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Shotgun proteomics for the identification of yeasts responsible for pink/red discoloration in commercial dairy products

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ABSTRACT

Pink/red discoloration encompasses a series of relatively common spoilage defects of commercial dairy products. In this study, we used shotgun proteomics to identify the microorganism responsible for the production of intensely red-coloured slimes found on the surface of freshly opened commercial spreadable cheese and yogurt samples. Proteome-wide characterization of microbial proteins allowed to identify 1042 and 687 gene products from *Rhodotorula* spp. in spreadable cheese and yogurt samples, respectively, while no significant protein scores from other microorganisms were recorded. Subsequent microbiological analyses and sequencing of the 26S rRNA gene region supported the proteomic results demonstrating that the microorganism involved was *Rhodotorula mucilaginosa*, a carotenoid - producing basidiomycetous that can be potentially pathogenic to humans, especially for immunocompromised individuals. This is the first time that shotgun proteomics has been used to identify a microorganism responsible for spoilage in dairy products, proposing it as a relatively fast, sensitive, and reliable alternative or complement to conventional methods for microbial identification.

1. Introduction

As highly nutritious growth media, rich in water, fat, proteins, vitamins, carbohydrates, and minerals, milk, cheese, butter, cream and others dairy products are ideal habitats for the development of numerous spoilage microorganisms (Lu & Wang, 2017). Microbial contamination can occur at any stage during the production, processing, and storage of dairy products (Martin et al., 2021). The microorganisms responsible for spoilage in dairy products include Gram-negative and -positive bacteria and a wide range of eumycetes (*i.e.*, yeasts and molds) that can result in either non-visible defects, such as unpleasant odours and tastes, or visible alterations, such as discoloration (production of pigments) or outgrowth on the product surface, which compromise shelf-life, safety and quality of the product (Cadwallader & Singh, 2009; Garnier et al., 2017; Martin et al. 2021). Pink/red discoloration is one of the most common visible signs of microbial contamination of milk and dairy products. More properly, pink/red discoloration encompasses a number of defects that can manifest themselves in multiple aspects depending on both nature of the contaminated dairy product and colonizing microbial species (Amato et al., 2012; Yeluri Jonnala et al., 2021). In spite of the extensive literature on the subject, conflicting and inconclusive indications about the microflora responsible and the causes of pink/red discoloration development are available (Daly et al., 2012).

Micrococcaceae, *Brevibacteriaceae* and *Corynebacteriaceae* are the dominant families in the complex ecosystem of red-smear cheeses. In particular, *Corynebacterium casei* has been considered a representative microorganism responsible of red-smear cheese surfaces (Amato et al., 2015). Quigley et al. (2016), attributed the pink-/red discoloration of continental type-cheese to *Thermus thermophilus* (formerly known as *Flavobacterium*), a thermophilic carotenoid-producing bacterial species found in hot water during milk manufacturing. The involvement of *T. thermophilus* in the pink discoloration of Swiss-type cheese was confirmed by other authors (Yeluri Jonnala et al., 2021). *Glutamicibacter arilaitensis* (basonym *Arthrobacter arilaitensis*) is one of the bacterial species responsible for the red pigmentation of cheese rinds, which was previously attributed to the biosynthesis of C_{50} carotenoids and subsequently to a set of water-soluble metal-free porphyrins (Sutthiwong et al., 2021). Under specific environmental conditions, *Serratia marcescens* synthesizes a red tripyrrole pigment named prodigiosin, which can produce an intense coloration of dairy products, including ricotta cheese

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(Alberghini et al., 2010). Infections with *S. marcescens*, a Gram-negative rod-shaped bacterium recently classified in the Yersiniaceae family, are a well-documented cause of mastitis outbreaks in dairy cattle herds (Friman et al., 2019). This species can also affect lactating mothers by contaminating breast milk (Quinn et al., 2018). Recently, *Serratia liquefaciens* was identified as the responsible for the pink discoloration of Pecorino Toscano cheese (Martelli et al., 2020). Among several spoilage yeasts already described (Geronikou et al., 2022), *Rhodotorula* spp. may confer an intense yellow/red pigmentation to the surface of dairy products through the production of a series of carotenoids (Kot et al., 2016).

