Rapid detection and characterization of postpasteurization contaminants in pasteurized fluid milk

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ABSTRACT

Microbial spoilage of pasteurized fluid milk is typically due to either (1) postpasteurization contamination (PPC) with psychrotolerant gram-negative bacteria (predominantly *Pseudomonas*) or (2) growth of psychrotolerant sporeformers (e.g., *Paenibacillus*) that have the ability to survive pasteurization when present as spores in raw milk, and to subsequently grow at refrigeration temperatures. While fluid milk quality has improved over the last several decades, continued reduction of PPC is hampered by the lack of rapid, sensitive, and specific methods that allow for detection of PPC in fluid milk, with fluid milk processors still often using time-consuming methods (e.g., Moseley keeping quality test). The goal of this project was to utilize a set of commercial fluid milk samples that are characterized by a mixture of samples with PPC due to psychrotolerant gram-negative bacteria and samples with presence and growth of psychrotolerant spore-forming bacteria to evaluate different approaches for rapid detection of PPC. Comprehensive microbiological shelf-life characterization of 105 pasteurized fluid milk samples obtained from 20 dairy processing plants showed that 60/105 samples reached bacterial counts >20,000 cfu/mL over the shelf-life due to PPC with gram-negative bacteria. Among these 60 samples with evidence of gram-negative PPC spoilage over the shelf-life, 100% (60/60) showed evidence of contamination with noncoliform, non-*Enterobacteriaceae* (EB) gram-negative bacteria (e.g., *Pseudomonas*), 20% (12/60) showed evidence of contamination with coliforms, and 7% (4/60) showed evidence of contamination with noncoliform EB. Among the remaining 45 samples, 28 showed levels of gram-positive bacteria above 20,000 cfu/mL and the remaining 17 samples did not exceed 20,000 cfu/mL over the shelf-life. Evaluation of the same set of 105 samples using 6 different approaches {all possible combinations of 2 different enrichment

protocols (13°C or 21°C for 18 h) and 3 different plating media [crystal violet tetrazolium agar, EB Petrifilm (3M, St. Paul, MN), and Coliform Petrifilm]} showed that enrichment at 21°C for 18 h, followed by plating on crystal violet tetrazolium agar provided for the most sensitive, accelerated detection of samples that reached >20,000 cfu/mL due to PPC with psychrotolerant gram-negatives (70% sensitivity). These results show that tests still required and traditionally used in the dairy industry (e.g., coliform testing) are not suitable for monitoring for PPC. Rather, approaches that allow for detection of all gram-negative bacteria are essential for improved detection of PPC in fluid milk.

Key words: postpasteurization contamination, rapid method, *Pseudomonas*, indicator organism

INTRODUCTION

Although research has shown that fluid milk quality has consistently improved over the last 2 decades (Carey et al., 2005; Martin et al., 2012), postpasteurization contamination (PPC) is still a hurdle for some processors. In fact, some studies suggest that ~40 to 50% of conventionally pasteurized fluid milk shows evidence of PPC (Ranieri and Boor, 2009; S. J. Reichler, Cornell University, Ithaca, NY, unpublished). Postpasteurization contamination has previously been associated with rapid bacterial outgrowth (Schröder et al., 1982; Ranieri and Boor, 2009; Martin et al., 2012) and unacceptable sensorial properties (Hayes et al., 2002; Martin et al., 2012), both of which often lead to premature spoilage before the labeled product shelf-life (defined here as the sell-by date provided by the manufacturer). Because premature spoilage is a contributing cause of food loss, which accounts for approximately one-third of the fluid milk processed in the United States, at a value of \$6.4 billion (Buzby et al., 2014), reducing PPC is of great importance from a business, consumer, and sustainability perspective.

Postpasteurization contamination can be introduced into the fluid milk continuum at various points, but several research studies indicate that the filling equipment is an area that is particularly susceptible to

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contamination often due to lapses in good manufacturing practices (Eneroth et al., 1998; Ralyea et al., 1998; Gruetmacher and Bradley, 1999). Additionally, although stringent cleaning and sanitation programs reduce the incidence of PPC, some contaminants that exist within resistant biofilms, or in niches that are inaccessible to cleaning and sanitizers, may not be effectively removed from processing equipment and lead to persistent PPC. Methods to detect PPC in fluid milk have primarily relied upon traditional indicator organisms used in the dairy industry. Coliforms have been used, for nearly a century, as indicator organisms in the dairy industry. For example, the US Pasteurized Milk Ordinance specifies a coliform limit of 10 cfu/mL for grade A pasteurized fluid milk by (FDA, 2015). Coliforms are heat labile and are very effectively eliminated by HTST pasteurization. Hence, coliform presence in pasteurized fluid milk can be expected to generally be due to re-introduction of these organisms after the heat step, unless coliforms were present in very high numbers (e.g., $>10^6$ cfu/mL) in raw milk. Detection of coliforms in fluid milk thus is often considered an indication of unhygienic conditions or PPC. Testing for coliforms is also relatively fast, a desirable quality for indicator organisms, with many methods (e.g., Coliform Petrifilm) taking 24 h or less.

