Internal transcribed spacer (ITS) sequencing reveals considerable fungal diversity in dairy products

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ABSTRACT

Fungi are important spoilage organisms in dairy products. However, little is known about the diversity of naturally occurring spoilage fungi in raw milk and processed dairy products, due at least in part to the fact that classical fungal identification methods require considerable expertise. To gain further insight into the fungal diversity in the dairy system, we isolated fungi from raw milk, raw and pasteurized milk cheese, and yogurt using the selective dichloran rose bengal chloramphenicol agar. In total, 361 fungal isolates were obtained and further characterized by DNA sequencing of the internal transcribed spacer (ITS) region and the nuclear ribosomal large subunit (LSU) rRNA gene if needed. We conducted BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) searches of the ITS region sequences against the UNITE Database (<https://unite.ut.ee/analysis.php>), and selected other databases if needed, which allowed identification to the species level of 183 isolates and to the genus level of 107 of the 346 isolates that were successfully ITS sequenced. The isolates characterized represented 3 phyla and 19 genera; the most common genera isolated were *Penicillium* (25% of isolates), *Debaryomyces* (18%), and *Candida* (9%). This study not only provides, by using modern molecular tools, a baseline understanding of the types of fungi in dairy products, but also confirms that ITS sequencing is a useful approach for identification of fungal organisms found in the dairy food chain.

Key words: fungi, internal transcribed spacer (ITS) sequencing, yeast, mold

INTRODUCTION

Dairy products have a long history of spoilage by yeasts and molds, but as shelf lives and distribution chains are extended, these microorganisms have become increasingly problematic for the dairy industry.

Some reports estimate that 5 to 10% of all food produced is lost to fungal spoilage worldwide (Pitt and Hocking, 2009). Although specific estimates for dairy product loss due to fungal spoilage do not appear to be available, cultured dairy products, such as yogurt and cheese, are well documented to be susceptible to spoilage by fungi because of the ability of many strains to grow at the low temperature and pH encountered in these products (Fröhlich-Wyder, 2003; Mayoral et al., 2005; Banjara et al., 2015). Additionally, many cheeses have reduced water activity and high salt concentrations, which provides a unique niche for the growth of yeasts and molds (Gardini et al., 2006; Ledenbach and Marshall, 2009). Fungal spoilage organisms produce degradative enzymes that break down lipids, proteins, and carbohydrates, leading to a variety of undesirable sensorial qualities (Ledenbach and Marshall, 2009). In addition, fungal spoilage is often easily visually detected by consumers; and with broad use of the internet, communication of fungal spoilage via pictures shared on social media is increasingly common and has considerable potential to damage the reputation of dairy products (Newkirk et al., 2012).

Sources of yeast and mold contamination of dairy products typically appear to be the air and other environmental sources in processing facilities and other environments (e.g., aging facilities, retail; Kure et al., 2001). Airborne mold has been reported to enter the processing environment from either the outdoor air via the ventilation system or from moist niches present in the process environment (Kure et al., 2008). In the presence of prolonged elevated indoor moisture content, some fungi can grow and sporulate, resulting in further contamination in the indoor environment (Pitkäranta et al., 2008). Fungal environmental contamination in processing plants has previously been documented (Lund et al., 2003; Temelli et al., 2006). For example, Lund et al. (2003) collected swab and air samples from cheese production plants, the processing environment, and contaminated cheese products and identified the packaging environment and the coating step as the major points for fungal environmental contamination. In another study, Temelli et al. (2006) reported the cold

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room and production room air as sources for fungal environmental contamination in Turkish white cheese. Based on these findings, air in the processing plant represents an important source of fungal contamination.

Yeasts and molds are also commonly found in the dairy farm environment and can appear as natural contaminants in raw milk (Fleet, 1990; Lavoie et al., 2012; Atanassova et al., 2016). Most authors suggest, however, that fungi found in raw milk are typically heat sensitive and that raw milk is thus not an important (direct) source of fungi found in dairy products (Jacques and Casaregola, 2008). A few studies have surveyed the diversity of fungi in raw milk and reported that yeasts are more frequently isolated from raw milk than molds (Callon et al., 2007; Delavenne et al., 2011; Panelli et al., 2013). For example, Panelli et al. (2013) tested 40 bulk milk samples across the Italian Alps and most frequently isolated *Kluyveromyces marxianus*, *Atrotorquata lineata*, and *Candida* spp. Importantly, these studies identified some fungal species that had not previously been found in raw milk, suggesting that the fungal diversity associated with raw milk remains to be fully understood.

Historically, methods used to identify fungal microorganisms have involved visual and labor intensive phenotypic characterization, which requires extensive training and experience to master (Pitkäranta et al., 2008). Standardized DNA sequencing methods, sometimes referred to as DNA barcoding, represent robust and rapid methods for fungal identification. Moreover, an accepted universal internal transcribed spacer (ITS) region barcode for fungi has been well documented to allow for reproducible and discriminatory DNA sequencing-based fungal identification (Mayoral et al., 2005; Schoch et al., 2012; Sulaiman et al., 2014).

