Journal of Pharmacology &Drug Development

eISSN: 2958-6801

OPEN ACCESS

DNA-Based Molecular Markers review for Identification and Characterization of Probiotic Organisms

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Received 9 Mar , Accepted 9 May , Published 1 Jun

ABSTRACT

Lactic acid bacteria (LAB) are essential in food generation and the maintenance of good health. There is a growing interest in these species in order to learn more about the many health advantages they may provide. LAB's activities are dependent on the number of bacteria present in the gastrointestinal system and their species and strain specific. Chemical preservatives and processed meals are causing a lot of anxiety among consumers. Products containing or treated with LAB, on the other hand, are widely acknowledged as a normal approach to keep food and enhance health. The current publication intended to summarize the research on the function of probiotic LAB in food preservation, gastrointestinal immunomodulation, and health benefits.

In food science and associated researches, the identification and categorization of helpful bacteria is critical. Traditional phenotypic techniques have several drawbacks, including the possibility of misidentification of a target, which limits their use. Genotyping techniques have a larger chance of succeeding, and they are commonly employed to differentiate microorganisms. The techniques used to genotype lactic acid bacteria (LAB) varies somewhat from each other, and each instrument has a set of benefits and drawbacks. This reviewing study covers different fingerprinting approaches used to identify and characterize LAB at the species, sub-species, and strain levels. The majority of such methods rely on restriction digestion, polymerase chain reaction amplification, as well as sequencing. Concerning cost, technique, and throughput DNA sequencing technologies have advanced significantly in recent years. A worldwide research effort is underway to produce enhanced versions of broadly usable and commercially feasible fingerprinting analysis technologies.

Keywords: Probiotics Organisms, LAB, Biotechnology, Food, Molecular technique.

INTRODUCTION

In light of growing understanding of how probiotics work, the main objective was to set them apart out of antibiotics by making it obvious that they are their antithesis. They were eventually described as "organisms and compounds that help maintain intestinal microbial balance" by scientists in 1992 [1].

There were numerous other descriptions and definitions prior to the Food and Agriculture Organization of the United Nations-World Health Organization (FAO-WHO) formally defining probiotics as "living microbes that when given in proper quantities provide a better health advantage on the host".

The (ISAPP International Scientific Association for Probiotics and Prebiotics) later approved this description, and it is presently the one that scientists use to define probiotics most frequently. Probiotic food cultures have gained popularity as a result of recognition of their positive impact on health [2] .

These helpful bacteria are purposely consumed and added to the intestinal microbiota during probiotic treatment. As a result, large numbers of helpful bacteria engage in nutritional competing with harmful ones and starve them out. Probiotics participate in a variety of physiological processes in humans that are good for health. Those microorganisms that stick to the intestinal epithelium provide the probiotic bacteria's positive effects when consumed [3],[4]. Numerous harmful bacteria are protected by a potent natural biological barricade that is made by probiotics stick to the mucous membrane of the intestines. So, the initial phase in colonization is considered to be adhesion. Receptor molecules and bacterium on the epithelial cells are examples of selective and non-specific types of adhesion to the epithelium, respectively. [5],[6].

1. Scope of research strategy for probiotic diagnosis and characterization

The Development of research strategy for an essay on the diagnosis and characterization of probiotics requires a multi-faceted comprehensive approach, emphasizing both traditional phenotypic methods and modern molecular techniques, while adhering to international guidelines for safety and functionality. Key criteria for probiotic characterization including strain identification, safety assessment, functional characterization and, technological properties. The methods selection for diagnostic is culture-dependent techniques by using selective media to isolate and enumerate potential probiotics (e.g., MRS agar for LAB), molecular techniques through utilizing high-throughput methods to quantify live cells and differentiate closely related strains and, omics technologies by incorporating metagenomics to profile gut microbiota changes and transcriptomic to understand metabolic pathways. The probiotic product consumption has recently increased with the prevalent intent to promote human and animal wellbeing. The complex selection process dealing with new-isolated probiotic candidates is the first challenge that has to be faced. From the isolation to the launch on the market, information about safety, tolerance to host physiological conditions, adhesion properties, genetics and interaction with the host has to be collected. Probiotics must be safe, survive to the exposition to bile salts and to gut transit, adhere to intestinal cells lining and colonize the lumen of the tract. The evaluation process of the possible probiotic health benefits is widely supported by in-vitro assays simulating the in-vivo conditions. The aim of this work is to summarize the classical models usually employed for the probiotic screening by underlying strengths and weaknesses of all models and to present some more recent molecular analysis tools used in the probiotic field characterization [7].

1.1 Niche-Specific Adaptation: The Intestinal Environment

The pioneering thoughtful notion of the ancestral (Lactic acid bacteria) LAB is to be the soil and vegetation and, later, the intestines of its carnivorous animals. The intestines of mammals are packed with 100 trillion of microorganisms (known "microbiota") which could be vital for health. The transformation from soil and vegetation to the intestines of an animal has 3 genomic adaptations: resist handling barriers, adhere to intestinal cells, and ferment other intestinal substrates [4].

Low pH and bile salts have an impact on the structure of the lipid membrane. Microarray analysis showed the manifestation of glycerophosphatase in *Lactobacillus reuteri* after acid shock, as well as increased acid sensitivity to *Lactobacillus reuteri* [7]. Extracellular lipopolysaccharides (LPS) have a key role in resisting; however, it is imprecise [4].

Peristalsis, excellent adhesion capability, and the existence of mucins to shield and lubricate the epithelial surface area are all factors that contribute to LAB adherence to intestinal cells. Since they may promote the synthesis of intestinal mucins, intestinal bacteria can stop harmful bacteria from adhering to epithelial cells in the gut. By increasing the mRNA synthesis of other mucins, Lactobacillus plantarum prevents enteropathogenic Escherichia coli from adhering to cells. Because LAB can access simple sugars and complex carbs, bacteria that have the genes necessary to break them down are probably better able to grow in the gut. A microbial barrier against microbial infections is provided by the gut microbiota. [8].

Lactobacillus and *Bifidobacterium spp*. from the human intestine producing antibodies which are effective in vivo and in vitro against enteropathogenic bacteria that cause diarrhea; these types can disrupt or block the pathogenic method of enteric viruses. numerous viruses, including enteropathogenic *Escheirichia coli, enterohemmorrhagic E. coli, Listeria monorovacytogenes, Salmonela-enterica Typhimurium*, and *S.flexors*, are impacted by the species of *Lactobacillus acidophilus, L. johnsonii, L. rhamnousus, L. casei, and L. acidophilius*. [9]. preventing the pathogenicity process of enteric bacteria is done without compromising their function, like inhibition of *Sallmonella-spp*, *S.flexnerri,* and *L monocytogenes* by *E.coli* strain Nissle 1917, and the blockade of *E.coli* LF82 adherent- attack of Crohn's disease [9].

1.2 Bio preservation of Food

Consumers could be too worry about chemical restrictions and foods that are processed, on the other hand, they readily consider LAB as a healthy and natural approach to preserve food. The development of bacteria that cause food deterioration and pathogens is inhibited by bacteriocins that are ribosomal generated from low proteins by LAB.; in addition, bacteriocin genic LAB has been linked and used as the first culture in food processing [10].

Bacteriocins are divided into 4 main groups. Grp. (1), lantibiotics, contains nisin as the 1st and most well-popular bacteriocin. Grp (2) is a huge group comprising three types of tiny heat-resistant proteins: i) subgroup (2a), active bacteriocins against *Listeria monocytogenes*, and pediocin PA-1, sakacinsA & P, leucocinA, bavaricinMN, and curvacinA members of this group; ii) subgroup (2b) requires pairs of various peptides to function, of which lactococcin G & M, and lactacinF are its components; and iii) subgroupIIc, like lactacinB, requires reduced cysteines to function [11].

In Group III they are divided into high-temperature labile proteins, like helveticens J and V, and lactaecins A and B. Leuconocin S, lactocin 27, and pediocin SJ-1 contain lipid or carbohydrate particles and are divided into groups I.V. Yang *et al*. [11] divided gram-positive bacteria into 3 classes: (1) (modified peptides, lantibiotics), (2) (unmodified peptides, non-lanthionine), and (3) (high protein), unstable temperature). The scientists divided Class (3) into 5 minor-classes. Bacterocin nisin is only available for supplement in pure form. Add to milk, cheese, and dairies, foods in cans, mayonnaise, and child food.

The genic cultures of Bacteriocin could be added to raw foods or mature ones like the original cultures. Because of the sensitivity of bacteria to other proteases, nonharmful bacteriocins may be digested. Therefore, bacteriocins could be safe food supplements after being discovered by the intestinal system [11].

