AN OVERVIEW OF SOME PCR-BASED MOLECULAR METHODS USED FOR IDENTIFYING AND DIFFERENTIATING CLOSELY RELATED SPECIES AND STRAINS OF LAB ASSOCIATED WITH FOOD AND INDUSTRY

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ABSTRACT

For a number of different species of LAB connected to food and dairy products, a revolution in the development of highly sensitive, quick, automated, and pcr-based molecular detection technologies has occurred in recent years. These days, several of these LAB strains are regarded as probiotics. The genome-based techniques may be used in conjunction with or instead of phenotypic techniques for the identification of bacteria. Numerous identification techniques have been developed over the years, including random amplified polymorphic DNA, RFLP, denaturing gradient gel electrophoresis, restriction enzyme analysis,amplification rDNA restriction analysis rRNA, emperature gradient gel electrophoresis,and PFGE. These techniques use primers that target various sequences, like the 16S ribosomal RNA (rRNA)-encoding gene, the 16S- This article's goal is to provide a summary of various quick and accurate polymerase chain reaction-based molecular techniques for detecting and distinguishing closely related species & strains of LAB linked to the food and industrial sectors.

Keywords: *lactic acid bacteria, molecular identification, polymerase chain reaction, lactobacilli.*

INTRODUCTION

Microbial culture that have utilized for many thousands of years in the fermentation of food and alcohol have been studied in the 20th century for their potential to treat and prevent illness. As a result, the word "probiotics" was created to describe these beneficial microorganisms. Probiotics are defined as "live bacteria that, when provided in suitable proportions, impart a health benefit to the host by enhancing the host's microbial balance." The human gut and urogenital tract are frequented by a plethora of LAB, including Lactobacilli and Bifidobacteria. Specific species of Lactobacillus, Bifidobacterium, and Enterococcus have been repeatedly isolated from the intestines of humans and animals and chosen for use as probiotics. [1]

Even though many probiotics are made from LAB that live in the intestines, there are notable exceptions. Starter cultures of Streptococcus, Lactococcus, Leuconostoc, and Pediococcus are often employed in the production of dairy products. It is vital to determine which microorganisms are present in a microbial ecosystem or which species are the most likely to have the potential protective

benefits, since various kinds of LAB might alter the human intestinal milieu in different ways. However, determining the exact species of these bacteria is not a simple process. Identifying Lactobacillus isolates phenotypically is challenging since it often requires the measurement of bacterial characteristics that cannot be accomplished with conventional fermentation procedures.[2]

LAB

Some of the bacterial genera that make up the LAB group are listed below. Others are included in the group by implication. Bacterial lactic acid bacteria (LAB) were first identified from milk but have subsequently been discovered in a wide variety of foods and fermented items, including meat, milk products, vegetables, drinks, and baked goods. Additionally, LAB has been utilized for millennia as a food preservative, taste enhancer, and textural modifier. Probiotic LAB like Lactobacillus lactis or Streptococcus thermophilus may prevent the formation of harmful bacteria in food, extending its shelf life and keeping its nutritional value. Just recently, the concept of using LAB metabolites as natural preservatives in food products has been brought forward for consideration. [3]

The creation of lactic and organic acids by LAB reduces the pH of their development environment, making it less favorable to the growth of microorganisms. Organic acids are able to pass through cell membrane and enter the cytoplasm when the pH is low enough to encourage their trans formation to soluble lipids. Some of the byproducts of LAB metabolism include compounds with potential antimicrobial activity, such as acetaldehyde, carbon dioxide, hydrogen peroxide, diacetyl, , polysaccharides, and bacteriocins. Probiotics containing LAB are often considered to be among the most important. Some strains of lactobacilli, lactococci, and bifidobacteria are thought to have positive effects on human health. Yet, the probiotic processes of gut microbiota remain mostly unexplored. In general, the use of LAB in several foods has a long and reputable history of safety. Thus, Lactococcus, Lactobacillus, and Bifidobacterium species have been classified as GRAS. Because of this, bacteria from the genera Lactobacillus or Bifidobacterium spp. are the focus of the majority of research into probiotic usage in the intestines.[4]

