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Established qPCR Method Using Strain-Specific Primers for Enumeration of Paraprobiotic *Lactobacillus plantarum* L137

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ABSTRACT

Lactobacillus plantarum, extensively present in fermented foods and the human intestinal tract, has numerous health advantages, including regulating systemic immunity, maintaining a balanced intestinal flora, and lowering the risk of tumour progression. Because the health benefits and safety are strain-specific, it is essential to have a trustworthy method for the specific detection and enumeration of *L. plantarum* L137 to verify probiotic efficacy. The real-time PCR tool was developed using strain-specific primer pairs from the specialized plasmid pLTK2 and successfully validated for specificity, precision, and amplification efficiency. The assay achieved 100% true positive and 0% false positive results by effectively amplifying the target sample and not amplifying any of the six non-target strains. The limit of quantification was established to be $1.7.10^3$ CFU/reaction. The reaction efficiency values were 86.7%, with an R^2 value of 0.9864. The repeatability's relative standard deviation (RSD) was 2.34%. The qPCR assay for *L. plantarum* L137 was found to meet the specific, precise, and efficient requirements. To meet compliance standards, it is crucial to have a reliable and approved assay for strain-specific identification and enumeration for probiotic products.

KEYWORDS: *Lactobacillus plantarum*, paraprobiotic, real-time PCR, quantification, validation

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INTRODUCTION

Lactic acid bacteria are rod-shaped, non-spore-forming, and Gram-positive bacteria that produce lactic acid [1]. With more than 237 species, the *Lactobacillus* genus is undoubtedly the most diverse and significant group of lactic acid bacteria, with the discovery of new species such as *L. metriopterae* and *L. timonensis* [2]. Certain *Lactobacillus* species are among the most frequently employed probiotics [2]. *L. plantarum* is a *Lactobacillus* member widely recognized due to its presence in diverse ecological habitats, including vegetables, fermented foods, and the healthy human digestive system [3]. The Food and Drug Administration has granted *L. plantarum* the qualified presumption of generally recognized as safe (GRAS) category [4]. Accumulating evidence has proposed that *L. plantarum* has beneficial effects on health, such as the prevention and treatment of irritable bowel syndrome [5], management of the fecal flora composition [4], inflammatory bowel disease [3], cancer [6], blood cholesterol level reduction [4], and host immunity promotion [7]. In general, the consumption of probiotic as a live active cell is subject to common potential risks such as infection by live microorganisms, long-term alteration of immune responses, or the emergence of multi-drug-resistant strains [8-11], and *L. plantarum* is no exception. A possible remedy is using inactivated probiotics, also referred to as paraprobiotics (not viable microbial cells or cell composition), which provide a comparable benefit to the consumer. In addition, their benefits include an extended shelf life, as the viability of the microorganisms is not dependent on the maintenance of the cold system. [12]. A plethora of scientific evidence has shown important probiotic activity from components present in inactivated *L. plantarum* cells. It has been found that peptidoglycan in the cell wall of *L. plantarum* ATCC 14917 inhibited macrophages from producing interleukin-12 (IL-12) via the Toll-like receptor 2, which is involved in inflammatory bowel disease [13]. The lipotechoic acid exhibited anti-inflammatory properties on the intestinal epithelium by attenuating the IL-8 expression induced by Pam2CSK [14]. The exopolysaccharides (EPS) attenuated the production of inflammatory cytokines (IL-6, IL-8, and MCP-

1) in porcine intestinal epithelial cells [15]. These structures strongly inhibited the proliferation of HT-29 colon cancer cells and induced Fas/Fasl-mediated apoptosis in murine CT26 cells [16,17]. Cell wall-anchored protein A and mannose-specific adhesin played an active role in the adhesion of probiotic bacteria to human intestinal epithelium [18], whereas moonlight proteins help compete for and eliminate pathogens [19,20]. With clarified probiotic properties, paraprobiotic *L. plantarum* has been studied and extensively commercialized in the functional food and food industry [21].

