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Identification and Characterization of Fungi Isolated from a Cheese Cave in the Eastern United States

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Abstract: Fungal spores are ubiquitous in indoor air, with the diversity of species geographically variable. Species present depend upon outdoor sources, physical conditions of the built environment, and human use of the built environment. Cheese is a microbial product produced through inoculation of milk with specific fungal and bacterial cultures. Indigenous fungi can also impact cheese flavor and quality. While there are studies investigating the microbiome of cheese and starter cultures, there are not many studies investigating the airborne fungal community in cheese caves. This study investigated the viable airborne fungi present in a man-made cheese cave in the Eastern United States. Fungi were captured passively on both general culture media and milk-based media using the open plate method. Thirty-one isolates were identified to genus by sequence of the nuclear ribosomal internal transcribed spacer (ITS) region. Most of the isolates were cheese-associated taxa: *Penicillium* (26 isolates) and *Scopulariopsis* (1 isolate). The only other taxon identified was *Cladosporium* (4 isolates), which is commonly isolated in surveys of both indoor and outdoor air and has been isolated from cheese. The airborne *Penicillium*-Fasciculata isolates captured from the cheese cave exhibit growth traits (conidiation, increased growth on malt extract media, and loss of casein hydrolysis) more similar to wild *Penicillium* fungi than the domesticated commercial cheese strain *Penicillium candidum*.

Keywords: Indoor fungi; cheese fungi; *Penicillium*.

Introduction

Fungi produce spores for both asexual and sexual reproduction. While some species rely on insects or other animals for spore dispersal, many species rely on wind or rain for dispersal. Approximately 3.5 teragrams of fungal spores are released into the air globally each year; across the United States, fungal spores are released into the air at an average rate of 62 spores m^{-2} s^{-1} (Janssen et al., 2021). The quantity and species of spores varies by geographic location and season, with rural environments generally more diverse than urban environments (Nevalainen et al., 2015). The air inside buildings also contains a variety of microbial particles, including spores of filamentous fungi (Flannigan et al., 2011). Genera commonly found in indoor air include *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* (Flannigan et al., 2011; Fradkin et al., 1987; Lee et al., 2021; Visagie, Hirooka, et al., 2014). The outdoor environment has a strong impact on the fungi found in indoor air, with spores or hyphal fragments of species found in the outside environment dominating the indoor air (Adams et al., 2013; Lee et al., 2021). However, the relative abundance of species found indoors differs from that found outside; *Cladosporium* is generally associated with outdoor sources, while *Penicillium* is associated with indoor sources (Li & Kendrick, 1995; Ren et al., 1999). Spore abundance is lower indoors than outside (Shelton et al., 2002).

Cheese is a microbial food product made from

fermentation of milk by a diverse microbiological community of bacteria, yeast, and filamentous fungi (Beresford et al., 2001). Cheese production is generally initiated with a mixed culture of bacteria that ferment the milk and produce lactic acid (Beresford et al., 2001). Different types of cheese are then ripened and aged with specific fungi: blue-veined cheeses are aged with the filamentous ascomycete *Penicillium roqueforti* and soft, surface-ripened cheeses use the filamentous ascomycete *Penicillium camemberti* and the yeast *Geotrichum candidum* (Beresford et al., 2001). Milk is the primary ingredient of cheese and the main protein in milk is casein, a large protein that gives milk its opaque white appearance. *Penicillium camemberti* and *Geotrichum candidum* work together to metabolize casein: casein is degraded to peptides by *P. camemberti* and the peptides are hydrolyzed by *G. candidum* (Boutrou & Guéguen, 2005). In addition to a role in cheese ripening and flavor, *G. candidum* also inhibits the growth of *Listeria monocytogenes*, a food-borne bacterial pathogen that can grow at refrigeration temperatures (Dieuleveux et al., 1998).

Fungi have been used for the production of cheese since at last 7500 BCE (Salque et al., 2012) and some of the fungi used in cheese production are domesticated. Compared to the wild progenitor *Penicillium fuscoglaucum*, the domesticated *P. biforme*, *P. camemberti*, and *P. caseifulvin* fungi exhibit decreased production of asexual spores (conidia), increased growth as fuzzy white hyphae, decreased production of the mycotoxin cyclopiazonic acid, faster growth in the cheese environment, and a greater ability to outcompete other microbes in the cheese environment (Ropars et al., 2020).

