Symposium review: Effect of post-pasteurization contamination on fluid milk quality¹

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ABSTRACT

Fluid milk quality in the United States has improved steadily over the last 2 decades, in large part due to the reduction in post-pasteurization contamination (PPC). Despite these improvements, some studies suggest that almost 50% of fluid milk still shows evidence of PPC with organisms that are able to grow at 6°C, even though PPC may be much less frequent in some facilities. Several gram-negative bacteria, when introduced as PPC, can grow rapidly at refrigeration temperatures around 6°C and can lead to bacterial levels above 20,000 cfu/mL (the regulatory limit for bacterial numbers in fluid milk in the United States) and spoilage that can be detected sensorially within 7 to 10 d of processing. Importantly, however, storage temperature can have a considerable effect on microbial growth, and fluid milk stored at 4°C and below may show considerably delayed onset of microbial growth and spoilage compared with samples stored at what may be considered mild abuse (6°C and above). Notable organisms that cause PPC and grow at refrigeration temperatures include psychrotolerant *Enterobacteriaceae* and coliforms, as well as *Pseudomonas*. These organisms are known to produce a variety of enzymes that lead to flavor, odor, and body defects that can ultimately affect consumer perception and willingness to buy. Detecting PPC in high temperature, short time, freshly pasteurized fluid milk can be challenging because PPC often occurs sporadically and at low levels. Additionally, indicator organisms typically used in fluid milk (i.e., coliforms) have been shown to represent only a fraction of the total PPC. Recent studies indicate that coliforms account for less than 20% of the total gram-negative organisms introduced into fluid milk after pasteurization. In contrast, *Pseudomonas*, which is not a coliform and there-

fore is not detected using coliform media, is the most commonly isolated genus in PPC fluid milk. To reduce PPC, processors must (1) use testing methods that can detect both coliforms and non-coliform gram-negatives (i.e., *Pseudomonas*) to understand true contamination rates and patterns, and (2) establish cleaning and sanitation protocols and employee and management behaviors that target persistent and transient PPC organisms.

Key words: fluid milk, post-pasteurization contamination, *Pseudomonas*, sanitation, *Enterobacteriaceae*

INTRODUCTION

Raw milk, even when produced under ideal circumstances, has a diverse bacterial ecology that reflects the lifestyle of the animal and the environment in which the commodity is produced. A wide range of gram-positive and gram-negative bacteria, pathogens, spoilage bacteria, and organisms that are commensal with the animal or cause animal disease are found in raw milk. Fortunately, pasteurization, which was widely adopted in the United States in the 1940s, reduces the levels of many of these organisms by up to 6 orders of magnitude (Vilamiel and de Jong, 2000). Certain heat-resistant or thermophilic bacteria (e.g., *Micrococcus*) are capable of surviving pasteurization conditions (e.g., 72°C/15s) in vegetative form, but these organisms are typically not able to grow under refrigeration (Gleeson et al., 2013). Additionally, spore-forming bacteria can survive pasteurization in spore form; importantly, several aerobic sporeformers that can grow under refrigeration conditions have been identified in both raw milk and HTST-pasteurized fluid milk (Ivy et al., 2012). When post-pasteurization contamination (PPC) occurs with organisms that can grow at refrigeration temperatures, the gram-negative organisms introduced typically cause spoilage and reach levels above the Pasteurized Milk Ordinance (PMO) limit of 20,000 cfu/mL before growth of psychrotolerant sporeformers occurs and appear to outcompete these sporeformers. In the absence of PPC with gram-negative, psychrotolerant organisms, aerobic psychrotolerant sporeformers present in raw milk typi-

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cally grow to spoilage levels after 14 d at 6°C (Ranieri and Boor, 2009). The predominant spore-forming bacteria capable of growing at refrigeration temperatures are certain strains of *Paenibacillus* and *Viridibacillus*, along with *Bacillus weihenstephanensis* (Ivy et al., 2012). We might expect that much of the fluid milk supply would be spoiled by these aerobic spore-forming bacteria that originate in raw milk and survive pasteurization, yet almost 50% of the fluid milk supply shows evidence of contamination with heat-labile gram-negative bacteria that originate from the processing facility environment and recontaminate fluid milk after pasteurization.