1.3 Stimulation of Immunity

Through peyer's patches or the epithelial layer, external microbes may access the gut wall. When native intestinal bacteria, such as lactobacilli, restart phagocytic activity, they can pass through the mucous membrane of the intestines and survive for many days in the spleen or other organs. The intestinal mucus layer's rigidity, physiological condition, and responsiveness to oral lactobacilli [12] are crucial for the immunological response. By attaching to certain receptors in cells that are immune and various tissues, like the intestinal epithelium, lactobacilli can induce an internal and adaptive immunological responsive reaction in the host. [13].

Such receptors stimulate producing the cytokines, chemokines and other natural substances: native T cells, regulating T cells, and the activity of dendritic DC cells and macrophages. Various types and strains of *lactobacilli* produce various effects, that may alter the immune response in various methods [11]. New methods of acquiring LAB antibodies have been used to protect the immune system. The contrasting genomic analysis to determine loci link to several phenotypes was performed with *Lactobacillus spp*. nonetheless, a large gap in understanding of immunomodulation processes by probiotic cells is their end inside the organism. Many probiotic research with various types of Lactobacilli were made in humans and rats and mice models with negative / incomplete or positive effects.

Good findings were obtained in the treatment of infectious diarrhea or other allergies such as atopic eczema or dermatitis. Such probiotics could boost the immunity of oral vaccines like rotavirus, polio, cholera and influenza [14].

1.4 Health Benefits

The goal of probiotic-containing food components is to get 107CFU/g at the finale of their shelf cycle; but the probiotic LAB ought to withstand certain pressures to make sure that it reaches sufficient amounts in the target area to produce its impact. The human gastrointestinal tract has cells ranging from 10^{13} - 10^{14}. It is an intricate ecosystem comprising intestinal epithelium, cells being immune and a living microbiota [15]. Simon and Gorbach estimate one generation or two times among 1 and 4 times a day. The 3 key components of the human intestinal Stomach system are the stomach, small and large intestines. Each category bears its unique microbiota **[16]**. contains mainly gram-positive aerobic microorganisms (<10^3 CFU / g). The *Lactobacillus*, *Bifidiobacterium*, *Bacteroides*, and *Streptococus* genera are found in the small intestine (103–104 CFU/g). Additionally, the large intestine contains high concentrations of *Bacteroides*, *Fusobactarium*, *Lactobacillus*, *Bifidiobacterium*, and *Eubacterium* (1011–1012 CFU/g). finally, LAB is a good candidate for developing vector oral novels for mucosal delivery techniques, forming some attractive mechanisms for the reduced bacteria. Bermúdez-Humarán *et al*. [17] made a research on the development of *Lactococcus lactis* gene expression for medicinal proteins like antigens, cytokines, and enzymes that are expressed heterologously. these researchers have found positive findings to human papillomavirus type 16 (HPV-16), inhibition of bovine b-lactoglobuline (BLG) -allergies reaction, as well

as weight control and diet. Use of L. lactis bringing DNA to mucosal level has also been improved [15], modifying the immune response.

1.5 Antibiotic Resistance

FAO / WHO has described the issue of antibiotic-resistant genes in probiotic species. The ability of probiotics to convey antibiotic-resistant genes could be an essential barrier to their selection and use must not be tolerated. When probiotics get into the intestine, interacting with native microbiota and possible gene transference, it has contributed to the transmission of antibiotic-resistant genes or pathogens exist in the intestinal system. Fukao and Yajima [18] re-viewed the key genes for resistance to probiotic lactobacili: tet, erm, or cat genetics of tetracycline, erythromycin, or chloramphenicol resistances, respectively. Aimmo et al. [19] and Patel *et al*. [20] pointed out other antibiotic-resistant genes, like str streptomycin and vanA vancomycin.

Detection of antibiotic resistance in probiotic species can be made by the phenotypic and genotypic methods:

i) determining the most crucial drugs' mini inhibitory concentrations (MICs) for each kind of bacterium.

ii) Using PCR-based methods and microarray assay.

2. Identification and characterization of bacterial lactic acid using molecular tools

Because of their variability and widespread nature, there is an increasing interest in identifying industrially related and helpful microbial strains. In order to capture a full diversity profile, modest morphological characterization of such bacteria is inadequate [21]. many phenotypic and genotypic typing techniques have been reported throughout time; nonetheless, a successful tool should be of high type ability as well as discriminatory power and for the microorganisms under research. Probiotics are living microbes which, when given in proper amounts, could be beneficial to the host's health. Surprisingly, certain LAB strains are categorized as GRAS (generally regarded as safe) bacteria. LAB are Gram-positive bacteria that are rod- or cocci-shaped, have a low G+C concentration, and share, metabolic, physiological, and morphological properties [22].

LAB are widespread in both fermented and unfermented foods, and they live in the human gastrointestinal system. Because of the extensive variety of apps of LAB, it is required to link genomic evidence to key traits in order to fully leverage metabolic applications and quickly and correctly identify new bacteria at all taxonomic levels. furthermore, new beginning species from the wild LAB pool are required for producing a wide range of food and medicinal goods aimed at human health [23]. traditional morphological, biochemical, and physiological assays are well-known for identifying and classifying LAB populations. The classification of several novel strain types solely based on phenotypic traits resulted in ambiguities, which were subsequently resolved using molecular methods [24]. furthermore, owing to the many intricate techniques, LAB's various nutritional and development demands, and poorer discriminatory strength, identifying a bacterial strain using these methodologies is highly challenging. due to the shortcomings of phenotypic techniques, genotypic methods to categorize LAB emerged. Furthermore, selecting the right genetic marker or gene for PCR amplification to differentiate the LAB classes is an important step in the molecular documentation of LAB. The main benefit of DNA-centered techniques is that they can reliably identify strains [25].

2.1 Types of Samples

Effective sample handling requires maintaining the sample's integrity from collection to analysis by adhering to specific temperature, aseptic, and documentation protocols based on the sample type. Food samples must be kept at storage temperatures, clinical specimens require sterile containers for patient safety, and probiotics need temperature control to maintain viability. [19].

i) **Food Samples**

Collecting samples aseptically using sterile gloves and equipment to prevent cross-contamination with temperature control maintains same temperature during transport as the original storage conditions (e.g., frozen or refrigerated). to ensure a chain of evidence must be documented the collection date, time, sample type, and temperature at the time of sampling.

ii) **Clinical Samples**

Wash hands before and after collection and wear appropriate personal protective equipment. use the correct specimen container, which may include specific additives, and

ensure it is securely sealed and labeled and deliver samples to the laboratory without delay to ensure accurate test results.

iii) **Probiotic Products**

Probiotics are sensitive to environmental conditions; cold storage is typically recommended throughout the production and transport chain to maximize strain viability. The mechanism of storage must monitor and control temperature and humidity to guarantee the total quantity of strains labeled on the product.

2.2 DNA extraction methods:

Advanced DNA extraction for Lactic Acid Bacteria (LAB) requires overcoming their thick, Gram-positive peptidoglycan cell walls. Effective methods include enzymatic treatment with lysozyme (often combined with ampicillin) for lysis, or advanced techniques like metagenomics shotgun sequencing for complex samples [22].

i) **Cell Wall Lysis Techniques**

- Enzymatic Lysis: Treating samples with lysozyme is critical to weaken the cell wall.
- Pretreatment: Weakening the cell wall with ampicillin before lysozyme treatment can significantly increase DNA yield.
- Mechanical Disruption: For complex samples like stool or soil, bead-beating is necessary to physically disrupt the cells.

ii) **Extraction Methodologies**

- Commercial Kits: Silica-based membrane kits (e.g., from Promega or Thermo Fisher) offer rapid, reliable DNA isolation, often with higher purity than traditional methods.
- Modified CTAB Method: A revised Cetyltrimethylammonium bromide (CTAB) method is effective for isolating DNA from LAB cultured in broth, such as MRS or M17.
- Phenol-Chloroform Extraction: A traditional, rigorous method useful for maximizing yield, involving chemical denaturation of proteins.

2.3 Purity, Integrity and Concentration of DNA Requirements

Maintaining specific absorbance A260/280 ratio is ~1.8 is typically required for high-purity DNA. Ratios below 1.6 indicate significant protein or phenol contamination. Integrity and physical characteristics molecular weight DNA is preferred, typically >100 kb on average. DNA must not be highly degraded using 1% agarose gel electrophoresis, where high-quality DNA appears as a distinct band near the top of the gel, while degraded DNA appears as a smear [24]. fluorometric quantification is recommended over spectrophotometry because it specifically measures double-stranded DNA (dsDNA) at minimum concentrations often exceed 50 ng/µl for preparations. long-term storage DNA sample at -20°C is recommended, preferably in a low TE buffer (e.g., <0.1 mM EDTA) to prevent fragmentation. DNA samples must be free of inhibitors common chemical, biological and environmental contaminations [25].

