Short Communication

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2010;18:156–161
DOI: 10.1159/000308518

Published online: April 13, 2010

Rapid Detection and Identification of Probiotic *Lactobacillus casei* ATCC 393 by Multiplex PCR

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Key Words
Lactic acid bacteria · *Lactobacillus casei* · Multiplex PCR · Probiotics

Abstract
Many functional foods containing probiotic strains have been developed recently. *Lactobacillus casei* ATCC 393 is one of the most frequently used cultures in probiotic products. The present study aimed to develop a method for the detection and identification of *L. casei* ATCC 393 based on genetic polymorphisms of the *hsp60* gene. A multiplex polymerase chain reaction (PCR) assay was designed, utilizing two novel strain-specific primer sets that enable identification of *L. casei* ATCC 393. The accuracy of our method was further confirmed by successful identification of our strain in probiotic cheese. The method described is an easy to use, rapid, inexpensive and accurate tool that may be readily applied to food, fecal and intestinal samples.

In recent years, the development and production of novel foods containing probiotic microorganisms have attracted tremendous interest due to their healthful properties. The Food and Agriculture Organization (FAO) and the World Health Organization (WHO) of the United Nations defined probiotics as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' [FAO, 2002]. Maintenance of the intestinal microbial homeostasis, prevention of pathogenic infections, stabilization of the gastrointestinal barrier function and production of anti-carcinogenic and anti-mutagenic compounds [Choi et al., 2006; Boirivant and Strober, 2007; Saulnier et al., 2008] are included among the beneficial effects of probiotic-based foods, mainly yogurt and other dairy products. Thus, a probiotic-rich diet is linked with the prevention and potential treatment of several severe digestive disorders, such as inflammatory bowel disease [Boirivant and Strober, 2007], colorectal cancer [Fotiadis et al., 2008] and obstructive jaundice [unpubl. data].

Lactobacillus and *Bifidobacterium* species are the most commonly used probiotic microorganisms. Among these lactic acid bacteria (LAB), the *Lactobacillus casei* ATCC 393 strain has been extensively incorporated into food products to confer probiotic properties [Kourkoutas et al., 2005, 2006; Li et al., 2009]. Although the taxonomic position of *L. casei* ATCC 393 (called *L. zeae* ATCC 393 in previous literature) was a subject of controversy in the past, the Judicial Commission recently affirmed that typification of *L. casei* is based on ATCC 393 [Judicial Com-

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L. zeae                 GGCGACCAAGGCAGCG   CTTCGGTTTCATCTTCC    CTGATTGCGGACGCC
L. paracasei            AGCAACTAAGGCTGCC   CGTCCGTTTCTTCCTCA    CTGATTGCCGACGCC
L. ingluviei            GGCTACGGCCGCAGCC   CTTCGATCTCTGCTGCG    TTGATTGCGGACGCA
L. kefiri               GGCTACCGAGACTGCT   CTTCTGTTTCATCAGCT    TTAATTGCTGATGCA
L. acetotolerans        AGCTACTGCAGCCGTA   CTTCAGTTTCATCATCT    TTAATCGCTGACGCT

L. casei ATCC 393 5'… GGCGACCAAGGCAGCG[78] CTTCGGTTTCATCTTCC[156] CTGATTGCGGACGCT[189] …3'
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Fig. 1. The *hsp60* gene sequence containing the diagnostic nucleotides (in bold) targeted by the novel primers designed in this study.

mission of the International Committee on Systematics of Bacteria, 2008].

Unfortunately, experimental analysis and quality control are hampered by the dire lack of a rapid, reliable and sensitive method for the detection of the above strain, in order to confirm and establish the probiotic character of a product. Identification and characterization of LAB in probiotic products are based on microbiological and molecular techniques. However, conventional microbiological and biochemical tests for phenotypical characterization are considered inadequate, as they have limitations in discriminating large numbers of isolates with similar physiological characteristics [Mohania et al., 2008]. On the other hand, several genotype-based methods have recently been developed. These methods are more accurate, sensitive, time-efficient and capable of identifying a wide spectrum of LAB in a single reaction [Mohania et al., 2008]. They are either directed at unique genes specific for a microbial species or at unique sequences of ubiquitous genes. Likewise, a number of studies focusing on the identification of *Lactobacillus* species developed polymerase chain reaction (PCR)-based molecular assays employing primers that target the variable regions of the 16S rRNA, the 23S rRNA, the 16S-23S rRNA intergenic spacer region (ISR) or other universal genes [Nour et al., 1998; Ward et al., 1999; Fasoli et al., 2002; Teanpaisan et al., 2006]. In parallel, a multiplex PCR assay system has been developed that allows the simultaneous amplification of two or more loci in the same reaction, thus increasing sensitivity and accuracy of discrimination [Song et al., 2000; Settanni et al., 2005; Sul et al., 2007].