Despite the longstanding use of coliforms as indicators of PPC in fluid milk, many studies have identified *Pseudomonas*, a noncoliform, as the primary causative agent of PPC (Ternström et al., 1993; Eneroth et al., 1998; Deeth et al., 2002). Importantly, *Pseudomonas* and other noncoliform gram-negative bacteria are not recovered on coliform media and therefore may go undetected by current indicator tests. Testing methods that allow for the detection of coliforms, *Pseudomonas*, and other gram-negative bacteria (e.g., plating on crystal violet tetrazolium agar, **CVTA**) are not as rapid as coliform testing methods, typically requiring 48 h until results. A variety of methods with varying complexity and time-to-result have hence been developed to detect PPC in fluid milk and other fluid dairy products including impedance measurements (Bossuyt and Waes, 1983), direct epifluorescent filter technique (Griffiths et al., 1984), and bioluminescence assays (Griffiths, 1993). Additionally, several researchers have evaluated various selective enrichment procedures for rapid enumeration of PPC (Byrne et al., 1989). Although some of these methods show high correlation with the shelf-life performance of fluid milk (e.g., $r = 0.91$ for impedance methods using selective media; White, 1993) and some have rapid time to result (e.g., <20 h for ATP testing following selective enrichment), many have high initial costs for equipment, require the use of numerous chemicals and reagents, or are complex to run. Importantly,

almost all of the research that has been conducted on rapid detection of PPC in fluid milk was conducted in the 1980s when milk quality was significantly different and when milk quality issues due to PPC may have been due to different organisms (e.g., more common contamination with coliforms) and may have represented different contamination patterns (e.g., higher levels of initial contamination; Carey et al., 2005). Therefore, the goals of this study were to determine the overall population of bacterial contaminants contributing to PPC in a set of contemporary fluid milk samples and to test the ability of various methodologies, specifically those that do not require specialized or expensive equipment and complex steps, to detect PPC in these samples.

MATERIALS AND METHODS

Sample Collection and Handling

Pasteurized milk samples ($n = 105$) were collected from 20 fluid milk processing facilities by Milk Quality Improvement Program (**MQIP**; Cornell University, Ithaca, NY) personnel from 2014 to 2015. Processing facilities were all enrolled in the Voluntary Shelf-Life Program, administered by MQIP, and were located in the northeast United States (New York, Maine, Vermont, New Hampshire, and Massachusetts). Facility size ranged from small, on-farm facilities (with processing capacities of approximately 0.5 million kg/yr) to large facilities (approximately 250 million kg/yr). Pasteurized fluid milk samples collected included whole-fat (minimum 3.25% milk fat, $n = 35$), reduced-fat (2% milk fat, $n = 22$), low-fat (1% milk fat, $n = 24$), and nonfat ($<0.2\%$ milk fat, $n = 24$) milk in 12-ounce (355 mL, $n = 2$), pint (473 mL, $n = 1$), quart (946 mL, $n = 10$), half-gallon (1.9 L, $n = 91$), or gallon (3.8 L, $n = 1$) containers. None of the processors fortified their milk with nonfat dry milk. All products were pasteurized via HTST (15 facilities; 94 samples) or vat pasteurization (5 facilities; 11 samples) and packaged in glass bottles ($n = 9$), high-density polyethylene jugs ($n = 92$), or paperboard cartons ($n = 4$). After being packed in coolers with ice packs or ice, milk samples were transported to the MQIP laboratory and stored at 4°C until the initial testing, performed within 48 h of sample collection.

Shelf-Life Analysis, Rapid Shelf-Life Screening, and Bacterial Isolation

On initial day of testing, pasteurized milk samples were handled and stored as described previously (Martin et al., 2012) in preparation for microbiological and organoleptic analyses, which were performed on each

test day (day initial, d 7, 10, and 14) in accordance with *Standard Methods for the Examination of Dairy Products* (Laird et al., 2004). Extended shelf-life testing (on d 17 and 21) was performed on samples from a subset of processors that have a history of manufacturing high-quality products. Microbiological analyses conducted on each test day included total bacteria count on standard plate count (SPC) agar and total gram-negative bacteria count on CVTA with incubation at 32°C and 21°C for 48 h, respectively. Samples were also inoculated onto 3M *Enterobacteriaceae* (EB) Petrifilm and 3M Coliform Petrifilm according to the manufacturer's instructions (3M, St. Paul, MN), followed by incubation at 32°C for 24 h.

Additionally, on initial day of testing, 100-mL portions of each sample received a preliminary incubation (PI) at (1) 13°C for 18 h with subsequent plating on CVTA, EB Petrifilm, and Coliform Petrifilm, and (2) 21°C for 18 h with subsequent plating on CVTA, EB Petrifilm, and Coliform Petrifilm, following the plating procedure detailed above.

For every sample that showed positive results on media selective and differential for different gram-negatives (i.e., CVTA, EB Petrifilm, and Coliform Petrifilm), 2 isolates with typical gram-negative morphologies (for each combination of test day and medium) were streaked on brain heart infusion (BHI) agar (Difco, Franklin Lakes, NJ), followed by incubation at 32°C for 24 h. Single colonies were subsequently grown in BHI broth at 32°C for 18 h; isolates were then frozen and stored in 15% glycerol at -80°C; all pertinent isolate information is cataloged in Food Microbe Tracker (<http://www.foodmicrobetracker.com>). This procedure was also used to recover and preserve isolates from all SPC plates with counts >20,000 cfu/mL, selecting 1 colony for each unique morphology per set of duplicate plates.

