J. Dairy Sci. 99:1–10
http://dx.doi.org/10.3168/jds.2016-11074

Evaluation of different methods to detect microbial hygiene indicators relevant in the dairy industry

C. J. Hervert, A. S. Alles, N. H. Martin, K. J. Boor, and M. Wiedmann[1]
Milk Quality Improvement Program, Department of Food Science, Cornell University, Ithaca, NY 14853

ABSTRACT

It is estimated that 19% of the total food loss from retail, food service, and households comes from dairy products. A portion of this loss may be attributed to premature spoilage of products due to lapses in sanitation and postpasteurization contamination at the processing level. Bacterial groups including coliforms, *Enterobacteriaceae* (EB), and total gram-negative organisms represent indicators of poor sanitation or postpasteurization contamination in dairy products worldwide. Although Petrifilms (3M, St. Paul, MN) and traditional selective media are commonly used for the testing of these indicator organism groups throughout the US dairy industry, new rapid methods are also being developed. This project was designed to evaluate the ability of different methods to detect coliforms, EB, and other gram-negative organisms isolated from various dairy products and dairy processing environments. Using the Food Microbe Tracker database, a collection of 211 coliform, EB, and gram-negative bacterial isolates representing 25 genera associated with dairy products was assembled for this study. We tested the selected isolates in pure culture (at levels of approximately 15 to 300 cells/test) to evaluate the ability of 3M Coliform Petrifilm to detect coliforms, 3M Enterobacteriaceae Petrifilm, violet red bile glucose agar, and an alternative flow cytometry-based method (bioMérieux D-Count, Marcy-l'Étoile, France) to detect EB, and crystal violet tetrazolium agar to detect total gram-negative organisms. Of the 211 gram-negative isolates tested, 82% (174/211) had characteristic growth on crystal violet tetrazolium agar. Within this set of 211 gram-negative organisms, 175 isolates representing 19 EB genera were screened for detection using EB selective/differential testing methods. We observed positive results for 96% (168/175), 90% (158/175), and 86% (151/175) of EB isolates when tested on EB Petrifilm, violet red bile glucose agar, and D-Count, respectively; optimization of the cut-off thresholds for the D-Count may further improve its sensitivity and specificity, but will require additional data and may vary in food matrices. Additionally, 74% (129/175) of the EB isolates tested positive as coliforms. The data obtained from this study identify differences in detection between 5 microbial hygiene indicator tests and highlight the benefits of EB and total gram-negative testing methods.
Key words: coliform, *Enterobacteriaceae*, gram-negative, indicator organism

INTRODUCTION

Since 1914, the United States has used coliform organisms to indicate the microbiological quality and safety of drinking water (US Treasury Department, 1914). Over the course of the next 100 yr, the use of coliforms as indicator organisms expanded, becoming the standard hygienic quality test for many food and beverage products. The dairy industry has long since used coliforms for this purpose as they are represented in over 20 genera of gram-negative, non-sporeforming rods, which lack the capability to survive typical milk heat treatments (e.g., HTST pasteurization) and can hence act as indicators of postpasteurization contamination (Imhoff, 2005; Masiello et al., 2016). The phenotypic characteristic that defines coliform bacteria is their ability to ferment lactose, resulting in gas and acid production within 48 h at 35°C (Feng et al., 2002). It is this property that distinguishes coliform organisms from other lactose nonfermenters (e.g., *Pseudomonas* sp.) when plated on selective and differential coliform media. Strict FDA requirements regarding coliform and total bacterial limits have been put in place to ensure minimum standards are met for the hygienic quality of dairy foods. These standards are outlined in the 2011 Pasteurized Milk Ordinance and require coliform counts in grade A pasteurized milk to not exceed 10 cfu/mL (FDA, 2011). In addition to coliforms being indicative of the hygienic status of dairy products and processing environments, they have been shown to have implications on the sensory quality of dairy products. Past

Received February 22, 2016.
Accepted May 29, 2016.
[1]Corresponding author: mw16@cornell.edu

studies demonstrate that select strains from common coliform genera grow at refrigeration temperatures and exhibit proteolytic and lipolytic capabilities (Wessels et al., 1989; Masiello et al., 2016). The production of proteolytic and lipolytic enzymes may have an influence on the consumer acceptance of dairy products, as pasteurized milk samples contaminated with coliforms are associated with significant decreases in sensory scores on d 14 of shelf life when compared with uncontaminated samples (Martin et al., 2012). These instances of postpasteurization contamination with spoilage microorganisms may contribute to the dairy product food loss observed at the retail, food service, and household levels (Gunders, 2012).

