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Diversity of Yeast and Mold Species from a Variety of Cheese Types

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Abstract

To generate a comprehensive profile of viable fungi (yeasts and molds) on cheese as it is purchased by consumers, 44 types of cheese were obtained from a local grocery store from 1 to 4 times each (depending on availability) and sampled. Pure cultures were obtained and identified by DNA sequence of the ITS region, as well as growth characteristics and colony morphology. The yeast *Debaryomyces hansenii* was the most abundant fungus, present in 79 % of all cheeses and 63 % of all samples. *Penicillium roqueforti* was the most common mold, isolated from a variety of cheeses in addition to the blue cheeses. Eighteen other fungal species were isolated, ten from only one sample each. Most fungi isolated have been documented from dairy products; a few raise potential food safety concerns (i.e. *Aspergillus flavus*, isolated from a single sample and capable of producing aflatoxins; and *Candida parapsilosis*, an emerging human pathogen isolated from three cheeses). With the exception of *D. hansenii* (present in most cheese) and *P. roqueforti* (a necessary component of blue cheese), no strong correlation was observed between cheese type, manufacturer, or sampling time with the yeast or mold species composition.

Introduction

The cheese microbiota has long been known to be the major contributor to cheese flavor, aroma, texture, and appearance [3, 21, 38]. This complex microbial ecosystem includes both culture organisms and adventitious bacteria, yeasts, and molds [45]. The diversity and number of specific types of organisms present in cheese depend on the microbial quality of the milk, handling and heat treatment of the milk, manufacturing and curd-handling conditions, temperature and humidity during ripening, amount and manner of salting, and exposure of the cheese to exogenous microorganisms during and after manufacture [47]. Cheeses that are aged for even a short period of time may experience a shift in the microbiota, with different organisms or groups of organisms succeeding others [21]. In particular, yeasts and molds are known to significantly affect succession events in several types of cheese [35].

Yeasts and molds may enter cheese from a variety of sources, including the starter culture, ambient air, brine, processing equipment, and workers [5, 34, 39]. Many yeasts can contribute to taste, flavor, and appearance; however, not all species produce beneficial effects, and a species may benefit one cheese while spoiling another. Particular yeasts on the surface of the cheese can cause spoilage or generate undesirable aromas, flavors, or other metabolic products that reduce the quality of cheese [2, 4, 23, 50]. Furthermore, while yeasts are rarely associated with foodborne infections, studies have shown the presence of medically relevant yeast species in various cheeses, including *Candida albicans* [11, 51], *Candida tropicalis* [13, 51], *C. krusei,* and *C. glabrata* [51].

Like yeasts, molds are often added to certain varieties of cheese to provide a characteristic appearance, consistency, and flavor, and prolong shelf life [16]. However, adventitious molds that contaminate cheese can produce mycotoxins and may present a potential health risk [9, 37, 43]. The species and abundance of non-starter yeasts and molds vary between different types of cheese, between cheeses of the same variety produced by different manufacturers, and even between different batches of the same cheese from the same manufacturer [30].

In this study, we sought to obtain a comprehensive overview of the viable fungi–yeasts and molds, beneficial, and detrimental—inhabiting cheeses from numerous categories and origins. To this end, we sampled a representative variety of the cheeses available at a local supermarket at each of four dates, spanning a period of a year and a half. In total, 44 cheeses in eight categories, produced in six countries and seven US states, were analyzed.

Materials and Methods

Sampling of Cheese

Forty four distinct cheeses (Table 1), representing the major cheese categories [12], were purchased from retailers in Lincoln, Nebraska, USA between 2012 and 2013. In some cases, the same cheese was purchased at multiple times (up to 4). Samples were maintained at 4 °C until analysis.

Isolation of Yeasts and Molds

Six samples of 10 g each were aseptically removed from each cheese, three each from rind and core. Samples were homogenized in 50 ml of sterile 1 % peptone water using a Stomacher Lab Blender 400 (Seward Laboratory Systems, Davie, FL, USA) for 5 min at normal speed. Samples were serially diluted and plated on YGC agar (yeast extract 0.5 %, glucose 2 %, agar 1 % and chloramphenicol 0.1 %) and incubated aerobically for 5 days at 25 °C. Individual colonies were selected based on morphology and color. The colonies were restreaked on YGC agar to ensure pure culture, incubated for 4 days at 25 °C, and maintained at 4 °C until processing.

DNA Isolation

Pure colonies were inoculated into YGC broth and incubated for 48 h at 25 °C. For yeasts, DNA was extracted following the method described by Harju and colleagues [17]. Briefly, cells were exposed to repeated freeze thawing to induce lysis, followed by chloroform extraction and ethanol precipitation. For molds, the CTAB-phenol–chloroform method described by Pitkin and colleagues [40] was used.

