International Journal of Food Microbiology 406 (2023) 110401

Contents lists available at ScienceDirect

International Journal of Food Microbiology

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

Epicoccum sp. as the causative agent of a reddish-brown spot defect on the surface of a hard cheese made of raw ewe milk

Javier Rodríguez, Lucía Vázquez, Ana Belén Flórez, Baltasar Mayo *

Departamento de Microbiología y Bioquímica, Instituto de Productos Lácteos de Asturias (IPLA), Consejo Superior de Investigaciones Científicas (CSIC), Paseo Río Linares s/n, 33300 Villaviciosa, Spain
Instituto de Investigación Sanitaria del Principado de Asturias (ISPA), Avenida de Roma s/n, 33011 Oviedo, Spain

ARTICLE INFO

Keywords:
Epicoccum
Moulds
Fungi
Cheese defect
Colour defect
Cheese mycobiota
Cheese microbiota

ABSTRACT

Colour defects can affect the appearance of cheese, its flavour, the safety of its consumption, and the price it can demand. This work reports the identification of five fungal isolates from a dairy plant where the surface of most cheeses was affected by patent, reddish-to-brown stains. One of these isolates was obtained from cheese, two from brine, and two from a bulk tank containing ewe milk. Molecular identification by partial amplification, sequencing, and database comparison of the concatenated sequence of the genes coding for the largest subunit of RNA polymerase II (RPB2), β-tubulin (β-TUB), and the large subunit of the rRNA molecule (LSU), plus the internal transcribed sequence (ITS) regions, assigned the isolates to *Epicoccum layuense*, *Epicoccum italicum*, and *Epicoccum mezzettii*. Features of the growth of these different species on different agar-based media, and of the morphology of their conidia following sporulation, are also reported. The strain isolated from cheese, *E. layuense* IPLA 35011, was able to recreate the reddish-brown stains on slices of Gouda-like cheese, which linked the fungus with the colour defect. In addition, two other strains, *E. italicum* IPLA 35013 from brine and *E. italicum* IPLA 35014 from milk, also produced stains on cheese slices. *Epicoccum* species are widely recognized as plant pathogens but have seldom been reported in the dairy setting, and never as human or animal pathogens.

1. Introduction

The microbiota of cheese comprises a vast array of prokaryotic and eukaryotic organisms and their viruses (Mayo et al., 2021). Cheese-associated eukaryotes include both yeasts and filamentous fungi. The majority of yeast species belong to the genera *Geotrichum*, *Debaryomyces*, *Kluyveromyces*, *Candida*, and *Yarrowia* (Bintsis, 2021; Fröhlich-Wyder et al., 2019), while the dominant filamentous fungi belong to *Penicillium* species, such as *P. camemberti*, *P. roqueforti*, and *P. commune*, etc. (Ropars et al., 2020). Other filamentous fungi of the genera *Fusarium* and *Scopulariopsis* may also be spread throughout cheeses, but in low numbers (Cenci-Goga et al., 2021; Moubasher et al., 2018). Via their complex enzyme systems, some of these organisms contribute to the biochemical reactions of ripening (protein breakdown, fat hydrolysis, and/or lactose metabolism) in certain cheese varieties, helping to produce their typical appearance, taste, and aroma (Afshari et al., 2020; Giraffa, 2021). However, unwanted fungal development can occur, leading to quality defects and even food safety issues by the production of mycotoxins (Ritschard et al., 2018).

The most common cheese-spoiling defects include discolorations, bitterness, crystal formation, and an open (split) texture (Agarwal et al., 2006; Bassi et al., 2015; Kamelamela et al., 2018). These might have different causes and can be modulated by intrinsic and extrinsic factors, such as temperature, pH, humidity, calcium or protein content, storage conditions, etc. Colour defects are usually caused by the accumulation of high concentrations of microbial pigments. Atypically coloured cheeses are frequently reported by both the industrial and small-scale cheese production sectors (Daly et al., 2012), and commonly entail problems of consumer acceptance and economic profitability. Little is known about these pigments or the organisms that produce them, although recently, species of *Proteus* and *Psychrobacter* have been associated with a purple colour defect (caused by indigo and indirubin production from tryptophan) in a surface-ripened cheese (Kamelamela et al., 2018). Similarly, *Thermus thermophilus* isolated from a pink discolouration defect in a Continental-cheese type was shown to produce lycopene, a red carotenoid (Quigley et al., 2016). The latter authors were able to recreate this colour defect using the *T. thermophilus* isolate in a cheese model, unequivocally linking the organism with the defect. In addition, an

