International Journal of Food Microbiology 246 (2017) 25–31

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Short communication

Survey of Penicillia associated with Italian grana cheese

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ARTICLE INFO

Article history:
Received 14 October 2016
Received in revised form 18 January 2017
Accepted 30 January 2017
Available online 31 January 2017

Keywords:
Cheese
Citrinin
Fungal identification
Ochratoxin A
Penicillium

ABSTRACT

The present work aimed to contribute information on the mycobiota associated with ripening grana cheese, with focus on the genus *Penicillium* as potential mycotoxin producers. Eighteen wheels of grana cheese, aged in different storehouses situated in Northern Italy, were sampled to isolate associated fungi. *Penicillium* spp. were commonly dominant; morphological observation and gene sequencing were applied to identify *Penicillium* at species level. *P. crustosum* and *P. solitum* were the dominant species. Citrinin and ochratoxin A mycotoxins were analysed and the latter was found in all grana cheese samples. These results confirmed that a polyphasic approach is mandatory for *Penicillium* identification at species level.

1. Introduction

Grana is an Italian hard cheese, made from unpasteurised, partially skimmed cow's milk, aged for a period ranging from nine months up to four years. The word "grana" comes from "grainy" to describe its fine granular texture. This cheese is of utmost importance in Italian food culture and economy. The Protected Designation of Origin (PDO) is attributed to "Grana Padano" and "Parmigiano Reggiano" brands. Nevertheless, information on the mycobiota associated with this type of cheese is poor, with only one paper published on this topic in recent decades (Dragoni et al., 1983). The development of uncontrolled moulds during ripening and ageing has been reported for other cheese types, causing spoilage and possibly leading to mycotoxin production (Ropars et al., 2012). The genera *Aspergillus*, *Cladosporium*, *Geotrichum*, *Mucor*, *Penicillium*, *Trichoderma* (Sengun et al., 2008) and *Fusarium* (Lund et al., 1995; Montagna et al., 2004) have been reported as cheese contaminants. In addition, some related mycotoxins such as citrinin (CIT) (Bailly et al., 2002; Cooper et al., 1982; Ostry et al., 2013; Pugazhenthi et al., 2000) and ochratoxin A (OTA) (Dall'Asta et al., 2008; Jarvis, 1983) have been detected in cheese. In particular, OTA contamination was recently reported in packed grated cheese, commonly produced with grana cheese (Biancardi et al., 2013). Mycotoxins are a main concern in food and feed: CIT showed nephrotoxic, immunosuppresive, teratogenic and mutagenic effects in past studies, and seems able to cause hemolysis of human erythrocytes (Ambrose and Deeds, 1946; Houbraken et al., 2010; IARC, 1986; Lurá et al., 2004; Ostry et al., 2013) while OTA is mainly toxic for the kidneys and liver (Richard, 2007) and classified in group 2B by the International Agency for Research on Cancer (IARC, 1987). Therefore, the present work aimed to contribute by filling in these gaps in knowledge with a first description of the mycobiota associated with ripening grana cheese, with focus on the genus *Penicillium*, as potential mycotoxin producers. A polyphasic approach, based on morphological and molecular observations and on mycotoxin analysis, was used.

2. Materials and methods

2.1. Chemicals

Mycological peptone, microbiological grade agar, malt extract and yeast extract were purchased from Himedia Laboratories (Mumbai, India); Dichloran Rose Bengal Chloramphenicol Agar (DRBC) and Czapek Dox Agar (CZ) ready prepared culture media were purchased from Oxoid (Basingstoke, U.K.); D(+) glucose was purchased from Sigma-Aldrich (Merck, Darmstadt, Germany); salts ($CuSO_4 \cdot 5H_2O$; $ZnSO_4 \cdot 7H_2O$; $K_2HPO_4 \cdot 3H_2O$; $MgSO_4 \cdot 7H_2O$; KCl; $FeSO_4 \cdot 7H_2O$) were purchased form Carlo Erba Reagents S. r. l. (Milano, Italy); a DNA "Plant II" extraction kit was purchased from Macherey-Nagel (Düren, Germany); PCR master mix was purchased from Promega Corporation (Madison, Wisconsin, USA); an Exosap cleaning kit was purchased from Euroclone (Pero (MI), Italy).

