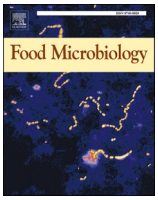
Food Microbiology 123 (2024) 104587

Contents lists available at ScienceDirect

Food Microbiology

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



Analysis of composition and molecular characterization of mycobiota occurring on surface of cheese ripened in Dossena's mine

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ARTICLE INFO

Keywords:
DNA based identification
Beta-tubulin
Metabarcoding
Natural environment
Fungi
Mine

ABSTRACT

Accurate identification of the fungal community spontaneously colonizing food products, aged in natural and not controlled environments, provides information about potential mycotoxin risk associated with its consumption.

Autochthonous mycobiota colonizing cheese aging in Dossena mines, was investigated and characterized by two approaches: microbial isolations and metabarcoding. Microbial isolations and metabarcoding analysis were conducted on cheese samples, obtained by four batches, produced in four different seasons of the year, aged for 90 and 180 days, by five dairy farms. The two approaches, with different taxonomical resolution power, highlighted *Penicillium biforme* among filamentous fungi, collected from 58 out of 68 cheeses, and *Debaryomyces hansenii* among yeasts, as the most abundant species (31 ÷ 65%), none representing a health risk for human cheese consumption. Shannon index showed that the richness of mycobiota increases after 180 days of maturation. Beta diversity analysis highlighted significant differences in composition of mycobiota of cheese produced by different dairy farms and aged for different durations. Weak negative growth interaction between *P. biforme* and *Aspergillus westerdijkiae* by *in vitro* analysis was observed leading to hypothesize that a reciprocal control is possible, also affected by natural environmental conditions, possibly disadvantageous for the last species.

1. Introduction

Moulds are generally considered as undesirable in food, because adversely affect the quality of the final product, through visible or non-visible defects, such as off-odor and -flavor, leading to significant food and economic waste and losses for the manufacturer. Furthermore, they may also affect the health of the consumer, if fungal colonizer is a toxigenic species in particular environmental conditions. However, in some cases, as meat, cheese and plant-fermented products, the spontaneous mycobiota may be considered an added value, because it represents the link from the geographical environment to food and leads development of characteristic colours, flavours and aromas (Capozzi et al., 2017).

In some cheeses, such as Gorgonzola, Camembert, Roquefort, moulds are deliberately added as secondary starters, for the ripening phase, to guarantee optimal maturation condition and appreciated organoleptic characteristics. However, undesired moulds could be introduced into dairy products through environmental cross-contamination pathways or by contaminated ingredients.

In Italy, many cheese traditionally manufactured are ripened in caves by artisanal dairies, exploiting spontaneous colonization by fungal community naturally occurring on cave walls, considered as the link with land of production and local traditions, as well as the responsible for both rheological and sensorial characteristics, highly appreciated by consumers. Mycobiota occurring in caves is mainly composed by *Debaryomyces* spp., *Yarrowia* spp., *Candida* spp., *Kluyveromyces* spp. (De Souza et al., 2021), as well as *Penicillium* and *Aspergillus* species, regrettably including also mycotoxigenic species (Decontardi et al., 2018; Ramos-Pereira et al., 2019; Anelli et al., 2019), namely able to produce toxic metabolites, like ochratoxin A (OTA), penitrem A (PA), roquefortine-C (ROQ-C) and sterigmatocystin (STG) (Dall'Asta et al., 2008; Fontaine et al., 2015; Kalinina et al., 2018; Pietri et al., 2022).

Among *Aspergillus* species contaminating ripened cheese products, the most feared and undesired toxigenic species is *A. westerdijkiae*, producing OTA, a potent renal carcinogen in several animal species (JECFA, 2002; Duarte et al., 2011). However, also *Penicillium* species

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https://doi.org/10.1016/j.fm.2024.104587
Received 28 March 2024; Received in revised form 4 June 2024; Accepted 18 June 2024
Available online 18 June 2024

associated with cheeses, could be toxigenic, such as *P. nordicum*, producing OTA, and *P. verrucosum*, producing OTA and/or citrinin (CIT) depending on growth and environmental conditions (Geisen et al., 2018), and *P. commune*, able to produce cyclopiazonic acid (CPA) and other secondary metabolites (i.e., rugulovasines and viridicatin) (Houbraken et al., 2020). Actually, *Penicillium commune* is not longer used, because it did not form a monophyletic group and has been reported as corresponding to either *P. biforme* or *P. fuscoglaucum* (Ropars et al., 2020), demonstrated to be able to produce also ergot alkaloids (Fabian et al., 2018). The European Union adopted maximum permissible limits for mycotoxins in several foodstuffs, but there are no regulations for mycotoxins in cheese, except for aflatoxin M_1, a hydroxylated derivative of aflatoxin B_1, a specific contaminant of milk, because excreted from lactating animals. However, because of few data available in literature report presence of OTA in cheese, the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM Panel) highlighted the need to increase data on this topic (Schrenk et al., 2020a, b).

With the aims to contribute to increase knowledge about complexity of fungal community associated with cheese ripened in natural and therefore uncontrolled environments, and to find a sustainable alternative to refrigerated cells for maturation, a pilot assay for cheese aging in natural environments was conducted in Dossena mine, for "Ol Minadùr", a typical semi-hard raw milk cheese produced in the Bergamo area.

In this scenario, the identification and characterization of autochthonous mycobiota, with a focus on toxigenic fungi, colonizing cheese ripened in Dossena's mine will contribute to increase knowledge about complexity of fungal community associated with cheese ripened in natural environments. Therefore, the present work investigated and characterized mycobiota of cheeses isolated from surface of a set of 68 cheeses, gathering as many representative samples as possible for different aging seasons (4), dairy farms (5), duration times, (2) in natural (mine) and controlled (refrigerate cell) environment for ripening. Analysing fungal community occurring on surfaces was conducted by metabarcoding approach on surfaces of 23 cheese samples and by microbial taxonomical identification by DNA-based approach, sequencing taxonomical informative DNA loci (beta-tubulin gene for filamentous fungi and ITS for yeasts) on a set of 216 filamentous fungi and 21 yeasts isolated by 68 cheese samples.