* Corresponding author at: Instituto de Productos Lácteos de Asturias (IPLA-CSIC), Paseo Río Linares s/n, 33300 Villaviciosa, Spain.
E-mail address: baltasar.mayo@ipla.csic.es (B. Mayo).

https://doi.org/10.1016/j.ijfoodmicro.2023.110401
Received 30 March 2023; Received in revised form 29 June 2023; Accepted 13 September 2023
Available online 16 September 2023

interaction between *Glutamicibacter arilaitensis* (a bacterium) and *Penicillium* species (fungi) has been shown to cause an overproduction of coproporphyrin III by the former organism (Cleary et al., 2018). This pigment has been identified as the cause of a pink marbling defect on the surface of some smear-ripened aged cheeses. Although bacteria are the cause of most defects, moulds growing on the surface of non-mouldy cheeses, or the development of non-starter strains inside or on the rind of mouldy cheese varieties, can cause aesthetic alterations and produce off-flavours (Daly et al., 2012; Ferrocino et al., 2022). The origin of contaminant moulds may be the starting milk, other ingredients (rennet, calcium chloride, colorants, salt, etc.), or the manufacturing and ripening environments (Garnier et al., 2017).

This work reports a fungus isolated from the surface of a Manchego-type hard cheese made from raw ewe milk in central Spain (Castile-Leon), that caused striking, red-brown spots on the cheese rind. Sampling performed in the cheesemaking factory identified further moulds of the same genus in the bulk tank milk and on equipment and tools in the manufacturing and ripening environments. Molecular identification assigned the isolates to different species of *Epicoccum*, a fungal genus associated with spots on the leaves of different plant species, but rarely reported in cheese.

2. Material and methods

2.1. Cheese sampling and culture conditions

Coloured patches of the cheese surface were sampled with sterile scalpels, and the material obtained was inoculated directly on agar plates or suspended in Ringer's solution (Merck, Darmstadt, Germany) to prepare ten-fold dilutions. Fungi were isolated on plate count agar with skimmed milk (PCAM). Isolates were purified and routinely grown on the same medium or on yeast-extract glucose chloramphenicol (YGC) agar plates (both media from Merck). Hyphae from moulds on the cheese surface were recovered in cotton swabs and inoculated directly into YGC plates. As controls, samples were taken and treated in the same way from non-coloured and non-mouldy areas of the cheese surface.

2.2. DNA extraction and purification

Fungal genomic DNA was extracted from the mycelium after growth on YGC agar plates. Extraction and purification were performed using the QIAmp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with minor modifications. In short, cells were lysed in 1.5 mL of lysis buffer containing 200 mM Tris-HCl, 2 mM EDTA, 2 mM NaCl, and 500 mM SDS. The mixture was then placed in a screw-cap tube containing 0.15 g of both 0.1 and 0.5 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK, USA) and subjected to mechanical breakage in a FastPrep FP120 Cell Disrupter (Qbiogene, Carlsbad, CA, USA) for three cycles at 5.5 m s^{-1} of 40 s, with cooling on ice for 30 s between cycles. This lytic suspension was used to continue with the DNA extraction using the QIAmp DNA Mini kit. Finally, the DNA was eluted with 100 μL of molecular grade water (Sigma-Aldrich, St. Louis, CA, USA) and stored at −20 °C until analysis.

2.3. Molecular identification

Molecular identification was achieved by amplification, sequencing, and comparison of concatenated sequences from four genomic regions against those held in databases. Ribosomal internal transcribed sequences (ITS) were amplified by PCR using primers ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4-R (5'-TCCTCCGCTTATTGATATGC-3'), as reported by Kageyama et al. (1997). Partial amplification of the gene coding for the largest subunit of RNA polymerase II (RPB2) was performed using PCR with primers RPB2-RF2 (5′-GGGGWGAYCAGAAGAAGGC-3′) (Sung et al., 2007) and RPB2-7Cr (5′-CCCATRGCTTGTYYRCCCAT-3′) (Liu et al., 1999). Partial amplification of the genes coding for β-tubulin was performed using the primers Btub2Fd (5′-GTBCACCTYCARACCGGYCARTG-3′) Btub4Rd (5′-CCRGAYTGRCCRAARACRAAGTTGTC-3′) (Woudenberg et al., 2009). Finally, partial amplification of the large subunit of the rRNA molecule (LSU) was accomplished with primers LR0R (5′-GTACCCGCTGAACTTAAGC-3′) and LR5 (5′-ATCCTGAGGGAAACTTC-3′) (Vilgalys and Hester, 1990). Amplification conditions were as reported in the respective referred articles. Amplicons were visualized via agarose (1 %) gel electrophoresis, purified, and sequenced at Macrogen (Madrid, Spain). Sequences were analysed using Chromas software (Technelysium, South Brisbane, Australia) and compared with those of reference taxa in NCBI GenBank using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4. Phylogenetic analyses