2.2. Sampling

Eighteen ripening storehouses for grana cheese were sampled in 2013, in five sites in northern Italy. Seven storehouses (1–7) were

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http://dx.doi.org/10.1016/j.ijfoodmicro.2017.01.019
0168-1605/

located in Brescia (BS), four in Cremona (8–11, CR), four in Mantova (12–15, MN), two in Piacenza (16 and 17, PC) and one in Verona (18, VR).

One ripening wheel was randomly chosen for each storehouse and sampled collecting 10 g of cheese rind, scratched from 5 different areas, to 0.5 mm depth.

2.3. Mycological analysis

One gram of scratched cheese rind was sub-sampled and diluted in 9 mL of physiological solution (1% peptone), accurately mixed and serially diluted up to 10^{-7}. Dilutions were plated in triplicate using DRBC agar medium and incubated for 7 days in the dark at 25 °C. Growing colonies were counted and reported as colony forming units per g of cheese rind (CFU/g) as a rough estimation of fungal bioload. Fungi were also identified to genus level using optical microscopy.

2.3.1. Strain selection

Three to five representative colonies of *Penicillium* were selected from each wheel, repeatedly transferred onto CZ to obtain pure cultures and subsequently managed to obtain monosporic strains destined for identification at species level.

Monosporic cultures were grown on Blakeslee Malt Extract Autolysate Agar (MEA, Samson and Frisvad, 2004; malt extract 30 g; mycological peptone 1 g; glucose 20 g; $CuSO_4 \cdot 5H_2O$ 0.005 g; $ZnSO_4 \cdot 7H_2O$ 0.01 g; agar 20 g; bidistilled water 1 L, adjusted to pH 5.3 ± 0.3), at 25 °C in the dark for one week. Plugs were then removed from each colony, put into bidistilled sterile water and stored at 4 °C until their use.

2.3.2. Morphological identification

All monosporic strains were 3-point inoculated on Czapek Yeast Agar (CYA, Samson and Frisvad, 2004; yeast extract 5 g; sucrose 30 g; $K_2HPO_4 \cdot 3H_2O$ 1.3 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; KCl 0.5 g; $FeSO_4 \cdot 7H_2O$ 0.01 g; $CuSO_4 \cdot 5H_2O$ 0.005 g; $ZnSO_4 \cdot 7H_2O$ 0.01 g; agar 15 g; bidistilled water 1 L, adjusted to pH 6.3 ± 0.2) and MEA and incubated at 25 °C in the dark for 7 days. At the end of incubation, they were observed for macroscopic (colony diameter, obverse and reverse colour, margins, presence/absence of exudate droplets) and microscopic characters (shape and size of penicillus and spores, roughness of the stipe) and tentatively grouped at species level.

Reference strains of the potential species of interest were obtained from culture collections (Table 1) and used as reference in the following steps of the study for comparisons with the strains selected according to Section 2.3.1.

2.3.3. Molecular identification

Monosporic strains were identified by *BenA* (encoding for β-Tubulin) and *COI* (encoding for Cytochrome Oxidase subunit I) gene sequencing. Samples were inoculated in 8 mL of Malt Extract Broth (MEB, Pitt, 1979; malt extract 30 g; mycological peptone 1 g; glucose 20 g; bidistilled water 1 L) and incubated at room temperature for 7 days in 15 mL Falcon® tubes; the tubes were kept on moving using an orbital shaker (100 rpm) for the whole incubation period.

DNA was extracted using a "Plant II" kit according to the handbook supplied by the producer. Diluted genomic DNA (1:10, about 2 ng/μL) was employed for PCR amplification using the primer pairs *Bt2a-Bt2b* (Glass and Donaldson, 1995) for *BenA* and *PenF1-AspR1* for *COI* (Seifert et al., 2007).