2. Materials and methods

2.1. Cheese sampling

"Ol Minadùr" a semi-hard raw milk cheese made by cow's milk, was produced by 5 different dairy farms (M, I, L, G, O) in Dossena's area and ripened simultaneously in factory's cell (C) and in a common cave of Dossena mine (M), for 2 duration times: 90 (T_1) and 180 (T_2) days. This data, related to the origin of sample, are joined to define 23 sample names, showed in Fig. 1. Cheese samples (68) included in the study (Table 1) belonged to 4 production batches: 1 (aged from November to May), 2 (aged from March to September), 3 (aged from May to November), and 4 (aged from July to January).

2.2. Rind cheese sample collection

Three pieces (7 cm^2 each) of rind were collected from lower, upper and lateral surfaces of each wheel of cheese, then soaked in 60 ml of peptone water (0.1%, added with 40 mg/L of streptomycin and 0.025% of Tween 80) and stirred at 200 rpm for 5 min at 20 °C. Tenfold dilutions ($10^{-1} \div 10^{-5}$) were plated on Dichloran Rose-Bengal Chloramphenicol (DRBC) and incubated for 7 days at 25 °C. Single spores pure colonies were obtained and collected to represent fungal and yeast morphological biodiversity, observed on DRBC.

The taxonomic identification at the genus level was conducted on

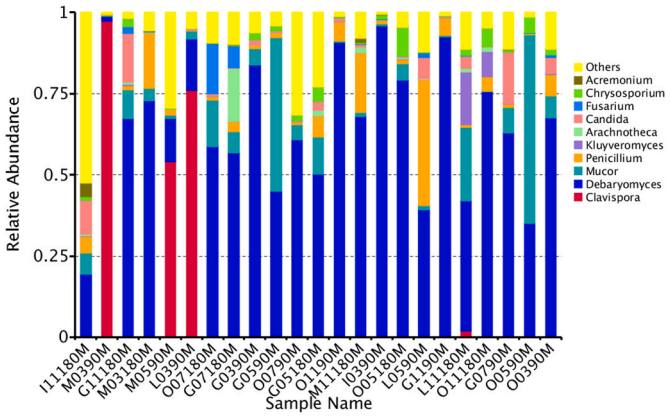

Fig. 1. *Relative abundance of top 10 fungal genera detected on 23 cheeses aged in Dossena's mine.* Genera were ranked based on their prevalence across all samples. The Y-axis represents "Relative Abundance", and X-axis "Samples Name". "Others" represents the cumulative relative abundance of genera outside the top 10. In sample name, first letter indicates the farm, the first two numbers indicate the month of batch production, the numbers 90 or 180 refer to days of aging, and latter letter indicates location of aging, M = mine.

Potato Dextrose Agar (PDA) medium according to the Samson identification keys (Samson, 2011). A set of representative strains, identified as explained below, was deposited in the culture collection at CNR-Institute of Sciences of Food Production (ITEM Collection, http://www.ispa.cnr.it/Collection).

2.3. Isolation and taxonomical identification of mycobiota from cheese surface

2.3.1. Pure culture-independent identification

Strains were cultured for 5–7 days on PDA, collected in 2.0 ml tubes, and subjected to DNA isolation with "Wizard® Magnetic Purification System for Food" kit (Promega, USA), according to manufacturer's protocol (50 mg of fresh mycelium for filamentous fungi or single colony for yeasts).

Regions of beta-tubulin (*BenA*) gene and ribosomal DNA, specifically Internal Transcribed Spacers (ITS), were sequenced for filamentous fungi and yeasts, regarded as reference *loci* in the taxonomy of fungi (Schoch et al., 2012; Samson et al., 2014; Visagie et al., 2014) and yeasts (Martorell et al., 2006; Nguyen et al., 2009). The *loci* were amplified using ITS4/ITS5 (White et al., 1990) and BT2a/BT2b (Glass and Donaldson, 1995). Amplicons were purified using EXO, Exonuclease I, and Thermo Sensitive Alkaline Phosphatase (Thermo Fisher Scientific Inc., USA) and subjected to bidirectional sequencing by Sanger-based sequencing technology, using the BigDye™ Terminator V3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., USA). Alignment of the two strands for each *locus* was performed using the BioNumerics 8.1 software package (Applied Maths, Belgium), with manual adjustments where necessary. After sequences cleaning and trimming, the DNA-based taxonomical identifications were performed by comparison with the non-redundant database, managed by the National Center for Biotechnology Information using the BLAST (Basic Local Alignment Search Tool), when similarity was ≥99%.

2.3.2. Mycobiota composition by metabarcoding analysis

A subset of 23 mine-aged cheese samples (G0390M, G05180M, G0590M, G07180M, G0790M, G11180M, G1190M, I0390M, I11180M, L0390M, L0590M, L11180M, M03180M, M0390M, M0590M, M11180M, O0390M, O05180M, O0590M, O07180M, O0790M, O11180M, O1190M) including representative samples for 4 production batches, 2 ripening times and 5 dairy farms, were analysed by metabarcoding approach, based on ribosomal ITS2 DNA. NGS amplicon-

Table 1
Sampling plan of cheeses and isolated strains.

PRODUCTION BATCH	PRODUCTION DATE – AGING DAYS	AGING PLACE	N° SAMPLES	N° FUNGI	N° YEASTS	ISOLATED SPECIES
1	November – 90d	CELL	4	16	8	*Debariomyces hansenii* *Penicillium biforme*
		MINE	5	21	9	*Debariomyces hansenii* *Penicillium biforme* *Penicillium solitum* *Scopulariopsis flava*
	November – 180d	CELL	4	17	2	*Clavispora lusitaniae* *Debariomyces hansenii* *Penicillium biforme* *Penicillium nalgiovense* *Penicillium solitum* *Yarrowia lipolitica*
		MINE	5	15	2	*Aspergillus versicolor* *Candida* spp. *Debariomyces hansenii* *Penicillium biforme* *Penicillium cvjetkovicii* *Penicillium polonicum* *Scopulariopsis asperula* *S. flava*
2	March – 90d	CELL	4	10	–	*Penicillium biforme* *Penicillium echinulatum*
		MINE	5	16	–	*Penicillium biforme* *Penicillium solitum*
	March – 180d	CELL	2	10	–	*Aspergillus creber* *Penicillium cvjetkovicii* *Penicillium solitum*
		MINE	5	10	–	*Aspergillus creber* *Penicillium solitum* *Schyzophillum* spp. *Scopulariopsis candida*
3	May – 90d	CELL	5	12	–	*Fusarium oxysporum* *Penicillium biforme* *Penicillium cvjetkovicii* *Penicillium solitum*
		MINE	5	17	–	*Penicillium biforme* *Penicillium citrinum* *Penicillium solitum*
	May – 180d	CELL	4	10	–	*Aspergillus creber* *A. ostianus* *Fusarium oxysporum* *Penicillium biforme*
		MINE	4	13	–	*Penicillium biforme* *Penicillium solitum* *Scopulariopsis flava*
4	July – 90d	CELL	4	13	–	*Aspergillus creber* *A. ostianus* *Fusarium oxysporum* *Fusarium proliferatum* *Penicillium biforme* *Penicillium cvjetkovicii*
		MINE	5	10	–	*Penicillium biforme* *Penicillium solitum* *Scopulariopsis flava.*
	July – 180d	CELL	3	8	–	*Aspergillus creber* *Fusarium proliferatum* *Penicillium biforme*
		MINE	4	18	–	*Penicillium biforme* *Penicillium solitum*
		total	**68**	**216**	**21**	