Appropriate taxa for the phylogenetic analysis were initially selected following BLAST searches of the GenBank database. Representative DNA sequences from *Epicoccum* species were selected and downloaded to be used as a reference in the phylogenetic analysis. Supplementary Table 1 summarises the list of species, strains, sources, geographical origins and GenBank accession numbers of the reference specimens consulted. Alignments of consensus sequences of the isolates and reference sequences were performed using Molecular Evolutionary Genetics Analysis (MEGA) v.11.0 software (Tamura et al., 2021), using standard settings and employing the MUSCLE algorithm. When necessary, alignments of our isolates were visually inspected and the sequences corrected manually. Finally, phylogenetic relationships of concatenated sequences of the different alignments were determined following the recommendations by Chai et al. (2019) using the maximum composite likelihood algorithm.

2.5. Morphology and phenotypic characterization

The morphological and cultural characteristics of the identified strains were carefully examined on malt extract agar (MEA) (Scharlab, Barcelona, Spain), oat agar (OA) (Merck), potato dextrose agar (PDA) (Merck), and CZAPEK agar (Merck), according to the recent recommendations of El-Sayed et al. (2020). Unless otherwise stated, all cultures were grown at 20 °C for 5–15 days. To allow for sporulation, the strains were incubated further at 25 °C under cycles of 12 h ultraviolet light and 12 h complete darkness for 3 extra days. Conidia were observed using a Leica DMi8 inverted microscope (Leica, Wetzlar, Germany) and photographed under bright field illumination using a Leica DFC 365FX camera.

2.6. Fungal development in cheese slices

Slices of a commercial Gouda-like cheese were exposed to UV light for 15 min in a laboratory cabinet. Slices were then rinsed with sterile water and placed on top of moistened filter papers on a large Petri dish. Finally, mould strains were inoculated on the centre of the cheese slice and incubated at 15–18 °C for up to 10 days.

2.7. Accession numbers

The DNA sequences obtained in this study of the ITS regions, and the LSU, β-TUB, and RPB2 genes from the IPLA 35011, IPLA 35013, IPLA 35014, IPLA 35015, and IPLA 35017 strains were deposited in GenBank database under accession numbers OQ568299-OQ568303, OQ570976-OQ570980, OQ599322-OQ599326, and OQ605790-OQ605794, respectively.

3. Results

The surface of the cheeses at the factory developed patent reddish-

brown spots while ripening in the cold room (Fig. 1, A1). Occasionally, these spots lay under a layer of white mould mycelium (Fig. 1, A2–A3) and were only visible after removing this coating. Development of the spots was usually stronger on cheeses out of the way of air currents generated by the refrigerators. They occupied a thin layer on the surface of the rind without entering the cheese matrix. The cheeses are brushed with olive oil before being sent to market, a process that hid the spots and would have safeguarded against appearance defects and negative purchase attitudes. However, the company wanted to identify the cause of the contamination in case of food safety issues.

Small sections of the spots on the surface of a mature cheese were removed using a sterile scalpel, and cultured on PCAM agar plates. At certain locations on the reverse of some plates, reddish-brown stains were seen against a background yeast-like lawn (Fig. 1, B). Separate yeast-like and filamentous fungus-like colonies were purified on YGC agar plates, but only the latter type produced coloured spots similar to those seen on the cheeses (Fig. 1, C). Amplification, sequencing, and database comparison of the ribosomal ITS regions identified the yeast-form isolate (IPLA 34018) as *Debaryomyces hansenii*, and that of the filamentous form (IPLA 35011) as *Epicoccum* sp. The development of fungal mycelium by *Epicoccum* was markedly reduced when (and thus the red colour of the spots more patent) in the presence of *D. hansenii* (Fig. 1, D). This suggests that the spots on the cheeses with no visible mycelium were formed on top of a strong *D. hansenii* background, which agrees with the large number of yeast colonies on the isolation plates. Association of the coloured spots with *Epicoccum* was demonstrated by inoculation of commercial Gouda-like cheese slices with this strain, which recreated the reddish-brown patches after 8–10 days of incubation at room temperature (Fig. 1, E1–E2).