Identification of Yeasts and Molds

Polymerase chain reaction (PCR) was performed using the fungal-specific forward primer ITS1F [14] and the universal eukaryotic reverse primer TW13 [53] which amplified the ITS1, 5.8S, ITS2, and the 5'–500 bp of the 28S regions of the nuclear ribosomal RNA genes. The amplification reaction was carried out in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with an initial denaturation step at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplification products were analyzed on 0.7 % agarose gel with 0.5 μg ml^{-1} ethidium bromide. PCR products were submitted for Sanger sequencing using ITS1F to the Michigan State University Research Technology Support Facility. Several colonies were sequenced from each cheese, and identical sequences from a single sample were considered to represent one strain and pooled. Samples were identified by sequence homology using nucleotide BLAST against the UNITE curated fungal ITS sequence database [25] and the curated FUNCBS database, as accessed through the Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl/Collections/BioloMICSSequences.aspx?file=all ; accessed June 2014).

The sequences identified as *D. hansenii* or *P. roqueforti* were aligned using Muscle in MEGA version 6.06 [46]. The maximum likelihood method was used to determine overall similarity. One thousand bootstrap replicates were performed; otherwise, default settings were used. *C. parapsilosis* (GenBank accession number KM115125) was used as an outgroup for *D. hansenii,* and *Penicillium chrysogenum*, *P. commune*, *P. verrucosum*, and *P. echinulatum* (KM115128, KM115167, KM115144, and KM115163, respectively) were used as an outgroup for P. *roqueforti*.

Results

The 44 cheese samples yielded more than 12,000 yeast and mold colonies; 239 colonies were obtained in pure culture, from which DNA was extracted and submitted for sequencing. When identical sequences from the same cheese sample were excluded, 127 unique sequences remained, and these were submitted to GenBank as accessions KM115040-KM115167. After BLAST analysis, 20 different yeast and mold species were identified (Table 1).

Diversity of Yeasts in Cheese

Eight different yeasts were isolated from 39 types of cheese (out of 44 sampled; Table 1). Among these, *D. hansenii* was the most abundant species and was present in 79 % of all cheeses and 63 % of all samples. The second most frequently isolated yeast species was *Galactomyces candidus*, present in 13 % of all cheeses. Other yeasts isolated were identified as *C. parapsilosis, C. sake, C. batistae, Kluyveromyces lactis*, *Pichia kudriavzevii*, and *Yarrowia lipolytica*.

Table 1. Yeasts and molds isolated from retail cheese samples in Nebraska, USA

Very hard cheese		
Asiago (Wisconsin, US; A)	1	*Debaryomyces hansenii*
	2	*Debaryomyces hansenii*
Asiago (Wisconsin, US)	1	*Debaryomyces hansenii*
Gruyere (Wisconsin, US; B)	1	*Debaryomyces hansenii*
	2	*Debaryomyces hansenii*, *Galactomyces candidus*, *Aspergillus* cf. *niger*, *Penicillium* cf. *camemberti*
	3	*Debaryomyces hansenii*
Parmesan-type (Wisconsin, US; A)	1	*Debaryomyces hansenii*
	2	*Debaryomyces hansenii*
	3	*Debaryomyces hansenii*, *Cladosporium cladosporioides*
Parmesan-type (Illinois, US)	1	*Galactomyces candidus*, *Penicillium verrucosum*
	2	**
Parmesan (Parma, Italy)	1	*Debaryomyces hansenii*, *Eurotium rubrum*
	2	**
Hard cheese		
Cheddar (Wisconsin, US)	1	*Debaryomyces hansenii*
Cheddar (Wisconsin, US)	1	*Debaryomyces hansenii*
Cheddar (Devon, UK)	1	**
	2	*Debaryomyces hansenii*
Cheddar, Goat (Missouri, US)	1	*Candida batistae*, *Candida parapsilosis*, *Galactomyces candidus*
Jarlsberg (Norway)	1	*Debaryomyces hansenii*, *Galactomyces candidus*
	2	*Debaryomyces hansenii*
	3	*Debaryomyces hansenii*, *Aspergillus phoenicis*
	4	*Debaryomyces hansenii*, *Penicillium* cf. *camemberti*
Romano (Wisconsin, US; A)	1	*Debaryomyces hansenii*
Swiss-type (Wisconsin, US)	1	*Debaryomyces hansenii*, *Galactomyces candidus*
	2	*Debaryomyces hansenii*
	3	*Candida parapsilosis*, *Debaryomyces hansenii*
	4	*Aspergillus flavus*
Semi-hard cheese		
Colby (Wisconsin, US; C)	1	*Debaryomyces hansenii*
Gouda (Missouri, US)	1	Candida sake, *Debaryomyces hansenii*
Gouda (Netherlands)	1	Candida sake, *Debaryomyces hansenii*
Gouda (Wisconsin, US)	1	*Debaryomyces hansenii*
Gouda, Smoked (Netherlands; D)	1	*Penicillium* cf. *camemberti*
	2	**
	3	*Debaryomyces hansenii*, *Aspergillus* cf. *niger*, *Cladosporium sphaerospermum*
Monterey Jack (Nebraska, US; E)	1	*Debaryomyces hansenii*, *Penicillium* cf. *roqueforti*
	2	*Debaryomyces hansenii*, *Penicillium* cf. *roqueforti*
	3	**
	4	*Debaryomyces hansenii*
Provolone (Wisconsin, US)	1	*Debaryomyces hansenii*
	2	*Debaryomyces hansenii*
	3	*Debaryomyces hansenii*
Raclette (Wisconsin, US; B)	1	*Debaryomyces hansenii*
Semi-soft cheese		
Fontina (Wisconsin, US; A)	1	*Debaryomyces hansenii*, *Penicillium verrucosum*
	2	**
	3	*Debaryomyces hansenii*