sequencing and bioinformatics analysis were commissioned to Novogene Co. (UK). DNAs for metabarcoding analysis were isolated using Clonit kit (CLONIT srl, Italy), according to the manufacturer's instructions, starting from 500 μl of surface washing solution (2.2). Quantity and purity of the total DNA were evaluated by spectrophotometric analysis (NanoDrop Technologies, USA), Qbit fluorimeter (Invitrogen, Carlsbad, USA) and agarose gel electrophoresis. ITS2 region amplification, library preparation and NGS sequencing were performed on each sample by Novogene.

Generated data from 23 samples were conveniently selected, varying the parameter to be correlated with composition of the fungal community, to obtain 3 sets of data, including samples for different starting season of aging period (data set A), aging time duration (data set B), and dairy farms (data set C). Libraries were sequenced on a paired-end Illumina platform to generate 250 bp paired-end raw reads. The library was checked with Qubit and real-time PCR for quantification and with bioanalyzer for size distribution detection, pooled and sequenced on Illumina platforms.

Paired-end reads were assigned to samples based on their unique barcode and truncated by cutting off barcode and primer sequence. Quality filtering on the raw reads was performed under specific filtering conditions to obtain the high-quality clean reads. Paired-end clean reads

were merged using FLASH (Fast Length Adjustment of SHort reads) software V1.2.7, based on the overlapping of reads (Magoč and Salzberg, 2011). Then, the merged tags were filtered to remove chimera and to obtain non-chimera tags. The pairs of reads obtained were also assembled in the case of DNA fragments shorter than twice the length of the reads. The tags were compared with the reference database (Unite V8.2), using the UCHIME algorithm, to detect of chimeric sequences, i.e. artefacts of PCR and sequencing (Edgar, 2013). Finally, the chimeras were removed from the assembled sequences and the Effective Tags finally obtained. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags according to the Qiime pipeline (V1.7.0) quality-controlled process (Caporaso et al., 2010).

Sequences analyses were performed by UPARSE (V7.0.1090) (Edgar, 2013) using all the effective tags. Sequences with ≥97% similarity were assigned to the same operational taxonomic units, or OTUs. Representative sequence for each OTU was screened for further annotation.

Sequences analyses were performed by BLAST with blastall (V2.2.25) and the Unite database V8.2 for species annotation. To obtain the phylogenetic relationship of all OTUs representative sequences, MUSCLE (V3.8.31) can compare multiple sequences rapidly. OTUs abundance information were normalized using a standard of sequence number corresponding to the sample with the least sequences, and expressed as value in the range 0–1. According to the results of OTU clustering, a taxonomic annotation is assigned to the representative sequence of each OTU, obtaining information on the corresponding taxa and their distribution. The top 10 taxa of each sample or group at each taxonomic rank (genus) were considered to obtain the distribution histogram of relative abundance of taxa.

OTUs were analysed for Alpha and Beta diversity analyses and community variance statistics (Adonis test), to obtain richness and evenness information in samples, common and unique OTUs information

among them. The differences between samples or among groups about structure of fungal community

is explained via dimension reduction (PCoA, PCA and NMDS) and UPGMA, using Unweighted and.

Weighted UniFrac as distance metrics. Statistic methods such as T-test, MetaStat, LEfSe, Anosim and MRPP test the significance of structure of microbial community differences between groups.

2.4. Determination of ergot alkaloids and epimers

Standards of ergometrine (EM), ergosine (ES), ergotamine (ET), ergocornine (ECO), ergocryptine (EKR), ergocristine (ECR), and their epimers ergometrinine (EMN), ergosinine (ESN), ergotaminine (ETN), ergocorninine (ECON), ergocryptinine (EKRN) and ergocristinine (ECRN) were purchased from Romer Laboratories (Getzersdorf, Austria).

Major EA and their -inine forms were measured in T_1-aged cheese surfaces (9 sample from batch1) and *Penicillium biforme* pure cultures (DS1, DS6, ITEM19058, DS15, DS19, DS23, DS28, DS33, DS37 isolated from the same cheeses) using Ultra-Performance Liquid Chromatography-Ion Mobility Separation-Quadruple Time-of-Flight MS (UHPLC-IMS-QTOF MS) mass spectrometer. Eeach fungal strain pure culture was performed as follows: a conidial suspension [10^5 spores/plate] was inoculated on Czapek Yeast Autolysate (CYA) agar (Pitt, 1979) and incubated at 25 °C for 14 days in the dark.

Standards of ES, ECO, EKR, ECR and the corresponding epimers, ESN, ECON, EKRN, ECRN, were purchased from Techno Spec (Spain), whereas EM, ET, EMN and ETN were obtained from Romer Labs (Austria). Standards were stored at −20 °C to avoid EA epimerization and reconstituted in the required amount of acetonitrile (MeCN) just before use. LC-MS grade MeOH and MeCN were purchased from Scharlab Italia S. r.l (Italy). Bi-distilled water was obtained using Milli-Q System (Millipore, Bedford, USA). Formic acid from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, USA) was also used. Dispersive sorbents used in the sample treatment procedure, namely Z-Sep+ and C18, were supplied from Supelco (Bellefonte, USA) and Agilent Technologies (Italy), respectively.

For sample treatment, cheeses and inoculated medium were freeze-dried prior to the sample preparation, then an aliquot (1.0 g) underwent the sample preparation described in our previous works (Carbonell-Rozas et al., 2021, 2022).

