

# Molecular approaches for identification and characterization of lactic acid bacteria

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The last few years have produced a revolution in the development of very sensitive, rapid, automated, molecular detection methods for a variety of various species of lactic acid bacteria (LAB) associated with food and dairy products. Nowadays many such strains of LAB are considered probiotics. The genome-based methods are useful in identifying bacteria as a complementary or alternative tool to phenotypical methods. Over the years, identification methodologies using primers that target different sequences, such as the 16S ribosomal RNA (rRNA)-encoding gene, the 16S-23S rRNA intergenic spacer region, the 23S rRNA-encoding, *recA* and

*ldhD* genes; randomly amplified polymorphic DNA, restriction fragment length polymorphism, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, amplification rDNA restriction analysis, restriction enzyme analysis, rRNA, pulse field gel electrophoresis and amplification fragment length polymorphism have played a significant role in probiotic bacteriology. Hence, the aim of this review is to provide an overview of some rapid and reliable polymerase chain reaction-based molecular methods used for identifying and differentiating closely related species and strains of LAB associated with food and industry.

**KEY WORDS:** lactic acid bacteria, lactobacilli, molecular identification, polymerase chain reaction, probiotics.

## INTRODUCTION

In the 20th century, microbial cultures that have been used for thousands of years in food and alcoholic fermentations, have undergone scientific scrutiny for their ability to prevent and cure various diseases. This

has led to the coining of the term 'probiotics'. Probiotics are the 'live microorganisms which when administered in adequate amounts confer a health benefit on the host by improving its microbial balance'.<sup>1</sup> Lactic acid bacteria (LAB), i.e., *Lactobacilli* and *Bifidobacteria* are common inhabitants of the human intestine<sup>2</sup> and urogenital tract.<sup>3</sup> Microbes that have been frequently isolated from human and animal intestines and selected as probiotics include species of the genera *Lactobacillus*, *Bifidobacterium* and *Enterococcus*. However, some other LAB that do not normally inhabit the intestinal tract are also sometimes used as probiotics. Most of these bacteria are used as starters in dairy products

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1 and include *Streptococcus*, *Lactococcus*, *Leuconostoc* and  
2 *Pediococcus* species. Since different types of LAB can  
3 affect the human intestinal microenvironment in  
4 different ways, it is important to identify which micro-  
5 organisms are present in a microbial ecosystem and  
6 which species are most likely to have the potential  
7 protective effects. Nevertheless, the precise identification  
8 of these bacteria at the species level is not an easy task.  
9 The identification of *Lactobacillus* isolates by phenotypic  
10 methods is particularly difficult because it requires, in  
11 several cases, the determination of bacterial properties  
12 beyond those available in common fermentation  
13 tests.<sup>4</sup>

14  
15 Over the past decades a number of strains of LAB have  
16 been incorporated in a wide range of food products for  
17 human and animal nutrition. As the probiotic capacities  
18 are strain-dependent, methods for reliably identifying  
19 LAB at the strain level are of great importance, especially  
20 for the quality control of approved strains, to avoid  
21 health risks and misleading claims, as well as for the  
22 description of new strains. These days the main focus  
23 for identification has moved from phenotypical to  
24 genotypical methods as the latter generate more  
25 sensitive and accurate results, as reported for LAB by  
26 several authors.<sup>5,6</sup>

