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Rapid identification and application of *Lactobacillus plantarum*, *Lactobacillus paracasei* and *Lactobacillus pentosus* using multiplex polymerase chain reaction and species-specific primers, targeting 16S ribosomal RNA and *recA* genes

Cheng-Chih Tsai*, Zhen-Yu Lai

Department of Food Science and Technology, HungKuang University, Shalu District, Taichung City 43302, Taiwan

Abstract

Background and Objective: Various products on the market contain probiotics such as lactic acid bacteria, which are promoted with a wide range of benefits. Functionality of these products is linked to the specific strains, bacterial species and viable cell counts. This study aimed to assess conformity of targeted lactic acid bacterial species and viable cell counts in commercially available probiotic products with their labeling, ensuring efficacy of the products.

Material and Methods: Multiplex polymerase chain reaction technique was developed using specific primers to effectively differentiate lactic acid bacteria in probiotic products. Therefore, strains used in the products were targeted and relevant nucleotide sequence data were searched to select two sets of polymerase chain reaction primer pairs of *L. pla*-F/R and *L. para*-F/R, targeting 16S ribosomal RNA genes, and *L .pen*-F/R, targeting *recA* genes.

Results and Conclusion: The individual primer sets produced the expected target products that matched the labeling for the tested strains of *Lactobacillus plantarum*, *Lactobacillus paracasei* and *Lactobacillus pentosus*. Then, specificity assessing was carried out using multiplex primer sets for single strains, pairwise combinations and triple combinations of lactic acid bacteria. After verifying specificity for all the three strains under similar polymerase chain reaction conditions, sensitivity of the multiplex polymerase chain reaction was investigated by assessing various dilutions of the three lactic acid bacterial strains and commercially available probiotic products. These findings demonstrated potential uses of multiplex polymerase chain reaction in lactic acid bacterial detection techniques. In conclusion, specific primer sets can be used in multiplex polymerase chain reaction to rapidly and effectively detect lactic acid bacterial strains in commercial products.

Conflict of interest: The authors declare no conflict of interest.

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Corresponding author:

Cheng-Chih Tsai

Department of Food Science and Technology, HungKuang University, No. 1018, Sec. 6, Taiwan Boulevard, Shalu District, Taichung City 43302, Taiwan.
Tel: 886-4-26318652-5082
Fax: 886-4-26319176
E-mail: tsaicc@sunrise.hk.edu.tw

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1. Introduction

Lactic acid bacteria (LAB) are addressed for their beneficial effects on the human gastrointestinal tract, enhancing overall immune health. There are recent interests in development of functional LAB products. Demands for the probiotic functional foods are rapidly increasing due to the increased consumer awareness of food effects on health [1]. Researchers have assessed potential uses of probiotics in dairy and nondairy products and their viabilities during storage [2]. They have concluded that the final product should contain a minimum of 10^6-10^7 viable cells per serving to benefit consumer health [2]. Their studies have shown that mislabeling of probiotic species is common in commercial products [3]. Lack of appropriate identification of the strains and false efficacy claims have led to confusion. Probiotic products available in the market often use mixed strains. Thus, there are needs to monitor conformities of the labeled bacterial species and viable cell counts.

Polymerase chain reaction (PCR) technique has successfully been used to detect and differentiate viruses and bacteria in various foods [4]. Rapid and reliable nature of PCR provides a valuable tool for distinguishing closely related species within two groups of lactobacilli [5]. This technique has been used to rapidly identify *Lactobacillus plantarum* in kimchi [6]. Oligonucleotide primers have been

developed from sequences between the 16S and 23S rRNA genes, enabling identification of various lactobacilli strains in dairy products and probiotics using PCR [7]. Furthermore, strain-specific PCR can be used for the rapid identification of lactobacilli isolated from food samples [8] and specific identification of ten common lactobacilli and bifidobacteria strains in fermented milks [9].

