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Original Article

Designing primers and evaluation of the efficiency of propidium monoazide – Quantitative polymerase chain reaction for counting the viable cells of *Lactobacillus gasseri* and *Lactobacillus salivarius*

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ARTICLE INFO

Article history:
Received 5 July 2016
Received in revised form
21 October 2016
Accepted 30 October 2016
Available online 29 November 2016

Keywords:
Lactobacillus gasseri
Lactobacillus salivarius
propidium monoazide
quantitative polymerase chain reaction
viable and heat-killed cells

ABSTRACT

The purpose of this study is to evaluate the efficiency of using propidium monoazide (PMA) real-time quantitative polymerase chain reaction (qPCR) to count the viable cells of *Lactobacillus gasseri* and *Lactobacillus salivarius* in probiotic products. Based on the internal transcription spacer and 23S rRNA genes, two primer sets specific for these two *Lactobacillus* species were designed. For a probiotic product, the total deMan Rogosa Sharpe plate count was 8.65 ± 0.69 log CFU/g, while for qPCR, the cell counts of *L. gasseri* and *L. salivarius* were 8.39 ± 0.14 log CFU/g and 8.57 ± 0.24 log CFU/g, respectively. Under the same conditions, for its heat-killed product, qPCR counts for *L. gasseri* and *L. salivarius* were 6.70 ± 0.16 log cells/g and 7.67 ± 0.20 log cells/g, while PMA-qPCR counts were 5.33 ± 0.18 log cells/g and 5.05 ± 0.23 log cells/g, respectively. For cell dilutions with a viable cell count of 8.5 log CFU/mL for *L. gasseri* and *L. salivarius*, after heat killing, the PMA-qPCR count for both *Lactobacillus* species was near 5.5 log cells/mL. When the PMA-qPCR counts of these cell dilutions were compared before and after heat killing, although some DNA might be lost during the heat killing, significant qPCR signals from dead cells, i.e., about 4–5 log cells/mL, could not be reduced by PMA treatment. Increasing PMA concentrations from 100 μM to 200 μM or light exposure time from 5 minutes to 15 minutes had no or, if any, only minor effect on the reduction of qPCR signals from their dead cells. Thus, to differentiate viable lactic acid bacterial cells from dead cells using the PMA-qPCR method, the efficiency of PMA to reduce the qPCR signals from dead cells should be notable.

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http://dx.doi.org/10.1016/j.jfda.2016.10.004

1. Introduction

Probiotics including lactic acid bacteria (LAB), such as *Lactobacillus* spp. and *Bifidobacterium* spp., are living microorganisms that, upon ingestion, exert health benefits on human and animals. Owing to the increasing use of lactobacilli in probiotic products and feed supplements, the manufacturers should declare the right LAB species and viable cell counts for total or each of the LAB strains in the product so that the consumers' rights and interests could be protected. Thus, correct identification and quantification of viable cells for each LAB strains in probiotic products are important. According to Coeuret et al [1], for human nutritional supplements, in general, viable cell counts of specific LAB strains are 8–9 log CFU/g. Since most of the probiotic products may contain two or more LAB species, rapid methods that allow simultaneous identification and quantification of viable cells of different LAB species are required.

Lactobacillus gasseri is one of the common species of the human gut flora [2]. Strains of this species have been found to have wide inhibitory activity against pathogenic and food-spoilage bacteria [3,4]. Reports regarding its anti-inflammatory properties and expression of superoxide dismutase using a mouse model [5], regulatory effect on gut environment and intestinal functionality [6], reduction of blood glucose levels and body weight in a mouse model of type 2 diabetes [7], as well as the protective effect against gastric ulcers [8] have been revealed. As for *Lactobacillus salivarius*, its influence on the incidences of dental health in children [9], and production of bacteriocins to inhibit the pathogens and influence the host immune system [10] have been reported. Recently a probiotic product, containing *L. salivarius* and *L. gasseri* and claiming to have antiallergic function for humans, has been commercialized in the market.