To assess the colonization and spreading potential of the filamentous *Epicoccum* strain, systematic screening was performed in the factory's manufacturing (milk and milk tanks, cheesemaking cubes, strainer, cheese cloths, presses, brines, etc.) and ripening (cheeses, boxes, cold room walls, olive oil, etc.) environments. Twenty-seven samples were taken directly from the surface of the cheeses, but no moulds grew on YGC agar plates when inoculated with mycelium from spot samples. This suggests poor regeneration of the (young) hyphae of this fungus. Among the yeasts and moulds growing on the plates from the screening of the factory's equipment, only four colonies with an *Epicoccum*-like morphology were recovered, two from brine (IPLA 35013 and IPLA

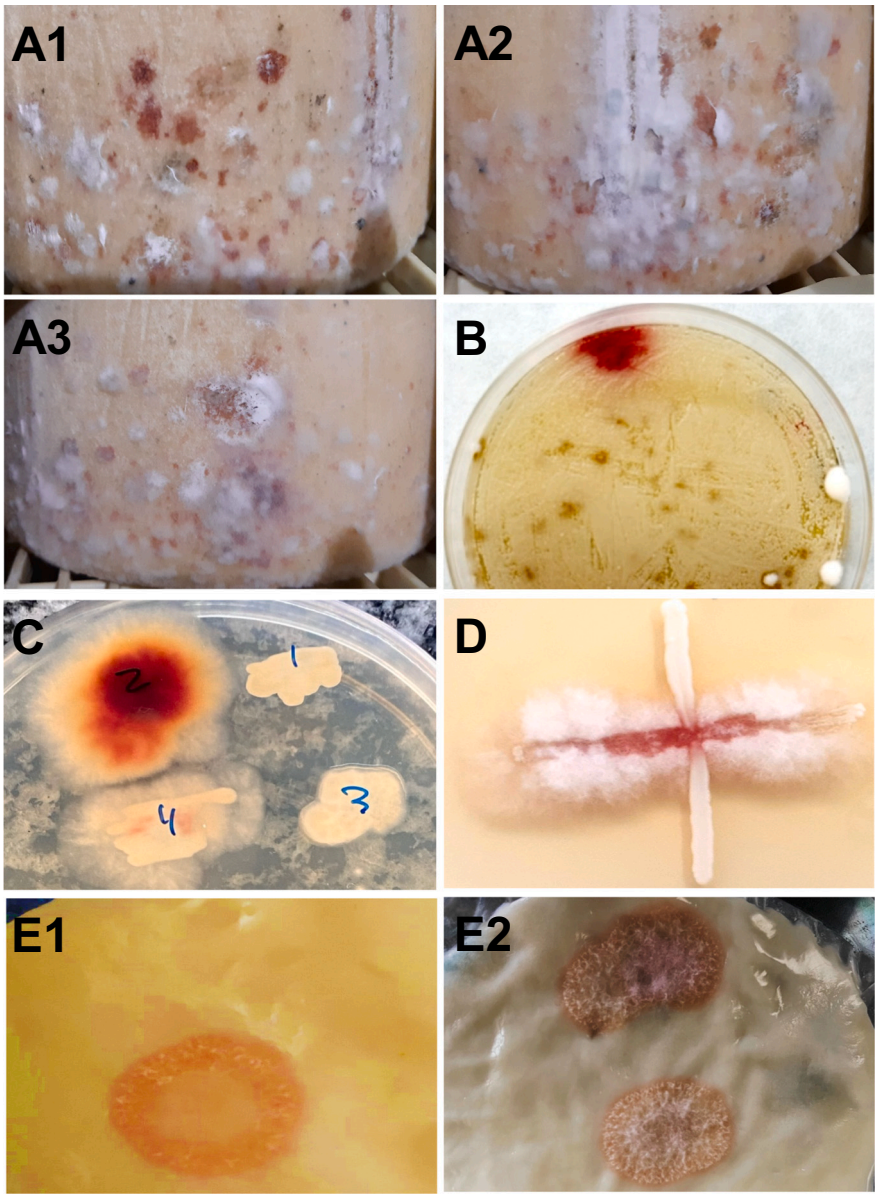

Fig. 1. Composite photography showing cheeses with the reddish-brown colour defect (A1–A3), and different phases of the isolation of specimen IPLA 35011: detection of a reddish-brown agglomerate on Yeast-extract Chloramphenicol Glucose (YCG) agar plates (B), growth of purified colonies of the mould and yeast on YGC plates (C), and cross growth on YGC agar of IPLA 35011 and *Debaryomyces hansenii* IPLA 34018 (D). Development and colour formation of IPLA 35011 (E1) and IPLA 35013 (E2) on Gouda-like cheese slices.