The objective of this study was (1) to implement molecular characterization methods for dairy-associated fungi isolated from raw milk, raw and pasteurized milk cheeses, and yogurt products; and (2) to use these methods to provide initial insights into the fungal diversity associated with different dairy products.

MATERIALS AND METHODS

Sample Collection

Using a convenience sampling approach, samples of bulk tank raw milk, cheese, and yogurt were collected between April and September 2015 and used for isolation of yeast and mold (see Table 1 for details on samples collected). Bulk tank raw milk samples (300 mL each) were collected from 8 dairy farms in New York State. Raw and pasteurized cheese samples (14

and 33, respectively) were collected from producers, supermarkets, wholesale distributors, and specialty shops in the state, excluding cheeses that were intentionally inoculated with fungi (e.g., mold-ripened cheeses). Yogurt samples ($n = 30$) representing plain, fruit, and other varieties were also conveniently collected from producers; none of the samples were visually spoiled at the time of collection. Samples were stored at 4°C until they were analyzed.

Fungal Isolation

Raw milk samples were plated directly on dichloran rose bengal chloramphenicol agar (DRBC; Becton, Dickinson and Co., Sparks, MD). Additionally, two 200-mL enrichments were prepared, each consisting of 100 mL of malt extract broth (Becton, Dickinson and Co.), with 40 mg/L streptomycin sulfate (Sigma, St. Louis, MO) and 100 mL of raw milk. Enrichments were prepared in sample bags and homogenized by hand for 30 s before incubation; one enrichment was incubated at $25 \pm 2^\circ\text{C}$ for 72 h and the other was incubated at $18 \pm 2^\circ\text{C}$ for 120 h. After these incubations, undiluted and serially diluted enrichments were spread plated on DRBC, followed by incubation at $25 \pm 2^\circ\text{C}$ for 120 h.

Cheese samples were tested by mixing approximately 20 g of cheese with 20 mL of malt extract broth (containing 40 mg/L streptomycin sulfate) in a sampling bag (prepared in duplicate), followed by homogenization at 260 rpm for 60 s in a Stomacher 400 Circulator (Seward Ltd., Worthing, UK). One homogenized enriched cheese sample was plated directly on DRBC and incubated at $25 \pm 2^\circ\text{C}$ for 120 h. In addition, the 2 homogenized sample enrichments were incubated at $25 \pm 2^\circ\text{C}$ for 72 h or $18 \pm 2^\circ\text{C}$ for 120 h. After these incubations, undiluted enrichments were spread plated on DRBC and then incubated at $25 \pm 2^\circ\text{C}$ for 120 h.

Yogurt samples were diluted 1:10 in PBS (Weber Scientific, Hamilton, NJ) in a sampling bag, followed by homogenization at 260 rpm for 60 s in a Stomacher 400 Circulator (Seward Ltd.). Each homogenized diluted yogurt sample was spread plated directly on DRBC and incubated at $25 \pm 2^\circ\text{C}$ for 120 h.

For all sample types, fungal colonies present on DRBC were visually examined, and colonies with distinct morphologies were selected and streaked for purity on malt extract agar (Becton, Dickinson and Co.). For each sample, DRBC plates were evaluated separately to select unique phenotypes from both direct plating and enrichment for further characterization. Per sample, between 0 and 10 isolates (average of 2 isolates) were collected from direct plating and between 0 and 11 isolates (average of 3 isolates) were collected from

sample enrichments (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-12635>). All isolates were then frozen at -80°C in 15% glycerol. Isolate characteristics, including nucleotide sequences, and relevant sample information are available through the Food Microbe Tracker database at www.foodmicrobetracker.com (Vangay et al., 2013); http://www.foodmicrobetracker.com/search/display_list.aspx?refid=505 provides a direct link to all 361 isolates reported here.

ITS Sequencing

Isolates obtained as described above were characterized by DNA sequencing of the ITS region, using the ITS primers described by Schoch et al. (2012). Briefly, purified DNA for PCR was prepared for each isolate from 120-h cultures on malt extract agar using the PowerSoil DNA Isolation Kit (MO BIO, Carlsbad, CA). The PCR conditions were slightly modified from

