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Identification and characterization of psychrotolerant coliform bacteria isolated from pasteurized fluid milk

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ABSTRACT

The presence of coliform bacteria in pasteurized fluid milk typically indicates that product contamination occurred downstream of the pasteurizer, but it may also indicate pasteurization failure. Although coliform detection is frequently used as a hygiene indicator for dairy products, our understanding of the taxonomic and phenotypic coliform diversity associated with dairy products is surprisingly limited. Therefore, using Petrifilm Coliform Count plates (3M, St. Paul, MN), we isolated coliforms from high-temperature, short-time (HTST)-pasteurized fluid milk samples from 21 fluid milk processing plants in the northeast United States. Based on source information and initial characterization using partial 16S rDNA sequencing, 240 nonredundant isolates were obtained. The majority of these isolates were identified as belonging to the genera *Enterobacter* (42% of isolates), *Hafnia* (13%), *Citrobacter* (12%), *Serratia* (10%), and *Raoultella* (9%); additional isolates were classified into the genera *Buttiauxella*, *Cedecea*, *Kluyvera*, *Leclercia*, *Pantoea*, and *Rahnella*. A subset of 104 representative isolates was subsequently characterized phenotypically. Cold growth analysis in skim milk broth showed that all isolates displayed at least a 2-log increase over 10 d at 6°C; the majority of isolates (n = 74) displayed more than a 5-log increase. In total, 43% of the representative isolates displayed lipolysis when incubated on spirit blue agar at 6°C for 14 d, whereas 71% of isolates displayed proteolysis when incubated on skim milk agar at 6°C for 14 d. Our data indicate that a considerable diversity of coliforms is found in HTST-pasteurized fluid milk and that a considerable proportion of these coliforms have phenotypic characteristics that will allow them to cause fluid milk spoilage.

Key words: coliform, HTST-pasteurized milk, milk spoilage, cold growth

INTRODUCTION

Preventing postpasteurization contamination (**PPC**) with spoilage microorganisms remains a major challenge for dairy processors (Ralyea et al., 1998; Ranieri and Boor, 2009; Martin et al., 2011). Along with *Pseudomonas* spp., coliform bacteria are frequently isolated PPC contaminants in pasteurized fluid milk (Martin et al., 2011). Coliforms are defined as aerobic or facultatively anaerobic, gram-negative, non-spore-forming rods capable of fermenting lactose, resulting in gas and acid production within 48 h at 35°C (Feng et al., 2002; Nörnberg et al., 2010). Traditionally, coliforms were considered to be represented by 4 genera: *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter* (Bergey et al., 1939). Today, over 20 bacterial genera include strains that have phenotypic characteristics that classify them as coliforms (Imhoff, 2005). Detection of coliforms plays an important role in the dairy industry because coliforms are frequently used as hygiene indicators, and there are clear regulatory limits for the presence of coliforms in finished dairy products. For example, the US Pasteurized Milk Ordinance limits the number of coliforms in pasteurized grade "A" milk to ≤10 cfu/mL (FDA, 2011).

Coliform bacteria that are psychrotolerant and capable of growing at refrigerated storage temperatures are of particular concern for the dairy industry, as psychrotolerant growth can result in physical degradation and unacceptable sensory characteristics of the product due to the production of lipolytic and proteolytic enzymes (Nörnberg et al., 2010). This is also supported by a recent 10-year study of HTST fluid milk quality in New York State (**NYS**); milk samples that tested positive for coliforms on the initial day of refrigerated shelf-life had significantly lower sensory scores on d 14 of refrigerated shelf-life compared with samples that were not positive for coliforms on the initial day (Martin et al., 2012). Postpasteurization contamination is not isolated to the United States; for example, previous research found 40% of fluid milk samples taken from Norway and Sweden had PPC with coliforms (Eneroth et al., 1998).