35017) and two from bulk tank ewe milk samples (IPLA 35014 and IPLA 35015). None of these, however, gave the exact colour tone produced on the plates by the isolate from cheese IPLA 35011 on YGC agar. As IPLA 35011, strains IPLA 35013 and IPLA 35014 also produced on cheese slices brown spots similar to those seen on cheese.

Purified DNA from all isolates was used as a template for the amplification and sequencing of four genome segments (ITS, RBP2, LSU, and β-TUB). The sequences obtained were concatenated and compared to those of *Epicoccum* species in databases. The final concatenated alignment contained 12 ingroup taxa (*E. nigrum* with two members) with a total of 1834 nucleotides including gaps (599 for RPB2, 474 for ITS, 473 nucleotides for LSU, and 288 for β-TUB). The phylogenetic clustering indicated the isolates belonged to three different *Epicoccum* species: *E. layuense* (IPLA 35011; isolated from cheese), *E. italicum* (IPLA 35013 and IPLA 35014, isolated from brine and ewe milk, respectively), and *E. mezzettii* (IPLA 35015 and IPLA 35017; isolated from ewe milk and brine, respectively) (Fig. 2).

Although the use of morphology to identify fungi may, in the past, have resulted in the description of an excessive number of species with few differential characteristics (Hibbett et al., 2007), morphology data are still used to characterize new isolates. The detected strains were thus inoculated into the centre of OA, PDA, MEA, and CZAPEK agar plates, and their development at 20 °C was recorded daily. Fig. 3 shows the appearance of the colonies after 10 days of incubation. Notable differences in the colour of the mycelium, as seen from under and above the agar plates, were recorded for the different strains on these media, ranging from white to black on the top side, and from brown to black on the bottom. Colony size, morphology, and overall appearance were also highly variable. Morphotype did not correlate with species; indeed, large differences were seen on some media between IPLA 35013 and IPLA 25014 (both *E. italicum*), and between IPLA 35015 and IPLA 35017 (both *E. mezzettii*).

Epicoccum isolates were grown on YGC plates and then subjected to alternate UV light and darkness cycles to force sporulation and characterize the spores (clamydospores). All isolates but one (IPLA 35017) produced visible black accretions on top of the colonies, and conidia with spores could be observed under the microscope (Fig. 4). The conidia of all isolates looked similar, i.e., spherical-globose, verrucose, dark brown, with a basal cell, and of a diameter of 12.5–28 μm. The appearance and size of the spores agree well with data reported in the literature for *Epicoccum* species (Jayasiri et al., 2017).

