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Viable cells differentiation improves microbial dynamics study of fermented milks

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ABSTRACT

Understanding the changes that occur during storage of fermented milk products is of significant importance to dairy producers. Here, we investigated the microbiota dynamics of two fermented milk products, yoghurt and kefir, using culture-independent methods in combination with the use of a viability dye and quantitative-polymerase chain reaction. Propidium monoazide (PMA) was used to selectively detect viable cells in the food matrix. Samples treated with PMA showed results comparable with culturing methods and a lower copy number than samples not treated with PMA. The use of two culture-independent approaches combined with PMA treatment showed a more complete picture of the viable microbiota in the fermented milks during storage. Significant differences in microbiota were detected in kefir samples treated with and without PMA whereas no such significant differences were found in yoghurt. The microbiota in kefir also showed a high variation between batches at the species level.

1. Introduction

Microbial stability is one of the major factors influencing the shelf life of fermented milks during storage. Lactic acid bacteria (LAB) represent the main group of bacteria used to ferment milk. Traditionally, natural acidification of milk was performed by indigenous LAB present in the milk. Nowadays, selected starter cultures are added to pasteurised milk to ensure safety and product stability during storage. The primary role of the starter cultures is the metabolism of lactose to lactic acid and a consequent decrease of the pH, which is associated with depletion of substrates. The low storage temperature contributes to the microbial stability of fermented milks (Nakasaki, Yanagisawa, & Kobayashi, 2008). Improved information on the viability of the microbiota is important for understanding the changes that occur during storage of fermented milk products.

Yoghurt and kefir are produced using different starter cultures and divergent microbial dynamics during storage have been indicated (Damin, Minowa, Alcantara, & Oliveira, 2008; Gronnevik, Falstad, & Narvhus, 2011). Yoghurt is produced using a thermophilic culture and contains two species of LAB: *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which remain stable during the storage and prolong the product's shelf life (Damin et al., 2008). Kefir is produced from kefir grains, by a backslopping technique or from freeze-dried cultures. Kefir grains contain a complex mixture of LAB, acetic acid bacteria and yeast (Chen, Wang, & Chen, 2008; Dobson, O'Sullivan, Cotter, Ross, & Hill, 2011; Gronnevik et al., 2011).

Molecular methods have been previously implemented to study fermented milks to explore the dynamics of single species by quantitative polymerase chain reaction (qPCR) (Furet, Quenee, & Tailliez, 2004; Postollec, Falentin, Pavan, Combrisson, & Sohier, 2011) or to explore the dynamics of microbial groups by culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE) (Cocolin, Alessandria, Dolci, Gorra, & Rantsiou, 2013; Leite et al., 2012; Wang et al., 2011). Of several culture-independent methods, automated Ribosomal intergenic spacer analysis (ARISA) has shown a great potential for the microbial characterisation in dairy products due to the low detection limit compared with DGGE and higher number of operational taxonomic units (OTUs) detected (Arteau, Labrie, & Roy, 2010; Chebenova-Turcovska, Zenisova, Kuchta, Pangallo, & Brezna, 2011; Ndoye, Rasolofo, LaPointe, & Roy, 2011; Porcellato et al., 2014).

Culture-independent methods are based on the isolation of DNA from all the microbial cells in the food matrix including dead and living cells from both the starter culture and environmental

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contaminants (Cocolin et al., 2013). PCR-based molecular methods have been explored to separate DNA from living cells by the use of covalent binding substrates such as ethidium and propidium monoazide (PMA) (Davis, 2014; Nocker, Sossa-Fernandez, Burr, & Camper, 2007). These dyes are able to penetrate dead cells and bind to their DNA and then prevent the amplification of this DNA by PCR. Previous studies on fermented milks were performed using PMA to assess the dynamics of starter cultures and were based on single species identification (Garcia-Cayuela, Tabasco, Pelaez, & Requena, 2009). However, to our knowledge, culture-independent methods applied following treatment of the cell pellets by PMA have not previously been performed in fermented milks.

The treatment of the cell pellets with PMA might increase the accurate identification of the live microbiota by preventing amplification of DNA from dead cells (Nocker, Cheung, & Camper, 2006). The starter culture, used in fermented foods, reaches a high number during the first stages of production and subsequent stabilisation and/or death of starter cultures cells in dairy products is a known fact (Giraffa, 2004). Thus, the extraction of DNA from dead cells might lead to an erroneous picture of the actual metabolically active microbiota. In this study, the microbial dynamics of two fermented milks, yoghurt and kefir, were investigated by the use of PMA and culture-independent methods. Furthermore, we explore the application of ARISA as possible additional culture-independent method associated with PMA treatment of cell pellets.

2. Materials and methods

2.1. Collection of samples and microbial counts

Yoghurt and kefir (produced from kefir grains) samples were obtained directly from the producer and were from three different production batches. Samples were stored at 4 °C and analysed weekly during the entire storage period and until 2 weeks after the best before date indicated by the producer. Samples were mixed by shaking the container for 3 min and by mixing with a sterile pipette. One gram of the mixed sample was diluted (1:10) in 2% sodium citrate water (w/v). Further serial dilutions were performed in Ringer's solution (Oxoid, Hampshire, United Kingdom). Microbial counts were performed on M17 agar (Merck, Darmstadt, Germany) for the enumeration of *Streptococcus* and *Lactococcus*, and MRS Agar (Merck) for enumeration of presumptive *Lactobacillus*.

