Journal of Digestive Diseases 2008; **9;** 190–198 doi: 10.1111/j.1751-2980.2008.00345.x

Molecular approaches for identification and characterization of *lactic acid bacteria*

Dheeraj MOHANIA,* Ravinder NAGPAL,† Manoj KUMAR,† Aarti BHARDWAJ,‡ Mukesh YADAV,§ Shalini JAIN,¶ Francesco MAROTTA,** Vinod SINGH,†† Om PARKASH‡‡ & Hariom YADAV§§

*Animal Biochemistry and Dairy Microbiology Division and †National Dairy Research Institute, Karnal Haryana, ‡Meerut Institute of Engineering and Technology, Meerut, Uttar Pradesh, §School of Studies in Chemistry, Jiwaji University, Gwalior, Madhya Pradesh, India, ¶Food Science and Human Nutrition, University of Illinois, Urbana, USA, **Nutraceutical-Nutragenomic Unit, GAIA Foundation, Milan, Italy, ††Department of Microbiology, Barkatullah University, Bhopal, Madhya Pradesh, ‡‡National JALMA Institute for Leprosy and Mycobacterial Diseases, Agra, Uttar Pradesh, India; and §§National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bethesda, MD, USA

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'.[1] Lactic acid bacteria (LAB), i.e., *Lactobacilli* and *Bifidobacteria* are common inhabitants of the human intestine[2] and urogenital tract.[3] 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

Correspondence to: Hariom YADAV, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA. Email: yadavh@mail.nih.gov

C D D 3 4 5 | Operator: Wang Jingjing | Dispatch: 01.09.08 | PE: Heidi Allen
Journal Name | Manuscript No. | Proofreader: Qiu Jing | No. of Pages: 9 | Copy-editor:

and include *Streptococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus* species. Since different types of LAB can affect the human intestinal microenvironment in different ways, it is important to identify which microorganisms are present in a microbial ecosystem and which species are most likely to have the potential protective effects. Nevertheless, the precise identification of these bacteria at the species level is not an easy task. The identification of *Lactobacillus* isolates by phenotypic methods is particularly difficult because it requires, in several cases, the determination of bacterial properties beyond those available in common fermentation tests.[4]

Over the past decades a number of strains of LAB have been incorporated in a wide range of food products for human and animal nutrition. As the probiotic capacities are strain-dependent, methods for reliably identifying LAB at the strain level are of great importance, especially for the quality control of approved strains, to avoid health risks and misleading claims, as well as for the description of new strains. These days the main focus for identification has moved from phenotypical to genotypical methods as the latter generate more sensitive and accurate results, as reported for LAB by several authors.[5,6]

LAB

The LAB group consists of a number of bacterial genera: *Lactobacillus*, *Lactococcus*, *Carnobacterium*, *Enterococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, *Weissella* and *Microbacterium*, *Bifidobacterium* and *Propionibacterium*.[7] LAB were first isolated from milk[8] and have since been found in foods and fermented products such as meat, milk products, vegetables, beverages and bakery products.[9] LAB has also been used for centuries as a flavoring and texturizing agent and as a preservative in food. LAB such as *Lactobacillus lactis* and *Streptococcus thermophilus* inhibit food spoilage and the growth of pathogenic bacteria, thereby preserving the nutritive qualities of raw food material for an extended shelf life.[9] Recently, the use of metabolites of LAB as biological preservatives in food-packaging materials has also been discussed.[10] The antimicrobial effect of LAB is mainly due to their lactic and organic acid production, which results in decreasing the pH of the growth environment.[11] A low pH induces the transformation of organic acids to soluble lipids, thereby making them diffuse through the cell membrane into the cytoplasm. LAB also produce acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins,[12] some of which may act as antimicrobials. LAB are regarded as a major group of probiotics.[13] 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.[14] 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'.[15] Consequently, the most commonly studied intestinal bacteria for potential probiotic use are members of the genera *Lactobacillus* and *Bifidobacterium* spp.[16]

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.[7] 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.[17]

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 fermentation of various carbohydrates, the methyl esters of fatty acids,[18] and the pattern of proteins in the cell wall[19] or in the whole cell.[20] Unfortunately, these typing methods are not completely accurate.[21,22] 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 investigations 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[23] 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.[24] 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.[25] 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.[26] The examples of such genes are the *dsr* gene for sulfate-reducing bacteria,[26] the *pmo*A gene for methanotrophs[27] and the *nif* H gene for cyanobacteria.[28]

