THE USE OF COLIFORMS, ENTEROBACTERIACEAE, AND GRAM-NEGATIVE ORGANISMS AS MICROBIAL HYGIENE INDICATORS IN THE DAIRY INDUSTRY

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by

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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 post-pasteurization contamination at the processing level. Bacterial groups including coliforms, *Enterobacteriaceae* (EB), and total Gram-negative organisms represent indicators of poor sanitation or post-pasteurization contamination in dairy products world-wide. While Petrifilms and traditional selective media are commonly used for the testing of these indicator organism groups throughout the U.S. dairy industry, new rapid methods are also being developed. The research presented in here was designed to evaluate the ability of different methods to detect dairy relevant coliforms, EB, and other Gram-negatives organisms in pure culture. 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 (i) 3M Coliform Petrifilm to detect coliforms, (ii) 3M *Enterobacteriaceae* Petrifilm, Violet Red Bile Glucose Agar, and the D-Count to detect EB, and (iii) Crystal Violet Tetrazolium Agar (CVTA) to detect total Gram-negative bacteria. Of the 211 Gram-negative isolates tested, 82% (174/211) had characteristic growth on CVTA. 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 the D-Count, respectively. Additionally, 74% (129/175) of the EB isolates tested positive as coliforms. The data obtained from this study identifies differences in detection between 5 microbial hygiene indicator tests and highlights the benefits of EB and total Gram-negative testing methods.

Limited information is available on the ability of coliform, EB, and non-EB Gram-negative organisms to (i) survive in low pH fermented dairy products, such as yogurt, and (ii) represent suitable microbial hygiene indicators. In order to identify suitable hygiene indicator groups and optimal detection methods for use in fermented dairy products, we screened 64 bacterial isolates of 24 dairy-relevant genera for survival and detection in Greek yogurt using 5 different testing methods. Prior to testing, isolates were inoculated into plain, 0% fat Greek yogurt (pH 4.35 to 4.65), followed by a 12 h hold period at 4 ± 1°C. Yogurts were subsequently tested using the 5 method evaluated in our pure culture study. Overall, the non-EB Gram-negative isolates showed significantly larger log reductions at 12 h after inoculation into Greek yogurt (based on bacterial numbers recovered on CVTA) as compared to the coliform and non-coliform EB isolates tested. The methods evaluated vary in their ability to detect different microbial hygiene indicators in Greek yogurt. Crystal Violet Tetrazolium Agar detected the highest portion of coliforms, while EB Petrifilm detected the highest portion of EB, as well as highest portion of total Gram-negative organisms. Additionally, the D-Count method allowed for a more rapid detection of EB in yogurt by generating results in approximately 13 h rather than the 24 h when using EB Petrifilm and Violet Red Bile Glucose Agar. Results from this study indicate

that the coliform and EB groups encompass the broadest range of dairy-relevant Gram-negative organisms with the capability to survive in Greek yogurt, thus validating their use as microbial hygiene indicator groups in low pH fermented dairy products.

BIOGRAPHICAL SKETCH

Clinton Hervert was born in Vail, Colorado on September 21, 1991 to his parents Jim and Susie Hervert. Clinton lived in the Vail Valley throughout his childhood where he attended Eagle County Charter Academy (Elementary and Middle School) and Battle Mountain High School. At Battle Mountain, Clinton graduated in the top 10% of his high school class, received the distinction of AP Scholar with Honor, was a 4 year member of the men's varsity soccer team, and received state and national recognition as the Colorado ProStart Student of the Year. Following graduation from high school in 2010, he went on to attend Cornell University with a major in Food Science and a minor in Nutrition. He obtained his degree of Bachelor of Science in May of 2014 and was accepted into a Masters program in the field of Food Science and Technology.

Clinton joined the Milk Quality Improvement Program Microbiology Laboratory during his freshman year at Cornell. Prior to his thesis research, Clinton worked alongside Rachel Miller to characterize the distribution of toxin genes amongst *Bacillus cereus* group isolates.

I dedicate this thesis to my parents Jim and Susie Hervert.

Thank you for the never-ending love, support, and values that you have poured into me. After over 20 years of schooling, you would be proud to know that I can now use a glue stick. I, however, still cannot see colors.

I love you both with all my heart.

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I would like to extend a special thanks to Nicole Martin. It is difficult for me to think of someone that has had such a large influence on my life and career path. I have always looked to you as role model, mentor, and someone that I can always share a laugh with. Thanks you for first hiring me to work as an undergraduate in the MQIP lab and taking me under your wing every year since. Not only have I learned a great deal from you about dairy microbiology, but you have also taught me the proper way to manage a team. I am happy to call you my friend.

A huge thanks to Sandy Alles for his friendship and assistance in completing my project. I am leaving Ithaca with many fond memories together and I look forward to having more of them in the future.

I would like to express my sincerest gratitude to all members of the MQIP and FSL laboratories. You have all served as my family over the past 6 years and I will always cherish the time that I have spent in your company. A special thanks to Rachel Miller for your mentorship and advice as an undergrad. You not only helped foster my love of science, but your advice was a major factor in my decision to stay for graduate school. I would also like to specifically thank Nancy, Maureen, Al, Dave, Sarah, Rachel E., Sam, Jeff, Jasna, and Miquela for your friendship and assistance in helping me complete my research. It would not have been possible without you all!

I would also like to thank the individuals at Chobani Inc. who generously funded my research and bioMérieux for their technical assistance throughout my graduate studies.

Lastly, I would like to thank my brother Cody, my sisters Haley and Hannah, my parents Jim and Susie, and my girlfriend Caroline. I was extremely lucky to have such a great support group throughout my time at Cornell. From seemingly endless days in the library and long nights in lab, you were all there to keep my motivation and spirits high. I cannot thank you enough for that.

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CHAPTER 1

INTRODUCTION

A majority of the milk and milk products sold in the United States undergoes heat treatment (e.g., high temperature, short time pasteurization) to eliminate pathogenic organisms that may be present in the raw milk (FDA, 2011). While the elimination of pathogens is the primary objective of pasteurization, it also inactivates many heat liable degradative enzymes and eliminates non-pathogenic, Gram-negative organisms present in the raw milk (Sørhaug and Stepaniak 1997; Fromm and Boor, 2004). In doing so, the shelf life of pasteurized milk is greatly extended when kept at refrigeration temperatures (Sørhaug and Stepaniak 1997; Fromm and Boor, 2004).

The Pasteurized Milk Ordinance (PMO) serves as a guideline for US dairy processors to ensure that safety and quality standards are met for grade "A" pasteurized milk and milk products. Specifically, grade "A" dairy products must not exceed 10 coliform organisms per mL of milk (FDA, 2011). While the PMO requires U.S. dairy processors to screen for coliform organisms in their grade "A" pasteurized milk and milk products, other parts of the world utilize alternative microbial hygiene indicator groups to evaluate their finished products and sanitation practices (European Communities Regulation, 2010). Such groups include the *Enterobacteriaceae* (EB) family of organisms, as well total Gram-negative genera, both of which are defined on a taxonomic basis (Figure 1.1). Coliform organisms, however, are defined on a phenotypic basis based on their ability to ferment lactose resulting in gas and acid production within 48 h at 32°C or 35°C (Feng et al., 2002; Davidson et al., 2004). Despite this, specific EB genera (e.g., *Enterobacter* and *Escherichia*) generally possess the ability to ferment lactose and will test coliform positive as a result (Imhoff, 2005; Figure 1.1).

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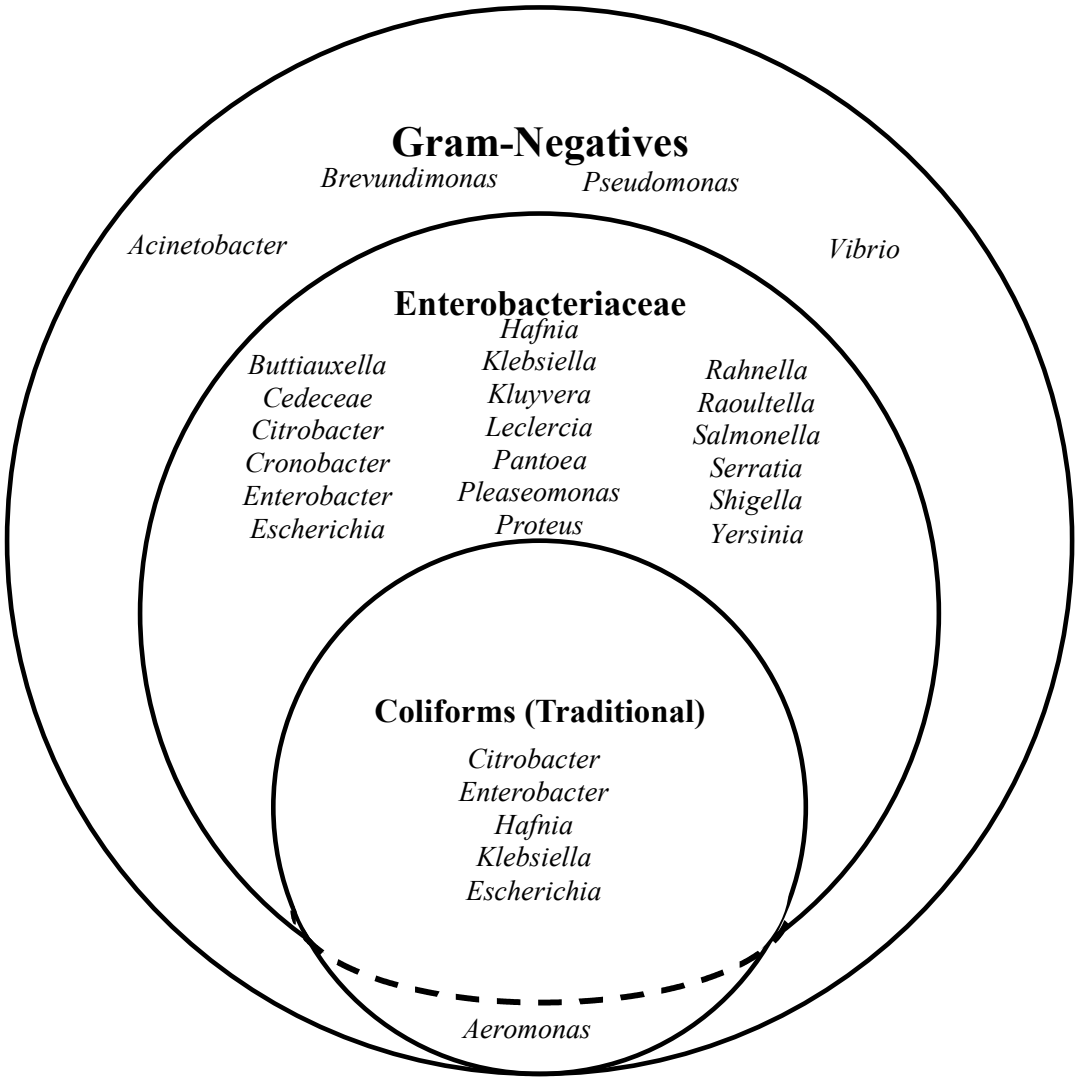

Figure 1.1. Distribution of dairy-associated Gram-negative genera into the coliform, *Enterobacteriaceae* (EB), and total Gram-negative groups. With the notable exception of specific strains of lactose fermenting *Aeromonas*, the EB group also encompasses classic coliform genera. The total Gram-negative group encompasses all EB and coliform genera, as well as a number of genera falling outside of the EB and coliform groups.