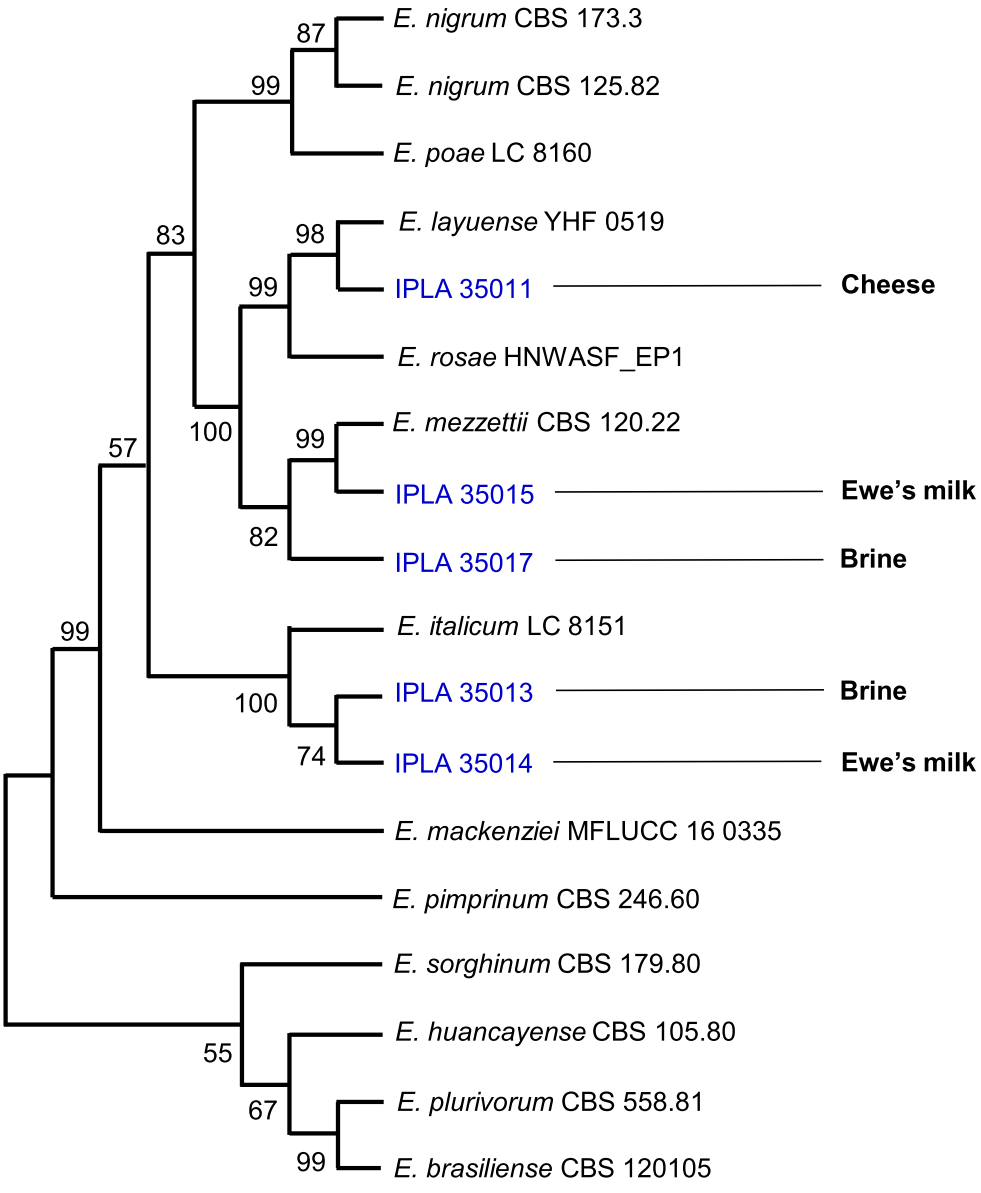

Fig. 2. Phylogenetic relationships of the five fungal isolates of this study (highlighted in blue) and *Epicoccum* specimens of 13 strains representing 12 ingroup taxa of the genus *Epicoccum*. The phylogenetic tree was inferred from the analyses of concatenated alignments of LSU, RPB2, ITS and β-TUB sequences by the Maximum Parsimony method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