Some taxa formed on the basis of phenotypical traits do not correlate with their evolutionary connections, as shown by the taxonomy of LAB based on comparative 16S ribosomal RNA sequencing studies. PCR- based techniques, such as rep-PCR fingerprinting and RFLP and PFGE, are considered crucial for the precise characterisation and identification of LAB strains. Analysis of the fecal 16S rDNA gene and rRNA amplicons by DGGE or TGGE has shown to be an effective method for detecting and monitoring the bacterial population in feces.[5]

Conventional morphological, biochemical, and physiological assays are used for identifying and classifying LAB populations. Misunderstandings arose from the phenotypic-only classification of several novel strain types, but these were resolved via the application of molecular techniques.[6]

LAB IDENTIFICATION METHODS

CLASSIFICATION BASED ON PHENOTYPE

Historically, LAB have been divided into subgroups based on phenotypical characteristics such as morphology, , temperature preferences, lactic acid configuration,mode of glucose fermentation fermentation of different carbohydrates, methoxy esters of fatty acids, and protein patterns in the cell wall or the whole cell. These techniques of typing are not perfect, unfortunately. Poor repeatability, ambiguity in certain procedures, costly logistics for large-scale investigations, and low discriminatory power are only some of the problems that come with using phenotypical methodologies. Another drawback of phenotypic analysis is that it does not take into account the influence of environmental factors on gene expression. All of these disadvantages make phenotype-based techniques for culture identification just at genus or species level less reliable.[7]

CLASSIFICATION OF THE GENOME

Genotyping methods may be used to distinguish between clonal strains of LAB or to identify specific species. These DNA-based typing systems excel primarily because of their discriminating strength and broad application. DNA-based techniques such as RAPD, DGGE, TGGE, and amplification rDNA restriction analysis can now be used to reliably distinguish closely related strains with comparable phenotypical features (ARDRA). Table 1 lists a few of these methods, and the following sections discuss how they have been used to identify probiotic strains.[8-10]

Table 1. Successful molecular techniques for the quick detection of lactic acid bacteria.

Serial no.	Technique	Species identified
1.	Restriction enzyme analysis	*L. acidophilus* *L. casei* *L. rhamnosus* *L. reuteri*
2.	Pulse-field gel electrophoresis	*Bifidobacteria* *L. casei* *L. acidophilus* *L. bulgaricus* *L. lactis* *L. fermentum* *L. helveticus* *L. plantarum* *L. rhamnosus* *L. sakei*
3.	Ribotyping	*L. reuteri* *L. fermentum* *L. casei* *L. acidophilus*
4.	RAPD profiling	*Bifidobacteria* *L. acidophilus* *L. rhamnosus* *L. fermentum*
5.	Amplified rDNA restriction analysis	*L. acidophilus* *L. casei* *L. delbrueckii* *L. fermentum* *L. helveticus* *L. plantarum* *L. reuteri* *L. rhamnosus* *L. sakei*
6.	Amplified fragment length polymorphism	*L. pentosus* *L. plantarum* *L. pseudoplantarum*
7.	Real-time PCR	*L. acidophilus* *L. casei* *L. delbrueckii* *L. fermentum* *L. paracasei* *L. plantarum* *L. reuteri* *L. rhamnosus*

- **Comparison Of Ribosomal DNA Sequencing With Other Chronometer**

It has been widely accepted that macromolecules serve as "records of evolutionary history," and also for decades they have been employed to investigate the phylogenetic variety and evolutionary relationships between various animals. For example, several studies of bacterial diversity examine the 16S rRNA gene. Its continuous and extremely limited functions, established at an early point in its development, make it a universal marker that is mostly resistant to changes in its surroundings. These features, in conjunction with the gene's relatively large size, make it a useful tool for measuring evolutionary time. The 16S rRNA gene is a universally conserved marker, however it does not come without drawbacks. When it comes to resolution, for example, the highly conserved 16S rRNA genes have a low cutoff. The 16S rRNA gene is indeed a universal identifier, yet its abundance varies amongst bacterial species. When employing 16S rRNA genes as targets, this causes an over- and under-representation of certain bacterial species. Studies of bacterial variety have also delved into a large number of additional genes outside those encoding for 16S rRNA. Some of them are shared genetic components shared by all bacteria but arranged in different ways in each species. Most universal genes are very conserved, with the majority serving the same purpose in all bacteria.[11]