Researchers have investigated the fungal species found in cheese, cheese rinds, and cheese starters (Martin & Cotter, 2023; Ropars et al., 2012), but there are not many studies that investigate the airborne fungi from cheese production facilities (Kandasamy et al., 2020). Airborne fungi could be advantageous, contributing to the flavor or texture of artisanal cheeses; airborne fungi could also be detrimental contaminants contributing to spoilage, off-flavors, or potential food safety concerns due to mycotoxin production (Martin & Cotter, 2023). However, mycotoxins are more of a problem in cereal products and, other than aflatoxin M, not generally of concern in cheese or dairy products (Garnier et al., 2017). Some of the species isolated in surveys of indoor air (Shelton et al., 2002) can be problematic to cheese production (Kure & Skaar, 2019).

Identification of unknown fungal isolates is difficult; expert knowledge within specific taxa and an integrated approach including microscopic morphology, biochemical analyses, and phylogenetic analyses is usually required for definitive identification to species (Lücking et al., 2020). For broad, exploratory studies that may involve species across the fungal kingdom, a DNA barcode approach is appropriate (Lücking et al., 2020). The nuclear ribosomal internal transcribed spacer (ITS) region is accepted as the DNA barcode marker with most utility for Fungi (Schoch et al., 2012).

In this study, we investigated culturable, airborne fungi present in man-made cheese caves in a small creamery in the eastern United States to see if the airborne fungal community included taxa commonly identified in indoor air surveys or if the airborne fungal community was dominated by cheese-associated taxa. We used a passive open-plate capture method with both general media that support growth of many fungi (malt extract yeast peptone agar and potato dextrose yeast agar) and milk agar to mimic the cheese environment. Captured fungi were identified by DNA barcode with the ITS region. We then investigated cheese-specific growth patterns of isolates captured from the cheese cave air to see if the airborne fungi exhibit growth patterns similar to a domesticated *Penicillium candidum* used for cheese production.

Materials and Methods

Cheese Cave Environment

Fungi were captured in two man-made cheese caves used for aging cheese in a small-scale creamery in the eastern United States. Human traffic in the caves is low: one to three people are in the caves one to five times daily for less than ten minutes at a time to adjust cheese or refill humidifiers. Caves receive little to no sunlight and artificial lighting is used only when people are working in the cave. One cave is used to age hard cheeses for up to one year; temperature in this cave is maintained between 8.9 – 9.5°C with 85% humidity. The second cave is used to age bloomy cheeses for 1 – 3 weeks and to store feta cheese in salt-water brine; temperature in this cave is maintained between 10 – 10.5°C with 95%

humidity. At the time of capture, hard cheese and feta were present, but not bloomy cheeses.

Capture and Isolation of Fungi

Airborne fungi were captured on three culture media: malt extract yeast peptone agar (MYPA), potato dextrose yeast agar (PDYA) and milk agar for capture (MA-c). MYPA consisted of 35 g malt extract agar (MP Biomedicals), 2 g yeast extract (Fisher Bioreagents), and 1 g peptone (Fisher Bioreagents) per liter. PDYA contained 39 g potato dextrose agar (Carolina Biological) and 2 g yeast extract (Fisher Bioreagents) per liter. MA-c contained 24 g milk agar (Oxoid) per liter. Two 100 mm culture plates of each medium were placed at four locations within two cheese caves (24 capture plates per cheese cave). Capture plates were left uncovered for 7.5 hours to allow airborne fungi to fall onto culture medium. Plates were closed and incubated in the cheese cave for two days, then transported to the laboratory and incubated at room temperature (~20-22°C) for one week.

Individual fungal colonies were transferred from capture plates to fresh culture medium to isolate pure cultures. Isolates were grown on the same culture medium used for capture. Three isolates (JCF-65, JCF-69, and JCF-70) required a second passage to achieve pure cultures. Pure cultures were frozen at -80°C in 30% glycerol.