Post-pasteurization contamination of fluid milk with psychrotolerant spoilage bacteria plays a significant role in limiting the quality and shelf life of conventionally pasteurized fluid milk. From the earliest days of pasteurization, recontamination of fluid milk after pasteurization has been identified as a problem. In 1920, milk inspectors were urged by Russel S. Smith of the Department of Agriculture to “not rest assured of a safe product because of the mere presence of a milk pasteurizing plant in their city. Special attention must be given to the operation of such a plant in view of the fact that unless it is properly operated it may become a chance source of infection” (Smith, 1920). Smith goes on to outline locations within the processing facility that should receive particular attention to prevent recontamination; namely, pumps, bottling machines, bottles, and milk cans (Smith, 1920). Despite the passage of nearly a century since the above advice to the dairy industry, PPC remains an important cause of fluid milk spoilage. This review focuses on the effects of PPC on fluid milk quality with special attention paid to the organisms commonly responsible for PPC, where they are typically introduced into fluid milk, and diagnostic tools for detecting and tracking them in product and processing environments.

PSEUDOMONAS IS THE PRIMARY CAUSATIVE AGENT OF PPC IN FLUID MILK

Pasteurization is designed to reduce the populations of the most heat-resistant vegetative pathogen found in milk, *Coxiella burnetii*, to levels that would not pose a public health risk (Holsinger et al., 1997). The resultant pasteurization parameters, a minimum of 72°C for 15 s for HTST processing, are reported to deliver a considerable reduction in psychrotolerant gram-negative bacteria (Champagne et al., 1994), and at least a 6-log reduction in some species of *Pseudomonas* (Villamiel and de Jong, 2000). Therefore, the presence of *Pseudomonas* and other gram-negative bacteria in pasteurized fluid milk is typically an indication that a contami-

nation event has occurred post-processing. However, pasteurization failures and presence of high levels of gram-negative bacteria (with subsequent survival of pasteurization of some bacterial cells) in raw milk may also be responsible for presence of gram-negative bacteria in finished HTST products. The PMO limits total bacterial counts in grade ‘A’ commingled raw milk to 300,000 cfu/mL (FDA, 2015). However, if this raw product is held for an extended period of time or at an elevated temperature before pasteurization, bacterial numbers may reach concentrations where even a 6-log reduction would result in residual bacterial cells in pasteurized finished product. Although these deviations (i.e., pasteurization failure and very high pre-pasteurization bacterial levels) are less likely to be an issue in countries with well-developed and sophisticated dairy industries, high levels of gram-negative bacteria in raw milk are not unusual in countries that lack effective on-farm cooling practices and an effective farm-to-processing plant refrigeration chain.

Four primary groups of psychrotolerant bacteria are important in PPC of fluid milk (Table 1): (1) *Pseudomonas*; (2) coliforms; (3) non-*Pseudomonas*, non-coliform gram-negative bacteria; and (4) gram-positive spore-forming bacteria. *Pseudomonas* is, by far, the most commonly reported organism responsible for PPC of HTST fluid milk in the United States (Ranieri and Boor, 2009; Martin et al., 2011b) and globally, including Sweden (Ternström et al., 1993; Eneroth et al., 1998), Australia (Juffs, 1973; Deeth et al., 2002), the UK (Schröder, 1984; Stevenson et al., 2003), and others. Several factors contribute to the success of *Pseudomonas* as an agent of PPC, the first being its ability to grow rapidly at low temperatures (Ternström et al., 1993). Ranieri and Boor (2009) reported that samples of HTST fluid milk contaminated with *Pseudomonas* had significantly higher bacterial numbers by 7 d after pasteurization than samples lacking PPC, with those samples contaminated with *Pseudomonas* reaching the PMO limit of 20,000 cfu/mL at d 8 after pasteurization, on average, compared with d 15 for samples with no PPC but with presence of psychrotolerant sporeformers. Trmčić et al. (2015) demonstrated that various *Pseudomonas* strains were capable of growing more than 4 log cfu/mL over 21 d at a slightly stressed refrigeration temperature (i.e., 6°C). Additionally, *Pseudomonas* are known to be particularly adept at outcompeting other spoilage microorganisms due in part to the ability of many strains to produce antibacterial and antifungal agents and siderophores (Gram et al., 2002), which are excreted into the growth medium where they bind and solubilize iron. *Pseudomonas* produce a variety of siderophores, notably pyoverdine (Brown and Luke,

2010). *Pseudomonas fluorescens* has been described as the predominant (Law, 1979; Dogan and Boor, 2003; Brown and Luke, 2010) and most important species of *Pseudomonas* found in HTST pasteurized milk. Other species that have been detected in fluid milk include *Pseudomonas fragi*, *Pseudomonas lundensis*, and *Pseudomonas putida* (Ternström et al., 1993; Eneroth et al., 2000b; Brown and Luke, 2010).

