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ORIGINAL ARTICLE

Development of a PCR-RFLP assay for the identification of *Lactococcus lactis* ssp. *lactis* and *cremoris*

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Abstract The development of new starter culture of *Lactococcus lactis* for the manufacture of fermented dairy products with unique characteristics usually requires the isolation and identification of *L. lactis* up to subspecies level. Therefore, a rapid and specific PCR-RFLP assay has been developed. Forward and reverse primer sets were designed targeting the conserved house keeping gene *htrA* and *yueF* encoding a trypsin-like serine protease and a non-proteolytic protein from peptidase family M16, respectively, of *L. lactis*. Amplicons of 265 bp and 447 bp of *htrA* and *yueF*, respectively, were subjected to restriction fragment length polymorphism analysis. Restriction of the 265 bp amplicons with *Taq*I produced DNA bands of 90 bp and 175 bp with ssp. *lactis*, and 66 bp and 199 bp with ssp. *cremoris*. Similarly, restriction of PCR product of 447 bp size with *Alu*I produced digested fragments of 125 bp and 322 bp with ssp. *lactis*, and 71 bp and 376 bp with ssp. *cremoris*. The designed primer sets were observed to be specific to *L. lactis* because other bacteria could not be amplified. The ssp. *lactis* and *cremoris* of *L. lactis* could be identified by restriction of PCR products of *htrA* and *yueF* with *Taq*I and *Alu*I, respectively.

Keywords *Lactococcus lactis* ssp. *lactis* · Polymerase chain reaction · Restriction fragment length polymorphism · Trypsin-like serine protease · Non-proteolytic protein · Peptidase family M16

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Introduction

Lactococcus lactis is an important member of the lactic acid bacteria (LAB) and is used in the dairy industry as a starter culture for the manufacture of fermented dairy products such as sour milk, cream, butter, fresh cheese and many varieties of cheeses, such as Cheddar, Colby, cottage cheese, cream cheese, Camembert, Roquefort and Brie (Ayad et al. 1999; Cogan 1995; Hugenholtz et al. 2000; Prodelalova et al. 2005). *L. lactis* influences flavor, texture and shelf-life of fermented dairy products, produces developed acidity in milk and converts milk protein into flavored components such as diacetyl, acetoin, 2,3-butanediol, lactic acid, acetic acid, aldehydes and carbon dioxide (Wouters et al. 2002). *L. lactis* has been further sub-divided into ssp. *lactis* and *cremoris*. In the dairy industry, *L. lactis* ssp. *lactis* is preferred for making soft cheese while ssp. *cremoris* is for hard cheese (Bolotin et al. 2001). However, one biovariant, *diacetylactis*, has been used to provide flavor in cottage cheese, cultured sour cream and cultured butter milk (Salama et al. 1995). The bio-variant differs from *L. lactis* ssp. *lactis* and *cremoris* in its ability to utilize citrate with the production of diacetyl, a compound with a strong buttery flavor highly prized in some dairy products (Hugenholtz et al. 2000). Furthermore, the development of new strains of *L. lactis* as starter culture for the manufacture of fermented dairy products with distinct characteristics can be carried out by the isolation and characterization of *L. lactis* from different sources.

The identification of bacterial species is based usually on a combination of phenotypic and genotypic techniques (Aquilanti et al. 2007; Temmerman et al. 2004). Moreover,

DNA-based techniques offer greater distinct discriminatory power than phenotypic methods in differentiating individual subspecies and strains of bacteria. The application of molecular tools has revolutionized the identification of many bacterial species, including LAB. Many of these techniques are based on the polymerase chain reaction (PCR), which uses oligonucleotide primers to amplify targeted DNA fragments. These PCR primers may be designed to differentiate the bacteria from genus-specific to species-specific to subspecies level. The whole genome sequence analysis of *L. lactis* has revealed genetic differences between the ssp. *lactis* and *cremoris*. It has been postulated on the basis of DNA hybridization stringency that 20–30 % divergence has occurred between ssp. *lactis* and *cremoris* (Godon et al. 1992). Since *L. lactis* ssp. *lactis* and *cremoris* have the potential metabolic properties for the fermentation of milk and the manufacture of fermented dairy products, it is pertinent to identify the bacteria up to subspecies level. Earlier studies had shown the application of PCR-RFLP-based discrimination of the two subspecies of *L. lactis* but one or more problems were encountered (Buist et al. 1995; Nomura et al. 2002; Ward et al. 1998). Therefore, this study attempted to develop a specific PCR-RFLP assay to differentiate *L. lactis* ssp. *lactis* and *cremoris* isolated from dairy and non-dairy sources.