- **Restriction Fragment Length Polymorphism (RFLP)**

For this reason, tiny restriction fragments could be recognized using the standard RFLP method due to its poor resolution. T-RFLP is an alternative to the traditional RFLP method that builds on the same principles. This technique involves the isolation of either DNA or RNA, amplification by PCR, digestion via enzyme, and identification of the resulting fragments. The production and isolation of restriction fragments follows the PCR amplification of a target gene using fluorescent-labeled primers. A DNA sequencer is used to count and sort fluorescently tagged terminal fragments of varying sizes, and the results are examined by comparing the bands or peaks of T-RFLP runs to the a database of recognized species. T-RFLP is a nucleic acid-based method that creates a "fingerprint" of a microbial community, much as DGGE does. Similar to RFLP, this method has advantages and disadvantages. When applied to a collection of bacteria, this approach excels in providing a profile of the bacteria in each species. In addition, the approach may be used to type a species of bacteria from the a mixed bacterial population without the need for any preliminary bacterial cultivation. More restriction enzymes may also be used to improve profile accuracy.[12]

- **Amplified Fragment Length Polymorphism (AFLP)**

Affinity purification (AFLP) was developed for the characterisation of plant genomes but is now widely utilized for microbial typing. One AFLP uses two different restriction endonucleases or PCR amplification primers, whereas the other uses only one restriction endonuclease and a single primer.[13-14]

AFLP incorporates both RFLP and PCR. Primers, also called adapters, are ligated to the target DNA after it has been digested with restriction enzymes, as in RFLP, in preparation for PCR amplification. This combination is then amplified selectively using a small number of primers. Two restriction enzyme, a frequent cutter, are used in AFLP for the digestion and genomic DNA. To limit the amount of fragments produced by a restriction endonuclease digestion, it is possible to do selective amplification by modifying the 3' ends of the PCR primers with particular nucleotides. First combinations with a single bp extension are used for the pre-amplification (first) PCR, whereas primers with up to three bp extensions are used for the selective (final) PCR amplification. Specific amplification is achieved because these primers anneal exclusively to DNA target fragments that share their adapter and changed nucleotide sequences. An agarose gel or a high resolution needed to break down polyacrylamide gel is used for electrophoresis of the fragments after amplification, and autoradiography is used to see the bands. When using a fluorescent-labeled PCR primer with an automated DNA sequencer, these fluorescent-labeled PCR primers have replaced the need for radioactive materials. Throughput, resolution, and discriminating power are all improved using this method.[15]

- **Ribotyping**

Ribotyping is the process of identifying ribosomal genes via the use of specific nucleic acid probes. Hybridization using a probe specific to either the 23S or 16S ribosomal RNA genes is used to generate restriction patterns after isolating bacterial chromosomal DNA. Southern blotting involves transferring chromosomal DNA from an agarose gel to a membrane & hybridizing it with probes specific for 23S and/or 16S rRNA. In most cases, patterns are more consistent and straightforward to interpret than REA results. The similarity of ribosome genes also has the added benefit of making it possible to utilize a common probe across all species, which greatly increases repeatability. [16]

Using Lactobacillus johnsonii, L. casei, L. rhamnosus, L. acidophilus, & L. fermentum as type and reference strains, Zhong et al. investigated the utility of ribotyping in this setting. Contrary to popular belief, ribotyping's discriminatory strength is best shown at the species level, not the strain level. Using an automated ribotyping equipment, a microbiological characterisation technology, Chun et al. identified 91 type or reference strains from the L. casei group as well as the L. acidophilus group. The Riboprinter technique provided fast, reliable, and repeatable genetic data for the identification of numerous strains, allowing for discrimination between most a few to the two groups at the species level.[17]