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

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

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

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

Pulse field gel electrophoresis (PFGE)

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

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 persistence at this site.[39] Later, Schillinger *et al.*[40] 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.[41] 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 produced in Argentina was studied using RAPD analysis with primers M13 and RAPD2 for the identification

and intraspecific differentiation of 100 strains of lactobacilli and *Micrococcaceae*.[42] Similarly, Weiss *et al.*[43] used RAPD-PCR with 14 arbitrary primers against potentially probiotic *L. reuteri* strains and found unique RAPD patterns specific to these strains. In a different investigation, LAB isolated from Spanish goat cheese were subjected to RAPD-PCR and the findings were compared with phenotypical characteristics.[44] Most of the isolates were identified as *L.s paracasei.* A high degree of genetic diversity was found for *L. paracasei* ssp. *paracasei*, *L. curvatus* and *L. plantarum*. Recently, Sanchez *et al.*[45] isolated 248 strains of predominant lactobacilli isolates during the manufacturing and ripening of artisanal Manchego cheese. RAPD-PCR determined the genetic diversity of 197 isolates and 42 distinct RAPD patterns were obtained, which grouped all the isolates into six major clusters at a similarity level of 54%. The RAPD-PCR DNA fingerprinting technique was used by Catzeddu *et al.*[46] to study the structure and diversity of LAB communities in the sourdough used to produce traditional bread. RAPD-PCR with a single primer followed by cluster analysis did not reveal the identity of isolates at a species level. However, a multidimensional sealing/bootstrapping approach on the RAPD-PCR patterns showed the diversity of the isolated LAB. Dal Bello and Hertel[47] revealed that the lactobacilli found in human fecal samples were autochthonous to the oral cavity by RAPD-PCR, which generated a similar type of banding patterns with each other. RAPD-PCR evaluated the biodiversity of lactobacilli from slightly fermented sausages.[48] The RAPD patterns thus generated were used to characterize 250 LAB isolated from low acid Spanish fermented sausages. This differentiated the LAB isolates into 144 different strains mainly belonging to *L. sakei*, *L. curvatus* and *Leuconostoc mesenteroides.* These findings were confirmed by species-specific PCR and by plasmid profiling of the isolates. Thus the efficiency of RAPD-PCR towards the typing of lactobacilli isolates at the species and strain level was substantiated.

RFLP or chromosomal DNA restriction analysis

Chromosomal DNA restriction analysis was the first of the chromosomal DNA-based typing schemes. The banding patterns that result after cutting and separating the DNA fragments by electrophoresis are referred to as DNA fingerprinting. Because of the high specificity of restriction enzymes and the stability of chromosomal DNA, a reproducible pattern of fragments is obtained after the complete digestion of the chromosomal DNA by a particular enzyme. These variations in the banding patterns between strains are ascribed to basic differences in the DNA base composition of the organism examined. One general criticism about this method is the complexity of banding pattern. Nevertheless, 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 microscope observation to distinguish cocci and rods cells, the strains of seven cocci and 12 lactobacilli species could be identified by the PCR-RFLP approach.[49] Mainville *et al.*[50] 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.[50] 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.*[51] 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[52] also used RFLP for the differentiation and rapid characterization of *Lactococcus lactis* strains producing exopolysaccharides 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.[53]

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.[54,55] The rRNA gene sequences from bacterial species in a mixed culture are first amplified using conserved bacterial primers that bracket a hypervariable

region of the rRNA gene, producing amplicons of the same length but with differing sequences that are specific to a given species. DGGE allows the separation of these amplicons, producing a molecular fingerprint of the bacterial species.[54,55] DGGE and other similar techniques have been shown to be useful to analyze human fecal microflora.[56,57] For example, DGGE may be useful to assess the effect of antibiotic therapy on the fecal microflora of hospitalized patients.[58] Since the bacterial microbiota of different individuals may vary significantly the ability to provide a detailed analysis of multiple individuals is limited.