Table 1. Yeasts and molds isolated from retail cheese samples in Nebraska, USA (*continued*)

Havarti (Wisconsin, US)	1	*Penicillium* cf. *camemberti*
Bel Paese (Italy)	1	*Debaryomyces hansenii*
	2	*Debaryomyces hansenii*
	3	*Debaryomyces hansenii*, *Penicillium* cf. *camemberti*, *Penicillium gladioli*
	4	*Debaryomyces hansenii*, *Penicillium* cf. *camemberti*
Muenster-type (Wisconsin)	1	*Debaryomyces hansenii*
Wensleydale (UK)	1	*Debaryomyces hansenii*
	2	*Debaryomyces hansenii*, *Penicillium* cf. *roqueforti*
	3	*Debaryomyces hansenii*, *Pichia kudriavzevii*, *Penicillium* cf. *camemberti*
Blue cheese		
Blue (Minnesota, US; F)	1	*Penicillium* cf. *roqueforti*
	2	*Debaryomyces hansenii*, *Penicillium* cf. *roqueforti*
Blue (Wisconsin, US)	1	*Penicillium* cf. *roqueforti*
	2	*Candida parapsilosis*, *Penicillium* cf. *roqueforti*
	3	*Penicillium* cf. *roqueforti*
Blue (Wisconsin, US; B)	1	*Penicillium* cf. *roqueforti*
	2	*Debaryomyces hansenii*, *Penicillium* cf. *roqueforti*
	3	*Debaryomyces hansenii*, *Penicillium* cf. *roqueforti*
Blue (Wisconsin, US; B)	1	*Debaryomyces hansenii*, *Penicillium* cf. *roqueforti*
Stilton (Leicestershire, UK)	1	*Debaryomyces hansenii*, *Penicillium* cf. *camemberti*, *Penicillium* cf. *roqueforti*
Gorgonzola-type (Minnesota, US; F)	1	*Penicillium* cf. *roqueforti*
	2	*Penicillium* cf. *roqueforti*
	3	*Penicillium* cf. *roqueforti*
Soft fresh cheese		
Cottage cheese (Iowa, US)	1	**
	2	**
	3	**
Cream cheese (Wisconsin, US)	1	**
	2	**
	3	**
Feta (Pennsylvania, US)	1	*Kluyveromyces lactis*
	2	**
	3	*Debaryomyces hansenii*, *Penicillium* cf. *roqueforti*
Mascarpone (Wisconsin, US; A)	1	**
	2	*Debaryomyces hansenii*
	3	*Penicillium* cf. *roqueforti*
Mozzarella (Wisconsin, US; A)	1	*Penicillium brevicompactum*, *Penicillium* cf. *camemberti*
	2	*Penicillium* cf. *roqueforti*, *Penicillium paneum*
Ricotta (Illinois, US)	1	**
	2	*Debaryomyces hansenii*, *Penicillium* cf. *roqueforti*
	3	**
Washed rind cheese		
Mold-ripened (Bavaria, Germany)	1	*Debaryomyces hansenii*
	2	*Debaryomyces hansenii*, *Galactomyces candidus*
	3	*Debaryomyces hansenii*, *Galactomyces candidus*, *Yarrowia lipolytica*
	4	*Debaryomyces hansenii*, *Galactomyces candidus*, *Yarrowia lipolytica*, *Penicillium* cf. *roqueforti*
Not otherwise classified		
Spanish-type fresh (Wisconsin, US)	1	*Debaryomyces hansenii*
Spanish-type fresh (Wisconsin, US; B)	1	*Debaryomyces hansenii*

Table 1. Yeasts and molds isolated from retail cheese samples in Nebraska, USA (*continued*)

Novelty (Wisconsin, US; C)	1	*Debaryomyces hansenii*
Novelty (Nebraska, US; E)	1	*Debaryomyces hansenii*
Cream Gouda (Netherlands; D)	1	*Debaryomyces hansenii*

Each cheese named represents a distinct type and manufacturer. Manufacturers from whom multiple cheeses were purchased are indicated as A–F. A cheese was purchased and sampled from 1 to 4 times, depending on availability
** Fungus was not detected

Diversity of Molds in Cheese

BLAST searches identified 12 different molds from 23 types of cheese (out of 44 sampled; Table 1). The most common molds were *Penicillium* (approximately 50 % of molds isolated, and present in 45 % of cheeses overall) and *Aspergillus* (15 % of molds isolated, in 11 % of cheeses). At the species level, *Penicillium* cf. *roqueforti* was the predominant mold, present in all blue cheese samples and 23 % of samples overall. *P.* cf. *camemberti* was present in ~10 % of all samples. *P. verrucosum* and *Aspergillus* cf. *niger* were each isolated from two cheeses, and seven mold species were isolated once each.

Fungal Distribution and Effect of Manufacturer

From 0 to 4 distinct fungal species were isolated from any individual cheese at any given sampling time. The largest number of species isolated from any single cheese was four. Fungi could not be cultured from two cheeses—cottage cheese and cream cheese, and one or two samples each from other soft fresh cheeses (Feta, Mascarpone, and Ricotta) yielded no cultures. Samples from which yeasts and molds were isolated contained $1.5 \times 10^2 – 7.95 \times 10^8$ CFU g^{-1} (median 8.5×10^3 CFU g^{-1}; data not shown).

