DEVELOPMENT OF THE REAL-TIME PCR METHOD FOR DETECTING *Lactobacillus acidophilus* IN SOME FUNCTIONAL FOODS MATRICES

Dang Thi Huong, Nguyen Thanh Trung[1] , Pham Nhu Trong, Tran Hong Ba, Le Thi Hong Hao
National Institute for Food Control

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Abstract

In the past decades, *Lactobacillus* species have been widely used in the market because they are thought to have biological properties and beneficial health effects. Conventional microbiological methods are often time consuming and labor-intensive. Real-time polymerase chain reaction (PCR) method will allow to identify and quantify rapidly *Lactobacilli* probiotics in food. In this study, the thermal shock protein target region (hsp60) was used to identify *Lactobacillus acidophilus* species. The limit of detection, specificity, and accuracy were of 10^4, 100 %, 100 %, respectively. This method is appropriate for application of detection of *L. acidophilus* bacteria in serveral food supplements.

Keywords: *Real-time PCR, Lactobacillus acidophilus*

1. INTRODUCTION

Lactobacillus acidophilus, a member of the group of lactic acid bacteria, is Gram-positive, rod-shaped, non-spore-forming, capable of aerobic and anaerobic fermentation, often present in the small intestine and helps balance the intestinal microflora. It is also considered a natural antibiotic against harmful microorganisms [5]. *Lactobacilli* strains with biological effects have been widely used in dairy products, such as yogurt as well as in food and pharmaceutical additives [2]. The variety of probiotic products available on the market is clear evidence of the health benefits of having those microbes in the body. Probiotics are defined as living micro organisms that bring health benefits to the host when administered in adequate amounts. For quality management reasons and in accordance with the European Health Requirements Regulation (EC 2007), a diagnostic tool is needed to identify and quantify beneficial bacterial strains in food. Currently, despite the economic impact, most of the tests being used to determine *lactobacilli* and employing conventional microbiological methods are often time-consuming, not easily standardized and sometimes error-prone. These methods include morphology, Gram staining and biochemical tests such as carbohydrate fermentation, grown at different temperatures and different salt concentrations. Morphological screening seems to be especially problematic for differentiation because lactobacilli are known to take different forms in the same species. Therefore, a quick and reliable identification tool is necessary, which uses genome characteristics to determine microorganisms at the species level: Realtime PCR. This method may allow rapid determination and quantification of *Lactobacilli* probiotics in food and can be applied for management and quality purposes. Real-time quantitative PCR represents a tool that has been established to specify and quantify species within a short time and is readily available in many laboratories [1]. In this study, the research group employed the heat shock protein target region (HSP60) to identify *Lactobacillus acidophilus* species. This protein region has been successfully used to access the identity of other bacteria.

[1] *Tel: 0349363269 Email: nguyenthanhtrung83@gmail.com*

2. MATERIALS AND METHODS

2.1. Research subject

Functional food products (nuggets, etc.)

2.2. Materials, chemicals, equipment

2.2.1. Materials

Reference strains: *Lactobacillus acidophilus* ATCC 4356, *Bifidobacterium longum V57*, *Bifidobacterium animalis V76*.

Lactobacillus phantarum, Lactobacillus paracasei, Lactobacillus casei V53, Lactobacillus rhamnosus V51, Lactobacillus acidophillus, Bifidobacterium lactic V59, Bifidobacterium longum, Bifidobacterium buve, Streptococcus facium, Lactococcus lactic, Bifidobacterium longum.

Sample isolation source: Varied food supplements.

2.2.2. Primary chemicals

TNES extraction buffer solution (Sigma); Proteinase K 20 mg/ml (Sigma), PowerUp SYBR Green master mix (Appliedbiosystems), ethanol, or Gene JET genomic DNA extraction kit from Thermo Fisher Scientific (code K0722), 100bp DNA ladder.

2.2.3. Equipment

Real-time PCR equipment CFX96 (Biorad), Nanodrop1000 (Thermo Scientific), Mikro 200 (Hettich) cold centrifuge, thermal blocks (Labnet), and other accessories.