2.4 Types of molecular tools

Numerous molecular approaches have been used extensively to describe probiotic or LAB species ever since the mid-1980s. Each technology has unique advantages, applications, and disadvantages. Notably, no one technique is capable of delivering entire details on the intra -and inter -species difference. Thus, the present method is to appropriately identify and characterize LAB strains by using a multiphase technique [26]. Molecular tools, as opposed to phenotypic techniques, are quicker, significantly more predictable, and can be reproduced. They could even discriminate between closely associated species that are phenotypically identical otherwise. [27], [28].

2.5 Random amplified polymorphic DNA (RAPD)

RAPD is a way of typing which adopts one random primer of 20–25 base pairs (bps). To identify polymorphisms, this primer haphazardly hybridizes to various sites on chromosomal DNA sequencer that have the most resemblance to the microbial genome [29]. DNA amplification fingerprinting and haphazardly primed PCR are RAPD variants depending on primer length, annealing temperature, and procedure length. Agarose gel electrophoresis separates the products of amplification, resulting in a bacterial fingerprint that may be employed

to recognize and describe microbial strains (Figure 1) [32]. When the sequencing data for freshly isolated strains is no found, RAPD assay is the pioneering technique employed to establish strain-specific primers [30].

Weiss *et al.* utilized and found RAPD patterns in *Lb. reuteri* strains, a possible probiotic bacteria. Correspondingly, the RAPD approach was adopted to characterize other *Lactobacillus* strains, including *Lb. plantarum*, *Lb. brevis*, and *Bacillus* species. The benefits of RAPD consist of high discriminatory power, broad application, and quick, low-cost, and simple performance [31]. through employing several primers, the discriminating power may be increased. Furthermore, no previous understanding of the sequencing related to target DNA is required, and just a little quantity of DNA that is bacterial is required for amplification.

The advantages of RAPD PCR marker is no required knowledge of DNA sequence, simple fast, cost-effective molecular technique that requiring small amounts of DNA, and high polymorphism detection for analyzing genetic diversity, phylogenetic studies, and creating genetic maps species identification. The limitations of RAPD PCR its low reproducibility across labs and it's a dominant marker nature meaning they cannot distinguish between homozygous and heterozygous individuals, sensitivity to contamination when low annealing temperatures and short primers causes the reaction sensitive to impurities and difficulty in interpretation of results through mismatches between primer and template can lead to weak or missing bands, leading to confusion in band scoring [32].

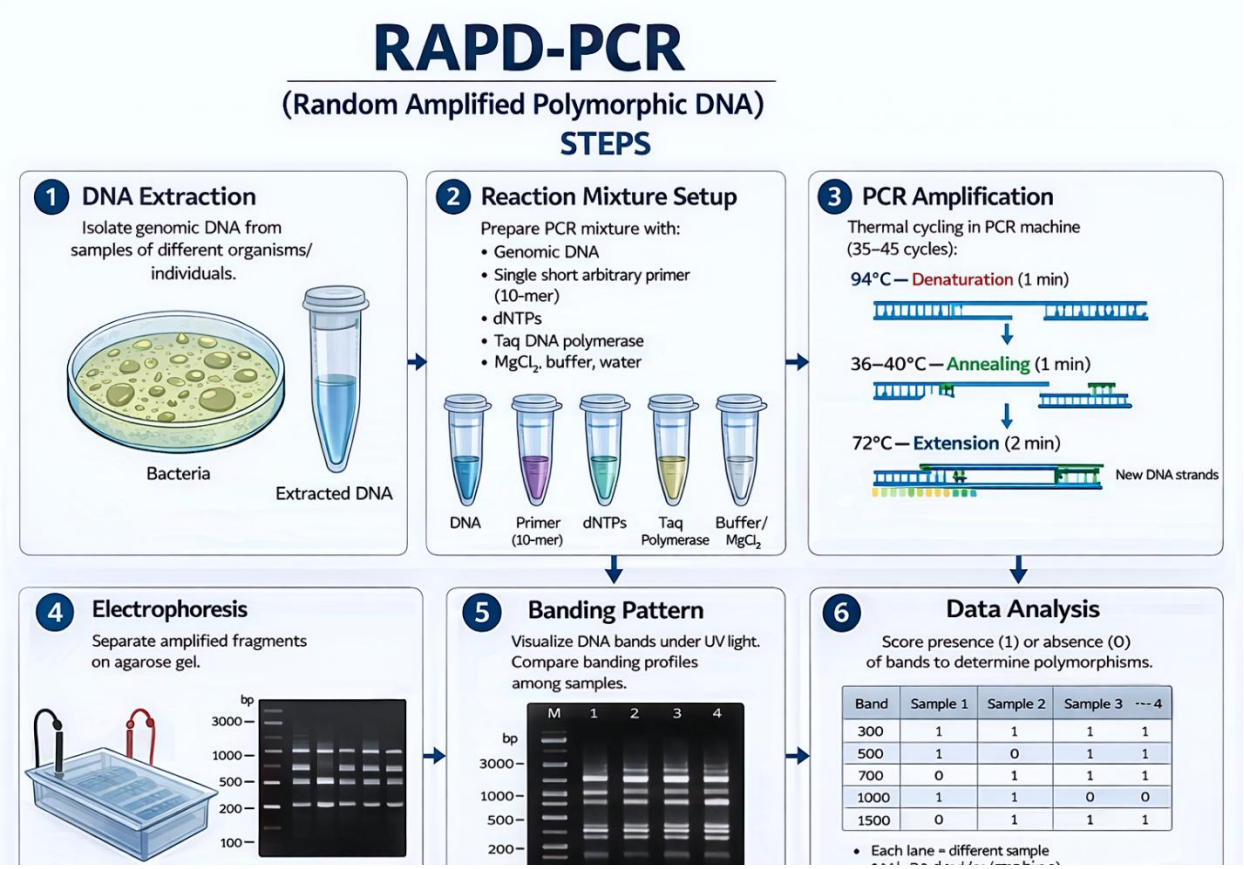

Figure 1: Scheme of Random Amplified Polymorphic DNA diagram [32] .

The main disadvantage of this approach is its low repeatability. Many variables influence reproducibility, including the DNA amount and quality, the PCR buffer, the concentration of primers, and the hardening temperature. It was proposed that correct optimization of the PCR procedure might solve this difficulty [33] in Table 1.

2.6 Amplified fragment length polymorphism (AFLP)

AFLP was initially intended to characterize plant genomes, which on the other hand has since gained popularity in the area of microbial typing. In fact, two distinct varieties of AFLP could be found: one that uses PCR amplification primers and two distinct restriction endonucleases, whereas the other that uses restriction endonuclease and just one primer. PCR and RFLP have combined to create AFLP. The target DNA is absorbed using restriction endonucleases, same as in RFLP, and then have ligation with adaptor primers for amplifying the PCR. (Figure 2). Using a restricted number of primers, amplification is applied selectively to the mixture [34].