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

ARDRA

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

Amplification fragment length polymorphism (AFLP)

AFLP analysis is based on the selective amplification of restriction fragments from total digests of genomic DNA, after which the DNA fragments are separated by polyacrylamide gel electrophoresis.[63] 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 epidemiological studies and in investigations aiming to distinguish virulence markers in food-borne pathogens (such as *Listeria* and *Salmonella* ssp.). However, species-level discrimination has been shown for the phylogenetically closely related species *Lactobacillus pentosus*, *L. plantarum* and *L. pseudoplantarum*, using this method.[35]

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*[64] and the different *Lactobacillus* species.[65,66] However, conventional PCR can be used only for semi-quantitative assessment due to endpoint analyses where limitations such as the plateau phase[67] and diminishing effects of differences in PCR product abundance prevail.[68] 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.[69] These results suggest that the melting curve analysis of PCR approach in this study is a rapid, simple and accurate method in distinguishing closely related strains of lactobacilli.

Future prospects: DNA chips

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

CONCLUSION

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

REFERENCES

1 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, Ontario: FAO, 2002.

2 Mitsuoka T. The human gastrointestinal tract. In: Wood, BJB, ed. *The Lactic Acid Bacteria in Health and Disease*, London: Elsevier Applied Science, 1992; pp 69–114.

3 Boris S, Barbes C. Role played by lactobacilli in controlling the population of vaginal pathogens. *Microbes Infect* 2000; **2**: 543–6.

4 Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Ng J, Munro K, Alatossava T. Identification of *Lactobacillus* isolates from the gastrointestinal tract, silage, and yoghurt by 16S–23S rRNA gene intergenic spacer region sequence comparisons. *Appl Environ Microbiol* 1999; **65**: 4264–7.

5 Lick S. Typing systems for lactobacilli. *Milchwissenschaft* 2003; **58**: 256–60.

6 Callon C, Millet L, Montel MC. Diversity of lactic acid bacteria isolated from AOC Salers cheese. *J Dairy Res* 2004; **71**: 231–44.

7 Holzapfel WH, Haberer P, Geisen R, Bjorkroth J, Schillinger U. Taxonomy and important features of probiotic microorganisms in food and nutrition. *Am J Clin Microbiol* 2001; **73**: 365S–73S.

8 Metchnikoff E. *Prolongation of Life: Optimistic Studies*. London: Heinemann, 1908: pp 161–83.

9 O'Sullivan L, Ross RP, Hill C. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie* 2002; **84**: 593–604.

10 Pirttijärvi TSM, Wahlström G, Rainey FA, Saris PEJ, Salkinoja-Salonen MS. Inhibition of bacilli in industrial starches by nisin. *J Ind Microbiol Biotechnol* 2001; **26**: 107–14.

11 Kuipers OP, Buist G, Kok J. Current strategies for improving food bacteria. *Res Microbiol* 2000; **151**: 815–22.

12 Rodriguez E, Arques JL, Rodriguez R, Nunez M, Medina M. Reuterin production by lactobacilli isolated from pig feces and evaluation of probiotic traits. *Lett Appl Microbiol* 2003; **37**: 259–63.

13 Tannock GW. Studies of the intestinal microbiota: a prerequisite for the development of probiotics. *Int Dairy J* 1998; **8**: 527–33.

14 Gibson GR, Fuller R. Aspects of *in vitro and in vivo* research approaches directed toward identifying probiotics and probiotics for human use. *J Nutr* 2000; **130**: 391S–95S.

15 Salminen S, Deighton MA, Benno Y, Gorbach SL. Lactic acid bacteria in health and disease. In: Salminen S, von Wright A, eds. *Lactic Acid Bacteria: Microbiology and Functional Aspects*. New York: Marcel Dekker, 1998; 211–54.

16 Nagpal R, Yadav H, Puniya AK, Singh K, Jain S, Marotta F. Potential of probiotics and prebiotics for synbiotic functional dairy foods: an overview. *Int J Probiotics Prebiotics* 2007; **2**: 75–84.

17 Zoetendal EG, Akkermans ADL, de-Vos WM. Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl Environ Microbiol* 1998; **64**: 3854–9.

18 Decallone J, Delmee M, Wauthoz P, El lioul M, Lambert R. A rapid procedure for the identification of lactic acid bacteria based on the gas chromatographic analysis of cellular fatty acids. *J Food Prot* 1991; **54**: 217–24.