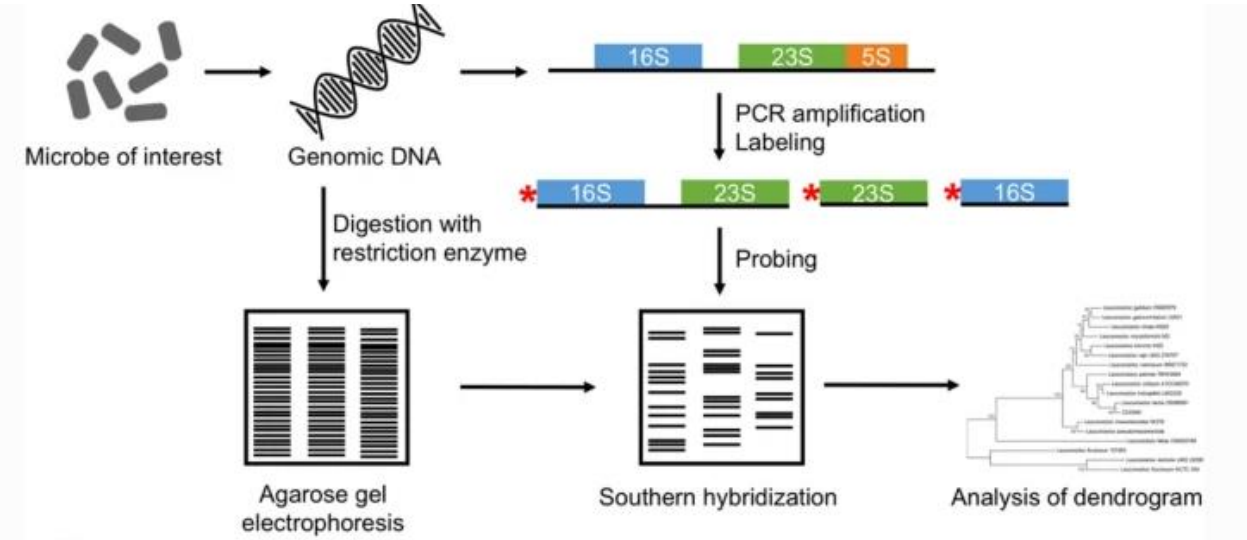

Fig.1 Ribotyping: a conceptual diagram.

- **Pulse Field Gel Electrophoresis (PFGE)**

The big DNA fragments generated from restriction digests using rare-cutting enzymes are separated by alternating field electrophoresis using progressively longer pulse periods; these fingerprint profiles may then be investigated for culture identification. So, compared to other fingerprinting methods, it may take longer to complete the process using this method. When compared to ribotyping, however, the discriminating potential of PFGE profiles is much higher since they reflect the whole genome. Several organisms, notably lactobacilli and bifidobacteria, have shown that PFGE provides good subspecies differentiation. Multiple examples are provided to evaluate the potential of PFGE for characterizing bacterial isolates, and it has been used to successfully classify bacterial strains inside a species. [18]

Furthermore, PFGE's use in tracking shifts in the predominance of human-origin bifidobacteria and lactobacilli populations within and across persons has been convincingly proven. Strain typing by PFGE has been accomplished for the L. acidophilus complex, L. casei, L. delbrueckii or its 3 subspecies, L. fermentum, L.s helveticus, L.plantarum, R. rhamnosus, and S. sakae. In general, PFGE has been proven to identify between strains from the same LAB species, to cluster strains within a species, to distinguish among strains of different LAB species, and to put isolates in distinct Lactobacillus species. Recent research has demonstrated that PFGE is superior than ribotyping and RAPD analysis for distinguishing between closely related strains of L. casei and L. rhamnosus. The selective potential of ribotype determination by using numerous ribo patterns has been shown in the past.[19]

