. Despite this being a hot topic, as demonstrated by the large amount of literature about it, the results of different proposed identification methods are often ambiguous and unsatisfactory. The aim of this study was to develop a more robust species-specific identification assay for differentiating the species of the L. casei group. A taxonomy-driven comparative genomic analysis was carried out to select the potential target genes whose similarity could better reflect genome-wide diversity. The gene mutL appeared to be the most promising one and, therefore, a novel species-specific multiplex PCR assay was developed to rapidly and effectively distinguish L. casei, L. paracasei and L. rhamnosus strains. The analysis of a collection of 76 wild dairy isolates, previously identified as members of the L. casei group combining the results of multiple approaches, revealed that the novel designed primers, especially in combination with already existing ones, were able to improve the discrimination power at the species level and reveal previously undiscovered intraspecific biodiversity.

INTRODUCTION

The widely heterogeneous genus Lactobacillus is the largest among lactic acid bacteria (LAB). It contains a range of species isolated from diverse sources, among which a number of closely related species groups share high resemblance, not only in their phenotypic traits, but also in ribosomal RNA sequence [1]. In particular, the species Lactobacillus casei, Lactobacillus paracasei and Lactobacillus rhamnosus are phylogenetically and phenotypically related and are regarded together as the L. casei group within the facultatively heterofermentative lactobacilli [2, 3]. Strains from this group potentially have beneficial probiotic effects on human and animal health, and also are a minor but important component of the human gut microbiota [4–7].

Bacteria from this group have been isolated from a variety of environments including fermented foods and, particularly, cheeses, from which the name casei derives. In different kind of cheeses, they can be naturally present or added as starter or adjunct cultures [8, 9] and they are commonly the dominant species of nonstarter LAB in long-ripened cheeses [10].

Despite their importance for the food industry, after many years of controversial nomenclature and taxonomic status, a decision of the judicial commission of the International Committee on Systematics of Prokaryotes (ICSP) has stated that the type strain of the species L. casei is strain ATCC 393, and that all strains similar to ATCC 334, proposed as the neotype strain of the species [11, 12], should be named...
L. paracasei [13, 14]. However, the systematics of the L. casei group is still under debate [9, 15] due to the fact that the three species L. casei, L. paracasei and L. rhamnosus are difficult to discriminate. Several techniques have been used to identify and characterize L. casei group isolates based on their physiological and/or genetic features [16–20]. However, these methods can still give ambiguous results or present disadvantages such as low reproducibility, high complexity and time-consuming protocols [16, 21].

In the present study, a genome-based rationale was applied to select a target gene for a robust species-specific PCR for the species of the L. casei group, with the aim of designing a novel set of primers. In particular, genes tuf [22], recA [23], rpoA [24], pepR [25], dnaK [26, 27] and dnal/dnaK [17] previously used for multi-locus sequence typing (MLST)/MLS analyses in the genus Lactobacillus, were considered as potential targets and compared. Their similarity percentages were calculated for the species in the L. casei group and compared to the genome similarity values [in silico DNA–DNA hybridization (isDDH) and average nucleotide identity (ANI)] to highlight genes with similarities most representative of whole-genome identity. Novel species-specific PCR primers were designed targeting the most promising molecular target, mutL. Effectiveness of the novel assay was determined by analysing a collection of 76 strains previously identified as belonging to the species of the L. casei group and results were critically evaluated.

**METHODS**

**Sequence retrieval and analysis**

The nucleotide sequences of 21 housekeeping genes used in MLST studies for the genus Lactobacillus [15, 28–41] were retrieved through BLASTN searches against the genomes of L. casei ATCC 393T (accession number: AP012544.1), *L. paracasei* JCM 8130T (AP012541.1), L. rhamnosus DSM 20021T (AZCQ01000001.1) and L. rhamnosus GG (FM179322.1). BLASTN searches were also performed against the genome of strain DSM 20178 (NZ_AZCT0000000.1), a former type strain of *Lactobacillus zeae* [42], for comparative purposes. The nucleotide sequences of Lactobacillus salivarius UCC 118 (NC_007929.1) or L. casei ATCC 393T (AP012544.1) were used as seeds. Each dataset was then aligned using MUSCLE [43] and the similarity percentage was calculated by using Genedoc software (http://www.nrbsc.org/old/gfx/genedoc/).