Six manufacturers, designated A, B, C, D, E, and F (see Table 1; Figures 1, 2) each produced multiple types of cheese in our survey. *D. hansenii* was isolated from all five cheeses produced by manufacturer A and in eight out of nine samples. The only sample from this manufacturer from which *D. hansenii* was not isolated was a blue cheese. *P.* cf. *roqueforti* was recovered from this cheese. For manufacturer B, *D. hansenii* was isolated from five out of six cheeses and eight out of 13 samples. Fungi were isolated from four of these samples, and *P.* cf. *roqueforti* alone was isolated from one sample. Mozzarella made by manufacturer A yielded multiple *Penicillium* species (including *P. brevicompactum, P. paneum, P.* cf. *camemberti*, and *P.* cf. *roqueforti*). Samples of cheese from manufacturer A also yielded *Cladosporium cladosporioides* and *P. verrucosum*. Manufacturers C–F each produced two cheeses in our survey, for a total of two samples (manufacturer C) to five samples (manufacturer F).

DNA Sequence Analysis

ITS sequence alignment was used to assess the similarity of *D. hansenii* and *P.* cf. *roqueforti* isolates from cheese samples. Fifty-eight *D. hansenii* ITS sequences were aligned to a length of 485 nucleotides, with 374 invariant positions. The majority of sequences did not differ significantly from the consensus sequence. For example, 23 sequences matched the consensus, and a further ten differed by only one nucleotide. However, ten sequences differed from the consensus by ten or more nucleotides (maximum of 20). Isolates formed many clades with high (>95 %) bootstrap support (Figure 1); however, this represents comparatively few shared derived characters, and is likely an artifact of long branch attraction. No significant clustering of isolate by cheese, type, manufacturer, or date of collection (data not shown) was observed.

Twenty nine *P.* cf. *roqueforti* sequences, from ten cheeses, produced an alignment of 619 nucleotides, 578 of which were invariant. Eighteen sequences did not differ from the consensus and four differed by only one nucleotide; only two sequences differed from the consensus at ten or more positions [12 (distinct) positions for both]. The likelihood tree (Figure 2) showed little resolution, congruent with the limited sequence variation between isolates. Comparison of database sequences suggested that *P. roqueforti*, *P. carneum,* and *P. paneum* could not be reliably distinguished by ITS sequence data; in our analysis, *P. carneum* isolates were indeed indistinguishable, while the two *P. paneum* isolates were clearly distinct from *P. roqueforti* and *P. carneum,* clustering with the outgroup taxa with 98 % bootstrap support. As for *D. hansenii* strains, sequences did not cluster significantly by cheese, type, manufacturer, or date of collection.

Discussion

In this survey of 44 cheeses, *D. hansenii*, present in 63 % of all samples, was clearly the dominant fungus. The prevalence of *D. hansenii* in cheese is likely due to several physiological properties. This organism is able to grow in high salt

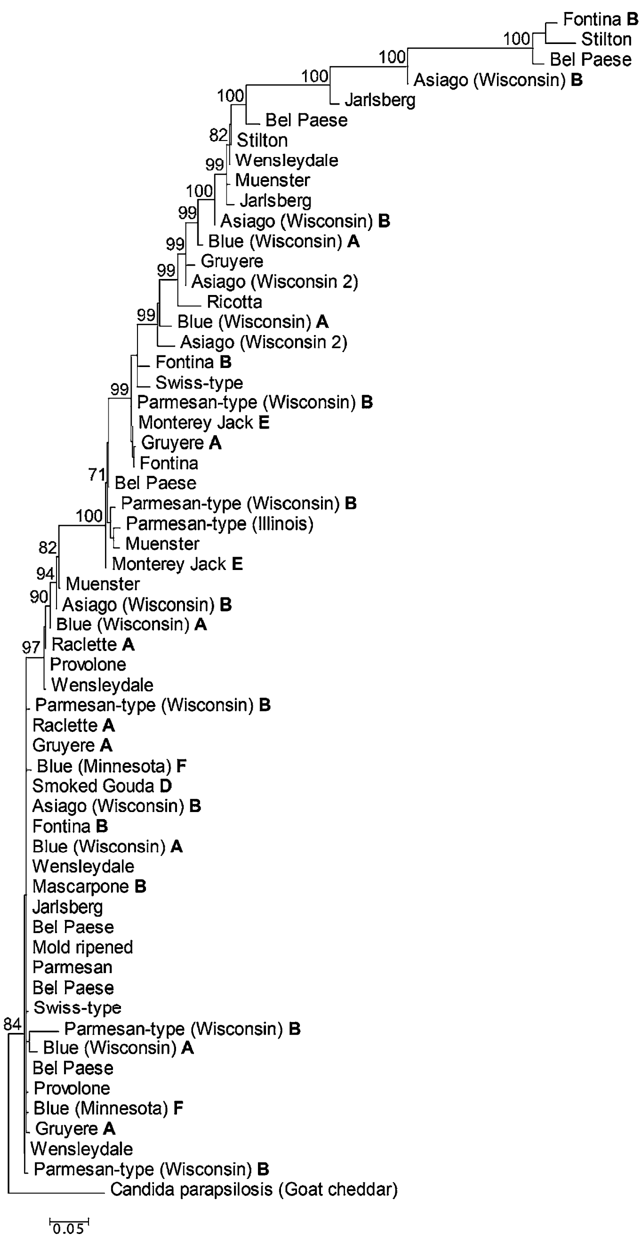

Figure 1. Maximum likelihood tree of *Debaryomyces hansenii* sequences from cheese. 1000 bootstrap replicates were performed; bootstrap support ≥70 % is shown.