Identification by DNA Barcode

Fungal isolates were grown from glycerol stocks. Mycelium and conidia (if present) were scraped from the surface of approximately one third a culture plate. DNA was extracted using the Quick DNA Fungal/Bacterial Miniprep Kit (Zymo Research), according to manufacturer's instructions, with the following modification: cell walls were disrupted using two 20-second cycles on a Sonibeast homogenizer (BioSpec). The nuclear ribosomal internal transcribed spacer (ITS) was PCR amplified using 2.5 µL extracted DNA as template, ITS1 and ITS4 primers (Fungal Primer Mix, Carolina Biological), and Illustra PuReTaq Ready-to-Go PCR beads. Amplification protocol consisted of 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C.

Crude PCR product was sequenced in both directions using the M13F and M13R primers (Genewiz/Azenta Life Science). Each sequence was compared to the Genbank rRNA/ITS fungi database using the blastn program to determine putative genera of fungal isolates. Sequences were aligned in BioEdit version 7.2.4 using the ClustalW Multiple Alignment accessory application (Hall, 1999). Sequences were additionally compared to sequences used for phylogenetic analyses of the *Penicillium* genus (Visagie, Houbraken, et al., 2014), *Scopulariopsis* genus (Woudenberg et al., 2017), and *Cladosporium* genus (Bensch et al., 2018). Sequences were submitted to Genbank.

DNA was extracted from the commercial cheese culture *Penicillium candidum* (Danisco) and the ITS region PCR-amplified and sequenced. *Penicillium candidum* is a synonym of *Penicillium camemberti*; the commercial Danisco cheese culture is referred to as *P. candidum* here.

Growth Assays

Malt Extract Agar contained 35 g malt extract agar (MP Biomedicals) per liter. Milk Agar for growth assays contained 100 g skim milk (Difco) and 15 g agar per liter. Salted Milk Agar contained 100 g skim milk, 15 g NaCl, and 15 g agar per liter. A few drops of red food coloring was added to both the Milk Agar and Salted Milk Agar to facilitate visualization of white fungal hyphae. Fungal isolates were grown from glycerol stocks on MYPA for one week, and then transferred to growth assay media. Colony diameter was measured after seven days growth at room temperature on Malt Extract Agar, Milk Agar, or Salted Milk Agar. Growth of fungal isolates was compared to the growth of commercial cheese cultures *P. candidum* and *Geotrichum candidum* (Danisco). Growth assays were conducted in triplicate. Statistical analyses conducted in GraphPad Prism included the Shapiro-Wilk test for normality and a one-way ANOVA with Tukey's multiple comparisons.

Results

Viable, airborne fungi were captured passively on milk agar, malt extract yeast peptone agar (MYPA), and potato dextrose yeast agar (PDYA) in two man-made cheese caves. One cheese cave is used for aging hard cheeses up to a year and the other cheese cave is used to age bloomy cheeses for 1-3 weeks and to store feta cheese in closed containers with saltwater brine. Hard cheeses and feta, but not bloomy cheeses, were present at the time of capture. More fungal isolates were captured in the hard

cheese cave than in the bloomy cheese cave and more fungal isolates were captured on milk agar than on MYPA or PDYA (Figure 1). Colony morphology differed between culture media, with more conidial colonies on milk agar and PDYA than MYPA.

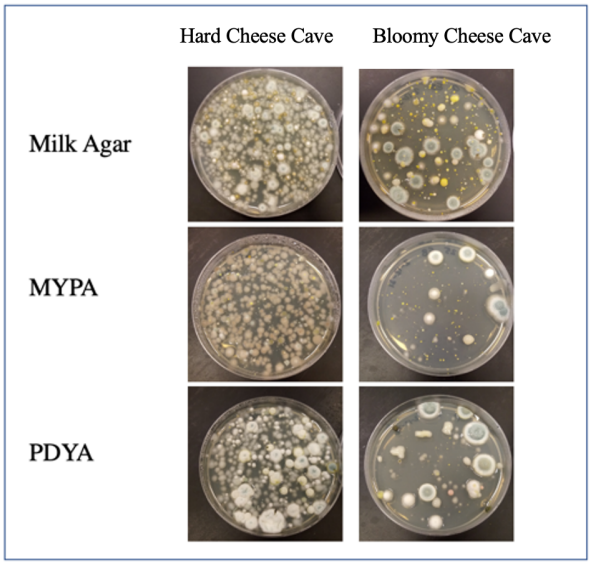

Figure 1. Capture Plates from Hard Cheese and Bloomy Cheese Caves. Select capture plates from each cave and each culture medium.