Dairy spoilage causes significant economic losses to the dairy industry. Even more, spoilage events could represent serious health concerns, since many of the above-mentioned microorganisms could be pathogenic to humans. For example, *S. marcescens* is believed to be harmless to immunocompetent people, while it can cause eye, respiratory, and urinary tract infections in hospitalized patients (Khanna et al., 2013). Similarly, several *Rhodotorula* yeast species are potential pathogens that can cause invasive fungemia in immunocompromised individuals (Nunes et al., 2013).

In view of the industrial needs of guaranteeing food quality and safety, there is a clear demand for rapid and reliable methods alternative to time-consuming conventional and culture-based strategies to identify microbial contaminants in foods during the production process and shelf-life (Abril et al., 2021).

Protein fingerprints generated by matrix-assisted laser desorption ionization (MALDI)-time-of-flight (TOF) MS are increasingly used a pattern recognition method for a rapid identification of microorganisms at the genus and species levels (Singhal et al., 2015). MALDI-TOFMS requires a dedicate mass spectrometer and opportune databases for the software assisted matching of actual MS profiles to those of previously characterized strains, with no need of identifying the proteins involved. Notwithstanding, the MALDI-TOF MS based approach is not without limitations, as it can suffer from poor discrimination between species or misidentification (Richert, 2019).

Shotgun proteomics is a versatile and informative bottom-up technique for the analysis of complex proteomes (Mazzeo et al., 2022). The workflow consists of the tryptic digestion of unfractionated protein extracts obtained from cell lysis and analysis of the resulting peptide mixture by liquid chromatography (LC) coupled with high-resolution tandem mass spectrometry (MS/MS). Proteins are individually inferred from a bioinformatics-assisted sequencing of peptides, achieved by matching experimental MS/MS spectra with theoretical ones generated from publicly available protein or genomic databases. Although the accurate identification of the microorganisms involved depends on the availability of genomic and proteins sequences, assignment at the genus level can also be obtained by homology. Nevertheless, shotgun proteomics has been used in a surprising low number of cases for microbial identification and its potential has not been fully explored (Dworzanski & Snyder, 2005; Tracz et al., 2013; Cheng et al., 2016; Abril et al., 2021).

In this work, we applied shotgun proteomics to reliably identify the microorganism(s) responsible for the pink/red discoloration in commercial fresh spreadable cheese and yogurt samples and compared the findings with those obtained with conventional culture methods associated with gene sequencing.

2. Materials and methods

Chemicals were purchased from Merck-Sigma (St. Louis, MI, USA) and HPLC-MS grade solvents were from Carlo Erba (Milan, Italy). Media for microbiological analysis were from Oxoid (Milan, Italy).

2.1. Sampling

Two dairy products of different brand, *i.e.*, fresh spreadable cheese and unflavoured yogurt, were acquired from two different local markets several months apart. The samples, opened before the "use by" date, exhibited an unusual red-coloured surface, as shown in Fig. 1 a, b. Both samples were individually used for proteomics and subsequent microbiological and genetic analyses.

2.2. Protein purification

Visible red microbial spots were carefully scraped from the surface using a spatula and collected in sterile tubes, forming ~ 70 μL pellets and stored in a refrigerator at 4 °C before analysis.

To minimize the presence of milk-derived proteins, the pellets were rinsed three times with 0.5 mL of phosphate saline buffer, pH 7.2, and centrifuged at 1,000 × *g*, 15 min, 4 °C.

Then, microbial cells were lysed by suspending the pellets in 0.5 mL of 100 mM triethyl ammonium bicarbonate (TEAB), 2% sodium dodecyl sulphate (SDS), pH 7.8, and sonicated 20 min at room temperature. The cell lysate was centrifuged at 12,000 × *g*, 15 min, 4 °C and the protein concentration in the supernatants was determined using the modified micro-Lowry assay (kit from Merck-Sigma). For each sample, 100 μg of proteins was transferred to new tubes and subjected to sequential Cys-reduction and alkylation with final 10 mM dithiothreitol, 1 h at 56 °C and 55 mM iodoacetamide, 30 min at room temperature in the dark, respectively. Afterwards, proteins were precipitated overnight at −20 °C with 600 μL of pre-chilled acetone. The protein pellets were rinsed twice with −20 °C chilled acetone, centrifuged at 12,000 × *g*, 15 min, 4 °C, and finally vacuum-dried. Proteins were suspended in 50 mM ammonium bicarbonate, pH 7.8, and sequentially digested with Lys-C, 6 h, 37 °C, enzyme-to-substrate ratio 1/100 (w/w) and with proteomic grade trypsin, overnight, 37 °C, enzyme-to-substrate ratio 1/50 (w/w). Proteolytic peptides were purified using Pierce C18 spin columns (Thermo Fisher Scientifics, Rockford, IL, USA), washed extensively with 0.1% trifluoroacetic acid (TFA) and eluted with 50 μL 70% acetonitrile (v/v)/0.1% TFA.