## 27 LAB

28  
29 The LAB group consists of a number of bacterial  
30 genera: *Lactobacillus*, *Lactococcus*, *Carnobacterium*,  
31 *Enterococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*,  
32 *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*,  
33 *Vagococcus*, *Weissella* and *Microbacterium*, *Bifidobacterium*  
34 and *Propionibacterium*.<sup>7</sup> LAB were first isolated from  
35 milk<sup>8</sup> and have since been found in foods and fermented  
36 products such as meat, milk products, vegetables,  
37 beverages and bakery products.<sup>9</sup> LAB has also been  
38 used for centuries as a flavoring and texturizing agent  
39 and as a preservative in food. LAB such as *Lactobacillus*  
40 *lactis* and *Streptococcus thermophilus* inhibit food spoilage  
41 and the growth of pathogenic bacteria, thereby preserving  
42 the nutritive qualities of raw food material for an  
43 extended shelf life.<sup>9</sup> Recently, the use of metabolites of  
44 LAB as biological preservatives in food-packaging  
45 materials has also been discussed.<sup>10</sup> The antimicrobial  
46 effect of LAB is mainly due to their lactic and organic  
47 acid production, which results in decreasing the pH of  
48 the growth environment.<sup>11</sup> A low pH induces the trans-  
49 formation of organic acids to soluble lipids, thereby  
50 making them diffuse through the cell membrane into  
51 the cytoplasm. LAB also produce acetaldehyde, hydrogen  
52 peroxide, diacetyl, carbon dioxide, polysaccharides  
53 and bacteriocins,<sup>12</sup> some of which may act as antimicro-

bials. LAB are regarded as a major group of probiotics.<sup>13</sup>  
Several lactobacilli, lactococci and bifidobacteria are  
considered to be bacteria that are beneficial to health.  
However, not much is known about the probiotic  
mechanisms of gut microbiota.<sup>14</sup> Generally, LAB have  
a long history of safe use in a variety of food products.  
Members of the genera *Lactococcus*, *Lactobacillus* and  
*Bifidobacterium* have thus been accorded the status of  
being 'generally recognized as safe'.<sup>15</sup> Consequently,  
the most commonly studied intestinal bacteria for  
potential probiotic use are members of the genera  
*Lactobacillus* and *Bifidobacterium* spp.<sup>16</sup>

The taxonomy of LAB based on comparative 16S  
ribosomal RNA (rRNA) sequencing analysis has  
revealed that some taxa generated on the basis of  
phenotypical features do not correspond with their  
phylogenetic relations. Molecular techniques, especially  
polymerase chain reaction (PCR)-based methods,  
such as rep-PCR fingerprinting and restriction fragment  
length polymorphism (RFLP) as well as pulse-field gel  
electrophoresis (PFGE) are regarded important for the  
specific characterization and detection of LAB strains.<sup>7</sup>  
Denaturing gradient gel electrophoresis (DGGE) and  
the temperature gradient gel electrophoresis (TGGE)  
analysis of the fecal 16S rDNA gene and its rRNA  
amplicons have shown to be powerful approaches in  
determining and monitoring the bacterial community  
in feces.<sup>17</sup>

## METHODS FOR IDENTIFYING LAB

### Phenotypical characterization

Traditionally, LAB have been classified on the basis of  
their phenotypical properties, e.g., their morphology,  
mode of glucose fermentation, growth at different  
temperatures, lactic acid configuration, the fermenta-  
tion of various carbohydrates, the methyl esters of fatty  
acids,<sup>18</sup> and the pattern of proteins in the cell wall<sup>19</sup> or  
in the whole cell.<sup>20</sup> Unfortunately, these typing  
methods are not completely accurate.<sup>21,22</sup> Phenotypical  
methods have inherent limitations such as their poor  
reproducibility, the ambiguity of some techniques  
(largely resulting from the plasticity of bacterial  
growth), the extensive logistics for large-scale investi-  
gations and their poor discriminatory power. Another  
disadvantage of phenotypical analysis is that the  
whole information potential of a genome is never  
expressed, i.e., gene expression is directly related to the  
environmental conditions (e.g., the growth conditions  
in the laboratory). All these drawbacks adversely affect  
the reliability of phenotype-based methods for culture  
identification at the genus or species level.

## Genotypical characterization

Many different genotyping techniques may be applied as tools for either species identification or differentiating the strains of LAB to their clonal level. The major advantages of these DNA-based typing methods lie in their discriminatory power<sup>23</sup> and in their universal applicability. Closely related strains with similar phenotypical features may now reliably be distinguished by DNA-based techniques such as randomly amplified polymorphic DNA (RAPD), RFLP, DGGE and TGGE and amplification rDNA restriction analysis (ARDRA). Some of these techniques are cited in Table 1, and their application in the identification of probiotic strains are reviewed in subsequent sections.