In this study, multiplex PCR method was developed to investigate applicability of molecular detection techniques for LAB using three sets of specific primers sourced from the literature. Moreover, 16S rRNA gene sequences were targeted to explore molecular LAB detection. By amplifying 16S rRNA and *rec*A gene sequences through PCR, this study simultaneously detected three LAB strains as well as mixed LAB strains in the products. Sensitivity of detection was assessed to establish a simple, reliable rapid method appropriate for the effective identification of LAB strains in probiotic products.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

The LAB strains were stored in a -80 °C freezer. Before the experiments, strains were activated twice using lactobacilli MRS broth (Difco, Detroit, MI, USA) supplemented with 0.05% (w/w) L-cysteine (Merck, Taipei, Taiwan). Strains were cultured under optimal growth conditions at 37 °C for 24 h. Reference strains were provided by the Bioresources Collection and Research Center (BCRC, Hsin-Chu, Taiwan). The *L. pentosus* BCRC 17972 and 17973, *L. plantarum* F7-1 and *L. paracasei* BCRC 12193, 12188, 12248 and 17002 were used in this study. Seven strains were used in the current study as well. The commercial probiotic product was purchased from Li-Fong, Tainan, Taiwan, for PCR detection. Each sachet of the bacterial powder contained a high level of viable probiotic cells with 5.0×10^{10} CFU.g^{-1}. Specific strains included in the product were *L. plantarum* LP112, *L. paracasei* LPC188 and *L. pentosus* LPE588. ThIS study was carried out at Testing and Analysis Center for Food and Cosmetics, HungKuang University, Taichung City, Taiwan.

2.2 Genomic DNA preparation and polymerase chain reaction primers

Total chromosomal DNA of the LAB cells was extracted using Blood and Tissue Genomic DNA Extraction Miniprep System (Viogene, Taipei, Taiwan) based on the manufacturer's instructions. Specific primer sequences for the LAB detection are shown in Table 1. Experiments were repeated thrice [10-12].

2.3 Polymerase chain reaction amplification

Method was carried out according to [13]. For each PCR cycle, denaturation, annealing and extension were carried out at 94 °C for 60 s, 57 °C for 60 s and 72 °C for 120 s, respectively. Final extension was carried out at 72 °C for 5 min.

2.4 Sensitivity of the polymerase chain reaction assay

A 24-h culture of the LAB strain was serially diluted 10-fold with sterile water. Purification of DNA was carried out as described in Section 2.2 [14].

2.5 Polymerase chain reaction detection in the commercial probiotic product

The probiotic product was purchased from Li-Fong, Tainan, Taiwan. After diluting the product to 10^8–10^6, 1 ml of the diluted sample was collected and DNA extraction was carried out. Then, 2 µl of the extracted DNA was used for multiplex PCR. Experiments were repeated thrice.

3. Results and Discussion

3.1 Multiplex Polymerase chain reaction

Figure 1 shows gel electrophoresis results of the multiplex PCR for DNA detection of individual LAB strains. Results demonstrated specificity of the three primer sets for their respective target genes in each strain. Small interferences were seen for *L. plantarum* F7-4 with no effects on amplification of other strains. Figure 1 shows gel electrophoresis results of multiplex PCR for DNA detection of two LAB strains. Results demonstrated that *L.pla*-F/R, *L.pen*-F/R and *L.para*-F/R primer sets could accurately amplify DNA from combination of two LAB strains. No nonspecific products were observed. Thus, 57°C was determined as the optimal annealing temperature for successful primer binding and DNA polymerase activity.

Table 1. Specific primer sequences used in this study for lactic acid bacteria the detection.

Target gene	Primer	Sequence (5′-3′)	Accession no.	Product size (bp)
16S rRNA	*L.pla*-F	TGATTGGTGCTTGCATCATG	KY88355	419
	L.pla-R	TGAACAGTTACTCTCAGATA		
*rec*A	*L.pen*-rF	AACAATTTCCAGCGGGTCAC	AJ621666	247
	L.pen-rR	ATCTGGTTGTGAAAGTAACAAA		
16S rRNA	*L.para*-1F	AAGATCACCCTCAAGCACCCT	CP014985	146
	L.para-1R	GCGTCAGCGGTTATGCGATGC		