Sensory Defect Analysis

For sensory defect analysis, samples were evaluated by a trained panel of 6 students and staff members from Cornell University as described in Martin et al. (2012). To participate on the defect judging panel, panelists were required to pass pre-screening and a test administered after completion of an initial defect judging training (representing approximately 15 h of training); panelists also received re-training (approximately 1 h) twice a year. On each testing day (except d 7), panelists assigned a flavor criticism(s) and flavor score to each sample, with an average overall flavor quality score computed by Compusense 5 (Compusense Inc., Guelph, ON, Canada). Only nonflavored milk products were evaluated.

Genus Identification by DNA Sequenced-Based Methods

A total of 880 gram-negative bacterial isolates and 10 nonsporeforming gram-positive bacterial isolates were characterized by 16S rDNA PCR and sequencing as described previously (Huck et al., 2007). Additionally, a total of 115 sporeformer isolates were characterized by *rpoB* PCR and sequencing as described previously with subsequent sequencing of the partial 16S gene for new *rpoB* allelic types (AT; Trmcic et al., 2015). Briefly, for both sequencing procedures, isolates were plated on BHI followed by incubation at 32°C for 24 h. For 16S rDNA PCR, 100 µL of distilled H₂O was inoculated with a colony and either (1) heated in a 1,200-W microwave for 30 s, or (2) heated in a thermal cycler at 95°C for 15 min. For *rpoB* PCR, lysis was performed by heating an undiluted colony in a 1,200-W microwave for 3 min. After PCR amplification, products were electrophoresed in 1.5% agarose gel at 120 V for 20 to 25 min. Upon treatment with ExoSap as previously described (Dugan et al., 2002), PCR products were sequenced using bidirectional Sanger sequencing as per manufacturer's instructions (Big Dye Sequencing Kit, Life Technologies Inc., Grand Island, NY). Partial 16S and *rpoB* sequences were used to classify isolates to the genus or genus and species level based on similarity searches against the Ribosomal Database Project database (Cole et al., 2005) or our in-house *rpoB* database (MQIP, Cornell University, Ithaca, NY), respectively, using the Basic Local Alignment Search Tool (BLAST; McGinnis and Madden, 2004). For *rpoB* AT, a new AT was assigned if the 632 nucleotide sequences differed from a type strain in the *rpoB* database by one or more nucleotides; species classification of a new *rpoB* AT included analysis of the 16S rRNA gene as described previously (Huck et al., 2007; Ivy et al., 2012).

Data Analysis

Data were managed in Microsoft Excel (version 2007, Microsoft Corp., Redmond, WA) and Microsoft Access (version 2016, Microsoft Corp.). All statistical modeling was performed in R (2016, R Foundation for Statistical Computing, Vienna, Austria) using the lme4 package. Bacterial count data were log-transformed before analyses. The detection limit was 1.0 log cfu/mL for SPC and CVTA and 0.0 log cfu/mL for EB and Coliform Petrifilm. A mixed-effect linear model was used to analyze the SPC data for samples with gram-negative PPC spoilage and gram-positive sporeformer spoilage (R). The model included test day, spoilage type, and corresponding interaction as fixed effects, and sample nested within plant as random effects (Bates et al.,

2014; Lenth, 2016; Wickham, 2016). To determine if sensory defect scores differed between spoilage types, a mixed-effects linear model was fitted to the 14 sensory scores with a single fixed effect of spoilage type. Random effects of plant, sampling, sample, panelist, sampling/plant interaction, and sampling/panelist interaction were included to account for the structure of the experiment. Standard errors and 95% confidence intervals for the spoilage type estimates and pairwise contrasts were generated using a semiparametric bootstrap method with the `bootMer` function of `lme4` in R.

Sensitivity was defined as the proportion of true positives that are correctly identified by a test [sensitivity = true positive/(true positive + false negative)], whereas specificity was defined as the proportion of true negatives that are correctly identified by a test [specificity = true negative/(true negative + false positive)] (Altman and Bland, 1994a). A positive predictive value was defined as the proportion of true positives among those that test positive [positive predictive value = true positive/(true positive + false positive)], whereas a negative predictive value was defined as the proportion of true negatives among those that test negative [negative predictive value = true negative/(true negative + false negative)] (Altman and Bland, 1994b).

RESULTS

Pasteurized Fluid Milk Sample Set Used Here Represents a Range of Gram-Negative and Gram-Positive Contamination Patterns

Among the 105 fluid milk samples tested here, 84% ($n = 88$) tested reached $>20,000$ cfu/mL during shelf-life (Table 1). For 60 of these 88 samples, bacteria representing gram-negative genera were isolated from shelf-life samples with $>20,000$ cfu/mL (spoilage classifications #1 and 3 in Table 1). These 60 samples were designated as samples with gram-negative PPC spoilage. Gram-negative bacteria were also isolated after pre-incubation from 4 further samples with $>20,000$ cfu/mL, even though these 4 samples yielded only gram-positive isolates from the actual shelf-life samples (spoilage classification #4 in Table 1). Therefore, a total of 64 samples showed evidence of contamination with gram-negative bacteria. Among these 64 samples, which reached $>20,000$ cfu/mL and showed evidence of PPC, 100% (64/64) showed evidence of contamination with noncoliform, non-EB gram-negative bacteria (e.g., *Pseudomonas*), 19% (12/64) showed evidence of contamination with coliforms, and 6% (4/64) showed evidence of contamination with noncoliform EB. Importantly, all of the samples that showed evidence of contamination with coliforms or noncoliform EB were

also contaminated with noncoliform, non-EB gram-negative bacteria, specifically *Pseudomonas* (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14216>).