### Sequencing analysis of ribosomal DNA and other chronometer

Macromolecules have been described as documents of evolutionary history and for decades they have been used to explore the phylogenetic diversity and evolutionary relatedness of organisms. The 16S rRNA gene is the most common gene targeted in bacterial diversity studies. It is a well-conserved universal marker with constant and highly constrained functions that were established at early stages in its evolution and it is relatively unaffected by environmental pressures. These facts, along with the size of the gene, make it a good evolutionary clock.<sup>24</sup> Though the 16S rRNA gene is a well-conserved universal marker, however, there are some shortcomings associated with its use. First, the 16S rRNA genes are so well conserved that it results in a limited resolving power.<sup>25</sup> Second, even though the 16S rRNA gene is a universal marker different bacterial species have different copy numbers of the gene. This leads to an over- and under-representation of some bacterial species when using 16S rRNA genes as targets. Additionally, many genes other than 16S rRNA genes have also been explored in bacterial diversity studies. Some of these are universal genes that every bacterium possesses, but with unique genetic sequential differences. Most of these universal genes are well conserved to the extent that they perform the similar functions in all bacteria. The advantage of using universal genes could be that they may have a more consistent copy number among bacterial species, thus giving a better quantitative representation of bacterial species. Some of these genes are taxa-specific, which reveals a greater genetic diversity between closely related species, i.e., such genes provide much sharper phylogenetic resolution compared to universal genes.<sup>26</sup> The examples of such genes are the *dsr* gene for sulfate-reducing bacteria,<sup>26</sup> the *pmoA* gene for methanotrophs<sup>27</sup> and the *nif H* gene for cyanobacteria.<sup>28</sup>

Table 1. Molecular approaches executed for the rapid identification of lactic acid bacteria

Serial no.	Technique	Species identified	Reference
1.	Restriction enzyme analysis	<i>L. acidophilus</i>	70
		<i>L. casei</i>	71
		<i>L. rhamnosus</i>	72
		<i>L. reuteri</i>	
2.	Pulse-field gel electrophoresis	<i>Bifidobacteria</i>	73
		<i>L. casei</i>	74
		<i>L. acidophilus</i>	70
		<i>L. bulgaricus</i>	34
		<i>L. lactis</i>	35
		<i>L. fermentum</i>	
		<i>L. helveticus</i>	
3.	Ribotyping	<i>L. plantarum</i>	
		<i>L. rhamnosus</i>	
		<i>L. sakei</i>	
		<i>L. reuteri</i>	75
4.	RAPD profiling	<i>L. fermentum</i>	76
		<i>L. casei</i>	32
		<i>L. acidophilus</i>	
5.	Amplified rDNA restriction analysis	<i>L. acidophilus</i>	73
		<i>Bifidobacteria</i>	77
		<i>L. acidophilus</i>	39
		<i>L. rhamnosus</i>	40
		<i>L. fermentum</i>	
		<i>L. casei</i>	35
		<i>L. acidophilus</i>	7
6.	Amplified fragment length polymorphism	<i>L. delbrueckii</i>	60
		<i>L. fermentum</i>	
		<i>L. helveticus</i>	
		<i>L. plantarum</i>	
		<i>L. reuteri</i>	
		<i>L. rhamnosus</i>	
7.	Real-time PCR	<i>L. sakei</i>	35
		<i>L. pentosus</i>	
7.	Real-time PCR	<i>L. plantarum</i>	
		<i>L. pseudoplantarum</i>	
		<i>L. acidophilus</i>	78
		<i>L. casei</i>	69
		<i>L. delbrueckii</i>	
		<i>L. fermentum</i>	
		<i>L. paracasei</i>	
<i>L. plantarum</i>			
		<i>L. reuteri</i>	
		<i>L. rhamnosus</i>	