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Although coliform testing continues to play an important role in the dairy industry, available data on the phenotypic and genotypic coliform diversity found in dairy products are surprisingly limited. Most reported studies characterized only small sets of coliform isolates and typically these isolates were obtained from only a few processing facilities. For example, researchers in Norway and Sweden assessed coliform levels from 3 different dairy processing plants (Eneroth et al., 1998), whereas other researchers utilized as little as 6 to as many as 75 coliform isolates to characterize dairy product-associated coliform bacteria (Juven et al., 1981; Wessels et al., 1989). An improved understanding of the diversity and spoilage potentials of fluid milk associated coliforms is vital to improve fluid milk quality. For example, better baseline data on coliform diversity associated with fluid milk may facilitate development and validation of more robust and faster detection methods and may facilitate source tracking of coliform contamination. Therefore, the objectives of this study were (1) to characterize the taxonomic diversity of psychrotolerant coliforms in pasteurized fluid milk obtained from a substantial number of processing plants, and (2) to characterize representative isolates for relevant phenotypic characteristics (i.e., cold growth capability and proteolysis and lipolysis during growth at refrigeration temperatures).

MATERIALS AND METHODS

Sample Collection and Handling

Pasteurized milk samples were collected from 2010 to 2011 from fluid milk processing facilities located in the northeast United States (NYS, Maine, Pennsylvania, Vermont, New Hampshire, and Massachusetts). All participating processing facilities were enrolled in the Voluntary Shelf-Life (VSL) program, which is administered by the Cornell University Milk Quality Improvement Program (MQIP; Martin et al., 2012). Processing capacity of facilities ranged from <1 million to >600 million lbs of fluid milk annually. Samples collected represented packaged pasteurized products that were processed at each facility via HTST pasteurization [at a minimum of 72°C (161°F), 15 s], including whole fat (minimum 3.25% milk fat), reduced fat (1.5 or 2% milk fat), low fat (1% milk fat), and nonfat (<0.2% milk fat) milk in quart (946 mL), half gallon (1.9 L), or gallon (3.8 L) containers. Containers used for packaging included high-density polyethylene jugs, paperboard cartons, or glass bottles. All milk samples were transported to the MQIP laboratory in coolers packed with ice packs or ice. Samples were held at 4°C until initial testing in the laboratory 24 to 48 h after sample collection. Of the 29 total fluid milk processing plants enrolled in the 2010–2011 VSL program, 21 plants had positive coliform samples and were included in the study reported here.

Coliform Isolation from Milk Samples

Upon arrival at the laboratory, samples were inverted 25 times within 7 s and for each milk sample ~400 mL of milk was aseptically distributed into each of 4 sterile 500-mL glass bottles. Bottles were stored at 6°C until the appropriate test day for each bottle. Samples were tested for microbiological and sensory quality on the initial day as well as at d 7, 10, and 14 postprocessing (Martin et al., 2012). The initial day was defined as the first day of testing, which varied from 0 to 6 d postprocessing due to the plant processing schedule. Samples from a subset of processors with histories of consistently manufacturing high-quality products were also tested at d 17 and 21 postprocessing (Martin et al., 2012). Coliform testing was performed on each test day by inoculating milk samples in duplicate on separate Petrifilm Coliform Count plates (3M, St. Paul, MN); plates were subsequently incubated at 32°C for 24 h according to the manufacturer's instructions. For each sample date (e.g., d 7) with a sample positive for coliforms, 2 coliform isolates were selected from the Petrifilm Coliform Count plates and streaked for purity on brain heart infusion (**BHI**) agar (Difco, Franklin Lakes, NJ), which was subsequently incubated at 32°C for 24 h. Isolates were confirmed as coliforms by inoculation in brilliant green bile broth (Difco); only isolates confirmed as coliforms were retained and further characterized. Pure cultures were grown overnight in BHI broth at 32°C and then frozen in 15% glycerol at −80°C and stored until further characterization. Before further characterization as described below, isolates were streaked for isolation from frozen stock onto BHI agar and grown at 32°C for 24 h. Detailed information on all isolates is available through Food Microbe Tracker (http://www.foodmicrobetracker.com).

Genus Identification Using Sequencing of Partial 16S rDNA

All isolates were characterized by sequencing a 616-bp fragment of the 16S rRNA gene as described previously (Huck et al., 2008). The resulting 16S rDNA sequence data were used to identify isolates to the genus level. Genus identification was performed using the Ribosomal Database Project classifier (Cole et al., 2005); genus identification was assigned based on a seqmatch score (S_ab) of 0.90 or higher (Cole et al., 2005). For 5 isolates that did not produce 16S rDNA