Controlling the presence and content of *L. plantarum* bacteria during the production process and testing prior to being distributed to the market is a reliable scientific foundation for demonstrating the manufacturer's compliance with the product's quality and effectiveness. The "gold standard" for enumerating probiotic cell viability has been the traditional plate count technique. Nevertheless, this method is limited to quantifying the number of cells capable of producing visible colonies and multiplying in vitro. Consequently, it is not applicable to products containing paraprobiotics [22,23]. In contrast, non-culture alternative techniques have broad applicability and reliable results on non-viable bacteria, such as real-time PCR, MALDI-TOF mass spectrometry, and flow cytometry [22]. The PCR technique has become a powerful tool for qualitative and quantitative analysis of different paraprobiotic microorganisms based on species-specific DNA sequences [24,25]. However, the 16S rRNA gene sequences of numerous Lactobacillus species are far too similar to be easily distinguished due to the diversity of *Lactobacillus* species and strains. Notably, the 16S rRNA gene sequences are notoriously challenging to differentiate between closely related species within *L. plantarum*, *L. paraplantarum*, and *L. pentosus*. To overcome this limitation, species-specific primer pairs were designed to target species-unique genes for detection and identification of *Lactobacillus* species [26]. Therefore, strain-specific gene sequences, which are frequently found on plasmids, are necessary for identification at the strain level. In this investigation, the strain *L. plantarum* L137 was identified and quantified by analyzing the region of the strain-specific plasmid pLTK2. A qPCR method was established and validated for quality control in products that contain the paraprobiotic *L. plantarum* L137.

MATERIALS AND METHODS

Microorganisms

The target strain used in this study was *Lactobacillus plantarum* L137. The reference strains were *Lactobacillus acidophilus* NCFM, *Lactobacillus casei* 431, *Lactobacillus reuteri* DMS 17938, *Bacillus subtilis* HU58, *Bifidobacterium bifidum* BB-12, and *Streptococcus thermophilus* ST-1.

DNA isolation from the food matrix

10 g of biscuit sample was blended into fine powder. 1 g of ground sample was put in a 15 ml tube containing 6 ml DTAB solution (DTAB 8.0%, NaCl 1.5M, tris buffer 0.1M, EDTA 0.05M, pH was adjusted 8.5-9.0 by NaOH solution), vortexing carefully before incubating at 80°C for 5 min. 7 ml of chloroform was added to the tubes, shaken well and centrifuged at 1600 g for 20 min. 60 µl of the supernatant was transferred to new Eppendorf with 1000 µl of CTAB solution (CTAB 0.5%, NaCl 0.04M and deionized water), vortexed, and incubated at 80 °C for 5 min. The pellet obtained after centrifuging at 14000 g for 10 min was dispersed in NaCl solution 1.2M and incubated at 80°C for 5 min. After centrifuging Eppendorf at 14000 g for 5 min; the supernatant was transferred into fresh Eppendorf prepared 300 µl of 95% ethanol to precipitate DNA. The DNA pellet was recovered after 14000 g centrifugation in 5 min and stored in 30 µl of deionized water. DNA concentration and purity were determined by a Nanodrop AA1000 spectrophotometer (Peak Instrument).

Establishment of the standard curve

The target strain, *L. plantarum*, was prepared in a series of ten-fold concentrations equivalent to 10^9 to 10^3 CFU/ml. The standard samples were extracted and quantified by real-time PCR to determine the Ct values. These values were used to construct a qPCR standard curve that depicts the correlation between the decimal logarithm of the *L. plantarum* concentration and the Ct value. Each concentration was performed in triplicate, and the average was calculated. The negative control sample was demineralized water without *L. plantarum* DNA. The linear line $y = ax + b$ (where y is Ct, x is the decimal logarithm of *L. plantarum* number, a is the slope of the regression line, and b is the y-intercept) was set up with the correlation coefficient $R^2 \geq 0.98$ [27].

