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The diversity and evolution of microbiota in traditional Turkish Divle Cave cheese during ripening

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ABSTRACT

The microbial diversity of traditional Turkish Divle Cave cheese was evaluated in three independent batches. Using molecular techniques, twenty three bacterial species were identified in the interior and outer part of the cheese on days 60 and 120. Bacilli and Gammaproteobacteria classes were predominant during early stages of ripening and Actinobacteria at later stages. Nineteen species of filamentous fungi and five yeast species were identified and the most frequently isolated species were *Penicillium polonicum, Penicillium biforme, Penicillium roqueforti, Penicillium chrysogenum* and *Debaryomyces hansenii*. The microbiota displayed similar communities among batches, but high diversity in different parts of the cheese during ripening. Novel cheese starter cultures could be developed after the technological characterisation of the isolated strains.

1. Introduction

The secondary microbiota contributes to cheese ripening in raw milk cheeses. Growth of this microbiota depends on intrinsic and/or external factors, and is usually unique to specific cheese varieties. There are some studies interested in isolating new wild strains of secondary cultures with novel properties from raw milk cheeses (Beresford, Fitzsimons, Brennan, & Cogan, 2001).

Divle Cave (DC) cheese is a semi-hard cheese made from raw ewes' milk, which has a crumbly texture and a strong flavour. It is produced in May and June in Karaman, a rural region in the middle of Turkey, with an estimated annual production of 70–80 tonnes (District Governor Erkan İsa Erat, personal communication). Ripening takes place in Divle cave, in the south of this region, where the average temperature is 5–10 °C with a humidity of 85–90%. The cheeses are produced without starter culture and ripened for 4 months in goatskin bags at a depth of 70 m. The bag surface turns from green to red during ripening and this red colour is the distinguishing characteristic of DC cheese (Supplementary Fig. S1). Studies on DC cheese so far focused on chemical composition and microbial counts (Gönç, 1974; Keleş & Atasever, 1996; Morul & İsleyici, 2012).

There is a lack of standardisation in production of DC cheese and it can be overcome by identification of the dynamics of microorganism communities and the determination of the predominant contributors to cheese ripening. If these strains can be used in productions under controlled conditions, standard and high quality cheeses can be produced. In the present study we examined the microbial community of DC cheese to provide insight into the species diversity and evolution from mid-to late ripening. The resulting data can be used to develop specific starter or adjunct cultures to accelerate the cheese ripening, obtain high diversity in cheese flavour and improve the organoleptic properties.

2. Materials and methods

2.1. Cheese production and sampling

The batches used in this study were produced by farmers at three different dairy farms in Karaman province. Two independent batches were produced per farm with an interval of 15 days. During production, semi-skimmed raw ewes' milk (4.5% fat content) was coagulated with calf rennet. The curd was broken into small pieces, dry-salted and 3–5 kg salted curd was tightly pressed into salted and dried goatskin bags. The goatskin bags were sewed firmly and kept under cool storage (14–15 °C) until whey drain off stopped (7–10 days). Finally, they were left in Divle cave for 4–5 months for ripening.

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Each sample was taken from cheese stuffed into goatskin bags. Eighteen cheeses coming from three different farms were analysed at each ripening stage. Analyses were performed in triplicate on the inner part (white core) and on outer crust of cheese inside the goatskin (subsurface).

2.2. Isolation of strains

Total aerobic mesophilic bacteria were determined on Plate Count Agar (Merck, Darmstadt, Germany) including 1% skim-milk at 35 °C for 48 h. Filamentous fungi and yeasts were grown on Potato Dextrose Agar (Merck) at 28 °C for 5–7 days. Lactobacilli were grown on de Man-Rogosa-Sharpe Agar (pH 5.4; Merck) incubated for 72 h at 30 °C under anaerobic conditions. Lactococci were grown on M17 agar (Merck) incubated for 48 h at 30 °C. *Enterobacteriaceae* and total coliforms were grown on Violet Red Bile Dextrose Agar (Merck) and Violet Red Bile Agar (Merck) respectively and incubated at 30 °C for 48 h. Staphylococci were grown on Baird-Parker Agar (Merck) supplemented with egg yolk tellurite solution (Merck), incubated at 37 °C for 24 h.

For the isolation of bacteria, aerobic strains were sub-cultured on Tryptone Soya Agar (Oxoid, Basingstone, Hamshire, UK) at 30 °C for 48 h and 15 °C for 5 days. Lactobacilli were sub-cultured on MRS agar, incubated at 30 °C for 72 h under anaerobic conditions containing a mixture of 90% CO_2 and 10% H_2 (Mart Anoxomat System, Drachten, The Netherlands). Lactococci and enterococci were sub-cultured on M17 agar. Filamentous fungi and yeasts were isolated on malt extract agar (Merck). Colonies were re-streaked for purification and used for DNA extraction. Each strain was stored in its respective broth mixed with 60% glycerol at −80 °C.