19 Gatti M, Fornasari E, Neviani E. Cell-wall protein profiles of dairy *thermophilic lactobacilli*. *Lett Appl Microbiol* 1997; **25**: 345–8.

20 Tsakalidou E, Manolopoulou E, Kabaraki E *et al*. The combined use of whole-cell protein extracts for the identification (SDS-PAGE) and enzyme activity screening of lactic acid bacteria isolated from traditional Greek dairy products. *Syst Appl Microbiol* 1994; **17**: 444–58.

21 William RAD, Sandler SA. Electrophoresis of glucose-6-phosphate dehydrogenase, cell wall composition and the taxonomy of heterofermentative lactobacilli. *J Gen Microbiol* 1971; **65**: 351–8.

22 Morelli L. Taxonomy and physiology of lactic acid bacteria, effects and function on nutrition. Report of a joint FAO/WHO expert consultation on evaluation on health and nutritional properties of probiotics in food including powder milk with lactic acid bacteria, 2001. (Online.) Food and Agricultural Organization of the United Nations, New York, NY Cited: 15 Aug 2008 Available from URL ftp://ftp.fao.org/es/esn/food/Morelli.pdf, 2001.
23 Farber JM. An introduction to the hows and whys of molecular typing. *J Food Prot* 1996; **59**: 1091–101.
24 Kimura K, McCartney AL, McConnell MA, Tannock GW. Analysis of fecal populations of bifidobacteria and lactobacilli, and investigation of the immunological responses of their human hosts to the predominant strains. *Appl Environ Microbiol* 1997; **63**: 3394–8.
25 Achenbach LA, Carey AJ, Madigan MT. Photosynthesis and phylogenetic primers for detection of anoxygenic phototrophs in natural environments. *Appl Environ Microbiol* 2001; **67**: 2922–6.
26 Chang YJ, Peacock AD, Long PE *et al*. Diversity and characterization of sulfate–reducing bacteria in groundwater at a uranium mill tailings site. *Appl Environ Microbiol* 2001; **67**: 3149–60.
27 Costello A, Lidstrom ME. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl Environ Microbiol* 1999; **65**: 5066–74.
28 Rosado AS, Dauarte GF, Seldin L, VanElsas JD. Genetic diversity of *nif* H gene sequences in *Paenibacillus azotofixans* strains and soil samples analyzed by denaturing gradient gel electrophoresis of PCR – amplified gene fragments. *Appl Environ Microbiol* 1998; **64**: 2770–9.
29 Charteris WP, Kelly PM, Morelli L, Collins JK. Selective detection, enumeration and identification of potentially probiotic *Lactobacillus and Bifidobacterium* species in mixed bacterial populations. *Int J Food Microbiol* 1997; **35**: 1–27.
30 Grimont F, Grimont PDA. Ribosomal ribonucleic acid gene restriction as potential taxonomic tools. *Annu Inst Pasteur Microbiol* 1986; **137B**: 165–75.
31 Zhong W, Millsap K, Bialkowska-Hobrzanska H, Reid G. Differentiation of *Lactobacillus* species by molecular typing. *Appl Environ Microbiol* 1998; **64**: 2418–23.
32 Chun SR, Czajka WJ, Lakamoto M, Benno Y. Characterization of the *Lactobacillus casei* group and the *Lactobacillus acidophilus* group by automated ribotyping. *Microbiol Immunol* 2001; **45**: 271–5.
33 O'Sullivan DJ, Kullen MJ. Tracking of probiotic bifidobacteria in the intestine. *Int Dairy J* 1999; **8**: 513–25.
34 Klein G, Pack A, Bonaparte C, Reuter G. Taxonomy and physiology of probiotic lactic acid bacteria. *Int J Food Microbiol* 1998; **41**: 103–25.
35 Giraffa G, Neviani E. Molecular identification and characterization of food associated lactobacilli. *Italian J Food Sci* 2000; **4**: 403–23.
36 Tynkkynen S, Satokari R, Saarela M, Mattila-Sandholm M, Saxelin M. Comparison of ribotyping, randomly amplified polymorphic DNA analysis, and pulse field gel electrophoresis in typing of *Lactobacillus rhamnosus* and *L. casei* strains. *Appl Environ Microbiol* 1999; **65**: 3908–14.