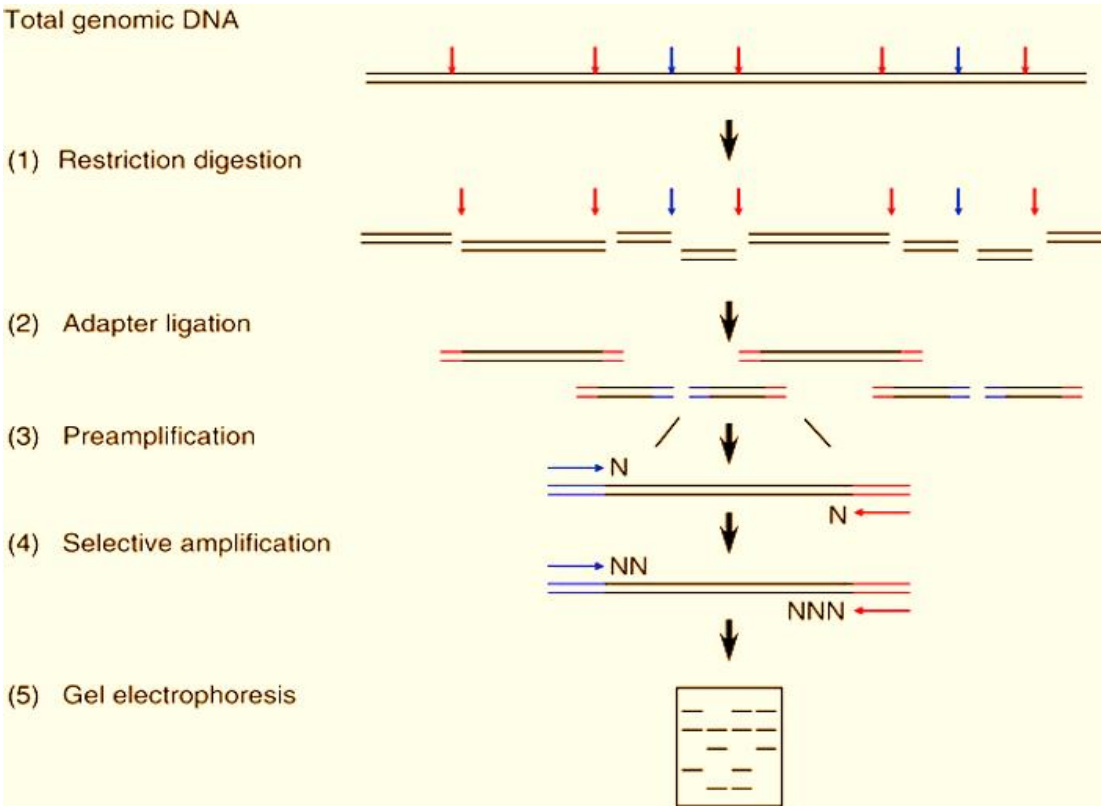

Figure 2: Amplified Fragment Length Polymorphism [34]

For digesting genomic DNA, AFLP makes use of a pair of restriction enzymes: a common cutter (such as MseI or TaqI) and an uncommon cutter (such as EcoRI or PstI). Following restriction endonuclease digestion, the 3-ends of the PCR primers are adjusted by the addition of certain nucleotides (one to three), which helps to reduce the amount of amplicons. One bp extension is used in the primer combinations used for pre-amplification (first) PCR, whereas up to three bp extensions are used in the primer combinations used for selective PCR amplification (final). [35]. With the aid of adaptors and changed nucleotides, such adjusted primers anneal to just target fragments of DNA, enabling focused amplification. [36]. the fragments amplified are detached by electrophoresis on a high-resolution denaturing polyacrylamide gel or an agarose gel with autoradiography. When using an automated DNA sequencer, fluorescent-identified PCR primers have arisen as an alternative to radioactive subject. This method provides a high level of discriminating power, resolution, and throughput.

The advantages of AFLP technique its repeatability and sensitivity, as well as is a PCR-based high-throughput technique for DNA fingerprinting that detects genetic variation without needing for previous knowledge of the sequence, ability to detect numerous polymorphisms in a single assay by provides a faster search of the entire genome. Highly sensitivity/resolution effective at distinguishing closely related strains and individuals, flexibility with large or small genomes and used for DNA profiling, genetic mapping, QTL analysis and evolutionary studies. The shortcomings of such approach are the low target DNA quality, a difficult approach with a high number of stages, a costly activity, Dominant markers cannot identify heterozygotes individuals,

needing high-quality, non-degraded DNA sequencer and lack of interlaboratory standardization lead to difficulties with complex data [37].

The AFLP approach was successfully used for genotyping and intra-species documenting the LAB isolated from several fermented foods and the human gastrointestinal system. According to one research, the gene shapes of *Lb. rhamnosus* in CB were more diverse than in MRS. The variable gene expresison levels in CB were most likely caused by the triggering of several metabolic paths in order to create a large quantity of energy. Another research found that AFLP may be utilized to create a link among carbohydrate consumption capacity and niche/genotype adaptability in *Lb. rhamnosus* isolates from people and food [38]. Furthermore, employing fluorescent AFLP, prior research distinguished numerous strains of *Lactobacillus* at the intra-species level (fAFLP). Another research created oligonucleotide primers for the Lb. brevis species-specific PCR assay, a fAFLP-resulting gene fragment (125 bps) encoding the aldo/keto reductase enzyme was used. [39] (Table 1).

2.7 Terminal (T)-restriction fragment length polymorphism (T-RFLP)

T-RFLP is now considered a classic fingerprinting technique used to study the structure and diversity of microbial communities Because the standard RFLP approach has a limited resolution, tiny restriction fragments could not be recognized. T-RFLP, which is based on the fundamental stages of RFLP, may be utilized as an alternative to the regular RFLP approach. Extracting DNA or RNA, amplifying PCR, digesting enzyme, and identifying pieces are all part of this approach. Typically, amplifying of a target gene by PCR is carried out using fluorescent-labeled primers [for instance, fluoresceinamidite (6- FAM)], and is trailed by producing and separating restriction fragments (often 4 base cutter restriction enzymes are employed). A DNA sequencer is used to separate fluorescent tagged terminal fragments of various sizes, which are then examined by making comparisons among a database of recognized species with peaks or bands of the T-RFLP runs (Figure 3) [40].

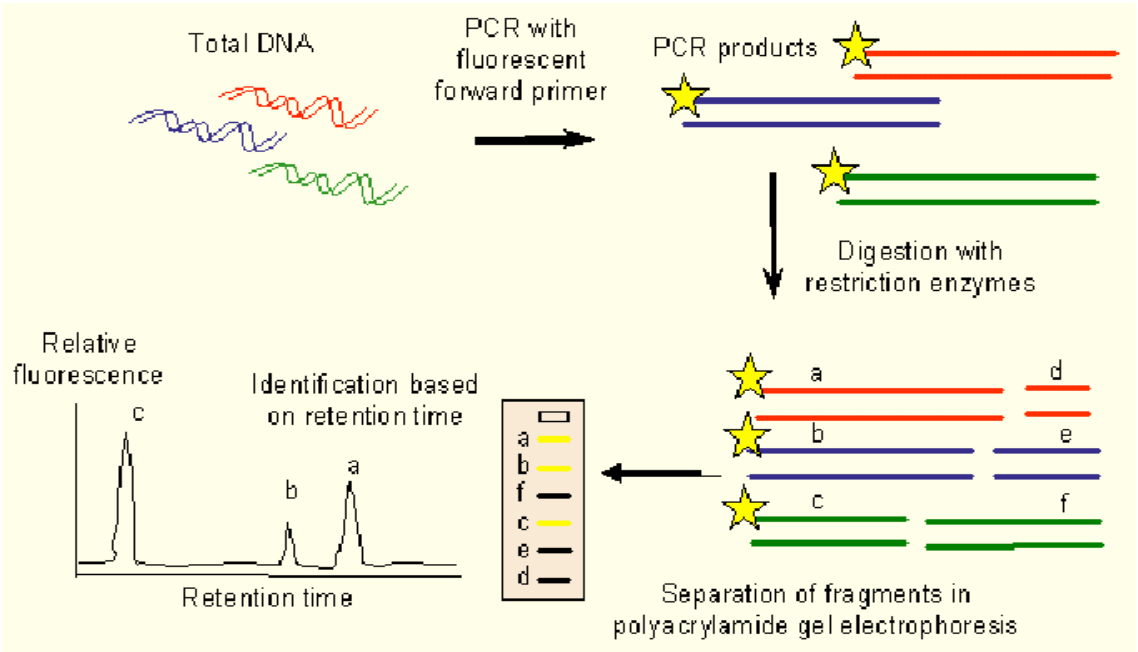

Figure 3: Terminal (T)-Restriction Fragment Length Polymorphism [40]

T-RFLP, like DGGE, is a nucleic acid-focused way that detects particular microbial communities and creates a microbial community fingerprint. This technique's advantages and disadvantages are similar to those of RFLP. This advantages are useful for generating a microbial profile in comparison of a varied bacterium sample. Furthermore, for typing a microbial species from a combined microbial community, this approach does not need previous bacterial culture, rapid, high-throughput data generation by T-RFLP uses automated sequencers to detect fluorescently labeled fragments, high reproducibility for repeated samples particularly, highly sensitive enable to identify many microbial species diversity that cannot be easily cultured based on functional genes, less expensive than Next-Generation Sequencing methods and the peak area/height data generated can be used for semi-quantitative assessment of microbial communities.

The disadvantages are incomplete or non-specific enzyme digestion can lead to inaccurate community profiling and overestimation of diversity, cannot identify organisms down to the species level, usually identifying them only to the genus level, T-RFLP only offers a "fingerprint" (peak sizes) therefore necessary to use clone libraries to identify sequence data of peaks, difficulty in data analysis and requires specialized software which is not always completely documented additionally, ineffective for in-depth analysis of highly complex diverse communities (e.g., soil samples) [41].