2.3. LC-MS/Ms

Before LC-MS/MS analysis, peptides were dried in speed-vac and resuspended in 100 μL of 0.1% formic acid. LC-MS/MS shotgun proteomic analysis was performed using an Ultimate 3000 nanoflow ultra-high performance liquid chromatograph (Dionex/Thermo Scientific, San Jose, CA) coupled with a Q Exactive Orbitrap mass spectrometer (Thermo Scientific). Peptides were loaded through Acclaim PepMap 100 (75 μm i.d. × 2 cm) trap columns (Thermo Scientific) using a Famos autosampler (Thermo Scientific) and separated using an EASY-Spray PepMap C18 column (2 μm, 25 cm × 75 μm) with 3 μm particles and 100 Å pore size (Thermo Scientific). Eluent A was 0.1% formic acid (v/v)

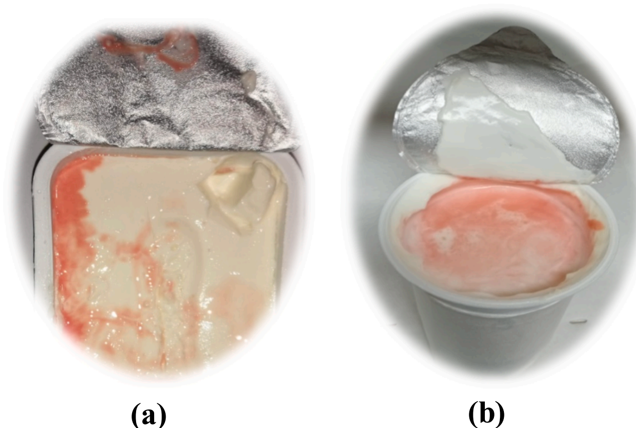


Fig. 1. Macroscopic alteration of fresh spreadable cheese (a) and yogurt (b) purchased from the retail commerce and opened before the expiration date.

in LC-MS grade water; eluent B was 0.1% formic acid (v/v) in 80% acetonitrile. Peptides were separated by applying a 2–45% gradient of B over 120 min at a constant flow rate of 300 nL min^{-1}. For each analysis, 2 μg of peptide mixture was injected. For the MS operating conditions, MS1 precursor spectra were acquired in the positive ionization mode scanning the 300–1600 *m/z* range, with a resolving power of 70,000 full width at half-maximum (fwhm), automatic gain control (AGC) target of 10^6 ions and maximum ion injection time (IT) of 100 ms. The spectrometer operated in top10 data-dependent acquisition, applying a 10 s dynamic exclusion.

Fragmentation spectra were obtained at a resolving power of 17,500 fwhm. Ions with one or more than six charges were excluded from MS/MS selection. Spectra were elaborated using the Xcalibur Software version 3.1 (Thermo Scientific). Each sample was analyzed in triplicate in a randomized sequence and alternated with the acquisition of blank samples.

2.4. Bioinformatics and computational proteomics

A preliminary identification of the protein components was carried out with the Protein Prospector Batch-Tag Web tool (https://prospector2.ucsf.edu), using the mgf files generated from the LC-MS runs with the MS Convert tool of the open-source ProteoWizard 3.0 software (https:// proteowizard.sourceforge.net/). Searches in the UniProtKB database were not taxonomically restricted and then, once the prevalent presence of *Rhodotorula* spp. proteins was ascertained, searches were restricted to the "microorganisms" to filter out bovine milk proteins. Afterwards, proteins were identified using the Proteome Discoverer software vers. 2.1 (Thermo Scientific) based on the Sequest algorithm. In this case raw spectra were searched against the *Rhodotorula* database downloaded from UniProtKB on December 2022 (unreviewed TrEMBL database, 74164 protein entries).