### Molecular ribotyping

Ribotyping simply refers to the use of nucleic acid probes to recognize ribosomal genes. In practice, bacterial chromosomal DNA is isolated and restriction patterns are created by hybridization with a 23S and 16S rRNA gene probe. Digestion of chromosomal DNA and

1 subsequent agarose gel electrophoresis is followed by  
2 Southern blotting, where the DNA is transferred to a  
3 membrane and hybridized with 23S and/or 16S rRNA  
4 probes. Generally, the fingerprint patterns are more  
5 stable and more easily interpretable than those obtained  
6 by restriction enzyme analysis (REA).<sup>29</sup> Another  
7 advantage lies in the high reproducibility and possibility  
8 of using a universal probe for all species because of the  
9 similarity of ribosomal genes.<sup>30</sup> In this context, Zhong  
10 *et al.*<sup>31</sup> examined the efficacy of ribotyping with  
11 *Lactobacillus* type and reference strains, namely, *L. johnsonii*,  
12 *L. casei*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, and  
13 *L. fermentum*. However, ribotyping shows high dis-  
14 criminatory power at the species level rather than on  
15 the strain level. Later, Chun *et al.*<sup>32</sup> characterized 91  
16 type and reference strains of the *L. casei* group and  
17 the *L. acidophilus* group by the automated ribotyping  
18 device Riboprinter Qualicon (Wilmington, DE, USA), a  
19 microbial characterization system. Most strains  
20 belonging to the two groups could be discriminated at  
21 the species level, and thus the Riboprinter system  
22 yielded rapid, accurate and reproducible genetic  
23 information for the identification of many strains.

#### 24 Pulse field gel electrophoresis (PFGE)

25 PFGE employs an alternating field of electrophoresis  
26 to allow the separation of the large DNA fragments  
27 obtained from restriction digests with rare-cutting  
28 enzymes, with increasing pulse times throughout  
29 the run, and the resulting fingerprint profiles can  
30 be explored for culture identification.<sup>7,33</sup> As such, the  
31 technique can be more time-consuming than other  
32 fingerprinting strategies. However, the profile generated  
33 by PFGE represents whole genome and this technique  
34 has a discriminatory power that is superior to ribotyping.  
35 Indeed, excellent subspecies differentiation has been  
36 shown using PFGE for a number of organisms, including  
37 lactobacilli and bifidobacteria.<sup>24,33</sup> In some cases PFGE  
38 has enabled the grouping of bacterial strains within a  
39 species, and there are various examples to assess the  
40 potential of this technique to characterize bacterial  
41 isolates as well. Further, the usefulness of PFGE has  
42 been adequately demonstrated in monitoring the  
43 changes in the predominant bifidobacterial and  
44 lactobacilli populations of human origin, both in  
45 individuals over time as well as between individuals.<sup>24</sup>  
46 Strain typing has been successfully achieved by PFGE  
47 for the *L. acidophilus* complex, *L. casei*, *L. delbrueckii*  
48 and its three subspecies (*bulgaricus*, *delbrueckii* and  
49 *lactis*), *L. fermentum*, *L. helveticus*, *L. plantarum*, *L. rhamnosus*  
50 and *L. sakei*.<sup>34,35</sup> Overall, PFGE has been shown to  
51 differentiate strains belonging to the same LAB species,  
52 to group strains within a species, to distinguish

between strains of different LAB species, and even to  
place isolates in specific *Lactobacillus* species. In a  
recent comparison, PFGE has been shown to be more  
discriminatory in typing closely related *L. casei* and  
*L. rhamnosus* strains than either ribotyping or RAPD  
analysis.<sup>36</sup> Use of multiple ribo patterns to determine  
the overall ribotypes of isolates has previously been  
shown to increase the discriminatory power of this  
technique.