The analysis was performed on an ACQUITY I-Class UPLC separation system coupled to a Vion IMS-QTOF mass spectrometer (Waters®, UK) equipped with an ESI interface. The chromatographic separation was carried out in an Acquity UPLC® System from Waters® using a reversed-phase C18 BEH ACQUITY column (50 × 2.1 mm, 1.7 μm particle size) from Waters® (Milford, USA). The analysis was performed under the same conditions previously validated in our work (Carbonell-Rozas et al., 2022). Applied conditions allowed for the stability of the EAs, to avoid epimerization during analysis.

2.5. In vitro growth interaction between P. biforme and A. westerdijkiae

Dual-culture assay was set up to investigate reciprocal antifungal activity between *P. biforme* and *A. westerdijkiae*. Fungal cultures were maintained in purity on PDA slants at +5 °C which were used for preparation of fresh cultures and inocula. The *P. biforme* (ITEM19058), isolated from batch1, and 4 *A. westerdijkiae* strains (ITEM18008, ITEM17419, ITEM17441, ITEM19080), previously isolated from cheese, were included in this assay (Table 2).

Two species agar plugs (Ø 6 mm) from fresh cultures with mycelium were transferred in Petri dishes of 90 mm containing 20 ml of PDA (dual-plate). Plugs were positioned symmetrically, on each side of the plate, at 4.5 cm apart from each other. All the co-cultures were incubated at 20 °C in the dark and the experiment was carried out in triplicate. As positive control, each isolate was individually inoculated on PDA. The radial growth of the tested fungus was recorded every day and measurements taken on the day before contact, or after 10 days if no contact between colonies occurred. The growth inhibition rate (IR) was then calculated, using the following formula: IR (%) = $(R_1–R_2)/R_1$ x 100; where R_1 is the furthest radial distance grown by *P. biforme* in the direction of *A. westerdijkiae*, measured from the center of the colony to the control Petri dish center (control value); R_2 is the distance fungal grown on a line between inoculation positions of the strains (inhibition value).

The type of colony interaction was evaluated visually and measured after 10 days' inoculation. Assessment was based on the following scale, previously reported by Skidmore and Dickinson (1976): 1. fungal mutual growth without macroscopic interaction areas; 2. fungal mutual growth, in which tested fungal growth is interrupted by the opposite fungus; 3. reciprocal growth, where tested fungus grows above or below the opposite fungus; 4. obvious mutual inhibition (inhibition zone ≈ 2 mm distance); 5. mutual growth inhibition (inhibition zone >2 mm distance).

Table 2
Fungal strains included in the dual-culture assay.

ITEM ID	SPECIES	GEOGRAPHICAL ORIGIN	MYCOTOXINS
19058	*P. biforme*	Dossena (Italy)	EA-
18008	*A. westerdijkiae*	Gravina di Puglia (Italy)	OTA+
17419	*A. westerdijkiae*	Martina Franca (Italy)	OTA-
17441	*A. westerdijkiae*	Altamura (Italy)	OTA+
19080	*A. westerdijkiae*	Gioia del Colle (Italy)	OTA+

EA: ergot alkaloids; OTA: ochratoxin A.
*The strains with ITEM numbers are from the culture collection of the Institute of Sciences of Food Production (ITEM Collection, http://www.ispa.cnr.it/Collection).

3. Results

3.1. Identification of representatives mycobiota isolated from mine- and cell-aged cheeses surfaces

The estimation of viable fungal population was determined by enumeration of fungal colonies on a selective medium for filamentous fungi (DRBC) after 7 days of incubation at 25 °C. Out of 68 analysed cheeses, 33 revealed total fungal counts ranging from $1{,}00x10^5$ to $0{,}99x10^6$ cfu/cm^2, 23 from $1{,}00x10^4$ to $0{,}99x10^5$ cfu/cm^2, 6 from $1{,}00x10^3$ to $0{,}99x10^4$ cfu/cm^2 and 6 from $1{,}00x10^6$ to $0{,}99x10^7$ cfu/cm^2 (Table 3).

The molecular identification revealed 216 isolates belonging to different genera: *Penicillium* (179), *Scopulariopsis* (18), *Aspergillus* (10), *Fusarium* (7), *Moniliella* (1) and *Schyzophillum* (1). Fungal genera assignments for 42 out of 216 mold strains were considered reliable based on the beta-tubulin gene sequence (Woudenberg et al., 2017; Houbraken et al., 2020), utilizing both BLAST and phylogenetic analyses (performed for 214 strains, excluding *Moniliella* spp. and *Schyzophillum* spp., Supplementary Fig. 1) including type or reference strains for species identified by BLAST or closely related: *A. creber*, *A. ostianus*, *A. versicolor*, *F. oxysporum*, *F. proliferatum*, *P. biforme, P. citrinum*, *P. cvjetkovicii*, *P. echinulatum*, *P. nalgiovense*, *P. polonicum*, *P. solitum*, *S. asperula*, *S. candida*, *S. flava*. .

Some of the identified species were isolated from a single cheese sample, as *A. versicolor* (1/68), *P. citrinum* (1/68), *P. echinulatum* (1/68), *P. nalgiovense* (1/68), *P. polonicum* (1/68), *S. candida* (1/68), *S. asperula* (1/68), while others, *P. biforme* (58/68), *P. solitum* (19/68) and *Scopulariopsis flava* (13/68), *A. creber* (3/68), *P. cvjetkovicii* (4/68), *F. oxysporum* (4/68), *F. proliferatum* (2/68), *A. ostianus* (2/68), were found in many of the 68 analysed cheeses (Table 1).

P. biforme was the species mainly occurring on cheese (25/30 cell-aged and 33/38 of mine-aged samples), followed by *P. solitum*, occurring mainly on cell-aged (16/30) and at lesser extent in mine-aged cheese samples (3/38), and *Scopularipsis* spp., occurring only on mine-aged cheese (14/38) (Table 1). Furthermore, *Fusarium* species have been isolated only from cell-aged cheese and *Aspergillus* species from both mine- and cell-aged cheese.

Similarly, limiting analysis to the batch1 (Table 1), the identification of 21 yeasts revealed the presence of *Debayomyces* in all tested samples, but also other genera were detected: *Clavispora*, *Candida* and *Yarrowia*, identified only on T_2 mine and cell-aged cheeses; *Debaryomyces* (99.8% identity with *D. hansenii* CBS767, MH545920), *Clavispora* (99.7% with *C. lusitaniae* CBS6936, HM156518), *Candida* (99.2% with *C. zeylanoides* CBS619, KY102539), *Yarrowia* (98.7% with *Y. lipolytica* CBS6124, AM279233).