Aside from the regulatory requirements set by the PMO, dairy processors use hygiene indicators to verify sanitation practices, detect and correct instances of post-pasteurization contamination, and evaluate the quality of their finished products (Craven and Macauley, 1992; Dogan and Boor, 2003; Masiello et al., 2016). Most commonly, processors utilize coliform tests (e.g., Coliform Petrifilm) for this purpose. However, over 50% of the Gram-negative genera isolated from pasteurized fluid milk fall outside of the coliform group (Ranieri and Boor, 2009). Specifically, *Pseudomonas* is the most commonly isolated Gram-negative genus from pasteurized milk and regularly demonstrates its ability to spoil pasteurized milk at refrigeration temperatures (Sørhaug and Stepaniak, 1997; Hayes et al., 2002; Ranieri and Boor, 2009). Therefore, by solely screening for coliforms, processors limit their ability to identify sanitation failures and correct instances of post-pasteurization contamination. The first aspect of my thesis highlights the advantages of screening for EB and total Gram-negative organisms as microbial hygiene indicators. To do this, I screen 211 dairy-relevant coliform, EB, and non-EB Gram-negative organisms for detection using 4 traditional detection methods and one alternative detection method. In doing so, I also evaluate the ability of the testes to detect their respective target group of organisms.

While fluid milk is the primary source of dairy in American's diets, the consumption of fermented milk products (e.g., Greek yogurt) has risen sharply over recent decades (USDA/ERS, 2015). As a result of its rise in popularity, an increasing amount of the US milk supply goes to the production of yogurt. Similar to fluid milk standards, the PMO requires that yogurt have ≤ 10 coliform organisms per gram of

product (FDA, 2011). However, existing data suggests that the low pH range of yogurt (i.e., 4.2 to 4.6) inhibits the growth of coliform organisms and limits their detection by traditional methods (Goel et al., 1971; Shaker et al., 2008). This presents a unique issue for processors looking to evaluate their finished products and sanitation practices using traditional coliform testing methods. Therefore, data on both the survival of microbial hygiene indicators in Greek yogurt and optimal detection methods would greatly benefit yogurt processors. The second aspect of my thesis evaluates the survival of 64 coliform, EB, and non-EB Gram-negative organisms following a 12 h hold in Greek Yogurt at refrigeration temperatures.

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CHAPTER 2

EVALUATION OF DIFFERENT METHODS TO DETECT MICROBIAL HYGIENE INDICATORS RELEVANT IN THE DAIRY INDUSTRY*

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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 post-pasteurization contamination at the processing level. Bacterial groups including coliforms, *Enterobacteriaceae* (EB), and total Gram-negative organisms represent indicators of poor sanitation or post-pasteurization contamination in dairy products world-wide. While Petrifilms and traditional selective media are commonly used for the testing of these indicator organism groups throughout the U.S. 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-negatives 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) to detect EB, and Crystal Violet Tetrazolium Agar to detect total Gram-negatives. 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 the 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 identifies differences in detection between 5 microbial hygiene indicator tests and highlights the benefits of EB and total Gram-negative testing methods.

INTRODUCTION

Since 1914, the United States has used coliform organisms to indicate the microbiological quality and safety of drinking water (U.S. Treasury Department, 1914). Over the course of the next 100 years, 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 utilized 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., high temperature, short time pasteurization) and can hence act as indicators of post-pasteurization 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 non-fermenters (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 to the sensory quality of dairy products. Past 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 day 14 of shelf life when compared to uncontaminated samples (Martin et al., 2012). These instances of post-pasteurization 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 U.S. dairy industry, recent work indicates that coliforms represent less than 50% of the bacterial contaminants involved in post-pasteurization 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 post-pasteurization 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.

While the EB group provides a more encompassing range of hygiene indicators when compared to coliforms, a number of the Gram-negative, post-pasteurization 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 post-pasteurization contamination, the growth of *Pseudomonas* and other non-EB Gram-negatives 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 post-pasteurization contamination, sanitation quality, and dairy shelf life when compared to 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 provides new information on potential use of these indicator organism groups in the dairy industry and identifies 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 2.1). Within the collection, 175 isolates from 19 genera were classified as falling into the EB family, while 36 isolates from 6 genera were classified as non-EB, Gram-negatives. 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 culture onto Brain Heart Infusion (BHI) agar (Becton Dickinson, Sparks, MD) and incubated for 24 h at 32°C ± 1°C. An isolated colony was selected for each isolate and used to inoculate a tube containing 5 mL of BHI broth (Becton Dickinson, Sparks, MD), followed by 18 h of incubation at 32°C ± 1°C. Incubated broths were diluted with phosphate buffer by a factor of 1,000 before spiral plating onto BHI agar using a automated spiral plater (Advanced Instruments, Inc., Norwood, MA). Following 24 h of incubation at 32°C ± 1°C, colony growth on the plates was enumerated on a Color Q-Count instrument (Advanced Instruments, Inc., Norwood, MA) to obtain count data on the 18 h BHI broth. A new set of BHI broths was then inoculated using the same isolated colonies that had been used for the initial experiments, followed by incubation for 18 h at 32°C ± 1°C. The count data was used to create serial dilutions of the new broths resulting in countable levels when plated on the various media types tested. These media types included Crystal Violet Tetrazolium Agar (Frank and Yousef, 2004), Violet Red Bile Glucose Agar (Becton Dickinson, Sparks, MD), 3M Coliform Petrifilm (3M, St. Paul, MN), 3M *Enterobacteriaceae* Petrifilm (3M, St. Paul, MN), as well as BHI agar to serve as a control media on which all isolates were expected to grow. Dilutions were plated on CVTA, VRBGA, and BHI agar using the non-exponential 100 μL mode of the automated spiral plater

resulting in colony counts of approximately 15 to 300 cfu/plate for most isolates. To obtain comparable cfu counts to those that were obtained using the automated spiral plater, 1 mL of a 10-fold further dilution was plated onto both the Coliform and EB Petrifilms for each respective isolate. Upon completion of plating, all VRBGA plates, BHI plates, Coliform Petrifilms, and EB Petrifilms were incubated aerobically for 24 h at 32°C ± 1°C and CVTA plates were incubated aerobically for 48 h at 21°C (Frank and Yousef, 2004). In addition to plating on traditional detection media, isolates were tested using a flow cytometry method (D-Count; bioMérieux, Marcy-l'Étoile, France) with a protocol designed to detect EB organisms. The "Presence/Absence Test of EB in Fermented Milk Products Containing *Bifidobacterium*" application of the D-count was used to test isolates according to the manufacture's protocols and procedures; this procedure includes an enrichment step, followed by flow cytometry to allow for EB detection in approximately 13 h rather than 24 h for EB Petrifilm and VRBGA. Briefly, for each test isolate, 1 mL of the serial dilution plated on Coliform and EB Petrifilms was inoculated into 9 mL of a proprietary EB selective broth. Inoculated broths were incubated for 13 h at 37°C ± 1°C prior to testing on the flow cytometry instrument. To test, 10 μL of enrichment broth were treated with reagents that label viable EB cells. The sample was then automatically injected into the flow cell analyzer of the D-count forming a narrow and laminar flow stream. Detectors within the analyzer counted the labeled cells, outputting a value in counts/mL of analyzed sample.

Media Interpretation

Following the incubation of plates and Petrifilms, colony growth was enumerated visually for Petrifilms, and with the aid of a Color Q-Count instrument for plates. Differential media types were also examined for typical or atypical growth characteristics indicating a positive or negative result for their respective differential capabilities. A positive result on EB Petrifilm was triggered through acid, gas, or gas and acid production generated from the fermentation of glucose. Per the manufacturer's instructions, these characteristics result in red colonies with yellow zones for acid producing isolates, red colonies with associated gas bubbles for gas producing isolates, and red colonies with yellow zones and associated gas bubbles for acid and gas producing isolates. EB glucose fermenters produce red colonies on VRBGA with red-purple halos (bile precipitation) in the presence of neutral red, a pH indicator. Isolates demonstrating lactose fermentation were classified as falling into the coliform group and were identified through formation of red colonies with associated gas bubbles when plated on Coliform Petrifilm, as outlined in the manufacturer's instructions. Characteristic growth of Gram-negative isolates was exhibited through the presence of dark red colony formation when plated on CVTA (Frank and Yousef, 2004). For the D-Count, a positive result for an EB organism was defined as having greater than 100 counts/mL upon completion of the test.

Data Analyses

All data were managed using Excel (version 14.5.4, Microsoft, Redmond, WA) and all linear models were created in RStudio (version 0.98.149, RStudio, Inc., Boston, MA). Isolates that showed no growth on a given selective and differential

media (i.e., 0 cfu/Plate) were not included in the calculation of slope and R^2 for the linear models. Sensitivity, or the true positive rate, is defined as the proportion of true positives that are correctly identified as such. Specificity, or the true negative rate, is defined as the proportion of true negatives that are correctly identified as such. The proportion of overall agreement (P_o) is the proportion of cases for which both testing methods agree and is calculated as follows:

$$= \frac{Positive\ Result\ Agreements + Negative\ Result\ Agreements}{Total\ Tests\ Performed}$$

RESULTS

Detection Methods Differed in Sensitivity and Specificity for their Respective Target Organisms with Varying Levels of Agreement Among EB Tests Evaluated

In this study, we evaluated testing methods for the detection of 3 dairy hygiene indicator organism groups including (i) Coliform Petrifilm for coliform detection; (ii) EB Petrifilm, VRBGA, and the D-Count for EB detection; and (iii) CVTA for detection of total Gram-negatives (see Supplemental Table 2.1 for detailed results on all 211 isolates tested). Out of the 211 Gram-negative organisms, 129 yielded positive results on Coliform Petrifilm and were hence classified as coliforms (Supplemental Table 2.1). On CVTA, 174 of the 211 Gram-negative isolates exhibited growth and characteristic colony morphology after incubation for 48 h for a sensitivity of 82% (174/211).

Based on data for 175 EB and 36 non-EB isolates, EB Petrifilm was the most sensitive of the EB specific tests, correctly detecting 96% (168/175) of the EB

isolates tested (Table 2.1). VRBGA and the D-Count were less sensitive, detecting 90% (158/175) and 86% (151/175) of EB isolates, respectively. On the other hand, the D-Count showed the highest specificity for non-EB organisms (100%; 36/36), followed by VRBGA (92%; 33/36) and EB Petrifilm (89%; 32/36). Out of the 168 EB isolates that tested positive on EB Petrifilm, no isolates exhibited gas production without acid production (Table 2.2). Isolates exhibiting both acid and gas production were the most common EB organisms on EB Petrifilm (83%; 139/168), followed by isolates exhibiting acid, but no gas production (17%; 29/168; Supplemental Table 2.1). It is also important to note that 4 *Aeromonas* isolates, which are not part of the family *Enterobacteriaceae*, tested positive on EB Petrifilm, whereas 2 *Aeromonas* and 1 *Acinetobacter* isolate tested positive on VRBGA (Supplemental Table 2.1**)**.

