International Dairy Journal 61 (2016) 18–21

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Short communication

Development of rapid and highly specific TaqMan probe-based real-time PCR assay for the identification and enumeration of *Lactobacillus kefiri* in kefir milk

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ARTICLE INFO

Article history:
Received 29 October 2015
Received in revised form
16 March 2016
Accepted 16 March 2016
Available online 9 April 2016

ABSTRACT

Lactobacillus kefiri is one of the key functional lactic acid bacteria in kefir milk. We designed a novel real-time PCR primer/probe set, LK_508 targeting the *rec*A gene, for the rapid identification and enumeration of *L. kefiri*. In inclusivity and exclusivity test using standard strains and kefir isolates, only the 9 tested *L. kefiri* strains were positive, with the remaining 38 closely related microorganisms testing negative, thus indicating 100% sensitivity and specificity of the assay. The population of *L. kefiri* was 3.77, 4.30, 4.79, and 5.63 log cfu mL^{-1} of kefir milk fermented at 25 °C with 5% grain-milk ratio for 12, 24, 36, and 48 h, respectively. The newly developed qPCR assay could be applied to investigate the quantitative relationship of kefir microbiota in fermentation process.

1. Introduction

Kefir is a natural, functional dairy product that has important beneficial health effects when consumed regularly (Guzel-Seydim, Kok-Tas, & Greene, 2011). The unique culture process combines both lactic acid and alcoholic fermentation of lactose in milk. Kefir is produced by the microbial activity of 'kefir grains', which contain a mixture of complex microbiota, including lactic acid bacteria, yeast, and acetic acid bacteria (Farnworth & Mainville, 2003).

Lactobacillus kefiri is one of the emerging probiotic species found in kefir milk (Kandler & Kunath, 1983). Recently, many beneficial effects of *L. kefiri* have been demonstrated, including antimicrobial activity, toxin neutralisation, inhibition of adhesion of foodborne pathogens to gut epithelium, prevention of hypercholesterolaemia, modulation of immune response, and production of exopolysaccharides (Carasi et al., 2014, 2015; Carasi, Trejo, Pérez, De Antoni, & Serradell, 2012; Golowczyc, Mobili, Garrote, Abraham, & De Antoni, 2007; Zheng et al., 2013).

Tools for the rapid species-specific quantification of the representative kefir microorganisms such as *L. kefiri* are now required to further investigate the microbial relationships in kefir fermentation and to optimise fermentation conditions to maximise the population of this bacterium (Garrote et al., 2005). As the Codex standard for fermented milks (CODEX STAN 243-2003) defines *L. kefiri* as a key lactic acid bacterium of kefir starter culture, it is important to identify and enumerate this bacterium in kefir products (www.codexalimentarius.net). However, conventional culture methods are time-consuming, and the differentiation and enumeration of individual species in samples such as kefir that contain mixed microbiota is difficult (Lee et al., 2015). To overcome these limitations, we designed a real-time PCR method specific for *L. kefiri* for the identification and quantification of the microorganisms in kefir milk to provide basic information on kefir microbiota during fermentation process.

2. Material and methods

2.1. Primer design

The primer/probe set targeting the *rec*A gene was designed as described previously (Chon et al., 2012; Kim et al., 2015; Seo, Valentin-Bon, Brackett, & Holt, 2004). The sequence of the gene was provided by GenBank (www.ncbi.nlm.nih.gov/Genbank/; Accession Number: AJ621650.1). Sequences unique to *L. kefiri* were

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http://dx.doi.org/10.1016/j.idairyj.2016.03.007
0958-6946/

compared with those of closely related strains, and potential target sites for specific detection were identified (Fig. 1). One primer/probe set was designed using Primer Express® Software v3.0 (Applied Biosystems, Foster City, CA, USA). The set was validated using NCBI BLAST (Basic Local Alignment Search Tool; www.ncbi.nlm.nih.gov/blast/). The sequences were as follows: LK_508F (forward primer): 5'- GGGAGATGCCCATGTTGGT-3'; LK_508R (reverse primer): 5'- AAGCTTTCGAAGTGCCTGTGA-3'; LK_508P (probe): 5'-FAM-TGCAAGCACGACTGAT-3'-MGB-NFQ. The amplicon size was 58 bases. The oligonucleotides were synthesised and purchased from Applied Biosystems.

