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Combination of culture-dependent and culture-independent molecular methods for the determination of lactic microbiota in sucuk

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ABSTRACT

In this study, the culture-dependent and culture-independent molecular methods were used for the identification of lactic acid bacteria (LAB) in sucuk a Turkish fermented dry sausage. On the one hand, the PCR-DGGE method targetting the V1 and V3 regions of 16S DNA was applied to DNA that was directly extracted from sucuk samples. On the other hand, rep-PCR fingerprinting was performed for the primary differentiation and grouping of the isolates, and the results were confirmed by sequencing of the 16S rDNA and 16S–23S rDNA intergenic spacer region. As a result of the PCR-DGGE analysis of all the samples, total 8 different lactic acid bacteria were identified, and *Lactobacillus sakei*, *Lactobacillus curvatus* and *Weissella viridescens* were the dominant microbiota among these bacteria. The culture-dependent approach indicated that the majority of the strains belonged to the *Lactobacillus* genera including *Lb. sakei*, *Lactobacillus plantarum*, *Lb. curvatus*, *Lactobacillus brevis*, *Lactobacillus farciminis* and *Lactobacillus alimentarius.* However, *Leuconostoc* and *Weisella* were also detected as minor genera. Again, *Lactococcus piscium*, *Weissella halotolerans*, *Staphylococcus succinus* and the comigrated *Staphylococcus piscifermentans/Staphylococcus condimenti/Staphylococcus carnosus* group were detected only with the culture-independent method while *Lb. plantarum*, *Leuconostoc mesenteroides* and *Leuconostoc citreum* were identified only by using the culture-dependent method. In the results, it was concluded that the combination of culture-dependent and culture-independent methods was necessary for reliable and detailed investigation of LAB communities in fermented food products.

1. Introduction

A Turkish fermented sausage, sucuk, is one of the traditional fermented meat products produced in Turkey. It usually contains beef, beef backfat, sheep tailfat, salt, sugar, garlic and various other spices, and it is produced through microbial fermentation and drying process (Aksu and Kaya, 2004). In the traditional production of sucuk, the fermentation, generally controlled by natural microbiota, is an essential stage for the development of desirable organoleptic characteristics and improving the hygienic stability and safety of the final product (Bozkurt and Erkmen, 2004; Gökalp, 1995). Nowadays, this artisanal and region-dependent flora, usually referred to as "house flora" (Garcia-Varona et al., 2000) in fermented products has attracted considerable attention for the development of new starter cultures with desirable fermentation characteristics (Babić et al., 2011; Talon et al., 2007). This is because, the metabolic activity and competitiveness of wild-type strains with undesired microbiota naturally well-adapted to the environment are higher in contrast to industrial starters, which are not well-adapted to the technology applied to the raw material and used in the production (Leroy et al., 2006; Talon et al., 2007). Therefore, artisanal fermented products are mostly of a higher quality compared to products fermented under controlled conditions and produced by using industrial starters.

In spontaneously fermented sucuk, lactic acid bacteria constitute the dominant flora and the members of the genus *Lactobacillus*; *Lb. sakei*, *Lb. curvatus*, *Lb. plantarum* and *Lb. brevis*, are commonly isolated from sucuk at the end of the ripening stage (Gürakan et al., 1995; Özdemir, 1999; Kaban and Kaya, 2008; Adiguzel and Atasever, 2009). It is important for food microbiologists to be able to correctly profile the LAB present in fermented foods because of their specific characteristics that lead to the development of typical textures and flavour, and putative health-conferring properties.

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Molecular techniques have been used as an effective method to identify and characterize the dominant flora in complex bacterial communities such as fermented food for the last 20 years. In particular, PCR-based genomic fingerprinting techniques are believed to have the highest potential for more rapid and reliable detection, identification and classification of LAB (Ben Amor et al., 2007; Olive and Bean, 1999; Temmerman et al., 2004). Culture-dependent PCR-based methods; random amplified polymorphic DNA (RAPD) (Comi et al., 2005), restriction fragment length polymorphism (RFLP) (Belgacem et al., 2009), and nucleotide sequence analysis of the 16S ribosomal DNA (rDNA) gene and repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprinting (Danilović et al., 2011) were performed to characterize and identify the LAB isolated from fermented sausages. In particular, the sequencing of the complete 16S rDNA gene has become the most widely used technique to study bacterial communities in food environment (Singh et al., 2009). Likewise, PCR amplification of repetitive bacterial DNA elements (rep-PCR fingerprinting), is considered to be a reliable tool for the classification and typing of very closely related species, especially in the differentiation of bacterial strains within one species (Gevers et al., 2001).

On the other hand some researchers who compared microscopic examination and culture media have stated that the majority of the microorganism in various ecosystems have not been cultivated (Langendijk et al., 1995; Suau et al., 1999; Wilson and Blitchington, 1996). For this reason, culture-dependent methods alone have not provided a completely accurate representation of these complex communities. However, molecular culture-dependent methods have been proved to be a better technique to provide more detailed information on microbial diversity in complex food samples. Among such methods, denaturing gradient gel electrophoresis (DGGE) is one of the most powerful tools for studying the composition of the microbiota of an ecosystem by sequence-specific separation of PCR-amplified 16S ribosomal DNA (rDNA) fragments (Cocolin et al., 2001; Ercolini, 2004; Muyzer et al., 1993).