Also, by adding additional restriction enzymes, profile accuracy may be improved [41]. The T-RFLP method was previously utilized to detect that when fermenting beverages, LAB is present at the species level. in a prior work. *Lactobacillus sp*. and *Lb. delbruecii* subsp. *bulgaricus* were determined to be the most common LABs [42] (Table 1).

2.8 Real-time PCR

Quantitative Real-time PCR (qPCR) is a molecular biology technique enables rapid, sensitive, and specific detection and quantification of nucleic acids by monitoring using fluorescent dyes or probes during amplification, often completing within 2-3 hours in "real-time". It calculates precise DNA quantities using Ct values—where fluorescence exceeds background levels. Two quantification Methods is Absolute Quantification using a standard curve created from a dilution series of a known template, such as plasmid DNA, to determine the exact copy number of an unknown sample and relative quantification it compares the difference in expression levels of a target gene relative to a reference gene, such as in gene expression studies.

The most significant development in using PCR was the ability to see DNA amplification in real-time using fluorescence emission. Real-time PCR may be employed for absolute (regular curve) or relative (comparative threshold technique) quantification, depending on the purpose of the investigation. Absolute quantification requires the creation of a regular curve employing diluted template samples of identified quantities, such as a plasmid carrying the desired gene, a single-sense synthesized oligonucleotide, whole RNA, genomic DNA, cDNA, or in vitro transcripts (Figure 4) [44].

The regular curve method is adopted to determine viral capacity or the precise quantity of DNA template in samples [32]. Relative quantification measures the relative manifestation of a target in comparison to a control using mathematical computations. It is used to examine the expression of gene and the relative quantity of DNA [45].

Real-time PCR is a DNA-centered technology frequently used to quantify LAB species in a variety of materials, such as milk, food, and excrement. It is centered on the idea of detecting the level of product fluorescence produced after each PCR amplification cycle, and this level of fluorescence is invariably relative to the rise in amplicon quantity at that moment [46].

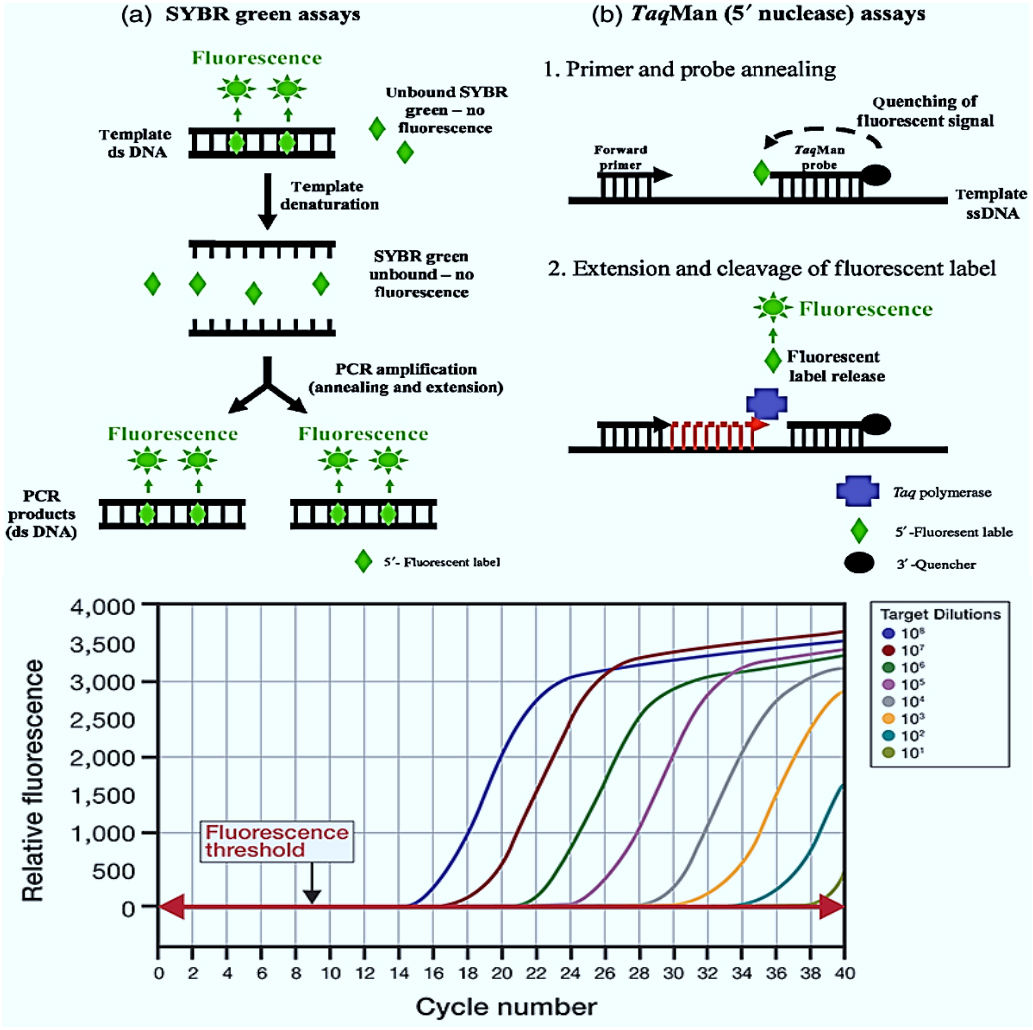

Figure 4: A scheme of a diagram for Real-Time PCR [44]

The methodology details include procedure, through involves optimizing DNA extraction, designing strain-specific primers, and using fluorescence-based chemistry (e.g., SYBR Green or probes) for real-time monitoring then data analysis, both absolute quantification (using standard curves) and relative quantification can be used. Real Time PCR technique working by two mechanisms: fluorescent dyes SYBR Green dye binds to double-stranded DNA (dsDNA) and emits fluorescence, which is measured to quantify DNA after each cycle. and probe-based (e.g., TaqMan) the specific probes allow for higher specificity and multiplexing, enabling the detection of multiple targets in one reaction [43].

The two methods employed the most frequently to track real-time target sequence amplification are fluorescent-labeled oligonucleotide probes (hydrolysis probes, such as those used in the TaqMan test, Molecular Beacons, and Scorpions), as well as non-sequence specified dual-standard DNA fluorescent attaching dyes (SYBR Green I). For real-time detection, a variety of PCR devices and probe types are available. The probe-centered technique has promise due to the lower likelihood of producing nonspecified PCR products (primer dimers) and improved specificity enabled by the additional oligonucleotide [47]. SYBR Green I dye, on the other hand, may be utilized to detect a target out of a combined population through PCR. Furthermore, real-time PCR allows for exact quantification of templates with a dynamic range of more than 107-fold. The main advantage of that approach is that it is of sensitivity and suited for quantifying LAB. It could be a better tool than traditional PCR-based approaches as it eradicates the necessity for post-PCR processing. Furthermore, no time-consuming selective bacterial cultivation is needed, as is required by previous genotypic approaches [48].

Moreover, the real-time PCR performance may be considerably increased in a multiplex setting by targeting further sequences at the same time. This may be done using the new closed-tube constructions, which reduce contamination in the course of real-time PCR[49],[50]. The incapacity to distinguish dead bacteria from active bacteria is the most serious drawback. This is possible owing to DNA may be augmented from dead cells as well. Another drawback is that the target DNA amplicons in the reaction tube are limited owing to the different types of lighting sources and the absence of as many fluorescent dyes [51]. Multiplex real-time PCR, a variation of this approach, allows for the amplification of several targets in one reaction utilizing separate reporters with varied fluorescence spectra (Table 1).

Aspects of q RT-PCR Reaction in Probiotics:

i) Rapid Quantification: qPCR allows for the simultaneous enumeration of species and strain-level identification, critical for quality control in multi-strain products.
ii) High Sensitivity & Specificity: It accurately counts low levels of probiotic cells without the need for preliminary enrichment.
iii) Addressing Viability: Standard qPCR cannot differentiate between live and dead cells. To address this, viability PCR (v-qPCR), such as PMA-qPCR (propidium monoazide), is used to selectively quantify only viable bacteria.
iv) Linear Dynamic Range: Validated qPCR assays can cover a wide linear range (e.g., 10^3-10^8 CFU/ml).
v) The Ct Value: Cycle Threshold (Ct) is the cycle number at which the fluorescence signal crosses a predefined threshold. It is directly related to the initial quantity of the target DNA—lower Ct values indicate higher initial amounts of target nucleic acid [52].