2.3. Research methods

DNA extraction: the method employed highly concentrated salt (high salt) or Gene JET kit. After homogenizing the samples, protease K was added to 50 - 100 mg of samples and incubated at 55^{o}C within five hours. DNA was collected using 100% ethanol.

The solution after OD extraction from 1.8 to 2.0 was used for real-time PCR analysis.

Real-time PCR for amplification of the target gene: The pair of primers in use is presented in Table 1.

Table 1. *Sequence species-specific primers based on hsp60 gene*

Species	***Primer***	***Size of amplicon (bp)***	***Melting temp (^{o}C)***	***Target***	***Reference***
L. acidophilus	LAcidoF: 5'–CTT TGA CTC AGG CAA TTG CTC GTG AAG GTA TG – 3' LAcidoR: 5'–CAA CTT CTT TAG ATGCTGVAAG AA CAG CAG CTA CG – 3'.	191 bp.	80.5^{o}C ± 0.5 ^{o}C	*hsp60*	Herbel SR1 and colleagues [1]

Table 2. *Amplification reaction mixture in the final volume/ concentration per reaction vial*

PowerUp SYBR Green master mix, 2X	10 µl
Sample DNA	1 µl
Forward primer	1 µl
Reverse primer	1 µl
Water added	20 µl

Table 3. *Procedure: Reaction conditions*

		Time (s)	Temperature (^oC)
Pre-PCR	Decontamination (optional)	120	50^{o}C
Pre-PCR: activation of DNA polymerase and denaturation of template DNA		120	95^{o}C
PCR (40 cycles)			
Step1	Denaturation	15	95^{o}C
Step 2	Annealing and extension	60	60^{o}C

Method validation [3, 4]: Validation of limit of detection (LOD), accuracy (AC), specificity (SP), sensitivity (SE) can be specified with the following formular:

$$AC = \frac{TP + TN}{N} \times 100 \qquad SE = \frac{TP}{TP + FN} \times 100$$

$$SP = \frac{TN}{TN + FP} \times 100$$

Where:

AC: Accuracy; SE: Sensitivity; SP: Specificity; TP: True positive; TN: True negative; FP: False positive; FN: False negative; N: total number of samples analyzed

3. RESULTS AND DISCUSSIONS

3.1. DNA extraction

Reference strains and microbiological samples were extracted by Gene JET DNA extraction kit, DNA products were tested for concentration on Nanodrop. The content was from 2 to 16 ng/µl, and high concentration of salt was from 200 - 500 ng/µl, purity was in the range of 1.8 to 2.1 ng/µl, the content and purity were ensured to be the template for real-time PCR reaction.

3.2. Limit of Detection (LOD)

LOD of the method is the lowest concentration of micro-organisms in the sample, where at least 90% of the samples is positive. To test the LOD, the sample was decimally diluted from the oncentration of 10^{-1} to 10^{-7} CFU/mL, each dilution was duplicated, the result was shown in Figure 1.