From the 64 samples with evidence of gram-negative contamination, a total of 880 gram-negative isolates were selected; these isolates were obtained from SPC, CVTA, EB Petrifilm, and Coliform Petrifilm plating media from both the PI methods and shelf-life testing. Sequence characterization and analysis of a 616-bp fragment of the 16S rDNA gene resulted in a subset of nonredundant gram-negative isolates ($n = 160$), which were classified into 23 genera. *Pseudomonas*, which was isolated from 55 of the 64 (86%) samples with evidence of gram-negative contamination, represented the dominant genus. Other gram-negative genera found in 5 or more samples with PPC, included *Acinetobacter* (22 samples), *Cedecea* (6 samples), and *Raoultella* (6 samples). Additionally, *Hafnia* was isolated from 4 samples that reached 20,000 cfu/mL during shelf-life as well as one additional sample that did not reach 20,000 cfu/mL during shelf-life but still showed evidence of gram-negative contamination (isolation of gram-negative bacteria from shelf-life; spoilage classification 5 in Table 1 and Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14216>).

For the 24 samples that exceeded 20,000 cfu/mL but did not show evidence of PPC with gram-negative bacteria either over the shelf-life or in PI samples (spoilage classification 2 in Table 1), gram-positive sporeforming bacteria represented the predominant bacterial contaminants, with a total of 60 gram-positive isolates obtained from these samples. Characterization with a combination of *rpoB* and 16S rDNA sequencing identified 56 isolates as gram-positive sporeformers in the family *Bacillales*, with *Paenibacillus* (43/56) the most prevalent followed by *Bacillus* (12/56) and *Viridibacillus* (1/56). The remaining gram-positive isolates were nonsporeforming gram-positive bacteria identified as *Leuconostoc* (4/4). Further, another 64 gram-positive bacteria were isolated from a subset of samples ($n = 26$) that exceeded 20,000 cfu/mL and also showed evidence of PPC (spoilage classification #3 in Table 1). These gram-positive isolates were identified as *Paenibacillus* (37/64), *Bacillus* (18/64), and *Viridibacillus* (1/64). The remaining gram-positive isolates were nonsporeforming gram-positive bacteria identified as *Lactococcus* (3/64), *Leuconostoc* (4/64), and *Okibacterium* (1/64).

Fluid Milk Samples with Presence of Gram-Negative Bacteria Showed Significantly Reduced Shelf-Life

A comparison of the mean SPC for each testing day for samples with gram-negative PPC spoilage and samples with gram-positive sporeformer spoilage over

Table 1. Microbial spoilage pattern for 105 pasteurized milk samples as well as detection pattern for 6 preliminary incubation approaches¹

Spoilage classification	Spoilage description	No. of samples	13°C/18 h			21°C/18 h		
			No. of samples positive on			No. of samples positive on		
			CVTA	EB Petrifilm	Coliform Petrifilm	CVTA	EB Petrifilm	Coliform Petrifilm
1	SPC >20,000 cfu/mL during shelf-life, only gram-negative bacteria isolated from shelf-life samples	34	15	4	2	29	9	6
2	SPC >20,000 cfu/mL during shelf-life, only gram-positive bacteria isolated from shelf-life samples	24	—	—	—	—	—	—
3	SPC >20,000 cfu/mL during shelf-life, gram-negative and gram-positive bacteria isolated from shelf-life samples	26	5	—	—	13	5	3
4	SPC >20,000 cfu/mL during shelf-life, only gram-positive bacteria isolated from shelf-life samples, gram-negative bacteria isolated only from preliminary incubation test	4	1	—	—	3	—	—
5	SPC <20,000 cfu/mL, gram-negative bacteria isolated from shelf-life samples	2	—	—	—	—	—	—
6	SPC <20,000 cfu/mL, no evidence of gram-negative bacteria in shelf-life samples	15	—	—	—	—	—	—
	Total	105	21	4	2	45	14	9

¹SPC = standard plate count; CVTA = crystal violet tetrazolium agar; EB = *Enterobacteriaceae*. Some samples were positive on more than 1 medium type (e.g., all 4 samples positive on EB Petrifilm after 13°C/18 h preliminary incubation were also positive on CVTA). EB Petrifilm and Coliform Petrifilm are from 3M (St. Paul, MN).

the shelf-life showed that samples with gram-negative bacteria had higher ($P < 0.05$) total bacterial counts at d 10, 14, 17, and 21 as compared with samples with gram-positive sporeformers. Mean SPC for samples with gram-positive sporeformer spoilage were 2.5, 2.5, 2.7, 3.6, 4.8, and 6.4 log cfu/mL on days initial, 7, 10, 14, 17, and 21, respectively, compared with mean SPC for samples with gram-negative PPC spoilage of 1.8, 3.6, 5.6, 6.9, 7.1, and 7.8 log cfu/mL on days initial, 7, 10, 14, 17, and 21, respectively.