The applications of q RT-PCR in Probiotic analysis through quality control to detecting and enumerating *Lactobacillus, Bifidobacterium*, and other strains in fermented foods, dairy products, and supplements, strain tracking by Specific primers and probes are designed to target unique genomic regions, such as the *tuf* gene, to differentiate closely related strains, regulatory compliance for ensuring products meet label claims of probiotic levels with studies finding qPCR often more reliable than culture methods for validating probiotic content and clinical trials using quantity fecal samples to track probiotic survival and persistence in the human gastrointestinal tract. Nucleic acid-based methods such as quantitative PCR (qPCR) have been widely applied to fields of biology, food science, environmental science for microorganisms' detection as it is rapid, specific, and highly sensitive [53]. However, its inability to distinguish between viable and dead cells limits its application. Fortunately, a novel dye named propidium monoazide (PMA) could be coupled with qPCR (PMA-qPCR) for viable cells quantification through selective staining based on membrane integrity. The PMA dye can only penetrate membrane damaged cells and covalently cross-link with DNA during photolysis, thus preventing PCR amplification of the DNA. Consequently, DNA from membrane-intact cells could be selectively amplified by the following PCR procedure. The PMA-qPCR shows its advantages for selectively detecting individual strains in compound probiotic products based on species specific primer design. Several crucial factors could affect the accurate numeration of viable cells by PMA-qPCR method, such as DNA extraction method, PMA treatment conditions, construction of standard curves, bacterial density, etc. All these factors should be considered and confirmed its suitability to the target strains to ensure accurate results [54]. other Main Applications of Real-Time PCR technique Gene Expression Analysis by measuring mRNA levels to understand how genes are turned on or off, Pathogen detection through rapidly detecting and quantifying viruses (e.g., COVID-19/SARS-CoV-2) and bacteria in clinical samples, Genotyping detecting SNPs, mutations, or deletions [55]. and food safety detecting allergens or genetically modified organisms (GMOs) [52].

Advantages of Real-time PCR Technique:

i) High sensitivity and specificity amplifies minute amounts of DNA (less than 100 copies) while limiting false positives.
ii) Rapid Results, saves time and reduces contamination risk through amplification is monitored in real-time, eliminating the need for gel electrophoresis.
iii) Quantitative capabilities of technique can be precise quantification of initial target concentration, crucial for gene expression studies and viral loads.
iv) Multiple targets enable detect in a single reaction using different fluorescent dyes.
v) Capable of detecting target concentrations across several orders of wide dynamic range. [56].

Limitation of Real-time PCR Technique:

i) The instrument itself is too costly (e.g., fluorescent probes) are expensive compared with conventional PCR.
ii) Limited multiplexing is still restricted compared to other methods.
iii) Amplification error detection as small experimental errors or non-specific amplification can be amplified and misinterpreted.
iv) High sensitivity to experimental conditions like temperature variations and reagent quality.
v) Sensitivity to inhibitors as samples like blood or sputum can contain inhibitors that affect results, demanding careful purification. [56].

3. Future Prospects: Next-generation sequencing (NGS) techniques

uses high-throughput, massive parallel sequencing to identify and characterize novel, often unculturable, human-derived microbes with potential therapeutic benefits.

3.1 16S rRNA sequencing

16S rRNA gene sequencing is a fundamental technique used in probiotic research to identify, characterize, and monitor the effects of beneficial microorganisms on host microbiota. It operates by targeting the highly conserved 16S rRNA gene present in all bacteria, allowing for identification down to the genus or species level without requiring laboratory culture. through the use of 16S rRNA sequencing, scientists can map the diversity of bacterial populations within the gut. This sequencing method allows for the tracking of how different bacterial species affect the overall balance of the microbiome, and how disruptions to this balance can lead to diseases like irritable bowel syndrome (IBS), obesity, or even autoimmune conditions (Table 1). By pinpointing the specific bacteria involved, researchers can better understand the mechanisms by which probiotics influence gut health and their potential therapeutic benefits, making 16S rRNA sequencing an invaluable tool in advancing probiotic research. the key applications of 16S rRNA in Probiotic Research: identify novel probiotic candidates from samples like infant feces or fermented foods, confirming their taxonomic affiliation to beneficial genera such as *Lactobacillus* or *Bifidobacterium*, Monitoring changes it allows for analyzing how probiotic administration alters the overall composition and structure of the gut microbiota in both human and animal studies, Assessing Probiotic efficacy It enables tracking changes in gut diversity before, during, and after probiotic intervention, often showing that probiotics can modulate microbial composition and reduce pathogens and Verify the presence and purity of live beneficial bacteria in commercial probiotic products or fermented foods as a quality control [57].

The standard 16S (V3–V4) is effective for broad screening, TSS is often required for precise, species-level quantification of administered probiotics, overcoming the quantitative bias inherent in standard 16S methods. Data analysis is Bioinformatics tools (such as QIIME 2) are used to analyze operational taxonomic units (OTUs) or amplicon sequence variants (ASVs), providing data on alpha- and beta-diversity. The limitations of 16S rRNA sequencing often lacks the resolution to distinguish between closely related species or specific strains. Certain variable regions can introduce amplification bias, underrepresenting specific taxa like *Bifidobacterium*. Standard 16S rRNA sequencing cannot distinguish between live (probiotic) and dead bacteria figure (5). [58].

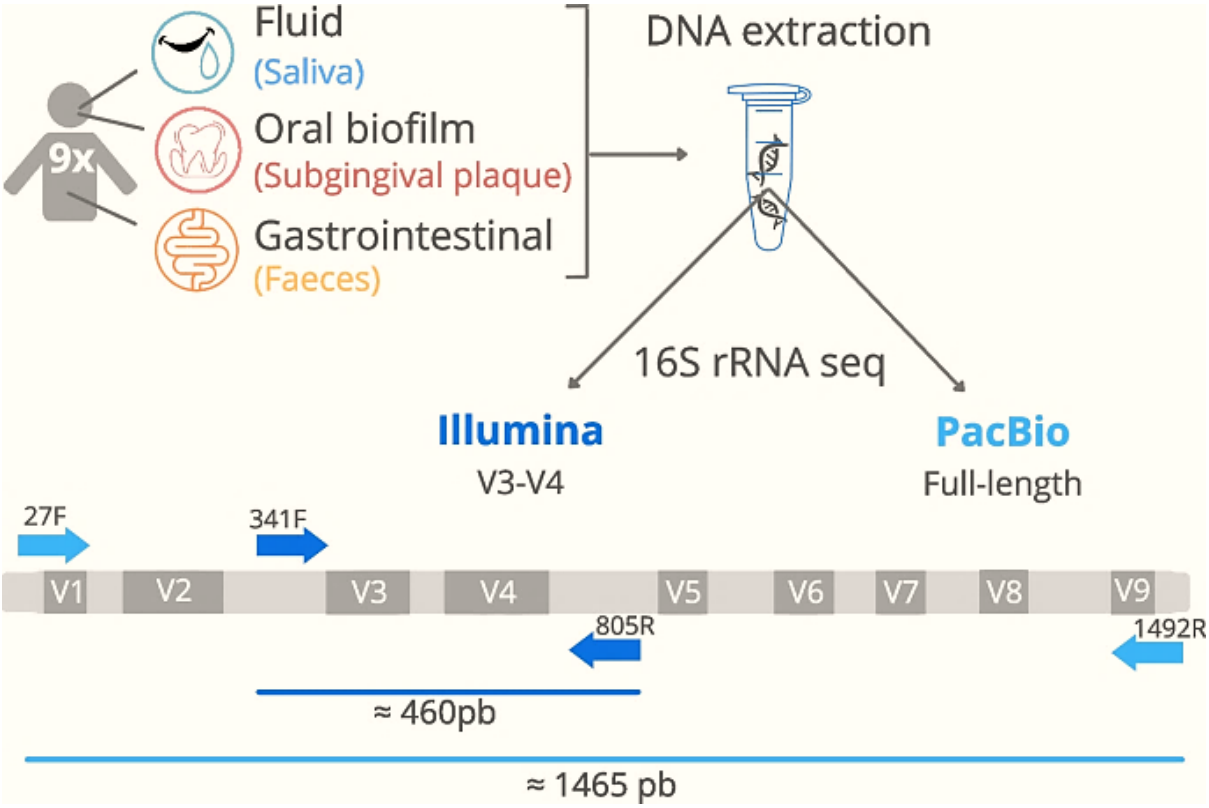

Figure 5: A scheme of a diagram 16S rRNA sequencing [58]

3.2 Whole Genome Sequencing (WGS)

Whole Genome Sequencing (WGS) is a powerful, high-resolution technique used to analyze the entire genetic makeup of probiotic strains, providing insights into their safety, functionality, and taxonomic classification. By analyzing the complete DNA sequence, WGS allows researchers to move beyond traditional in vitro testing to identify specific genes responsible for beneficial traits and potential safety risks, such as antibiotic resistance. The applications of WGS in Probiotics mention: WGS provides high-resolution taxonomic classification, accurately identifying strains down to the single nucleotide level, which is crucial for distinguishing between closely related strains, Safety Assessment to identifying virulence factors, toxic metabolites, and transmissible antibiotic resistance genes (ARGs). This allows for the selection of safe strains, Probiotic property analysis including Adhesion genes enabling colonization in the gut mucosa, Stress tolerance genes allowing survival in harsh conditions like acid and bile and, Antimicrobial action identification of bacteriocin-producing genes Figure 6 [59].