For both the search engines, the parameters were as follows: carbamidomethylation of cysteines as a static modification; methionine (Met) oxidation, pyroglutamic acid at N-terminus glutamine (Gln), Gln and asparagine (Asn) deamidation, and serine/threonine (Ser/ Thr) phosphorylation, as variable modifications; mass tolerance value of 10 ppm for precursor ion and 0.02 ppm for MS/MS fragments; trypsin as the proteolytic enzyme with missed cleavage up to 2. Protein identification scores were calculated by Target Decoy Peptide Spectrum Matches (PSMs) filtering working at a 1% peptide-level false discovery rate (FDR) that matched proteins identified at 1% protein-level FDR.

2.5. Functional analysis

For Gene Ontology (GO) analysis, the FASTA sequences obtained from the union of the yeast proteins identified in fresh spreadable cheese and yogurt by Batch-Tag Web (841 entries) were imported into OmicsBox release 3.0.25 (BioBam Bioinformatics, Valencia, Spain) and blasted using the blastp search ($E < 1.0 \times 10^{-3}$) against NCBI non-redundant databases with taxonomy restriction to *Fungi*. GO terms were mapped with Blast2GO. Enriched GO terms were functionally annotated according to biological process, cellular component, and molecular function.

2.6. Microbiological analysis

To identify microorganisms potentially responsible for discoloration, the red-colored part of the spoiled samples was scraped from the surface, transferred to a sterile plastic tube and homogenized 1:10 with physiological solution (9 g/L NaCl), using a blender (BAG MIXER 400, Interscience, France) for 2 min.

The sample dilutions were then plated in triplicate on different growth media, as described below. For total microbial count, Plate Count Agar (PCA) were incubated at 30 °C for 24–48 h. For the count of *Pseudomonadaceae*, Pseudomonas Agar Base supplemented with CFC-selective supplement (SR103, Oxoid) was used, incubating plates at 20 °C for 48 h, as reported by Reale et al. (2008). Yeast and molds were counted on Potato Dextrose Agar supplemented with 25 mg/L of chloramphenicol (Sigma-Aldrich, St. Louis, MO). Plates were incubated at 28 °C for 48–72 h.

The results are expressed as log CFU/g or mL and are reported as mean value ± standard deviation.

2.7. Microbial isolation from pink/red areas

Six pink or reddish colonies present only on Potato Dextrose Agar (PDA) plates were selected and isolated through two purification steps on the same medium. The purity of the isolates was checked by verifying the morphology using an optical microscope Olympus BX40 (Olympus, Tokyo, Japan), then stored at 4 °C before genetic identification.

2.8. Genotypic identification of isolated yeasts

The isolated yeasts were identified by sequencing the 26S rRNA gene region, as reported by Zotta et al. (2022). Briefly, the DNA of the selected isolates was amplified using the universal primers NL1 (5′-GCCATATCAATA AGCGGAGGAAAAG-3′) and NL4 (5′GGTCCGTGTTTCA AGACGG-3′) according to the protocol of Kurtzman and Robnett (1998). After purification (QIAquick PCR purification kit, QIAGEN GmbH, Hilden), the amplicons were sequenced (Eurofins Genomics, Ebersberg, Germany), and sequence homology and identification were carried out as described by Reale et al. (2013). Identified yeasts were stored as frozen stocks (50% v/v glycerol) at −20 °C, in the Microbial Culture Collection of the Institute of Food Sciences – National Research Council (ISA-CNR; Avellino, Italy).

2.9. Confirmation of pink/red discoloration production on fresh dairy products

The isolates were inoculated onto different commercial fresh spreadable cheeses and yogurts of different brands to confirm their ability to produce pink/red discoloration. Briefly, overnight yeast cultures (16 h), capable of producing the red pigment in Potato Dextrose Agar, were inoculated (final concentration of ca. 3 log cfu/ml) on the surface of fresh dairy products. Also, negative control samples with uninoculated dairy products were included. The samples were then incubated at 4 °C for 7 days and the presence of pigmentation was monitored. The appearance of pink/red pigment colour during the assay was evaluated visually as presence/absence.

3. Results and discussion

3.1. Proteomic analysis

After scraping of the red-discolored slime from the two dairy products (Fig. 1 a, b), peptide mixtures resulting from protein extraction and tryptic digestion were analyzed by LC-MS/MS. For a rapid pre-screening, the LC-MS/MS raw files were preliminarily converted to mgf files and analyzed with the Batch-Tag Web of the open-source protein prospector suite (https://prospector.ucsf.edu/), searching the taxonomically unrestricted UniProtKB protein database. This tool can perform relatively rapid analyses, though not less rigorous, compared to other search engines and it is particularly suited for searches in large databases (Chalkley et al., 2005). Apart for the expected presence of some of the abundant bovine milk-derived proteins, among which caseins, β-lactoglobulin, α-lactalbumin, xanthine dehydrogenase/oxidase, and lactotransferrin, the most represented protein entries were gene products of the *Rhodotorula* genus yeasts. To refine the characterization of the microbial proteome, searches were reiterated with taxonomic restriction to microorganisms. Overall, 608 and 468 gene products in the spreadable cheese and yogurt samples, respectively, were from yeasts

and more than 90% of these were from *Rhodotorula* spp. (Fig. 2a).