The aim of the present study was to develop a multiplex PCR assay to detect and unequivocally identify the *L. casei* ATCC 393 strain in a single reaction. Our methodology was based on the *hsp60* gene sequence polymorphisms. Polymorphic sites within the *hsp60* gene have been used successfully as a molecular marker [Goh et al., 1996; Blaiotta et al., 2008]. The main advantage of this gene over other ubiquitous genes is the higher resolution that may be achieved in species discrimination, due to the great variability of the *hsp60* nucleotide sequences of different *Lactobacillus* species and the wealth of sequence data available in GenBank.

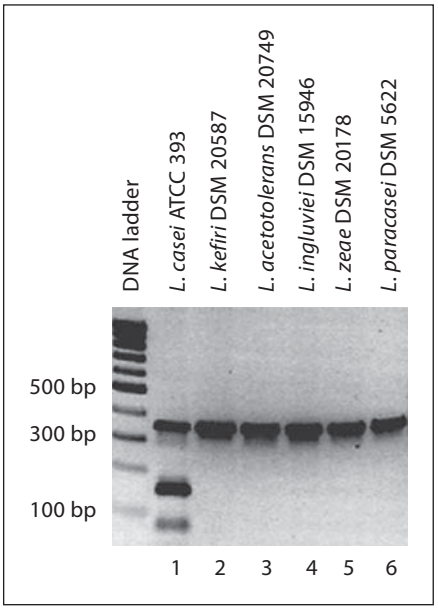

Fig. 2. Agarose gel electrophoresis of PCR products from multiplex PCR assay.

The *hsp60* gene sequences of all 56 *Lactobacillus* strains available in GenBank, including the *L. casei* ATCC 393 strain, were obtained by the National Center for Biotechnology Information (NCBI) (table 1). The sequences were aligned against each other using the DNA sequence alignment tool, ClustalW2 (European Bioinformatics Institute – EMBL). Extensive analysis allowed the identification of polymorphic sites that are unique for the *L. casei* ATCC 393 strain. The development of a multiplex PCR assay that enables straightforward detection of the strain of interest was based on the interrogation of combinations of three nucleotide sites, at positions 78, 156 and 189 (position +1 corresponds to the first nucleotide of the cDNA). These sites were selected because they have the greatest discriminatory value. As shown in table 1, *L. casei* ATCC 393 was the only strain with a G-C-T base