**In silico genome comparisons**

isDDH and ANI were calculated to determine genome relatedness among the sequenced strains. isDDH was calculated using the Genome-to-Genome Distance Calculator (GGDC) [44, 45], uploading the genome sequences of *L. casei* ATCC 393T, *L. paracasei* JCM 8130T, *L. zeae* DSM 20178T, *L. rhamnosus* DSM 20021T and *L. rhamnosus* GG to the GGDC 2.0 server (http://ggdc.dsmz.de/distcalc2.php). Formula 2 alone was used for analysis, since it is the only one independent of genome length and it is recommended by the authors of GGDC for use with any incomplete genomes [44, 45].

Identity values were calculated among the type strains of the species of the L. casei group by estimating the ANI for the same genomes using the ANI calculator, which supports both complete and draft genomes. The genomes were uploaded to the web-based ANI calculator (http://enve-omics.ce.gatech.edu/ani/index) and the calculation was carried out with default parameters.

Similarity values were compared with isDDH and ANI values for the same strains to determine which gene(s) could be considered the best indicator(s) of genome-wide diversity.

**Strains of the L. casei group investigated and DNA isolation**

Seventy-six wild strains isolated from dairy samples, previously identified by different molecular techniques as belonging to the species *L. casei*, *L. paracasei* and *L. rhamnosus*, were investigated (Table 1). Nine reference strains, namely *LMG 6904T*, *ATCC 393T*, *LMG 23516* (*L. casei*), *ATCC 334* (*L. paracasei*), *LMG 6400T*, *LMG 23522T*, *LMG 23304T*, *ATCC 53103* (strain GG) (*L. rhamnosus*), were included in the study and *Lactobacillus plantarum* ATCC 14917T was used as negative control.

The strains were maintained as frozen stocks in MRS broth (Sigma-Aldrich) containing 20% (v/v) glycerol at −80°C. The isolates were recovered in MRS broth by overnight sub-culturing twice at 37°C under anaerobiosis and their purity was verified by microscopic observation.

For extraction of genomic DNA from the isolates, 0.5 ml of overnight broth culture was used. Cells were centrifuged (10 min, 10,300 g) and DNA isolation was performed by using a DNeasy Qiagen Blood and Tissue kit (Spin-Column Protocol Purification of Total DNA from Animal Tissues with Gram-positive pretreatment; Qiagen) according to the manufacturer’s recommendations. The concentration of the DNA was estimated spectrophotometrically (Jasco V-530).

**Species-specific PCRs**

The strains of the *L. casei* group were identified by species-specific PCRs according to the method described by Ward and Timmins [46] after slight modifications. Briefly, PCR reactions were performed in a final volume of 20 µl, with 1× PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 2.5 U *Taq* DNA polymerase (Invitrogen Life Technologies), 0.4 mM of each primer and 1 µl template DNA (20 ng µl⁻¹). Initial denaturation at 94°C for 5 min was followed by 30 cycles consisting of denaturation at 95°C for 45 s, annealing for 30 s at 60, 58 or 55°C for the primer pairs *casei/Y2*, *para/Y2* and *rham/Y2*, respectively, and extension at 72°C for 2 min. A final extension step of 72°C for 10 min was used. PCR reactions were carried out in a 2720 Applied Biosystems Thermal Cycler (Applied Biosystems). Reaction products were visualized by electrophoresis on SYBR Safe (Invitrogen Life Technologies)-stained 1.5% agarose gel.
Table 1. Bacterial strains, source of isolation, technique of first identification

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A novel mutL-targeted multiplex PCR was developed. Briefly, PCR primers were designed on sequences available for the type strains by using Primer3 software (http://primer3.ut.ee/) and primer characteristics (self complementarity and primer dimer formation) were checked with OligoAnalyzer (https://eu.idtdna.com/calc/analyzer). Three forward primers specific for each species, CZfor (5'-CAGCGCTGTTGGAAGACTTG-3'), PC2a (5'-GGATTGGGTATTGCTGATTGTCG-3') and RHfor (5'-GAC TTCTCAACCAGCGCAGCAGA-3'), for L. casei, L. paracasei and L. rhamnosus, respectively, and a unique reverse primer, CPRrev (5'-TGAGACTTTGCAAGACTTG-3'), were finally chosen. Expected amplicon lengths were 266 bp for L. casei, 253 bp for L. paracasei and 801 bp for L. rhamnosus. Optimization of PCR parameters such as Mg, template DNA concentrations, as well as annealing temperature was performed and the optimized conditions for multiplex PCR were as follows. Reactions were performed in a final volume of 20 µl, with 1x PCR buffer, 25 µM dNTPs, 1.5 mM MgCl₂, 0.25 µM of each primer, 0.025 U Taq-DNA polymerase (Promega), and 1 µl template DNA (20 ng µl⁻¹). Amplification reactions were performed using a 2720 Applied Biosystems Thermal Cycler with the following temperature profile: initial denaturation at 94 °C for 5 min; 35 cycles consisting of denaturation at 94 °C for 30 s, annealing for 30 s at 68 °C, and extension at 72 °C for 45 s. A final extension step of 72 °C for 5 min was used. Reaction products were also visualized by electrophoresis on SYBR Safe (Invitrogen Life Technologies)-stained 1.5 % agarose gel. The same conditions could be applied for single PCR targeting only one species on the basis of the chosen primer pair.