Despite the longstanding use of coliforms as hygiene indicators in the US dairy industry, recent work indicates that coliforms represent less than 50% of the bacterial contaminants involved in postpasteurization contamination of fluid milk (Ranieri and Boor, 2009). An alternative group of indicators used widely across Europe are organisms within the taxonomic family *Enterobacteriaceae* (**EB**; European Communities Regulation, 2010). This group of organisms is composed of gram-negative, heat-labile, glucose fermenters and represents a broad range of dairy-related genera with the potential to indicate postpasteurization contamination. With the notable exception of specific strains of lactose-fermenting *Aeromonas* (Abbott et al., 2003), the EB group also encompasses classic coliform genera (Imhoff, 2005). Typical media for the enumeration of EB include violet red bile glucose agar (**VRBGA**) and EB Petrifilm, though new methods for EB detection are also being developed.

Although the EB group provides a more encompassing range of hygiene indicators when compared with coliforms, several the gram-negative, postpasteurization contaminants found in fluid milk (e.g., *Pseudomonas*) do not fall into this group. Prior studies indicate that *Pseudomonas* spp. are dominant among gram-negative organisms isolated from pasteurized milk (Ranieri and Boor, 2009) and generate unsatisfactory sensory defects through the production of proteases and lipases (Sørhaug and Stepaniak, 1997; Hayes et al., 2002). Subsequent to postpasteurization contamination, the growth of *Pseudomonas* and other non-EB gram-negative organisms at refrigeration temperatures has been shown to be indicative of the shelf life and overall consumer acceptance of milk (Dogan and Boor, 2003). Additionally, a recent study highlighted the unique spoilage potential of certain biovars of pigment-producing *Pseudomonas* isolated from fresh, low-acid cheese (Martin et al., 2011). It is for this reason that the "blanket-like" approach of screening for total gram-negative organisms offers a more comprehensive indicator of postpasteurization contamination, sanitation quality, and dairy shelf life when compared with other indicator organism groups. Crystal violet tetrazolium agar (**CVTA**) is the standard method for enumerating gram-negative organisms including *Pseudomonas* in dairy products (Frank and Yousef, 2004), while inhibiting gram-positive growth through the inclusion of crystal violet.

The objective of this study was to screen a diverse collection of dairy-relevant coliform, EB, and general gram-negative organisms for detection on Coliform Petrifilm, EB Petrifilm, VRBGA, CVTA, as well as by an alternative flow cytometry-based method. The resulting data provide new information on potential use of these indicator organism groups in the dairy industry and identify optimal detection methods for different indicator organism groups and gram-negative genera.

MATERIALS AND METHODS

Isolate Selection

Through utilization of the Food Microbe Tracker database (www.foodmicrobetracker.com; Vangay et al., 2013), a collection of 211 gram-negative bacterial isolates representing a broad range of organisms commonly associated with dairy products and processing environments was assembled for the purpose of this study. Isolation sources included pasteurized milk (117/211), dairy processing plant environment/dairy food product (42/211), raw milk (16/211), cheese (11/211), environment/food (7/211), unspecified (6/211), infant formula (6/211), laboratory heat-treated raw milk (3/211), pasteurized chocolate milk (2/211), and clinical (1/211; Supplemental Table S1; http://dx.doi.org/10.3168/jds.2016-11074). Within the collection, 175 isolates from 19 genera were classified as falling into the EB family, whereas 36 isolates from 6 genera were classified as non-EB, gram-negative. Genus identification information for isolates was obtained through the Food Microbe Tracker database based on previously performed partial 16S DNA sequencing, as described in prior studies (Huck et al., 2007). Additionally, 50% (106/211) of the isolates were previously described in one or more studies (Marie Yeung et al., 2003; Ranieri and Boor, 2009; Martin et al., 2011; Van Tassell et al., 2012; Ivy et al., 2013; Masiello et al., 2016).

Enumeration, Preparation, and Testing of Pure Cultures

Prior to undergoing selective and differential testing, the selected isolates were first streaked from frozen