A subset ($n = 90$) of the samples evaluated also underwent sensory defect analysis; samples were scored on overall acceptability on a scale from 1 to 10, with a scores of <6 designated as poor, 6 to 7 as fair, and 8 to 10 as good (Bodyfelt et al., 1988). The estimated d 14 mean sensory score for samples with gram-positive sporeformer spoilage ($n = 22$) was 7.98 [95% CI (7.74,

8.20)], whereas the estimated d 14 mean sensory score for samples with gram-negative PPC spoilage ($n = 46$) was 5.78 [95% CI (5.62, 5.93)]. Samples with total bacterial counts below 20,000 cfu/mL on d 14 ($n = 17$) had an estimated mean sensory score of 8.60 [95% CI (8.34, 8.85)], whereas the remaining 5 samples were not tasted on d 14. Our results also showed that on the final day of shelf-life (either d 14 or 21 depending on previous shelf-life data) the predominant defects identified among the 47 samples with gram-negative PPC spoilage were “coagulated” ($n = 24$), “lacks freshness” ($n = 11$), and “bitter” ($n = 10$). Comparatively, the predominant defects identified among the 22 samples with gram-positive sporeformer spoilage were “lacks freshness” ($n = 8$) and “not clearly defined” ($n = 6$); “coagulation” was only rarely identified among these samples ($n = 2$; Figure 1).

Pre-incubation at 21°C Followed by Plating on CVTA Had a Sensitivity of 70% for Accelerated Detection of Gram-Negative Bacteria

In parallel to the shelf-life testing detailed above, the 105 milk samples were used to evaluate the ability of combinations of different PI schemes (either 13 or 21°C for 18 h) with subsequent plating on 3 different media that allow for detection of gram-negative organism groups (Coliform Petrifilm, EB Petrifilm, and CVTA) to detect PPC in commercially processed milk. The comprehensive data shown above supported that this set of 105 samples was appropriate for evaluation of different approaches for accelerated detection of gram-negative bacteria in pasteurized milk as these samples represented a wide range of spoilage profiles and different spoilage organisms.

Among the 6 approaches evaluated, a PI of 21°C for 18 h followed by plating on CVTA was the most sensitive, correctly detecting 70% (42/60) of samples with gram-negative PPC spoilage over the shelf-life (Table 2, Figure 2a). This approach also detected gram-negative contamination in 3 additional samples that did not show evidence of gram-negative PPC spoilage over the shelf-life, resulting in a specificity of 93% (42/45;

spoilage classification #4 in Table 1). The second most sensitive approach for detection of gram-negative PPC spoilage was a PI of 13°C for 18 h followed by plating on CVTA; this approach correctly identified 33% (20/60) of samples with evidence for gram-negative PPC spoilage over the shelf-life. This approach resulted in a single false positive (i.e., detection of gram-negative organisms with no evidence of gram-negative PPC spoilage over the shelf-life), resulting in a specificity of 98% (44/45) for detection of gram-negative PPC spoilage (Table 2, Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-14216>). Hence, an overall 4 samples yielded gram-negatives in one of the PI tests, but did not yield gram-negatives in samples tested over the shelf-life [these samples represent spoilage classification 4 (Table 1, Supplemental Table S1, <https://doi.org/10.3168/jds.2017-14216>)]; in these cases the medium used after PI correctly identified gram-negative bacteria, but the positive tests did not correctly identify samples that spoiled due to PPC. The least sensitive test for PPC spoilage was a PI of 13°C for 18 h followed by plating on Coliform Petrifilm; this approach only correctly identified 3% (2/60) of samples with evidence for gram-negative PPC spoilage over the shelf-life (Table 2, Figure 2b).

Figure 1. Comparison of sensory defect attributes identified by panelists for samples with gram-negative postpasteurization contamination (PPC) spoilage (n = 47) or gram-positive sporeformer spoilage (n = 22) at the end of the shelf-life (d 14 or 21).

Figure 2. Scatter plots displaying log bacterial counts for samples plated on (a) crystal violet tetrazolium agar (CVTA) after a preliminary incubation at 21°C for 18 h and (b) Coliform Petrifilm (3M, St. Paul, MN) after a preliminary incubation at 13°C for 18 h with corresponding maximum standard plate count (SPC) through shelf-life. Data points are categorized into (1) samples with gram-negative postpasteurization contamination (PPC) spoilage; (2) samples with gram-positive sporeformer spoilage (“sporeformer”); and (3) samples with bacterial counts below 20,000 cfu/mL (“below spoilage”). The detection limit was 1.0 log cfu/mL for SPC and CVTA and 0.0 log cfu/mL for coliforms. Data points for samples that showed no growth on selective media are shown here at the detection limit. A line at 20,000 cfu/mL (4.3 log cfu/mL) marks the US Pasteurized Milk Ordinance regulatory limit of 20,000 cfu/mL. Equivalent scatter plots for other media/enrichment combinations can be found in Supplemental Figure S1 (<https://doi.org/10.3168/jds.2017-14216>).