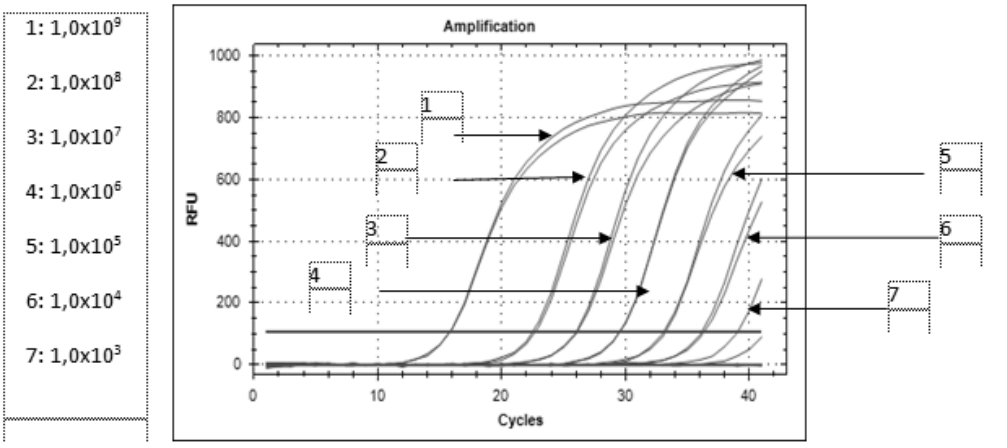


Figure 1. *Method detection limit survey result*

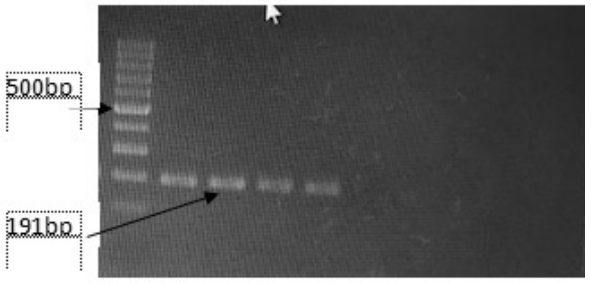
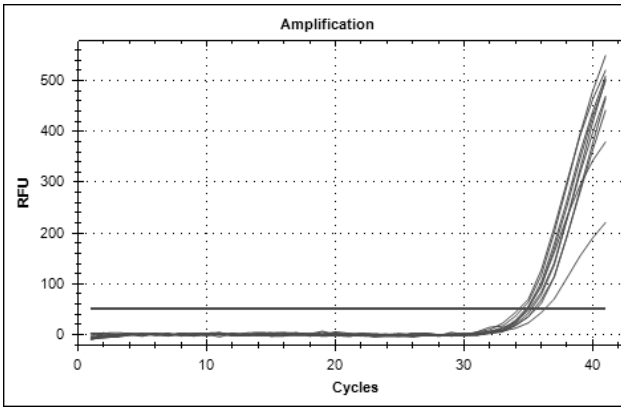


Figure 2. *Electrophoresis image at 10^5, 10^4, 10^3, from left to right respectively*

Comment: The amplification result showed that microbiological concentration of 10^3 at one time gave negative value and at another time, the value of Ct = 39.06 (40 cycles) and the electrophoresis image did not contain any band of 191bp. Hence, the highest probability of positive result when repeating 10 times will be 80% (< 90%). The survey at the microbial concentration of 10^4 was additional conducted. The results obtained were shown in Figures 3, 4.



Well	Fluor	Target	Content	Sample	Cq
C01	SYBR		Unkn	10^4	35.0
C02	SYBR		Unkn	10^4	35.5
C03	SYBR		Unkn	10^4	34.5
C04	SYBR		Unkn	10^4	34.8
C05	SYBR		Unkn	10^4	35.6
C06	SYBR		Unkn	10^4	34.3
C07	SYBR		Unkn	10^4	35.2
C08	SYBR		Unkn	10^4	35.1
D01	SYBR		Unkn	10^4	36.3
D02	SYBR		Unkn	10^4	34.9

Figure 3. *Amplified image and threshold value (Ct) of L. acidophilus for 10 times at microbial concentration of 10^4*

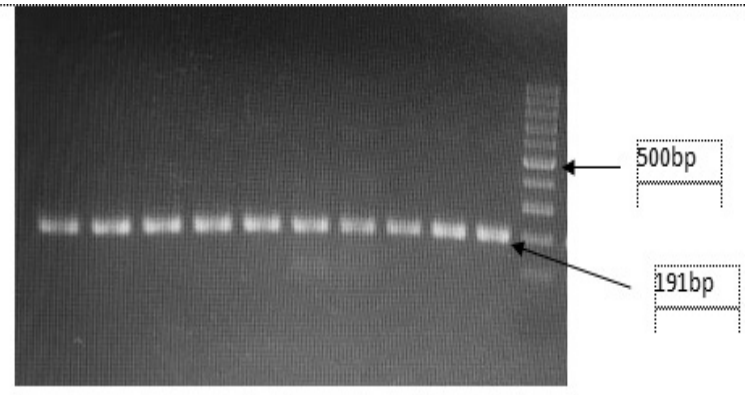


Figure 4. *Electrophoresis result DNA size of L.acidophilus bacteria (191bp)*

From the above results, the probability of positive result at the concentration of 10^4 CFU/mL microorganisms and was of 100%. Therefore, the detection limit of the method (LOD) was 10^4 CFU/mL.