Table 3
Plate counts of viable fungal populations. In sample name, first letter indicates the farm, the first two numbers indicate the month of batch production, the numbers 90 or 180 refer to days of aging, and latter letter indicates location of aging, M = mine and C = refrigerated cell.

FUNGAL COUNT RANGES (cfu/cm^2)	CHEESE SAMPLES
$1{,}00x10^3 \div 0{,}99x10^4$	G11180C, I1190C, L07180C, O0390C, O05180C, O11180C
$1{,}00x10^4 \div 0{,}99x10^5$	O1190M, G0390C, G0390M, G07180C, G07180M, G0790C, G1190C, I03180M, I07180M, I11180C, M03180M, M0390C, M05180C, M0590C, M07180M, M11180M, O03180C, O03180M, O05180C, O07180M, O0790C, O11180M, O1190C
$1{,}00x10^5 \div 0{,}99x10^6$	G03180M, G05180C, G05180M, G0590C, G0590M, G0790M, G11180M, G1190M, I0390M, I05180C, I05180M, I0590C, I0590M, I0790M, I11180M, I1190M, L05180M, L0590C, L0590M, L0790M, L11180C, L11180M, L1190C, L1190M, M0390M, M0790C, M0790M, M1190M, O0390M, O05180M, O0590M, O07180C, O0790M
$1{,}00x10^6 \div 0{,}99x10^7$	L03180C, L03180M, L0390C, L0390M, L0790C, M0590M

3.2. Mycobiota composition of mine-aged cheeses by metabarcoding analysis

The relative abundance of biodiversity of mycobiota at the genus level (Fig. 1), confirmed the presence of *Debaryomyces*, and highlighted it as the most spread genus, occurring in all tested samples. In 17/23 samples it was also the most prevalent genus (>0.4, in the range 0,96 ÷ 0,02) while in 6/23 it was detected at lower levels.

Mucor was the second most spread taxa on tested cheeses, included in all analysed samples. In 2/23 samples it was also the most detected taxa (>0,4, in the range 0,580 ÷ 0,001) and in 21/23 it was present at lower levels. Only in 3 samples *Clavispora* was the most detected taxa (>0,4, in the range 0,97 ÷ 0), and in 6/23 samples all aged in mine was not detected at all.

Other genera were detected at low levels (<0,4) in a variable range of relative abundance: *Penicillium* (0,37 ÷ 0), *Candida* (0,17 ÷ 0), *Kluyveromyces* (0,16 ÷ 0), *Arachnotheca* (0,16 ÷ 0), *Fusarium* (0,16 ÷ 0), *Chrysosporium* (0,09 ÷ 0), *Acremonium* (0,04 ÷ 0). The analysis identified also additional genera not listed among the first 10 ones, generically grouped in "Others" and ranging from 0,52 to 0 in the cheese samples.

The taxonomic annotation of the OTUs (based on abundance top 10 genera) allowed identifications at species level (data not shown), even though in same cases results should be considered under reserve, due to the limited power of ITS in species resolution.

Focusing on potential toxigenic fungal genera, *Fusarium armeniacum* and *Penicillium camemberti* were detected. Among yeast species, *Debaryomyces prosopidis* was the most abundant species, followed by *Mucor lanceolatus, Clavispora lusitaniae*, *Candida sake*, *Candida santamariae*, *Candida zeylanoides*, *Chrysosporium sulphureum.*

3.2.1. Mycobiota related to seasons of cheese production (data set A)

The data set generated by 23 mine-aged cheeses was analysed to evaluate possible differences in composition of fungal community related to 4 cheese production seasons. The 23 samples were grouped in 4 batches, produced in November, March, May and July.

Alpha and beta diversity indexes did not show any significant differences according to seasons of cheese production. At the class level, *Mucoromycetes* were 20.7% more abundant in the May group than in the July group (8%). *Sordariomycetes* were more common in the July group (14.9%) than in May (4%). *Eurotiomycetes* also showed a significant difference, with higher relative abundance in May (13.8%) than in July (8.2%). Further dissecting the data at the genus level, *Clavispora* stood out as being more abundant in May (9%) than in July (0.02%). *Debaryomyces*, on the other hand, was more abundant in July (59.6%) compared to May (43.3%). *Mucor* also exhibited a significant variation, with a predominance of 20.7% in May and 8.3% in July.

The relative abundance of fungal biodiversity at the genus level (Fig. 2), revealed the *Debaryomyces* as the most spread and abundant (>0,4, in the range 0,65 ÷ 0,43) genus through all batches, followed by other genera with different distributions among the seasons. *Debaryomyces* showed the highest relative abundance in cheeses included in batch1, aged from November to May (0,65), and the lowest in cheeses of batch3, aged from May to November (0,43). Other genera were detected at low levels (<0,4) in a variable range of relative abundance: *Clavispora* (0,29 ÷ 0), *Mucor* (0,21 ÷ 0,03), *Penicillium* (0,08 ÷ 0,02), *Fusarium* (0,06 ÷ 0), *Candida* (0,04 ÷ 0,02), *Kluyveromyces* (0,04 ÷ 0), *Arachnotheca* (0,04 ÷ 0), *Chrysosporium* (0,03 ÷ 0,01), *Acremonium* (0,01 ÷ 0). The analysis identified also additional genera not listed among the first 10 ones, generically grouped in "others" and ranging from 0,16 to 0,05 in the cheese samples.

3.2.2. Mycobiota related to aging time duration (data set B)

The data set generated by 23 mine-aged cheeses was analysed to

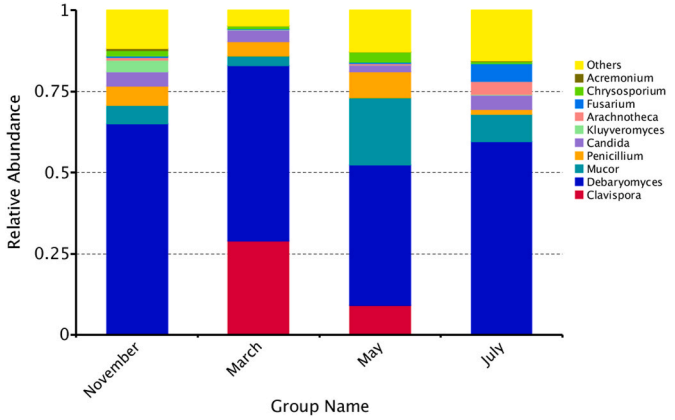

Fig. 2. *Relative abundance of top 10 fungal genera detected for data set A, related to seasons of cheese production.* Genera were ranked based on their average prevalence within each season. The Y-axis represents "Relative Abundance" and X-axis represents "Group Name" (November = batch1, March = batch2, May = batch3, July = batch4). "Others" represents the cumulative relative abundance of genera outside the top 10.