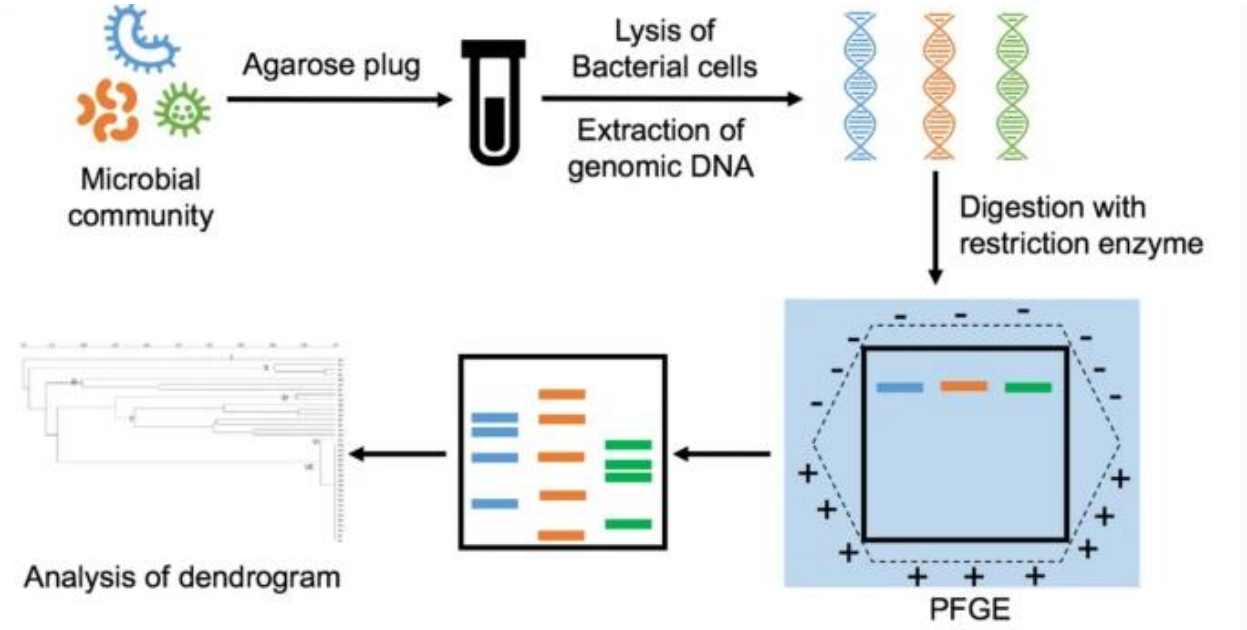

Fig.2 Pulsed field gel electrophoresis: a conceptual diagram.

- **RAPD Fingerprinting**

Short arbitrary primers enter the nucleus to several random target sequences, creating diagnostically useful patterns in the RAPD technique, which is based on polymerase chain reaction (PCR). Since the targeted sequence(s) to be amplified are unknown in RAPD analysis, a primer containing a made-up sequence is created. Low-stringency annealing conditions are utilized in PCR reactions once these sequences have been generated, leading to the amplification of DNA fragments of varying sizes. The possibility of using this technique to identify LAB, particularly probiotic strains, is now being researched. Because RAPD patterns may be hard to replicate, the testing environment must be carefully managed. RAPD has been used by a number of organizations for the purpose of identifying and characterizing LAB strains in human, food, and milk samples.[20]

It has also been possible to evaluate the probiotic persistence of L. rhamnosus GR-1, L. fermentum RC-14, & L. rhamnosus GG in the human vagina by using RAPD-PCR. To determine which strains of L. casei and L. acidophilus are most often found in probiotic yogurts, Schillinger et al. later investigated group-specific PCR & RAPD-PCR investigations. The RAPD patterns of the 20 Lactobacillus strains were matched to those of 11 reference strains from the L. acidophilus and L. casei group to determine the species and strains of lactobacilli present. Later, researchers used RAPD with two randomized primers (OPL-05 and ArgDei-F) to analyze 149 Lactobacillus isolates and found that the electrophoretic patterns they produced were useful for separating the different species. Researchers found a large range of genetic diversity across L. plantarum isolates.[21-22]