PCR reactions for both genes were performed in 7 μL reaction mixtures containing 3.5 μL Promega master mix (Promega Corporation, Wisconsin, USA), 1.5 μL genomic DNA, 0.25 μL of each primer (0.5 μM) and 1.5 μL bidistilled sterile water. Amplification conditions were the following: 95 °C for 2 min, 35 cycles at 95 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min, 72 °C for 10 min and rest at 4 °C.

Cleaning of the PCR product was performed using an Exosap kit according to the protocol provided by the producer. Sequencing was performed using a 48-capillary 3730 DNA Analyser (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Sequences (forward and reverse) provided in AB1 format file were opened using the pattern analysis software package Bioedit Sequence Alignment Editor v.7.2.5.0 (Alzohairy, 2011) and processed; identification at the species level was performed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Dendrograms were then deduced opening the final sequence using the pattern analysis software package Mega7 (Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets; Kumar et al., 2016) and using the Neighbor-Joining (NJ) method for dendrogram design (Saitou and Nei, 1987). The robustness of tree topology for each analysis was evaluated by 1000 bootstrap replicates in both cases.

Sequences of reference strains coming from official culture collections were obtained from the website of the respective collection, from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/), or by sequencing according to Section 2.3.3 if not available, and included in the dendrograms for clustering. More sequences were obtained from the GenBank database in case unexpected species were detected (type or neotype strains were chosen when possible).

Table 1
Reference strains from fungal collections used in this work.

Fungal collection	Strain code	Origin	Species
CBS	115992	The Netherlands	*P. citrinum*
CBS	122396	USA	*P. citrinum*
CBS	101025	Portugal	*P. crustosum*
CBS	115503	Scotland	*P. crustosum*
BFE	856	Italy	*P. nordicum*
CBS	110769	Spain	*P. nordicum*
CBS	112573	Italy	*P. nordicum*
CBS	323.92	Denmark	*P. verrucosum*
CBS	325.92	Denmark	*P. verrucosum*

CBS: CBS-KNAW Fungal Biodiversity Centre, The Netherlands.
BFE: Federal Research Centre for Nutrition and Food, Germany.

2.4. Mycotoxin analysis

CIT and OTA were analysed in the 18 cheese samples, according to Somorin et al. (2016), and reported in μg/kg. Briefly, 9 g of scratched cheese rind were used; CIT was extracted using 50 mL of ortho-phosphoric acid and 10 mM methanol solution (3:7 v/v) using a rotary-shaking stirrer; then, after filtration through a folded filter paper, 2 mL of the filtrate were diluted with 20 mL of PBS and purified through immunoaffinity columns (R-Biopharm, Darmstadt, Germany). The column was washed with 2 mL of PBS and CIT slowly eluted (0.5 mL/min) with 4 mL of methanol into a graduated glass vial; the eluate was then concentrated under a gentle stream of nitrogen, brought to 1 mL with methanol and water solution (3:7 v/v) and vortexed for a few seconds. The extract was filtered before LC-MS/MS analysis. The instrumental analysis was carried out using a LC-MS/MS system in positive mode consisting of a LC 1.4 Surveyor pump, a Quantum Discovery Max triple-quadrupole mass spectrometer (Thermo-Fisher Scientific, San Jose, California, USA) and a PAL 1.3.1 sampling system (CTC Analitycs AG, Zwingen, Switzerland). Excalibur 1.4 software (Thermo-Fisher Scientific, San Jose, CA, USA) controlled the whole system. Limits of detection (LOD) and of quantification (LOQ) were 0.5 and 1.5 μg/kg, respectively.

OTA was quantified using a HPLC-FLD (fluorescence detector) system Perkin Elmer 200 (Perkin Elmer, Norwalk, Connecticut, USA) after extraction with 100 mL of sodium bicarbonate 0.13 M methanol (1:1 v/v) using a stirrer. The suspension was then filtered and 5 mL of the filtrate were diluted with PBS (50 mL) and purified through immunoaffinity columns (Ochratest WB, Vicam, Watertown, MA, USA), which was washed with 2 mL PBS so that OTA was slowly eluted (0.5 mL/min) with 3 mL of acetonitrile into a glass vial. The eluate was then concentrated under a gentle stream of nitrogen, brought to 1 mL with