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Application of Next Generation Sequencing for Rapid Identification of Lactic Acid Bacteria

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Abstract The rapid identification of lactic acid bacteria, which are essential microorganisms in the food industry, is of great significance for industrial applications. The identification of lactic acid bacteria traditionally relies on the isolation and identification of pure colonies. While this method is well-established and widely used, it is not without limitations. The subjective judgment inherent in the isolation and purification process introduces potential for error, and the incomplete nature of the isolation process can result in the loss of valuable information. The advent of next generation sequencing has provided a novel approach to the rapid identification of lactic acid bacteria. This technology offers several advantages, including rapidity, accuracy, high throughput, and low cost. Next generation sequencing represents a significant advancement in the field of DNA sequencing. Its ability to rapidly and accurately identify lactic acid bacteria strains in samples with insufficient information or in the presence of multiple lactic acid bacteria sets it apart as a valuable tool. The application of this technology not only circumvents the potential errors inherent in the traditional method but also provides a robust foundation for the expeditious identification of lactic acid bacteria strains and the authentication of bacterial powder in industrial applications. This paper commences with an overview of traditional and molecular biology methods for the identification of lactic acid bacteria. While each method has its own advantages, they are not without limitations in practical application. Subsequently, the paper provides an introduction of the principle, process, advantages, and disadvantages of next generation sequencing, and also details its application in strain identification and rapid identification of lactic acid bacteria. The objective of this study is to provide a comprehensive and reliable basis for the rapid identification of industrial lactic acid bacteria strains and the authenticity identification of bacterial powder.

Key words Lactic acid bacteria, Rapid identification, Next generation sequencing

1 Introduction

Lactic acid bacteria (LAB) are a group of bacteria with specialized metabolic functions that produce lactic acid by fermenting lactose or glucose. These microorganisms are present in globular or rod-shaped forms, exhibit Gram-positive characteristics, lack spores, and exhibit facultative anaerobic or anaerobic biological properties[1]. In the natural environment, a diverse array of lactic acid bacteria exists, with approximately 40 genera and over 300 species having been identified to date[2]. *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Pediococcus* and *Leuconostoc* are the most common lactic acid bacteria species. These species are particularly prevalent in the food industry[3]. Lactic acid bacteria are common ingredients in the food industry. In the dairy industry, lactic acid bacteria are used to ferment a variety of products, including yogurt, cream, and cheese. This process gives these products their distinctive flavor and nutritional value. Furthermore, lactic acid bacteria are utilized in the production of cereal products, such as tempeh. It is noteworthy that lactic acid bacteria are also a type of probiotic bacteria present in the human body. These bacteria enhance immunity and have health-care effects, such as improving bodily functions[4].

Nevertheless, the extensive utilization of lactic acid bacteria in the food industry has led to the emergence of a pressing need to develop rapid and accurate methods for their identification. The identification of lactic acid bacteria traditionally relies on classical microbiological methods, which are reliable but time-consuming, subjective, not easily standardized, and of limited accuracy[5]. In recent years, with the development of molecular biology, there has been a notable advancement in the identification methods of lactic acid bacteria based on molecular biology techniques. The aforementioned methods encompass a number of techniques, including PCR-based DNA fingerprinting, rDNA sequence-based molecular marker technique, interspecies-specific PCR, and MALDI-TOF MS based on protein mapping. Although these molecular techniques are highly effective in characterizing lactic acid bacteria, they also have certain limitations. For instance, they necessitate the isolation and purification of pure colonies for sample pre-treatment. Additionally, they require a high degree of experimental manipulation, the acquisition of high-quality DNA, and the inclusion of a sufficiently large number of standardized maps in the library[6].

The continuous development of sequencing technology has led to the emergence of next generation sequencing (NGS), which re-

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presents a novel solution for the rapid identification of lactic acid bacteria. NGS technology is employed primarily for resequencing strains or individuals containing reference gene sequences with the objective of collecting and analyzing genetic diversity within microbial species. Furthermore, the NGS technology allows for the analysis of novel sequences without the necessity of prior knowledge regarding the presence or absence of information related to the sequence within the sample[7]. We conduct a review of the current status of the application of NGS technology in strain identification and discuss its potential application in the rapid identification of lactic acid bacteria.