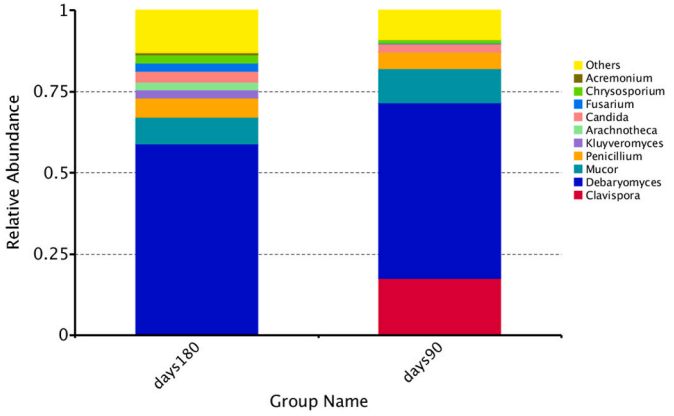

Fig. 3. *Relative abundance of top 10 fungal genera detected for data set B, related to aging time duration.* Genera were ranked based on their average prevalence within each aging time group. The Y-axis represents "Relative Abundance" and X-axis represents "Group Name" (T_1 = days90 and T_2 = days180). "Others" represents the cumulative relative abundance of genera outside the top 10.

evaluate possible differences in composition of fungal community related to 2 aging duration times. The 23 samples were grouped in 2 batches, one including cheese ripened for 90 days (T_1) and one cheese ripened for 180 days (T_2).

Alpha diversity indexes did not show any significant differences in mycobiota composition according to the aging time duration. The only significant difference concerned the Shannon index, which showed that the microbial community was richer at T_2. According to beta diversity analysis, there were significant differences between mycobiota of cheese with different aging time duration. At the class level, *Saccharomycetes* were more abundant at T_1 (77.3%) than T_2 (66.2%). *Mucoromycetes* were slightly more common at T_1 (10.5%) than T_2 (8.1%). *Sordariomycetes* and *Eurotiomycetes*, on the other hand, showed differences, with *Sordariomycetes* being more abundant at T_2 (10.1%) than T_1 (1.8%), and *Eurotiomycetes* being more abundant at T_2 (12.1%) than T_1 (7.3%). At the genus level, *Clavispora* showed a significant difference, being substantially more prevalent at T_1 (17.5%) than T_2 (0.25%). *Penicillium* abundance increased somewhat from T_1 to T_2 (5.9% vs 4.9%). *Kluyveromyces* showed a significant difference, with substantially greater abundance at T_2 (2,5%) compared to T_1 (0,097%). Similarly, *Arachnotheca* showed increased abundance at T_2 (2.4%) compared to T_1 (0.096%).

Debaryomyces genus (0,59 ÷ 0,54) showed the highest relative abundance, followed by *Mucor* (0,10 ÷ 0,08) and *Penicillium* (0,06 ÷ 0,04), both on T_1 and at T_2-aged samples. Differentely, *Kluyveromyces* (0,03 ÷ 0,00), *Chrysosporium* (0,03 ÷ 0,01), *Candida* (0,03 ÷ 0,02) genera showed low values of relative abundance on T_1-aged cheeses while *Clavispora* (0,18 ÷ 0,00) showed higher values on T_2-aged cheeses (Fig. 3). *Arachnotheca*, *Fusarium*, and *Acremonium* detection is negligible both on T_1 and at T_2-aged samples (≤0,02). The analysis identified also genera not listed among the first 10 ones, generically grouped in "Others" and ranging from 0,13 to 0,09 in the cheese samples.

3.2.3. Mycobiota related to dairy farms (data set C)

The data set generated by 23 mine-aged cheeses was analysed to evaluate possible differences in composition of fungal community related to 5 dairy farms. The 23 samples were grouped in 5 batches, produced by 5 different dairy farms.

Alpha diversity indexes did not show any significant differences in mycobiota composition according to the dairy farms. According to beta diversity analysis, there were significant differences between mycobiota of cheese produced by different dairy farms. At the class level, *Saccharomycetes* were found to be more prevalent in dairy farm M (84.8%) than other, such as G (71.1%), L (69.9%), and O (68.9%). *Mucoromycetes* were more abundant in G and O (12.6% each) than M (1.6%) and L (8.8%). G, L, and O have a higher prevalence of *Sordariomycetes* (2–5%) than M (0.8%). *Eurotiomycetes* were more abundant in M and L (11.5% and 16.9%, respectively) than G and O (7–8%). At the genus level, *Clavispora* was substantially more abundant in M and L (37.9% and 25.9%, respectively) than in G (0.008%) and O (0.02%). *Debaryomyces* was much more abundant in G and O (65.2% and 65%, respectively) than M (39.2%) and L (31%). *Mucor* was more abundant in G and O (12.6% in both), compared to M (1.5% and L, 8.8%). L and M had greater *Penicillium* abundances (12.5% and 9.5%, respectively) than G and O (2.8%).

