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Development of a rapid and reliable TaqMan probe-based real-time PCR assay for the detection and enumeration of the multifaceted yeast *Kluyveromyces marxianus* in dairy products

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ABSTRACT

Kluyveromyces marxianus is a multifaceted yeast used in the dairy, biotechnological, and probiotic industries, and has been implicated in the spoilage of dairy products. Currently, *K. marxianus* detection involves laborious and time-consuming culture methods. To develop a rapid and reliable detection method for *K. marxianus* in pure cultures and dairy products, we designed a novel real-time PCR primer/probe set, KM55, which targets the internal transcribed spacer (ITS) rRNA gene. In the inclusivity and exclusivity tests, 11 *K. marxianus* strains tested positive, while 28 closely related microorganisms tested negative; this confirmed 100% sensitivity and specificity of the assay. Further, the KM55 set enabled a linear *K. marxianus* detection in the range of 10^0–10^5 colony-forming units (CFU) per reaction in phosphate-buffered saline, 10% skim milk, and commercial yogurts, with no cross-reactivity with microorganisms of kefir origin. The enumeration of *K. marxianus* in kefir revealed 5.1 to 6.9 logCFU ml^{-1} of kefir, depending on the fermentation time, temperature, and grain-milk ratio. *K. marxianus* was successfully detected in artificially contaminated yogurt samples within 2 h. These results suggest that this method could be an effective and sensitive presumptive screening tool for detecting and enumerating *K. marxianus* in dairy products.

1. Introduction

Kluyveromyces marxianus is a homothallic, hemiascomycetous, lactose-fermenting yeast (Lane & Morrissey, 2010) that has been conventionally recognized as *Candida kefyr* (more formerly known as *Candida pseudotropicalis*); however, *C. kefyr* has been recently reported to be an asexual (anamorph) form of *K. marxianus* (teleomorph, the sexual form) since a comparison of their nucleotide sequences suggests that they are the same microorganism (Dufresne et al., 2014; Underhill & Iliev, 2014). This yeast is a type species of the genus *Kluyveromyces* and is phylogenetically related to *Saccharomyces cerevisiae*, the brewer's and baker's yeast (Lane & Morrissey, 2010). Interestingly, several studies recognize *K. marxianus* as an emerging probiotic, fermentation starter, and a bioreactor, as well as a spoilage yeast, thus setting its status as a multifaceted yeast (Kim et al., 2015a; Papon, Courdavault, Clastre, & Bennett, 2013; Wu et al., 2016; Xie et al., 2015).

Upon gaining the Generally Regarded as Safe (GRAS) status in the United States and Qualified Presumption of Safety (QPS) status in the United Kingdom, *K. marxianus* has been used as a key fermentation starter in the production of traditional dairy fermented beverages, such as kefir and koumiss, because of its unique ability to ferment lactose as well as glucose (Fasoli, Barrio, Tofalo, Suzzi, & Belloch, 2016; Lane & Morrissey, 2010; Maccaferri, Klinder, Brigidi, Cavina, & Costabile, 2012; www.codexalimentarius.net). It is noteworthy that the acquisition and incorporation of *K. marxianus* into the kefir grain enhance the microbial activity of lactic acid bacteria and yeast in kefir and improve its flavor, taste, and texture (Gethins et al., 2016). *K. marxianus* has also been found to possess many probiotic attributes, including the ability to survive in the gastric and intestinal environments, growth promotion of the broiler chicken, adhesion to the gut epithelium, cholesterol-lowering and immunomodulatory activities, antimicrobial and antitumor effects, and anti-inflammatory and anti-

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oxidative effects (Ham et al., 1999; Kumura, Tanoue, Tsukahara, Tanaka, & Shimazaki, 2004; Maccaferri et al., 2012; Romanin et al., 2015; Xie et al., 2015; Yoon, Na, & Kim, 2003; You, Cho, Ha, Kim, & Heo, 2006). Furthermore, this yeast species is used in the biotechnological industry for the production of bioethanol from produce waste, based on its ability to use various substrates, thermotolerance, and rapid growth (Fasoli et al., 2016; Fonseca, Heinzle, Wittmann, & Gombert, 2008; Lane & Morrissey, 2010; Wu et al., 2016). In these applications, it is important to monitor the quantity of *K. marxianus* during various fermentation and processing steps (Kim et al., 2015c, 2016).

On the other hand, because of its ability to ferment lactose, and hydrolyze milk fats and proteins, and wide range of growth temperatures from 5 °C to 52 °C, *K. marxianus* is frequently reported as one of the causes of yeast contamination of dairy products (Valderrama, de Silóniz, Gonzalo, & Peinado, 1999; Lane & Morrissey, 2010). Yeast contamination is one of the main factors affecting the stability and quality of dairy products, including yogurts, potentially leading to economic losses and industrial wastage (Canganella et al., 1998; Mayoral et al., 2005; Viljoen, Lourens-Hattingh, Ikalafeng, & Peter, 2003). Many laboratories employ traditional culture methods to detect and enumerate this spoilage yeast (Mayoral, Martin, Hernández, González, & Garcia, 2006). Although a differential medium for the isolation of *K. marxianus* and *Kluyveromyces lactis* has been developed, the culturing method is laborious and time-consuming (requiring 7–10 d from the initial isolation to final confirmation) (Valderrama et al., 1999). Hence, fast and reliable methods are needed to detect yeast spoilage and to effectively identify the possible route of contamination.