Thirty-one fungal isolates were selected to represent all the distinct morphologies observed. Eleven isolates were selected from the bloomy cheese cave capture plates, including four on milk agar, three on MYPA, and four on PDYA. Twenty isolates were selected from the hard cheese cave capture plates, including two on milk agar, ten on MYPA, and eight on PDYA. All isolates were passaged to pure culture and then tentatively identified to genus using the sequence of the nuclear ribosomal internal transcribed spacer (ITS) region Twenty-six isolates were identified as *Penicillium*, four isolates were identified as *Cladosporium*, and one isolate was identified as *Scopulariopsis* (Table 1). Putative identification was refined to section through comparison with sequences used for phylogenetic analyses of *Penicillium* (Visagie, Houbraken, et al., 2014). Isolate JCF-64 was identified to the Citrina section of *Penicillium*; the remaining 25 *Penicillium* isolates belong to the Fasciculata section. The *Penicillium*-Fasciculata isolates fell into two groups that differed in sequence at two nucleotides across the approximately 530-bp ITS region. Based on the ITS sequence, the Danisco *Penicillium candidum* also belongs to the *Penicillium* Fasciculata section.

Table 1. Genbank Accession Number and Putative Identification of Fungal Isolates.

Isolate	Accession	Putative genus or species complex
JCF-62	OQ437260	*Scopulariopsis* genus
JCF-63	OQ437261	*Penicillium* - Fasciculata
JCF-64	OQ437262	*Penicillium* - Citrina
JCF-65	OQ437263	*Penicillium* - Fasciculata
JCF-66	OQ437264	*Penicillium* - Fasciculata
JCF-67	OQ437265	*Penicillium* - Fasciculata
JCF-68	OQ437266	*Penicillium* - Fasciculata
JCF-69	OQ437267	*Penicillium* - Fasciculata
JCF-70	OQ437268	*Penicillium* - Fasciculata
JCF-71	OQ437269	*Penicillium* - Fasciculata
JCF-72	OQ437270	*Penicillium* - Fasciculata
JCF-73	OQ437271	*Cladosporium sphaerospermum*
JCF-74	OQ437272	*Penicillium* - Fasciculata
JCF-75	OQ437273	*Penicillium* - Fasciculata
JCF-76	OQ437274	*Cladosporium cladosporioides*
JCF-78	OQ437275	*Penicillium* - Fasciculata
JCF-79	OQ437276	*Penicillium* - Fasciculata
JCF-80	OQ437277	*Penicillium* - Fasciculata
JCF-81	OQ437278	*Penicillium* - Fasciculata
JCF-82	OQ437279	*Penicillium* - Fasciculata
JCF-83	OQ437280	*Penicillium* - Fasciculata
JCF-84	OQ437281	*Penicillium* - Fasciculata
JCF-85	OQ437282	*Penicillium* - Fasciculata
JCF-86	OQ724820	*Penicillium* - Fasciculata
JCF-87	OQ437283	*Penicillium* - Fasciculata
JCF-88	OQ437284	*Penicillium* - Fasciculata
JCF-93B	OQ437285	*Penicillium* - Fasciculata
JCF-94	OQ437286	*Penicillium* - Fasciculata
JCF-95	OQ437287	*Cladosporium cladosporioides*
JCF-96	OQ437288	*Cladosporium cladosporioides*
JCF-97	OQ437289	*Penicillium* - Fasciculata

ITS sequence is insufficient to resolve *Cladosporium* to species; unknown isolates can be tentatively identified to one of three species complexes based on high similarity over the ITS region to multiple species within a given complex (Bensch et al., 2018). JCF-76, JCF-95, and JCF-96

tentatively belong to the *Cladosporium cladosporioides* species complex based on high similarity to multiple species within this complex. The ITS region of JCF-73 had strong similarity to multiple species within the *Cladosporium sphaerospermum* species complex.

It is difficult to identify unknown isolates from indoor samples of fungi to species within the Microascaceae family, which includes the genera *Microascus* and *Scopulariopsis*; only five species within the *Scopulariopsis* genus can be identified definitively by ITS sequence (Woudenberg et al., 2017). The ITS sequence of JCF-62 was most similar to Genbank accession numbers LM652463, KX924020, and LM652484, which have been identified as *S. asperula* (LM652463), *S. caseicola* (KX924020), and *S. candida* (LM652484) (Woudenberg et al., 2017).