Materials and methods

Samples

Approximately 60 milk samples from cow (34), goat (8) and buffalo (17) were collected from local herds in Varanasi (Uttar Pradesh) India. Among non-dairy sources, seven samples of buffalo hair, ten from cow dung, eight from cattle fodder and ten samples of poultry feces were collected. In addition, eight samples of cheddar cheese were obtained from the experimental dairy of the National Dairy Research Institute, Karnal, (Haryana), India.

Bacterial strains

The reference strains *Lactococcus lactis* ssp. *lactis* NCDC 094, *Lactococcus lactis* ssp. *cremoris* NCDC 086, *Lactobacillus acidophilus* NCDC 015, *Leuconostoc mesenteroides* NCDC 029, *Lactobacillus plantarum* NCDC 021, *Enterococcus faecalis* NCDC 114, *Enterococcus faecium* NCDC 124, *Bacillus subtilis* NCDC 215 and *Staphylococcus aureus* NCDC 237 were obtained from National Collection of Dairy Cultures (NCDC), National Dairy Research Institute, Karnal (Haryana), India. ATCC strain of *L. lactis* ssp. *cremoris* (19297) was purchased from Himedia Laboratories, India. *Lactococcus, Lactobacillus* and *Leuconostoc* strains were grown in MRS broth (Himedia Laboratories, India) at 30°C for 24–48 h. *Bacillus*, *Enterococcus* and *Staphylococcus* strains were grown in Nutrient broth (Himedia Laboratories) and incubated at 37°C for 24 h. All strains were maintained in glycerol stocks at −20°C and sub-culturing of strains was carried out at 15-day intervals in MRS agar.

Phenotypic and molecular identification

Gram-positive cocci, homo-fermentative, catalase negative and acid-producing LAB were isolated from dairy and non-dairy sources as per the method of Mirhosaini et al. (2006). Isolates were analyzed further at the genus level. Litmus milk reduction, and methyl red reduction tests and the growth of bacterial cultures at different temperatures (10° C, 40°C and 45°C) were carried out as suggested by Corroler et al. (1998). The isolates were tested for the ability to ferment various carbohydrates (glucose, sucrose, lactose, maltose, galactose, fructose and mannitol) as described by Nomura et al. (1999). The isolates thus identified as members of the genus *Lactococcus* were characterized further at subspecies level by performing an arginine hydrolysis test and growth at pH 9.2 and 4 % (w/v) NaCl (Klijn et al. 1995).

Genotypic identification of phenotypically characterized strains was carried out by PCR amplification of a fragment of *gadB* gene followed by RFLP using *Ase*I as described by Nomura et al. (2002). Moreover, a set of eight representative strains from each isolation sources of dairy and non-dairy origin were also identified by gene sequencing of a fragment (>500 bp) of 16S rRNA (B27F 5′-AGA GTT TGA TCC TGG CTC AG-3′) and (U1492R 5′-GGT TAC CTT GTT ACG ACT T- 3′). GenBank accession numbers were allocated by NCBI for the sequences of 16S rRNA of a total of eight strains of *L. lactis* ssp. *lactis*.

Oligonucleotide primers for new PCR-RFLP assay

Two sets of primer pairs were designed from the nucleotide sequences encoding the serine protease and non-proteolytic protein, peptidase family M16 of *L. lactis* ssp. *lactis* IL1403 and *L. lactis* ssp. *lactis* KF147, respectively published in the NCBI database (http://www.ncbi.nlm.nih.gov/) and synthesized by Metabion (Martinstried, Germany). One of the target genes to be amplified was a fragment of the *htrA* gene encoding for the trypsin-like serine protease—a unique surface housekeeping gene in *L. lactis*. The PCR primer set was named serF (5′-CTG TCG TTT CTG TTA TGA AT-3′) and serR (5′-GTG TAT TCA TCA TAA CCA AC-3′), corresponding to positions 262 to 281 and 507 to 526, respectively, of the *htrA* gene and producing an amplicon of 265 bp. Another target gene to be amplified was a fragment of the *yueF* gene encoding the non-proteolytic