Table 1. List of *Lactobacillus* strains included in this study

Reference strains	Collection No.	Accession No.	Bases at diagnostic sites (78, 156, 189)
Lactobacillus acetotolerans	**DSM 20749**	**AF429666**	**A-T-T**
Lactobacillus acidipiscis	DSM 15836	AJ621719	T-A-T
Lactobacillus acidophilus	DSM 20079	AY424311	T-A-T
Lactobacillus alimentarius	DSM 20249	AY424318	T-A-T
Lactobacillus amylophilus	DSM 20533	AY424319	G-A-C
Lactobacillus amylovorus	DSM 20531	AY424312	C-A-T
Lactobacillus bifermentans	DSM 20003	AY424320	T-T-C
Lactobacillus brevis	DSM 20054	AY424329	T-A-C
Lactobacillus buchneri	DSM 20057	AY571676	T-C-A
Lactobacillus casei	**ATCC 393**	**AY424336**	**G-C-T**
Lactobacillus casei	**ATCC 11578**	**n.a.**	**n.a.**
Lactobacillus casei	**DN114001**	**n.a.**	**n.a.**
Lactobacillus casei	**BB-12**	**n.a.**	**n.a.**
Lactobacillus casei	CCC B1241	AF429642	C-A-C
Lactobacillus casei	DSA-FB2	AY424335	C-A-C
Lactobacillus casei	ATCC 4913	AF429654	T-A-C
Lactobacillus casei	CCC B1205	AF429631	C-A-C
Lactobacillus casei	DSA 145	AY424333	C-A-C
Lactobacillus casei	ATCC 334	AY424332	C-A-C
Lactobacillus casei	CCC 95G2L	AF429701	C-A-C
Lactobacillus casei	DSA 15	AY424334	C-A-C
Lactobacillus casei	I18C	AF429649	C-A-C
Lactobacillus casei	CCC B9657	AF429644	C-A-C
Lactobacillus casei	I03	AF429647	C-A-C
Lactobacillus coryniformis	DSM 20001	AY424321	C-T-T
Lactobacillus crispatus	DSM 20584	AY424313	T-T-T
Lactobacillus curvatus	DSA 32Y	AY424348	T-T-A
Lactobacillus cypricasei	DSM 15353	AJ621720	T-A-T
Lactobacillus delbrueckii subsp. lactis	DSM 20072	AY424322	C-T-C
Lactobacillus durianis	DSM 15802	AJ621721	T-T-T
Lactobacillus farciminis	DSM 20184	AY424323	A-T-C
Lactobacillus fermentum	DSA FB5	AY424325	C-T-C
Lactobacillus fructivorans	DSM 20203	AF429681	C-C-A
Lactobacillus gallinarum	DSM 10532	AY571675	T-T-T
Lactobacillus gasseri	DSM 20243	AY424314	T-T-A
Lactobacillus helveticus	DSM 20075	AY424315	T-T-T
Lactobacillus hilgardii	DSM 20571	AF429699	T-T-A
Lactobacillus homohiochii	ATCC 15434	AF429685	T-T-T
Lactobacillus ingluviei	**DSM 15946**	**AB267877**	**C-G-A**
Lactobacillus jensenii	DSM 20557	AF429687	T-T-T
Lactobacillus johnsonii	DSM 20553	AY424317	T-T-A
Lactobacillus kefiranofaciens	DSM 5016	AF429691	C-A-T
Lactobacillus kefiri	**DSM 20587**	**AF429688**	**T-T-A**
Lactobacillus kimchii	DSM 13961	AY571674	T-T-C
Lactobacillus mali	DSM 20444	AF429692	A-C-A
Lactobacillus parabuchneri	DSM 5708	AF429638	A-C-A
Lactobacillus paracasei	**DSM 5622**	**AY424340**	**C-A-C**
Lactobacillus paracasei	**DSM 20006**	**n.a.**	**n.a.**
Lactobacillus paracasei	**DSM 20207**	**n.a.**	**n.a.**
Lactobacillus paracasei	**DSM 46331**	**n.a.**	**n.a.**
Lactobacillus paracasei	**DSM 20312**	**n.a.**	**n.a.**
Lactobacillus paracasei	**DSM 20008**	**AF429694**	**C-A-C**
Lactobacillus paraplantarum	DSM 10667	AY424357	G-A-C
Lactobacillus pentosus	DSM 20314	AY424353	G-A-C
Lactobacillus plantarum	DSM 13273	AY571677	G-A-C

Table 1 (continued)

Reference strains	Collection No.	Accession No.	Bases at diagnostic sites (78, 156, 189)
Lactobacillus reuteri	DSM 20016	AY424326	T-T-A
Lactobacillus rhamnosus	DSM 20021	AY424337	C-T-A
Lactobacillus sakei	LTH 673	AY424352	T-T-T
Lactobacillus salivarius	DSM 20554	AY424328	A-T-C
Lactobacillus sanfranciscensis	DSM 20451	AY700220	C-C-C
Lactobacillus thermotolerans	DSM 14792	AJ621723	C-A-A
Lactobacillus vaccinostercus	DSM 20634	AJ621724	T-T-C
Lactobacillus zeae	**DSM 20178**	**AY571673**	**G-C-C**

The collection number relates to the *Lactobacillus* strain registered to the microbial banks. The accession number applies to the *hsp60* gene of each strain. The strains used in the present study are indicated in bold.
n.a. = Accession number and sequence data not available in NCBI.