JCF-62 (*Scopulariopsis*) was captured on milk agar in the hard cheese cave. JCF-64 (*Penicillium* – Citrina section) was captured on milk agar in the bloomy cheese cave. Two of the *Cladosporium* isolates (JCF-73 and JCF-76) were captured on MYPA in the hard cheese cave and two of the *Cladosporium* isolates (JCF-95 and JCF-96) were captured on PDYA in the bloomy cheese cave. The 26 *Penicillium*-Fasciculata isolates were captured from both the hard cheese cave and the bloomy cheese cave, and were captured on all three culture media.

Three *Penicillium*-Fasciculata isolates (JCF-63, JCF-65, and JCF-69), the *Penicillium*-Citrina isolate (JCF-64), one *Cladosporium* isolate (JCF-73), and the *Scopulariopsis* isolate (JCF-62) were selected for growth assays (Figures 2 and 3). Growth of these representative fungal isolates on malt extract agar, milk agar, and salted milk agar was compared to the growth of *Penicillium candidum* and *Geotrichum candidum*, two commercial fungi used in cheese production (Figure 2). The commercial cheese strain *Penicillium candidum* exhibits almost no growth on malt extract agar; but grows well on milk agar and salted milk agar. All the fungal isolates show significantly larger colony diameter at seven days growth on malt extract agar compared to the commercial cheese strain *Penicillium candidum* and significantly smaller colony diameter compared to *G. candidum* (ANOVA $p<0.0001$, Tukey's pairwise comparison adjusted $p<0.01$).

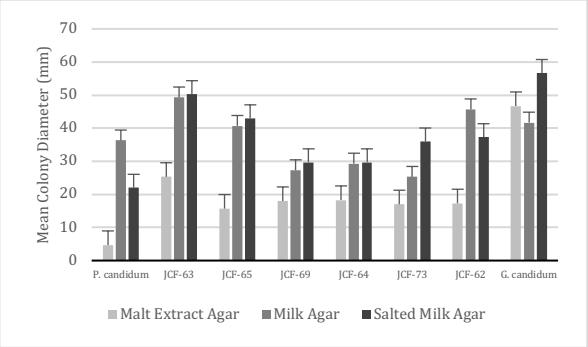

Figure 2. Growth on Malt Extract Agar, Milk Agar, and Salted Milk Agar. Mean (n=3) colony diameter plus standard error for *Penicillium candidum*, JCF-63 (*Penicillium)*, JCF-65 (*Penicillium*), JCF-69 (*Penicillium*), JCF-64 (*Penicillium*), JCF-73 (*Cladosporium*), JCF-62 (*Scopulariopsis*), and *Geotrichum candidum* grown on Malt Extract Agar, Milk Agar, and Salted Milk Agar at room temperature for seven days.

Casein is a large protein in milk that gives milk its opaque, white appearance; when casein is hydrolyzed, milk-based culture medium becomes clear. *Penicillium candidum* exhibits a large clearing (arrow in panel A, Figure 3); however, the *Penicillium*-Fasciculata isolates JCF-63, JCF-65, and JCF-69 do not exhibit casein hydrolysis (Figure 3, panels B, C, and D). The *Penicillium*-Citrina isolate JCF-64 exhibits moderate casein hydrolysis (Figure 3, panel F). The commercial cheese fungus *Geotrichum candidum*, the *Cladosporium* fungal isolate JCF-73, and the *Scopulariopsis* fungal isolate JCF-62 do not exhibit casein hydrolysis (Figure 3, panels E, G, and H).