2 Research progress of rapid identification of lactic acid bacteria

The early identification of lactic acid bacteria was primarily conducted through traditional microbiological methods. In general, researchers employed specific lactic acid bacteria isolation media, such as MRS media and M17 media, to isolate and initially screen samples for lactic acid bacteria under aerobic or anaerobic environmental conditions[8]. Subsequently, they may employ physiological and biochemical identification techniques, such as API identification and VITEK 2 identification, to further corroborate the species of lactic acid bacteria. However, these traditional methods often prove challenging to provide accurate identification results. For instance, Zhang Lei *et al.*[9] identified 79 *Lactobacillus* strains using the API identification technique, yet ultimately achieved a valid identification rate of only 60.8%.

The advancement of molecular biological technology has led to a notable enhancement in the accuracy of lactic acid bacteria identification. Currently, mapping-based identification technology and molecular marker-based identification technology represent the dominant approaches. The application of these novel techniques has not only enhanced the precision of identification but also markedly reduced the time required for identification. Table 1 provides a comprehensive overview of the various identification methods for lactic acid bacteria and their salient characteristics, offering a valuable reference for researchers engaged in related fields.

Table 1 Identification methods for lactic acid bacteria and their salient characteristics

Identification method	Reproducibility	Repeatability	Resolution capability	Experimental cycle//d	Cost	Convenience of experimental operation	Sample requirement
Classical microbiological method	Moderate	Moderate	Low	>3	Low	Convenient for operation	Pure culture
rep-PCR	Good	Good	Moderate	1	Moderate	Convenient for operation	Pure culture
AFLP	Good	High	High	2	High	Difficult for operation	Pure culture
RAPD	Moderate	Moderate	Good	1	Low	Convenient for operation	Pure culture
MALDI-TOF MS	High	High	Low	>3	High	Convenient for operation	Pure culture
16S rDNA	High	High	Moderate	>3	Moderate	Moderate level	Pure culture
MLST	High	High	Moderate	>3	High	Difficult for operation	Pure culture
Real-time PCR	High	High	High	1	High	Moderate level	Pure culture

2.1 Mapping-based identification techniques At present, the principal techniques employed for the identification of lactic acid bacteria are DNA fingerprinting-based mapping and protein fingerprinting-based mapping. The former category encompasses repetitive extragenic palindromic PCR (rep-PCR), genomic DNA restriction fragment length polymorphism (RFLP), random amplified polymorphism DNA (RAPD), and other similar techniques[10]. The latter category includes matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS).

The identification technique based on DNA fingerprinting primarily capitalizes on the distinctions observed in the genomes of distinct strains. This approach involves polymerase chain reaction (PCR) and the analysis of electrophoretic profiles following agarose gel electrophoresis, which enables the differentiation of strains. For instance, the rep-PCR technique primarily employs the disparities in brief repetitive sequences within the bacterial genome to categorize and identify them[11]. Lee Chinmei *et al.*[12] successfully identified 8 *Lactobacillus* strains to the species level by comparing the REP sequence, ERIC sequence, BOX sequence, and (GTG)5 sequence in the genome short sequences using the PCR method. The RFLP technique primarily employs the distinctions in restriction enzyme cleavage sites on the bacterial genome, which result in variations in the length of the DNA restriction fragments for species classification and identification. In a study by Li Jia *et al.*[13], the 16S rDNA of six morphologically diverse lactic acid bacteria species was successfully amplified using universal primers for 16S rDNA. After enzymatic treatment with *Hae*III, *Hin*f I, and *Hha* I endonucleases, precise maps for enzymatic analysis were obtained. Through careful comparison and identification, four strains of lactic acid bacteria were finally identified. The RAPD technique is primarily based on the PCR amplification of genomic DNA with random primers, followed by a comprehensive analysis of the resulting PCR products by gel electrophoresis. This approach can be utilized to classify and identify the strains of bacteria. This technique was employed by Jiang Houyang *et al.*[14], who successfully characterized 27 strains of lactic acid bacteria isolated from yak cheese in Tibet and accurately distinguished two subspecies, *L. casei* and *L. paracasei*. These studies not only demonstrate the application of modern molecular biological techniques in the identification of lactic acid bacteria, but also provide substantial technical support for further research and utilization of lactic acid bacteria.

The identification technology based on protein mapping is specific to the characteristics of microbial nuclear protein systems, which are primarily controlled by genetic factors and less affected by external factors such as environmental and cultural conditions[15]. The process of rapid identification of microorganisms is achieved through the comparison of their respective fingerprints with those stored in a database[16]. Wei Chao *et al.*[17] employed the MALDI-TOF MS identification technique to identify *L. delbrueckii* in natural yak yogurt and obtained good identification results.