Probiotic WGS typically utilizes a combination of high-throughput sequencing technologies:

i) Short-Read Sequencing (e.g., Illumina): Used for high-accuracy identification of small variations and gene identification.
ii) Long-Read Sequencing (e.g., Oxford Nanopore): Used to produce high-quality, complete genome assemblies.
iii) Bioinformatics Analysis: Specialized software is used to analyze the raw data to determine genome structure, gene function, and evolutionary relationships.

The advantages over conventional methods comprehensive insight it unlike in vitro tests which only show phenotypic action, WGS provides a "genetic map" to predict and understand the full potential of a probiotic strain, Safety assurance of WGS is more efficient at detecting hidden potentially harmful genes that might not be detected through traditional susceptibility testing. (Table 1). High-throughput risk assessment of WGS is a rapid and, with decreasing costs, cost-effective way to evaluate probiotics for industrial,

agricultural, and clinical use. WGS is now considered a key component in the modern, holistic approach to characterizing and validating probiotic potential for consumer safety [60].

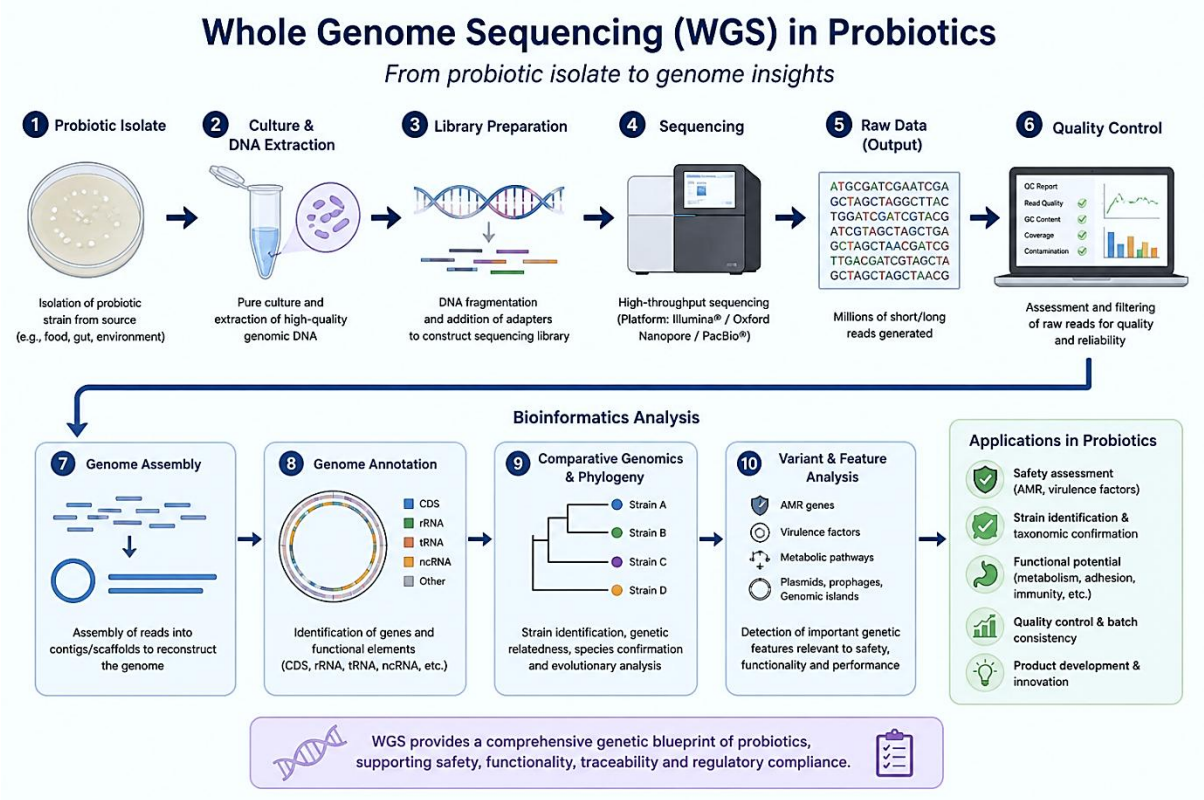

Figure 6: A scheme of a diagram Whole Genome Sequencing (WGS) [59]

3.3 MALDI-TOF MS Method

The (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry) provides rapid, accurate identification of probiotic strains in food and supplements by generating unique protein "fingerprints". It offers high-throughput, cost-effective analysis, often achieving 92%–97.5% specificity for species-level identification within minutes compared to traditional methods. the advantages of this method is rapid identification for same-day identification of probiotics in products, which is critical for quality control, as shown by specialized solutions like Axcess® MALDI-TOF, Highly accuracy reliable for characterizing probiotic genera like *Lactobacillus* and *Bifidobacterium*, with studies showing high concordance with molecular methods and addition quality control of Probiotic by detecting discrepancies between label claims and actual bacterial content in commercial products figure (7) (Table 1) . The disadvantages are Database dependence lead to identification is limited by the extensiveness of the spectral database, Strain differentiation while strong at the species level, distinguishing specific strains can require advanced methods, though it can sometimes identify subspecies and, Extraction Needs direct of colony method can be improved by adding formic acid extraction for higher confidence in Identification [61] , [62].

The MALDI-TOF MS analysis of Lactobacillus strains performed after protein extraction with formic acid/acetonitrile showed the great potential of this technique in the taxonomic characterization of lactobacilli. When compared to the genomic analysis, MALDI-TOF MS allowed to correctly identify at the species level Lactobacillus strains, unlike the 16S rRNA gene sequencing that provided only species-level identification (*L. paracasei* LC10 and *L. delbrueckii* FV13), MALDI-TOF MS analysis allowed to obtain information at subspecies level (*L. paracasei* subsp. *paracasei* and *L. delbrueckii* subsp. *delbrueckii*) [63], [64].

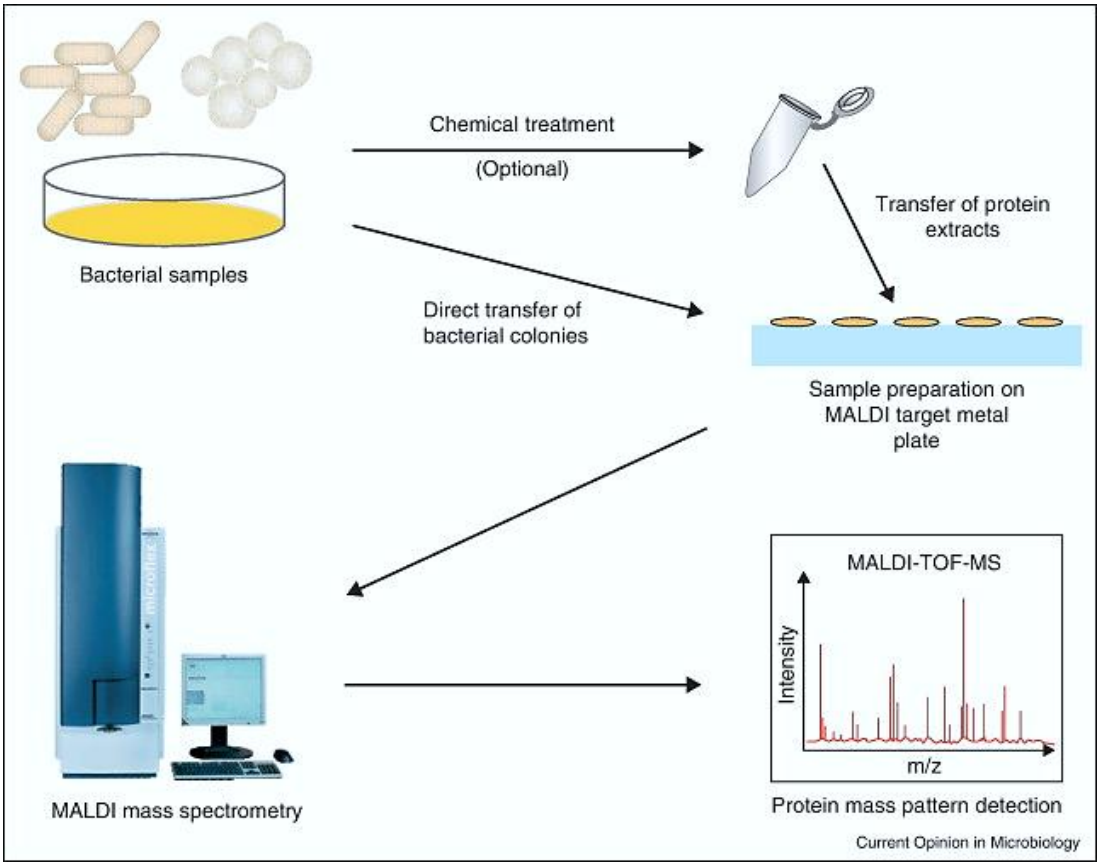

Figure 7: A scheme of a diagram MALDI-TOF MS Method [61]