Table 2. Oligonucleotide primers used in this study

Primer	Primer sequence (5′→3′)	Length bp	Optimal amount pmol
p78F	GGCGACCAAGGCAGC**G**	16	10
p156F	CTTCGGTTTCATCTTC**C**	17	50
p189R	GGCCAACTTTTTCCAT**A**	17	50
LacF	AGCAGTAGGGAATCTTCCA	19	10
LacR	ATTYCACCGCTACACATG	18	10

Table 3. Specificity of primers designed for multiplex PCR on *Lactobacillus* strains

Reference strain	Specificity of primer pairs		
	p78F/ p189R (144 bp)	p156F/ p189R (67 bp)	LacF/ LacR (340 bp)
L. casei ATCC 393	+	+	+
L. acetotolerans DSM 20749	–	–	+
L. ingluviei DSM 15946	–	–	+
L. paracasei DSM 5622	–	–	+
L. zeae DSM 20178	–	–	+

The expected size of PCR products is indicated.

composition at these three positions respectively. Thus, we designed two sets of primers (p78F-p189R and p156F-p189R) that are complementary to the sequence of the *hsp60* gene of the strain of interest (table 2). All primers end at diagnostic nucleotide positions. Some other *Lactobacillus* strains may carry the same base as *L. casei* at one or at two but not at all three of these diagnostic positions. Therefore, multiplex PCR allows the exclusive identification of *L. casei* with enhanced accuracy and reliability. According to the *hsp60* gene sequences of all strains examined, these primers do not generate any PCR fragment unless the *L. casei* ATCC 393 strain genomic DNA is used as template. In addition, a set of primers (LacF and LacR) that recognize the *16S rRNA* gene of all *Lactobacillus* strains was included in the present study as a positive control marker [Walter et al., 2001]. The specificity of the primers and the expected size of PCR products are presented in table 3. The annealing positions of these primers were designed so that they generate fragments which can be easily resolved in a standard agarose gel electrophoresis.

In order to rigorously test the reliability of our PCR-based methodology, we employed 5 strains selected in such a way that their sequences represent every possible nucleotide composition at the diagnostic positions (fig. 1; table 1). The strains were provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) microbial bank (Braunschweig, Germany) and grown on MRS broth (Merck, Darmstadt, Germany). Genomic DNA from liquid cell cultures was extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The amount of extracted DNA was determined by absorbance at 260 nm using a UV spectrophotometer (Eppendorf). All reactions were carried out in a total volume of 50 μl, containing 5 units Taq DNA polymerase (HyTest Ltd.), 400 μM each dNTPs (Promega), 1.5 mM $MgCl_2$ (HyTest Ltd.) and 100 ng template DNA. Optimized amounts of primers