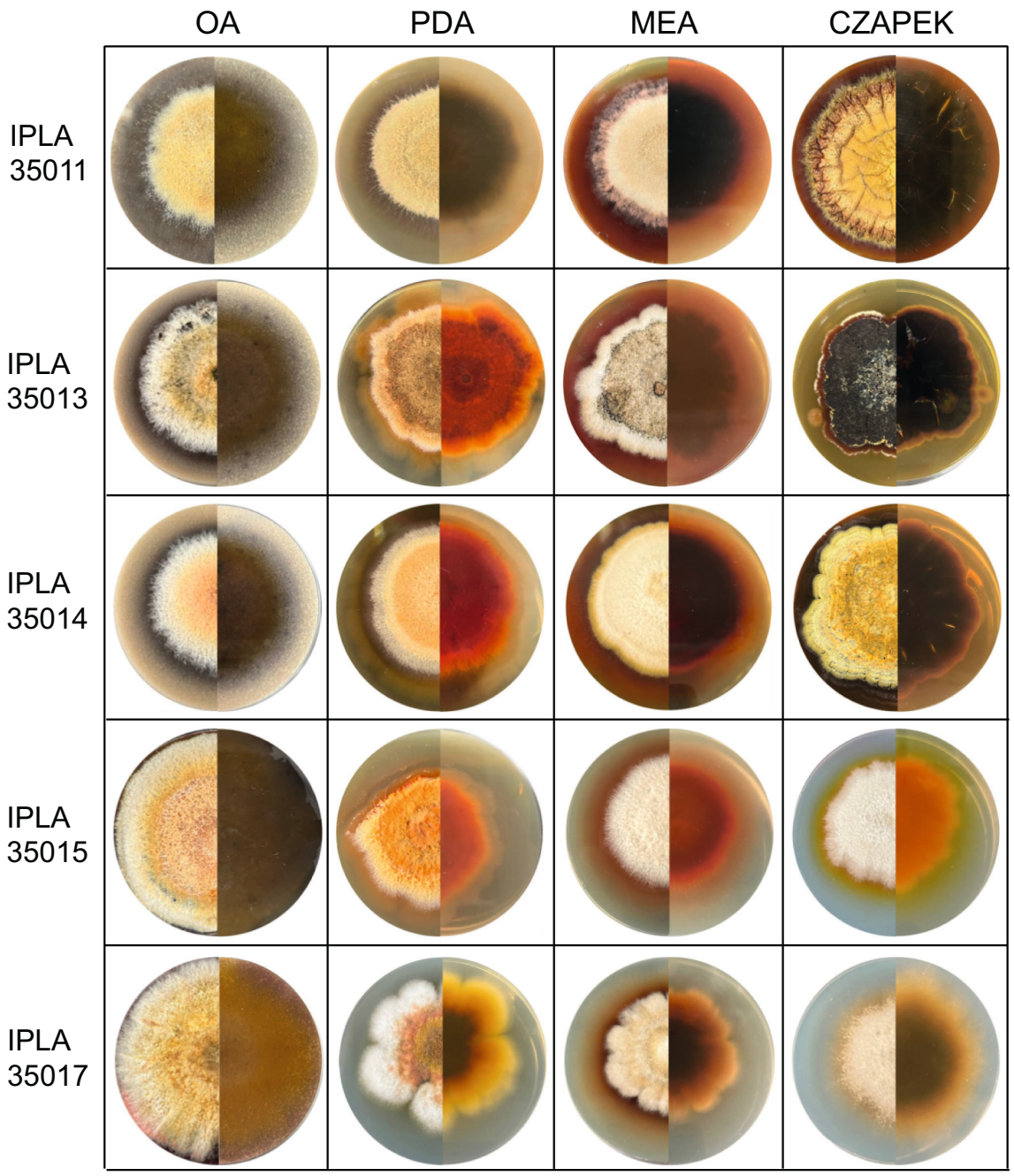

Fig. 3. Colony morphology from front (left) and reverse (right) sides of the plates of the five *Epicoccum* strains identified in this study grown on plates of Oat Agar (OA), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA) and CZAPEK agar, at 20 °C for 5 to 15 days.

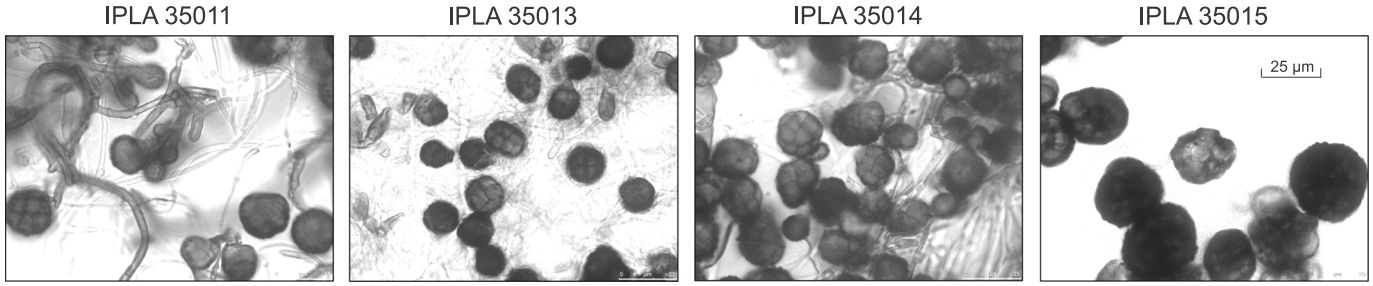

Fig. 4. Optical microscopy of fungal spores from four out of the five *Epicoccum* specimens identified in this study. Magnifications are identical; the scale bar on the right panel applies to all pictures. (Spores for the IPLA 35017 strain were not obtained).

4. Discussion

In this work, five *Epicoccum* isolates belonging to three species were recovered from a cheese factory. *Epicoccum* is a ubiquitous genus typically found in air, soil, and decaying plant material (Braga et al., 2018). Although it can lead to an endophytic lifestyle (Chen et al., 2017), *Epicoccum* has been recently recognized as a widespread plant pathogen. In agreement with this, strains of *Epicoccum sorghinum* have been reported to produce tenuazonic acid (TeA), a potent phytotoxin blocking the photosynthetic pathway (Oliveira et al., 2019). Tenuazonic acid is considered unlikely to be of human health concern (EFSA, 2011). *Epicoccum* species can cause different types of spots (colour, size, etc.) on the leaves (Bernardi et al., 2022; Guo et al., 2020; Han et al., 2021; Liu et al., 2020; Mahadevakumar et al., 2014; Niu et al., 2022; Xie et al., 2022) and other organs of plants (Xiao et al., 2022; Xu et al., 2022).