Total Gram-Negative Test (i.e., CVTA) Detected a Greater Diversity of PPC than EB and Coliform Tests

Overall, 59 of the 60 samples (98%) that showed evidence of gram-negative PPC spoilage over the shelf-life also showed bacterial growth on CVTA on at least one of the samples tested over the shelf-life (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14216>). In contrast, EB and Coliform Petrifilm were only positive for 13 (22%) and 12 (20%) of the 60 samples, respectively. Bacterial isolates from these 60 samples as well as the additional 28 samples that exceeded 20,000 cfu/

mL during shelf-life due to gram-positive bacteria, were selected from SPC agar and 3 selective and differential gram-negative medium types (i.e., CVTA, EB Petrifilm, and Coliform Petrifilm) for molecular characterization. Specifically, among a total of 1,005 gram-negative and gram-positive isolates collected from these 88 samples, a subset ($n = 378$) of isolates was selected to represent the nonredundant diversity of isolates. Only one isolate with a given *rpoB* sequence or 16S rDNA genus obtained for each sample, medium type, and test (i.e., PI or shelf-life) combination was included in this nonredundant set (to avoid overrepresentation of multiple identical isolates from the same test). These 378 nonre-

Table 2. Sensitivity, specificity, positive predictive value, and negative predictive value for 6 different approaches for accelerated detection of fluid milk samples that spoil due to postpasteurization contaminants in 105 pasteurized fluid milk samples

Medium type ¹	Preliminary incubation temperature (°C)	Sensitivity ² (%)	Specificity ³ (%)	Positive predictive value ⁴ (%)	Negative predictive value ⁵ (%)
CVTA	21	70 (42/60)	93 (42/45)	93 (42/45)	70 (42/60)
EB Petrifilm	21	23 (14/60)	100 (45/45)	100 (14/14)	49 (45/91)
Coliform Petrifilm	21	15 (9/60)	100 (45/45)	100 (9/9)	47 (45/96)
CVTA	13	33 (20/60)	98 (44/45)	95 (20/21)	52 (44/84)
EB Petrifilm	13	7 (4/60)	100 (45/45)	100 (4/4)	44 (45/101)
Coliform Petrifilm	13	3 (2/60)	100 (45/45)	100 (2/2)	44 (45/103)

¹CVTA = crystal violet tetrazolium agar; EB = *Enterobacteriaceae*. EB Petrifilm and Coliform Petrifilm are from 3M (St. Paul, MN).

²Sensitivity = $TP / (TP + FN) \times 100\%$ (TP: true positive, FN: false negative).

³Specificity = $TN / (TN + FP) \times 100\%$ (TN: true negative, FP: false positive).

⁴Positive predictive value = $TP / (TP + FP) \times 100\%$.

⁵Negative predictive value = $TN / (TN + FN) \times 100\%$.

Table 3. Genera isolated from 88 pasteurized fluid milk samples that exceeded 20,000 cfu/mL during shelf-life on various media

Genus ¹	No. of isolates obtained on ²				Total isolates	% Total isolates
	SPC	CVTA	EB Petrifilm	Coliform Petrifilm		
<i>Pseudomonas</i>	51	84	—	—	135	35.7
<i>Paenibacillus</i>	82	—	—	—	82	21.7
<i>Bacillus</i>	31	—	—	—	31	8.2
<i>Acinetobacter</i>	5	19	—	—	24	6.3
<i>Hafnia</i>	2	4	4	3	13	3.4
<i>Cedecea</i>	—	2	5	3	10	2.6
<i>Raoultella</i>	—	—	4	6	10	2.6
<i>Lelliottia</i>	2	—	4	3	9	2.4
<i>Obesumbacterium</i>	2	1	2	4	9	2.4
<i>Leuconostoc</i>	6	—	—	—	6	1.6
<i>Aeromonas</i>	1	2	3	—	6	1.6
<i>Rahnella</i>	1	—	2	2	5	1.3
<i>Janthinobacterium</i>	1	3	—	—	4	1.1
<i>Citrobacter</i>	—	—	2	2	4	1.1
<i>Serratia</i>	—	1	2	1	4	1.1
<i>Lactococcus</i>	2	1	—	—	3	0.8
<i>Buttiauxella</i>	1	1	1	—	3	0.8
<i>Escherichia/Shigella</i>	—	1	1	1	3	0.8
<i>Klebsiella</i>	—	1	1	1	3	0.8
<i>Viridibacillus</i>	2	—	—	—	2	0.5
<i>Stenotrophomonas</i>	1	1	—	—	2	0.5
<i>Comamonas</i>	—	2	—	—	2	0.5
<i>Yersinia</i>	—	—	2	—	2	0.5
<i>Okibacterium</i>	1	—	—	—	1	0.3
<i>Brevundimonas</i>	—	1	—	—	1	0.3
<i>Enterobacter</i>	—	—	—	1	1	0.3
<i>Flavobacterium</i>	—	1	—	—	1	0.3
<i>Limnohabitans</i>	—	1	—	—	1	0.3
<i>Providencia</i>	—	—	1	—	1	0.3
Total	191	126	34	27	378	100

¹Genus identification based on partial 16S rDNA and *rpoB* sequence data. The *rpoB* data were used for genus identification of the majority of gram-positive isolates.