However, mapping-based identification techniques also have drawbacks that can not be ignored. For instance, RAPD has the disadvantage of poor reproducibility of identification, AFLP has the disadvantage of cumbersome steps and high operational requirements, and MALDI-TOF has the disadvantages of being dependent on the quality and coverage of databases for accuracy, having equipment that is overpriced, and needing specialized training for operation, which is not conducive to large-scale application.

2.2 Molecular marker-based identification techniques At present, molecular marker-based identification techniques for lactic acid bacteria encompass sequence analysis based on the 16S rDNA gene, multilocus sequence typing (MLST) methodology, and real-time quantitative fluorescence PCR (Real-time PCR) identification techniques. The 16S rDNA has been extensively utilized in the identification of lactic acid bacteria due to its distinctive properties, including a moderate number of nucleotides, a moderate molecular size, a ubiquitous presence across all organisms, and a highly conserved yet interspecies specificity in structure and function[18]. Bostan *et al.*[19] employed 16S rDNA gene sequence analysis to ascertain the composition of lactic acid bacteria in a traditional cone-shaped yogurt. Their findings indicated that the *L. bulgaricus* present in this yogurt was derived from pine cones added to the yogurt. Xia Xuejuan *et al.*[20] employed 16S rDNA gene sequence analysis to identify 12 isolates as *Lactobacillus* spp. out of 14 isolates obtained by isolation and purification from traditional dairy products in Tibet to the species level. This provided a basis for the study of the diversity of *Lactobacillus* spp. in Tibet.

MLST is a typing technique that analyzes the nucleotide sequences of microbial genomes based on multiple housekeeping gene fragments to distinguish microorganisms between genera[21]. Housekeeping genes are an appropriate means of strain identification as their expression levels are less affected by environmental factors and they are conserved but vary among species or strains. The principle of selecting housekeeping genes is based on the premise of saving time and effort, with the objective of ensuring the availability of an accurate typing resolution. In general, 6 – 10 housekeeping genes are selected for this purpose[22]. Liu Wenjun *et al.*[23] examined 30 strains of *Streptococcus thermophilus* and observed distinct phylogenetic clustering based on housekeeping genes and acetaldehyde production. This clustering was identified using the MLST technique, which involved the selection of 7 housekeeping genes (*Ak*, *lld*, *pdc*, *glyA*, *nod*, *pfl*, *pdh*). The results of this study were found to be promising.

Real-time quantitative fluorescent PCR developed on the basis of PCR characterization, is a process that involves the design of specific primers, the detection of the entire process in real time by the accumulation of fluorescent signals during PCR, and finally, the quantification of the unknown templates by standard curves[24]. Shehata *et al.*[25] successfully achieved the quantitative detection of *L. gasseri* BNR17 and *L. reuteri* LRC from the products by designing primers specific for these strains through real-time quantitative fluorescence PCR, which met the requirements in terms of sensitivity, repeatability, and specificity.

It is evident that molecular marker-based identification techniques are not without shortcomings. One such shortcoming is the necessity to design appropriate primers and fluorescent probes in order to achieve the effect of specific amplification. Additionally, the PCR reaction conditions must be optimized over an extended period of time. Furthermore, the quality of DNA templates will also affect the final results.

3 Next generation sequencing

3.1 Development of next generation sequencing Next generation Sequencing (NGS) represents the second generation high-throughput sequencing and the third generation sequencing, developed from the first generation of Sanger sequencing[26].

The initial generation of Sanger sequencing employs ddNTP and radioisotope labeling to ascertain the DNA sequence of the subject sample following gel electrophoresis and radiography[27]. This first generation sequencing is straightforward and expeditious, yet it is also characterized by limitations in terms of throughput and time efficiency[28]. Consequently, the second and third generations of sequencing have been developed based on this technology, collectively designated as next generation sequencing.

Second generation sequencing, also known as high throughput sequencing (HTS), is a method that allows for the massively parallel sequencing of the same sample template in a single run, thereby generating a large amount of data[29]. Subsequently, third generation sequencing, also known as sequencing by synthesis, was developed. This method allows for the acquisition of longer DNA fragment readings at a reduced cost, without the need for PCR amplification, and is not susceptible to the biases introduced by PCR amplification[30].