Another common cause of PPC in fluid milk is contamination with coliforms (Kaloianov and Gogov, 1977; Wessels et al., 1989; Martin et al., 2012). Coliforms are defined not by taxonomic relationships but by common phenotypic characteristics (Martin et al., 2016). Specifically, coliforms are a group of aerobic and facultatively anaerobic, gram-negative, non-spore-forming rods that are capable of fermenting lactose to produce gas and acid within 48 h at 32 to 35°C (Davidson et al., 2004). Most coliforms are within the *Enterobacteriaceae* family, but at least one organism, *Aeromonas*, can produce a positive reaction on coliform medium and is in the family *Aeromonadaceae* (Abbott et al., 2003). Coliforms have been used by the dairy industry for nearly a century as indicators of hygienic conditions in fluid milk (Tortorello, 2003), although there has been some discussion as to whether coliforms are the best indicators to use in fluid milk (Martin et al., 2016). In the United States, coliforms are limited to no more than 10 cfu/mL in grade 'A' pasteurized milk (FDA, 2015).

Coliforms, because of their method-defined nature, are a very diverse group of microorganisms. A study of microbiological quality of pasteurized fluid milk in New York State from 2001 to 2010 reported that 7.6 to 26.6% of samples were positive for coliforms in a given year (Martin et al., 2012). In a study of coliform contaminants found in HTST-pasteurized milk from 21 processors in the northeastern United States, Masiello et al. (2016) found that *Enterobacter* was the most prevalent coliform, compromising 42% of isolates collected. *Hafnia*, *Citrobacter*, *Serratia*, *Raoultella*, *Buttiauxella*, *Cedecea*, *Kluyvera*, *Leclercia*, *Pantoea*, and *Rahnella* were also found. Another group found *Enterobacter*, *Klebsiella*, and *Citrobacter* to be the predominant coliform genera in fluid milk and other dairy products in South Africa (Wessels et al., 1989).

In addition to *Pseudomonas*, other non-coliform gram-negative bacteria linked to fluid milk spoilage are known to contaminate HTST fluid milk, including *Aeromonas*, *Flavobacterium*, *Alcaligenes*, *Acinetobacter*, and others (Sørhaug and Stepaniak, 1997). Like *Pseudomonas* and coliforms, this group of bacteria has been shown to include several species and strains that can grow at low temperatures and produce a variety of enzymes that lead to fluid milk degradation (Michener and Elliott, 1964). Non-coliform *Enterobacteriaceae* are

Table 1. Key groups of spoilage bacteria known to contaminate pasteurized fluid milk and their characteristics

Group	Examples of genera/species	Overview of key phenotypic characteristics relevant to fluid milk spoilage	Importance in PPC ¹
<i>Pseudomonas</i>	<i>P. fluorescens</i> <i>P. fragi</i> <i>P. lundensis</i> <i>P. putida</i>	Many strains can grow at 4°C and below; many strains can produce extracellular enzymes (e.g., proteases and lipases); some strains produce pigmented siderophores (e.g., pyoverdine)	Primary cause of PPC fluid milk
Coliforms	<i>Enterobacter</i> <i>Klebsiella</i> <i>Citrobacter</i> <i>Hafnia</i> <i>Serratia</i>	Many strains can grow at 4°C and below; many strains can produce extracellular enzymes (e.g., proteases and lipases)	Indicator used in US dairy industry; PMO does not allow for coliforms levels >10 cfu/mL
Non-coliform, non- <i>Pseudomonas</i> gram-negatives	<i>Aeromonas</i> ² <i>Flavobacterium</i> <i>Acinetobacter</i> Non-coliform	Many strains can grow at 4°C and below; many strains can produce extracellular enzymes (e.g., proteases and lipases)	Includes indicator organisms (i.e., EB) used in European dairy testing
Gram-positive sporeformers	<i>Enterobacteriaceae</i> (EB) <i>Bacillus cereus</i> group members <i>Paenibacillus</i>	Some <i>B. cereus</i> group lineages (e.g., <i>B. weihenstephanensis</i>) and several <i>Paenibacillus</i> strains can grow at temperatures as low as at least 6°C; some strains produce extracellular enzymes (e.g., proteases and lipases)	Difficult to differentiate between contamination resulting from spores in raw milk and PPC

¹PPC = post-pasteurization contamination; PMO = Pasteurized Milk Ordinance.