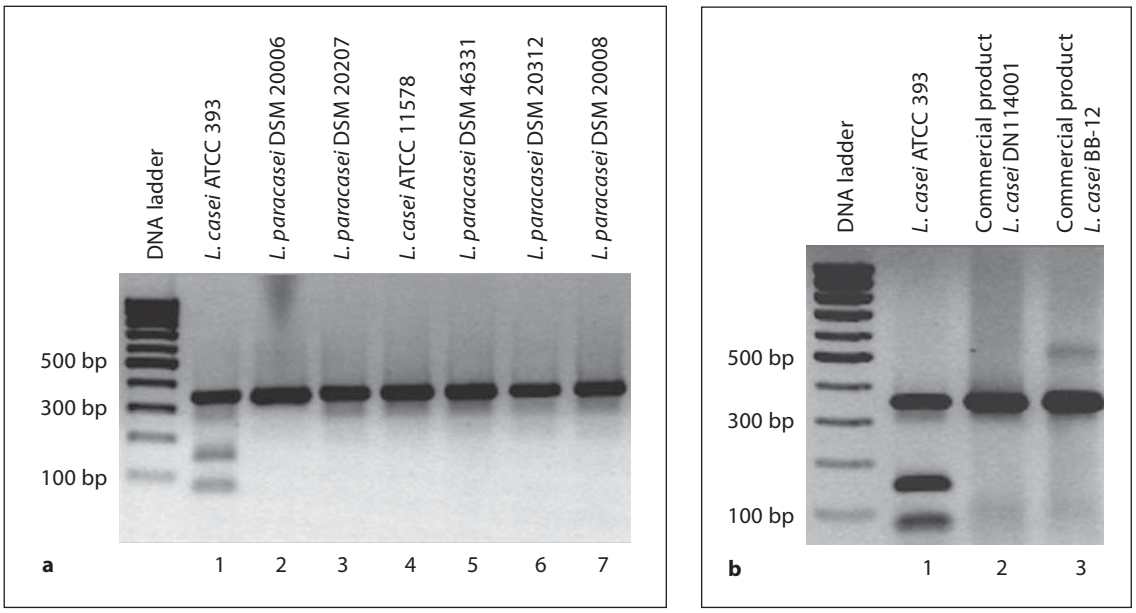

Fig. 3. Multiplex PCR assay of *L. casei* and *L. paracasei* strains (**a**) and two commercial probiotic products containing *L. casei* DN114001 and *L. casei* BB-12 (**b**).

per 50-μl reaction are shown in table 2. Amplification was carried out in a Thermal Cycler (Mastercycler Eppendorf) under the following conditions: 94°C (2 min), followed by 25 cycles of 94°C (15 s), 51°C (15 s), 72°C (30 s), followed by a final extension step at 72°C (1 min). The PCR products were separated on 1.5% w/v agarose gels, visualized under UV illumination and photographed with a digital camera (Gel Doc EQ System Biorad).

To optimize primer concentrations and cycling conditions (extension and annealing time and temperature), single PCR reactions were initially performed on *L. casei* ATCC 393, whereas the 5 representative *Lactobacillus* strains were used as negative controls. Subsequently, optimization of the multiplex PCR assays was performed (data not shown) [Henegariu et al., 1997]. Consistently, the multiplex PCR assays generated three products (67, 144 and 340 bp) for *L. casei* ATCC 393 and only one (340 bp) for any other *Lactobacillus* strain tested (fig. 2). The two shorter fragments (67 and 144 bp) originate from the *hsp60* gene sequence that is characteristic of the *L. casei* ATCC 393, whereas the 340-bp fragment corresponds to the 16S rRNA sequence common in all *Lactobacillus* strains and serves as a positive control marker [Walter et al., 2001].

Under all conditions tested, *L. casei* was readily detectable. No false-negative result was encountered. Importantly, no false-positive results were obtained when analysis was performed on 6 additional *L. casei* and *L. paracasei* strains (*L. casei* ATCC 11578, *L. paracasei* DSM 20006, *L. paracasei* DSM 20207, *L. paracasei* DSM 46331, *L. paracasei* DSM 20312 and *L. paracasei* DSM 20008) in liquid cultures (fig. 3a) and in 2 commercial probiotic products (fig. 3b). It is worth mentioning that for most of the above strains no sequence data was available (table 1). We then tested whether our method could be used for the detection of *L. casei* ATCC 393 in probiotic cheese prepared as described previously [Kourkoutas et al., 2006]. Cheese samples were blended with sterilized ¼ Ringer solution and total DNA was extracted from the liquid cell suspension as described above. Then, a multiplex PCR assay was performed using our novel strain-specific primers. As expected, three PCR products were generated (data not shown), corresponding to the unique pattern of *L. casei* ATCC 393 (fig. 2), thus confirming the presence of our strain in the cheese. Similarly, the applicability of our method for the detection of *L. casei* ATCC 393 in intestinal and fecal samples has been tested successfully. Of note, the proposed method was able to detect the strain of interest on the above samples even at levels $\leq 10^1$ cfu/g. This application illustrates the advantage of our approach over classical microbiological methods that require multiple dilutions and culture in order to detect LAB content in mixed microbial populations. Our multiplex assay readily identifies the exact strain without any require-

ment for separation of colonies. Our work paved the way for future attempts to develop quantitative multiplex PCR assays which may allow determination of the exact microbial load in food or biological samples.

In conclusion, the proposed multiplex PCR method proved an efficient tool for accurate, convenient and reliable identification of the *Lactobacillus casei* ATCC 393 strain. Our approach is rapid, inexpensive and does not require sophisticated lab equipment. It can be immediately adopted for detection and identification of this probiotic strain in food products, intestinal samples and feces. Additionally, our methodology can be easily adapted for other probiotic LAB species and strains, thus providing a powerful tool of molecular discrimination that could be invaluable in food industry.