3.2 Principle and process of NGS At present, the most representative second generation sequencing techniques include pyrosequencing, reversible terminator sequencing, and sequencing by ligation. All second generation sequencing techniques comprise two principal steps: template preparation and sequencing. Template preparation encompasses a number of processes, including nucleic acid extraction from the template, preparation of the DNA library, and amplification of the template. The process of template

preparation can be tailored to the specific characteristics of the research object. For instance, nucleic acid extraction can be conducted using different nucleic acid extraction kits, depending on the type of nucleic acid being extracted. Similarly, library construction can be performed using various physical/mechanical methods, enzymatic techniques, and so forth, depending on the intended platform of use. The most significant distinction between the first and second generations of sequencing technique lies in the underlying sequencing principle. Pyrosequencing technology employs a polymerase chain reaction (PCR) approach, whereby each deoxyribonucleotide triphosphate (dNTP) is polymerized on the primer, resulting in the release of a fluorescence signal[31]. This signal is then amplified by a DNA polymerase, ATP synthase, luciferase, and adenosine triphosphate diphosphatase, which are activated upon annealing of the primer and template DNA. The intensity and duration of the fluorescence signal are used to determine the sequence of the DNA. Reversible terminator sequencing technology is employed primarily for single molecule array sequence analysis[32]. This approach involves the use of bridge PCR and fluorescent reversible terminator synthesis in conjunction with sequencing. The platform arrays single molecules on a small chip to perform a bridge PCR reaction. This is achieved through the use of reversible blocking technology, which allows for the synthesis of only one base at a time. Laser excitation of fluorescent groups is then employed to capture the fluorescence signal and read the base information. Sequencing by ligation is based on the sequential ligation synthesis of four-color fluorescently labeled oligonucleotides. Prior to sequencing, the template is amplified by emulsified PCR, and microbeads modified at the 3' end can be deposited on the slide. The substrate utilized for sequencing by ligation is an 8-base fluorescent probe mixture. Depending on the position of the sequence, the sample DNA can then be labeled with the probe. DNA ligase exhibits a preferential affinity for the probe paired with the template, thereby initiating the generation of a fluorescent signal at that specific site. The current platforms based on second generation sequencing techniques encompass pyrophosphate sequencing (454 Roche), reversible terminator sequencing (Illumina Hiseq 2500 series, NextSeq, Miseq, MiniSeq), and sequencing by ligation (SOLID and others).

At present, third generation sequencing techniques are primarily comprised of single molecule real-time sequencing (SMRT) and nanopore sequencing. As single-molecule real-time sequencing technology employs a template that is captured by a polymerase, four distinct fluorescently labeled deoxynucleoside triphosphates (dNTPs) enter the detection region and bind to the polymerase. The time required for the bases matching the template to form chemical bonds is considerably longer than the residence time of other bases. Consequently, the duration of the fluorescent signal can be quantified for the purpose of sequencing[33]. Nanopore sequencing technology employs a protein nanopore to convert chemical bases into electrical signals. The nanopore protein is fixed on a resistance film, and the double-stranded DNA is unchained into a single strand. The motor protein is used to pull the single strand of DNA through the nanopore, as different bases have different charges. The current on the resistance film is thus altered by the passage of the single strand of DNA, and the bases are identified by the resulting current change[34]. The current platforms based on third generation sequencing techniques include the single-molecule real-time sequencing technique, the Pacific Biosciences platform, the nanopore sequencing technique, and the Oxford Nanopore Technologies platform. Table 2 compares the performance of several common sequencing platforms.

Table 2 Performance comparison of several common sequencing platforms

Sequencing platform	Read length//bp	Flux Gb/run	Error rate//%
454 Roche	400	0.5	1
Hiseq 2500	2X125	720 – 800	0.26
NextSeq	2X100	100 – 120	0.8
Miseq	2X300	13 – 15	0.8
MiniSeq	2X150	6.5 – 7.5	0.8
SOLID	2X60	155	0.01
Pacific Biosciences	46 000	3.2	13
Oxford Nanopore Technologies	2 000	1	38