2.2. Inclusivity and exclusivity test using standard strains

For inclusivity and exclusivity tests of the designed sequences, real-time PCR was performed in 26 standard strains. *Lactobacillus acidophilus* ATCC4357, *Lactobacillus brevis* ATCC8287, *Lactobacillus crispatus* KCTC3174, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC11842, *Lactobacillus gasseri* KCTC3163, *Lactobacillus helveticus* KCTC3545, *Lactobacillus jensenii* KCTC5194, *Lactobacillus johnsonii* JCM1022, *Lactobacillus kefiranofaciens* subsp. *kefiranofaciens* ATCC43761, *L. kefiranofaciens* subsp. *kefirgranum* DSM10550, *Lactobacillus kefiri* ATCC35411, *Lactobacillus buchneri* ATCC9460, *Lactobacillus casei* ATCC393, *Lactobacillus parabuchneri* ATCC11307, *L. parabuchneri* ATCC49374, *Lactobacillus paracasei* KCTC13169, *Lactobacillus parakefiri* KCTC5087, *L. parakefiri* KCTC5044, and *Lactobacillus fermentum* ATCC11739 were grown on De Man, Rogasa, and Sharpe agar (MRS; Oxoid, Basingstoke, Hampshire, UK) at 30 °C for 48–72 h, anaerobically. *Leuconostoc mesenteroides* KCTC13302 and *Streptococcus salivarius* subsp. *thermophilus* KCTC5091 were grown on Rogosa agar (Oxoid) at 30 °C for 48–72 h, anaerobically. *Bifidobacterium adolescents* ATCC15703 and *Bifidobacterium longum* BL-720 were grown on BL agar (Difco Laboratories, Detroit, MI, USA) at 37 °C for 48 h, anaerobically. *Acetobacter aceti* ATCC23746, *Enterococcus faecalis* ATCC19433, and *Escherichia coli* ATCC25922 were grown on tryptic soy agar (Difco Laboratories) at 37 °C for 24–48 h. After subculture twice, each microorganism was subjected to DNA extraction.

2.3. Isolation of kefir microorganisms

For the isolation of lactic acid bacteria in kefir milk, a loopful of kefir milk was streaked on MRS and rogosa agar, and incubated at 30 °C for 72 h, anaerobically. Colonies were subject to catalase test using 3% hydrogen peroxide (Sigma, St. Louis, MO, USA; 4 mg L^{-1}) and catalase-negatives were regarded as lactic acid bacteria. After subculture 2 times on MRS agar at 30 °C for 72 h, anaerobically, colonies were subjected to 16S ribosomal RNA (rRNA) sequencing.

2.4. DNA extraction

Genomic DNA extraction was performed using the NucliSENS easyMAG instrument (Biomérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. Briefly, each colony was added to 1 mL of lysis buffer, and the mixture was incubated for 10 min at room temperature. The lysed sample was then transferred to the well of a plastic vessel with 50 μL of magnetic silica and subjected to automatic magnetic separation. DNA was extracted in 50 μL of elution buffer.

2.5. Sequencing

DNA was extracted from the isolates grown on MRS agar and the partial sequence of 16S rRNA of lactic acid bacteria was amplified and sequenced using the primer pair 27F/1492R (Lane, 1991). PCR products were sequenced using the above-mentioned PCR primers and ABI BigDye v3.1 terminator sequencing chemistry according to the manufacturer's instructions of a BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, CA, USA) in an Applied Biosystems 3730xl DNA Analyzers at Cosmo Genetech, Korea. A BLAST search (http://www.ncbi.nlm.nih.gov.blast) was used to identify the lactic acid bacteria isolated from kefir milk. The sequences of each isolate were searched in the GenBank DNA database (www.ncbi.nlm.nih.gov/Genbank/) by using the BLAST system. The identities of the isolates were determined on the basis of highest score.

2.6. Real-time PCR

The extracted DNA fluids (5 μL) were transferred into 20 μL of PCR mix, consisting of TaqMan® Universal PCR Master Mix (Applied Biosystems, 12.5 μL), LK_508F (2.5 μL, 300 nM), LK_508R (2.5 μL, 900 nM), and LK_508P (2.5 μL, 250 nM). The 96-microwell plate was sealed with optical adhesive covers (BioRad Laboratories, Hercules, CA, USA) and placed in a real-time PCR system (CFX96, BioRad). The reaction was run at 50 °C for 2 min and then 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

2.7. Standard curve

L. kefiri ATCC35411 was used as the standard strain for the LK_508 primer/probe set. *L. kefiri* was grown on MRS agar anaerobically at 30 °C for 72 h. Typical colonies were suspended, serially diluted in phosphate buffered saline (Amnesco, Solon, OH, USA), and plated on MRS agar for enumeration of bacteria. The genomic DNA of *L. kefiri* ATCC35411 was extracted simultaneously as described in 2.3. Standard curves were generated using CFX manager software v3.1 (BioRad).

2.8. Quantification of lactic acid bacteria, yeast, and L. kefiri in kefir milk

Viable kefir grains were obtained from a private household in Korea. A total of 50 g of kefir grains were inoculated in 1000 mL sterilised milk (Seoul Milk Co. Ltd., Seoul, Korea; 5% w/v) and cultured at 25 °C for 48 h. The pH and number of lactic acid bacteria, yeast, and *L. kefiri* was measured every 12 h. Lactic acid bacteria and yeast in kefir were enumerated using MRS agar at 30 °C for 48–72 h in anaerobic condition and potato dextrose agar (Oxoid) at 25 °C for 48–72 h, respectively. For the quantification of *L. kefiri*, 1 mL of kefir milk was centrifuged at 15,770 × *g* for 3 min and the pellet was used for DNA extraction. Real-time PCR was performed on extracted DNA, and the CFX manager software v3.1 was used for quantification of *L. kefiri* using the standard curve described in 2.7. The experimental procedures were repeated three times.

2.9. Data analysis

Excel 2010 (Microsoft, Redmond, WA) was used for data analysis. Colony counts in food samples were converted to $\log_{10}$ cfu mL^{-1}.

3. Results

3.1. Inclusivity and exclusivity of newly developed real-time PCR

With the exception of *L. kefiri*, there were no positive reactions for this primer set with any unrelated microorganisms. The assay achieved 100% sensitivity and specificity for the detection of *L. kefiri* at the species level.