²Numbers indicate the number of isolates collected on each medium type either from shelf-life samples or from positive stress test samples; numbers specifically represent the nonredundant diversity of isolates obtained for each sample, medium type, and test (i.e., stress test or shelf-life) combination (meaning only 1 isolate was counted if multiple isolates with the same 16S rDNA or *rpoB* sequence type were obtained from a given sample, medium type, and test combination). SPC = standard plate count agar; CVTA = crystal violet tetrazolium agar; EB petrifilm = *Enterobacteriaceae*. EB Petrifilm and Coliform Petrifilm are from 3M (St. Paul, MN).

dundant isolates were used to compare isolation of different genera and *rpoB* AT from different media (Table 3). A total of 191 isolates representing 16 genera were isolated from SPC agar, with the predominant genera being *Paenibacillus* (82/191), *Pseudomonas* (51/191), and *Bacillus* (31/191). Among the media that detect gram-negative organisms, CVTA detected the largest number of genera (17 genera among 126 isolates), followed by EB Petrifilm (14 genera among 34 isolates), and Coliform Petrifilm (11 genera among 27 isolates; Table 3). The CVTA captured 9 genera that went undetected by EB Petrifilm and 11 that went undetected on Coliform Petrifilm, with *Pseudomonas* (84/126) and *Acinetobacter* (19/126) as the predominant organisms detected on CVTA and not detected on either of the Petrifilm media. Whereas CVTA detected the largest number of genera, EB Petrifilm and Coliform Petrifilm detected 6 and 4 genera, respectively, that were not detected by CVTA (Table 3). It should be noted that in addition to the ability of each medium to support the

recovery and growth of different bacterial genera, the isolate selection procedures used in this study may have also contributed to the observation that some genera were only recovered on some media; with characterization of a larger number of isolates from each medium one would expect to see fewer genera that are only detected on a single given medium (specifically EB Petrifilm and Coliform Petrifilm).

DISCUSSION

Total Gram-Negative Testing Represents a Viable Indicator Test for PPC of Fluid Milk

Coliforms have been used in the dairy industry since the beginning of the 20th century (Tortorello, 2003) to identify milk that has been processed under unsanitary conditions and contaminated after pasteurization. Coliforms are a method defined group of organisms, primarily within the EB family (Davidson et al., 2004),

that have been shown historically to play an important role in fluid milk spoilage (Martin et al., 2016). Testing for coliforms in fluid milk is rapid (i.e., 24–48 h depending on method) and inexpensive. Despite the advantages of using coliforms as indicators of PPC, current research indicates that coliforms are decreasingly prevalent in fluid milk (Carey et al., 2005; Martin et al., 2012). Carey et al. (2005) found that 21 to 34% of fluid milk samples tested from 1991 to 2000 were positive for coliform, whereas the same group found that from 2001 to 2010 the number of samples positive for coliform on any day of shelf-life ranged from 7.6 to 26.6% in a given year (Martin et al., 2012). Further, a survey of fluid milk from across the United States found that among 175 gram-negative isolates collected over the shelf-life, only 16.5% (29/175) were EB and the remainder were non-EB gram-negatives, with *Pseudomonas* the predominant contaminant ($n = 122$; Ranieri and Boor, 2009). In the current study, 84% of samples reached spoilage level of bacteria (defined here as $>20,000$ cfu/mL), due to coliforms, noncoliform EB, non-EB gram-negative bacteria, and gram-positive bacteria, with many samples yielding multiple genera over the shelf-life. Coliforms represented one of the least common groups of contaminants in this study (second only to noncoliform EB, which were only detected in 4 samples; Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14216>).

Similarly to previous studies, *Pseudomonas* was found in over 86% of the samples tested here that showed evidence of gram-negative contamination (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14216>). *Pseudomonas*, in the family *Pseudomonadaceae*, is not a coliform and therefore not detected using coliform test methods but represents a major postpasteurization contaminant in this study and others (Ternström et al., 1993; Eneroth et al., 1998; Deeth et al., 2002). The presence of *Pseudomonas* in fluid milk not only indicates that PPC has occurred, but also typically has a dramatic effect on the shelf-life of the product. Many *Pseudomonas* species are not only capable of growing rapidly at refrigeration temperatures (Ternström et al., 1993; Ranieri and Boor, 2009), but also express several enzymes, including proteases and lipases, that degrade the quality of fluid milk (Dogan and Boor, 2003; Nörnberg et al., 2010).

Rapid Detection of Low Level Gram-Negative Contamination that Leads to Product Spoilage Remains a Challenge

Rapid detection of indicator organisms in fluid milk and accurate shelf-life prediction have long been goals of the dairy industry. The Moseley keeping-quality test