2.2. DNA extraction

For DNA extraction, 1 mL from the first dilution of each product was collected in duplicate. One sample was used for total DNA extraction and the other was used for total DNA extraction following treatment with propidium monoazide (PMA) (Biotium, Hayward, United States). Samples were centrifuged at 16,000 × *g* for 5 min and washed three times by resuspending the pellet in 1 mL of citrate water (2%, w/v). The pellet to be treated with PMA was resuspended in 500 μL 2% sodium citrate water and PMA added to give a final concentration of 0.05 mM (Biotium), and treated according to manufacturer's instructions. DNA was extracted using the GenElute Bacterial Genomic DNA Kit from Sigma–Aldrich, following the manufacturer's instructions with some modifications. DNA was added 80 μL of cetyltrimethylammonium bromide (CTAB) solution (10% CTAB in 0.7 M NaCl) after the proteinase K step. The samples were mixed and centrifuged at 13, 000 × *g* for 3 min and the supernatant used for DNA extraction. DNA was stored at −18 °C until analysis.

2.3. qPCR and standard curve construction

Quantitative PCR was performed using the LightCycler 480 System (Roche, Germany) in a final volume of 20 μL containing 1× of the LightCycler 480 High Resolution Melting Master Mix (Roche) buffer, 2 mM $MgCl_2$, 0.4 μM each primer and 1 μL of DNA. The primer pairs used in this experiment were LAC1F-LAC2R (Walter et al., 2001) for the detection of *Lactobacillus*, *Leuconostoc* and *Pediococcus* spp. and LAC3F-LAC2R (Endo & Okada, 2005) for the detection of *Lactococcus*, *Enterococcus*, and *Streptococcus* spp. These primers amplify the V3 region of the 16S rRNA gene. PCR cycles were performed as described previously (Porcellato, Gronnevik, Rudi, Narvhus, & Skeie, 2012). *Lactococcus lactis* YIMM1 and *Lb. rhamnosus* YIMM2 were used to construct the standard curve for the quantification of the number of copies for each primer set. Standard curves were created from the PCR product obtained with the same conditions described previously for each primer set. The PCR product was purified with QIAquick PCR Purification Kit (Qiagen, Venlo, Netherlands) following the manufacturer's instructions and quantified by Qubit (Life Technologies Corporation, United States). The number of copies of PCR product was calculated based on the length and amount of DNA. Serial dilutions of the PCR product were used to obtain the CP value and to estimate the number of copies present in the samples.

2.4. DGGE analysis and band sequencing

DGGE was performed using the IGENYphorU-2 system (Ingeny International BV, Goes, Netherland) as described previously by Porcellato et al. (2012). Twenty microlitres of PCR product obtained from the qPCR described above was loaded in a 30–55% denaturing gradient gel. DGGE was performed as previously described. After the run, the gel was stained with GelRed (Biotium) by immersing the gels in a GelRed 3× solution for 30 min and was then visualised under UV light. DGGE bands were selected and excised from the gel with a sterile scalpel blade and incubated at 37 °C for 4 h with 50 μL 0.1× TE buffer, to allow diffusion of the DNA. The eluted DNA was used for sequencing after PCR amplification under the same conditions described previously. The PCR products were purified as described previously and sequenced at GATC (GATC biotech AG, Konstanz, Germany).

2.5. ARISA and database construction

ARISA was performed with the methods described previously by Porcellato et al. (2014). Two microlitres of PCR product was diluted (1:10 in water), obtained with the primer set described by Fisher and Triplett (1999), was mixed with 10 μL highly deionised formamide (Applied BioSystems, Carlsbad, California) and mixed with 0.025 μL GeneScan™ 1200 Liz® dye size standard. Samples were submitted to the Institute for Molecular Medicin Finland (FIMM, Helsinky, Finland) for capillary electrophoresis analysis in an ABI 3700 Genetic Analyzer. Peaks were analysed for length and area with a Peak Scanner™ Software v1.0 (Applied BioSystems) with the conditions described previously by Porcellato et al. (2014). Identification of the starter and non-starter microbiota was performed by comparison of each peak length with a database of ITS lengths and compared with the length of the ITS obtained from the pure strains. The pure strains used in this analysis were: *Lactococcus lactis* YIMM1, *Lactobacillus rhamnosus* YIMM2, *Lb. plantarum* INF15D, *Lb. casei* INF448, *Enterococcus hirae* INF-E1, *Streptococcus thermophilus* TAR-1 and *Leuconostoc mesenteroides* TAR-2. The database used was previously described in Porcellato et al. (2014). Improvement of the database was performed by in silico analysis of genome sequences, publicly available at NCBI (http://www.ncbi.nlm.nih.gov/) using an