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Pseudomonas fluorescens group bacterial strains are responsible for repeat and sporadic postpasteurization contamination and reduced fluid milk shelf life

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ABSTRACT

Postpasteurization contamination (PPC) of high temperature, short time-pasteurized fluid milk by gram-negative (GN) bacteria continues to be an issue for processors. To improve PPC control, a better understanding of PPC patterns in dairy processing facilities over time and across equipment is needed. We thus collected samples from 10 fluid milk processing facilities to (1) detect and characterize PPC patterns over time, (2) determine the efficacy of different media to detect PPC, and (3) characterize sensory defects associated with PPC. Specifically, we collected 280 samples of high temperature, short time-pasteurized milk representing different products (2%, skim, and chocolate) and different fillers over 4 samplings performed over 11 mo at each of the 10 facilities. Standard plate count (SPC) as well as total GN, coliform, and *Enterobacteriaceae* (EB) counts were performed upon receipt and after 7, 10, 14, 17, and 21 d of storage at 6°C. We used 16S rDNA sequencing to characterize representative bacterial isolates from (1) test days with SPC >20,000 cfu/mL and (2) all samples with presumptive GN, coliforms, or EB. Day-21 samples were also evaluated by a trained defect judging panel. By d 21, 226 samples had SPC >20,000 cfu/mL on at least 1 d of shelf life; GN bacteria were found in 132 of these 226 samples, indicating PPC. Crystal violet tetrazolium agar detected PPC with the greatest sensitivity. Spoilage due to PPC was predominantly associated with *Pseudomonas* (isolated from 101 of the 132 samples with PPC); coliforms and EB were found in 27 and 37 samples with spoilage due to PPC, respectively. Detection of *Pseudomonas* and *Acinetobacter* was associated with lower flavor scores; coagulated, fruity fermented, and unclean defects were more prevalent in d-21 samples with PPC. Repeat isolation of *Pseudomonas fluorescens* group strains with identical partial 16S rDNA sequence types was observed in 8 facilities. In several facilities, specific lines, products, or processing days were linked to repeat product contamination with *Pseudomonas* with identical sequence types. Our data show that PPC due to *Pseudomonas* remains a major challenge for fluid milk processors; the inability of coliform and EB tests to detect *Pseudomonas* may contribute to this. Our data also provide important initial insights into PPC patterns (e.g., line-specific contamination), supporting the importance of molecular subtyping methods for identification of PPC sources.

Key words: postpasteurization contamination, *Pseudomonas*, shelf-life, spoilage, crystal violet tetrazolium agar

INTRODUCTION

Spoilage due to bacterial growth is a cause of consumer complaints and a contributor to dairy product waste, which claims an estimated 15% of production in the industrialized world at the consumer level (Gustavsson et al., 2011). Fluid milk spoilage specifically has been valued at $6.4 billion per year in the United States (Buzby et al., 2014). Microbial spoilage is of particular concern and can be caused by either psychrotolerant sporeformers, which likely originate from raw milk, or by postpasteurization contamination (**PPC**). Whereas gram-positive (**GP**) bacteria can also recontaminate milk after pasteurization (Eneroth et al., 2001; Salustiano et al., 2009), bacteria responsible for PPC leading to fluid milk spoilage are typically gram-negative (**GN**) and are thought to originate from the processing environment (Schröder, 1984). Although several recent studies have explored the on-farm sources of psychrotolerant sporeformers (Miller et al., 2015a; Masiello et al., 2017), less information is available on sources of PPC in contemporary fluid milk processing facilities. Defects associated with GN-PPC include coagulation, bitter and astringent flavors (Bassette et al., 1986; Harwalkar et al., 1993), rotten, barny, cheesy, and fruity odors

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(Hayes et al., 2002), and pigment production (Seitz et al., 1961; Evanowski et al., 2017). Whereas the regulatory limit on bacterial counts in HTST-pasteurized fluid milk in the United States is 20,000 cfu/mL, sensory defects due to GN-PPC typically require bacterial growth to >5,000,000 cfu/mL (Punch et al., 1965), a level often reached during shelf life by milk with PPC (Martin et al., 2012).