To assess consistency in detection results between the 3 EB testing methods, we calculated the proportion of overall agreement (P_o). The D-Count and VRBGA showed the highest proportion of agreement ($P_o = 0.93$) with 148 positive result agreements and 14 negative result agreements out of the 175 tests performed (Figure 2.1). We observed lower proportions of overall agreement for EB Petrifilm and VRBGA ($P_o = 0.89$), and the D-Count and EB Petrifilm ($P_o = 0.85$). EB isolates that were detected on VRBGA, but not on EB Petrifilm included *Buttiauxella* (n = 5), whereas EB isolates that were detected on EB Petrifilm, but not VRBGA included *Rahnella* (n = 8), *Serratia* (n = 4), *Citrobacter* (n = 1), *Plesiomonas* (n = 1), and *Raoultella* (n = 1). EB isolates that were detected on VRBGA, but not the D-Count included *Hafnia* (n = 1) and *Yersinia* (n = 9), whereas EB isolates that were detected using the D-Count, but not VRBGA included *Citrobacter* (n = 1), *Rahnella* (n = 1),

Table 2.1. Detection of the study's 211 isolates using select methods with reference to test sensitivity, specificity, and % coliforms detected

Detection Method	Detection of		Sensitivity (%)	Specificity (%)	Coliforms Detected (%)[4]
	EB (n = 175)	Non-EB (n = 36)			
Brain Heart Infusion (BHI) Agar	175	36	100[1]	N/A	100
Crystal Violet Tetrazolium (CVTA) Agar	155	19	82[1]	N/A	97
Violet Red Bile Glucose Agar	158	3	90[2]	92[3]	91
Enterobacteriaceae (EB) Petrifilm	168	4	96[2]	89[3]	100
D-Count	151	0	86[2]	100[3]	93

[1]Sensitivity calculations for BHI and CVTA were based on all 211 Gram-negative isolates tested.
[2]Sensitivity calculations for the EB specific assays were based on the 175 EB isolates included in the isolate set used.
[3]Specificity calculations for the EB specific assays were based on the 36 non-EB isolates included in the isolate set used.
[4]Proportion of coliform isolates detected is based on the 129 coliform isolates included in the isolate set used.

Table 2.2. Isolate growth characteristics on *Enterobacteriaceae* (EB) and Coliform Petrifilm

Genera	Number of Isolates Tested	Detected on[1]		Phenotype on EB Petrifilm[1]			Gas formation on Coliform Petrifilm[1]
		EB Petrifilm	Coliform Petrifilm	Gas formation only	Acid formation only	Acid and gas formation	
Acinetobacter	9	-	-	-	-	-	-
Aeromonas	7	57%	-	-	29%	29%	-
Brevundimonas	6	-	-	-	-	-	-
Buttiauxella (EB)	10	50%	50%	-	-	50%	50%
Cedecea (EB)	10	+	+	-	-	+	+
Citrobacter (EB)	14	+	+	-	-	+	+
Cronobacter (EB)	14	+	93%	-	7%	93%	93%
Enterobacter (EB)	12	+	92%	-	8%	92%	92%
Escherichia (EB)	12	83%	83%	-	-	83%	83%
Flavobacterium	3	-	-	-	-	-	-
Hafnia (EB)	10	+	+	-	-	+	+
Klebsiella (EB)	11	+	+	-	-	+	+
Kluyvera (EB)	9	+	+	-	-	+	+
Leclercia (EB)	2	+	+	-	-	+	+
Pantoea (EB)	9	+	11%	-	89%	11%	11%
Plesiomonas (EB)	1	+	-	-	+	-	-
Proteus (EB)	3	+	-	-	+	-	-
Pseudomonas	10	-	-	-	-	-	-
Rahnella (EB)	10	+	60%	-	30%	70%	60%
Raoultella (EB)	12	+	+	-	-	+	+
Salmonella (EB)	9	+	-	-	-	+	-
Serratia (EB)	10	+	+	-	-	+	+
Shigella (EB)	7	+	71%	-	29%	71%	71%
Vibrio	1	-	-	-	-	-	-
Yersinia (EB)	10	+	-	-	+	-	-

[1] + indicates 100% of isolates positive; - indicates 100% of isolates negative; otherwise % of isolates positive is indicated

Figure 2.1. Comparison of *Enterobacteriaceae* (EB) detection results for EB Petrifilm, Violet Red Bile Glucose Agar (VRBGA), and the D-Count. Brain Heart Infusion (BHI) agar represents the nonselective media control upon which all EB (n = 175) isolates exhibited growth.

		D-Count		VRBGA		EB Petrifilm	
		Positive Detection	Negative Detection	Positive Detection	Negative Detection	Positive Detection	Negative Detection
BHI Agar	Positive Detection	151	24	158	17	168	7
	Negative Detection	0	0	0	0	0	0
EB Petrifilm	Positive Detection	146	22	153	15		
	Negative Detection	5	2	5	2		
VRBGA	Positive Detection	148	10				
	Negative Detection	3	14				

and *Serratia* (n = 1). EB isolates that were detected using the D-Count, but not EB Petrifilm included *Buttiauxella* (n = 5), whereas EB isolates that were detected on EB Petrifilm, but not the D-Count included *Yersinia* (n = 9), *Rahnella* (n = 7), *Serratia* (n = 3), *Hafnia* (n = 1), *Plesiomonas* (n = 1), and *Raoultella* (n = 1)

EB and Total Gram-negative Tests Detected a Broader Range of Dairy-relevant Gram-negative Indicator Organisms than Coliform Tests

In the previous section, we highlighted the ability of each test to detect their respective indicator organism groups through calculation of sensitivity and specificity. In addition to these measures of assay performance, we also evaluated the ability of the 5 tests to detect dairy associated Gram-negative bacteria included in our collection of 211 isolates assembled for this study. This is highly relevant as detection of any Gram-negative organisms in pasteurized dairy products may be an indication of potential hygiene issues (e.g., post-pasteurization contamination) and as a number of the organisms included in our test set of 211 isolates have been linked to dairy spoilage. Coliform Petrifilm yielded positive results with 61% (129/211) of the organisms tested, representing 15 genera. The 82 organisms that tested negative on Coliform Petrifilm represent a broad range of dairy associated Gram-negative isolates encompassing 17 of the 25 genera tested (Table 2.2). Among these isolates, 46 were classified as EB organisms (including *Salmonella* [n = 9] and *Yersinia* [n = 10]) and 36 were classified as non-EB Gram-negatives (including *Pseudomonas* [n = 10] and *Acinetobacter* [n = 9]).

Consistent with the fact that EB represent a broader range of dairy-associated Gram-negative organisms, the 3 EB methods detected a higher proportion of the 211 Gram-negative dairy isolates, including typical non-coliform EB genera (e.g., *Salmonella*, *Yersinia*), as compared to a coliform test. With 172 positive results, EB Petrifilm detected the highest number of Gram-negative organisms of the EB tests (82%; 172/211). Of the positive results, 168 were EB (true positives) and 4 were non-EB (false positives; Table 2.1). The 39 isolates not detected included 7 EB (false negatives; *Buttiauxella* [n = 5] and *Escherichia* [n = 2]) and 32 non-EB Gram-negatives (true negatives). VRBGA detected 161 of the 211 isolates with 158 true positives and 3 false positives (Table 2.1). The 50 negatives observed on VRBGA consisted of 33 true negatives and 17 false negatives. Notably, *Rahnella* and *Serratia* accounted for 12 of the 17 false negatives observed on VRBGA (Supplemental Table 2.1). All 151 Gram-negative organisms detected using the D-Count were true positives, whereas the 60 undetected isolates consisted of 36 true negatives and 24 false negatives (Table 2.1). Non-EB Gram-negative organisms that were consistently negative across all EB detection methods included *Brevundimonas*, *Flavobacterium*, *Pseudomonas*, and *Vibrio*.

Despite being the standard method for the detection of dairy-relevant Gram-negative organisms, CVTA only detected 2 more Gram-negative isolates than EB Petrifilm (174/211). However, CVTA detected a number of isolates that went undetected by all other detection methods. This included non-EB Gram-negative isolates with dairy spoilage significance in the genera *Pseudomonas* (n = 8), *Aeromonas* (n = 3), *Acinetobacter* (n = 2), and *Brevundimonas* (n = 1). The 37 Gram-

negative isolates that CVTA failed to detect included 17 non-EB Gram-negative isolates represented in the genera *Acinetobacter* (n = 6), *Brevundimonas* (n = 5), *Flavobacterium* (n = 3), *Pseudomonas* (n = 2), and *Vibrio* (n = 1). The remaining 20 isolates came from EB genera including *Yersinia* (n = 9), *Escherichia* (n = 4), *Shigella* (n = 4), *Plesiomonas* (n = 1), *Proteus* (n = 1), and *Salmonella* (n = 1).

EB and Total Gram-negative Testing Methods Detected Up to 100% of the Coliforms Represented in 15 of the 25 Genera Tested

As previously noted, plating on Coliform Petrifilm revealed that 129 organisms from 15 genera possessed the ability to ferment lactose, thus classifying them as coliforms. The more expansive EB and total Gram-negatives tests were also successful in detecting coliforms with EB Petrifilm detecting 100% (129/129) of the lactose-fermenting isolates (Table 2.1). CVTA, VRBGA, and the D-Count were also highly successful at detecting coliforms, with all methods detecting over 90% of the 129 coliform isolates tested (Table 2.1).

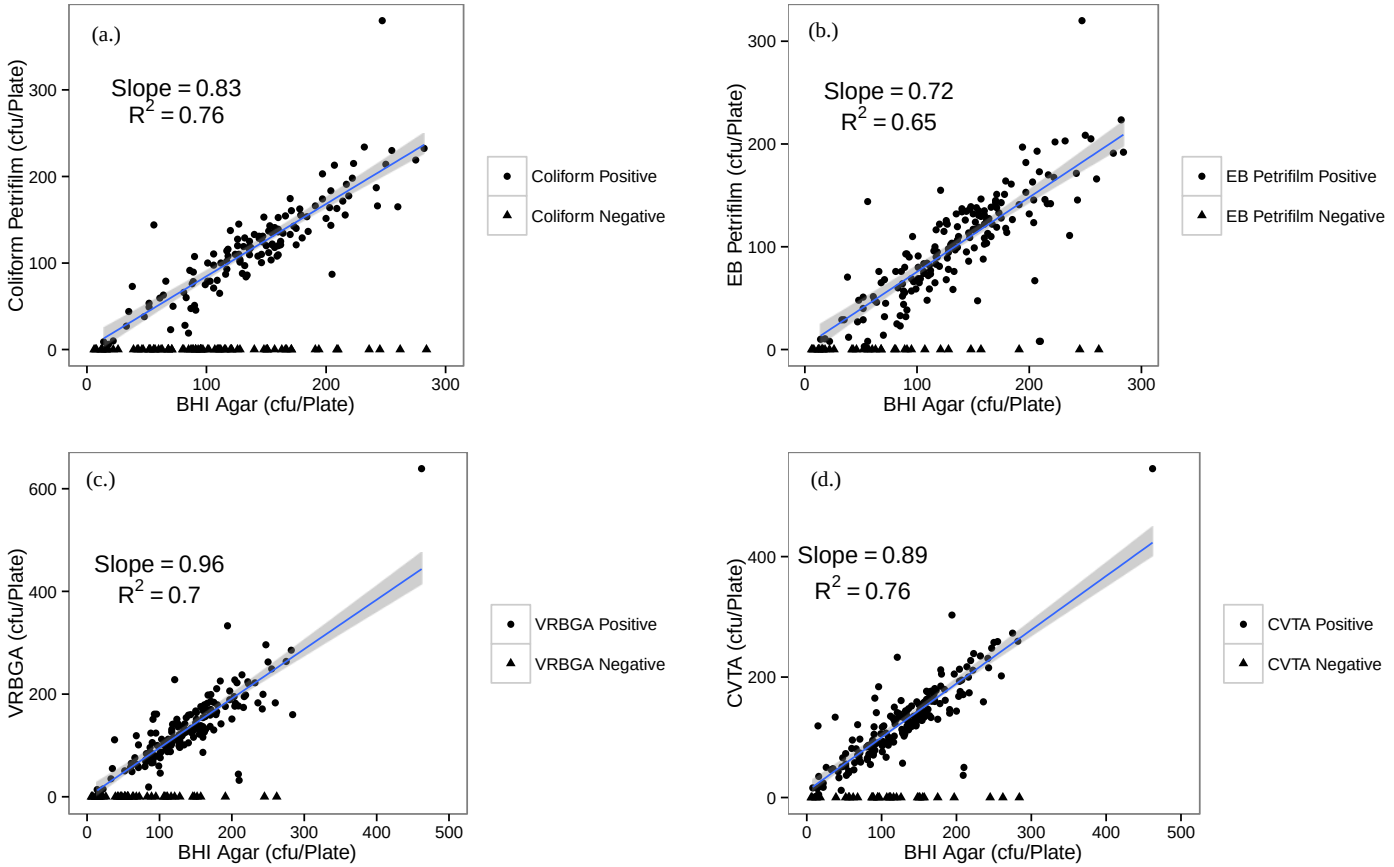
All isolates that tested positive as coliforms were classified into EB genera and represented 74% (129/175) of the study's 175 EB isolates. Coliforms were represented in 15 of the 19 EB genera, though individual genera differed in the proportion of isolates that were identified as coliforms. From the 15 genera containing coliform organisms, we observed 8 genera where all isolates were characterized as coliforms (e.g., *Cedecea* and *Klebsiella*; Table 2.2). On the other hand, for a total of 4 EB genera (e.g., *Salmonella* and *Yersinia;* Table 2.2), none of the isolates tested as coliforms. Finally, 7 genera included both coliform and non

coliform isolates, including the genus *Pantoea* (with 1/9 isolates classified as coliforms) and the genus *Cronobacter* (with 13/14 isolates classified as coliforms; Table 2.2).