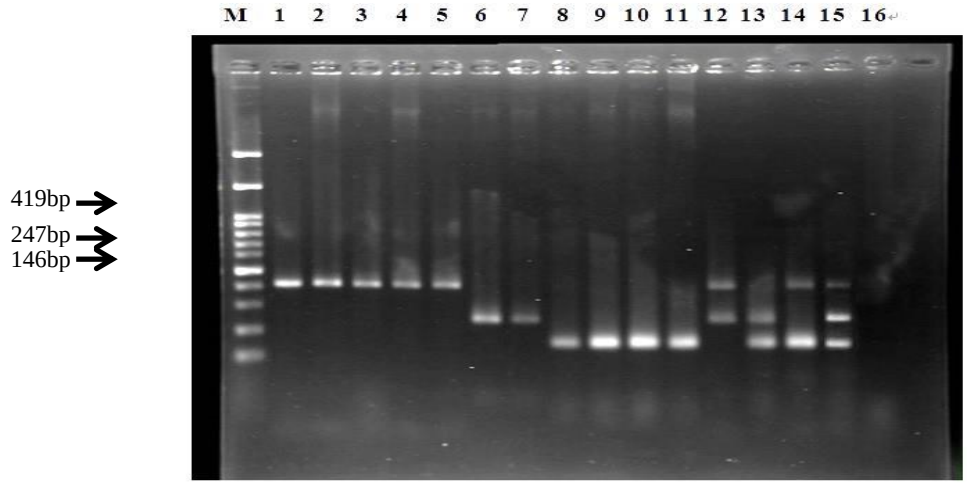
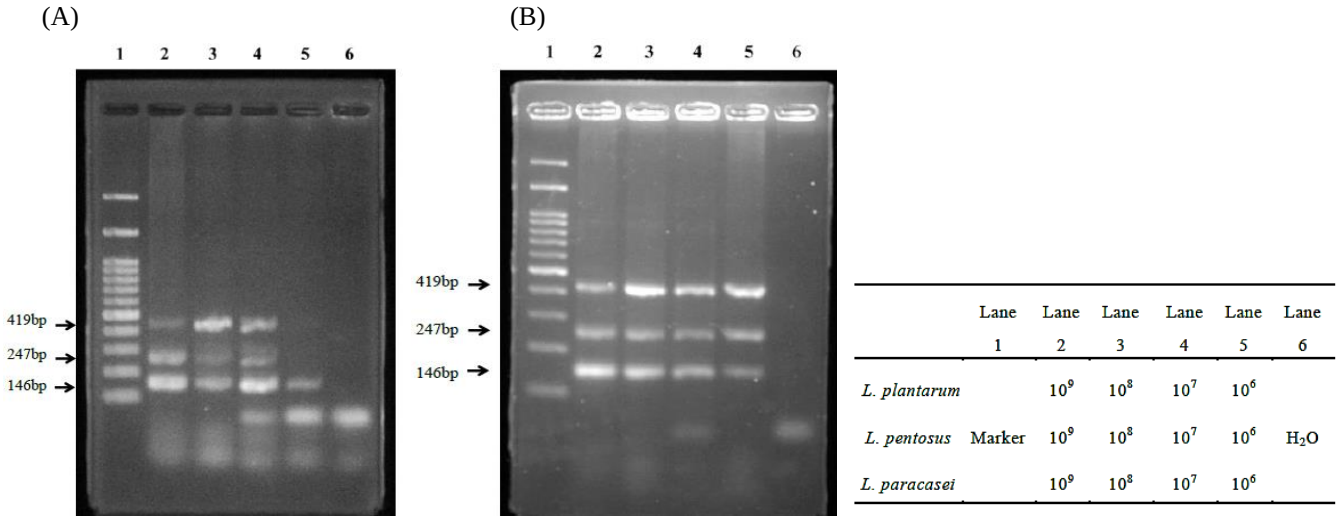


Figure 1. Polymerase chain reaction results of the three primers: verification and detection using multiplex Polymerase chain reaction. M, 100 bp ladder marker; Lanes 1–5, polymerase chain reaction products from five *L. plantarum* strains BCRC 10069 and 12251, F7-1, F7-4 and J1-3; Lanes 6 and 7, two *L. pentosus* strains BCRC 17972 and 17973; Lanes 8–11, four *L. paracasei* strains BCRC 12193, 12188, 12248 and 17002; Lane 12, *L. plantarum* F7-1 and *L. pentosus* BCRC 17973; Lane 13, *L. pentosus* BCRC 17973 and *L. paracasei* BCRC 12188; Lane 14, *L. plantarum* F7-1 and *L. paracasei* BCRC 12188; Lane 15, *L. plantarum* F7-1, *L. pentosus* BCRC 17973 and *L. paracasei* BCRC 12188; and Lane 16, blank control.