concentrations and low pH, can use lactate as the main carbon source [15], produces proteolytic and lipolytic enzymes capable of metabolizing milk proteins and fat, and grows at low temperature and low water activity [8, 52]. The majority of *D. hansenii* isolates were highly similar (Figure 1), and no clear clustering by cheese, type, manufacturer, or date was detected. With a few exceptions, *D. hansenii* isolated in this study may represent a single domesticated adjunct culture strain, or closely related strains in widely separated locations [28].

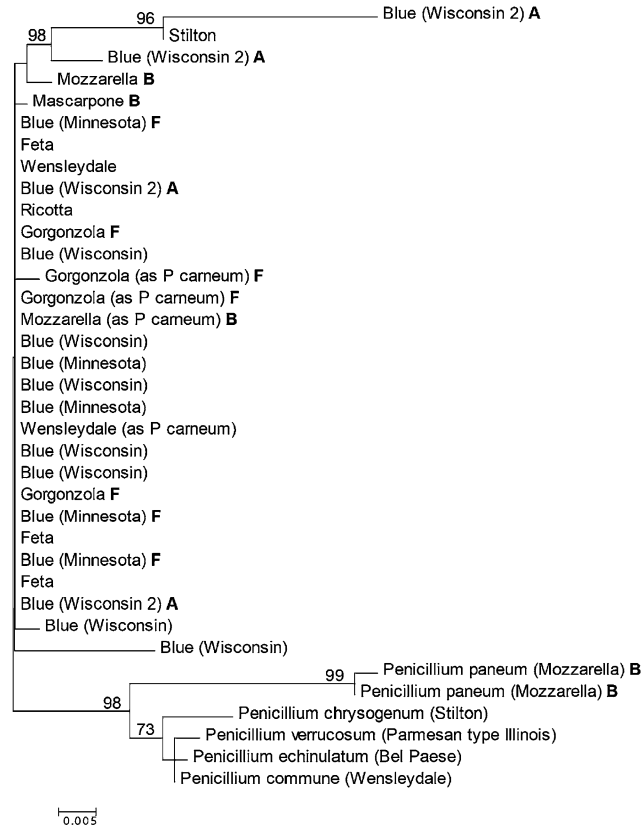

Figure 2. Maximum likelihood tree of *Penicillium* cf. *roqueforti* sequences from cheese. 1000 bootstrap replicates were performed; bootstrap support ≥70 % is shown.

The second most common yeast, *Galactomyces candidus* (= *Geotrichum candidum*), was isolated from Gruyere-type, Parmesan-type, goat's milk Cheddar, Swiss-type, Jarlsberg, and a mold-ripened, washed rind cheese. *G. candidus* is used as a starter culture in surface mold-ripened cheeses [32, 54] and is generally recognized as safe. *Pichia kudriavzevii* was isolated from a single sample of Wensleydale cheese. This yeast is a common species in Graukase cheese and has also been isolated from brines [28]. *Kluyveromyces lactis* was also isolated only once, from a single sample of feta cheese. Its presence in cheese may contribute to flavor development, increasing the concentration of odorous compounds and changing the flavor. It is also known for its ability to assimilate lactose [10].

Mold-ripened cheeses are known to contain not only the widely used production strains (e.g., *P. roqueforti* and *P. camemberti*), but also other species of *Penicillium* and other fungal genera. *Penicillium* species diversity may be underreported in this study; BLAST searches gave top hits to *P. brevicompactum*, *P. camemberti*, *P.* carneum, *P.* chrysogenum, *P. commune, P. gladioli, P. paneum, P. roqueforti*, *P. solitum,* and *P. verrucosum*. However, ITS sequence is insufficient to accurately distinguish *P. chrysogenum, P. commune* and *P. solitum* from *P. camemberti*, or *P. carneum,* and some isolates of *P. paneum* from *P. roqueforti*. In this survey, the abundance of *P.* cf. *roqueforti* isolates varied greatly among the different types of cheese. As expected, all blue cheese samples contained *P.* cf. *roqueforti* due to the use of *P. roqueforti* as a secondary culture [20]. Little differentiation was observed between strains isolated from different blue cheeses (Figure 2). The homogeneity of strains in blue cheese is likely due to use of the same strains as adjunct cultures; similar strains in non-blue cheeses (Mozzarella, Mascarpone) may represent within-plant contamination.