In general, proteins found as derived from "yeast" species likely belonged to *Rhodotorula* spp., because the publicly available genomic and proteomic resources for this genus are still fragmentary. In this study, the highest number of proteins were from *Rhodotorula graminis*, because this is the most extensively sequenced species within the *Rhodotorula* genus. Nevertheless, the top scorer protein entry, namely ADP/ATP carrier of the mitochondrial inner membrane (UniProtKB accession: F8SL17) identified with 28 unique peptides, and two additional proteins (P10248 phenylalanine ammonia-lyase and F8SL13 phosphoglucomutase) within the 13 top scorer proteins, were from *Rhodotorula mucilaginosa*, also classified as *Rhodotorula rubra*. These findings suggest that the species involved could be *R. mucilaginosa*. The relatively low number of proteins identified for this species depends on the poorly annotated protein database, since *R. mucilaginosa* has only 88 catalogued protein sequences, compared to 7255 ones of *R. graminis*.

The unfiltered lists of proteins identified in the two samples by Batch-Tag Web are reported in Supplementary Table 1.

Proteomic analyses were repeated with the proprietary Proteome Discoverer vers 2.1 software (Thermo Scientific) using as input the raw files of the LC-MS/MS runs and restricting the searches to the unreviewed UniProtKB database for *Rhodotorula* spp. After merging the proteins identified in triplicate analyses, homology filtering, and including only razor protein matches with > 2 sequenced unique peptides, 1042 and 687 non redundant gene products *Rhodotorula* spp. were identified with high confidence (FDR threshold 1%) for spreadable cheese and yogurt samples, respectively. In particular, 352 proteins were common to both samples, while 690 and 335 proteins were specifically identified in samples from cheese and yogurt, respectively, as shown in the Venn diagram of Fig. 2a. The list of filtered *Rhodotorula* proteins identified by Proteome Discoverer is reported in Supplementary Table 2. The different performances of proteomic search engines are a well-established issue (Audain et al., 2017). The analysis with the Proteome Discoverer search engine enlarged the inventory of proteins identified with high confidence. However, the findings from both the bioinformatic analyses substantially indicated that the identified gene products stemmed from a homogeneous genome of *Rhodotorula* spp. Most likely, the number of protein entries would have been higher if the protein and gene repositories for *Rhodotorula* were complete. On the other hand, the possibility of redundant entries cannot be ruled out, due to the use of an unreviewed protein database. The significant difference in the number of identified proteins can be attributed to the manual sampling of the microbial slime that could result in an uneven distribution of yeast cells and milk proteins between cheese and yogurt samples. Nonetheless, the analysis of both samples unequivocally showed that *Rhodotorula* yeast was the sole or largely predominant microorganism for both contaminated products.

Thus, a proteome-wide characterization using shotgun proteomics allowed to obtain a rapid and reliable identification of the microorganism potentially responsible for the pink-red discoloration of fresh spreadable cheese and yogurt.

3.2. Gene Ontology mapping and functional analysis

The genus *Rhodotorula* includes carotenoid-producing yeasts well-known for their intracellular biosynthesis of β-carotene, torulene and torularhodin (Davoli et al., 2004; Libkind & van Broock, 2006; Maldonade et al., 2008). The biosynthetic pathway of carotenoids in *Rhodotorula* has been described in detail (Tang et al., 2019). In this study, functional analysis was performed primarily to map possible gene products involved in the biosynthesis of carotenoids. Using Blast2GO with taxonomic restriction to *Fungi* 806 gene products out of 841 overall identified with Batch Tag Web were mapped with annotation of GO terms. The GO terms were classified according to biochemical process (BP), molecular function (MF) and cellular component (CC), as summarized in Fig. 3a.