Gel electrophoresis results validated effectiveness and specificity of the multiplex PCR for identifying and discriminating various LAB strains. These findings demonstrated applicability of the developed primer sets and verified their suitability for use in multiplex PCR. The optimized annealing temperature ensured robust amplification without nonspecific amplification products.

3.2 Sensitivity assessment of the lactic acid bacterial strains using multiplex polymerase chain reaction

In Figure 2A, DNA extracted directly from the mixed cultures of three LAB strains (10^9, 10^8 and 10^7 cfu ml^{-1}) are shown. At a concentration of 10^6 cfu ml^{-1}, only *L. paracasei* (BCRC 12188) demonstrated amplifications in multiplex PCR, indicating that detection sensitivity of *L. plantarum* and *L. pentosus* was limited to 10^7 cfu ml^{-1}. Figure 2B shows mixed cultures of the three LAB strains at similar concentrations after preculture in MRS broth (37 °C, 24 h). Multiplex PCR detected all the three strains at a sensitivity of 10^6 cfu ml^{-1}, suggesting that *L. plantarum* and *L. pentosus* proliferated and could be detected. Figure 2 shows detection limits and proliferation capabilities of the three LAB strains using multiplex PCR. Results indicated that preculture of the mixed cultures in MRS broth improves detection sensitivity, enabling accurate identification of *L. plantarum* and *L. pentosus* strains at lower concentrations.



	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6
L. plantarum		10^9	10^8	10^7	10^6	
L. pentosus	Marker	10^9	10^8	10^7	10^6	H_2O
L. paracasei		10^9	10^8	10^7	10^6	

Figure 2. Sensitivity of three lactic acid bacteria using (A) direct detection and (B) preculture multiplex polymerase chain reactions.

3.3 Preculture and mixed various concentrations of lactic acid bacterial strains using multiplex polymerase chain reaction

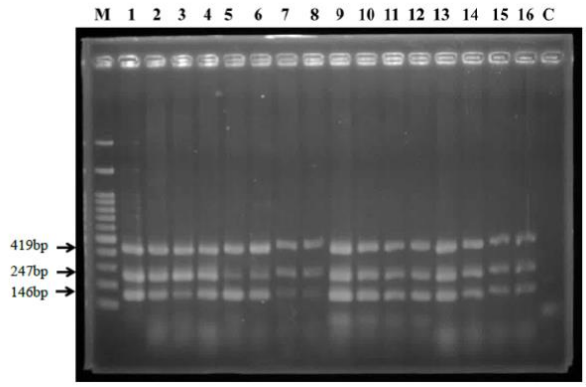
Figure 3A shows gel electrophoresis results of the multiplex PCR carried out on DNA samples extracted from a mixture of *L. plantarum* (10^9 cfu ml^{-1}) with two other LAB strains after preculture for 24 h. In Lanes 5–8, amplification products of *L. pentosus* and *L. paracasei* diluted to 10^6 cfu ml^{-1} were less expressed. The PCR amplification products of 10^6 cfu ml^{-1} DNA could be observed. The *L. pentosus* BCRC 17973 demonstrated a bacterial count of ~10^9 cfu ml^{-1} after 24 h of cultivation, whereas *L. paracasei* BCRC 12188 showed increased bacterial count, suggesting that preculture could enhance detection rate of low-concentration bacterial strains. Figure 3B shows gel electrophoresis results of the multiplex PCR on DNA extracted from a mixture of *L. plantarum* (10^8 cfu ml^{-1}) with two other LAB strains after preculture for 24 h. The PCR products in Lanes 1–16 indicated that all the three sets of species-specific primer pairs yielded the expected PCR products for various concentrations of the bacterial suspensions after 24 h of preculture. Figure 3 shows effectiveness of the multiplex PCR in detecting *L. plantarum* at various concentrations in a mixed culture with other LAB strains. Results highlighted effects of preculture on enhancing assay detection rate and sensitivity.