Table 1 Isolation of *Lactococcus lactis* ssp. *lactis* from dairy and non-dairy samples. *LAB* Lactic acid bacteria

Sample	No. of samples	No. of LAB	No. of *L. lactis* ssp. *lactis*	Percentage of *L. lactis* ssp. *lactis* /sample
Dairy	59	56	14	23.72
Cow milk	25	25	7	28.00
Buffalo milk	17	16	4	25.00
Goat milk	9	7	2	22.22
Cheddar cheese	8	8	1	12.50
Non-dairy	39	26	7	17.94
Poultry feces	10	6	2	20.00
Cattle dung	10	8	2	20.00
Cattle hair	11	6	2	18.18
Cattle fodder	8	7	1	12.50

protein, peptidase family M16. The forward primer pepF (5′-TCC TGA TTC ACA GTA AAT CC-3′) and reverse primer pepR (5′-GAT GAA TGG ACT TTT GGG-3′) correspond to positions 29 to 48 and 458 to 475, respectively, of the *yueF* gene and producing an amplicon of 447 bp. Conserved regions were identified by multiple sequence alignment of the *htrA* and *yueF* gene sequences from *L. lactis* ssp. *lactis* (GenBank accession number: AE005176.1, CP002365.1 and CP001834.1) and *L. lactis* ssp. *cremoris* (GenBank accession number: AM406671.1, CP00425.1 and CP002094.1). The selected sequences were aligned using the multiple sequence alignment program BLASTN 2.2.24+ (Altschul et al. 1997). Alignment was carried out with published NCBI databases revealed no matches other than those for the sequence of the gene encoding *L. lactis htrA* and *yueF*.

Table 2 Phenotypic characteristics of isolates from dairy and non-dairy samples

Phenotypic characteristic	Test isolate	*L. lactis* ssp. *lactis* NCDC 094	*L. lactis* ssp. *cremoris* ATCC 19297 and NCDC 086
Gram stain	+ (21/21)	+	+
Cell shape	Cocci (21/21)	Cocci	Cocci
Catalase test	− (21/21)	−	−
Cultural characteristics	White and circular colony with entire margin	White and circular colony with entire margin	White and circular colony with entire margin
Cell arrangement	Chain/ double /single (21/21)	Chain/ double /single	Chain/ double /single
Fermentation type	Homo-fermentative (21/21)	Homo-fermentative	Homo-fermentative
Growth at or in			
10°C	+ (21/21)	+	+
40°C	+ (21/21)	+	–
45°C	− (21/21)	−	−
4 % NaCl and pH 9.2	+ (21/21)	+	−
Litmus milk reduction test	+ (21/21)	+	+
Methyl red reduction test	+ (21/21)	+	+
Arginine utilization test	+ (21/21)	+	−
Fermentation of			
Glucose	+ (21/21)	+	+
Lactose	+ (21/21)	+	+
Sucrose	+ (21/21)	+	−
Maltose	+ (21/21)	+	−
Galactose	+ (21/21)	+	+
Fructose	+ (21/21)	+	+
Mannitol	− (21/21)	−	−
Identified as	*Lactococcus lactis* ssp. *lactis* (21/21)		