Real-time PCR

Real-time PCR amplification and detection were performed in a real-time MyGo Pro system (IT-IS International, Novacyt Group). The reaction mixture (22.5 µl) was composed of 12.5 µl SYBR Premix Ex Taq II 2X, 1 µl each primer 10 µM, 5 µl template DNA, and 3 µl deionized water. For the quantification of *L. plantarum*, primers were used to target a region of the major plasmid specified for L137 strain (Forward primer: 5'-GGCAGAAGGCTCTTAAAAGCTC-3', and reverse primer: 5'-CGCTTCAACGTTCACAATCG-3'). The amplification program consisted of one cycle of 95°C for 30 s, then 40 cycles of 95°C for 5 s, 60°C for 30 s, and finally one cycle of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s for melting curve analysis [26].

Validation of analysis process

Specification test

The assay specificity test was verified to ensure that it could only amplify the *L. plantarum* L137 strain and not reference strains. Initially, an in silico BLAST analysis was used to determine the specificity of the amplicon against the

gene sequences of probiotic species. Second, specificity was evaluated using one target strain, three closely related strains (*Lactobacillus acidophilus* NCFM, *Lactobacillus casei* 431, and *Lactobacillus reuteri* DMS 17938), and three common probiotic strains (*Bacillus subtilis* HU58, *Bifidobacterium bifidum* BB-12, and *Streptococcus thermophilus* ST-1). All target and non-target samples were normalized to 1 ng/µl for the DNA concentration. The real-time PCR parameters previously described were employed to assess the samples. Melting temperature assessment of PCR products was carried out to ascertain the analytical specificity after PCR amplification cycles [25].

Precision test

Precision (repeatability) represents the closeness of conformity between the reference value and successive test results that are obtained using the same method, test material, and experimental conditions (equipment, operator, laboratory and short time period). The DNA sample was extracted six times, and the qPCR reaction was performed with each extraction measured three times, and the average Ct values were recorded. Repeatability is expressed as relative standard deviation (%RSD) and evaluated through a value of %RSD ≤ 25% [28].

Amplification efficiency evaluation

Amplification efficiency (E%) is evaluated based on the formula: $E\% = (10^{-1/slope} - 1) \times 100\%$, in which slope is derived from the linear regression line, so it should be in the range from -3.9 to -2.9, equivalent to an amplification efficiency ranging from 80-120%, which is the ideal efficiency range of the reaction [25].

Limit of quantification

From the results of building a real-time PCR linear curve, the limit of quantification is the point corresponding to the lowest number of CFU/reaction on the linear curve. LOQ is determined as the smallest amount of analyte that can be measured and quantified with precision under experimental conditions according to a validated method (CV < 25%) [28].

Data analysis

The Ct values were automatically calculated by MyGo Pro PCR software 3.6, which is accompanied by a real-time PCR MyGo Pro system. Data analysis was performed using Microsoft Excel. All experiments were conducted in triplicate and have average values.

RESULTS AND DISCUSSION

DNA isolation method

The CTAB-DTAB method was highly effective in extracting *L. plantarum* DNA from food samples, resulting in an average DNA concentration of 1767.54 ng/µl and an A260/280 ratio that ranged from 1.96 to 1.99. The CTAB-DTAB method employs the cationic surfactant CTAB to precipitate DNA in place of SDS, which gives higher purity values and improved yields. A robust detergent, such as DTAB, is implemented for the rigid samples. It is reported that the combination of CTAB and DTAB yields excellent DNA extraction efficiency for analysis with PCR or restriction enzymes. DTAB and CTAB disrupt cell membranes, triggering the release of intracellular components. DTAB-CTAB subsequently forms complexes with nucleic acids at high temperatures or with proteins and polysaccharides at low temperatures. The CTAB-DTAB method has been demonstrated to be effective in the extraction of *L. plantarum* DNA from complex sample matrices in the present study, as well as in numerous other studies, including fish intestine, meat, and plant samples. Despite the widespread commercialization of rapid extraction tools, most researchers prefer to use traditional methods to obtain purer DNA yields, particularly from complex samples.