²*Aeromonas* strains have been shown to ferment both lactose and glucose, thereby testing positive on coliform and EB media.

an important group of bacteria in this category and include organisms such as *Proteus* (Hervert et al., 2016), which are less frequent contaminants in pasteurized fluid milk. Also in this group are bacteria belonging to genera that include strains that ferment lactose (coliforms) and those that do not (non-coliform). A recent study that surveyed growth of *Enterobacteriaceae* (EB) and coliforms isolated from dairy products on EB and Coliform Petrifilm (3M, Minneapolis, MN) showed that even some strains from genera well known as coliforms do not ferment lactose and therefore do not meet the criteria to be coliforms (Hervert et al., 2016). For example, the authors reported that, of 10 *Rahnella* isolates tested, only 6 were able to ferment lactose (Hervert et al., 2016). *Rahnella* has been implicated in a smoky/phenolic defect in chocolate milk as a result of guaiacol production (Jensen et al., 2001). These strain variations highlight one reason some groups advocate using EB or total gram-negative testing as more comprehensive indicators of PPC.

Gram-positive bacteria, including aerobic gram-positive sporeformers, are also capable of contaminating milk after pasteurization; however, several gram-positive bacteria also survive pasteurization, either in vegetative (e.g., *Micrococcus*; Gleeson et al., 2013) or spore form (e.g., *Paenibacillus*; Postollec et al., 2012), making it more complicated to determine whether these types of organisms, when found in finished products, originated from raw milk or PPC. A study conducted in Brazil showed that the same subtypes, as determined by ribotyping, of *Bacillus cereus* were found in finished products and on equipment swabs downstream from the pasteurizer (Salustiano et al., 2009). A similar study used randomly amplified polymorphic DNA (RAPD) to assess subtypes of *Bacillus* found in fluid milk along various points in 2 processing facilities in Sweden and in corresponding sealed consumer packages. The authors found that some RAPD types were found in consumer packages and in samples after the pasteurizer, but not in samples taken before the pasteurizer, concluding that these types were evidence of PPC (Eneroth et al., 2001). Although these studies do not necessarily prove that *Bacillus* were introduced by PPC in these cases, they do raise the possibility of PPC with *Bacillus* and other sporeformers and point to need for additional studies on occurrence and importance of PPC with spore-forming bacteria that cause fluid milk spoilage. If PPC with aerobic gram-positive sporeformers is suspected, the subtyping tools used by these authors (i.e., ribotyping and RAPD), and others discussed below, may be useful in determining whether the contamination occurred at the farm or processing plant.

PPC HAS A SIGNIFICANT EFFECT ON BACTERIAL LEVELS AND SENSORIAL PROPERTIES OF FLUID MILK

It has been extensively reported that fluid milk with reduced shelf life (i.e., <10–14d) is virtually always characterized by presence and growth of microorganisms introduced by PPC (Schröder et al., 1982; Griffiths et al., 1988; Ranieri and Boor, 2009; Martin et al., 2012). In these studies, shelf life was defined either microbiologically (number of days under refrigerated storage to reach the PMO limit of 20,000 cfu/mL) or by milk defect judging or sensory evaluation. In the absence of PPC, the limiting biological agents in fluid milk are aerobic psychrotolerant spore-forming bacteria that originate in the farm environment, enter the fluid milk continuum on the farm, survive pasteurization in spore form, and then grow at refrigeration temperatures (Huck et al., 2008). Fluid milk reaching spoilage levels due to aerobic spore-forming bacteria typically have shelf lives of greater than 14 d (Ranieri and Boor, 2009), in contrast to those with PPC, which routinely reach spoilage levels after 7 to 10 d of refrigerated storage at around 6°C (Ranieri and Boor, 2009). Martin et al. (2012), in a survey of fluid milk over a 10-yr period in New York State, indicated that samples with PPC, specifically with coliform bacteria, showed significantly higher total bacteria counts at 14 d after processing than samples with no coliform contamination. Another study showed that many psychrotolerant coliform strains are capable of growing more than 5 log in refrigerated fluid milk at 6°C over 10 d (Masiello et al., 2016). Others have shown that, even at refrigeration temperatures of 4°C and at temperatures below 0°C, *Pseudomonas* and other psychrotolerant post-pasteurization contaminants are capable of growing in and spoiling (i.e., producing degradative enzymes) pasteurized fluid milk (Michener and Elliott, 1964; Sørhaug and Stepaniak, 1997). Although some spoilage microorganisms can grow in milk even at temperatures of 4°C and below, storage temperature is a known factor affecting growth rates of *Pseudomonas* and other gram-negative contaminants (e.g., coliforms) in pasteurized fluid milk (Schröder et al., 1982). Temperature may also play a role in the relative populations of psychrotolerant contaminants in fluid milk over shelf life (Schröder et al., 1982). For example, Schröder et al. (1982) found that the predominant psychrotolerant organisms present in pasteurized fluid milk from 4 processors in the UK after storage at 5°C were gram-negative rods, whereas after storage at 11°C, the predominant organisms detected were psychrotolerant sporeformers. However, another study found that when PPC was present, there were no