As reported in the literature (Braga et al., 2018; Oliveira et al., 2019), many *Epicoccum* species secrete during growth pigments such as β- and γ-carotene, epicocconones, epipyrones, and others. Some of these are thought to be the cause of the reddish spots on the present cheeses. Stimulation of carotenoid production in the yeast *Xanthophyllomyces dendrorhous* by the fungus *Epicoccum nigrum* has been reported (Echavarri-Erasun and Johnson, 2004). However, in the present work, pigment production in the filamentous *E. layuense* IPLA 35011 was favoured when co-cultured with the yeast form *D. hansenii* IPLA 34018. *D. hansenii* is among the yeast species with the highest prevalence on the surface of ripened cheeses. Antagonistic and synergistic effects of the yeast on growth and enzyme activity of several mould species have been reported (Fröhlich-Wyder et al., 2019). Most pigments and secondary *Epicoccum* metabolites (e.g., diketopiperazines, epicorazines, epicoccolides, epicocconigrones, epicocconones, epicolactone dimers, epipyrones, flavipins, triornicins, etc.) show strong antimicrobial activity against bacteria and fungi (Lee et al., 2020), for which property certain *Epicoccum* strains have been assessed as biocontrol agents for crops (Braga et al., 2018; Taguiam et al., 2021). Other secondary metabolites made by *Epicoccum* species (e.g., epicoccamides, meroterpenoids, taxol, etc.) have potent activity against cancer cells (El-Sayed et al., 2020; Perveen et al., 2017), and could thus provide a source of novel anticancer drugs.

The isolation of *Epicoccum* moulds from Spanish cheeses has never been reported. Indeed, literature on the detection of *Epicoccum* species in dairy products is scarce. An *Epicoccum purpurascens* isolate was identified in Fontina cheese as part of a microbial consortium associated with a pink discolouration (Carini et al., 1979). Single strains of *E. nigrum* were isolated by Hocking and Faedo (1992) and Kure and Skaar (2000) from Cheddar (out of 195 mould isolates) and Norvegia (out of 159 mould isolates) cheeses, respectively. Two *Epicoccum* sp. isolates have been reported in the fungal communities of milk from cows with mastitis (Costa et al., 1993). Furthermore, few or no *Epicoccum* reads have been reported as components of the cheese mycobiota when examined using high throughput sequencing (HTS) methods (Anelli et al., 2019; Biolcati et al., 2022). As in this study, significant variations on the macro- and micro-morphological features of *Epicoccum* isolates from the same environment have been already reported, with no association to the phylogenetic results (Oliveira et al., 2019).

Certainly, *Epicoccum* is not considered a dairy food spoilage agent (Cenci-Goga et al., 2021; Garnier et al., 2017; Kure et al., 2008). This genus is not included either in the list of priority fungal pathogens by the World Health Organization (WHO, 2022), and no report of its pathogenicity towards animals or humans was found in databases. Nevertheless, *E. nigrum*, a common species in air and dust, can colonize the nasal tissue of humans causing allergic sinusitis (Noble et al., 1997), and indoor exposure to this mould has been correlated with less asthma control (Segura-Medina et al., 2019). Therefore, if any of these symptoms were shown to increase among the workers at the dairy plant, protective measures in the factory should be taken.

5. Conclusions

The fact that a very large number of cheeses in the affected factory showed reddish-brown spots suggests *Epicoccum* had colonized the whole cheesemaking environment. To eliminate such extensive contamination, prolonged, purposeful action will surely be required, perhaps including the treatment of equipment and cheeses with highly active, commercial antifungal agents such as pimaricin (natamycin). One of the strains (*E. layuense* IPLA 35011) was readily isolated from a tainted spot on cheese and could reproduce the colour defect on cheese slices, as did the two *E. italicum* strains (IPLA 35013 and IPLA 35014). Genome analysis of these strains, and comparison with *Epicoccum* isolates of different origins, might provide clues as to whether adaptations in these biotypes allowing colonization of the dairy environment have occurred.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110401.

Declaration of competing interest

The research was conducted in the absence of any conflict interests.

Data availability

No data was used for the research described in the article.

Acknowledgements

This research was supported by projects from the Spanish Ministry of Science and Innovation (PID2019-110549RB-I00/AEI/10.13039/501100011033) and the Principality of Asturias (AYUD/2021/50916; AYUD/2021/57336). A Ph.D. grant from the Severo Ochoa Program of the Principality of Asturias was awarded to J.R. (BP19-098).

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