Table 1. Dairy products sampled for yeast and mold

Sample type	Species	Origin	Raw or pasteurized milk	Type	No. of samples	No. of isolates obtained by: ¹	
						Direct plating	Enrichment
Raw milk	Cow	New York State dairy farms	Raw	Bulk tank	8	10	28
Cheese	Cow	United States	Pasteurized	Semi-hard	1	2	7
	Cow	New York, United States	Pasteurized	Soft, washed rind	1	1	0
	Cow/sheep	Italy	Pasteurized	Soft, washed rind	1	1	0
	Cow	United States	Raw	Hard, washed rind, brined	2	3	6
	Cow	United States	Raw	Semi-hard, natural rind	2	5	8
	Cow	United States	Raw	Hard, washed rind	1	0	4
	Cow	New York, United States	Raw	Soft, washed rind	2	7	16
	Goat	Portugal	Pasteurized	Hard	1	3	1
	Goat	Switzerland	Pasteurized	Hard	1	2	7
	Goat/sheep	France	Pasteurized	Hard	1	1	4
	Goat	Portugal	Pasteurized	Semi-hard	1	2	3
	Goat	Spain	Pasteurized	Semi-hard	1	1	2
	Goat	The Netherlands	Pasteurized	Semi-hard Gouda	1	2	3
	Goat	The Netherlands	Pasteurized	Semi-hard, natural rind	1	1	2
	Goat	Spain	Pasteurized	Semi-hard, washed rind	2	5	5
	Goat	France	Pasteurized	Semi-hard, washed rind	1	1	6
	Goat	Canada	Pasteurized	Brined	1	1	3
	Goat	Canada	Pasteurized	Fresh	1	2	3
	Goat	France	Pasteurized	Soft	1	1	1
	Goat	Italy	Raw	Hard	1	2	4
	Goat	Switzerland	Raw	Semi-hard	1	2	4
	Sheep	Italy	Pasteurized	Hard	2	4	7
	Sheep	Spain	Pasteurized	Hard	2	1	6
	Sheep	Canada	Pasteurized	Hard, natural rind	1	1	5
	Sheep	France	Pasteurized	Hard, natural rind	1	3	4
	Sheep	Cyprus	Pasteurized	Semi-hard	1	2	2
	Sheep	France	Pasteurized	Semi-hard	1	1	3
	Sheep	Italy	Pasteurized	Semi-hard	2	4	5
	Sheep	Italy	Pasteurized	Semi-hard, natural rind	2	3	7
	Sheep	The Netherlands	Pasteurized	Semi-hard, natural rind	1	1	3
	Sheep	France	Pasteurized	Semi-hard, washed rind	1	1	5
	Sheep	France	Pasteurized	Brined	1	1	4
	Sheep	Greece	Pasteurized	Brined	1	1	2
	Sheep	New York, United States	Pasteurized	Fresh	1	1	0
	Sheep	Italy	Raw	Hard	1	3	5
	Sheep	United States	Raw	Hard, natural rind	1	2	5
	Sheep	Spain	Raw	Semi-hard	1	2	2
	Sheep	Spain	Raw	Semi-hard, natural rind	1	1	4
	Sheep	Spain	Raw	Soft	1	3	2
Yogurt	Cow	United States	Pasteurized		22	75	NA ²
	Cow	United States	Pasteurized	Added fruit	2	2	NA
	Cow	United States	Pasteurized	Added fruit and other	6	6	NA

¹Detailed information on the number of isolates obtained by direct plating and enrichment of the different samples can be found in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-12635>).

²NA = enrichments were not performed for yogurt samples. All isolates from yogurt samples were obtained from direct plating.

Schoch et al. (2012) and consisted of 1 cycle of 95°C for 5 min; 35 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min; 1 cycle of 72°C for 10 min, followed by a 4°C hold. Amplification of PCR products was confirmed by gel electrophoresis and PCR products were purified with the ExoSAP method (Affymetrix, Santa Clara, CA). Sanger sequencing with PCR primers was performed at Cornell University's Life Sciences Core Laboratory Center (Ithaca, NY) using the ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). The DNA sequences were assembled and proofread in Sequencher (version 5.3, Gene Codes Corporation, Ann Arbor, MI), and high-quality, double-stranded sequence data were used for further analysis.

Sequences were aligned using MUSCLE (Edgar, 2004), and ITS fragments, ranging in length from 251 to 724 nucleotides (nt), corresponding to 2 internal transcribed spacers and the 5.8S gene between them (hereafter referred to as the ITS region), were used for subsequent analyses.

Nuclear Ribosomal Large Subunit Sequencing

Isolates that could not be amplified using the ITS region primers were characterized by sequencing part of the gene that encodes the D1/D2 domain of the nuclear large subunit (LSU) (26S) ribosome, using primers NL1 and NL4 (Kurtzman and Robnett, 1998). The PCR conditions were slightly modified from Kurtzman and Robnett (1998) and consisted of 1 cycle of 94°C for 5 min; 36 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 2 min; 1 cycle of 72°C for 10 min, followed by a 4°C hold. The PCR products were purified and sequenced as described above for ITS sequencing.

Allelic Type Assignment

The ITS allelic types (AT) were assigned using BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). A unique ITS AT was assigned to every gene sequence that differed by ≥ 1 nucleotides from any previously obtained sequence. The first isolate of each new ITS AT was designated the reference strain for that AT.

Alignment, Tree Construction, and Species Identification

For species identification, the ITS region sequences for each unique ITS AT were queried against ITS region sequences in the UNITE database (<https://unite.ut.ee/analysis.php>; Kõljalg et al., 2005). The LSU sequences were queried against LSU sequences in Mycobank (<http://www.mycobank.org/>; Crous et al., 2004). An isolate with an identity score of $\geq 99.00\%$ to exactly

one type strain was assigned the species ID of that type strain. Isolates that had an identity score of $\geq 99.00\%$ against more than one type strain in the same phyla were assigned the finest classification rank available (typically a genus designation but in some cases only family designations could be assigned). Identity scores between 97.00 and 99.00% were assigned a phylum; no isolates showed identity scores $< 97.00\%$.