major differences in the populations found in pasteurized milk held at 6°C or at 10°C (Griffiths et al., 1988). More research is needed to better understand the effect of storage temperature on specific population changes that occur in fluid milk over its shelf life.

In addition to growth to high numbers during storage, several PPC organisms (e.g., *Pseudomonas*) also produce a variety of enzymes that lead to sensorial defects in fluid milk. Production of proteases and lipases that break down milk components have been described in *Pseudomonas* (Corrêa et al., 2011), a variety of coliform bacteria (Masiello et al., 2016), and in psychrotolerant spore-forming bacteria (Trmčić et al., 2015). For example, Dogan and Boor (2003) reported that of 338 *Pseudomonas* isolates, representing 42 unique ribotypes collected from processed milk, raw milk, and dairy plant environments, 51% were protease positive, 47% were lecithinase positive, and 67% were lipase positive. They further report that enzyme production appeared to be strain dependent, with the majority (69%) of *P. fluorescens* being positive for all 3 enzymes and 87.5% of *P. putida* being negative for all 3 enzymes. Another group assessed proteolytic and lipolytic activity of 37 *P. fluorescens* isolates from pasteurized milk and reported that all isolates were positive for protease and lipase activity (Rajmohan et al., 2002). Species and strain variations are consistent with other studies that report that different *Pseudomonas* species produce different sensory defects in skim and whole milk (Hayes et al., 2002). Specifically, Hayes et al. (2002) found that *P. putida* produced fruity fermented odors, whereas *P. fluorescens* did not. Reports indicate that most enzyme production by *Pseudomonas* occurs when bacterial concentrations reach $\sim 10^6$ cfu/mL or higher. However, some strains are known to produce these enzymes at much lower concentrations (i.e., 10^4 cfu/mL; Law, 1979; Schröder et al., 1982; Sørhaug and Stepaniak, 1997). Psychrotolerant coliforms have also been reported to vary in their ability to produce lipolytic and proteolytic enzymes. Masiello et al. (2016) reported that of 10 *Buttiauxella* isolates collected from pasteurized fluid milk, none were positive for lipolysis, whereas all *Serratia* isolates ($n = 17$) from the same study were positive for lipolysis. Similar to the variation seen between different *Pseudomonas* species, strains within the same genera of psychrotolerant coliforms also show varying levels of enzyme production. For example, among the 17 *Serratia* isolates characterized by Masiello et al. (2016), 4 were negative for proteolytic activity, 6 had moderate activity, and 7 were highly proteolytic. Wessels et al. (1989) observed a similar variation in capacity to produce proteolytic and lipolytic enzymes in strains of coliforms isolated from various dairy products in

South Africa. Specifically, they found that some strains of *Enterobacter* and *Klebsiella* were proteolytic at 7°C, whereas some strains of *Enterobacter*, *Klebsiella*, and *Serratia* showed lipolytic activity at 30°C (Wessels et al., 1989).

The flavor and odor defects resulting from the production of extracellular enzymes are varied. Hayes and colleagues (2002) investigated the odor defects produced by 6 strains of *Pseudomonas* (2 strains each of *P. fluorescens*, *P. fragi*, and *P. putida*) and found that odor defects such as fruity, barny, rotten, cheesy, and others were produced and differed by strain, milk fat level (e.g., skim or whole), and time of storage. The accumulation of small peptides resulting from bacterial proteolysis have also been reported to cause bitterness (Ma et al., 2000; Clark et al., 2009) and astringency (Harwalkar et al., 1989). Lipolytic activity, causing the release of free fatty acids, may cause rancidity (Shipe et al., 1978) and unclean and soapy flavors (Dogan and Boor, 2003), all of which are common defects in fluid milk contaminated after pasteurization with psychrotolerant gram-negative bacteria.