The aim of this study was to develop a novel TaqMan probe-based real-time PCR method for a rapid and sensitive detection and quantification of *K. marxianus*. The novel method was used to enumerate *K. marxianus* during kefir fermentation and to detect the yeast in artificially contaminated yogurt samples, to determine its applicability in the dairy industry.

2. Materials and methods

2.1. Primer design

The novel primer/probe set targeting the *ITS* rRNA region (KM55) was designed by the procedures described previously (Kim et al., 2015c, 2016). The sequence of the gene was obtained from the GenBank (www.ncbi.nlm.nih.gov/Genbank/; accession number NR111251). Sequences unique to *K. marxianus* were compared with those of closely-related strains, and potential target sites for specific detection were identified (Fig. 1). The primer/probe set was designed using the Primer Express software v 3.0 (Applied Biosystems, Foster City, USA). The set was validated using the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST; www.ncbi.nlm.nih.gov/blast/). The primer and probe sequences were as follows: KM55_F: 5′-TTTGGGTTTGGTAGTGAGTGATAC-3′ (forward primer; melting temperature = 56 °C; GC contents = 42%); KM55_R: 5′-GGCAACGGCTAGCCACTT-3′ (reverse primer; melting temperature = 58 °C; GC contents = 61%); and KM55_P: 5′-FAM-CGTCTCGGGTTAACTT-3′ MGB-NFQ (probe; melting temperature = 68 °C; GC contents = 50%). The amplicon size was 61 bases. The oligonucleotides were synthesized and purchased from Applied Biosystems.

2.2. Inclusivity and exclusivity tests using standard strains

For the inclusivity and exclusivity tests of the designed primers/probe set, real-time PCR was performed using DNA from 21 standard and 18 wild-type strains from dairy products as templates (Table 1). Each microorganism was grown on potato dextrose agar (PDA; Oxoid, Basingstoke, UK); De Man, Rogasa, and Sharpe agar (MRS; Oxoid); tryptic soy agar (Oxoid), and *Bifidobacterium* selective agar (Difco, Spark, USA) before DNA extraction.

2.3. DNA extraction

Genomic DNA was extracted using the NucliSENS easyMAG instrument (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's instructions. Briefly, each colony was suspended in 1 ml of lysis buffer (BioMerieux), 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 (BioMerieux) and subjected to automatic magnetic separation. DNA was extracted into 50 μl of elution buffer (BioMerieux).

2.4. Real-time PCR

The extracted DNA (5 μl) was transferred to 20 μl of a PCR mix consisting of TaqMan Universal PCR master mix (12.5 μl; Applied Biosystems), KM55_F (2.5 μl, 50 nM), KM55_R (2.5 μl, 50 nM), and KM55_P (2.5 μl, 50 nM). A 96-microwell plate was sealed with optical adhesive covers (Applied Biosystems) and placed in the 7500 real-time PCR system (Applied Biosystems). The reaction was run for 2 min at 50 °C, followed by 10 min at 95 °C, and then, 40 cycles of 15 s at 95 °C and 60 s at 59 °C.

2.5. Standard curve in phosphate-buffered saline (PBS), milk, and yogurt

K. marxianus ATCC 46537 was used as the standard strain for the KM55 primer/probe set assay. The cells were grown on PDA, aerobically at 37 °C for 24 h. Typical colonies were suspended and then serially diluted in phosphate-buffered saline (PBS; Sigma, St. Louis USA), and plated on PDA to enumerate the yeast. Pasteurized 10% skim milk (Sigma) and commercial yogurt (Maeil Dairy Inc., Seoul, Korea) were used to generate the standard curves for the *K. marxianus* enumeration with the KM55 set in milk and yogurt.

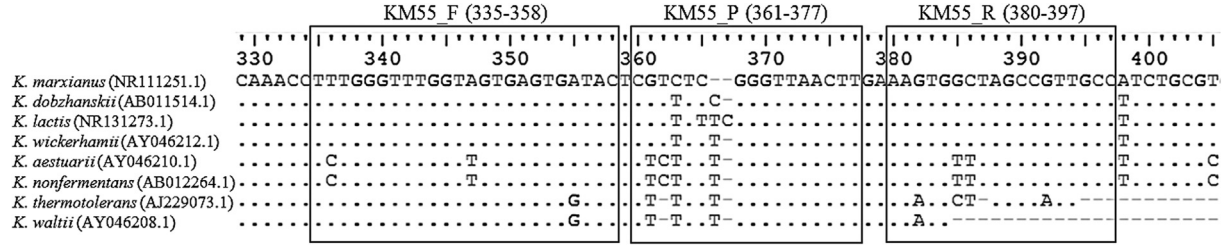

Fig. 1. Differences between the partial internal transcribed spacer (*ITS*) rDNA sequence alignment of eight strains of *Kluyveromyces* species. The annealing sites for the designed primers and the probe are boxed.