All Selective Media-based Detection Methods Exhibited a Reduced Recovery of Pure Cultures when Compared to Nonselective BHI Agar

All isolates selected for this study exhibited growth when plated on nonselective BHI agar. To analyze the recovery of the isolates when plated on the selective/differential media types, we generated linear models and compared the slopes of the regression lines (Figure 2.2). A slope of 1 indicates that, on average, the same number of colonies exhibit growth on the nonselective BHI agar as the selective/differential media. Therefore, slope values of < 1 indicate a lower average recovery of pure cultures grown on selective/differential media types when compared to the nonselective BHI medium. VRBGA demonstrated a recovery most similar to that of BHI as indicated by a slope of 0.96. We observed lower recoveries for CVTA, Coliform Petrifilm, and EB Petrifilm with slopes of 0.89, 0.83, and 0.72, respectively. Furthermore, we computed R^2 values for isolates demonstrating growth on both the selective/differential media and nonselective BHI to evaluate the variability of selective/differential media plate counts and the overall fit of the regression line to the data (an R^2 of 1 indicates a perfect fit). CVTA and Coliform Petrifilm exhibited the least amount of plate count variability with R^2 values of 0.76. Greater plate count variability was observed for VRBGA and EB Petrifilm with R^2 values of 0.70 and 0.65, respectively.

Figure 2.2. Colony count comparisons between selective/differential detection methods and nonselective Brain Heart Infusion (BHI) agar. Selective/differential detection methods include Coliform Petrifilm (a.), *Enterobacteriaceae* Petrifilm (EB) (b.), Violet Red Bile Glucose Agar (VRBGA) (c.), and Crystal Violet Tetrazolium Agar (CVTA) (d.). Isolates exhibiting growth on both the selective/differential detection method and BHI are indicated with a ●, whereas isolates only exhibiting growth on BHI are indicated with a ▲. Slope values of < 1 indicate a lower average recovery of pure cultures grown on selective/differential media types when compare to the nonselective BHI medium. R^2 values indicate the variability of selective/differential media plate counts and the overall fit of the regression line to the data (an R^2 of 1 indicates a perfect linear fit). Isolates that showed no growth on a given selective and differential media (i.e., 0 cfu/Plate) were not included in the calculation of slope and R^2. Isolates included in the statistical model, but not pictured due to high plate count values include A5-0095, C4-0023, C4-0012, and W5-0630 for Figure 2.2 (a.) & (b.) and A5-0095, C4-0023, and C4-0012 for Figure 2.2 (c.) & (d.).


(a.)
Slope = 0.83
R2 = 0.76
Coliform Petrifilm (cfu/Plate)
BHI Agar (cfu/Plate)
Coliform Positive
Coliform Negative
(b.)
Slope = 0.72
R2 = 0.65
EB Petrifilm (cfu/Plate)
BHI Agar (cfu/Plate)
EB Petrifilm Positive
EB Petrifilm Negative
(c.)
Slope = 0.96
R2 = 0.7
VRBGA (cfu/Plate)
BHI Agar (cfu/Plate)
VRBGA Positive
VRBGA Negative
(d.)
Slope = 0.89
R2 = 0.76
CVTA (cfu/Plate)
BHI Agar (cfu/Plate)
CVTA Positive
CVTA Negative

DISCUSSION

Despite numerous advances in both processing technology and sanitation practices in the dairy industry over the past 25 years (Goff and Griffiths, 2006; Marriott and Gravani, 2006), prevention of post-pasteurization contamination remains a challenge for many dairy processors. While the occurrence of coliforms in milk has been linked to unhygienic processing conditions and decreased sensory scores of pasteurized milk during product shelf life (Wessels et al., 1989; Martin et al., 2012; Masiello et al., 2016), several studies show non-coliform Gram-negatives to be the primary culprits of post-pasteurization contamination in fluid milk (Craven and Macauley, 1992; Dogan and Boor, 2003). For example, a study evaluating the bacterial ecology of HTST milk in the United States found that approximately 75% of Gram-negative organisms isolated from pasteurized milk fall into common non-coliform genera (i.e., *Pseudomonas*, *Acinetobacter*, and *Flavobacterium*; Ranieri and Boor, 2009). Despite the dominating presence of non-coliform Gram-negative organisms in pasteurized milk, screening for coliforms remains the standard method by which most dairy processors evaluate the hygienic status of their products and processing environments (Frank and Yousef, 2004). Our data indicates that (i) coliform tests fail to detect a number of dairy-relevant Gram-negative organisms responsible for product spoilage and (ii) EB and total Gram-negative testing methods detect a large proportion of dairy associated genera without excluding traditional coliform organisms.

Coliform Tests Fail to Detect a Large Number of Dairy-relevant Gram-negative Organisms, Including Some Responsible for Dairy Product Spoilage and Sensory Defects

This study highlights a principal shortcoming of coliform tests, i.e., their inability to detect key dairy spoilage organisms commonly isolated from finished dairy products. The 39% (82/2l1) of isolates that went undetected on Coliform Petrifilm represent a diverse group of organisms from 17 genera, including 10 genera where all isolates went undetected on Coliform Petrifilm. As Gram-negative organisms, the isolates that went undetected on Coliform Petrifilm possess the ability to indicate the hygienic status of dairy products or dairy processing environments. The exclusion of non-coliform organisms with regards to post-pasteurization contamination screening is emphasized by the fact that a majority of the coliform-negative isolates had been isolated directly from pasteurized milk (63%; 56/82). This not only indicates their presence in the U.S. retail milk supply, but also highlights that these organisms would not have been detected during routine coliform testing. Among the group of organisms not detected on Coliform Petrifilm were isolates classified into EB genera with economic and food safety significance to the dairy industry, notably *Yersinia* and *Salmonella* and *Shigella* (Tacket et al., 1984; CDC, 1985; García-Fulgueiras et al., 2001). Additional organisms that are not coliforms and were not detected on Coliform Petrifilm include *Pseudomonas* and *Acinetobacter*, genera commonly associated with milk spoilage and sensory defects (Dogan and Boor, 2003; Hantsis-Zacharov and Halpern, 2007). By solely screening for coliforms, processors thus limit their ability to detect and correct instances of post-pasteurization contamination.

Enterobacteriaceae and Total Gram-negative Tests Detect a Large Proportion of Dairy Associated Genera and up to 100% of Coliform Isolates

Our results demonstrate that testing for EB offers a more comprehensive indicator for the hygienic status of dairy products and processing environments when compared to coliform organisms. By screening for EB, up to 100% of coliform organisms are detected along with other dairy-related EB genera that typically lack the ability to ferment lactose (e.g., *Yersinia* and *Salmonella*; Imhoff, 2005). Hence, replacement of coliform with most EB tests will continue to detect coliforms, but will allow for improved detection of the organisms whose presence indicates the same type of hygiene issues identified by coliform testing. When considering sensitivity, convenience, and ease of differentiating between positive and negative test results, EB Petrifilm offers distinct advantages over other EB detection methods. Our results were similar to those found by Silbernagel and Lindberg (2002) in that recovery rates of EB isolates on EB Petrifilm were over 95% and exceeded those of the VRBGA standard. Additionally, a false positive rate of 16% was observed for EB Petrifilm in the Silbernagel and Lindberg (2002) study compared to a false positive rate of 11% observed here. By also detecting 100% of isolates that tested coliform positive, EB Petrifilm offers a more complete screening of potential dairy-related indicator organisms without excluding the conventionally utilized coliform group. VRBGA proved to be sensitive and specific in the detection of EB organisms, though the additional space and materials needed for this method make it less convenient. Our results agree with past studies in that certain species within the *Aeromonas* genus have the ability to ferment glucose and thus may test as false positives when plated on EB

selective and differential media (Abbott et al., 2003; Erdem et al., 2011). Similarly, previous studies demonstrate the ability of select *Acinetobacter* isolates to utilize glucose with acid production, thus potentially testing positive when using EB detection methods (Constantiniu et al., 2004). Despite this, the high specificities of EB testing methods evaluated in this study validate the ability of the tests to correctly identify the vast majority of true negatives.

With time to result proving to be a driving force for the development of alternative indicator organism tests, rapid detection methods (e.g. the D-Count) can have distinct advantages over the traditional media types. Where traditional EB detection methods such as EB Petrifilm and VRBGA require a 24 h incubation period before obtaining results, the D-Count provides results in approximately 13 h through a selective enrichment step and flow cytometry. Though the 13 h incubation period of the D-Count led to a reduced end-result detection time, the test did have the lowest sensitivity of the EB detection methods assessed. However, optimization of the cut-off threshold for the D-Count may further improve its sensitivity and specificity for the use of testing on various food matrices, but will require additional data. The D-Count was also unique amongst the EB detection methods in that we observed no instances of false positive test results at a cut-off of 100 counts/mL. Our data thus supports the potential for development of more rapid tests for EB, which may further improve adoption of EB as dairy hygiene indicators in countries that currently prefer coliform tests.

Screening for total Gram-negatives allows for detection of an even broader range of organisms with the potential to indicate the hygienic status of dairy products and processing environments when compared to the EB and coliform groups. In this study, we included *Acinetobacter*, *Aeromonas*, *Brevundimonas*, *Flavobacterium*, *Pseudomonas*, and *Vibrio* to represent dairy-related Gram-negative organisms falling outside of the EB and coliform groups. These organisms are largely undetected when using coliform or EB testing methods and are predominant dairy spoilage organisms whose presence indicates hygiene issues (Ternström et al., 1993; Dogan and Boor, 2003; and Ranieri and Boor, 2009).