Real-time PCR for *L. plantarum* enumeration

Standard curve and limit of quantification

L. plantarum L137, a probiotic strain that has been exhaustively studied and is widely used, was initially isolated from traditional fermented food produced in the Philippines [29]. As the health benefits of probiotics are strain-specific, it is imperative to establish an assay for the precise identification of the *L. plantarum* L137 strain. Strain-specific assays for *L. plantarum* were developed in certain prior investigations. Primer pairs for PCR amplification using SYBR Green dye included 147 bp amplicon from unique gene and 120 bp amplicon from gene encoding transporter were developed based on pan-genome analysis [26,30]. Both studies relied on specific sequence regions of the genomes for species/subspecies distinguishing. Identification to the strain level requires the use of strain-specific sequences such as gene portion located on specialized plasmids. In this investigation, strain-specific primers were designed to amplify a 50 bp amplicon for *L. plantarum* L137 from a unique gene-coding major plasmid. DNA was extracted from a known quantity of *L. plantarum* L137 bacterial cells, and serial decimal dilutions were generated. The Ct value obtained in qPCR for each dilution was used to establish a calibration curve. The equation retrieved from the calibration curve was $y = -3.688x + 42.472$ ($R^2 = 0,9864$). Based on the obtained Ct value, this equation is appropriate for enumerating the number of bacterial cells from the commercial samples. The DNA concentration in the samples should be between $1.7.10^3$-$1.7.10^8$ CFU/reaction. The slope values were -3.688, referred the amplification efficiency value was 86.7%, within the optimal reaction efficiency range (80 to 120%) [25].

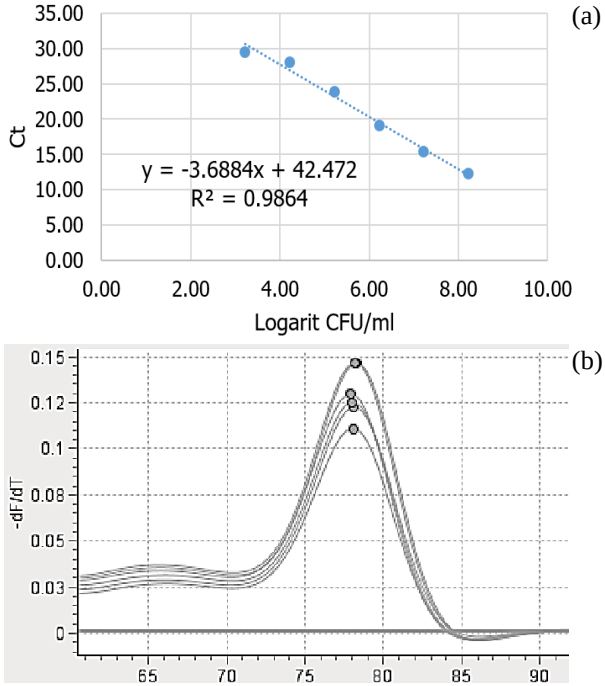

Figure 1. *L. plantarum* quantification standard curve
(a) Melting curve analysis of PCR product specificity;
(b) Analysis of melting curve derivative

The data determined the LOQ value to be $1.7.10^3$ CFU, corresponding to the average Ct value of 29.52, and CV = 0.24%, showing that the qPCR reaction was sensitive and reliable. When analyzing the melting curve of the product after amplification, at the concentration of $1.7.10^3$-$1.7.10^8$ CFU/reaction, the melting peak value was 78.02-78.29ºC, and at the concentration of $1.7.10^2$ CFU/reaction, the melting peak had a low intensity close to the threshold line; these were non-specific products. This shows that $1.7.10^3$ CFU/reaction is the lowest amount of *L. plantarum* employed in the PCR, so the signal can be amplified and accurately measured.

Analytical specification

Assay specificity was initially evaluated in silico against probiotic strains. The amplicon amplified from this assay did not exhibit significant similarities to any of the six non-target sequences, including three closely related and three genera-different strains. The selection of non-target genera or species was based on their close genetic relationship to the target species on the phylogenetic tree (e.g., *L. acidophilus*, *L. casei*, and *L. reuteri*) and species that are commonly associated with Lactobacillus in commercial dietary supplements (*Bacillus subtilis*, *Bifidobacterium bifidum*, and *Streptococcus thermophilus*). Therefore, these non-target species are likely to cross-react with the primers used. The results expressed target strain amplification with mean Ct values ranging from 21.48 to 23.88 and averaging 22.68. Non-target samples either did not exhibit no amplification or showed late discrepant amplification after 32 cycles. The assay was found to be specific for the strain-level identification of *L. plantarum* L137, with a true positive rate of 100% and false negative and false positive rates of 0%, using the given threshold Ct.