Safety concerns raised by adventitious fungi include the potential for infection by opportunistic pathogens, and the potential of various molds to produce mycotoxins. *C. parapsilosis,* isolated from Swiss-type, blue cheese, and goat's milk Cheddar, is an emerging human pathogen capable of causing invasive candidiasis due to exogenous acquisition [33, 48]; however, infection due to consumption of contaminated food has not been documented. *C. parapsilosis* has been isolated from a wide variety of environmental sources, including hands of workers, soil, freshwater, marine water, and plants [33], and has been recorded as a dominant species in the Italian cheese Manteca [44]. *Yarrowia lipolytica*, isolated from two samples of a washed rind cheese, is also an emerging opportunistic pathogen, although cases are rare [22].

Aspergillus and *Penicillium* species are capable of producing a wide range of mycotoxins. Of molds isolated in this study, *Aspergillus flavus*, isolated from a single sample of Swiss cheese and capable of producing aflatoxins, presents the greatest potential public health concern. Hayaloglu and Kirbag reported *A. flavus* from a single sample of Turkish Kuflu cheese; however, this fungus is much more commonly associated with foods of plant origin [18]. Aflatoxin in dairy is an acknowledged problem, but is associated with the metabolism of aflatoxin B1 in lactating cattle to produce the milk-associated aflatoxin M1, and not generally with the growth of fungi in dairy products. *A. flavus* is capable of growth and aflatoxin production following artificial inoculation on Cheddar [29], so the risk, while low, exists. *A. niger*, isolated from smoked Gouda, is used in industrial fermentations and such strains have generally recognized as safe (GRAS) status; however, some strains have been reported to produce ochratoxin [1]. *P. roqueforti* isolates can produce PR-toxin, roquefortine C, mycophenolic acid, and andrastin A [6, 36] but PR-toxin is unstable in cheese and the toxicity and role of the other potential mycotoxins in human health have not been conclusively demonstrated [6]. Wild *Penicillium* related to *P. roqueforti* and *P. camemberti* may produce patulin, cyclopaldic acid [7], and cyclopiazonic acid in culture [27, 41]. Cyclopiazonic acid has been produced on cheese agar [27], but has not been implicated in human poisoning [41].

Food spoilage occurs when microorganisms, including starter culture strains, grow in excess or in products where they are not desired. *Penicillium* species are the primary spoilage agents of refrigerated cheese, and prolonged storage of refrigerated packaged cheese creates an environment which favors *P. roqueforti* [26]. *P.* cf. *roqueforti* was isolated from one sample each of Wensleydale, Mascarpone, Feta, Ricotta, a mold-ripened washed rind cheese, an alpine style cheese, and two samples of Monterey Jack, in which it is more likely a contaminant than an ingredient [49]. *P. commune* is among the most common spoilage organisms on cheese [21, 50] and growth results in discoloration at the surface and production of off flavors [31]. *Aspergillus niger* is commonly isolated from foods and can cause thread mold spoilage of cheese [19]. Of the yeasts we isolated, only *C. parapsilosis* is considered a serious spoilage organism in dairy, due to its production of lipolytic and strong proteolytic enzymes [42], a trait notably shared with *Yarrowia lipolytica* [52]. *C. parapsilosis* represents a potential spoilage agent in the cheeses from which we isolated it (goat's milk cheddar, Swiss-type, and blue); however, it should be noted that none of the cheeses in our study were noticeably spoiled. We only detected *Y. lipolytica* in two samples of a mold-ripened cheese, a substrate from which this yeast has been previously reported as of high abundance, and in which it is considered a defect [55].

With the exception of *P. roqueforti* and blue cheese, and the near-ubiquity of *D. hansenii*, there was no strong correlation between cheese (i.e., Cheddar), cheese type (i.e., hard cheese), manufacturer, or sampling date (data not shown), with the yeast or mold species isolated. No manufacturer could be said to possess an obvious "house blend" of fungi, although the intentional use of *D. hansenii* by manufacturers A, B, and possibly C is probable, while the intentional use of *P. roqueforti* in blue cheeses is certain.

Blue cheeses and soft fresh cheeses were the only categories in which *D. hansenii* was lacking from the majority of samples. Soft fresh cheeses, such as cottage cheese and cream cheese, do not undergo a prolonged ripening process, nor are yeasts or molds commonly used as starters for these cheeses. Due to the high moisture content and low pH, soft fresh cheeses are particularly prone to fungal spoilage [24] and, while both *D. hansenii* and *P. roqueforti* were isolated from these cheeses (along with more overt spoilage species such as *P. carneum*), it may be assumed that these fungi were contaminants.

Our sampling obtained a broad diversity of mold and yeast species; however, some species may be unculturable on the chosen media. Others may have been present at such low numbers, or grown so slowly, that they were concealed by the dominant species. Colonies were selected based on morphology, color, shape and size, so species with similar gross morphology may have been under-sampled or undetected.

It is difficult to know whether species are present from the starter culture (if used) or if they are natural contaminants. This may be due to contamination from different sources such as the environment, workers, ripening environment, handling sources, and sampling bias. We did not consider the length of storage prior to sale in our analyses, which may change the abundance of fungal species. The longer the storage time, the higher the chance of cheese contamination.