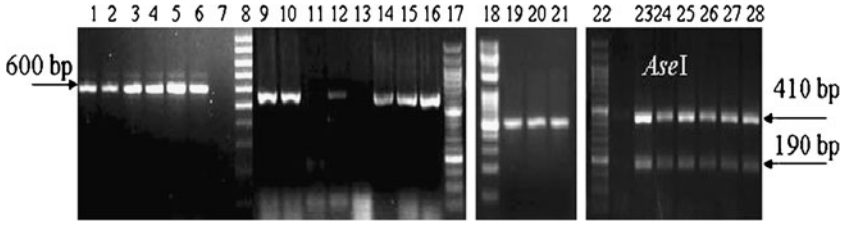

Fig. 1 PCR amplification of a fragment of the glutamate decarboxylase (*gadB*) gene and *gadB–Ase*I of *Lactococcus lactis* ssp. *lactis* and *cremoris*. Lane 1–5, 9–10, 12 and 15–16=test isolates (600 bp), 7=*E. faecalis* NCDC 114, 11=*Leuconostoc mesenteroides* NCDC 029,13=*B. subtilis* NCDC 215, 6 and 14=*L. lactis* ssp. *lactis* NCDC 094, 19=*L. lactis* ssp. *cremoris* ATCC 19297, 20=*L. lactis* ssp. *cremoris* NCDC 086 (560 bp), 8=100 bp DNA ladder, 17 & 22=50 bp DNA ladder, 18=1 kb DNA ladder, 23–27=*gad B-Ase*I in test isolates 21=*gad B-Ase*I in *L. lactis* ssp. *cremoris* ATCC 19297 and 28=*gad B-Ase*I in *L. lactis* ssp. *lactis* NCDC 094

Genomic DNA extraction

The genomic DNA of test and reference strains was extracted as per the method of Ho et al. (1991). The band of extracted genomic DNA of test and reference strains was observed using 0.8 % (w/v) agarose gel electrophoresis.

PCR conditions

The PCR was carried out using reagents obtained from Bangalore Genei, India. A reaction mixture (25 μL) containing 10–15 ng bacterial genomic DNA, 2.5 μl 10× *Taq* buffer, 1.5 mM of $MgCl_2$, 10 pmol of each primer, 100 μM dNTP mixture and 2.5 U *Taq* DNA polymerase was prepared for each PCR reaction. Amplification was performed in a PCR thermal cycler (BioRad laboratories India). The amplification reaction involved initial denaturation at 94°C for 4 min, 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 51°C for 1 min for both the primer sets, extension at 72°C for 1 min and a final extension step consisting of 72°C for 10 min. A volume of 10 μl of each PCR product was analyzed by 1.8 % (w/v) agarose (G-Biosciences, Maryland Heights, MO) gel electrophoresis in 1×TAE buffer (pH 8.0) at 65 V for 1 h using horizontal gel electrophoresis (Tarsons Products, India). The gel was visualized using a gel documentation system (Biorad laboratories, India).

Restriction fragment length polymorphism

The digestion of PCR products was carried out using a total of seven fast-digest restriction endonuclease enzymes (*Alu*I, *BamH*I, *Hind*III, *Hin*fI, *Pvu*II, *Sac*I and *Taq*I; Fermentas, Hanover, MD) in appropriate buffered solution as recommended by the manufacturer. The restriction fragments were separated on a 2.5 % (w/v) agarose gel.

Results

Isolation and identification of *Lactococcus lactis*

During the isolation of *L. lactis* from a total of 98 dairy and non-dairy samples, 82 (84 %) isolates were identified as LAB, which were characterized as Gram-positive cocci arranged as chains/double/single, catalase-negative, homofermentative and with the ability to reduce litmus milk to a white curd with pinkish band. The yield of LAB was 95 % (56/59) and 67 % (26/39) from dairy and non-dairy samples, respectively (Table 1). A total of 14 isolates of *L. lactis* (24 %, 14/59) could be isolated from dairy products, and 7 (18 %, 7/39) from non-dairy products. The phenotypic characterization of 21 isolates of *L. lactis* by a battery of test substrates is presented in Table 2. A genotypic identification by PCR

Table 3 Identification of a group of phenotypically characterized strains of *L. lactis* ssp. *lactis* by sequencing of a portion (>500 bp) of the universal 16S rRNA

Accession no. of isolate	Isolation source	Maximum identity (%)	*E*-value	Total score	Maximum similarity
JQ319711	Cow milk	94	0.0	4,627	*L. lactis* ssp. *lactis* KF147, complete genome (Accession no.- NC013656.1)
JQ319712	Buffalo milk	98	0.0	5,169	
JQ319713	Goat milk	98	0.0	5,192	
JQ319714	Cheddar cheese	98	0.0	5,203	
JQ319715	Poultry feces	97	0.0	5,125	
JQ319716	Cattle dung	95	0.0	4,593	
JQ319717	Cattle hair	96	0.0	4,826	
JQ319718	Cattle fodder	96	0.0	4,970	