Debaryomyces is the most spread and abundant genus (0,65 ÷ 0,31) among the 23 cheese samples produced by the 5 dairy farms, while *Clavispora* (0,38 ÷ 0) was detected only on cheeses produced by dairy farms M and L (Fig. 4). Other genera were detected at low levels (<0,38) in a high variable range of relative abundance: *Mucor* (0,13 ÷ 0,02), *Penicillium* (0,13 ÷ 0,03), *Fusarium* (0,02 ÷ 0), *Candida* (0,05 ÷ 0), *Kluyveromyces* (0,05 ÷ 0), *Arachnotheca* (0,03 ÷ 0), *Chrysosporium* (0,03 ÷ 0). *Acremonium* detection is negligible among the 23 cheeses produced by the 5 dairy farms (<0,02). The analysis identified also genera, not listed among the first 10 ones, generically grouped in "Others" and

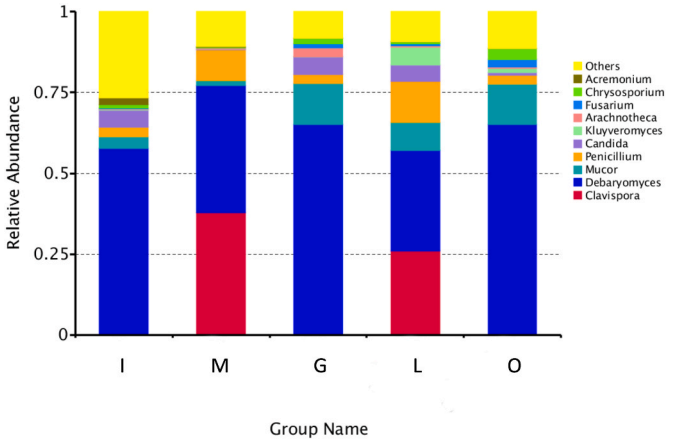

Fig. 4. *Relative abundance of top 10 fungal genera detected for data set C, related to dairy farms.* Genera were ranked based on their average prevalence across samples from different farms. The Y-axis represents "Relative Abundance" and X-axis represents "Group Name" (M, I, L, G, O). "Others" represents the cumulative relative abundance of genera outside the top 10.

ranging from 0,27 to 0,08 in the cheese samples.

3.3. Determination of ergot alkaloid in fungal cultures and cheese samples

None of the *P. biforme* tested strains (9) were able to produce ergosine (ES), ergocristine (ECR), ergocryptine (EKR), ergocornine (ECO), ergometrine (EM) and ergotamine (ET) and related epimers.

Levels of tested EA and related epimers, potentially produced by *P. biforme* and defined by the European Food Safety Authority as those that are the most common and physiologically active, were not detected neither in cheese rinds.

3.4. In vitro growth interaction between P. biforme and A. westerdijkiae

The interaction between *P. biforme* (strain ITEM19058) and *A. westerdijkiae* (strains ITEM18008, ITEM17419, ITEM17441, ITEM19080) was investigated, in order to evaluate the possible *in vitro* trophic relationship between the 2 species. The test was conducted for 10 days, when the two isolates growth was balanced, no further alterations were highlighted, and the growth inhibition rate (%IR) was about 30% for both. In particular, for all tested strains of *A. westerdijkiae*, OTA producers and non-producers, the growth inhibition rate was 31.4% while for *P. biforme* it was 31.1% The reciprocal growth inhibition area between the two fungi (Fig. 5), calculating as 5 mm distance (type 5 interaction), resulted therefore as a weak mutual growth negative interaction.

4. Discussion

Cheese is one of the most widely consumed dairy products worldwide, especially in Europe, and is one of the excellences of Italy, the third EU producer, accounting 1.3 million tonnes of cheeses in 2022 (Eurostat, 2022). It is generally regarded as an unfavourable matrix for mycotoxin contamination (Delavenne et al., 2011; Vacheyrou et al., 2011), except for aflatoxin M1, as carryover of the milk (Vaz et al., 2020). However, ochratoxin A, produced by filamentous fungi in *Aspergillus* and *Penicillium* genera has been reported in cheese (Anelli et al., 2019; De Souza et al., 2021). In natural-aged cheeses, traditionally the autochthonous mycobiota is generally considered as safe and has role of added value, representing the link between product and local environment and traditions, providing specific sensory properties. However, it is not excluded that autochthonous mycobiota could include mycotoxigenic species, therefore the use of safe and pro-technological moulds, as secondary starters, should be encouraged in traditional and uncontrolled dairy chain. Selection of those strains should be based on

Fig. 5. *In vitro growth interaction between P. biforme* (DS10=ITEM19058) and *A. westerdijkiae* (EL166 = ITEM19080). Dual-plate assay on PDA, showing a mutual inhibition zone after 10 days incubation at 20 °C.

atoxigenicity and pro-technological characteristics, e.g. lipolitic proteolitic abilities, to keep the link to environment and traditions, without renouncing quality and safety of product (De Miranda et al., 2023; Martin and Cotter, 2023).

In this study, identification and characterization of autochthonous mycobiota colonizing cheese ripened in Dossena's mine was conducted with the aims to: contribute to increase knowledge about complexity of fungal community associated with cheese ripened in natural, and therefore not controlled, environments; to find natural locations, like Dossena mine, exploitable for a sustainable step in the dairy chain; isolate indigenous strains able to colonize "Ol Minadùr" cheese in Dossena's mine and simultaneously able to contrast the presence of undesirable toxigenic species, *A. westerdijkiae*, frequently reported on cheese and associated with OTA contamination.

Attention was focused on occurrence of toxigenic fungal species and therefore isolations were addressed to strains with morphological features ascribable to *Aspergillus* and *Penicillium,* commonly occurring on cheeses (Anelli et al., 2019; Ramos-Pereira et al., 2019; De Souza et al., 2021).

The microbiological isolations allowed specific taxonomical assignments, leading to unambiguous species identification, consisting mainly of *Penicillium*, some *Aspergillus* species, and few species belonging to *Fusarium* and *Scopulariopsis*.

The most widespread species on the 68 tested cheeses resulted *Penicillium biforme*, a sister species of *P. camemberti,* hypothesized to be the result of domestication on cheese of wild *P. fuscoglaucum* (Ropars et al., 2020), acquiring whiter colour, faster growth on cheese medium under cave conditions, and lower amounts of toxin production. Indeed, even though *P. biforme* has functional biosynthetic pathway for biosynthesis of EAs, their accumulation is not reported in cheese for this species, likely due to a nutrition-driven gene regulation preventing production in dairy products (Fabian et al., 2018). In this work, the ability of *P. biforme* to produce alkaloids deriving from lysergic acid (ergopeptine), the regulated alkaloids in barley, wheat, spelled, rye and oats and in processed cereals foods (Regulation EU, 2021/1399), was also evaluated. The presence of those metabolites was not detected, neither in synthetic medium (CYA), neither in cheese samples naturally contaminated by *P. biforme*, confirming it as safe species for cheeses aging process.

Also *P. solitum* was isolated either in cell-aged and mine-aged cheeses, but it was not considered as potential species to guide cheese aging, because it can also cause cheese spoilage and because it is a potential producer of metabolites of toxicological interest, due to the presence in its genome of secondary metabolites gene clusters (Wu et al., 2019).