Although most facilities perform microbial tests on HTST-pasteurized fluid milk, the methods most commonly used by industry either do not differentiate between GN bacteria and psychrotolerant sporeformers (e.g., SPC) or only detect a subset of GN bacteria responsible for PPC, such as coliforms and *Enterobacteriaceae* (**EB**). Whereas the *Standard Methods for the Examination of Dairy Products* (Frank and Yousef, 2004) specifies crystal violet tetrazolium agar (**CVTA**) for the enumeration of total GN bacteria, this method is not routinely used by dairy processing facilities, possibly because it uses a complex medium not available in ready-to-use form. However, CVTA has been well established as an effective medium for detection of GN bacteria (Randolph et al., 1973; Hervert et al., 2017), including *Pseudomonas* spp., which are a common cause of PPC, but are not detected with coliform and EB tests. After PPC detection, characterization and subtyping of isolates can provide important information on spoilage organism sources, transmission patterns, and persistence. Molecular subtyping methods have been used to identify sources of food-borne pathogens, but use of these tools for spoilage organisms is less common. Although many molecular methods are available, cost and ease of use are crucial factors. Hence, despite the availability of highly discriminatory subtyping tools, such as pulsed field gel electrophoresis (Nogarol et al., 2013) and whole genome sequencing (Andreani et al., 2015a), use of less discriminatory and cheaper methods (e.g., single-gene sequencing) can provide initial data that can be used to identify instances that may require follow-up with more expensive and discriminatory methods. Though practicality precludes the use of these methods for routine quality checks, they are valuable for troubleshooting when defect events occur and for understanding the mechanisms of product contamination and spoilage.

Though PPC is still commonly observed today (Martin et al., 2011a), little current information is available regarding the identity, prevalence, and quality effect of PPC bacteria from dairy processing facilities. Hence, the aim of our study was to use a longitudinal sampling approach and contemporary methods to characterize PPC and provide industry with data and tools needed to better control PPC.

MATERIALS AND METHODS

Sample Collection and Handling

Samples were collected from 10 dairy processing facilities producing packaged HTST-pasteurized fluid milk (see Table 1); facilities were located across the northeastern United States. For each facility, sample collections were performed on 4 separate occasions between July 2015 and May 2016. Samples of raw milk and finished product were either collected in-person by Milk Quality Improvement Program personnel or collected by facility personnel and delivered via overnight shipping. Samples collected at each collection date included (1) consumer packages representing HTST-pasteurized nonfat (<0.2% milk fat), reduced fat (2% milk fat), and chocolate milk, as well as (2) a sample of raw milk from the silos used to produce the pasteurized products tested. Whenever possible, each of the pasteurized products was obtained from up to 3 different fillers per facility. This resulted in a diversity of packaging container volumes [237 mL to 3.8 L (8 fluid ounces to 1 gallon)] and materials (high-density polyethylene, polyethylene terephthalate, and paperboard; Table 1). The number of pasteurized samples collected was between 3 and 9 per facility per sample collection, depending on the products manufactured and the number of fillers at each facility. Milk samples were transported to the Milk Quality Improvement Program laboratory in coolers packed with ice or ice packs, and were received at temperatures at or below 6°C. Following receipt, samples were held at or below 4°C without freezing. Within 48 h of receipt, samples were divided into aliquots and stored at 6°C for shelf-life and sensory testing, as described by Martin et al. (2012).

Microbiological Analysis of Raw Milk and Pasteurized Fluid Milk Samples

Testing of raw milk was performed at the same time as initial testing for pasteurized products; tests performed included SPC and coliform count. Microbiological testing of pasteurized products took place on the initial day (the first day of testing, 0–5 d postprocessing) and 7, 10, 14, 17, and 21 d postprocessing; tests performed included SPC, coliform count, EB count, and total GN count. All tests were performed with 2 technical replicates per sample. The SPC was performed by spiral plating (Autoplate 4000, Advanced Instruments, Norwood, MA) onto standard methods agar (EMD Millipore Corporation, Billerica, MA) in accordance with *Standard Methods for the Examination of Dairy Products* (Laird et al., 2004). Coliform and EB counts