Table 1: molecular techniques used in probiotic research and their main characteristics

Molecular tool	Primary Use	Discriminatory Power	Reproducibility	Cost	Speed	Labor Intensity	Requires Culturing	Key Advantage	Key Limitation
RAPD	Strain Typing	High (Strain)	Low-Moderate	Low	Fast	Low-Moderate	Yes	Quick, cheap typing	Poor inter-lab reproducibility
AFLP	High-Res Strain Typing	Very High (Strain)	High	High	Slow	High	Yes	Unambiguous strain typing	Complex, expensive
T-RFLP	Species/Strain Typing	Moderate (Species/ Strain)	High	Moderate	Moderate	Moderate	Yes	Reliable, stable fingerprints	Laborious
Real Time PCR	Quantification & ID	High (Species)	Very High	Moderate-High	Very Fast	Low-Moderate	No	Rapid quantification	Needs specific probes
16SrRNA Sequencing	Species Identification	Low/ Moderate (Genus/ Species)	High	Moderate	Slow (Days)	High	Yes/No	Gold standard for ID	Low resolution for closely related species
Whole Genome Sequencing (WGS)	Comprehensive Strain ID & Function	Highest (Strain/ Subspecies)	Very High	Very High	Slow (Days/ Weeks)	Moderate-High	Yes	Full genetic potential/ safety	Highest cost and data analysis
MALDI-TOF MS	Rapid Species/Strain ID	High (Species/ Subspecies)	High	Moderate (High Setup)	Fastest (Minutes)	Low	Yes	Instant protein fingerprint	Requires good database, culture time

RAPD: random amplified polymorphic DNA; AFLP: augmented fragment length polymorphism; T-RFLP: terminal (T)-restriction fragment length polymorphism; Real-time: Real-time PCR; 16S rRNA Sequencing; (WGS): Whole Genome Sequencing ; MALDI-TOF MS: Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. [35] [57] [60] [63].

CONCLUSION

Probiotics have been widely researched and used in a variety of goods across the globe. Probiotics showed that they have favorable impacts on human and animal health in recent research. Infantile, antibiotic-related, and traveler's diarrhoea have all been the focus of clinical probiotic research. Nonpathogenic organisms utilized as probiotics come in a variety of species and subspecies, and the capacity to attach to, make colonies, and modify the human gastrointestinal tract is not a universal pro perty. The primary probiotic bacteria are Lactobacillus and Bifidobacterium. However, there have been findings on probiotic biotic groupings and yeast potential. Anti-inflammatory, anti-allergic, and other important qualities are found in several of the discovered probiotic strains. Furthermore, both dairy and non-dairy food boost immunity in various ways.

Probiotics have become a major health problem across the globe. Many typing techniques were devised to recognize and categorize probiotics or LAB strains, as well as to detect a genetic relationship between these helpful bacteria, as previously discussed. Traditional phenotypic techniques have advantages and disadvantages that influence their usefulness. Despite the fact that a variety of molecular approaches have made it easier to type LAB strains, a polyphasic strategy using a pair of typing tools or more is preferable for accurate recording of LAB strains. Moreover, the development and availability of NGS methods, such as WGS, has enabled the investigation of DNA sequence variations. These technologies are expected to replace conventional typing devices in the near future. These sequencing-based procedures, on the other hand, are only accessible in well-equipped labs and need highly trained workers. In our perspective, less creative approaches will still be employed for the investigation of a small number of strains, necessitating developing simple, rapid, and costing procedures with maximum discriminatory power.

RECOMMENDATIONS

Adherence to the standards of scientific organizations such as FAO/WHO (Organization of the United Nations-World Health Organization) guidelines for probiotics, ISAPP (International Scientific Association for Probiotics and Prebiotics) using more than one molecular technique to obtain reliable results and connecting molecular findings with functional properties (functional validation) by practical laboratory steps at future:

1- Select reliable sources (dairy, fermented products, human/animal samples), purify bacterial isolates and obtain pure cultures and confirm primary phenotypic characteristics (Gram staining, morphology).
2- High-quality (A260/A280) DNA extraction.
3- Identification using molecular techniques for the 16S rRNA gene by (Sequencing) then sequence comparison with the databases: NCBI (BLAST)SILVA database for species-level identification, Whole Genome Sequencing (WGS) and (MALDI-TOF MS) Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.
4- Using MLST (Multi-Locus Sequence Typing) for high-precision strain analysis.
5- Evaluation of functional genes by detecting genes for acid tolerance genes, bile salt resistance, bacitracin production using PCR or q RT-PCR technique.
6- Molecular safety assessment to detection of virulence genes, antibiotic resistance genes (ARGs) This is very important before approving a strain as a probiotic.
7- Data analysis using software: bioinformatics tools (MEGA, BLAST) and building a phylogenetic tree to confirm identity.

ACKNOWLEDGMENT

Thanks and appreciation to the Deanship of the College of Pharmacy/ Al-Nahrain University

FUNDING Non

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مراجعة العلامات الجزيئية القائمة على DNA لتحديد وتوصيف الكائنات البروبيوتية

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الخلاصة

تعد بكتيريا حمض اللاكتيك (LAB) ضرورية في توليد الغذاء والحفاظ على الصحة الجيدة. هناك اهتمام متزايد بهذه الأنواع لمعرفة المزيد عن الفوائد الصحية العديدة التي قد تقدمها. تعتمد أنشطة المختبر على عدد البكتيريا الموجودة في الجهاز الهضمي ونوعها ونوعها ونوع السلالة المحددة. المواد الحافظة الكيميائية والأطعمة المصنعة تسبب الكثير من القلق بين المستهلكين. أما المنتجات التي تحتوي على أو معالجة ب LAB، فهي معترف بها على نطاق واسع كنهج طبيعي للحفاظ على الطعام وتعزيز الصحة. يهدف المنشور الحالي إلى تلخيص الأبحاث حول وظيفة LAB البروبيوتيك في حفظ الغذاء، وتعديل المناعة الهضمي، والفوائد الصحية.

في علوم الغذاء والأبحاث المرتبطة بها، يعد تحديد وتصنيف البكتيريا المفيدة أمرا بالغ الأهمية. التقنيات الظاهرية التقليدية لها عدة سلبيات، منها احتمال الخطأ في تحديد الهدف، مما يحد من استخدامها. تقنيات التصنيف الجيني لديها فرصة أكبر للنجاح، وتستخدم عادة لتمييز الكائنات الدقيقة. تختلف التقنيات المستخدمة لتشكيل بكتيريا حمض اللاكتيك (LAB) إلى حد ما عن بعضها البعض، ولكل أداة مجموعة من الفوائد والسلبيات. تغطي هذه الدراسة المراجعة طرق البصمة المختلفة المستخدمة لتحديد وتوصيف LAB على مستويات الأنواع والنوع الفرعي والسلالات. تعتمد غالبية هذه الطرق على الهضم التقييدي، وتضخيم تفاعل البوليميراز المتسلسل، بالإضافة إلى التسلسل. فيما يتعلق بالتكلفة والتقنية والإنتاجية، تطورت تقنيات تسلسل الحمض النووي بشكل كبير في السنوات الأخيرة. تجري جهود بحثية عالمية لإنتاج نسخ محسنة من تقنيات تحليل البصمة القابلة للاستخدام والتجارية بشكل عام.

الكلمات المفتاحية: الكائنات البروبيوتيكية، المختبر، التكنولوجيا الحيوية، الغذاء، التقنية الجزيئية.