3.4 Polymerase chain reaction detection of the commercial probiotic product

Figure 4 demonstrates results of the multiplex PCR. Whether directly detected or precultured, the three LAB strains provided DNA amplification products from 10^7 to 10^9 cfu ml^{-1}. It was suggested that 10^7 cfu ml^{-1} was the detection limit of the product (not detected when diluted to 10^6 cfu ml^{-1}).

The PCR-based species identification is a critical highly valuable tool for detecting and identifying bacteria. It offers advantages, including time efficiency and reliability in microbial identification. Compared to traditional methods such as culture-based techniques, PCR can provide results in a relatively short time. It eliminates the need of time-consuming cultivation of bacteria, allowing for further rapid identification and subsequent decision-making processes. In industrial uses, species identification is critical in selecting and developing bacterial strains appropriate for specific purposes.

(A)

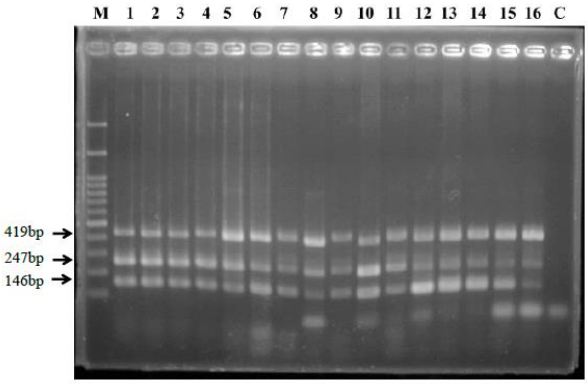


	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
L. plantarum	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9	10^9
L. pentosus	10^9	10^9	10^9	10^9	10^6	10^6	10^6	10^6	10^7	10^7	10^7	10^7	10^8	10^8	10^8	10^8
L. paracasei	10^9	10^8	10^7	10^6	10^9	10^8	10^7	10^6	10^9	10^8	10^7	10^6	10^9	10^8	10^7	10^6

Lane M : 100bp maker

C : blank control (dd H_2O)

(B)



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
L. plantarum	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8	10^8
L. pentosus	10^9	10^9	10^9	10^9	10^8	10^8	10^8	10^8	10^7	10^7	10^7	10^7	10^6	10^6	10^6	10^6
L. paracasei	10^9	10^8	10^7	10^6	10^9	10^8	10^7	10^6	10^9	10^8	10^7	10^6	10^9	10^8	10^7	10^6

Lane M: 100bp maker

C: blank control (dd H_2O)

Figure 3. Detection sensitivity of DNA extraction from various diluents of the three strains after preculture for 24 h using multiplex polymerase chain reaction.

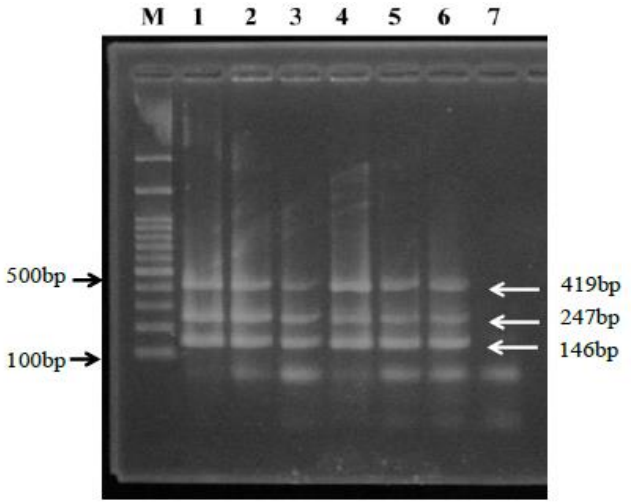


Figure 4. Multiplex polymerase chain reaction sensitivity for the detection of three various lactic acid bacteria in the probiotic product. Lane M, 100 bp marker; Lane 1, probiotic products of directly extract DNA 10^9 cfu mL^{-1}; Lane 2, probiotic products of directly extract DNA 10^8 cfu mL^{-1}; Lane 3, probiotic products of directly extract DNA 10^7 cfu mL^{-1}; Lane 4, probiotic products of preculture for 24 h, extraction DNA of 10^9 cfu ml^{-1}; Lane 5, probiotic products of preculture for 24 h, extraction DNA of 10^8 cfu ml^{-1}; Lane 6, probiotic products of preculture for 24 h, extraction DNA of 10^7 cfu ml^{-1}; and Lane 7, blank control (double-distilled H_2O).