A second level classification according to BP and MF is represented in Fig. 3b and 3c, respectively. The most represented gene products were involved in the cell energetic metabolism, cell reproduction and primary metabolism, while the key enzymes involved in the biosynthetic pathways of secondary metabolites were missing. On the other hand, manual inspection of the lists of identified proteins demonstrated the presence of phytoene desaturase from *R. mucilaginosa* (UniProtKB accession A0A2I8BIC4) and isopentenyl-diphosphate D-isomerase from *Rhodotorula* spp. (A0A4U0W0M1), which were identified with 3 and 2 unique peptides, respectively. Additional *Rhodotorula* spp. enzymes of the biosynthetic pathways of isoprenoids, such as lycopene cyclase/phytoene synthase (A0A194S0H1), squalene/phytoene synthase (A0A4U0VYF5), and mevalonate kinase (A0A4U0W0G1), were retrieved with only one matching peptide and therefore they had been removed from the lists during filtering. These findings suggested that the enzymes of the biosynthetic pathways of carotenoids were present but expressed at low levels.

3.3. Microbiological analysis and genotypic identification of the yeasts

Microbiological analyses were performed on the fresh spreadable cheese and yogurt, and the results showed a total mesophilic count of 6.5 $\pm$ 0.2 log cfu/g and 8.0 $\pm$ 0.5 log cfu/ml, respectively. The samples had values $<$ 1 log cfu/g for both molds and *Pseudomonas* spp. Yeasts were found in concentrations of 6.1 $\pm$ 0.3 log cfu/g, and 5.3 $\pm$ 0.4 log cfu/mL in the fresh spreadable cheese and yogurt, respectively. Six colonies (three from fresh spreadable cheese and three from yogurt samples) were randomly picked up from the plates at the highest dilutions of PDA.

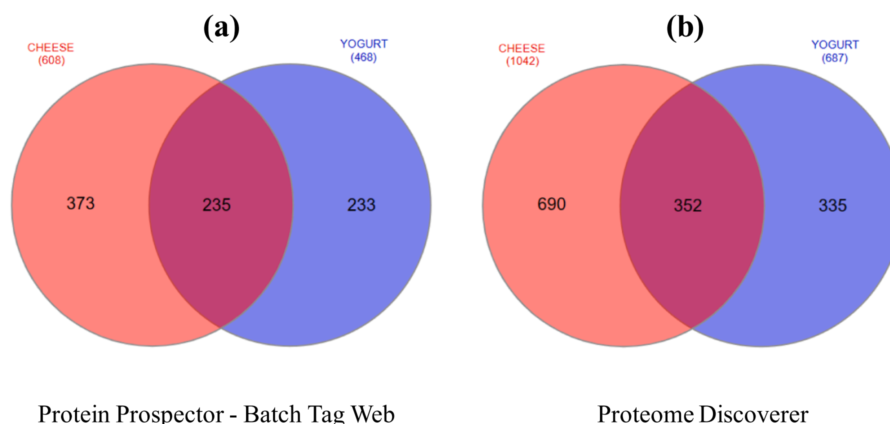


Fig. 2. Venn diagrams reporting the number of proteins from *Rhodotorula* spp. identified with high confidence in cream and yogurt samples, using the Protein Prospector – Batch Tag Web (a) and Sequest-based Proteome Discoverer (b) search engines.

GO Distribution by Level - Top 20

(a)

#Seqs

0 100 200 300 400 500 600

BP: cellular process; biological regulation; positive regulation of biologi...; homeostatic process; reproductive process; multicellular organismal proce...

MF: catalytic activity; ATP-dependent activity; molecular function regulator a...; antioxidant activity; transcription regulator activi...; cytoskeletal motor activity

CC: cellular anatomical entity

Direct GO Count (MF)

(b)

#Seqs

0 20 40 60 78

translation; cytoplasmic translation; intracellular protein transpor...; translational elongation; rRNA processing; electron transport chain; proton motive force-driven mit...; cellular oxidant detoxificatio...; proton motive force-driven ATP...; protein transport; others

Direct GO Count (BP)

(c)