As a selective media for total Gram-negative organisms, we evaluated CVTA for its ability to detect all coliform, EB, and non-EB Gram-negative isolates. While a majority of isolates exhibited typical growth and detection on CVTA, a number of organisms exhibited atypical growth or no growth at all when plated on CVTA (18%; 37/211). This lead to CVTA having the lowest sensitivity (relative to its target organisms, i.e., all Gram-negatives for CVTA) of the 5 indicator organism testing methods analyzed. Notably, approximately half of the non-EB, Gram-negative isolates did not exhibit expected growth characteristics on CVTA. This demonstrates the need for a more sensitive total Gram-negative testing method. Despite the lack of sensitivity, screening for total Gram-negatives using CVTA detects a more expansive group of potential hygiene indicators than the somewhat limiting coliform group. This is largely due to the inclusion of *Pseudomonas* in total Gram-negative testing. In this study, EB Petrifilm and CVTA detected a similar number of dairy relevant Gram-negative organisms (172 and 174, respectively). However, previous studies reveal that

Pseudomonas dominate the Gram-negative microflora of pasteurized milk and commonly cause dairy product spoilage and sensory defects (Ternström et al., 1993; Ranieri and Boor, 2009). In this study, CVTA detected a majority of the selected *Pseudomonas* isolates (8/10). On the other hand and as expected, as they are not members of the EB family or coliform group, all *Pseudomonas* isolates were not detected with EB and coliform testing methods. Hence, a total Gram-negative test, such as CVTA, has distinct advantages over EB and coliform testing methods if aiming to detect the maximum number of Gram-negative hygiene indicators relevant to pasteurized fluid milk. While further evaluation of CVTA for exclusivity (i.e., absence of detection of Gram-positive bacteria), may be valuable, we have not identified issues with detection of Gram-positive bacteria when using CVTA for pasteurized fluid milk testing.

CONCLUSIONS

Through the testing of 211 dairy-related isolates falling into the coliform, EB, and Gram-negative groups, we evaluated standard and alternative hygiene indicator organism tests. Out of the testing methods evaluated, EB Petrifilm was the most sensitive and the D-Count was the most specific for the detection of EB. By monitoring for EB or total Gram-negatives, traditional coliform organisms are detected along with a broad range of dairy-related quality indicators lacking the ability to ferment lactose (yet causing spoilage in dairy products). While testing for total Gram-negatives in fluid milk offers advantages to other hygiene indicator groups due to the inclusion of non-EB Gram-negative organisms (e.g., *Pseudomonas*), further

studies must evaluate their use as indicators in fermented dairy products. This study highlights the wide range of methods available for the detection of EB and total Gram-negative organisms, however, our data also demonstrates the need for continued development of dairy indicator detection methods that are rapid, reliable, and yield accurate results. For researchers interested in further validation of testing methods, Cornell University and the Food Microbe Tracker database (www.foodmicrobetracker.com) offer access to over 50,000 bacterial isolates, including the 211 isolates evaluated here.

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CHAPTER 3

SURVIVAL AND DETECTION OF COLIFORMS, ENTEROBACTERIACEAE, AND GRAM-NEGATIVE ORGANISMS IN GREEK YOGURT*

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ABSTRACT

Despite the widespread use of coliforms as indicator organisms, increasing evidence suggests that the *Enterobacteriaceae* (EB) and total Gram-negative groups more accurately reflect the hygienic status of high temperature, short time pasteurized milk and processing environments. If introduced into milk as post-pasteurization contamination, these organisms may grow to high levels and produce a wide range of sensory-related defects. Limited information is available on the ability of these organisms to (i) survive in low pH fermented dairy products, such as yogurt, and (ii) represent suitable microbial hygiene indicators. In order to identify suitable hygiene indicator groups and optimal detection methods for use in fermented dairy products, we screened 64 bacterial isolates of 24 dairy-relevant genera for survival and detection in Greek yogurt using 5 different testing methods. Prior to testing, isolates were inoculated into plain, 0% fat Greek yogurt (pH 4.35 to 4.65), followed by a 12 h hold period at 4 ± 1°C. Yogurts were subsequently tested using (i) 3M Coliform Petrifilm to detect coliforms, (ii) 3M *Enterobacteriaceae* Petrifilm, Violet Red Bile Glucose Agar, and the D-Count to detect EB, and (iii) Crystal Violet Tetrazolium Agar (CVTA) to detect total Gram-negative bacteria. Overall, the non-EB Gram-negative isolates showed significantly larger log reductions at 12 h after inoculation into Greek yogurt (based on bacterial numbers recovered on CVTA) as compared to the coliform and non-coliform EB isolates tested. The methods evaluated vary in their ability to detect different microbial hygiene indicators in Greek yogurt. Crystal Violet Tetrazolium Agar detected the highest portion of coliforms, while EB Petrifilm detected the highest portion of EB, as well as highest portion of total Gram-negative

organisms. Additionally, the D-Count method allowed for a more rapid detection of EB in yogurt by generating results in approximately 13 h rather than the 24 h when using EB Petrifilm and Violet Red Bile Glucose Agar. Results from this study indicate that the coliform and EB groups encompass the broadest range of dairy-relevant Gram-negative organisms with the capability to survive in Greek yogurt, thus validating their use as microbial hygiene indicator groups in low pH fermented dairy products.

INTRODUCTION

Due to the fact that Gram-negative organisms typically do not possess the capability to survive heat treatment (e.g., high temperature, short time pasteurization), their presence in pasteurized dairy products indicates inadequate sanitation, instances of post-pasteurization contamination, and the overall inadequate hygienic status of finished products (Schröder, 1984; Ranieri and Boor, 2009). Coliform bacteria fall into this group of Gram-negative organisms and have a long history of use as microbial hygiene indicators in the United States dairy industry. Despite this, a recent study showed that a wide range of dairy-relevant hygiene indicator organisms go undetected on coliform selective and differential media (Hervert et al., 2016). Alternative indicator groups suggested for use in the dairy industry include the *Enterobacteriaceae* (EB) family and total Gram-negative organisms (Hervert et al., 2016). The primary advantage of testing for these alternative indicator groups is that EB and total Gram-negative tests detect the traditional coliform group of organisms, as well as all other non-coliform Gram-negative groups that represent common post-pasteurization contaminants (Hervert et al., 2016).

By definition, coliforms are aerobic or facultatively anaerobic, Gram-negative, non-spore-forming rods capable of fermenting lactose resulting in gas and acid production within 48 h at either 32°C or 35°C (Feng et al., 2002; Davidson et al., 2004). Traditional coliform genera include *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter*, though organisms in over 20 Gram-negative genera meet the phenotypic criteria of coliforms (Imhoff, 2005; Masiello et al., 2016). While EB share many of the phenotypic traits that define coliforms, classification into the EB family is on a taxonomic basis (Imhoff, 2005). The differential capabilities of most traditional EB detection methods rely on the ability of EB organisms to ferment glucose resulting in gas, acid, or gas and acid production. The EB family, on the other hand, encompasses the vast majority of coliform organisms (Imhoff, 2005) and also includes a number of dairy associated genera typically lacking the ability to ferment lactose (e.g., *Salmonella* and *Yersinia*; Imhoff, 2005). Because the presence of any Gram-negative organisms in a pasteurized dairy product indicates the occurrence of post-pasteurization contamination, total Gram-negative tests encompass the broadest range of relevant dairy hygiene indicator organisms (Hervert et al., 2016). In addition to encompassing all coliform and EB organisms, the total Gram-negative group includes genera such as *Pseudomonas* and *Acinetobacter*. Previous studies indicate that organisms outside of the coliform and EB groups (e.g., *Pseudomonas* and *Acinetobacter*) dominate the Gram-negative microflora of pasteurized milk and go undetected when using coliform and EB detection methods (Ranieri and Boor, 2009; Hervert et al., 2016).

The majority of published research on dairy hygiene indicators focuses on coliforms in fluid milk and cheese (Martin et al., 2012; Masiello et al., 2016; Trmčić et al., 2016). Perhaps the most critical ability of microbial hygiene indicators is their capability to survive in the food matrix to the point of selective and differential testing. For example, pasteurized milk, with its pH close to neutral (approximately 6.7), contains key nutrients that are ideal for sustaining growth and survival of microbial hygiene indicators over the course of product shelf life. While previous studies that evaluated the quality of pasteurized milk utilized coliforms, EB, and total Gram-negative organisms as microbial hygiene indicators (Van Tassell et al., 2012; Masiello et al., 2016), limited research exists on the use and detection of microbial hygiene indicator organism groups in low pH, fermented dairy products. While the research that does exist typically evaluated a limited diversity of species representing different indictors, it generally suggested a limited ability of coliforms and EB to survive in low pH, fermented dairy products (Goel et al., 1971; Shaker et al., 2008). Yogurt, while still possessing key nutrients required for microbial growth, typically ranges in pH between 4.2 and 4.6. The relatively low pH of yogurt and other fermented dairy products is a result of the fermentation of milk lactose into lactic acid through the activity of lactic acid bacteria usually added as starter cultures (Vedamuthu, 2007). Yogurt starter cultures contain *Streptococcus salivarius* subsp. *thermophiles* and *Lactobacillus delbrueckii* subsp. *bulgaricus* that largely out-compete other bacteria present in milk due the inhibitory effect of the lactic acid, utilization of the primary carbohydrate source (lactose), as well as other mechanisms (Vedamuthu, 2007). Yogurt processors also commonly add other dairy cultures, for example probiotics

(e.g., *Bifidobacterium* sp. and *Lactobacillus* sp.), to potentially achieve additional health promoting effects (Vedamuthu, 2007). The low pH of yogurt is the main mechanism that eliminates most bacterial spoilage organisms giving yogurt a shelf life of up to 2 months compared to the 2 to 3 weeks observed in pasteurized fluid milk (Vedamuthu, 2007; Cruz et al., 2010; Fromm and Boor, 2004). Contamination of yogurts with acid tolerant yeast via post-pasteurization contamination or added non-dairy ingredients represents the primary cause of premature spoilage in yogurt (Fleet, 1990). This is largely due to the ability of yeasts to survive and grow at a lower pH range than most of their bacterial spoilage counterparts as well as utilize lactic acid as carbon source (Rohm et al., 1992; Lourens-Hattingh and Viljoen, 2002). However, detection of microbial indicator organisms (e.g., coliforms) also provides an important tool for the detection of post-pasteurization contamination and provides faster results than yeast and mold testing.

In a recent study by Hervert et al. (2016), 211 dairy-relevant coliform, EB, and Gram-negative isolates were screened for detection in pure culture on Coliform Petrifilm, EB Petrifilm, Violet Red Bile Glucose Agar (VRBGA), Crystal Violet Tetrazolium Agar (CVTA), and a rapid flow cytometry-based method. For the study reported here, we selected a 64 isolate subset from the original 211 isolates used in the previous study to undergo inoculation into Greek yogurt, followed by testing using the indicator organism detection methods listed above. The resulting data provides important new information on the survival of a diverse set of coliforms, EB, and Gram-negative isolates in Greek yogurt over a 12 h hold at refrigeration temperatures, as well as comprehensive data on the ability of different methods to detect different

groups of indicator organisms that would allow for detection of post-pasteurization contamination of yogurt.

MATERIALS AND METHODS

Isolate Selection

Based on results from Hervert et al. (2016) and information listed in the Food Microbe Tracker Database (www.foodmicrobetracker.com; Vangay et al., 2013), a collection of 64 Gram-negative bacterial isolates representing a broad range of dairy-relevant organisms was assembled for the study reported here. The selected isolate set contained 54 EB isolates from 19 genera and 10 non-EB Gram-negative isolates from 5 genera. Identification of isolates to genus level was based on previously completed 16S rDNA sequencing as described in prior studies (Huck et al., 2007). Among the 54 EB isolates selected, 42 were identified as coliform organisms based on their ability to ferment lactose resulting in colonies with associated gas bubbles when plated on Coliform Petrifilm (Hervert et al., 2016). Additionally, isolates were selected based on growth characteristics observed in the pure culture testing experiment with preference in selection given to isolates whose plate counts on selective and differential media corresponded well with those expected based on 18 h BHI broth enumerations. Isolates were also selected to represent dairy-associated isolation sources, with all but one isolate coming from dairy products or processing environments.