3.3. Accuracy (AC), specificity (SP) and sensitivity (SE)

Prepare three sample groups: Lactobacillus acidophilus strain, mixture of 10 microorganisms (mix 10 bags including: *Lactobacillus phantarum, Lactobacillus paracasei, Lactobacillus casei, Lactobacillus. rhamnosus, Lactobacillus acidophillus, Bifidobacterium lactic, Bifidobacterium longum, Bifidobacterium buve, Streptococcus facium, Lactococcus lactic, Bifidobacterium longum)*.

The analysis was repeated twice, the results were displayed in Figure 5.

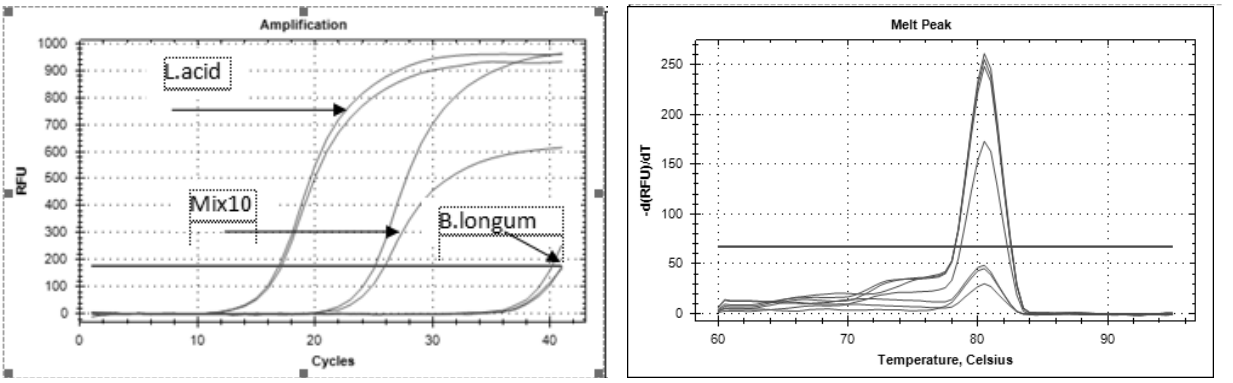


Figure 5. *Sample analysis results of AC, SE, SP*

According to the results in Figure 5, accuracy (AC), specificity (SP), sensitivity (SE) were all 100%, LOD was also consistent with Herbel et al's research [7].

3.4. Analysis of actual samples

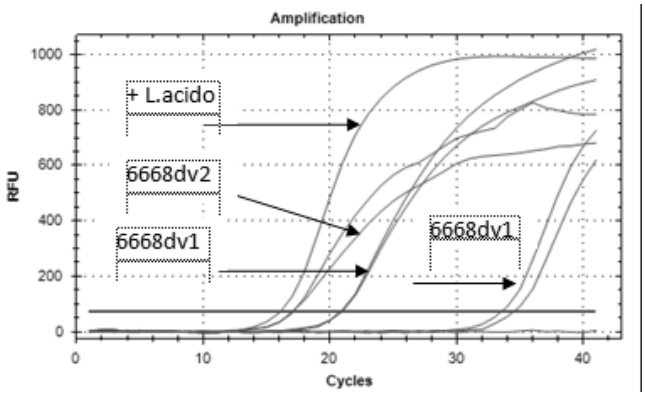
The method was applied to identify some of the commercial functional food products from the market including:

- Probiotics: Lactomin plus (code 6668dv1): including on three bacteria species *Lactobacillus acidophilus, Lactobacillus plantarum, Bifidobaterium animalis subsp. lactis*

- Probiotics: Lactomin (code 6668dv2): including on three bacteria species *Lactobacillus acidophilus, Lactobacillus plantarum, Bifidobaterium animalis subsp. lactis*