#Seqs

0 30 60 90 120 150 180

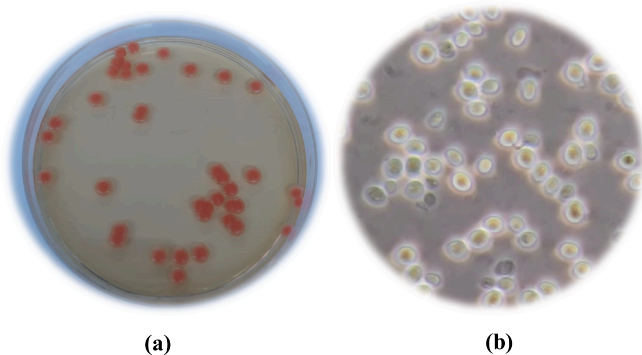
GO: ATP binding; ATP hydrolysis activity; hydrolase activity; ATP-dependent protein folding ...; unfolded protein binding; oxidoreductase activity; transferase activity; rRNA binding; proton-transporting ATP syntha...; methyltransferase activity; others

Fig. 3. First level functional analysis of proteins identified by proteomics and mapped by Blast2GO (a), and second level classification according to MF, molecular function, (b) and BP, biochemical process (c).

The isolates were purified by streaking on fresh medium and subjected to genetic identification. After at least 72 h of incubation, all the isolates developed a smooth surface with pink/red colonies (Fig. 4a) which on microscopic observation were oval or round in shape with asexual reproduction, indicating multi-lateral budding and the absence of ascospores (Fig. 4b).

The isolates were, then, subjected to PCR analysis and 26S rRNA sequencing and were identified as *Rhodotorula mucilaginosa* (Table 1). The results of 26 sRNA sequencing were consistent with those of shotgun proteomics and confirmed that the species involved was *R. mucilaginosa*.

Rhodotorula spp., a basidiomycetous yeast belonging to the phylum *Basidiomycota* and the family *Sporidiobolaceae*, is an emerging opportunistic fungal pathogen that can be found in several natural environments (Wirth & Goldani, 2012; Ioannou et al., 2019). Indeed, it has been isolated from a variety of sources, including air, soil, salt water, plants, and domestic environment (Cespedes et al., 2022). Several studies have also recorded the presence of *Rhodotorula* species in dairy products (Viljoen,



(a) (b)

Fig. 4. a) Morphology of *Rhodotorula mucilaginosa* colonies developed on Potato Dextrose Agar after 3 days incubation at 28 °C. b) Yeast observation by light microscope Olympus BX40.

Table 1
Identification, based on blast comparison in GenBank, of yeast strains isolated from yogurt (Y1, Y2, Y3) and fresh spreadable cheese (PH1, PH2, PH3).

Strain	Amplicon size (bp)	Closest relative	% identity	Source[a]
Y1	839	*Rhodotorula mucilaginosa*	99.65%	LT598656
Y2	628	*Rhodotorula mucilaginosa*	99.65%	MF783067
Y3	612	*Rhodotorula mucilaginosa*	98.25%	HE660055
PH1	606	*Rhodotorula mucilaginosa*	99.76%	MG707723
PH2	621	*Rhodotorula mucilaginosa*	98.07%	MT550663
PH3	762	*Rhodotorula mucilaginosa*	98.07%	LT598656

[a] Accession number of the sequence of the closest relative found by blast search.

et al., 2003; Callon et al., 2007; Moubasher et al., 2018; Kazou et al., 2023) with *Rhodotorula mucilaginosa, Rhodotorula glutinis* and *Rhodotorula diffluens* species being isolated mainly from unripened dairy products (Garnier et al., 2017).

Raw milk, equipment surfaces, and processing environment are potential sources of contamination of dairy product (Garnier et al., 2017). *Rhodotorula* spp. can serve as an indicator of a lack of hygienic standards during the production and storage of the products.

In the present study, the samples appeared to have intact packages, indicating that product contamination likely occurred during production and the microorganisms were able to grow during the shelf-life of the product. Fortunately, product contamination is easily detected due to the typical pink/red colouration of the colonies.

To test the ability of the isolated yeast to produce a pink/red discoloration, fresh cheese and yogurt samples were inoculated and incubated at 4 °C for 7 days. Macroscopic changes due to inoculation of *R. mucilaginosa* in the samples are shown in Fig. 5, highlighting the ability of the isolates to reproduce a pink/red discoloration under controlled experimental conditions. These results confirm that dairy products are substrates for the growth of *Rhodotorula* and that refrigeration does not inhibit the growth of this microorganism. As secondary metabolites, carotenoids are generally biosynthesized during the late logarithmic phase of yeast growth, while they accumulate during the stationary phase (Tang et al., 2019). This aspect justifies the late appearance of visible colonies of *R. mucilaginosa* and suggests that these might go undetected during the processing stages and confectioning, only becoming visible when the consumers open the diary products' package.

