DIVERSITY OF YEASTS AND MOULDS IN DAIRY PRODUCTS FROM UMBRIA, CENTRAL ITALY

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MATERIALS AND METHODS

Sampling and fungal isolation

A total of 69 samples were taken from 3 dairy-plants located in Umbria, central Italy, over a period of 3 months. The following product categories were analysed: bovine and ovine raw milk (12 samples), curd (11 samples), cream cheese (10 samples), fresh cheese (12 samples), aged cheese at the medium and high end of the spectrum (24 samples). Samples were collected in sterile containers and immediately transported to the laboratory in a refrigerated container for subsequent analysis. The analysis procedure followed the ISO regulation number 21527-1 for samples with a water activity of over 0.95 and the ISO regulation number 21527-2 for samples with a water activity below or equal to 0.95. In brief, 10 ml or 10 g of sample were homogenised in a sterile bag with 90 ml of peptone water (PW, Difco, Detroit, MI, USA) using a Stomacher 400 (PBI International, Milan, Italy). Samples were inoculated in triplicate on Dichloran Rose Bengal Chloramphenicol Agar (DRBC, HiMedia, Einhausen, Germany), Dichloran Glycerol Medium (DGM, HiMedia) and Chloramphenicol Yeast Glucose Agar (CYG, HiMedia) using the spread plate technique and incubated at 25°C for 3-5 days. Yeasts and moulds were isolated and purified on the same agar media. Isolates were preliminarily characterised at the genus level, using phenotypic methods, including macro- and microscopic observations. The isolates morphology was studied macroscopically by observing the colony features (color, shape, size and hyphae), and microscopically by a compound microscope with a digital camera using a lactophenol cotton blue (LCB) stained slide mounted with a small portion of the mycelium (conidia, conidiophores and arrangement of spores for moulds, cells shape for yeasts).

Identification of fungal isolates

Moulds. Part of the pure isolated fungal colony (about ½ cm^2) was taken by disposable scalpel and placed in a 1.7 ml tube containing 200 µl of lysozyme buffer (20mg/mL) and subjected to incubation at 37 °C over-night in a stirring thermomixer. DNA extraction was performed using gram positive bacteria protocol following the manufacturer's instructions (Qiagen, Hilden, Germany). DNA was subsequently subjected to the amplification of a portion of the internal transcribed spacer (ITS) region using ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') primers (Larena et al., 1999). DNA amplification was conducted in a total volume of 50 µl containing 1X PCR Buffer, 1.25 mM of $MgCl_2$, 0.25 mM of dNTPs, 0.5 µM of each primer, 1.25 U of Taq Hot Start (Promega, Madison, WI, USA) and 3 µl of DNA. The amplification conditions used were as follows: 95°C for 10 min, followed by 35 cycles at 94°C for 1 min, 53°C for 1 min, 72°C for 2 min and final extension at 72°C for 7 min. PCR products were analyzed by 2% agarose gel electrophoresis and visualised by colouring with Midori Green Advance DNA stain (NIPPON Genetics Europe, Dueren, Germany). The expected amplified portion was approximately 800 base pairs (bp). Positive PCR reactions were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing was performed by automated Sanger method with ITS4 and ITS5 primers using the BigDye Teminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions. The reactions were separated by 3500 Genetic Analyzer (Applied Byosistems) and the consensus sequences, generated using the BioEdit Sequence Alignment Editor software v 7.0.9.0, were aligned with the Genbank database.

Yeasts. A yeast colony was taken and diluted in a 1.7 mL tube containing 200 µL of physiological solution. DNA extraction was performed by boiling and the amplification of the D1/D2 region of the large-subunit of rRNA gene was carried out using NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTCAAGACGG-3') primers (Leaw et al., 2006). DNA amplification was conducted in a total volume of 50 µl containing

1X PCR Buffer, 2.5 mM of $MgCl_2$, 0.25 mM of dNTPs, 0.5 µM of each primer, 2.5 U of Taq Hot Start (Promega) and 3 µl of DNA. The amplification conditions used were as follows: 95°C for 10 min, followed by 30 cycles at 94°C for 30 secs, 58°C for 30 secs, 72°C for 30 sec and final extension at 72°C for 10 min. PCR products were analysed by 2% agarose gel electrophoresis and visualised by colouring with Midori Green Advance DNA stain (NIPPON Genetics Europe); the expected amplicon was approximately 650 base pairs (bp). Positive PCR products were sequenced as described for moulds.