The yeasts and molds isolated differed between cheeses, cheese types, and samplings, as would be predicted. Yeast and mold species that were not consistently detected between samplings or within a manufacturer's products are likely contaminants [28, 47]. The source(s) might be predicted based on fungal ecology (i.e., farm environment, factory environment, or personnel), but cannot be conclusively traced. It is important to examine the production line and identify possible points in the process where the cheeses are exposed to mold and yeast contamination. However, the majority of molds and yeasts detected in this study were likely starter culture organisms and unlikely to cause serious harm.

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References

1. Abarca ML, Bragulat MR, Castellá G, Cabañes FJ (1994) Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. Appl Environ Microbiol 60:2650–2652
2. Atanassova M, Choiset Y, Dalgalarrondo M, Chobert JM, Dousset X, Ivanova I (2003) Isolation and partial biochemical characterization of a proteinaceous anti-bacteria and anti-yeast compound produced by *Lactobacillus paracasei* subsp. *paracasei* strain M3. Int J Food Microbiol 87:63–73
3. Beresford TP, Fitzsimons N, Brennan NL, Cogan TM (2001) Recent advances in cheese microbiology. Int Dairy J 11:259–274
4. Bintsis T, Papademas P (2002) Microbiological quality of white-brined cheeses: A review. Int J Dairy Tech 55:113–120
5. Borelli BM, Ferreira EG, Lacerda ICA, Franco GR, Rosa CA (2006) Yeast populations associated with the artisanal cheese produced in the region of Serra da Canastra, Brazil. World J Microbiol Biotechnol 22:1115–1119
6. Bourdichon F, Casaregola S, Farrokh C, Frisvad JC, Gerds ML, Hammes WP et al (2012) Food fermentations: Microorganisms with technological beneficial use. Int J Food Microbiol 154:87–97
7. Boysen M, Skouboe P, Frisvad J, Rossen L (1996) Reclassification of the *Penicillium roqueforti* group into three species on the basis of molecular genetic and biochemical profiles. Microbiology 142:541–549
8. Breuer U, Harms H (2006) *Debaryomyces hansenii*—An extremophilic yeast with biotechnological potential. Yeast 23:415–437
9. Creppy EE (2002) Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicol Lett 127:19–28
10. De Freitas I, Pinon N, Berdagué J, Tournayre P, Lortal S, Thierry A (2008) *Kluyveromyces lactis* but not *Pichia fermentans* used as adjunct culture modifies the olfactory profiles of cantalet cheese. J Dairy Sci 91:531–543
11. El-Sharoud WM, Belloch C, Peris D, Querol A (2009) Molecular identification of yeasts associated with traditional Egyptian dairy products. J Food Sci 74:M1–M6
12. Fox PF, McSweeney PLH (2004) Cheese: An overview. In: Fox PF, McSweeney PLH, Cogan TM, Guinee TP (eds.) Cheese: chemistry, physics and microbiology, vol 1, 3rd edn. Elsevier Academic, Amsterdam, pp 1–18
13. Gadaga TH, Mutukumira AN, Narvhus JA (2000) Enumeration and identification of yeasts isolated from Zimbabwean traditional fermented milk. Int Dairy J 10:459–466
14. Gardes M, Bruns TD (1993) ITS primers with enhances specifocity for basidiomycetes — Application to the identification of mycorrhizae and rusts. Mol Ecol 2:113–118
15. Gori K, Sorensen LM, Petersen MA, Jespersen L, Arneborg N (2012) *Debaryomyces hansenii* strains differ in their production of flavor compounds in a cheese-surface model. MicrobiologyOpen 1:161–168
16. Haasum I, Nielsen PV (1998) Physiological characterization of common fungi associated with cheese. J Food Sci 63:157–161
17. Harju S, Fedosyuk H, Peterson KR (2004) Rapid isolation of yeast genomic DNA: Bust n' grab. BMC Biotechnol 4:8
18. Hayaloglu AA, Kirbag S (2007) Microbial quality and presence of moulds in Kuflu cheese. Int J Food Microbiol 115:376–38
19. Hocking AD, Faedo M (1992) Fungi causing thread mold spoilage of vacuum packaged Cheddar cheese during maturation. Int J Food Microbiol 16:123–130
20. Houbraken J, Frisvad JC, Samson RA (2010) Sex in *Penicillium* series *roqueforti*. IMA Fungus 1:171–180
21. Irlinger F, Mounier J (2009) Microbial interactions in cheese: Implications for cheese quality and safety. Curr Opin Biotechnol 20:142–148
22. Jacques N, Casaregola S (2008) Safety assessment of dairy microorganisms: The hemiascomycetous yeasts. Int J Food Microbiol 126:321–326
23. Jakobsen M, Narvhus J (1996) Yeasts and their possible beneficial and negative effects on the quality of dairy products. Int Dairy J 6:755–768
24. Kindstedt PS (2013) The basics of cheesemaking. Microbiol Spectr 1:CM-0002–CM-0012
25. Kõljag U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M et al (2013) Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol 22:5271–5277
26. Kure CF, Wasteson Y, Brendehaug J, Skaar I (2001) Mould contaminants on Jarlsberg and Norvegia cheese blocks from four factories. Int J Food Microbiol 70:21–27

27. Larsen TO, Gareis M, Frisvad JC (2002) Cell cytotoxicity and mycotoxin and secondary metabolite production by common Penicillia on cheese agar. J Agric Food Chem 50:6148–6152