Whether in food, agriculture or biotechnology industries, PCR-based species identification enables researchers to screen and select the most appropriate bacterial species for desired characteristics and functions.

The 16S rRNA gene in the ribosomal RNA of prokaryotes is the best molecular marker for bacterial evolutionary analysis. This is due to several gene characteristics, including its presence across various species, abundance, sufficient sequence length and presence of conserved and variable loci. The 16S rRNA gene is widely used in identifying lactobacilli and a commonly rapid technique for bacterial classification and identification in dairy products. Caro et al. reported that partial sequencing of 16S rRNA genes is often used for lactobacilli identification [15]. The RecA protein is a DNA recombinase that plays critical roles in DNA repair and recombination processes of bacteria. It is encoded by the *rec*A gene in the genomes of various prokaryotic microorganisms. The *rec*A gene and its corresponding protein have extensively been studied and used in various research, including evolutionary analysis, phylogenetic studies and identification of bacterial strains. Conserved nature and functional importance of the RecA protein make it a valuable molecular marker for understanding genetic relatedness and evolutionary relationships within bacteria. By comparing the *rec*A gene sequences of various bacterial strains, researchers can have insights into their genetic diversity, evolutionary history and phylogenetic classification.

Lu et al. developed a multiplex PCR method that could effectively be used in key vaginal microbiota evaluation in women with bacterial vaginosis [16]. You et al. used multiplex PCR to detect six species of *L. acidophilus* group [17]. Petri et al. used multiplex PCR for the rapid identification of wine-associated LAB [18]. Settanni et al. introduced a method and reported its state-of-the-art uses for microbial identification in foods and beverages [19]. Sciancalepore et al. described use of a simple, low-cost, rapid sensitive method based on droplet-based multiplex PCR directly on food matrices for the simultaneous detection of bacterial genes involved in biogenic amine synthesis [20]. Specific primers were designed based on similar sequences and multiplex PCR was optimized for the simultaneous identification of *L. plantarum*, *L. pentosus* and *L. paraplantarum* [21]. Sul et al. developed a multiplex PCR to detect *Lactobacillus* and *Bifidobacterium* spp. in commercial probiotic products [13].

Previously, Gram-negative broth enrichment was used in studies, followed by immunomagnetic separation multiplex PCR to enable the simultaneous detection of *Salmonella* spp. and enterohemorrhagic *Escherichia coli* in food samples, irrespective of their significant discrepancy in cell counts [22]. A PCR primer set derived from the sequence in 16S to 23S internal transcribed spacer (ITS) region was also developed for the specific detection of *B. adolescentis* in probiotics. This primer set included potentials for inspecting dairy food and environmental samples [14]. To ensure food safety during direct vat inoculation of Paocai, a propidium monoazide-based quantitative PCR method was developed to quantify *L. plantarum* NCU116 fermentation starter, as well as *Saccharomyces* spp. and potentially present pathogenic bacteria [23]. Plate counting was carried out and demonstrated similar results to quantitative PCR analysis, indicating appropriateness and effectiveness of absolute quantitative PCR for rapidly detecting microbial composition in the Paocai system [23]. In a recent study, surveillance of *Lactobacillus* bacteremia was carried out using biochemical and conventional-PCR assays. However, these methods could not provide target quantification and might lead to false-positive results [24]. To address this limitation, a *L. rhamnosus*-specific quantitative PCR assay was developed. This assay delivers accurate and reproducible results, leveraging specificity of a TaqMan probe, targeting unique 16S rDNA sequences of *L. rhamnosus* [24].

4. Conclusion

In summary, the three sets of PCR primer combinations, targeting various LAB strains, demonstrated specificity and generated the expected amplicons during PCR amplification. Use of multiplex PCR in LAB genomic detection showed potentials and was appropriate for detecting various species in food products. Multiplex PCR decreased

experimental cost and time, eliminating the need of time-consuming sequencing processes. Therefore, this method is expected to contribute to the reliability of probiotic labeling systems by facilitating strain identification. This molecular technique offers a valuable tool for quality control, product development and microbial monitoring of probiotic strains in various fields.