In addition to flavor and odor defects generated by PPC organisms in fluid milk, some cause severe body defects as well. A major defect associated with the growth of organisms introduced after pasteurization is coagulation, which can occur via 2 major pathways. The first pathway is via production of acid as a byproduct that destabilizes the protein matrix. Many common PPC organisms, including some strains of *Pseudomonas* and many psychrotolerant coliforms produce acid and thereby coagulate milk (Komagata, 1961). Another cause of coagulation is via proteolytic activity and it is commonly associated with psychrotolerant gram-negative bacteria such as *Pseudomonas* (Nörnberg et al., 2010). This defect, typically called “sweet-curdling” because it occurs in the absence of acidification, is also caused by some gram-positive spore-forming bacteria (Collins, 1981). Another body defect associated with PPC is “ropiness,” which results from the production of exopolysaccharides that cause the product to develop a slimy consistency. This defect is caused by several organisms, including *Klebsiella* and other common post-pasteurization contaminants (Cheung and Westhoff, 1983). Finally, certain PPC organisms are known to produce pigments that cause color defects in fluid milk (Palleroni, 1984). For example, Evanowski et al. (2017) described a gray pigment defect in conventionally pasteurized fluid milk that was contaminated with *Pseudomonas azotoformans*. This organism is closely related to *Pseudomonas fluorescens*, which has been implicated in several color-related defects in dairy products (Martin et al., 2011a; Nogarol et al., 2013).

PROCESSORS COMMITTED TO REDUCING PPC WILL USE TOTAL GRAM-NEGATIVE TESTING

In the United States and many countries, indicator organisms are used to determine the hygienic quality of pasteurized milk. Current US standards require total plate counts of less than 20,000 cfu/mL and coliforms no greater than 10 cfu/mL in grade 'A' pasteurized fluid milk (FDA, 2015). In Europe, *Enterobacteriaceae* are the primary indicators used for pasteurized milk and milk products (European Communities Regulation, 2010). Importantly, total plate counts do not provide an indication of PPC because high counts with this method could be due to bacteria surviving HTST (as detailed above) or to PPC. Methods approved for coliform enumeration in grade 'A' pasteurized fluid milk include (1) coliform plate count on violet red bile agar (VRBA); (2) Petrifilm Coliform Count or High Sensitivity Coliform Count (3M, St. Paul, MN); (3) TEMPO CC-Coliform Count (BioMerieux, St. Louis, MO); and (4) Peel Plate *E. coli* and Coliform and/or Peel Plate *E. coli* and Coliform High Volume Sensitivity (Charm Sciences Inc., Lawrence, MA; FDA, 2015). These methods, although approved for coliforms, are not able to detect all PPC because they do not detect lactose non-fermenters (e.g., *Pseudomonas*), which are known to compromise the bulk of PPC. Van Tassell et al. (2012) specifically demonstrated that VRBA, Petrifilm Coliform Count plates, and MacConkey agar were ineffective at recovering a panel of 12 dairy-associated *Pseudomonas* isolates. However, pour plating with crystal violet tetrazolium agar (CVTA) showed the highest detection efficiency for the presence of PPC (determined by end of shelf life testing for gram-negative bacteria) compared with a nonselective standard plate count agar ($R^2 = 0.95$). Another recent study has shown that plating pasteurized fluid milk on CVTA following an enrichment step (21°C/18 h) resulted in significantly higher detection of PPC than other methods (e.g., plating on coliform medium following the same enrichment protocol; Alles et al., 2016). The primary driver of the increased sensitivity for CVTA-based methods is the ability of CVTA to detect total gram-negative bacteria including *Pseudomonas*, which represented ~50% of the isolates identified in milk with PPC by the study cited above (Alles et al., 2016), as well as traditional indicators (i.e., coliforms).