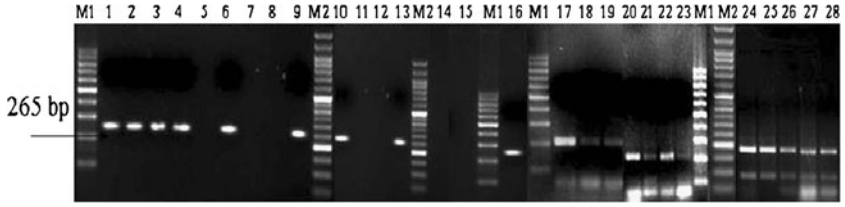

Fig. 2 PCR-RFLP of a fragment of the serine protease (*htr A*) gene of *L. lactis* ssp. *lactis* and *cremoris* with *Taq*I. Lane 1–3, 9 and 25–28=test isolates, 4 and 17–19=*L. lactis* ssp. *cremoris* ATCC 19297, 5=*Lactobacillus acidophilus* NCDC 015, 6, 16 and 24=*L. lactis* ssp. *lactis* NCDC 094, 7=*Leuconostoc mesenteroides* NCDC 029, 8=*Lactobacillus plantarum* NCDC 021, 10,13 and 20–23=*L. lactis* ssp. *cremoris* NCDC 086, 11=*E. faecalis* NCDC 114, 12=*E. faecium* NCDC 124, 14=*S. aureus* NCDC 237, 15=*B. subtilis* NCDC 215, M1=100 bp DNA ladder and M2=50 bp DNA ladder

amplification of a fragment of *gadB* gene from genomic DNA followed by restriction digestion with *Ase*I is presented in Fig. 1, which depicts the amplification of a 600 bp fragment of the *gadB* gene and digestion by *Ase*I into two fragments of 410 bp and 190 bp by test and reference strain *L. lactis* ssp. *lactis* NCDC 094. However, amplification of a 560 bp fragment with no digestion by *Ase*I was observed only with the reference strains *L. lactis* ssp. *cremoris* ATCC 19297 and *L. lactis* ssp. *cremoris* NCDC 086. Further, the maximum sequence similarity of a fragment of 16S rRNA from a total of eight strains with the GenBank reference strain *L. lactis* ssp. *lactis* KF147 is shown in Table 3. All 21 strains tested exhibited typical phenotypic and genotypic characters of *L. lactis* ssp. *lactis*. However, none of strains tested was phenotypically and genotypically identified as *L. lactis* ssp. *cremoris*.

Characterization of *L. lactis* ssp. *lactis/cremoris* by new genotypic method

The PCR amplification of chromosomal DNA of the identified strains, positive reference strains *L. lactis* ssp. *lactis* NCDC 094, *L. lactis* ssp. *cremoris* ATCC 19297, *L. lactis* ssp. *cremoris* NCDC 086 and other reference strains *Lactobacillus acidophilus* NCDC 015, *Leuconostoc mesenteroides* NCDC 029, *Lactobacillus plantarum* NCDC 021, *E. faecalis* NCDC 114, *E. faecium* NCDC 124, *S. aureus* NCDC 237 and *B. subtilis* NCDC 215 using the designed primer sets targeting a fragment of nucleotide sequence encoding for serine protease (*htrA*) and non-proteolytic protein, peptidase family M16 (*yueF*) resulted in the amplicons of 265 bp (Fig. 2) and 447 bp (Fig. 3). Amplification was obtained with the chromosomal DNA of the test and reference strains *L. lactis* ssp. *lactis* NCDC 094, *L. lactis* ssp. *cremoris* ATCC 19297 and *L. lactis* ssp. *cremoris* NCDC 086. However, no amplicon was generated with the chromosomal DNA of *Lactobacillus acidophilus* NCDC 015, *Leuconostoc mesenteroides* NCDC 029, *Lactobacillus plantarum* NCDC 021, *E. faecalis* NCDC 114, *E. faecium* NCDC 124, *S. aureus* NCDC 237 or *B. subtilis* NCDC 215. Therefore, our results confirmed the specificity of primer sets (serF/R and pepF/R) under the described PCR conditions for the identification of *L. lactis* ssp. *lactis/cremoris*.