#### RAPD fingerprinting

The RAPD technique is a PCR-based discrimination  
method in which short arbitrary primers anneal to  
multiple random target sequences, resulting in patterns  
of diagnostic value. In RAPD analysis, the target  
sequence(s) to be amplified is unknown and a primer  
with an arbitrary sequence (a 10-base pair sequence or  
a 10-bp sequence randomly generated by computer) is  
designed and synthesized. After these sequences have  
been synthesized they are used in PCR reactions with  
low-stringency annealing conditions, which results in  
the amplification of randomly sized DNA fragments.  
This method is currently being explored for the  
identification of LAB including probiotic strains. As  
the reproducibility of RAPD patterns is occasionally  
poor; this method needs to be performed under  
carefully controlled conditions. Various groups have  
adopted the use of RAPD to identify and characterize  
LAB strains from various sources, i.e., human, food  
and milk samples.<sup>37,38</sup>

RAPD-PCR has also been used to detect *L. rhamnosus*  
GR-1, *L. fermentum* RC-14 and *L. rhamnosus* GG (a  
commercially available intestinal probiotic) in the  
human vagina in order to assess their probiotic per-  
sistence at this site.<sup>39</sup> Later, Schillinger *et al.*<sup>40</sup> studied  
group-specific PCR and RAPD-PCR analyses to identify  
strains of the *L. casei* and *L. acidophilus* groups most  
commonly used in probiotic yogurts. For identification  
of lactobacilli to species or strain level, RAPD profiles  
of the 20 *Lactobacillus* strains were compared with 11  
reference strains of the *L. acidophilus* and *L. casei* group.  
In a later study 149 *Lactobacillus* isolates were subjected  
to RAPD with two random primers, OPL-05 and  
ArgDei-F.<sup>41</sup> The electrophoretic patterns generated were  
suitable for strain discrimination. The investigators  
reported a considerable degree of genomic diversity in  
*L. plantarum* isolates.

The dynamics of the microbial community responsible  
for the artisanal fermentation of dry sausages pro-  
duced in Argentina was studied using RAPD analysis  
with primers M13 and RAPD2 for the identification

1 and intraspecific differentiation of 100 strains of  
2 lactobacilli and *Micrococcaceae*.<sup>42</sup> Similarly, Weiss *et al.*<sup>43</sup>  
3 used RAPD-PCR with 14 arbitrary primers against  
4 potentially probiotic *L. reuteri* strains and found  
5 unique RAPD patterns specific to these strains. In a  
6 different investigation, LAB isolated from Spanish goat  
7 cheese were subjected to RAPD-PCR and the findings  
8 were compared with phenotypical characteristics.<sup>44</sup>  
9 Most of the isolates were identified as *L. s. paracasei*. A  
10 high degree of genetic diversity was found for *L. paracasei*  
11 ssp. *paracasei*, *L. curvatus* and *L. plantarum*. Recently,  
12 Sanchez *et al.*<sup>45</sup> isolated 248 strains of predominant  
13 lactobacilli isolates during the manufacturing and  
14 ripening of artisanal Manchego cheese. RAPD-PCR  
15 determined the genetic diversity of 197 isolates and 42  
16 distinct RAPD patterns were obtained, which grouped  
17 all the isolates into six major clusters at a similarity  
18 level of 54%. The RAPD-PCR DNA fingerprinting  
19 technique was used by Catzeddu *et al.*<sup>46</sup> to study the  
20 structure and diversity of LAB communities in the  
21 sourdough used to produce traditional bread. RAPD-PCR  
22 with a single primer followed by cluster analysis did  
23 not reveal the identity of isolates at a species level.  
24 However, a multidimensional scaling/bootstrapping  
25 approach on the RAPD-PCR patterns showed the  
26 diversity of the isolated LAB. Dal Bello and Hertel<sup>47</sup>  
27 revealed that the lactobacilli found in human fecal  
28 samples were autochthonous to the oral cavity by  
29 RAPD-PCR, which generated a similar type of banding  
30 patterns with each other. RAPD-PCR evaluated the  
31 biodiversity of lactobacilli from slightly fermented  
32 sausages.<sup>48</sup> The RAPD patterns thus generated were  
33 used to characterize 250 LAB isolated from low acid  
34 Spanish fermented sausages. This differentiated the  
35 LAB isolates into 144 different strains mainly belonging  
36 to *L. sakei*, *L. curvatus* and *Leuconostoc mesenteroides*.  
37 These findings were confirmed by species-specific PCR  
38 and by plasmid profiling of the isolates. Thus the  
39 efficiency of RAPD-PCR towards the typing of lacto-  
40 bacilli isolates at the species and strain level was  
41 substantiated.