For isolates that could not be assigned genus and species identifications from the UNITE database, Mycobank was used to allow for similarity searches against additional fungal databases. Isolates with an identity score of $\geq 99.00\%$ to exactly one type strain were assigned the species ID of that type strain. For the genera *Penicillium*, *Mucor*, and *Geotrichum*, where known species complexes exist and database queries may result in unreliable identifications, ITS maximum-likelihood phylogenetic trees were constructed with sequences for type strains and isolates characterized in the study reported here. *Penicillium* type strains were acquired from the most recent verified reference database for the genus (Visagie et al., 2014). For *Mucor* and *Geotrichum*, no recent published verified reference databases are available. For these genera, the most recent publications providing DNA barcodes for type strains were hence used (De Hoog and Smith, 2004; Walther et al., 2013). Phylogenetic trees were constructed using the rapid maximum-likelihood algorithm RAXML (Stamatakis, 2006) with rapid bootstrapping and 100 bootstrap replicates (Supplemental Figures S1, S2, and S3; <https://doi.org/10.3168/jds.2017-12635>). Species-level identification was only assigned to isolates if the ITS sequence representing a specific AT associated with a given isolate clearly clustered with an ITS sequence of a single type specimen with a bootstrap value of > 50 (Supplemental Table S2; <https://doi.org/10.3168/jds.2017-12635>). The same approach to construct phylogenetic trees was used to construct an ITS maximum-likelihood phylogenetic tree with all the ITS AT identified in the study reported here (Figure 1).

RESULTS AND DISCUSSION

ITS Sequencing for Rapid and Reliable Characterization of Yeasts and Molds

A total of 361 isolates obtained from raw milk and different dairy products as described above were characterized by ITS PCR amplification and sequencing; 346 isolates yielded ITS products that could be successfully sequenced. The remaining 15 isolates, none of which yielded PCR products with primers ITS4 and ITS5, were characterized by sequencing part of the gene that encodes D1/D2 domain of the nuclear large

Tree scale: 0.1

Source

- raw milk
- raw milk cheese
- pasteurized milk cheese
- yogurt

Figure 1. Midpoint-rooted maximum-likelihood (ML) phylogenetic tree of internal transcribed spacer (ITS) region sequences from fungal species isolated from raw milk (black), raw milk cheese (dark gray), pasteurized milk cheese (medium gray), and yogurt (light gray). Numerical values indicate the percentage of bootstrap replications (out of 100 total replications) that support the corresponding node. Species identification of allelic types (AT) was based on ITS region sequence analyses as described in Materials and Methods. Allele types that could not be identified to the species level with a $\geq 99.00\%$ identity score were assigned the finest classification rank.

subunit (LSU) (26S) rRNA. Thirteen of these isolates were successfully characterized by sequencing the D1/D2 domain, whereas 2 isolates could not be successfully sequenced using either ITS or LSU primers. The 359 isolates that could be characterized by either ITS or LSU sequencing represented the phyla *Ascomycota* (347 isolates), *Basidiomycota* (1 isolate), and *Mucoromycota* (11 isolates). It is important to note that the phylum-level classification of zygomycete fungi remains to be fully defined and clarified; although *Mucoromycota* represents the most current name for these organisms (Spatafora et al., 2016), the taxonomic classification of these organisms into phyla is likely to change.

Overall, the ITS sequence data for the 346 dairy-associated isolates from raw milk, raw and pasteurized milk cheeses, and yogurt yielded 81 unique ITS AT. Using the UNITE database (and Mycobank as needed), all 346 isolates that yielded ITS sequence data could be identified to at least the phylum level; 183 isolates could be assigned a species (based on an identity score of $\geq 99.00\%$ against ITS region sequences in the UNITE Database) and 107 could be assigned a genus but not a species. The most common AT (AT9, 55 isolates) was classified as a *Penicillium* species, whereas the second-most common AT (AT5, 50 isolates) was classified as *Debaryomyces hansenii*. Importantly, though, ITS AT-based species identifications should be interpreted carefully as current uncertainties as well as likely future changes in fungal taxonomy will affect ITS sequencing-based species identification. Furthermore, ITS sequencing does not provide high-resolution power for species complexes such as the *Penicillium camemberti* clade, *Mucor circinelloides*, and *Geotrichum*. Additional characterization methods, discussed below, are necessary to confidently assign species names for these organisms.