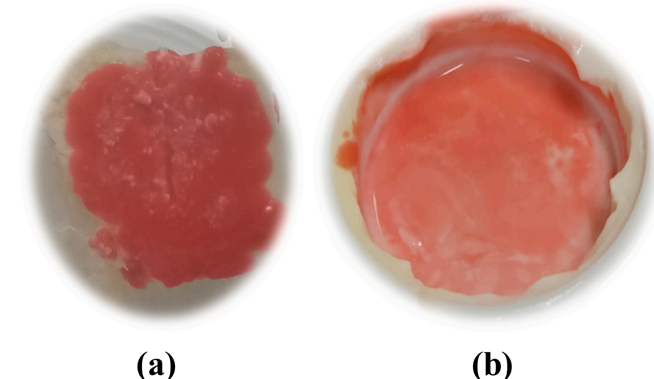


Fig. 5. Macroscopic alteration of fresh spreadable cheese due to inoculation of *Rhodotorula mucilaginosa* PH2. Red discoloration on the surface of spreadable cheese (a) and yogurt (b) after 7 days incubation at 4 °C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This microorganism must be kept under control because, in addition to economic damage, it can cause health risks by colonizing the skin, fingernails, respiratory, genital-urinary tracts and causing diarrhoea (Stratford, 2006). *Rhodotorula* species have been linked various diseases, including endocarditis, peritonitis, meningitis, endophthalmitis, lymphadenitis, and fungemia in immunocompromised individuals (Tuon & Costa, 2008; Nunes et al., 2013; Cespedes et al., 2022).

In general, the presence of these spoilage yeasts in products of the typology investigated is due to post-heat-treatment contamination. Consistent with our results, other studies have found different yeasts capable of altering yogurt and heat-treated products, such as *Candida* spp., *Debaryomyces*, *Kluyveromyces*, *Pichia*, *Yarrowia*, *Galactomyces*, *Torulaspora* and *Saccharomyces* spp. (Nielsen et al., 2021).

Results of this study demonstrate that *R. mucilaginosa* can be a common spoiler of dairy products, highlighting the importance of hygienic standards during production to prevent contamination and growth of potentially harmful microorganisms.

4. Conclusions

In this study, we exploited shotgun proteomics to characterize the microorganisms causing an intense pink/red discoloration of fresh dairy products. To our knowledge, this is the first time that shotgun proteomics has been used to identify the microorganisms responsible for the spoilage of dairy products. Overall, the shotgun proteomics analysis unequivocally showed that the microorganisms causing the red discoloration in the spoiled samples of fresh spreadable cheese and yogurt belonged to *Rhodotorula* genus.

The strategy was relatively fast as sample analysis and bioinformatics data processing extended to the entire microorganism taxa were performed in just over thirty hours. However, time range might be shortened reducing the time of trypsin proteolysis (Tracz et al., 2013) or restricting the databases to be searched only to a set of possible taxa (Lasch et al., 2020).

Genetic analysis allowed to identify the spoiling microorganism at species level as *R. mucilaginosa*, while microbiological assays confirmed the ability of the yeast to reproduce the alteration.

Shotgun proteomics proved to be a rapid and reliable strategy to identify food spoiling microorganisms, although it may suffer from limitations imposed by the incompleteness of microbial genomic and proteomic sequence databases.

As genomic and proteomic repositories are compiled, the specificity of the bottom-up proteomic approaches could increase, enabling the accurate characterization of microorganisms at the species and even at strain level. Overall, the choice of a specific method for identifying microbial contaminants in dairy products should rely on the desired level of accuracy, the speed and cost of the analysis, and the available resources and expertise. Shotgun proteomics has the potential to become an attractive and unbiased identification method in laboratories where high-resolution mass spectrometers are available or also to support the monitoring of microbial contamination during the phases of food processing, so facilitating timely interventions and preventing health risks and economic losses for dairy industry.

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CRediT authorship contribution statement

Tiziana Di Renzo: Conceptualization, Methodology, Writing – review & editing. **Anna Reale:** Conceptualization, Methodology, Writing – review & editing. **Stefania Nazzaro:** Formal analysis, Investigation. **Francesco Siano:** Formal analysis, Investigation. **Francesco Addeo:** Supervision, Writing – review & editing. **Gianluca Picariello:** Conceptualization, Methodology, Software, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112945.

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