PCR assays proposed by Ventura et al. [22] and Iacumin et al. [17] were applied following the protocols presented by the respective authors.

Reference strains for species-specific PCRs were L. casei (LMG 6904 T, ATCC 393 T, LMG 23516 T), L. paracasei (ATCC 334), L. rhamnosus (LMG 6400 T, LMG 23522 T, LMG 23304 T, GG). L. plantarum ATCC 14917 T was used as negative control.

## RESULTS

In the past, several genes, namely tuf [22], recA [23], rpoA [24], pepR [25], dnaK [26, 27] and dnaJ/dnaK [17], were selected for species identification as the target for gene sequencing and comparison or for specific PCR amplification. In general, the reasons for those choices were the usefulness of genes as phylogenetic markers and their degree of conservation in bacteria, as protein-coding genes have higher discrimination ability than rRNA gene sequences, first proposed as universal markers [42].
### Table 2. Nucleotide similarity of 22 MLST genetic targets of the type strains of the *L. casei* group

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<td>mutL2</td>
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thanks to the availability of recently published genome sequences of lactobacilli [15], we decided to apply a systematic and pragmatic approach for gene selection: all the genes previously used for MLST/MLS analyses in the genus Lactobacillus were considered as potential targets and compared. In particular, their similarity percentages were (1) calculated for the species in the L. casei group and (2) compared to the genome similarity values (isDDH and ANI) to highlight genes the similarity of which was most representative of whole-genome identity.

**Genome sequence analysis, selection of target gene and primer design**

Housekeeping genes previously used in MLST studies for different species of the genus Lactobacillus [47–53] were retrieved, aligned and identity values were calculated among the three type strains of the recognized species of the L. casei group, strain DSM 20178, former type of species ’L. zeae’, as well as L. rhamnosus strain GG (Table 2). Remarkably, similarity values shared by strain DSM 20178 with ATCC 393T did not always support previous hypotheses that they could constitute a single species. On the contrary, strain GG always shared identity values higher than 97 % with the type strain of L. rhamnosus, thus confirming that it belongs to the latter species. The similarity of L. casei group species and associated strains was also assessed through the analysis of total genome content through distance-based methods, such as isDDH and ANI (Table 3). Based on these parameters, strains of the same species share idDDH >70 % and ANI >94 %. The taxonomic groups (L. casei, L. paracasei, L. rhamnosus and ’L. zeae’) established by isDDH and ANI values (Table 3) were used as a reference scheme using which the similarity values of housekeeping genes (reported in Table 2) were compared.

Based on this rationale, the analysis revealed that, among the other housekeeping genes, the locus mutL was the best indicator of genome-wide diversity, while dnaJ/dnaK, rpoA and tuf, as well as pepR, showed higher similarities.

Novel species-specific PCR primers were designed targeting mutL; moreover, since the expected amplicon size could effectively distinguish the type strains of L. casei (666 bp), L. paracasei (253 bp) and L. rhamnosus (801 bp), primers were combined in a multiplex PCR assay (Fig. 1), faster than the three simple separate tests. After verification of correct identification of nine reference strains (not shown) and lack of amplification of the negative control strain (L. plantarum), a wider collection of strains was analysed.

**Molecular identification of wild strains of the L. casei group**

The collection comprised 76 strains belonging to the collection of the University of Parma and isolated from dairy samples (milk and differently ripened cheeses) over the years and during different research projects. They were formerly identified as belonging to the L. casei group by different genotypic methods, such as 16S rRNA sequencing [54] or tRNA(Ala)–23S ribosomal DNA restriction fragment length polymorphism (tRNA(Ala)–23S rDNA-RFLP) [55] (Table 1).

