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Diversity and dynamics of antibiotic-resistant bacteria in cheese as determined by PCR denaturing gradient gel electrophoresis

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ABSTRACT

This work reports the composition and succession of tetracycline- and erythromycin-resistant bacterial communities in a model cheese, monitored by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE). Bacterial 16S rRNA genes were examined using this technique to detect structural changes in the cheese microbiota over manufacturing and ripening. Total bacterial genomic DNA, used as a template, was extracted from cultivable bacteria grown without and with tetracycline or erythromycin (both at 25 μg ml^{-1}) on a non-selective medium used for enumeration of total and viable cells (Plate Count agar with Milk; PCA-M), and from those grown on selective and/or differential agar media used for counting various bacterial groups; i.e., lactic acid bacteria (de Man, Rogosa and Sharpe agar; MRSA), micrococci and staphylococci (Baird–Parker agar; BPA), and enterobacteria (Violet Red Bile Glucose agar; VRBGA). Large numbers of tetracycline- and erythromycin-resistant bacteria were detected in cheese samples at all stages of ripening. Counts of antibiotic-resistant bacteria varied widely depending on the microbial group and the point of sampling. In general, resistant bacteria were 0.5–1.0 Log_{10} units fewer in number than the corresponding susceptible bacteria. The PCR-DGGE profiles obtained with DNA isolated from the plates for total bacteria and the different bacterial groups suggested *Escherichia coli*, *Lactococcus lactis*, *Enterococcus faecalis* and *Staphylococcus* spp. as the microbial types resistant to both antibiotics tested. This study shows the suitability of the PCR-DGGE technique for rapidly identifying and tracking antibiotic resistant populations in cheese and, by extension, in other foods.

1. Introduction

Antibiotic resistance increases the cost of treatment of infections and can be the cause of therapeutic failure (Andersson and Hughes, 2010). The spread of antibiotic resistance to human and animal pathogens is therefore of great concern. The food chain is thought to be one of the main routes via which such resistance spreads (Rossi et al., 2014). The transfer of genes from resistant to susceptible bacteria may occur during food manufacture or during transit through the gastrointestinal tract (Rossi et al., 2014; Gazzola et al., 2012). Fermented foods, such as cheese, in which several bacterial types grow to high cell densities, are key players in the transmission of antibiotic resistance between beneficial/commensal and pathogenic bacteria (Nawaz et al., 2011). Complex bacterial communities composed of the natural cheese microbiota plus an array of environmental microorganisms develop and change in fermented foods over time, particularly in starter-free, raw-milk cheeses (Flórez and Mayo, 2006).

Cheeses made from raw milk have been reported to sometimes contain high antibiotic resistance gene loads (Flórez et al., 2014; Manuzon et al., 2007). The characterisation of the bacterial species involved via conventional, culture-dependent analysis can, however, be difficult due to the intrinsic limitations of this approach (it is time consuming, expensive, and has a high manpower demand, etc.) (Devirgiliis et al., 2013; Gazzola et al., 2012; Nawaz et al., 2011). To overcome this, a number of culture-independent molecular methods have been developed in recent decades, including conventional polymerase chain reaction (PCR) and quantitative PCR (qPCR) amplification, temporal temperature gel electrophoresis (TTGE), denaturing gradient gel electrophoresis (DGGE), and the construction and analysis of metagenomic libraries (Devirgiliis et al., 2014; Flórez et al., 2014; Manuzon et al., 2007).

Since its first use in Microbial Ecology research in the early 90s (Muyzer et al., 1993), DGGE analysis of rRNA-encoding genes amplified by PCR (PCR-DGGE) has become a widely used tool for investigating the microbial diversity of food ecosystems, including milk, cheese and other dairy products [for recent reviews see Cocolin et al. (2013) and Quigley et al. (2011)]. Given the source of the nucleic acids used in PCR-DGGE, the technique can be performed in two ways: DNA or RNA can be extracted directly from the food matrix (direct PCR-DGGE), or be purified from cultivable bacteria harvested from non-selective and/or selective/differential media (indirect PCR-DGGE). The technical aspects, advantages and biases of these two alternatives have been discussed

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elsewhere (Ercolini, 2004). Direct PCR-DGGE has already been applied to analyse the polymorphism of tetracycline resistance genes in dairy and environmental samples (Flórez et al., 2014; Aminov et al., 2001; Chee-Sanford et al., 2001), as well as for tracking antibiotic resistant transconjugants in an experimentally-inoculated food (Gazzola et al., 2012). The indirect PCR-DGGE method has been successfully used for studying the microbiology of water buffalo Mozzarella- and Gouda-type cheeses (Ricciardi et al., 2014; van Hoorde et al., 2010; Ercolini et al., 2004).

The present work examines the diversity and dynamics of antibiotic-resistant bacterial communities of a traditional cheese during the manufacture and ripening, using the PCR-DGGE technique. Tetracycline and erythromycin were selected as the target antibiotics since resistance to them is widely spread among lactic acid bacteria species (Ammor et al., 2007), thus having the highest risk of horizontal transfer. Cabrales, a Spanish traditional, blue-veined cheese made from raw milk without the use of starter and ripening cultures (Flórez and Mayo, 2006) was selected as the cheese model. The PCR-DGGE technique was used after harvesting cultivable bacteria grown on a non-selective medium and on selective/differential media (for counting bacteria of different groups). To our knowledge, this is the first study in which indirect PCR-DGGE has been used to characterise the bacterial populations involved in antibiotic resistance in cheese along manufacturing and ripening stages. The technique was found valuable for identifying, quantifying and tracking tetracycline- and erythromycin-resistant communities.