Overall, our data indicate that ITS sequencing allows straightforward initial characterization of the majority of fungal isolates obtained from dairy products: 96% of isolates yielded sequence data suitable for searches against the UNITE ITS database, with 107 and 183 isolates identified to the genus and species levels, respectively. Although we were able to find one large study that characterized 610 yeast and mold isolates from raw milk and raw milk cheeses (Lavoie et al., 2012), our study represents the largest molecular study characterizing yeast and mold isolates across diverse dairy products. Most other previous studies (Sulaiman et al., 2014; Banjara et al., 2015; Garnier et al., 2017) that used ITS sequencing for characterization of dairy isolates included fewer than 175 isolates. Importantly, our findings are consistent with previous studies that have shown that “ITS barcoding” provides for a standardized ITS AT nomenclature that can transcend taxonomic changes. In addition, our data showed considerable AT

diversity among the dairy isolates characterized. We also found that ITS AT data can sometimes differentiate subtypes within a given species; for example, *Clavispora lusitaniae* included 4 different AT. This indicates that ITS-based AT data (even for isolates that cannot be identified to the genus or species level) can be used for isolate characterization, which can help dairy producers assess whether isolates obtained at different times or locations (e.g., in ingredients and finished product) may or may not have a common source. In some cases, this might allow for exclusion of a source, but more sensitive subtyping methods are needed to more definitively identify contamination sources. Specifically, even though ITS sequencing has high reliability for initial characterization and identification (given appropriate databases), it does not provide the high-resolution power of other characterization and subtyping techniques (Stielow et al., 2015). Furthermore, intragenomic heterogeneities involving the ITS region have been documented in different fungal species such as *Geotrichum candidum* (Alper et al., 2011) and other yeast species including *Candida glabrata*, *Candida tropicalis*, *Pichia norvegensis*, and *Saccharomyces cerevisiae* (Zhao et al., 2015), potentially complicating ITS-based AT and species assignment. Importantly, sequencing different regions such as protein-coding genes may provide higher resolution at the species level. For example, Garnier et al. (2017) characterized 41 unique fungal species from 175 French yogurt and cheese isolates by using ITS sequencing as a preliminary step, followed by sequencing of protein-coding genes (e.g., partial β -tubulin gene and partial elongation factor 1- α) for higher resolution power. If further discrimination beyond the species levels (i.e., subtyping) is required for fungal organisms, fragment analysis techniques such as amplified fragment length polymorphism (AFLP), microsatellites, and random amplified polymorphic DNA (RAPD) may be appropriate tools (Almeida and Araujo, 2013; Saghrouni et al., 2013).

Ascomycetous Yeasts Are Primary Fungal Contaminants in Raw Milk

The 38 fungal isolates obtained from raw milk samples (Table 2) all represented yeast, with no molds identified. For 4 isolates, the ITS PCR did not yield an amplicon, and LSU sequencing was successful for 3 of these isolates, which were classified as *Pichia*, *Candida*, and *Ascomycota*. The 34 isolates with ITS sequence data represented 21 different ITS AT; the most common AT among these isolates (AT7; 7 isolates) was classified as *Candida*. The remaining 20 AT found among the raw milk isolates each represented 3 or fewer isolates. Interestingly, one isolate was identified with LSU se-

quence data as the yeast *Pichia rhodanensis*, a species that has not been previously reported as being isolated from raw milk (although other *Pichia* species have been reported from raw milk; Cocolin et al., 2002; Lavoie et al., 2012; Panelli et al., 2013). The most common genera identified among the 23 isolates with genus-level identification were *Candida*. The predominance of yeast isolates was not surprising because a recent review suggests that yeast populations of 10¹ to 10³ cfu/mL of raw milk are typical (Fleet, 2011). The fact that *Candida* was a common genus identified among raw milk isolates is also consistent with previous reports (Cocolin et al., 2002; Lavoie et al., 2012). For example, Lavoie et al. (2012) found *Candida* species in 44% of the 111 raw milk samples collected from farms in Quebec, Canada. Although *Candida* species have been linked to bovine mastitis (Watts, 1988; dos Santos and Marin, 2005), it is likely that yeasts such as *Candida* are introduced into raw milk from the dairy farm environment because *Candida* species are widely distributed in the environment and air (Torkar and Vengust, 2008). In a study of 16 dairy farms in France, Vacheyrou et al. (2011) found that the same yeast species isolated from 9 stable and 3 milking parlor environments were also isolated from bulk tank raw milk of 14 of the farms. Importantly, *Candida* species are heat sensitive and easily killed

during processing; studies indicate that most *Candida* strains, at 10⁵ cells/mL, can survive 20 min at 55C but not 10 min at 62.5C (Put et al., 1976).

Debaromyces Is a Major Contributor to Fungal Microflora of Raw and Pasteurized Milk Cheeses

Overall, 90 yeasts and molds isolated from 14 raw milk cheese samples and 150 yeasts and molds isolated from 33 pasteurized milk cheese samples (Table 1) were initially characterized by ITS sequencing. A total of 47 different ITS AT were identified among the 240 isolates. Among the 90 raw milk cheese isolates, the most common AT were classified as *Debaryomyces hansenii* (AT5; 21 isolates), *Penicillium* spp. (AT9; 16 isolates), *Penicillium* spp. (AT82; 9 isolates), and *Dipodascaceae* spp. (AT10; 8 isolates) (Table 2). The remaining 16 AT found among the raw milk cheese isolates each represented 6 or fewer isolates. Among the 150 pasteurized milk cheese isolates, the most common AT were classified as *Debaryomyces hansenii* (AT5; 28 isolates), *Penicillium* spp. (AT9; 27 isolates), *Penicillium* spp. (AT82; 11 isolates), and *Dipodascaceae* spp. (AT10; 8 isolates) (Table 2). The remaining 32 AT found among the pasteurized milk cheese isolates each represented 7 or fewer isolates. Consistent with our results, Wolfe et al. (2014)