2.3. Molecular identification of bacteria and mycobiota

2.3.1. DNA extraction and PCR amplification of bacteria

Extraction of DNA was performed by boiling the loopful culture in 500 μL in sterile demineralised water. PCR amplification of the partial 16S rDNA was performed in 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA) with PCR GoTaq Green Master Mix (Promega, Madison, WI, USA). The conditions were: denaturation at 94 °C for 2 min and 36 cycles at 94 °C for 1 min, annealing 54 °C for 1 min, amplification at 72 °C for 2 min and final amplification at 72 °C for 5 min.

2.3.2. DNA extraction and PCR amplification of filamentous fungi and yeasts

Isolates of filamentous fungi and yeast strains were sub-cultured on Malt Extract Agar (Merck) and incubated for 5–7 days at 25 °C. The extraction of DNA was performed using the Microbial DNA Isolation Kit (MoBio Inc., Solana Beach, CA, USA) according to the manufacturer's protocol. The ITS barcode and a part of the β-tubulin genes (BenA) were amplified according to the parameters mentioned for bacteria amplification.

2.3.3. Sequencing, data analysis and identification

Sequencing was performed with the BigDye terminator chemistry (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instruction and analysed on an ABI 3730 XL Genetic Analyzer (Applied Biosystems). Sequences were edited and trimmed using SeqMan software in the Lasergene package (DNASTAR Inc., Madison, WI, USA). A homology search with the generated partial 16S rRNA, ITS and BenA gene sequences was performed on GenBank and/or internal databases of the CBS-KNAW Fungal Biodiversity Centre.

3. Results and discussion

In total, 98 bacteria, 101 filamentous fungi and 27 yeast isolates were picked from plates. Among them, 23 bacterial, 19 filamentous fungi and 5 yeast species were identified (Tables 1 and 2). GenBank accession numbers and culture collection numbers of bacterial and fungal species are given in Supplementary Table S1. Both bacterial and fungal biota demonstrated low diversity in different dairy farms; however they showed high diversity between two ripening stages.

3.1. Diversity among bacterial microbiota

Three different bacterial classes were identified, Bacilli, Gammaproteobacteria and Actinobacteria (Table 1). Bacteria were mostly detected from the interior part of cheese. Gammaproteobacteria and Bacilli were largely detected and predominant at day 60 in all batches. Bacilli were mainly represented by the following species: *Staphylococcus equorum* subsp. *equorum, Lactobacillus paraplantarum, Lactococcus lactis* subsp. *lactis, Enterococcus faecium, Staphylococcus warneri and Lactobacillus brevis*. These species were also detected as predominant species in ewes' milk cheese (Pangallo et al., 2014), and traditional raw milk cheeses (Fontana, Cappa, Rebecchi, & Cocconcelli, 2010).

With regard to Gammaproteobacteria, *Psychrobacter glacincola* demonstrated dominance both in the interior and outer layer of cheese at day 60. *Psychrobacter* species are described as aerobic, halotolerant, psychrophilic bacteria and particularly isolated from soil and moist habitats, as well in dairy products (Hantsis-Zacharov & Halpern, 2007; Pangallo et al., 2014).

The Bacilli and Gammaproteobacteria detected decreased drastically at day 120 and were replaced by Actinobacteria as the most abundant group on day 120. Until the 60th day of ripening, due to the growth of yeast strains, an increase was observed in pH, presumably because of the utilization of lactate and in some cases production of ammonia by yeasts (Gori, Mortensen, Arneborg, & Jespersen, 2007). The increase of pH favours growth conditions of fungi and Actinobacteria in the following days of ripening until day 120. In our study, chemical composition data (Supplementary Tables S2, S3) confirmed these results. In the beginning of ripening pH of cheese was low, but a rise was seen collaterally with the rise in yeast number when ripening progressed (Supplementary Table S4). The yeast species and *Brevibacterium* spp. among Actinobacteria contribute to the cheese flavour and cheese colour (Arfi, Leclercq-Perlat, Spinnler, & Bonnarme, 2005). Actinobacteria were represented by *Brevibacterium antiquum, Brachybacterium tyrofermentas, Micrococcus luteus, Kocuria salcicia, Microbacterium oryzae, Arthrobacter arilaitensis, Microbacterium gubbenense, Microbacterium halotolerans, Brevibacterium* spp., and *Brachybacterium* spp. Similar results were obtained in Italian Taleggio cheese (Panelli, Buffoni, Bonacina, & Feligini, 2012). Actinobacteria are able to hydrolyse casein (Collins, 2006), so could be effective for proteolysis and ripening. Some species of *Brevibacterium* were seen as white colonies when incubated at 30 °C and produced orange pigment at 15 °C.

The genus *Arthrobacter* produces a great variety of pigments, e.g., yellow, red, green, and is commonly found in soil, water, air, food and dairy products (Sutthiwong et al., 2014). *Arthrobacter arilaitensis* has been isolated from smear ripened cheeses (Mounier, Monnet, Jacques, Antoinette, & Irlinger, 2009), but to our knowledge it is the first time that it is isolated from a semi-hard or hard cheese. The other Actinobacteria species found in this study were previously detected in raw milk cheese (Hantsis-Zacharov & Halpern, 2007), traditional Italian cheeses produced without using any starter culture (Fontana et al., 2010), matured