- **Real-Time PCR**

DNA-based real-time PCR uses fluorescence as a readout to track the progress of amplification of target DNA throughout real time. In addition to processing, identifying, and confirming pathogens in numerous samples at once, real-time PCR can also be used to quantify bacteria from the a wide variety of materials, including milk, excrement, food, and water. The procedure is modified in real time, decreasing the number of false positives seen with conventional PCR. [23]

Since the dynamic range is so great, even a single instance of the target DNA may be recognized. Conventional PCR has adequate sensitivity for detecting Lactobacillus and other species in the genus. 65,66 However, endpoint studies, including the plateau phase, and declining effects of changes in PCR product density mean that traditional PCR can only be utilized for semi-quantitative evaluation. Modern quantitative real-time PCR permits monitoring of the whole amplification, therefore avoiding the problems inherent in PCR endpoint analysis. Recently, a fast and accurate method was established for identifying LAB strains by exploiting the 16S rRNA gene. Differentiation of L. casei, L. paracasei, and L. rhamnosus was achieved by the use of a PCR method including hybridization probes created in accordance with differences in their respective 16S rRNA genes, and melting curve of a hybridization probe. Due to the presence of the identical 16S rRNA sequence in both L. paracasei and L. casei, this method was only able to accurately identify L. paracasei or L. rhamnosus. Based on these findings, the melting curve analysis of PCR methodology used in this work seems to be a straightforward, efficient, and reliable tool for distinguishing between closely related lactobacilli strains.[24]

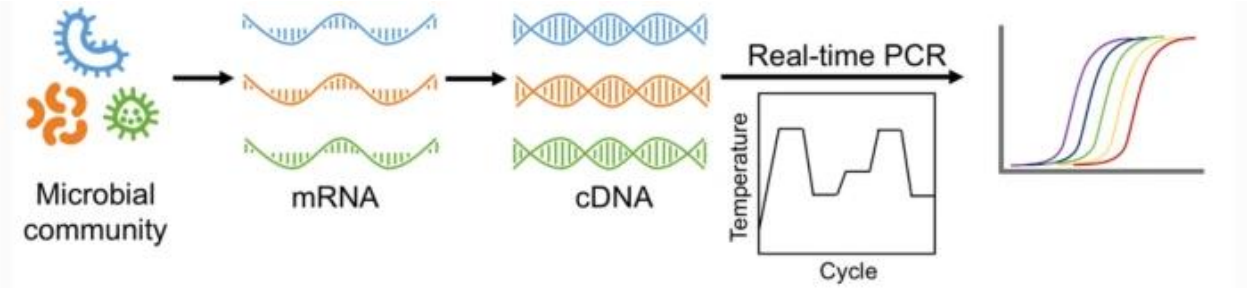

Fig.3 Real-time polymerase chain reaction diagram.

CONCLUSION

Probiotics are a topic of intense interest in the health care systems of both industrialized and developing nations. Phenotypical and biochemical characteristics are used extensively in the identification of probiotic LAB. These features may not be sufficient to definitively attribute a strain to a specific species when used for routine identi fication of isolates. In order for these beneficial microorganisms to be effectively used in enhancing human health via probiotic fermented foods, there has been an urgent need for the development of novel, quick, and reliable analytical procedures to detect them. Although the development of new, more effective methods for detecting food-borne

microorganisms has been aided by the use of polymerase chain reaction (PCR)-based techniques, and these offer advancements in detecting & characterizing microbes further than classical plating & phenotypical methods, there is still a great deal of work to be done to increase the capacity of these potent techniques in comparison to the laboratory setting. Information and understanding in this field might benefit greatly from this.

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