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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

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another study, Temelli et al. (2006) reported the cold 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 ± 2°C for 72 h and the other was incubated at 18 ± 2°C for 120 h. After these incubations, undiluted and serially diluted enrichments were spread plated on DRBC, followed by incubation at 25 ± 2°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 ± 2°C for 120 h. In addition, the 2 homogenized sample enrichments were incubated at 25 ± 2°C for 72 h or 18 ± 2°C for 120 h. After these incubations, undiluted enrichments were spread plated on DRBC and then incubated at 25 ± 2°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 ± 2°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