Recently the combination of culture-dependent and culture-independent molecular methods have become the preferred approach for determining and analysing the species composition of targetted microbial communities (Ercolini et al., 2001a; Temmerman et al., 2004; Silvestri et al., 2007).

In order to protect the traditional aspects of sucuk and to detect autochthonous starter cultures that have potential for the use in production, it is essential to understand the microbial populations responsible for the ripening (Rantsiou and Cocolin, 2006). For this reason, the aim of this study was to determine the lactic acid bacterial profile of the sucuk from different regions of Turkey by using different molecular strategies, based on both culture-dependent and culture-independent methods. In the research, first of all, PCR-DGGE analysis of the PCR-amplified V1 and V3 region of the 16S rRNA gene (rDNA) were performed to determine the total microbial diversity. Secondly, rep-PCR fingerprinting technique using a $(GTG)_5$ primer was employed to identify and characterize LAB strains isolated from the sucuk samples and cultivated on opportune culture media. Thirdly, the results of rep-PCR fingerprinting were validated by the sequencing of the 16S rDNA and 16S–23S rRNA intergenic spacer regions. This paper is assumed to be the first report concerning the microbial characterization of sucuk using above PCR based molecular techniques or the polyphasic approach.

2. Materials and methods

2.1. Sampling procedures

The sucuk samples, produced by the traditional method without using starter culture were obtained from 8 local meat factories in Kayseri and Afyon in Turkey. Triplicate samples of ready-for-sale sucuks from a single batch for each plant were collected at the end of the ripening process. The samples were labeled as M, KS (from Afyon) and G, K, U, B, BA and N (from Kayseri). No substantial differences in formulations and fermentation conditions were found between sucuk samples obtained from different plants. From the label information, all of the sucuk samples were found to consist of common ingredients such as powdered red pepper, garlic, salt, sugar, cumin, black pepper, pimento, antioxidant and sodium nitrite (E 250) in addition to beef and beef fat. All of the sucuk mixtures were stuffed into artificial casings and fermented for 3 days at 22–25 °C and relative humidity (RH) 85–90% followed by a gradual reduction of temperature to 16–19 °C and RH to 75–80% during the next 5–7 days, and ripening process was completed by a step at 15–16 °C and 60–65% RH for 1–2 days. After the sampling process, the pH values of samples were measured by pH meter (inoLab® pH 720 WTW, GmbH Germany) combined with a glass electrode and then sucuk samples were subjected to traditional isolation on agar plates and DNA extraction followed by PCR amplification.

2.2. Isolation and enumeration of lactic acid bacteria (LAB)

Twenty-five grams of each of the sucuk samples was diluted with 225 ml of Ringer solution (Sigma), and homogenized for 2 min with a Stomacher machine. Serial decimal dilutions were prepared with the same diluent and subjected to the agar plate method for enumeration and isolation of LAB on duplicate agar plates. Lactobacilli were enumerated on MRS agar (Merck, GmbH, Darmstadt, Germany) incubated in anaerobic conditions (Anaerocult C, GasPak Merck, Germany) at 30 °C for 48 h. Lactococci were counted on M17 agar (Merck, GmbH, Darmstadt, Germany) incubated at 30 °C for 48 h. To inhibit yeast growth 200 ppm cycloheximide (Sigma) was added to both of the agars. After the incubation period, the colonies grown on the plates were counted and the means and standard deviations were calculated. From each sucuk sample, 10–15 colonies showing different morphological characteristics were selected from the MRS and M17 plates and plated on new plates in order to purify the colonies for further characterization. In total 96 Gram-positive and catalase negative pure isolates were selected from all the samples and were stored at −80 °C in the corresponding isolation medium as glycerol stocks.

2.3. Extraction of bacterial DNA from sucuk samples and pure cultures

Total DNA extraction from sucuk samples and pure cultures was performed using a commercial DNA isolation kit (QIAamp DNA Mini kit, Qiagen, Germany) according to the manufacturer's protocol for Gram-positive bacteria. 20 g of each sucuk sample was homogenized in a stomacher bag with 20 ml of saline-peptone water for 3 min. 2 ml of the homogenized sucuk suspensions and 2 ml of each overnight bacterial culture were used for total DNA extraction. Bacterial cells were pelleted by centrifugation for 10 min at 10 000 ×g and the pellets were then resuspended in 180 µl of the enzyme solution (20 mg/ml lysozyme, 20 mM Tris·HCl, pH 8.0; 2 mM EDTA; 1.2% Triton X-100). After incubation for 1 h at 37 °C, genomic DNA was extracted by the silica membrane-based method.

2.4. PCR-DGGE analysis of sucuk samples

The V1 and V3 regions of the 16S rDNA gene were amplified by using isolated DNA from each sucuk sample and pure cultures. The primers V1f (5′-GCGGCGTGCCTAATACATGC-3′) and V1r (5′-TTCCCCACGCGTTACTCACC-3′) (Klijn et al., 1991) were used to amplify the V1 region while the amplification of the V3 region was carried out by using the primers V3f (5′-CCTACGGGAGGCAGCAG-3′) and V3r (5′-ATTACCGCGGCTGCTGG-3′) (Muyzer et al., 1993). Two different GC-clamps were attached to the V1f and V3f primers according to Sheffield et al. (1989) and Muyzer et al. (1993) respectively. All of PCR amplifications were performed in a final volume of 50 µl, containing 25 µl of commercial PCR master mix (Qiagen GmbH, Germany),