Among *Aspergillus* species, *A. ostianus*, an OTA non-producer species, because of lack of OTA biosynthetic gene cluster, even though previous studies reported it as OTA weak producer (Gil-Serna et al., 2020). For some other *Aspergillus* strains, identification with beta-tubulin gene allowed assignment to *A. versicolor* and *A. creber*, including possible sterigmatocistin producers (Jurjević et al., 2013). A precise identification could be possible using multiple DNA markers, but we overlook it because strains came from cell-aged cheese.

Other species sporadically isolated from tested cheeses, mainly cell-aged, belong both to *Penicillium* (<2%) and *Aspergillus* (4.8%) genera: *P. citrinum*, able to produce citrinin, as well as many other extrolites (Houbraken et al., 2020); *P. echinulatum*, often isolated on lipid-rich substrates (e.g. butter, margarine, and cheese), known to not produce mycotoxins with a significant impact on humans or animals; *P. nalgiovense*, frequently isolated from cheese, able to produce nalgiovensin and nalgiolaxin, and traces of penicillin; *P. polonicum*, able to tolerate NaCl as low as 5%, and to produce penicillic acid and verrucosidine (Frisvad and Samson, 2004); *P. cvjetkovicii,* not known as producing mycotoxins species (Peterson et al., 2015).

Among *Fusarium* species, we reported *F. oxysporum* and *F. proliferatum*, known as plant pathogenic agents, but not as human and

animal mycotoxins source (Munkvold, 2017). However, their occurrence was detected only on cell-aged cheese.

Yeasts isolated and identified resulted *Debaryomyces hansenii*, widely spread both on mine- and cell-aged cheeses, ripened at T_1 and T_2. Its presence is congruent with related literature, reporting this species as associated with European and North American cheeses (Wolfe et al., 2014), and commonly and deliberately added in the cheesemaking process (Fröhlich-Wyder et al., 2019). Single strains of *Clavispora lusitaniae, Candida zeylanoides*, *Yarrowia lipolytica* were also isolated from T_2 cheese samples.

In consideration of a possible and undesired co-occurrence of toxigenic species, e.g. *A. westerdijkiae*, frequently reported on cheese and related to its contamination by OTA (Sakin et al., 2018; Anelli et al., 2019), we investigated possible reciprocal behaviours of *A. westerdijkiae* and *P. biforme*, highlighting a weak mutual growth negative interaction between them at 20 °C, a growth permissive temperature for both species, currently not yet reported in literature. Actually, *P. biforme* and *A. westerdijkiae* in Dossena mines were exposed to a lower temperature (8–10 °C), enhancing *P. biforme* growth (Ropars et al., 2020) and disadvantaging *A. westerdijkiae* growth and OTA production (Gil-Serna et al., 2020). Therefore, the absence of *A. westerdijkiae* could be due to the natural environmental parameters of Dossena mines.

Additional information at taxonomical level, but not quantitative, were retrieved from metabarcoding approach. That analysis was conducted to explore mycobiota of representative of mine-aged cheese, trying to put data in relation to 3 different parameters, considered in the setup of the study: duration time of aging (90 and 180 days), year seasons aging and dairy farms. Metabarcoding, based on the ribosomal ITS2 DNA region, provided a picture on the fungal community of "Ol Minadùr" cheese, confirming identification obtained by microbiological isolations, considered more accurate, because based on more informative locus than ribosomal regions used in metabarcoding. Alpha diversity did not show any significant differences in mycobiota composition of all groups; the only significant difference concerned the Shannon index of the ripening time, which showed that the microbial community is richer after 180 days. According to beta diversity analysis, there were significant differences between mycobiota of cheese with different dairy farms and different ripening time, while it appeared that the mycotic community is quite unaffected by the ripening season.

Debaryomyces prosopidis was the most abundant species in all aging seasons (data set A), at T_1 and T_2 aging times (data set B), and for all the cheese factories involved in the study (data set C). A deeper resolution identification in *Debaryomyces* genus is not possible, because of low power of ITS2 region (Nguyen et al., 2009), therefore the 18 isolated strains were assigned to *D. hansenii*, because high identity with CBS767 (MH545920), well adapted to grow on milk and cheese (Irlinger and Monnet, 2021; Quijada et al., 2020).

Mucor lanceolatus, *Clavispora lusitaniae*, *Candida sake*, *Candida santamariae*, *Candida zeylanoides*, *Chrysosporium sulphureum* were also found, confirming their link with natural environment (Dugat-Bony et al., 2015; Bintsis, 2021).

5. Conclusions

The dual approach highlighted that microbiological isolations and metabarcoding are complementary for the characterization of the whole mycobiota associated with a specific food, but not powerful if considered as stand-alone. ITS metabarcoding returns information about composition of fungal community, while microbiological isolations and consequent sequencing of appropriate DNA marker allow a correct species identification and offer the advantage to save the viable fungal strains, available for future applications, as driving safe aging stage in dairy chain.

Environmental features, enhancing *P. biforme* and disadvantaging *A. westerdijkiae* growth, combined with the evidence of absence of EAs production, make *P. biforme* an eligible species for guided cheese aging in natural environments comparable to Dossena's mine, and state Dossena's mine as eligible area for sustainable aging of cheese.

Funding

This work was supported by CheeseMine project (201801063265), funded by Lombardy Region (Italy) within FEASR program (Programma di Sviluppo Rurale 2014–2020, MISURA 16 – "COOPERAZIONE", SOTTOMISURA 16.1 – "Sostegno per la costituzione e la gestione dei Gruppi Operativi del PEI in materia di produttività e sostenibilità dell'agricoltura" OPERAZIONE 16.1.01 – "Gruppi Operativi PEI").

CRediT authorship contribution statement

Pamela Anelli: Writing – original draft, Visualization, Investigation. **Chiara Dall'Asta:** Writing – review & editing, Resources, Methodology, Formal analysis. **Giuseppe Cozzi:** Methodology, Formal analysis. **Filomena Epifani:** Data curation. **Daria Carella:** Data curation. **Davide Scarpetta:** Writing – original draft, Formal analysis. **Milena Brasca:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Antonio Moretti:** Writing – review & editing, Supervision. **Antonia Susca:** Writing – review & editing, Writing – original draft, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Milena Brasca reports financial support was provided by CheeseMine project (201801063265), funded by Lombardy Region (Italy) within FEASR program. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2024.104587.

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