#### 42 RFLP or chromosomal DNA restriction analysis

43 Chromosomal DNA restriction analysis was the first of  
44 the chromosomal DNA-based typing schemes. The  
45 banding patterns that result after cutting and separating  
46 the DNA fragments by electrophoresis are referred to  
47 as DNA fingerprinting. Because of the high specificity  
48 of restriction enzymes and the stability of chromosomal  
49 DNA, a reproducible pattern of fragments is obtained  
50 after the complete digestion of the chromosomal DNA  
51 by a particular enzyme. These variations in the  
52 banding patterns between strains are ascribed to basic  
53

differences in the DNA base composition of the  
organism examined. One general criticism about this  
method is the complexity of banding pattern. Never-  
theless, there are researchers who believe that using the  
right enzyme and specified conditions RFLP could still  
be a relatively rapid and reliable technique. PCR-RFLP  
has been successfully used to identify lactic acid bacteria  
species commonly isolated from wine. Preceded by  
colony isolation on solid selective medium and micro-  
scope observation to distinguish cocci and rods cells,  
the strains of seven cocci and 12 lactobacilli species  
could be identified by the PCR-RFLP approach.<sup>49</sup>  
Mainville *et al.*<sup>50</sup> also isolated and characterized the  
LAB of kefir using phenotypical, biochemical, and  
genotypical methods. Polyphasic analyses of the  
results permitted the identification of the microflora to  
the strain level, indicating that a RFLP-based polyphasic  
analysis approach increased confidence in strain  
determination by helping to confirm strain groupings,  
and hence, could have an impact on the phylogeny of  
the strains.<sup>50</sup> A simple and accurate protocol, based on  
the direct amplification from the colony of 16S rDNA  
and later digestion with restriction enzymes, to identify  
species of LAB isolated from grape must and wine has  
been executed by Rodas *et al.*<sup>51</sup> The technique was able  
to discriminate LAB reference species tested and  
allowed the successful identification of 342 isolates  
from musts and wines. Deveau and Moineau<sup>52</sup> also  
used RFLP for the differentiation and rapid characteri-  
zation of *Lactococcus lactis* strains producing exopoly-  
saccharides by analysis of their DNA restriction  
patterns, and concluded that the availability of such an  
effective RFLP-based cataloging system could benefit  
research aimed at identifying lactococcal strains.  
Hence, 16S rDNA-RFLP offers an effective and rapid  
method to isolate and distinguish the LAB coccus from  
their genera, such as the *Enterococcus* genus, the *Lactococcus*  
genus and the *Leuconostoc* genus, and may offer more  
correct results.<sup>53</sup>

#### DGGE and TGGE

The general principle of DGGE and TGGE is the  
separation of individual rRNA genes based on differences  
in their chemical stability or melting temperature.  
Polyacrylamide gels consisting of a linear denaturing  
gradient formed by urea and formamide are employed  
for DGGE, whereas a linear temperature gradient is  
used during TGGE. DGGE of PCR-amplified rRNA  
gene amplicons is a useful technique for monitoring  
dynamic changes in mixed bacterial populations over  
time.<sup>54,55</sup> The rRNA gene sequences from bacterial  
species in a mixed culture are first amplified using  
conserved bacterial primers that bracket a hypervariable