PCR-RFLP of serine protease and non-proteolytic protein, peptidase family M16 gene fragment

The PCR-RFLP patterns of the 265 bp and 447 bp amplicons encoding the serine protease (*htrA*) and non-proteolytic protein, peptidase family M16 (*yueF*), respectively, of the identified test and reference strains *L. lactis* ssp. *lactis* NCDC 094, *L. lactis* ssp. *cremoris* ATCC 19297 and *L. lactis* ssp. *cremoris* NCDC 086 with seven restriction endonucleases (*Alu*I, *BamH*I, *Hind*III, *Hinf*I, *Pvu*II, *Sac*I and *Taq*I) are presented in Tables 4 and 5. The amplification of PCR products and restriction of DNA bands are depicted in Figs. 2 and 3. The digestion of PCR product of 265 bp and 447 bp with *Taq*I and *Alu*I, respectively, revealed discriminatory restriction patterns between ssp. *lactis* and *cremoris*. The restriction of 265 bp amplicons with *Taq*I

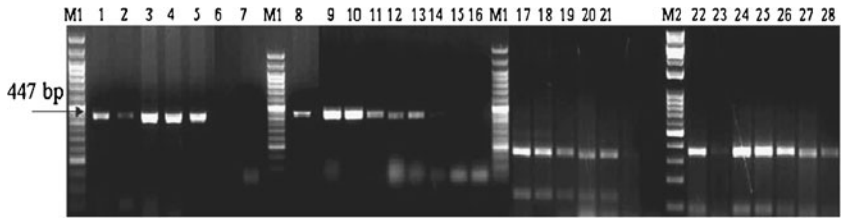

Fig. 3 PCR-RFLP of a fragment of non-proteolytic protein, peptidase family M16 (*yueF*) gene of *L. lactis* ssp. *lactis* and *cremoris* with *Alu*I. Lane 1–3, 11–13 and 18–21=test isolates, 4, 9 and 22–25=*L. lactis* ssp. *cremoris* ATCC 19297, 5, 10 and 17=*L. lactis* ssp. *lactis* NCDC 094, 6=*E. faecalis* NCDC 114, 7=*E. faecium* NCDC 124, 8 and 26–28=*L. lactis* ssp. *cremoris* NCDC 086, 14=*Lactobacillus acidophilus* NCDC 015, 15=*Leuconostoc mesenteroides* NCDC 029, 16=*Lactobacillus plantarum* NCDC 021, M1=50 bp DNA ladder and M2=1 Kb DNA ladder

Table 4 PCR-RFLP patterns of a 265 bp fragment encoding the serine protease (*htrA*) of *L. lactis* ssp. *lactis* and *cremoris*

Strain	PCR product (bp)	*Alu*I	*BamH*I	*Hind*III	*Hinf*I	*Pvu*II	*Sac*I	*Taq*I
L. l. ssp. *lactis* NCDC 094	265	NF[a]	NF	30, 235	NF	NF	NF	90, 175
L. l. ssp. *cremoris* ATCC 19297	265	87,178	NF	NF	NF	NF	NF	66, 199
L. l. ssp. *cremoris* NCDC 086	265	87,178	NF	NF	NF	NF	NF	66, 199
Test isolates	265	NF	NF	30, 235	NF	NF	NF	90, 175

[a] Not found

produced DNA bands measuring 90 bp and 175 bp with test strains and *L. lactis* ssp. *lactis* NCDC 094, while 66 bp and 199 bp were obtained with *L. lactis* ssp. *cremoris* ATCC 19297 and *L. lactis* ssp. *cremoris* NCDC 086. Similarly, the restriction of amplicons of 447 bp with *Alu*I produced DNA bands measuring 125 bp and 322 bp with ssp. *lactis*, and 71 bp and 376 bp with *L. lactis* ssp. *cremoris* ATCC 19297 and *L. lactis* ssp. *cremoris* NCDC 086. Thus, it was observed that ssp. *lactis* and *cremoris* of *L. lactis* could be identified by restriction of the PCR products of gene *htrA* and *yueF* with the single restriction enzymes *Taq*I and *Alu*I, respectively.