Inoculation and Subsequent Testing of Greek Yogurts

Isolates were streaked onto Brain Heart Infusion (BHI) Agar (Becton Dickinson, Sparks, MD) from frozen culture and placed into a 32 ± 1°C incubator for 24 hours. Individual colonies were then used to inoculate 5 mL of BHI broth (Becton Dickinson), followed by 18 hours of incubation (no aeration) at 32 ± 1°C. Based on previously generated growth data (Hervert et al., 2016), BHI broth cultures were serially diluted using sterile phosphate buffer to concentrations that facilitated inoculation at target levels of approximately 300 to 2,000 cfu/g of 0% fat, plain Greek yogurt (actual inoculation levels enumerated on BHI are shown in Supplemental Table 3.1). Isolates that did not yield colonies on at least one of the media used for detection at the targeted yogurt inoculation levels were retested at ≥ 1 log greater inoculum concentrations. The sealed yogurts were inoculated from the base of the cup using a sterile 18-gauge needle and syringe as to not alter the headspace of the packaging. Immediately following inoculation, the hole from the needle was sealed using sterile, adhesive foil. Inoculated yogurts were then held for 12 h at 4 ± 1°C prior to testing using the selective and differential detection methods. These methods included (i) Coliform Petrifilm (3M, St. Paul, MN) for coliform detection; (ii) EB Petrifilm (3M), VRBGA (Becton Dickinson), and a proprietary flow cytometry instrument for EB detection (D-Count; bioMérieux, Marcy-l'Étoile, France); and (iii) CVTA (Frank and Yousef, 2004) for detection of total Gram-negative organisms. To plate on Coliform Petrifilm, EB Petrifilm, VRBGA, and CVTA, a 1:10 serial dilution of the yogurt was prepared with sterile phosphate buffer and the pH of the resulting dilution was adjusted to between 6.6 and 7.2 using 1N NaOH. A 1 mL aliquot of each pH adjusted yogurt dilution was plated in duplicate on Coliform Petrifilm and EB Petrifilm, while

250 μL aliquots were plated in duplicate on CVTA and VRBGA using the non-exponential mode of an automated spiral plater (Advanced Instruments, Inc., Norwood, MA). Thus, Coliform and EB Petrifilms both had detection limits of 10 cfu/g of yogurt, while CVTA and VRBGA plates had detection limits of 20 cfu/g of yogurt. Coliform Petrifilms, EB Petrifilms, and VRBGA plates were incubated for 24 hours at 32 ± 1°C while CVTA plates were incubated for 48 hours at 21 ± 1°C prior to enumeration (Frank and Yousef, 2004). In addition to plating on traditional detection media, yogurts were tested in duplicate using a flow cytometry method (D-Count) with a protocol designed to detect EB organisms (protocol title "Presence/Absence Test of EB in Fermented Milk Products Containing *Bifidobacterium*"); this procedure includes an enrichment step, followed by flow cytometry to allow for EB detection in 13 h rather than 24 h. Briefly, for each isolate tested, 1 g of the inoculated yogurt was transferred into 9 mL of a proprietary EB selective broth. Inoculated broths were incubated for 13 h at 37°C ± 1°C prior to testing on the D-Count. To perform the final test, 10 μL of enrichment broth were treated with reagents that label viable EB cells. The sample was then automatically injected into the flow cell analyzer of the D-count and the detectors within the analyzer counted the labeled cells, outputting a value in counts/mL of analyzed sample.

Interpretation of Growth Results

Test methods were interpreted as outlined by Hervert et al. (2016). Briefly, following the incubation of plates and Petrifilms, colony growth was enumerated visually for Petrifilms, and with the aid of a Color Q-Count (Advanced Instruments)

instrument for plates. Differential media types were also examined for typical or atypical growth characteristics indicating a positive or negative result for a media type's respective differential capabilities. For EB Petrifilm, a positive result was indicated by either acid, gas, or gas and acid production generated through the fermentation of glucose. Per the manufacturer's instructions, these characteristics resulted in red colonies with yellow zones for acid producing isolates, red colonies with associated gas bubbles for gas producing isolates, and red colonies with yellow zones and associated gas bubbles for acid and gas producing isolates. EB glucose fermenters produced red colonies on VRBGA with red-purple halos (bile precipitation) indicating acidification identified by neutral red, a pH indicator. Lactose fermenting isolates were classified as coliforms and were identified through formation of red colonies with associated gas bubbles when plated on Coliform Petrifilm, as outlined in the manufacturer's instructions. On CVTA, dark red colonies indicated characteristic growth of Gram-negative isolates (Frank and Yousef, 2004). For the D-Count, a positive result for an EB organism was defined as an instrument reading indicating ≥ 100 counts/mL.

Data Analyses

All data were managed using Excel (version 14.5.4, Microsoft, Redmond, WA) and all models were created in RStudio (version 0.98.149, RStudio, Inc., Boston, MA). Sensitivity, or the true positive rate, was defined as the proportion of true positives that are correctly identified as such. Specificity, or the true negative rate, was defined as the proportion of true negatives that are correctly identified as such. For the

purpose of this study, isolate detection refers to the growth of an isolate with typical characteristics when evaluated using a given testing method. On the other hand, atypical growth on CVTA (i.e., colonies without red color) was used in some cases (i.e., 9 isolates) to provide enumeration data to assess isolate survival. An ANOVA and a Tukey's honest significant difference test were performed to compare the log differences between bacterial levels inoculated into Greek yogurt and those recovered on CVTA for coliforms, non-coliform EB, and non-EB Gram-negative bacteria.

RESULTS AND DISCUSSION

A total of 64 Gram-negative bacterial isolates were inoculated into Greek yogurt, followed by incubation at 4 ± 1°C for 12 h; inoculation levels were determined by enumeration of the inoculum on BHI. After incubation of the inoculated yogurt, bacterial numbers were enumerated using plate counts determined on Coliform Petrifilm, EB Petrifilm, VRBGA, and CVTA. The resulting data were used to assess (i) bacterial survival in Greek yogurt over 12 h and (ii) the ability of different media to recover bacterial isolates that could be introduced into yogurt through post-pasteurization contamination.

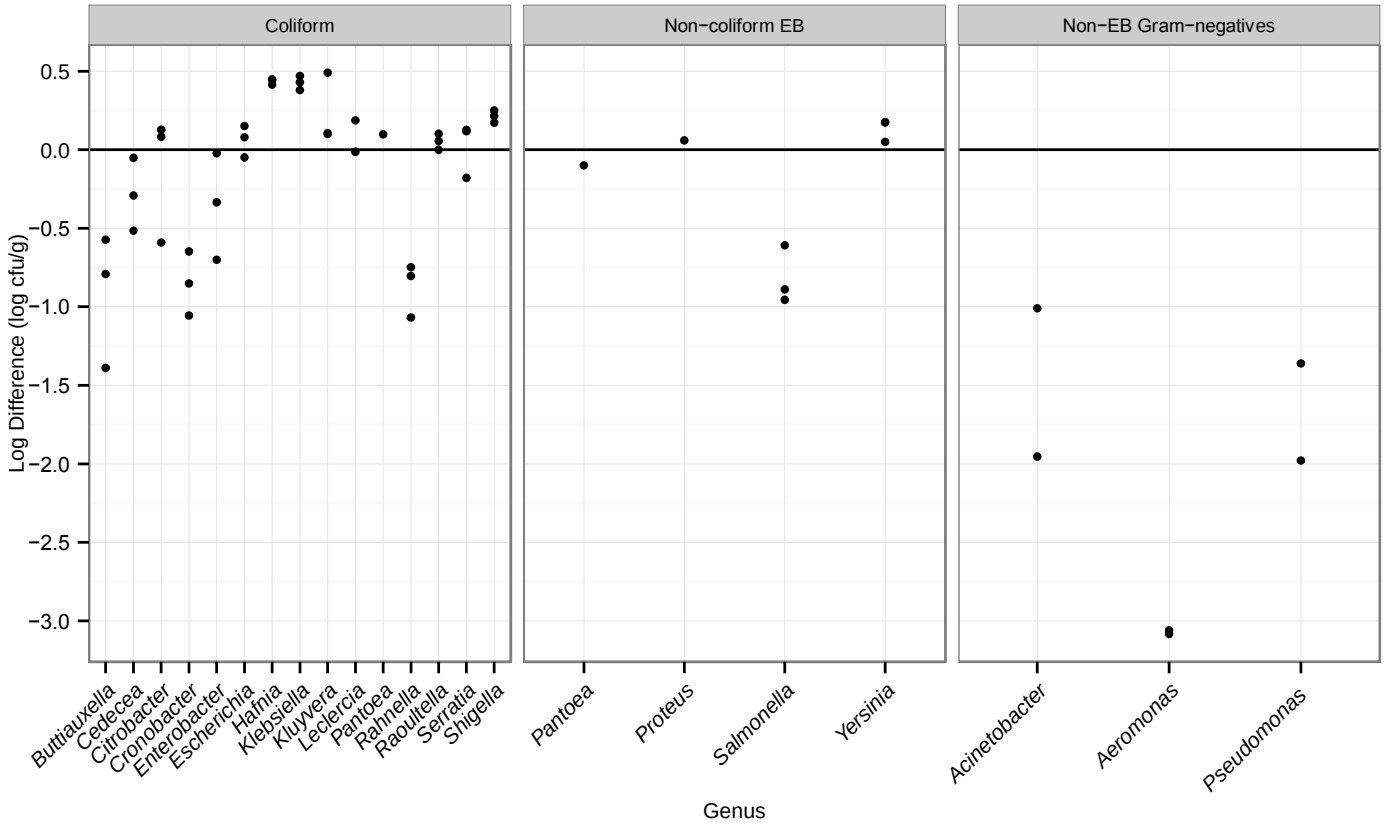
In Comparison to the Coliform and EB Isolates, Non-EB Gram-negative Organisms Tested in Greek Yogurt Exhibit Rapid Die-off with Limited Recovery on Selective and Differential Media

Bacterial enumeration data on CVTA were used to assess growth and survival of Gram-negative organisms in Greek yogurt at 12 h after inoculation. Survival was assessed based on enumeration data generated on CVTA, which selects for total

Gram-negative organisms. Among the 64 Gram-negative isolates inoculated into Greek yogurt, no appropriate enumeration data to assess growth or survival were generated for 8 isolates. These 8 isolates represent (i) 3 isolates (*Plesiomonas* isolate FSL Y1-0254; *Brevundimonas* isolates FSL C4-0016 and C4-0057) known to not exhibit growth on CVTA; (ii) 2 isolates (*Flavobacterium* isolates FSL R5-0497 and R5-0610) that grew with atypical growth on CVTA (brown film-like growth that did not present as countable colonies), and (iii) 3 isolates (*Pantoea* isolate FSL P4-0767; *Proteus* isolates FSL A5-0110 and A5-0127) for which inoculation levels could not be determined due to spreading colony morphologies on BHI. The 56 isolates with enumeration data on CVTA could be grouped into 3 categories including (i) no change in bacterial numbers (< 0.5 log increase or decrease in numbers); (ii) moderate die-off (between 0.5 and 1.0 log decrease in numbers); and (iii) > 1.0 log die-off. *Aeromonas* isolates FSL C4-0005 and R5-0758 showed no countable colonies; as these isolates grew with typical colony characteristic when tested in pure culture (Supplemental Table 3.1), this suggests that their numbers dropped below the detection limit during the 12 h hold in Greek yogurt. Reduction of these isolates was thus calculated, using the detection limit on CVTA, as > 3.08 log and > 3.06 log for FSL C4-0005 and R5-0758, respectively; these isolates were thus classified into the > 1 log die-off category.