37 Oh-Sik K. Characterization of isolated *Lactobacillus* spp. and classification by RAPD-PCR analysis. *J Microbiol* 2002; **38**: 137–44.
38 Spano G, Beneduce L, Tarantino D, Zapparoli G, Massa S. Characterization of *Lactobacillus plantarum* from wine must by PCR species-specific and RAPD-PCR. *Lett Appl Microbiol* 2002; **35**: 370–4.
39 Gardiner GE, Heinemann C, Bruce AW, Beuerman D, Reid G. Persistence of *Lactobacillus fermentum* RC-14 and *Lactobacillus rhamnosus* GR-1 but not *L. rhamnosus* GG in the human vagina as demonstrated by randomly amplified polymorphic DNA. *Clin Diagn Lab Immunol* 2002; **9**: 92–6.
40 Schillinger U, Yousif NM, Sesar L, Franz CM. Use of group-specific and RAPD-PCR analyses for rapid differentiation of *Lactobacillus* strains from probiotic yogurts. *Curr Microbiol* 2003; **47**: 453–6.
41 Sanchez I, Sesena S, Palop L. Polyphasic study of the genetic diversity of lactobacilli associated with 'Almagro' eggplants spontaneous fermentation, based on combined numerical analysis of randomly amplified polymorphic DNA and pulsed-field gel electrophoresis patterns. *J Appl Microbiol* 2004; **97**: 446–7.
42 Fontanaa C, Cocconcelli PS, Vignoloa G. Monitoring the bacterial population dynamics during fermentation of artisanal Argentinean sausages. *Int J Food Microbiol* 2005; **103**: 131–42.
43 Weiss A, Lettner HP, Kramer W, Mayer HK, Kneifel W. Molecular methods used for the identification of potentially probiotic *Lactobacillus reuteri* strains. *Food Technol Biotechnol* 2005; **43**: 295–300.
44 Sanchez I, Sesena S, Poveda JM, Cabezas L, Palop L. Phenotypic and genotypic characterization of lactobacilli isolates from Spanish goat cheese. *Int J Food Microbiol* 2005; **102**: 355–62.
45 Sanchez I, Sesena S, Poveda JM, Cabezas L, Palop L. Genetic diversity, dynamics, and activity of Lactobacillus community involved in traditional processing of artisanal Manchego cheese. *Int J Food Microbiol* 2006; **107**: 265–73.
46 Catzeddu P, Mura E, Parente E, Sanna M, Farris GA. Molecular characterization of lactic acid bacteria from sourdough breads produced in Sardinia (Italy) and multivariate statistical analyses of results. *Syst Appl Microbiol* 2006; **29**: 138–44.
47 Dal Bello F, Hertel C. Oral cavity as natural reservoir for intestinal lactobacilli. *Syst Appl Microbiol* 2006; **29**: 69–76.
48 Aymerich T, Martin B, Garriga M, Vidal-Carou MC, Bover-Cid S, Hugas M. Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *J Appl Microbiol* 2006; **100**: 40–9.
49 Claisse O, Renouf V, Lonvaud-Funel A. Differentiation of wine lactic acid bacteria species based on RFLP analysis of a partial sequence of *rpoB* gene. *J Microbiol Meth* 2007; **69**: 387–90.
50 Mainville I, Robert N, Lee B, Farnworth ER. Polyphasic characterization of the lactic acid bacteria in kefir. *Syst Appl Microbiol* 2005; **29**: 59–68.
51 Rodas AM, Ferrer S, Pardo I. 16S-ARDRA: A tool for identification of lactic acid bacteria isolated from grape must and wine. *Syst Appl Microbiol* 2003; **26**: 412–22.
52 Deveau H, Moineau S. Use of RFLP to characterize *Lactococcus lactis* strains producing exopolysaccharides. *J Dairy Sci* 2003; **86**: 1472–5.
53 Yanagida F, Chen Y, Shinohara T. Isolation and characterization of lactic acid bacteria from soils in vineyards. *J Gen Appl Microbiol* 2005; **51**: 313–8.
54 Muyzer G, DeWaal EC, Uitterlinden AG. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 1993; **59**: 695–700.
55 Muyzer G, Brinkhoff T, Nubel U, Santegoeds C, Schafer H, Wawer C. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology. *Molecular Microbial Ecology Manual* 1998; **3.4.4**: 1–27.
56 Millar MR, Linton CJ, Cade A, Glancy D, Hall M, Jalal L. Application of 16S rRNA gene PCR to study bowel flora of