Discussion

During discrimination o *L. lactis* ssp. *lactis* and *cremoris* by PCR-RFLP, the PCR product of 447 bp from both subspecies was subjected to a battery of restriction endonucleases. It was observed that *Alu*I was able to generate 125 bp and 322 bp fragments from ssp. *lactis* while ssp. *cremoris* yielded 71 bp and 376 bp fragments. Two more endonucleases (*Pvu*II and *Taq*I) could restrict only the amplicons of ssp. *lactis*. Another conserved housekeeping gene, *htrA* encoding for trypsin like serine protease, was targeted to discriminate the two subspecies of *L. lactis* by restriction analysis of 265 bp amplicons with endonucleases. The restriction of amplicons with *Taq*I yielded fragments of 90 bp, 175 bp from ssp. *lactis* and 66 bp, 199 bp from ssp. *cremoris*. Further, *Alu*I and *Hin*dIII could discriminate ssp. *cremoris* and ssp. *lactis*, respectively. Other endonucleases such as *Bam*HI, *Hin*fI and *Sac*I could not act on the amplicons of *yueF* and *htrA* and therefore were not useful for the discrimination of subspecies of *L. lactis*. Larger restriction fragments were observed with *yueF*-*Alu*I as compared to *htrA*-*Taq*I and therefore were better visualized in agarose gels.

Earlier studies had already discriminated the two subspecies of *L. lactis* using PCR-RFLP assay. Buist et al. (1995) designed a set of primers (PALA-4 and PALA-14) targeting the *L. lactis* N-acetylmuramidase (*acmA*) gene, which differentiated ssp. *lactis* from ssp. *cremoris*. However, these results could not be confirmed by Prodelalova et al. (2005) with the primers designed by Buist et al. (1995).

Ward et al. (1998) designed a primer pair from the conserved sequence of 16S rRNA for the discrimination of ssp. *lactis* and *cremoris*. But the smaller size of fragments, measuring 23 bp, 65 bp and 257 bp in 16S rRNA-*Mbo*II, was difficult to visualize in agarose gels. Later, Nomura et al. (2002) also designed a primer set targeting the glutamate decarboxylase gene (*gadB*) to discriminate between two subspecies of *L. lactis*. The amplicons produced by ssp. *lactis* and *cremoris* were 600 bp and 560 bp, respectively, which were also difficult to discriminate easily. Therefore, this method was found best suitable for species level identification. Further, upon restriction digestion of *gadB* amplicons by *Ase*I, two fragments of 410 bp and 190 bp were generated in ssp. *lactis* but there was no restriction site in ssp. *cremoris*. However, the restriction site could be observed with one strain *L. lactis* ssp. *cremoris* MG1363 (Nomura et al. 2002).

Therefore, it may be concluded from the present study that amplicons targeting *yueF* and *htrA* subjected to restriction by *Alu*I and *Taq*I, respectively, are good enough to discriminate

Table 5 PCR-RFLP patterns of a 447 bp fragment encoding the non-proteolytic protein, peptidase family M16 (*yueF*) of *L. lactis* ssp. *lactis* and *cremoris*

Strain	PCR product (bp)	*Alu*I	*BamH*I	*Hind*III	*Pvu*II	*Sac*I	*Taq*I
L. l. ssp. *lactis* NCDC 094	447	125, 322	NF	NF	125, 322	NF	91, 356
L. l. ssp. *cremoris* ATCC 19297	447	71, 376	NF	NF	NF	NF	NF
L. l. ssp. *cremoris* NCDC 086	447	71, 376	NF	NF	NF	NF	NF
Test isolates	447	125, 322	NF	NF	125, 322	NF	91, 356

[a] Not found

L. lactis ssp. *lactis* and *cremoris* and hence PCR-RFLP assay could be used routinely for the identification of subspecies of *L. lactis* from dairy and non-dairy sources. Further, the detection of *L. lactis* ssp. *lactis* from dairy and non-dairy samples confirms their survival in dairy as well as non-dairy environments such as poultry farms and on cattle (Klijn et al. 1995). However, no detection of ssp. *cremoris* strains in the tested samples indicated its rare occurrence or survival. Our study also supports the observation that *L. lactis* strains present in starter cultures and in spontaneous milk fermentations may originate from cattle and their surroundings. However, it was observed that the number of lactococci in non-dairy environments was significantly lower than in dairy environments. Therefore, the survival of most starter cultures could be difficult in non-dairy environments.

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