For the quantification of viable cells, recently, the use of selective nucleic acid intercalating dyes, such as propidium monoazide (PMA), has been suggested as a method to reduce polymerase chain reaction (PCR) signals from DNA in dead cells. The approach is based on the difference of membrane integrity between viable and nonviable cells [11]. Ideally, PMA should only penetrate into membrane-compromised dead cells and intercalate with double-strand DNA in the cells. This method has been used to differentiate viable cells from dead cells for different bacterial species, such as *Escherichia coli* and *Campylobacter* [12,13]. For LAB, it has been used for the quantification of viable cells (>10^5 CFU/g) in spray-dried probiotic lactobacilli [14], and differentiation of viable as well as heat-killed cells of specific strains of *Bifidobacterium breve* and *Bifidobacterium bifidum* cells (10^{10} cells/mL) added in human feces [15,16]. In this study, based on the 16S and internal transcription spacer (ITS)–23S rRNA sequences, we designed PCR primers specific for *L. gasseri* and *L. salivarius*, and attempted to use these primers for simultaneous identification and quantification of viable LAB cells in the probiotic product. Meanwhile, the possible loss of DNA during heat killing of these two LAB species cells and the efficiency of PMA treatment in the reduction of quantitative polymerase chain reaction (qPCR) signals from heat-killed cells of these two *Lactobacillus* species were evaluated.

2. Materials and methods

2.1. Bacterial strains and heat-killed cells

Strains used in this study include strains of *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp., *Bacillus* spp., and other bacterial species, such as those of the family of Enterobacteriaceae (Table 1). These strains were obtained from Bioresources Collection and Research Center (BCRC; Hsin-Chu, Taiwan) and American Type Culture Collection (Manassas, VA, USA). All LAB strains were maintained at −80°C as 25% glycerol stocks. Strains of *Lactobacillus* spp. were grown in deMan Rogosa Sharpe (MRS) broth (Merck, Darmstadt, Germany), while strains of *Bifidobacterium* spp. were grown in MRS broth plus 0.05% L-cystine under anaerobic conditions at 37°C. By contrast, strains of *Enterococcus* spp. were grown in Brain Heart Infusion broth (Difco, Detroit, MI, USA) at 37°C. For all these LAB strains, the culture time was 16–20 hours. For bacteria other than the LAB strains, Luria broth (yeast extract 5 g, tryptone 10 g, NaCl 5 g, and sterile H_2O to 1000 mL) was used and the culture time was 20–24 hours at 37°C.

For probiotic samples, a market-available probiotic product with antiallergic activity (Kan-Min 2) manufactured by Promed Biotech (Tainan, Taiwan) was used. This product was labeled with only two LAB species, i.e., *L. gasseri* and *L. salivarius*, but not with their viable cell counts. It was in the form of capsule (0.6 g/capsule). To prepare the heat-killed product, 1 g/mL of the sample in phosphate-buffered saline (PBS) was mixed with 9 mL PBS, while for reference strains, *L. gasseri* (BCRC 14619) and *L. salivarius* (BCRC 12574) in PBS, with different cell counts, were heated at 100°C for 30 minutes and then cooled at 4°C for 10 minutes, followed by centrifugation at 6000 *g* (Eppendorf Cat. No. 5424) for 10 minutes to collect the cells and supernatant.

2.2. Preparation of genomic DNA from bacterial strains

For LAB cells, total chromosomal DNA was prepared from the overnight culture as described earlier using the Blood & Tissue Genomic DNA Extraction Miniprep System according to the bacterial protocol in the instruction sheet from the manufacturer (Viogene Laboratories, Taipei, Taiwan). Briefly, cells collected from 500 μL of the overnight culture were washed with 1× PBS. After spinning down the cells, the cell pellet was mixed with 200 μL lysozyme (20 mM Tris–HCl, pH 8.0, 2 mM EDTA, 20 mg/mL lysozyme). After incubation at 37°C for 60 minutes, 20 μL proteinase K (10 μg/mL) and 200 μL EX buffer were mixed, followed by incubation at 60°C for 60 minutes until the solution became clear. After incubation at 37°C for 30 minutes, total DNA was prepared by mixing the solution with 400 μL absolute ethanol followed by separation of DNA with B/T Genomic DNA minicolumn according to the manufacturer's manual. These DNA samples were then stored at −20°C.

For bacterial species other than LAB, total DNA was prepared from 100 μL of the overnight cell culture after 10-fold dilution with sterile water. The bacterial suspension was boiled (100°C for 30 minutes) to decompose the cells, followed by cooling (−20°C for 10 minutes). After centrifugation at