1 region of the rRNA gene, producing amplicons of the  
2 same length but with differing sequences that are  
3 specific to a given species. DGGE allows the separation  
4 of these amplicons, producing a molecular fingerprint  
5 of the bacterial species.<sup>54,55</sup> DGGE and other similar  
6 techniques have been shown to be useful to analyze  
7 human fecal microflora.<sup>56,57</sup> For example, DGGE may  
8 be useful to assess the effect of antibiotic therapy on  
9 the fecal microflora of hospitalized patients.<sup>58</sup> Since  
10 the bacterial microbiota of different individuals may  
11 vary significantly the ability to provide a detailed analysis  
12 of multiple individuals is limited.

13 DGGE of PCR-amplified rRNA gene amplicons offers  
14 several potential advantages over culture techniques as  
15 a method to monitor mixed bacterial populations.  
16 The development of molecular techniques based on  
17 sequence variability in 16S and 23S rRNA genes has  
18 led to an improved understanding of the microbial  
19 communities present in a variety of ecosystems,  
20 including the intestinal tract.<sup>59–61</sup> Although more than  
21 400 different species of anaerobes may be present in  
22 the human colon, phylogenetic analysis of 16S rRNA  
23 gene sequences has revealed that most of these organisms  
24 could be categorized into merely four phylogenetic  
25 clusters.<sup>60</sup> These clusters included the genus *Bacteroides*,  
26 the genus *Bifidobacterium*, *Clostridium coccooides* and  
27 relatives and a cluster including *Clostridium leptum* and  
28 relatives, fusobacteria and the *Atopobium* group.<sup>60</sup>  
29 Others have used similar techniques to identify many  
30 anaerobic species in the colon that have not previously  
31 been cultured.<sup>61</sup>

### 33 ARDRA

34 ARDRA is essentially the reverse of ribotyping, i.e., the  
35 RFLP of 16S rRNA PCR amplicons. However ribotyping  
36 generally affords greater discriminatory power than  
37 ARDRA due to the inclusion of the flanking regions of  
38 the 16S rRNA genes in the fingerprint. This approach  
39 has successfully differentiated various species or  
40 strains within the *Lactobacillus acidophilus* complex,  
41 *L. casei*, *L. delbrueckii*, *L. fermentum*, *L. helveticus*, *L.*  
42 *plantarum*, *L. reuteri*, *L. rhamnosus* and *L. sakei*.<sup>7,35</sup>  
43 ARDRA has been used to differentiate a variety of  
44 lactobacilli at species level, including *L. delbrueckii* and  
45 its three subspecies (*bulgaricus*, *delbrueckii* and *lactis*),  
46 *L. acidophilus* and *L. helveticus*.<sup>62</sup>

### 49 Amplification fragment length polymorphism (AFLP)

50 AFLP analysis is based on the selective amplification of  
51 restriction fragments from total digests of genomic  
52 DNA, after which the DNA fragments are separated by  
53 polyacrylamide gel electrophoresis.<sup>63</sup> AFLP methods

rapidly generate hundreds of highly replicable markers  
from the DNA of the organism, thus allowing high-  
resolution genotyping of fingerprinting quality. The  
time, cost efficiency, replicability and resolution of  
AFLP is of high quality. Originally developed for plant  
systematics, AFLP has been found to be a very useful  
fingerprinting technique for bacteria that is applicable  
for both species resolution and strain differentiation.  
To date, AFLP has been employed mostly in epidemio-  
logical studies and in investigations aiming to distinguish  
virulence markers in food-borne pathogens (such as  
*Listeria* and *Salmonella* spp.). However, species-level  
discrimination has been shown for the phylogenetically  
closely related species *Lactobacillus pentosus*, *L. plantarum*  
and *L. pseudoplantarum*, using this method.<sup>35</sup>