Table 2. Unique internal transcribed spacer (ITS) allelic types (AT) isolated from raw milk, cheese, and yogurt

Genus and species (or finest classification rank possible) ¹	ITS AT	No. of isolates with a given AT obtained from:				Total no. of isolates
		Raw milk	Raw milk cheese	Pasteurized milk cheese	Yogurt	
<i>Ascomycota</i>						
<i>Ascomycota</i> ²	1	1				1
<i>Ascomycota</i> ²	3	1				1
<i>Ascomycota</i> ²	38			1		1
<i>Ascomycota</i> ²	69		3	6		9
<i>Ascomycota</i> ²	79		3			3
<i>Ascomycota</i> ²	115	1				1
<i>Ascomycota</i> ²	137				1	1
<i>Candida orthopsilosis</i>	88			2		2
<i>Candida parapsilosis</i>	30	2			2	4
<i>Candida parapsilosis</i>	113			1		1
<i>Candida sojae</i>	56				5	5
<i>Candida</i> spp.	7	7		1		8
<i>Candida</i> spp.	91			1	1	2
<i>Candida tropicalis</i>	8	2				2
<i>Candida tropicalis</i>	62	1				1
<i>Candida umkomasiana</i>	70		2			2
<i>Candida zeylanoides</i>	12		3	4		7
<i>Clavispora lusitaniae</i>	4	3				3
<i>Clavispora lusitaniae</i>	53				2	2
<i>Clavispora lusitaniae</i>	57				1	1
<i>Clavispora lusitaniae</i>	61				14	14
<i>Cyberlindnera fabianii</i>	84	2				2
<i>Debaryomyces hansenii</i>	5	1	21	28		50
<i>Debaryomyces hansenii</i>	6	1	2			3
<i>Debaryomyces prosopidis</i>	11			6		6
<i>Debaryomyces</i> spp.	112			3		3

Continued

Table 2 (Continued). Unique internal transcribed spacer (ITS) allelic types (AT) isolated from raw milk, cheese, and yogurt

Genus and species (or finest classification rank possible) ¹	ITS AT	No. of isolates with a given AT obtained from:				Total no. of isolates
		Raw milk	Raw milk cheese	Pasteurized milk cheese	Yogurt	
<i>Debaryomyces</i> spp.	127			1		1
<i>Debaryomyces</i> spp.	128			1		1
<i>Debaryomyces</i> spp.	129			1		1
<i>Dipodascaceae</i> ³	10	1	8	8		17
<i>Dipodascaceae</i> ³	63	1		7		8
<i>Dipodascaceae</i> ³	73	1				1
<i>Dipodascaceae</i> ³	74	1				1
<i>Dipodascaceae</i> ³	80	1				1
<i>Dipodascaceae</i> ³	114	1				1
<i>Dipodascaceae</i> ³	116	1				1
<i>Dipodascaceae</i> ³	117	2				2
<i>Dipodascaceae</i> ³	118	1				1
<i>Dipodascaceae</i> ³	120			1		1
<i>Dipodascaceae</i> ³	125			1		1
<i>Geotrichum</i> cf. <i>restrictum</i> ⁴	98				1	1
<i>Hanseniaspora uvarum</i>	99				1	1
<i>Hypocreaceae</i> ³	96			1		1
<i>Kazachstania servazzii</i>	81			1		1
<i>Kluyveromyces lactis</i>	66			1		1
<i>Kluyveromyces lactis</i>	89			2		2
<i>Kluyveromyces marxianus</i>	2	2		2		4
<i>Lachancea fermentati</i>	138				1	1
<i>Nectriaceae</i> ³	72			1		1
<i>Nectriaceae</i> ³	85			2		2
<i>Penicillium brevicompactum</i>	39				1	1
<i>Penicillium citrinum</i>	16				1	1
<i>Penicillium</i> spp.	9		16	27	12	55
<i>Penicillium</i> spp.	19		2		7	9
<i>Penicillium</i> spp.	35		1	1		2
<i>Penicillium</i> spp.	82		9	11		20
<i>Penicillium</i> spp.	122			2		2
<i>Sarocladium kiliense</i>	119			1		1
<i>Trichocomaceae</i> ³	25		2	2		4
<i>Torulaspora delbrueckii</i>	58				1	1
<i>Torulaspora delbrueckii</i>	59				1	1
<i>Torulaspora delbrueckii</i>	60				25	25
<i>Torulaspora delbrueckii</i>	101				1	1
<i>Wickerhamomyces anomalus</i>	102				2	2
<i>Yarrowia lipolytica</i>	64			1		1
<i>Yarrowia lipolytica</i>	67		6	6		12
<i>Yarrowia lipolytica</i>	71		1			1
<i>Yarrowia lipolytica</i>	77		1			1
<i>Yarrowia lipolytica</i>	87			2		2
<i>Yarrowia lipolytica</i>	92			1		1
<i>Yarrowia lipolytica</i>	95		1			1
<i>Yarrowia lipolytica</i>	121		1			1
<i>Yarrowia lipolytica</i>	123			1		1
<i>Basidiomycota</i>						
<i>Sistotrema</i> spp.	136				1	1
<i>Mucoromycota</i>						
<i>Mucor circinelloides</i>	86		4			4
<i>Mucor circinelloides</i>	93		1			1
<i>Mucor</i> cf. <i>fuscus</i> ⁴	124			2		2
<i>Mucor</i> cf. <i>fuscus</i> ⁴	126			1		1
<i>Mucor plumbeus</i>	28				1	1
<i>Mucor</i> spp.	90		1			1
<i>Rhizopus arrhizus</i>	31				1	1