5. Conflict of Interest

The authors report no conflict of interest.

6. Authors Contributions

Conceptualization, TCC; methodology, LZY; data curation, LZY; writing, TCC.

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شناسایی سریع و کاربرد *لاکتوباسیلوس پلانتاروم، لاکتوباسیلوس پاراکازی و لاکتوباسیلوس پنتوسوس* با استفاده از واکنش زنجیره‌ای پلیمراز مالتی پلکس و پرایمرهای اختصاصی گونه، هدف قرار دادن ژن‌های S۱۶ ریبوزومی RNA و recA

چنگ-چیه تسای*، ژن یو-لای

گروه علوم و صنایع غذایی، دانشگاه هانگ کوانگ، ناحیه شالو، شهر تایچونگ ۴۳۳۰۲، تایوان

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چنگ-چیه تسای

گروه علوم و صنایع غذایی، دانشگاه هانگ کوانگ، شماره ۱۰۱۸، بخش ۶، بلوار تایوان، ناحیه شالو، شهر تایچونگ ۴۳۳۰۲، تایوان

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پست الکترونیک:
tsaicc@sunrise.hk.edu.tw

چکیده

سابقه و هدف: فرآورده‌های گوناگونی حاوی زیست‌یارهایی[1] مانند باکتری‌های لاکتیک اسید در بازار وجود دارند که به لحاظ داشتن فواید بسیار، ترویج می‌شوند. عملکرد این فرآورده‌ها به سویه‌های خاص، گونه‌های باکتریایی و تعداد سلول‌های زنده مربوط است. این مطالعه با هدف ارزیابی انطباق گونه‌های باکتریایی لاکتیک اسید و تعداد سلول‌های زنده در محصولات زیست‌یار تجاری موجود با برچسب‌گذاری آنها، حصول اطمینان از اثربخشی محصولات انجام شد.

مواد و روش ها: تکنیک واکنش زنجیره‌ای پلیمراز چندگانه با استفاده از آغازگرهای خاص برای تمایز موثر باکتری-های لاکتیک اسید در محصولات زیست‌یار به‌کار گرفته شد. بنابراین، هدف سویه‌های مورد استفاده در فرآورده‌ها بود و داده‌های مربوط به توالی نوکلئوتیدی برای انتخاب دو مجموعه از جفت آغازگر واکنش زنجیره‌ای پلیمراز L. pla-F/R و L.para-F/R، که ژن‌های RNA ریبوزومی S۱۶ ، و ژن های recA و L. pen-F/R که recA genes را هدف قرار می‌دهند، مورد بررسی قرار گرفت.

یافته‌ها و نتیجه‌گیری: مجموعه پرایمرهای جداگانه فرآورده‌های مورد نظر را تولید شد که با برچسب گذاری سویه های آزمایش شده *لاکتوباسیلوس پلانتاروم، لاکتوباسیلوس پاراکازی* و *لاکتوباسیلوس پنتوسوس* مطابقت داشت. سپس، ارزیابی ویژگی با استفاده از مجموعه پرایمرهای چندگانه برای سویه‌های منفرد، ترکیب‌های زوجی و ترکیب‌های سه‌گانه باکتری‌های لاکتیک اسید انجام شد. پس از تایید ویژگی برای هر سه سویه تحت شرایط واکنش زنجیره ای پلیمراز مشابه، حساسیت واکنش زنجیره‌ای پلیمراز مالتی پلکس با ارزیابی رقت‌های مختلف سه سویه باکتری لاکتیک اسید و محصولات زیست‌یار تجاری مورد بررسی قرار گرفت. این یافته ها کاربردهای بالقوه واکنش زنجیره‌ای پلیمراز مالتی پلکس را در روش‌های تشخیص باکتری لاکتیک اسید نشان داد. در نتیجه، مجموعه پرایمرهای خاص را می‌توان در واکنش زنجیره‌ای پلیمراز مالتی پلکس برای شناسایی سریع و موثر سویه‌های باکتری لاکتیک اسید در محصولات تجاری استفاده کرد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] probiotics