(Moseley, 1980; Duncan et al., 2004), often considered the gold standard, has shown good correlation with fluid milk shelf-life with a study by Bishop and White (1986) reporting a correlation coefficient of -0.77 ; however, this test takes 7 to 9 d to obtain results. Other tests to predict fluid milk shelf life and detect PPC include a variety of PI methods (e.g., selective enrichment using crystal violet tetrazolium in milk incubated for 18 h at 21°C ; White, 1993), which typically rely on selective agents to restrict the growth of gram-positive bacteria either in the enrichment or on the plating medium (Griffiths et al., 1984). The current standard method for the PI method, as detailed in the *Standard Methods for the Examination of Dairy Products* (Duncan et al., 2004), relies on enrichment of 10 mL of pasteurized milk at 21°C for 18 h followed by plating on standard methods agar, using no selective agents during enrichment or plating (Duncan et al., 2004). Use of selective agents, however, represents a valuable approach that can be used to inhibit the growth of one group of organisms, while still allowing for growth of a targeted set of organisms (e.g., gram-negatives), facilitating simple screening approaches that do not require molecular or other advanced techniques for organism identification. The current study hence tested variations of the standard PI approach on a set of contemporary milk samples that represent current predominant bacterial contaminants, examining different incubation temperatures (i.e., 13°C and 21°C) and the use of selective and differential media (i.e., CVTA, EB Petrifilm, and Coliform Petrifilm), all with pre-enrichment of a higher volume of milk (i.e., 100 mL) than the standard method. A larger sample volume was specifically used to enhance the sensitivity of the PI method to potentially 1 bacterium per 100 mL, while being realistic about the ability to routinely use large milk volumes in a PI test on larger samples sets. The variations of the PI tests evaluated here showed substantial differences in the sensitivity and specificity of the tests, with regard to early identification of samples that spoiled due to PPC with gram-negative bacteria. Despite the sizeable improvement in sensitivity for detection of gram-negative PPC spoilage by using enrichment at 21°C for 18 h followed by plating on CVTA (70% sensitivity) over the other methods tested (e.g., sensitivity of 3% for PI at 13°C for 18 h followed by enumeration on Coliform Petrifilm), several samples ($n = 18$) still showed PPC over the shelf-life, but were not identified with this modified PI test (Table 2, Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14216>). We hypothesize that the primary reason for the lack of detection in some samples is a low initial contamination level (around or below 1 cell per 100 mL) where 100-mL sub-samples are not consistently positive. We specifically speculate

that this was the case for 4 of the samples tested here (spoilage classification #4 in Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14216>), which allowed for detection of gram-negative bacteria from the enrichment vial (containing 100 mL of milk), but did not test positive for a gram-negative organism throughout shelf-life. Importantly, although freshly pasteurized milk has been reported to frequently have a total gram-negative count below 1 cfu/mL (the typical detection limit for direct plating; Schröder, 1984), over the course of refrigerated storage even contaminants at these lower levels can grow to spoilage levels. In theory, as few as 1 bacterial cell introduced after pasteurization per container could result in premature spoilage (Schröder, 1984). In addition to challenges with detection of low levels of gram-negative bacteria, CVTA may also not allow for detection of some gram-negative organisms, as demonstrated in previous work (Hervert et al., 2017); our study here, however, suggests that this may not be a common issue.

The study reported here also clearly demonstrated that, among the media tested here, CVTA recovered the most comprehensive set of gram-negative organisms (Table 3). This is consistent with the predominance of *Pseudomonas*, which will not grow on EB or Coliform Petrifilm, in fluid milk samples that spoil due to PPC. Van Tassell et al. (2012) also found that CVTA outperformed Coliform Petrifilm, violet red bile agar, and MacConkey agar when enumerating common dairy associated *Pseudomonas*. Yet, even CVTA has been shown in other studies not to detect certain gram-negative organisms. Specifically, Hervert et al. (2016) found that of a set of 211 previously characterized dairy associated gram-negative bacteria including EB and non-EB strains, 37 failed to grow in pure culture on CVTA. Importantly, Hervert et al. (2016) also found that all of the selective media (CVTA, Coliform and EB Petrifilm, and violet red bile glucose agar) used to evaluate indicator organisms relevant to dairy products showed reduced recovery compared with the nonselective media, BHI agar. Hence, despite the ability of CVTA to detect a more comprehensive set of dairy-associated gram-negative postpasteurization contaminants, as compared with coliform or EB media, further research is needed to identify alternative medium solutions that will detect a broader range of gram-negative organisms associated with contemporary fluid milk PPC, which would improve sensitivity of culture-based tests with regard to detection of PPC.

CONCLUSIONS

Reducing PPC and thereby providing consumers with the highest quality fluid milk is not only important for

the dairy industry to retain customers, but would also play a role in reducing food loss, which is a major issue in the United States. The current study shows that relying on coliform testing is insufficient to detect PPC and leads to underreporting of PPC. To reduce PPC, processors must be able to rapidly and accurately detect PPC events, a goal that clearly is not achievable with current industry testing practices (i.e., coliform testing), particularly as the organisms responsible for PPC of milk continue to shift from coliforms to primarily *Pseudomonas*. The test presented here (i.e., enrichment of 100 mL of fluid milk at 21°C for 18 h followed by enumeration on CVTA) not only allows for detection of PPC with a range of organisms, including *Pseudomonas*, but also is more rapid (allowing for completion in less than 3 full days) relative to traditional methods such as the Moseley keeping quality test, which takes 7 to 9 d. While the PI test detailed above already provides a valuable tool for industry, further research is needed to continue to develop PPC screening methods that have improved sensitivity, increased speed, and increased ease of use; ease of use is a particular concern as the PI method detailed here requires CVTA plates. Briefly, an ideal PPC indicator method should (1) detect low level contamination, which may require testing of larger volumes of product; (2) detect a comprehensive set of PPC organisms, in particular *Pseudomonas*; (3) have a short time to result, ideally less than 24 h, but certainly less than 48 h, to improve processor response time to contamination events; and (4) be accessible to processors of all sizes and resource levels (favoring methods that are affordable and easy to use).

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