4 Application status of NGS in bacteria

4.1 Application status of NGS in identification, traceability and diversity analysis of bacteria NGS offers a number of advantages over traditional methods for strain identification. For instance, NGS is able to rapidly identify strains, and it is also highly efficient and capable of providing highly accurate identification. For instance, Gong Yanwen *et al.*[35] employed the conserved sequences at both ends of the V1 and V3 variable regions of bacterial 16S rRNA and the pyrosequencing technique to complete the identification of 96 strains of bacteria preserved in their laboratory within 4 h. The results obtained were 100% accurate in comparison with the traditional microbial physiological and biochemical identification results. In a study by Garcia-Garcia *et al.*[36], nanopore sequencing was employed to rapidly and accurately identify 32 invasive strains of *Streptococcus pneumoniae* in approximately 4 h, a significant reduction in time compared to Sanger DNA sequencing and traditional identification methods. In the context of traceability analysis, particularly with regard to pathogen traceability, NGS plays a pivotal role, offering greater efficiency and accuracy compared to traditional methods. For instance, Ashton *et al.*[37] employed the Illumina Hiseq second-generation sequencing platform to identify the source of an outbreak of *Salmonella typhimurium* infections in Jersey, UK, in 2013. Their findings indicated that the pathogen originated from mayonnaise prepared with raw eggs. Underwood *et al.*[38] investigated the traceability of an outbreak of human infection with enterohemorrhagic *Escherichia coli* on a farm using the 454 and Illumina second generation sequencing platforms. Their findings indicated that the widespread distribution of the pathogen in the farm's animals and the environment was the

primary cause of the event. NGS offers significant advantages in microbial diversity analysis compared to traditional approaches reliant on pure culture. The V3-V4 region of bacterial 16S rDNA was employed as a template by Ding Ruixue *et al.* [39] in conjunction with the Illumina Miseq second-generation sequencing platform to investigate the dynamic changes of microorganisms in pasteurized milks under different storage conditions. The study concluded that the emergence of *Paenibacillus* and *Serratia* were the key determinants of spoilage in the quality of pasteurized milk. Similarly, Doyle *et al.* [40] employed the V3-V4 region of bacterial 16S rDNA as a template to investigate microbial dynamics in raw milk using the Illumina MiSeq second generation sequencing platform. Their findings indicated that the stage of lactation, whether middle or late, has a significant impact on milk microbial diversity.

4.2 Application of NGS in identification of lactic acid bacteria NGS is also employed in the identification of lactic acid bacteria, particularly in the investigation of the diversity and abundance of lactic acid bacteria strains within samples. For instance, Cai Hongyu *et al.* [41] employed SMRT third generation sequencing to investigate the diversity of *Lactobacillus* flora in a total of 19 samples of fresh mare milk and koumiss from Xinjiang. The findings revealed that 52 distinct *Lactobacillus* strains were identified. From the koumiss, the predominant strains were identified as *L. helveticus* (abundance 61.77%) and *L. kefiranofaciens* (abundance 14.91%), with five other strains also present in notable quantities. A total of 103 *Lactobacillus* strains were isolated from fresh mare milk. Of these, 13 strains were identified as predominant strains, including *L. helveticus* (abundance 35.52%) and *S. salivarius* (abundance 10.11%). Zheng Yuqian *et al.* [42] employed the Illumina MiSeq second generation sequencing platform to investigate the bacterial diversity in 16 types of commercially available low-temperature yogurts. They utilized the V3-V4 variable region of bacterial 16S rRNA sequences as templates and discovered that the actual bacterial strains in different commercially available yogurts differed from those indicated on their corresponding labels. Additionally, the researchers identified bacterial strains that did not belong to the yogurt category, suggesting the possibility of environmental contamination during the production process.

5 Conclusions and prospects

NGS, which offers significant advantages in terms of rapid identification, high throughput, and the absence of the need for pure culture, has demonstrated considerable potential for application in the fields of rapid strain identification, traceability, and microbial diversity analysis. This technique offers a novel approach to the rapid identification and traceability of lactic acid bacteria, as well as the analysis of the diversity of lactic acid bacteria in samples. It represents a significant improvement over traditional methods in terms of analytical efficiency and accuracy.

Although NGS is still deficient in certain respects, such as the limitation of short length of sequencing read DNA fragments in second generation high-throughput sequencing, which presents certain challenges for subsequent bioinformatics analysis[43]. Furthermore, third generation sequencing necessitates the presence of high-quality samples, such as nucleic acids, during the sequencing process, thereby placing significant demands on the operator's level of expertise. Despite these demands, the sequencing error rate remains relatively high[44]. Nevertheless, as sequencing technology continues to evolve and innovate, there is reason to believe that these shortcomings and bottlenecks will gradually be overcome.

As technology continues to evolve, NGS will become an increasingly crucial tool for the rapid identification of lactic acid bacteria and the authentication of bacterial powder, playing a pivotal role in supporting the sustainable development of the food industry. The implementation of this technology will not only enhance production efficiency, but also help to safeguard product quality and safety, thereby further promoting the innovation and progress of the entire industry.

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