The use of CVTA for detection of total gram-negative bacteria is outlined in the *Standard Methods for the Examination of Dairy Products* (Frank et al., 1992). Crystal violet has been shown to inhibit gram-positive bacteria while not significantly suppressing gram-negative bacteria (Smith and Witter, 1979) and has been used for detecting PPC since the 1960s (Thomas,

1969). Despite the half century since this method was first used in the dairy industry, very little additional methodological development, in particular in the area of rapid and automated methods, has occurred for detecting total gram-negative bacteria in fluid milk. This is in stark contrast to the numerous methods developed and widely used for detecting total viable organisms and coliforms in fluid milk, including dehydrated film media (Ginn et al., 1986), flow cytometry (Loss et al., 2012), and optical-based detection methods (Firstenberg-Eden et al., 2002). The limited availability of rapid and automated methods for detection and enumeration of total gram-negative bacteria in fluid milk is a major barrier to the dairy industry's ability to quickly identify and resolve contamination events and ultimately deliver the highest quality product to consumers. Further research and development at both the academic and diagnostic industry level is needed to fill this gap.

FILLERS ARE A MAJOR SOURCE OF PPC BUT TRACKING PPC SOURCES REQUIRES DISCRIMINATORY MOLECULAR SUBTYPING METHODS

Many factors contribute to the occurrence of PPC in fluid milk, including problems with hygienic design of equipment, cleaning and sanitization procedures, preventative maintenance, control of plant air, and prevention of cross contamination. To identify and resolve PPC events, processors must perform root-cause analysis that includes establishing whether the contamination is persistent or transient in nature, as this will inform the necessary steps to resolve the contamination. Persistent contamination occurs when an organism is introduced into, and continues to live in, the facility or equipment over time without being removed by cleaning and sanitation. A common vehicle of persistent contamination is biofilms, which are communities of bacteria that attach to processing equipment and are resistant to cleaning and sanitation, leading to continued contamination of the product over time (Marchand et al., 2012). Many organisms have been found to inhabit biofilms in dairy processing facilities, including gram-positive (e.g., *Bacillus*) and gram-negative (e.g., *Escherichia coli*) bacteria (Salustiano et al., 2009; Shi and Zhu, 2009; Simões et al., 2010; Cherif-Antar et al., 2016). Biofilms are likely to occur when cleaning and sanitation and preventative maintenance programs are ineffectively designed or implemented. This may be in the form of dead ends in equipment, incorrect concentrations of cleaning and sanitizing chemicals, and cracked or pitted rubber filler components. Even correctly used clean-in-place systems may allow development of biofilms in dairy processing equipment that

cannot be subsequently removed (Simões et al., 2010). However, persistent bacterial communities may not necessarily have to represent biofilms; sanitary design issues with equipment and facilities may provide niches where bacteria are protected from sanitizer and survive in “non-biofilm communities.” For example, it is conceivable that pipe dead ends may contain planktonic bacterial communities or sessile bacteria without the extracellular matrix that is typical of biofilms.

Filling equipment has been identified by several studies as a primary source of persistent PPC in fluid milk. For example, Eneroth et al. (1998) took samples of HTST fluid milk at various sites along the processing continuum (e.g., silo tank, immediately preceding and following the pasteurizer, buffer tank, filler and consumer package) and found that the majority of PPC was occurring at the filling step. Similarly, another study used molecular subtyping tools, specifically ribotyping, to track the source of *Pseudomonas* PPC of HTST pasteurized fluid milk to filler nozzles, which had cracks and other evidence of deterioration upon manual compression that were not evident in the nozzles during cleaning and sanitation (Ralyea et al., 1998). A study conducted in the UK showed that although instances of PPC originated from milk storage tanks, the majority of PPC originated at the filling step and occurred at low levels (1–50 psychrotolerant gram-negative bacteria per 100 mL; Schröder, 1984). Gruetzmacher and Bradley (1999) also found that the filling equipment was a major source of PPC. Those authors sampled milk just before it flowed through the filling machine head and immediately after, finding that the milk that had not passed through the filling equipment had a 20-d-longer shelf life at 7°C (Gruetzmacher and Bradley, 1999).

Transient contamination occurs when an organism that is present in the equipment or facility is introduced onto food contact surfaces or directly into the product but is subsequently removed with effective cleaning and sanitation. Primary modes of transient contamination are through worker contact, especially when proper handwashing frequency and technique are not adhered to (Montville et al., 2002), and via biological aerosols (Kang and Frank, 1989). Aerosols are suspensions of microscopic solid or liquid particles in air or gas (Kang and Frank, 1989); when they carry bacteria, fungi, or other microorganisms, they are considered biological aerosols. In the case of biological aerosols, contamination that may be present in non-food contact areas (e.g., floors or drains) is aerosolized by a variety of mechanisms, including hose use during production (Kang and Frank, 1989). One study found that hose use, drains, and personnel activity were all associated with an increase in total aerobes and staphylococci found in dairy processing facility air (Ren and Frank,

1992). In addition to hose use causing potential PPC through aerosolization, direct use of water on fillers in dairy plants has been found to be associated with higher levels of PPC (Eneroth et al., 2000a).