28. Lavoie K, Touchette M, St-Gelais D, Labrie S (2012) Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec. Dairy Sci Technol 92:455–468

29. Lie JL, Marth EH (1967) Formation of aflatoxin in cheddar cheese by *Aspergillus flavus* and *Aspergillus parasiticus*. J Dairy Sci 50:1708–1710

30. Lund F, Filtenborg O, Frisvad JC (1995) Associated mycoflora of cheese. Food Microbiol 12:173–180

31. Lund F, Nielsen AB, Skouboe P (2003) Distribution of *Penicillium commune* isolates in cheese dairies mapped using secondary metabolite profiles, morphotypes, RAPD and AFLP fingerprinting. Food Microbiol 20:725–734

32. Marcellino N, Benson (2013) The good, the bad and the ugly: Tales of mold-ripened cheese. Microbiol Spectr 1:CM-0005–CM-0012

33. Moran G, Coleman D, Sullivan D (2012) An introduction to the medically important Candida species. In: Calderone RA, Clancy CJ (eds) Candida and candidiasis, 2nd edn. ASM Press, New York, pp 11–25

34. Mounier J, Goerges S, Gelsomino R, Vancanneyt M, Vandemeulebroecke K, Hoste B et al (2006) Sources of the adventitious microflora of a smear-ripened cheese. J Appl Microbiol 101:668–681

35. Mounier J, Monnet C, Vallaeys T, Arditi R, Sarthou AS, Helias A, Irlinger F (2008) Microbial interactions within a cheese microbial community. Appl Environ Microbiol 74:172–181

36. Nielsen KF, Dalsgaard PW, Smedsgaard J, Larsen TO (2005) Andrastins A-D, *Penicillium roqueforti* metabolites consistently produced in blue-mold-ripened cheese. J Agric Food Chem 53:2908–2913

37. O'Brien NM, O'Connor TP, O'Callaghan JO, Dobson ADW (2004) Toxins in cheese. In: Fox PE, McSweeney PLH, Cogan TM, Guinee TP (eds) Cheese: Chemistry, Physics and Microbiology, vol 1, 3rd edn. Elsevier Academic, Amsterdam, pp 562–571

38. Ogier JC, Son O, Gruss A, Tailliez P, Delacroix-Buchet A (2002) Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis. Appl Environ Microbiol 68:3691–3701

39. Pereira-Dias S, Potes ME, Marinho A, Malfeito-Ferreira M, Loureiro V (2000) Characterisation of yeast flora isolated from an artisanal Portuguese ewes' cheese. Int J Food Microbiol 60:55–63

40. Pitkin JW, Panaccione DG, Walton JD (1996) A putative cyclic peptide efflux pump encoded by the TOXA gene of the plant-pathogenic fungus *Cochliobolus carbonum*. Microbiology 142:1557–1565

41. Pitt JI, Cruickshank RH, Leistner L (1986) *Penicillium commune*, *P. camembertii*, the origin of white cheese moulds, and the production of cyclopiazonic acid. Food Microbiol 3:363–371

42. Pitt JI, Hocking AD (2009) Fungi and food spoilage, 3rd edn. Springer, New York, p 519

43. Sengun IY, Yaman DB, Gonul SA (2008) Mycotoxins and mould contamination in cheese: A review. World Mycotoxin J 1:291–298

44. Suzzi G, Schirone M, Martuscelli M, Gatti M, Fornasari ME, Neviana E (2003) Yeasts associated with Manteca. FEMS Yeast Res 3:159–166

45. Swearingen PA, O'Sullivan DJ, Warthesen JJ (2001) Isolation, characterization, and influence of native, nonstarter lactic acid bacteria on cheddar cheese quality. J Dairy Sci 84:50–59

46. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

47. Torkar KG, Teger SG (2006) The presence of some pathogen micro organisms, yeasts and moulds in cheese samples produced at small dairy-processing plants. Acta Agric Slov 88:37–51

48. Trofa D, Gacser A, Nosanchuk JD (2008) *Candida parapsilosis*, an emerging fungal pathogen. Clin Microbiol Rev 21:606–625

49. Tsai WJ, Liewen MB, Bullerman LB (1988) Toxicity and sorbate sensitivity of molds isolated from surplus commodity cheeses. J Food Prot 51:457–462

50. Vasdinyei R, Deák T (2003) Characterization of yeast isolates originating from Hungarian dairy products using traditional and molecular identification techniques. Int J Food Microbiol 86:123–130

51. Wanderley L, Bianchin A, Arruda Teo CRP, Meneghello Fuentefria A (2013) Occurrence and pathogenicity of *Candida* spp. in unpasteurized cheese. Braz J Biosci 11:145–148

52. Welthagen JJ, Viljoen BC (1998) Yeast profile in gouda cheese during processing and ripening. Int J Food Microbiol 41:185–194

53. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: A guide to methods and applications. Academic Press, San Diego, pp 315–322

54. Wolfe BE, Button JE, Santarelli M, Dutton RJ (2014) Cheese rind communities provide tractable systems for *in situ* and *in vitro* studies of microbial diversity. Cell 158:422–433

55. Wyder M-T, Puhan Z (1999) Investigation in the yeast flora in smear ripened cheeses. Milchwissenschaft 54:330–333