¹Column subheaders (e.g., *Ascomycota*, *Basidiomycota*, and *Mucoromycota*) represent phyla.²These ITS AT could only be identified to the phylum level.³Represents a family-level classification.⁴cf. [short for the Latin “confer” (“compare with”)] signifies AT that resemble the given named species but where identification represents considerable uncertainty.

previously reported isolation of a considerable diversity of fungal organisms from surfaces of different cheeses. When studying 137 cheese rinds from 10 different countries, this group identified 10 fungal genera that were consistent across all samples. Although we elected to not include cheeses that were clearly inoculated with fungal organisms (e.g., brie, blue cheese, bloomy rind cheeses, where the rind is intentionally inoculated with fungal organisms), we still reported considerable fungal diversity (at least 9 genera across 47 cheese samples). It is possible, however, that some cheeses included in our study reported here were deliberately inoculated through the washed rind process. Fungal organisms such as *Debaryomyces hansenii*, *Geotrichum candidum*, and *Yarrowia lipolytica* (all of which were isolated from cheeses in our study) can be included in the brine solution that is used to wash the cheese rind or may be directly sprayed to the cheese surface (Petersen et al., 2002; Bokulich and Mills, 2013). These organisms can be desirable in cheese and provide proteolytic and lipolytic enzymes as well as aroma compounds during the ripening process (Fleet, 1990). Thus, the frequency of the isolates from cheese reported below may include contaminants as well as deliberately inoculated organisms.

Fisher's exact test showed that there was no significant difference in the distribution of AT among raw and pasteurized milk cheese samples ($P > 0.05$); for this test, AT that occurred <5 times (combined frequency for raw and pasteurized cheese isolates) were combined into a single category ("uncommon ATs"). Consequently, combined results for raw and pasteurized milk cheese data are discussed below. Importantly, the observation that yeast and mold AT distribution does not differ between raw and pasteurized milk cheese samples suggests that contamination with these organisms occurs predominantly from the processing environment, consistent with the fact that the genera isolated here from cheeses are typically heat sensitive. For example, Wallace and Tanner (1931) showed that the conidia of *Penicillium* species are killed after heating for 5 min at 60°C.

Among the raw and pasteurized milk cheese fungal isolates, the most common AT were identified as *D. hansenii* (AT5; 49 isolates), *Penicillium* spp. (AT9; 43 isolates), and *Penicillium* spp. (AT82; 20 isolates). The frequent occurrence of *D. hansenii* in a variety of cheeses is consistent with previous studies (Gardini et al., 2006; Capece and Romano, 2009; Padilla et al., 2014; Atanassova et al., 2016). For example, Capece and Romano (2009) sampled the curd and rind of 2 Pecorino di Filiano cheeses throughout each cheese's ripening time of 120 d and reported that 96% of cheese samples tested positive for *D. hansenii*. The occurrence of *D. hansenii*

can be attributed to the ability of this species to grow in extreme environments such as high salt concentrations, low temperature, and low pH (Gori et al., 2012), and its ability to metabolize lactic and citric acids (Capece and Romano, 2009). *Debaryomyces hansenii* has also been isolated from brine solutions, raw milk, and dairy environments (Gori et al., 2012). In another study, Mounier et al. (2006) isolated the same *D. hansenii* strain from the surface of an Irish smear-ripened cheese as well as from the brine solution, processing equipment, and the hands of the workers in the dairy plant. These results mirror our results of frequent isolation of *D. hansenii* and suggest that it can colonize the surface of washed-rind cheeses. The *Penicillium* section Fasciculata, series *Camemberti* clade includes dairy-relevant species such as *P. camemberti* and *P. commune*. These species are typically distinguished by their phenotypic characteristics: *P. commune* has blue-green conidia whereas *P. camemberti* has white conidia. Although *P. camemberti* is a desired organism in mold-ripened cheeses, *P. commune* is reported as one of the most frequently occurring spoilage molds in cheese; dairy isolates classified into this species have also been shown to grow rapidly at refrigeration temperatures (Lund et al., 1995; Kure et al., 2001; Hayaloglu and Kirbag, 2007). Growth of *P. commune* on cheese has been reported to result in discoloration of the surface and production of off-flavors (Lund et al., 2003). Overall, *Penicillium* species (e.g., *P. commune*, *P. biforme*) closely related to AT9 and AT82, which were found here to be common among raw and pasteurized milk cheeses isolates, have been shown to cause product defects that consumers can detect (Lund et al., 1995; Pitt and Hocking, 2009; Giraud et al., 2010). Prevention of contamination with these fungi (e.g., from environmental and raw material sources) is thus important to prevent quality issues with raw milk and pasteurized milk cheeses.