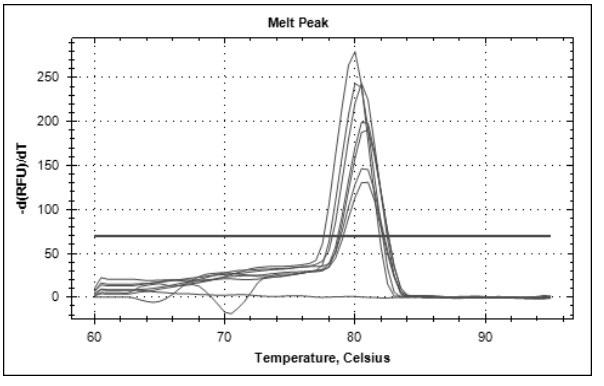
- Gabulin probiotics tablet (code 6735dv4): including on two bacteria species: *Bacilus subtilis, Lactobacillus acidophilus.*

Results of the analysis were given in Figures 6 and 7.



Well	Fluor	Target	Content	Sample	Cq
B01	**SYBR**		**NTC**		**N/A**
B02	SYBR		Unkn	L.a	16.12
B03	SYBR		Unkn	6668dv1	20.85
B04	SYBR		Unkn	6668dv1	21.02
B05	SYBR		Unkn	6668dv2	17.16
B06	SYBR		Unkn	6668dv2	17.07
B07	SYBR		Unkn	6735dv4	34.55
B08	SYBR		Unkn	6735dv4	33.57

Figure 6. *Amplification results and Ct threshold of samples*



Well	Fluor	Target	Content	Sample	Melt Temp
B01	SYBR		NTC		None
B02	SYBR		Unkn	L.a	80.50
B03	SYBR		Unkn	6668dv1	80.00
B04	SYBR		Unkn	6668dv1	80.00
B05	SYBR		Unkn	6668dv2	80.50
B06	SYBR		Unkn	6668dv2	81.00
B07	SYBR		Unkn	6735dv4	81.00
B08	SYBR		Unkn	6735dv4	80.50

Figure 7. *Melt curve peak chart and melting cuver temperatre on samples*

The results showed that all three samples supplemented with *Lactobacillus acidophilus* DNA were amplified and melted within the permitted range (80.5 ± 0.5).

4. CONCLUSIONS

The study has successfully developed a real-time PCR method to detect *L. acidophilus* bacteria in several functional food products with limit of detection of 104 CFU/mL, sensitivity of 100%, specificity of 100%, and accuracy of 100%. This is a suitable method for detection of *L. acidophilus* bacteria in functional food products.

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Tóm tắt

XÂY DỰNG PHƯƠNG PHÁP PHÁT HIỆN *LACTOBACILLUS ACIDOPHILUS* BẰNG REAL-TIME PCR TRONG MỘT SỐ NỀN THỰC PHẨM CHỨC NĂNG

Đặng Thị Hường, Nguyễn Thành Trung, Phạm Như Trọng, Trần Hồng Ba, Lê Thị Hồng Hảo
Viện Kiểm nghiệm An toàn Vệ sinh thực phẩm Quốc gia

Trong những thập kỷ qua, các loài *Lactobacillus* đã được sử dụng rộng rãi trên thị trường vì chúng được cho là có đặc tính sinh học và có tác dụng tốt cho sức khỏe. Các phương pháp vi sinh cổ điển, thường tốn thời gian, tốn nhiều công sức, cho kết quả chậm. Phương pháp phản ứng chuỗi polymerase thời gian thực (PCR) cho phép nhanh chóng xác định và định lượng men vi sinh *Lactobacilli* trong thực phẩm. Trong nghiên cứu hiện tại, chúng tôi đang sử dụng vùng protein sốc nhiệt đích (hsp60) để xác định loài *Lactobacillus acidophilus*. Giới hạn phát hiện, độ đặc hiệu, độ chính xác lần lượt là 104, 100%, 100%. Phương pháp thích hợp để áp dụng phát hiện vi khuẩn *L. acidophilus* trong một số thực phẩm bổ sung.

Từ khóa: *Real-time PCR, Lactobacillus acidophilus*