The species-specific PCR developed by Ward and Timmins [46] has been frequently and recently used [17, 55, 56]; therefore, in the present study, that protocol was adapted to laboratory conditions and considered the standard test. When applied to the analysis of the nine reference strains, it produced satisfactory results. However, 10 out of the 76 wild strains (approx. 13 %) gave a PCR product with more

<table>
<thead>
<tr>
<th>Strain</th>
<th>MLST gene</th>
<th>Nucleotide similarity (%)</th>
</tr>
</thead>
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<td></td>
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<td>ANI</td>
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<td>81.24</td>
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<td>L. paracasei JCM 8130T</td>
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<td>100</td>
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<td>L. rhamnosus DSM 20021T</td>
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<td>100</td>
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</table>

\*ND, Not detected; T, type strain.

Table 3. DDH and ANI values of the comparison among type and reference strains

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than one primer pair (Table 4). When tested with the novel mutL-targeting primers, eight out of the ten strains previously not identified by 16S rDNA-based PCR were successfully classified, six as L. rhamnosus (strains 2407, 2411, 2466, 1019, 2101 and 2352 supported by amplification results with the primers dnaKRHf/dnaKRHr, dnaKCPf/dnaKCPr and dnaJPAf/dnaJPar [17]), two as L. paracasei (strains 2167 and 2471, the latter not supported by amplification results with the primers dnaKRHf/dnaKRHr, dnaKCPf/dnaKCPr and dnaJPAf/dnaJPar [17]) and only two gave ambiguous (strain 1473 showed amplicons for L. casei and L. rhamnosus, while strain 2465 was not amplified) (Table 4, figure not shown); moreover, the identification of 23 strains out of the remaining 66 (approx. 35 %) was in disagreement with the original identification (Table 5).

As for the other 66 strains, 16S rDNA-targeting assay produced the following results (Fig. S1, available in the online Supplementary Material): 19 strains were identified as L. casei (amplified with the casei/Y2 primer pair), 35 were assigned to L. paracasei (para/Y2) and 12 were identified as L. rhamnosus (rham/Y2). Unexpectedly, when tested with the novel mutL-targeting assay, only 53 strains (80 %) confirmed the same identification (Table 5). This incongruence was further investigated applying at least one of the previously reported PCRs [17, 22] and results were compared (data not shown) and can be summarized as follows, grouped with respect to species determined with 16S rDNA-based primers:
- **L. casei**: 16 of the 19 strains identified with 16S rDNA-PCR were confirmed; one strain (strain 2154) was identified as L. rhamnosus with mutL primers (identical to first identification and confirmed by dnaKRHf/dnaKRHr, dnaKCPf/dnaKCPr and dnaJPAf/dnaJPar primer amplification [17] PCR); two strains gave ambiguous results (2046 and 2349), also highlighted with dnaKRHf/dnaKRHr, dnaKCPf/dnaKCPr and dnaJPAf/dnaJPar primer amplification [17] (data not shown).
- **L. paracasei**: 31 strains out of the 35 were confirmed; two strains (2243 and 2246) were assigned to L. casei, confirming previous identification and supported by

![Fig. 1. Multiplex PCR amplification results of L. casei group reference strains. The novel species-specific PCR mutL-targeting primers yielded amplicons clearly distinguishable on 1.5 % agarose gel. Predicted amplicon size were 253 bp for L. paracasei (Lp, NCFB 151T), 666 bp for L. casei (Lc, ATCC 393T), and 801 bp for L. rhamnosus (Lr, LMG 6400T). The ladder used (Marker) was O’GeneRuler (ThermoFisher Scientific). Negative control was represented by L. plantarum (Lpl, ATCC 14917T).](image-url)

### Table 4. Comparison of amplification results for 10 strains not clearly identified with 16S rDNA-based PCR [46]

<table>
<thead>
<tr>
<th>Strain</th>
<th>Original ID</th>
<th>Amplicons of the 16S rDNA assay [46]</th>
<th>Amplicons of the mutL-targeting assay</th>
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NA, Not Amplified
Table 5. Comparison of amplification results for 66 strains unambiguously identified with 16S rDNA-based PCR [46]

For better readability, strains are listed separated in species as determined by 16S rDNA-targeting PCR. Strains underlined are those whose original identification differed from that determined by 16S rDNA-targeting PCR (see text).