Determining the source, type (i.e., persistent vs. transient), and causative agents of PPC cannot be achieved with traditional microbiological methods alone. Molecular subtyping tools, which have been used in source tracking in foodborne disease outbreaks for decades (Sabat et al., 2013), provide a sensitive tool for the dairy industry to track and resolve PPC. Previously used subtyping techniques for tracking PPC include pulsed field gel electrophoresis (PFGE; Martin et al., 2011a), ribotyping (Martin et al., 2011a), multilocus sequence typing (MLST; Andreani et al., 2014), RAPD (Eneroth et al., 2000a), and DNA-based sequencing techniques (e.g., *rpoB* allelic typing; Huck et al., 2007). For example, Martin et al. (2011a) used DNA sequencing, ribotyping, and PFGE as molecular subtyping tools to track PPC causing a blue discoloration in a fresh, low-acid cheese product and identified environmental sources that were responsible for contamination of the finished products. Ultimately, PFGE was found to be sufficiently discriminatory to distinguish between *P. fluorescens* strains capable of causing the defect and those that did not, as well as to determine that the source of the organism was an agitator track above a cheese vat. Likewise, RAPD was used to identify the primary sources of gram-negative contamination in 3 dairies in Sweden (Eneroth et al., 2000b). Those authors found that the same persistent RAPD types (most of which were identified as *Pseudomonas*) were found in condensed water on the filling nozzles, in wastewater at the bottom of the filling machine, and in the air surrounding the filling machine as were found in pasteurized packaged milk (Eneroth et al., 2000b).

Finally, in the case of suspected PPC with organisms that can both originate in raw milk and potentially contaminate product after pasteurization (i.e., gram-positive sporeformers such as *Paenibacillus*), these highly sensitive molecular subtyping tools are necessary to distinguish the source of the contamination. A 2007 study (Huck et al., 2007) demonstrated that gram-positive spore-forming bacteria can be tracked from the farm throughout a HTST fluid milk processing facility, and that certain *rpoB* allelic types appear to be introduced at various points throughout the process. However, although single-gene methods (such as *rpoB* allelic typing) provide for good characterization and identification, these methods typically have limited discriminatory power and hence may not always be the best source-tracking tool. Interestingly, another study showed that unique RAPD subtypes of gram-positive sporeformers (*Bacillus cereus* s.l. and *Paenibacillus*

odorifer) were detected over time in extended-shelf-life fluid milk products processed in Germany but were never isolated from raw bulk tank milk (Doll et al., 2017). Although this finding may suggest that contamination with these organisms represented PPC, it could also be due to difficulties detecting low levels of these organisms in raw milk. These studies highlight the need for sensitive and discriminatory subtyping tools to better identify and characterize PPC sources and transmission of both gram-positive and gram-negative spoilage organisms. Although application of emerging whole-genome sequencing methods will likely provide valuable tools for these purposes, the importance of well-designed sampling plans and schemes cannot be underemphasized.

CONCLUSIONS

Although PPC remains an important cause of fluid milk spoilage, some processing facilities have been highly successful at minimizing PPC (Martin et al., 2012), indicating that effective control is possible, even though contamination of a milk container with a single organism that can grow at refrigeration temperatures is sufficient to cause product spoilage over shelf life. Efforts to develop and deploy more effective tools to detect PPC, trace it to a source, and ultimately prevent PPC are, however, essential in improving the quality and shelf life of HTST-pasteurized fluid milk. Specific areas of need include (1) development of better methods for detection and trace-back of PPC; (2) validation and implementation of improved procedures to prevent PPC (e.g., sanitation standard operating procedures, procedures for mid-shift clean-up); and (3) sanitary equipment design. Lessons learned from the control of environmentally transmitted foodborne pathogens (primarily *Listeria monocytogenes*) might be translatable to improved control of PPC. For example, the use of “seek and destroy” type approaches developed for *Listeria* control (Malley et al., 2015) could be modified to identify and eliminate environmental sources of PPC. Similarly, data indicating that mid-shift clean-up actually increases the risk of *Listeria* contamination could be applied to PPC and spur further studies on the effectiveness of different in process cleaning strategies that may currently be used in HTST plants.

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