References

Blaiotta G, Fusco V, Ercolini D, Aponte M, Pepe O, Villani F: *Lactobacillus* strain diversity based on partial *hsp60* gene sequences and design of PCR-restriction fragment length polymorphism assays for species identification and differentiation. Appl Environ Microbiol 2008;74:208–215.

Boirivant M, Strober W: The mechanism of action of probiotics. Curr Opin Gastroenterol 2007;23:679–692.

Choi SS, Kim Y, Han KS, You S, Oh S, Kim SH: Effects of *Lactobacillus* strains on cancer cell proliferation and oxidative stress in vitro. Lett Appl Microbiol 2006;42:452–458.

FAO/WHO: Food and Agriculture Organization of United Nation and World Health Organization Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food. London, FAO, 2002.

Fasoli S, Marzotto M, Rizzotti L, Rossi F, Dellaglio F, Torriani S: Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. Int J Food Microbiol 2003;82:59–70.

Fotiadis CI, Stoidis CN, Spyropoulos BG, Zografos ED: Role of probiotics, prebiotics and synbiotics in chemoprevention for colorectal cancer. World J Gastroenterol 2008;14:6453–6457.

Goh SH, Potter S, Wood JO, Hemmingsen SM, Reynolds RP, Chow AW: Hsp60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. J Clin Nutri 1996;36:818–823.

Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH: Multiplex PCR: Critical parameters and step-by-step protocol. BioTech 1997;23:504–511.

Judicial Commission of the International Committee on Systematics of Bacteria: The type strain of *Lactobacillus casei* is ATCC 393, ATCC 334 cannot serve as the type because it represents a different taxon, the name *Lactobacillus paracasei* and its subspecies names are not rejected and the revival of the name '*Lactobacillus zeae*' contravenes Rules 51b (1) and (2) of the International Code of Nomenclature of Bacteria. Opinion 82. Int J Syst Evol Microbiol 2008;58:1764–1765.

Kourkoutas Y, Ksolis V, Kallis M, Bezirtzoglou E, Kanellaki M: *Lactobacillus casei* immobilization on fruit pieces for probiotic additive, fermented milk and lactic acid production. Process Biochem 2005;40:411–416.

Kourkoutas Y, Bosnea L, Taboukos S, Baras C, Lambrou D, Kanellaki M: Probiotic cheese production using *Lactobacillus casei* cells immobilized on fruit pieces. J Dairy Sci 2006;89:1439–1451.

Li XY, Chen XG, Cha DS, Park HJ, Liu CS: Microencapsulation of a probiotic bacteria with alginate-gelatin and its properties. J Microenc 2009;26:315–324.

Mohania D, Nagpal R, Kumar M, Bhardwaj A, Yadav M, Jain S, Marotta F, Singh V, Parkash O, Yadav H: Molecular approaches for identification and characterization of lactic acid bacteria. J Digest Dis 2008;9:190–198.

Nour M: 16S–23S and 23S–5S intergenic spacer regions of lactobacilli: nucleotide sequence, secondary structure and comparative analysis. Res Microbiol 1998;149:433–448.

Saulnier D, Spinler J, Gibson G, Versalovic J: Mechanisms of probiosis and prebiosis: considerations for enhanced functional foods. Curr Opin Biotechnol 2009;20:1–7.

Song Y, Kato N, Liu C, Matsumiya Y, Kato H, Watanabe K: Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. FEMS Microbiol Lett 2000; 187:167–173.

Sul SY, Kim HJ, Kim TW, Kim HY: Rapid identification of *Lactobacillus* and *Bifidobacterium* in probiotic products using multiplex PCR. J Microbiol Biotechnol 2007;17:490–495.

Teanpaisan R, Dahlen G: Use of polymerase chain reaction techniques and sodium dodecyl sulphate polyacrylamide gel electrophoresis for differentiation of oral *Lactobacillus* species. Oral Microbiol Immunol 2006; 21:79–83.

Walter J, Hertel C, Tannock GW, Lis CM, Munro K, Hammes WP: Detection of *Lactobacillus, Pediococcus, Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. Appl Environ Microbiol 2001;67:2578–2585.

Ward LJ, Timmins MJ: Differentiation of *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction. Lett Appl Microbiol 1999;29: 90–92.