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<th>Identification according to 16S rDNA assay [46]</th>
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Table 5. cont.

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NA, Not amplified.

PAR, CAS, RHA, CPR primer [17, 22] amplification (data not shown); one strain (2077) was identified as L. rhamnosus; one strain (2081) produced ambiguous results with mutL and dnaK primers [17] (data not shown).

- L. rhamnosus: only six out of 12 strains identified as L. rhamnosus were confirmed, one strain (2177) was identified as L. casei and this identification was supported by dnaKRH/dnaKRHr, dnaKCPf/dnaKCPr and dnaJPAl/dnaJPAr primer [17] amplification (data not shown); one strain (2233), assigned to L. casei, produced an amplification profile of L. rhamnosus with dnaKRH/dnaKRHr, dnaKCPf/dnaKCPr and dnaJPAl/dnaJPAr primers [17] (data not shown). Finally, four strains (1200, 2114, 2118, 2362) produced no amplification with mutL-targeting primers, but were confirmed as L. rhamnosus with dnaKRH/dnaKRHr, dnaKCPf/dnaKCPr and dnaJPAl/dnaJPAr primers [17] (data not shown).

Considering the whole collection, with the novel mutL-targeting species-specific primers, the 76 strains were identified as follows: 20 strains as L. casei, 33 as L. paracasei and 14 as L. rhamnosus (Tables 4 and 5). Among these, 16, 31 and six strains, respectively, confirmed previous 16S rRNA gene PCR results. Finally, nine strains could not be clearly

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identified with the mutL-targeting amplification. To further investigate their identity, previously described dnaJK- and tuf-targeting PCRs were applied and the results were not always satisfactory, suggesting that the strains under study could probably be atypical from a more general molecular viewpoint.

DISCUSSION

Identification at the species level of wild isolates is often not an easy task, and this is particularly true when considering closely related species such as L. casei, L. paracasei and L. rhamnosus. However, when strains belonging to taxa with applied importance are characterized, their clear identification is required for scientific and regulatory reasons [57, 58].

In the present paper, a pragmatic approach to PCR target selection was presented, starting from deposited genome sequences to highlight a gene, mutL, which could better represent the genome-wide diversity of the three species and was therefore selected as a target for specific PCR primers.

Results presented in the present paper suggest that mutL-targeting primers were able to clearly identify 67 strains out of the 76 of the collection and, in particular, to improve characterization of eight out of the 10 strains not clearly identified with 16S rDNA-based primers described in [46]. Interestingly, when conflicting amplification results were investigated with an additional species-specific PCR assay on a different genotypic target [17, 22], results agreed with 16S or mutL patterns or were ambiguous (as for strain 2240), depending on the cases, even though they generally agreed more with mutL than 16S rDNA results.

Furthermore, the species-specific PCRs described in the literature and in the present study produce ambiguous results when analysing wild strains, and the percentage of ambiguities revealed analysing 16S RNA gene and mutL were very similar. However, mutL-targeting primers are probably more accurate in unravelling the species diversity, thanks to the rationale of selection based on the genome-wide comparison, and the multiplex assay described here is a fast and accurate method to allot wild strains to species, especially when 16S rRNA gene primers fail. This higher discriminatory ability of mutL revealed a certain degree of biodiversity within the three species which is overlooked when targeting 16S rRNA gene sequences and that might be conflicting with results produced targeting other protein-coding genes. Most probably this is not a limitation of mutL as a target gene, but could be due to the biodiversity of strains, which makes the assignment of specific strains to one of the three closely related species intrinsically difficult when only one single target gene is taken as the only reference. Different results obtained when targeting different genes could be explained by horizontal gene transfer among strains sharing the same ecological niche. The ongoing horizontal gene transfer among L. casei group strains is also supported by the comparative genomic analysis of L. casei group species [9], which showed the presence of transferred specific genomic islands, thus reflecting the genomic adaptation of these strains to various niches [9]. For this reason, when conflicting results are obtained in the preliminary identification step comparing the results of at least two PCR assays, our suggestion is that the strains giving ambiguous results should be selected for genome sequencing. This change in perspective, i.e. the assay is not failing, but it is revealing something peculiar, could be very useful in a biotechnological perspective, since comparing the results of different techniques could help in revealing unexpected biodiversity, enlarging our comprehension of species structure and evolution and characterizing strains which could have phenotypic peculiarities behind genotypic ones.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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