All 42 coliform isolates tested in this study showed growth on the total Gram-negative media, CVTA. A majority of these isolates (71%; 30/42) showed no change in bacterial numbers over the 12 h hold period in Greek yogurt (Figure 3.1). The remaining coliform isolates showed either moderate die-off (21%; 9/42) or > 1 log die-off (7%; 3/42). The 30 coliform isolates with no change in bacterial numbers

Figure 3.1. Recovery of 42 coliform, 8 non-coliform *Enterobacteriaceae* (EB), and 6 non-EB Gram-negative isolates on Crystal Violet Tetrazolium Agar (CVTA). Plotted values represent the log difference between the cfu inoculated into Greek yogurt and the cfu recovered on CVTA per g of yogurt.


Coliform
Non−coliform EB
Non−EB Gram−negatives
Log Difference (log cfu/g)
0.5
0.0
−0.5
−1.0
−1.5
−2.0
−2.5
−3.0
Buttiauxella
Cedecea
Citrobacter
Cronobacter
Enterobacter
Escherichia
Hafnia
Klebsiella
Kluyvera
Leclercia
Pantoea
Rahnella
Raoultella
Serratia
Shigella
Pantoea
Proteus
Salmonella
Yersinia
Acinetobacter
Aeromonas
Pseudomonas
Genus

represented 12 different genera, including 3 *Hafnia* and 3 *Klebsiella* isolates, which all showed numerical increase close to 0.5 log (ranging from 0.38 to 0.47 log; Figure 3.1). *Hafnia* was previously identified as part of the microbial population of raw milk cheeses and can even represent the dominant bacterial population in some cheese varieties made from raw milk (Trmčić et al., 2016; Wolfe et al., 2014). While these previous data support *Hafnia*'s ability to survive mild acidification, to our knowledge, the ability of representatives of this genus to survive very low pH values typical for yogurt had not been previously reported. Additionally, we did not identify other studies that reported the effective survival of *Klebsiella* in yogurt. The ability of *Hafnia* and *Klebsiella* to readily survive the low pH of Greek yogurt suggests that representatives of these genera, and specifically the isolates tested here, may be appropriate in for use in challenge studies involving microbial hygiene indicators in low pH dairy foods. Interestingly, all 3 *Escherichia* isolates tested also survived over the 12 h hold period. By comparison, a previous study reported a reduction of *E.coli* in inoculated yogurt stored at 4°C of 3.8 log cfu/g to 1.9 log cfu/g for a nonpathogenic *E. coli* strain and 4.4 log cfu/g to 3.6 log cfu/g for a pathogenic *E. coli* strain over a 72 h hold period (Bachrouri et al., 2002). The pathogenic and nonpathogenic strain levels declined below detection limit (10 cfu/mL) after 312 and 168 h of incubation, respectively (Bachrouri et al. 2002). The < 1 log reduction seen for the pathogenic *E. coli* strain over 72 h supports the ability of *E. coli* to tolerate the pH stress encountered in yogurt (Bachrouri et al. 2002).

Notably, 9 of the 12 coliform isolates showing moderate (between 0.5 and 1.0 log) or > 1.0 log decreases in numbers were classified into the genera *Buttiauxella*,

Cronobacter, or *Rahnella*; the remaining 3 coliform isolates represented the genera *Cedecea*, *Citrobacter*, and *Enterobacter*. These results were consistent with previous studies (Goel et al., 1971; Shaker et al., 2008) evaluating the survival of typical coliform genera in fermented dairy products. For example, one study that assessed the survival of *Enterobacter sakazakii* (now *Cronobacter sakazakii*) in yogurt suggests approximately a 2 log reduction in *E. sakazakii* populations over the 20 h after the yogurt reaches a pH of about 4.7 (Shaker et al., 2008). Another study found that *Aerobacter (Enterobacter) aerogenes* rapidly dies-off following its inoculation into yogurt with approximately 1 to > 2 log reductions over 24 h of refrigerated storage (Goel et al., 1971).

Of the 56 total isolates included in our analysis of survival in Greek yogurt, 8 represented non-coliform EB organisms in the genera *Pantoea*, *Proteus*, *Salmonella*, and *Yersinia*. Within this set, 5 isolates showed no change in numbers over the hold period while 3 showed moderate decreases in numbers (Figure 3.1). Statistical analysis showed no significant difference between the log differences of the coliform and non-coliform EB isolates tested ($P = 0.8090$). Notably, the 3 isolates showing moderate die-off were classified as *Salmonella*. This is consistent with a previous study reporting a > 1 log reduction in *Salmonella* numbers over 48 h in yogurt (Nassib et al., 2006). On the other hand, the 3 *Yersinia* isolates tested in this study showed the ability to survive in Greek yogurt with numerical increases ranging from 0.05 log to 0.18 log. Consistent with these observations, a previous study evaluating the survival of *Yersinia enterocolitica* in yogurt held at 4°C reported a 0.5 log increase in *Y.*

enterocolitica numbers during the first 5 days of storage at before decreasing > 2.5 log over the remaining 21 days of the study (Aykut and Oezbas, 1994).

Finally, 6 of the 56 total isolates included in our survival analysis fell into one of 3 non-EB Gram-negative genera (i.e., *Acinetobacter*, *Aeromonas*, and *Pseudomonas*). All 6 isolates of this group showed high die-off in Greek yogurt with > 1.0. log decrease in numbers over the 12 h hold period (Figure 3.1). Overall, the non-EB Gram-negative isolates showed significantly larger log reductions at 12 h after inoculation into Greek yogurt (based on bacterial numbers recovered on CVTA) as compared to the coliform ($P < 0.0001$) and non-coliform EB isolates tested ($P < 0.0001$). These results suggest that non-EB Gram-negative organisms may be more susceptible to the low pH of yogurt than coliform and EB. The 2 *Acinetobacter* isolates (FSL C4-0013 and C4-0087) showed reductions of 1.01 log and 1.95 log, respectively; we are not aware of other studies that evaluated the survival of *Acinetobacter* in yogurt. Both *Aeromonas* isolates tested in this study did not exhibit recovery on CVTA even though the preliminary tests in pure culture showed that both isolates can be recovered on this medium (Hervert et al., 2016). This suggests that they did not survive the 12 h hold period; alternatively, cell injury could have prevented growth on CVTA during the 48 h incubation period. A previous study testing the survival of *Aeromonas* in yogurt saw similar results in that *A. hydrophilia* numbers decreased > 6 log over the first 5 days of storage at 4°C (Aykut and Oezbas, 1994). The 2 *Pseudomonas* isolates tested in this study showed > 1 log reduction over the 12 h hold in the yogurt (1.36 and 1.98 log for isolates FSL R5-0318 and W7-0098, respectively). Though limited research exists on the survival of *Pseudomonas* in

yogurt, a previous study noted the ability of *Pseudomonas paucimobilis* to survive up to 45 days in yogurts inoculated at levels of approximately 10^8 cfu/mL (Canganella et al., 1999). However, Canganella et al. (1999) acknowledge that *Pseudomonas paucimobilis* is not a common contaminant of fermented milk and is more commonly associated with non-dairy environments. Consistent with the pH survival results obtained here, previous studies found that *Pseudomonas* isolated from cottage cheese exhibit limited to no growth at pH values below 4.6. (Brocklehurst and Lund, 1988; Martinez-Rios et al., 2016). Despite their rapid die off in yogurt, screening for non-EB Gram-negative organisms using CVTA may be useful in fermented dairy products with pH values higher than yogurt (e.g., cottage cheese). For example, *Pseudomonas* spp. regularly are linked to spoilage of cottage cheese through the formation of surface films and the production of degradative enzymes and metabolites (Cousin, 1982; Brocklehurst and Lund, 1985).

CVTA Detects the Highest Portion of Coliforms While EB Petrifilm Detects the Highest Portion of EB and Total Gram-negative Organisms in Inoculated Yogurt

Among the 64 isolates, 4 (*Aeromonas* FSL C4-0005 and R5-0758; *Brevundimonas* FSL C4-0016 and C4-0057) did not show detectable growth on any of the 4 media and were not detected on the D-Count. Assessment of the different testing methods evaluated here was thus performed based on data from 60 different isolates. The 60 isolates in this data set included 42 coliforms. Each of the 5 methods evaluated yielded positive results with ≥ 90% of the 42 coliform isolates inoculated into Greek yogurt (Table 3.1) Crystal Violet Tetrazolium Agar yielded the highest % detection for

Table 3.1. Percent detection of hygiene indicator organism groups on Crystal Violet Tetrazolium Agar (CVTA), Violet Red Bile Glucose Agar (VRBGA; Becton Dickinson, Sparks, MD), *Enterobacteriaceae* (EB) Petrifilm (3M, St. Paul, MN), the D-Count (bioMérieux, Marcy-l'Étoile, France), and Coliform Petrifilm (3M)

Detection Method	% Coliforms Detected (n=42)[1]	% *Enterobacteriaceae* Detected (n=54)[2]	% Gram-Negative Bacteria Detected (n=60)[3]
CVTA	98	83	80
VRBGA	90	91	82
EB Petrifilm	93	94	85
D-Count	90	93	83
Coliform Petrifilm	93	72	65

[1]42 isolates were classified as coliforms based on positive results when pure culture was tested on Coliform Petrifilm.
[2]54 isolates were classified as EB based on sequencing of partial 16S rDNA.
[3]Of the total 64 Gram-negative isolates tested, 60 isolates survived the 12 h hold in Greek yogurt and were detected using one or more testing method. Isolates were classified as Gram-negative organisms based on sequencing of partial 16S rDNA.

coliforms (98%; 41/42 isolates; Table 3.1). Among the 60 isolates, 54 represented EB; this includes all 42 coliform isolates. Crystal Violet Tetrazolium Agar and Coliform Petrifilm yielded positives results with 83% (45/54) and 72% (39/54), respectively, of the EB isolates inoculated into Greek yogurt. By comparison, the EB specific detection methods (i.e., EB Petrifilm, VRBGA, and the D-Count) all detected > 90% of the EB isolates tested (Table 3.1). Among the total isolate set of 60 Gram-negatives, EB Petrifilm showed the highest % detection (85%; 51/60). The remaining tests were less successful at detecting the 60 Gram-negative isolates that survived the hold period with % detection ranging from 65% (Coliform Petrifilm) to 83% (D-Count; Table 3.1). Notably, CVTA only detected 80% (48/60) of the Gram-negative isolates; the 12 isolates not detected with CVTA included 1 coliform, 8 non-coliform EB, and 3 non-EB Gram-negative isolates.

All Methods Evaluated Showed 100% Specificity and Detected ≥ 80% of Their Respective Target Organisms

The sensitivity (true positive rate) and specificity (true negative rate) of each test was evaluated using data for 60 Gram-negative isolates (which included 42 coliforms and 54 EB), as detailed in the previous section (Table 3.2). Observed sensitivities of the detection methods ranged from 80 to 94 % (Table 3.2). Coliform Petrifilm, the only coliform detection method evaluated, detected 93% (39/42) of the coliforms inoculated into Greek yogurt. All 3 EB detection methods exhibited comparable results with observed sensitivities of 91% (49/54), 93% (50/54), and 94% (51/54) for VRBGA, the D-Count, and EB Petrifilm, respectively (Table 3.2). While EB Petrifilm and VRBGA require 24 h time-to-results, the D-Count delivers results in 13 h, indicating the potential for more rapid time-to-result with flow cytometry-based methods. Crystal Violet Tetrazolium Agar, the only detection method for total Gram-negative organisms, had the lowest sensitivity of the 5 methods evaluated (80%; 48/60; Table 3.2). No false positive test results were found for any of the detection methods evaluated, indicating 100% specificity with the isolates tested (Table 3.2). By comparison, a previous pure culture study of a larger isolate set found lower specificities for some methods (i.e., 89% and 92 % for EB Petrifilm and VRBGA, respectively; Hervert et al. 2016).