2. Material and methods

2.1. Cheese samples

One batch of Cabrales cheese was made following the traditional manufacturing procedure, which involves the use of raw milk but no commercial starter culture or mould spores. Samples were taken from the cheese at days 3, 7, 15, 30 and 60 of ripening. Cubes of 10 g from the cheese core were cut aseptically and homogenized at 45 °C for 1 min in a Colworth Stomacher 400 (Seward Ltd., London, UK) with 90 ml of a sterile 2% (w/v) sodium citrate solution.

2.2. Enumeration of total and antibiotic-resistant bacteria

Ten-fold dilutions of homogenized cheese samples were prepared with sterile Ringer's solution (VWR International). Aliquots (100 μl) were plated in duplicate on non-selective and selective/differential solid media: total aerobic mesophilic bacterial counts were determined on Plate Count agar with Milk (1%) (PCA-M) (VWR International) after incubation at 32 °C for 48 h; lactic acid bacteria (LAB) counts were made on de Man, Rogosa and Sharpe agar (MRSA) (VWR International) after incubation at 32 °C for 48 h; Baird–Parker agar (BPA) (VWR International) was used to enumerate staphylococci and micrococci after incubation at 37 °C for 48 h; and enterobacteria were counted on Violet Red Bile Glucose agar (VRBGA) (VWR International) after incubation at 37 °C for 24 h. The respective antibiotic-resistant populations were enumerated on the same media as above supplemented with 25 μg ml^{-1} of either tetracycline or erythromycin.

Bacterial cells from plates showing semi-confluent colonies were harvested by vigorous washing in sterile phosphate-buffered saline (PBS). Cells were then suspended in Brain Heart Infusion broth (BHI) (VWR International) supplemented with 25% glycerol (Merck) and maintained at −80 °C until use.

2.3. DNA extraction from cultivable bacteria

Total DNA from cultivable bacteria (susceptible and/or resistant) was isolated from 180 μl of the BHI–glycerol suspensions. Cell pellets were collected by centrifugation and suspended in the same volume of a lysis buffer containing 20 mg ml^{-1} lysozyme (Merck), 200 U mutanolysin (Sigma-Aldrich), 50 μg ml^{-1} lysostaphin (Sigma-Aldrich), 20 mM Tris–HCl (pH 8.0), 2 mM EDTA and 1.2% Triton-X-100 (Merck). This lysis suspension was then incubated at 37 °C for 1 h and the DNA extracted using the DNeasy Blood and Tissue kit (Qiagen), according to the manufacturer's protocol. The DNA concentration was adjusted to 100 ng μl^{-1} and stored at −20 °C until analysis. Genomic DNA was also extracted and purified from well-identified bacteria strains, which have been previously isolated from Cabrales cheese (Flórez and Mayo, 2006). PCR amplicons from these strains were used as DGGE standards.

2.4. PCR-DGGE amplification and electrophoresis conditions

Purified total and genomic DNA was used as a template for the amplification of the V3 region of the bacterial 16S rRNA gene by PCR using two universal primers: 357F (5′ CCTACGGGAGGCAGCAG 3′) and 518R (5′ GTATTACCGCGGCTGCTGG 3′). A GC clamp of 40 nucleotides (CGCC CGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG) was attached to the 5′ end of the forward primer (357F-GC), as described by Muyzer et al. (1993). The PCR reaction mixtures contained 3 μl of total DNA, 25 μl of Taq Master Mix (Ampliqon), 1 μl of each of the primers (10 μM) and 20 μl of H_2O in a total volume of 50 μl. The PCR amplification conditions were as follows: an initial cycle at 95 °C for 5 min, 30 cycles at 95 °C for 30 s, 56 °C for 30 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min.

DGGE was performed in a DCode apparatus (Bio-Rad) using 8% polyacrylamide gels with denaturing ranges of 40–60%. Electrophoresis ran at 60 °C and 75 V for 17 h. The resulting gels were stained in an ethidium bromide solution (0.5 μg ml^{-1}) for 15 min, rinsed with water, and photographed under UV light using a G-Box system (Syngene).

2.5. Identification of PCR-DGGE bands

PCR-DGGE bands were identified by comparing their migration with that of amplicons from standards. Further, DNA from the bands was extracted by cutting out part of each using a micropipette tip and allowing their contents to diffuse out overnight at 4 °C into sterile, nuclease-free water (Sigma-Aldrich). The resulting DNA solution was used as a template for re-amplification, using the same pair of primers but without the GC clamp, and under the same PCR conditions as above. The presence of amplification products was checked in 1% agarose gel after ethidium bromide staining. Amplicons were then purified to remove any unincorporated primers and nucleotides using the ATP Gel/PCR Extraction kit (ATP Biotech). The purified amplicons were sequenced using the 357F primer, and the resulting sequences compared with those deposited in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) and Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp) databases. As reported elsewhere (Stackebrandt et al., 2002), sequences sharing a percentage identity of 97% or higher to those in the databases were considered to belong to the same species.

3. Results

3.1. Counting of bacterial populations in cheese

Large numbers of antibiotic-resistant bacteria were detected in samples at all stages of ripening. Both tetracycline- and erythromycin-resistant counts varied widely (from 10^2 to $>10^8$ cfu g^{-1}) depending on the bacterial group in question and the sampling point (Fig. 1). The presence of antibiotics caused a significantly decrease in counts ($p \leq 0.05$; Student's *t*-test) of all bacterial groups. In general, counts of resistant bacteria were around 1.0 Log_{10} units fewer in number than those corresponding to susceptible bacteria. The numbers of erythromycin-resistant enterobacteria were, however, equal to those recovered from PCA-M plates without the antibiotic. Resistant and susceptible populations reached a maximum on about day 3, and