preterm infants with and without necrotizing enterocolitis. *J Clin Microbiol* 1996; **3**: 2506–10.
57 Favier CF, Vaughan EE, De Vos WM, Akkermans AD. Molecular monitoring of succession of bacterial communities in human neonates. *Appl Environ Microbiol* 2002; **68**: 219–26.
58 Donskey CJ, Hujer AM, Das SM, Pultz NJ, Bonomo RA, Rice LB. Use of denaturing gradient gel electrophoresis for analysis of the stool microbiota of hospitalized patients. *J Microbiol Methods* 2003; **54**: 249–56.
59 Wilson KH, Blitchington RB. Human colonic biota studied by ribosomal DNA sequence analysis. *Appl Environ Microbiol* 1996; **62**: 2273–8.
60 Wilson KH, Ikeda JS, Blitchington RB. Phylogenetic placement of community members of human colonic biota. *Clin Infect Dis* 1997; **25**: S114–6.
61 Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Dore J. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. *Appl Environ Microbiol* 1999; **65**: 4799–07.
62 Roy D, Sirois S. Molecular differentiation of *Bifidobacterium* species with amplified ribosomal DNA restriction analysis and alignment of short regions of the *idh* gene. *FEMS Microbiol Lett* 2001; **191**: 17–24.
63 Vos P, Hogers R, Bleeker M *et al*. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res* 1995; **23**: 4407–14.
64 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**: 2578–85.
65 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**: 167–73.
66 Walter J, Tannock GW, Tilsala-Timisjarvi A *et al*. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl Environ Microbiol* 2000; **66**: 297–303.
67 Morrison C, Gannon F. The impact of the PCR plateau phase on quantitative PCR. *Biochim Biophys Acta* 1994; **1219**: 493–8.
68 Mathieu-Daude WFJ, Vogt T, McClelland M. DNA rehybridization during PCR: the 'Cot effect' and its consequences. *Nucleic Acids Res* 1996; **24**: 2080–6.
69 Kao YT, Liu YS, Shyu YT. Identification of *Lactobacillus* spp. in probiotic products by real-time PCR and melting curve analysis. *Food Res Int* 2007; **40**: 71–9.
70 Roussel Y, Colmin C, Simonet JM, Decaris B. Strain characterization, genome size and plasmid content in the *Lactobacillus acidophilus* group. *J Appl Bacteriol* 1993; **74**: 549–56.
71 Ahrne S, Molin G. Restriction endonuclease analysis of total chromosomal DNA of *Lactobacillus. Microecol Ther* 1997; **26**: 27–30.
72 Stahl M, Molin G. Classification of *Lactobacillus reuteri* by restriction endonuclease analysis of chromosomal DNA. *Int J Syst Bacteriol* 1994; **44**: 9–14.
73 Roy D, Ward P, Champagne G. Differentiation of *Bifidobacteria* by use of pulsed-field gel electrophoresis and polymerase chain reaction. *Int J Food Microbiol* 2004; **29**: 11–29.
74 Ferrero M, Cesena C, Morelli L, Scolari G, Vescovo M. Molecular characterization of *Lactobacillus casei* strains. *FEMS Microbiol Lett* 1996; **140**: 215–9.
75 Ning W, Mackie RI, Gaskins HR. Biotype and ribotype diversity among *Lactobacillus* isolates from mouse ileum. *Syst Appl Microbiol* 1997; **20**: 423–31.
76 Rodtong S, Tannock GW. Differentiation of *Lactobacillus* strains by ribotyping. *Appl Environ Microbiol* 1993; **59**: 3480–4.
77 Du Plessis EM, Dicks LMT. Evaluation of random amplified polymorphic DNA (RAPD) -PCR as a method to differentiate *Lactobacillus acidophilus, Lactobacillus crispatus, Lactobacillus amylovorus, Lactobacillus gallinarum, Lactobacillus gasseri,* and *Lactobacillus johnsonii. Curr Microbiol* 1995; **31**: 114–8.
78 Haarman M, Knol J. Quantitative real-time PCR analysis of fecal *Lactobacillus* species in infants receiving a prebiotic infant formula. *Appl Environ Microbiol* 2006; **72**: 2359–65.