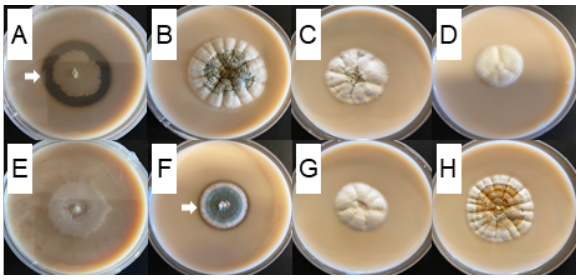

Figure 3. Casein Hydrolysis. Fungal isolates grown on Milk Agar for seven days at room temperature. Arrows indicate a clearing in the culture medium. Panel A: *Penicillium candidum*; B: JCF-63 (*Penicillium*); C: JCF-65 (*Penicillium*); D: JCF-69 (*Penicillium*); E: *Geotrichum candidum*; F: JCF-64 (*Penicillium*); G: JCF-73 (*Cladosporium*); H: JCF-62 (*Scopulariopsis*)

Most species in the *Pencillium* genus reproduce asexually through the production of conidia, which are usually green; these green conidia make the center of a colony appear green while the edge of the

colony (not yet producing conidia) appears white or tan from hyphal growth. The commercial cheese strain *Penicillium candidum* does not form conidia on milk agar (Figure 3, panel A) or on malt extract agar (data not shown). JCF-63 (*Penicillium*-Fasciculata) produces ample conidia on milk agar (Figure 3, panel B), as does JCF-64 (*Penicillium*-Citrina; Figure 3, panel F). *Scopulariopsis* produces conidia in shades of brown; JCF-62 exhibits plentiful conidiation on milk agar (Figure 3, panel H).

Discussion

This study found cheese-associated fungi to be the dominant fungi found in the cheese cave environment. Most of the fungi (26 of 31 isolates) captured in the cheese cave environment belong to the *Penicillium* genus, with 25 of these in the Fasciculata section that includes the commercial *Penicillium candidum* used for cheese culture. In addition, *Scopulariopsis*, commonly found in indoor environments and often associated with cheese production was also isolated (Ropars et al., 2012; Woudenberg et al., 2017). Several *Penicillium* and *Scopulariopsis* species have only been found in the cheese environment (Ropars et al., 2012).

Only five of the 31 isolates captured in the cheese cave environment are environmental species not usually associated with cheese. One isolate belongs to the *Penicillium* Citrina section; *Penicillium citrinum* is commonly found in surveys of both indoor and outdoor air (Flannigan et al., 2011). *Penicillium citrinum* generally poses no risk to people, though there are rare reports of infection (Flannigan et al., 2011).

Four isolates belong to the *Cladosporium* genus; *Cladosporium* are among the most commonly identified fungi in both indoor and outdoor surveys (Fradkin et al., 1987; Nevalainen et al., 2015; Ren et al., 1999; Shelton et al., 2002; Visagie, Hirooka, et al., 2014). Species from all three *Cladosporium* species complexes (*C. cladosporioides*, *C. herbarum*, and *C. sphaerospermum*) have been identified in indoor environments (Bensch et al., 2018). *Cladosporium herbarum* is the most commonly isolated fungus in surveys of indoor air in temperate zones (Flannigan et al., 2011). Because the maximum growth temperature of *C. herbarum* is 32°C, *C. herbarum* is not a health problem for humans (Flannigan et al., 2011). A few studies have investigated the fungal diversity in the air of cheese-production facilities, and found that environmental fungi can contaminate cheese (Garnier et al., 2017; Kandasamy et al., 2020). All three *Cladosporium* species complexes have been isolated from spoiled dairy products (Garnier et al., 2017).

This study also investigated the cheese-related growth patterns of selected fungal isolates captured in the cheese cave. Interestingly, the *Penicillium*-Fasciculata fungi that we captured in the cheese cave environment do not exhibit the same growth characteristics as the commercial cheese strain *Penicillium candidum*. *P. candidum* exhibits poor growth on malt extract agar compared to milk-based media, hydrolyzes casein, and does not produce conidia. In contrast, our *Penicillium*-Fasciculata cheese cave isolates grow well on malt extract agar and do not hydrolyze casein. Some of our *Penicillium*-Fasciculata isolates produce prolific conidia. Our *Penicillium*-Fasciculata cheese cave isolates cannot be distinguished from the commercial cheese strain *P. candidum* by ITS sequence. However, if these airborne isolates derive from the commercial cheese-production strain, they have lost some of the growth characteristics associated with cheese production. Microbes associated with cheese production are typically cultivated on bread, then used to inoculate the cheese medium; the cheese-production microbes are not repeatedly passaged on cheese (Steensels et al., 2019). Airborne spores of cheese-production fungi may indicate incomplete domestication (Steensels et al., 2019).

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