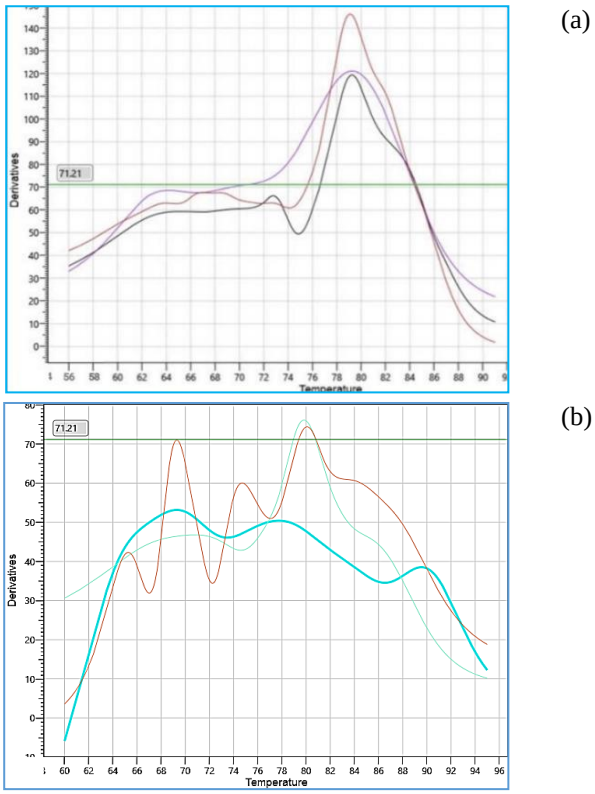

Figure 2. Melting temperature curve in specificity test
(a) Standard sample containing *L. plantarum*; (b) Samples without *L. plantarum*

Additionally, the melting temperature of the amplified products was determined during the slow heating phase, which served as evidence of the qPCR reaction's specificity. Consequently, the negative control samples (devoid of *L. plantarum*) did not exhibit any peaks, and no non-specific peaks were observed. The melting peak diagram (Figure 2) reveals that the amplified products exhibit a single peak at 78.03-78.29ºC at all dilutions, indicating the high specificity of the amplified products and used primer pairs that could not form dimers.

Analytical precision

The developed qPCR reaction exhibited good consistency between the analytical procedure repeats, as evidenced by the RSD value of 2.34% (< 25%). Precision or repeatability measures the consistency of results obtained from an experiment when repeated under the same conditions. The qPCR method was demonstrated to be stable, accurate, and repeatable in this study by achieving a %RSD within the acceptable range (Table 1).

Table 1. Validation results of analytical precision test

Extraction samples	***Ct values***				
	1	***2***	***3***	***Mean***	***RSD (%)***
1	22.33	22.79	22.56	22.56	2.34%

2	20.39	19.73	20.17	20.10
3	25.77	25.38	25.90	25.68
4	24.16	23.86	23.30	23.77
5	19.79	19.32	19.74	19.62
6	19.23	19.64	19.42	19.43

The development and standardization of qualitative and quantitative method for microorganisms is an important issue. The PCR-based assay described has been demonstrated to be highly reproducible, amplification efficient, and specific, which will be widely accepted for commercial products. All of these criteria are crucial for a testing procedure that is utilized for product quality control. The specialized gene on the small plasmid is considered a marker gene useful for the accurate identification of *L. plantarum* L137, which overcomes the limitations of 16S rDNA-targeted primers since the high similarity of up to 98% between *L. plantarum* and genetically close species [26]. The method is predicated on real-time PCR, eliminating the need for post-PCR processing with cross-contamination.

CONCLUSION

The assay developed and validated for the specific identification and enumeration of the *L. plantarum* L137 strain exhibited high specificity, amplification efficiency, and precision. Access to trustworthy techniques for strain-specific identification is vital to verifying the compliance of paraprobiotic products.

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