***Torulaspora delbrueckii* Is a Major Contributor to Fungal Contamination in Yogurt**

In total, 59 yeasts and 24 molds were isolated from 30 yogurt samples (Table 1). Twenty-two different ITS AT were identified among these 83 isolates (Table 2). The most common AT represented *Torulaspora delbrueckii* (AT60; 25 isolates), *Clavispora lusitaniae* (AT61; 14 isolates), *Penicillium* spp. (AT9; 12 isolates), and *Penicillium* spp. (AT19; 7 isolates). The remaining 18 AT found among the yogurt isolates each represented 5 or fewer isolates.

Consistent with common identification of yeasts (e.g., *Candida* and *Kluyveromyces*) among yogurt isolates, yeasts are well documented as a major cause of spoilage in yogurt because the low pH of the product selects

for their growth (Rohm et al., 1992; Mataragas et al., 2011; Krisch et al., 2014). For example, Suriyarachchi and Fleet (1981) reported that among 128 yogurt samples, the yeasts *Candida famata* and *Kluyveromyces marxianus* were isolated from 16 and 9% of samples, respectively, and represent the most frequently isolated species in their study. Likewise, Green and Ibe (1987) reported similar findings for 100 yogurt samples, with *Clavispora lusitanae*, *K. marxianus*, and *Candida krusei* isolated from 65, 48, and 51 of samples, respectively.

Previous studies have reported the presence of *T. delbrueckii* in dairy products such as cheese (Westall and Filtenborg, 1998); however, to our knowledge, the current study is the first report of the presence of *T. delbrueckii* in yogurt. This finding could be attributed to the different methods used to identify the fungal isolates here and in previous studies. For example, Suriyarachchi and Fleet (1981) used physiological tests such as carbohydrate fermentation, nitrogen assimilation, and casein hydrolysis to characterize yeast isolates and then identified the isolates to the species level with the use of a standard key for yeast identification. The standard key used in Suriyarachchi and Fleet's study contained only 434 yeast species. In contrast, the UNITE database, which we used to query ITS region sequences, contains sequence identification information for over 24,000 ascomycetes, many of which would be classified as yeasts (Köljalg et al., 2005). Consequently, it is possible that *T. delbrueckii* was previously isolated but not identified as this species. Importantly, this illustrates the advantages of using ITS sequencing, along with fungal sequence databases with high-quality reference ITS sequences, such as those in UNITE, for fungal characterization and identification.

The occurrence of molds in yogurt has not been widely reported. In one study, Sulaiman et al. (2014) documented *Rhizomucor variabilis* in 15 recalled Greek yogurt samples. In another study, Snyder et al. (2016) characterized *Mucor circinelloides* spoilage in yogurt. In this study, we documented the presence of *Penicillium*, *Mucor*, *Rhizopus*, and *Sistotrema* in yogurt. The occurrence of these genera in yogurt samples could suggest contamination from environmental sources, consistent with previous studies. Moist environments commonly found in dairy plants can support the growth of molds, which can lead to mold growth on floors, ceilings, walls, and drains (Sørhaug, 2011). Once in the dairy environment, mold spores can become airborne and contaminate the product (Salustiano et al., 2003). For example, Fleet (1990) reported that fungi may be present in the processing environment and can enter the product during packaging. Moreover, Snyder et al. (2016) hypothesized that *M. circinelloides* contamination in yogurt represented postprocessing contamination because

the species is heat sensitive. Thus, these studies and our results suggest that fungal contaminants may be present in the dairy processing environment and can contaminate the product after heating.

CONCLUSIONS

Our data show that ITS sequencing is a useful and standardized tool for fungal classification, facilitated by a high PCR and sequencing success rate across a broad range of fungi as well as a large fungal sequence database (UNITE) with high-quality reference ITS sequences. However, additional databases and phylogenetic analyses are sometimes needed to identify fungal isolates from milk and dairy products. Ongoing realignments of fungal taxonomy can represent a challenge for meaningful identification of some isolates based on ITS sequences. Importantly however, ITS sequence data transcends taxonomic changes and isolate identification based on ITS data can easily be revised and updated as taxonomic changes are made. By using DNA sequence-based approaches, we demonstrated that fungi in dairy represent a broad diversity over multiple phyla. Overall, our data represent a step in developing new tools and approaches to better control dairy product spoilage due to fungal contaminants.

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