### Real-time PCR

Real-time PCR is a DNA-based technique that monitors  
the amplification of the target DNA in real time by  
monitoring fluorescence. Real-time PCR can be used  
to quantify bacteria from various samples including  
milk, feces, food and water, and it can be used for  
processing, detecting and confirming pathogens in  
multiple samples at any one time. Real time modifies  
the technique in a way that reduces (by 99.9%) the  
chance of false positives observed in traditional PCR.  
Even a single copy of target DNA can be detected due  
to a high dynamic range. The conventional PCR is  
sufficiently sensitive to detect the genus *Lactobacillus*<sup>64</sup>  
and the different *Lactobacillus* species.<sup>65,66</sup> However,  
conventional PCR can be used only for semi-quantitative  
assessment due to endpoint analyses where limitations  
such as the plateau phase<sup>67</sup> and diminishing effects of  
differences in PCR product abundance prevail.<sup>68</sup>  
Contemporary quantitative real-time PCR allows the  
monitoring of the complete amplification and, as a  
consequence, overcomes the limitations correlated  
with endpoint analyses of the PCR process. Recently a  
rapid and reliable technique to identify the strains of  
LAB has been developed using the 16S rRNA gene as  
the target. A PCR approach has been developed with  
hybridization probes that were designed according to  
the differences among the 16S rRNA genes of *L. casei*,  
*L. paracasei* and *L. rhamnosus*, and a melting curve analysis  
of the hybridization probe was used to distinguish  
them. This approach could identify *L. paracasei* and  
*L. rhamnosus* correctly but could not separate *L. paracasei*  
from *L. casei* due to the existence of same 16S rRNA  
sequence in both species.<sup>69</sup> These results suggest that  
the melting curve analysis of PCR approach in this  
study is a rapid, simple and accurate method in distin-  
guishing closely related strains of lactobacilli.

## 1 Future prospects: DNA chips

2 DNA chips or DNA arrays, which are nothing but  
3 ordered arrays of oligonucleotides immobilized on an  
4 organic substrate, provide new opportunities for assessing  
5 genetic diversity among microorganisms. These arrays  
6 rely on the hybridization of isolated microbial DNA to  
7 large sets of oligonucleotides or DNA fragments present  
8 at a precise location on a miniaturized inorganic  
9 substrate (e.g., a glass slide). DNA chips are now being  
10 investigated for bacteriological testing by various groups.  
11 The number of oligonucleotides spotted on a chip can  
12 vary from several hundred to almost a million.  
13 Oligonucleotides can be derived from every region of  
14 the genome and point mutations (single nucleotide  
15 polymorphisms) are targeted as well. Such DNA chips  
16 will certainly be commercially available in the near  
17 future for large-scale testing. The only difficulty this  
18 method may face is the cost of a chip, which can only  
19 be used once, and the need to purchase expensive equip-  
20 ment for hybridization and analysis. Nevertheless, this  
21 technique will be the method of choice to identify  
22 dairy organisms in the near future because of its high  
23 degree of reliability in terms of specificity and sensitivity.

## 24 CONCLUSION

25 These days the concept of probiotics has become a  
26 major area of considerable health concern in both  
27 developed and developing countries. The identification  
28 of probiotic LAB is largely based on phenotypical and  
29 biochemical characters. Practically, for routine identi-  
30 fication of isolates, these characteristics may not be  
31 enough to assign a strain definitely to a particular  
32 species. Hence, the need for developing innovative,  
33 rapid and reliable analytical techniques to identify  
34 these good bugs has been an urgent step towards their  
35 successful application in improving human health  
36 through probiotic fermented foods. Though the use of  
37 PCR-based techniques has facilitated the innovation of  
38 more efficient methods to detect microorganisms  
39 associated with foods, and they offer improvements in  
40 detecting and characterizing microbes beyond classical  
41 plating and phenotypical methods, much research,  
42 however, is still underway to further improve the  
43 capability of these powerful techniques vis-à-vis LAB.  
44 This eventually could result in generating very useful  
45 information and knowledge in this particular area.

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