Overall, 4 methods exhibited reduced sensitivities for target organisms inoculated into Greek yogurt when compared to those expected based on pure culture testing for the same isolates (Hervert et al., 2016). Crystal Violet Tetrazolium Agar

Table 3.2. Expected and observed sensitivities and specificities for Crystal Violet Tetrazolium Agar (CVTA), Violet Red Bile Glucose Agar (VRBGA; Becton Dickinson, Sparks, MD), *Enterobacteriaceae* (EB) Petrifilm (3M, St. Paul, MN), the D-Count (bioMérieux, Marcy-l'Étoile, France), and Coliform Petrifilm (3M)

	Sensitivity (%)		Specificity (%)	
Detection Method	Observed[1]	Expected[2]	Observed[1]	Expected[2]
CVTA	80[3]	87[3]	N/A	N/A
VRBGA	91[4]	93[4]	100[6]	100[6]
EB Petrifilm	94[4]	100[4]	100[6]	100[6]
D-Count	93[4]	89[4]	100[6]	100[6]
Coliform Petrifilm	93[5]	100[5]	100[7]	100[7]

[1]Observed results in inoculated yogurt tests.
[2]Expected results for inoculated yogurt tests based on pure culture results.
[3]Total isolates detected using one or more testing method included in the calculation (n = 60).
[4]EB isolates included in the calculation (n = 54).
[5]Coliform isolates included in the calculation (n = 42).
[6]Non-EB isolates detected using one or more testing method included in the calculation (n = 6).
[7]Non-coliform isolates detected using one or more testing method included in the calculation (n = 18)

and Coliform Petrifilm showed the largest difference between expected and observed sensitivities with decreases of 7 percentage points, respectively (Table 3.2). Discrepancies between expected and observed sensitivities represented 16 instances of unexpected negative test results across each of the 5 detection methods (Supplemental Table 3.1). Unexpected negative results represented either (i) isolates that yielded positive results when tested in pure culture, but did not exhibit growth or detection when tested in Greek yogurt (11 isolates; Supplemental Table 3.1), or (ii) isolates that yielded positive results with typical colony characteristics when tested in pure culture, but exhibited atypical colony characteristics yielding negative results when tested in Greek yogurt (5 isolates; Supplemental Table 3.1). The 11 instances of unexpected absence of growth occurred on CVTA (2/11), Coliform Petrifilm (3/11), EB Petrifilm (4/11), VRBGA (1/11), and the D-Count (1/11). The 5 instances of unexpected atypical colony characteristics included 3 *Salmonella* isolates (FSL A5-0214, A5-

0218, and A5-0285), one *Yersinia* isolate (FSL L6-0049), and one *Rahnella* isolate (FSL P4-0879) that exhibited colorless colonies rather than red colonies on CVTA. The reduced recovery of injured cells following exposure to stress conditions is a well-documented occurrence that is commonly encountered with selective and differential media types (Ray, 1986; Smith et al., 2013). We hypothesize that the lack of recovery or typical colony characteristics on selective and differential media may be attributed to cell injury induced from the low pH environment of the yogurt.

Surprisingly, 3 isolates (*Yersinia* FSL R5-0761 and R5-0600; *Plesiomonas* FSL Y1-0254) showed positive test results on the D-Count when tested from Greek yogurt, even though they tested negative on the D-Count in pure culture (Hervert et al., 2016); these instances lead to the D-count method showing a higher observed sensitivity for the testing of isolates inoculated into Greek yogurt, as compared to the expected sensitivity based on pure culture testing (Table 3.2). We hypothesize that these isolates may benefit from the 1g addition of yogurt to the enrichment broth, thus allowing them to grow to detectible levels when tested on the D-Count. Additionally, one *Acinetobacter* isolate (FSL C4-0087) grew with atypical colony characteristics on CVTA when tested in pure culture, but grew with typical red colonies when tested in Greek yogurt.

CONCLUSIONS

Our data indicate that Gram-negative organisms show a range of survival capabilities over a 12 h incubation in Greek yogurt. While presence of any Gram-negative bacteria in commercially produced yogurt indicates post-pasteurization

contamination (or, much less likely, pasteurization failure), our data indicate that EB testing methods allow for sensitive detection that yields positive results with a majority of the genera likely encountered in low pH, fermented dairy products. The fact that *Pseudomonas* spp. do not present positive results with EB detection methods represents a concern for higher pH dairy products (fresh cheeses, fluid milk) where *Pseudomonas* spp. are a considerable spoilage concerns. However, use of methods that do not detect *Pseudomonas* is less of a concern in yogurt since *Pseudomonas* seem to show rapid die-off and are not linked to spoilage issues. Overall, the use of EB testing methods appears to provide an improved approach for monitoring the hygienic status of yogurt.

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CHAPTER 4

CONCLUSIONS

The goal of my studies as described in chapters 2 and 3 was to (i) provide new information on the use of coliforms, Enterobacteriaceae (EB), and total Gram-negatives as microbial hygiene indicator organisms in the dairy industry, (ii) evaluate detection methods for each group of organisms, and (iii) evaluate the survival and detection of Gram-negative organisms in a low pH, fermented dairy product.

To accomplish these goals, I first highlighted the shortcomings of coliform detection methods in their inability to detect a wide range of bacterial post-pasteurization contaminants responsible for fluid milk spoilage and sensory defects. My data in Chapter 2 showed that Coliform Petrifilm only detected 61% of the 211 total Gram-negative isolates tested and missed key genera with relevance to the dairy industry. Among the isolates that went undetected on Coliform Petrifilm were EB organisms with food safety significance (i.e., *Salmonella* and *Yersinia*), as well as non-EB Gram-negative genera representing approximately 75% of the post-pasteurization contamination of fluid milk (i.e. *Pseudomonas*, *Acinetobacter*, and *Flavobacterium*; Ranieri and Boor, 2009). The EB testing methods (i.e., EB Petrifilm, VRBGA, and D-Count) evaluated in Chapter 2 detected ≥ 90% of the 175 EB isolates and up to 100% of the coliform isolates tested. Specifically, EB Petrifilm showed the highest sensitivity for EB organisms (96%) while the D-Count showed the highest specificity (100%) for this same group. Although EB testing methods detected a number of genera that went undetected on Coliform Petrifilm (e.g., *Salmonella* and

Yersinia) and up to 82% of the total 211 isolates tested in Chapter 2, CVTA detected the greatest number of Gram-negative genera associate with post-pasteurization contamination. Notably, CVTA was the only method that detected *Pseudomonas*, the most commonly isolated Gram-negative genus from pasteurized fluid milk (Ranieri and Boor, 2009). My results in Chapter 2 indicate that adoption of a total Gram-negative test, such as CVTA, would greatly benefit fluid milk processors aiming to better evaluate the quality of their products and maximize detection of post-pasteurization contamination. However, the development of a more rapid and sensitive detection method for total Gram-negative is critical to facilitate adoption in industry settings. Advanced flow cytometry-based technologies, such as the D-Count, show promise for use in the dairy industry through their detection of microbial hygiene indicators up to 11 h sooner than competing media-based methods.

The results in Chapter 3 indicate that optimal microbial hygiene indicator groups differ depending on the dairy product undergoing evaluation. While non-EB Gram-negative organisms (e.g. *Pseudomonas*) represent the primary bacterial post-pasteurization contaminants of fluid milk, my results highlight that representative isolates in this group showed >1 log reduction in numbers over a 12 h hold period in Greek Yogurt. On the contrary, 70% of the EB isolates survived the hold period in Greek Yogurt with <0.5 log increases or decreases in bacterial numbers. Additionally, all 3 EB detection methods (i.e., EB Petrifilm, VRBGA, and the D-Count) showed sensitivities ranging from 91% to 94%. Thus, the results in Chapter 3 suggest that EB testing methods allow for sensitive detection that yields positives results with a

majority of the Gram-negative genera that likely survive in low pH, fermented dairy products.

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APPENDIX

Supplemental Table 2.1. Genus identification, isolation source, and test interpretation results for the 211 study isolates on Brain Heart Infusion (BHI) Agar, Crystal Violet Tetrazolium Agar (CVTA), Enterobacteriaceae (EB) Petrifilm, the D-Count, and Coliform Petrifilm

Genus	FSL #	Isolation Source	BHI (cfu/Plate)	CVTA (cfu/Plate)	EB Petrifilm (cfu/Plate)	VRBGA (cfu/Plate)	D-Count (Counts/mL)	Coliform Petrifilm[1] (cfu/Plate)
Acinetobacter	J3-0127	Pasteurized Milk	157	0	0	0	0	0
	R5-0198	Pasteurized Milk	46	12	0	0	0	0
	R5-0599	Pasteurized Milk	89	0	0	0	0	0
	W5-0630	Raw Milk	462	546	0	639	0	0
	A5-0068	Cheese	15	119	0	0	0	0
	C4-0013	Pasteurized Milk	262	0	0	0	0	0
	C4-0023	Pasteurized Milk	2,904	0	0	0	0	0
	C4-0087	Pasteurized Milk	56	0	0	0	0	0
	C4-0085	Pasteurized Milk	7	0	0	0	0	0
Aeromonas	C4-0004	Pasteurized Milk	16	5	0	0	0	0
	C4-0005	Pasteurized Milk	12	2	0	0	0	0
	C4-0059	Pasteurized Milk	209	37	8	44	0	0
	C4-0061	Pasteurized Milk	210	50	8	32	0	0
	L1-0014	Unspecified	63	46	0	0	0	0
	R5-0758	Pasteurized Milk	56	44	8	0	0	0
	R5-0759	Pasteurized Milk	53	37	3	0	0	0
Brevundimonas	J3-0142	Pasteurized Milk	191	140	0	0	0	0
	C4-0006	Pasteurized Milk	245	0	0	0	0	0
	C4-0012	Pasteurized Milk	1,889	0	0	0	0	0
	C4-0016	Pasteurized Milk	107	0	0	0	0	0
	C4-0057	Pasteurized Milk	148	0	0	0	0	0
	C4-0010	Pasteurized Milk	18	0	0	0	0	0
***Buttiauxella* (EB)**	J3-0007	Pasteurized Milk	85	85	23	78	5,785	19
	P4-0809	Pasteurized Milk	70	66	14	60	589	23
	W4-0259	Pasteurized Milk	91	79	39	73	7,608	46
	J3-0009	Pasteurized Milk	82	87	25	84	7,631	28
	P4-0876	Pasteurized Milk	52	56	51	51	390	54
	P4-0920	Pasteurized Milk	68	71	0	64	1,427	0
	P4-0923	Pasteurized Milk	91	88	0	67	2,881	0
	P4-0927	Pasteurized Milk	81	66	0	69	1,214	0
	P4-0921	Pasteurized Milk	80	84	0	59	584	0

	P4-0922	Pasteurized Milk	60	82	0	57	1,468	0
***Cedecea* (EB)**	A5-0474	Environment, Food or Food; Dairy	243	216	146	200	486,694	166
	A6-0012	Environment, Food or Food; Dairy	204	193	124	177	465,906	144
	A6-0007	Environment, Food or Food; Dairy	216	200	142	174	559,598	156
	A6-0123	Environment, Food or Food; Dairy	179	185	118	166	713,288	156
	A6-0091	Environment, Food or Food; Dairy	219	228	142	198	333,153	178
	A6-0106	Environment, Food or Food; Dairy	173	178	133	165	604,189	143
	J3-0055	Pasteurized Milk	242	231	172	171	813,705	187
	W4-0284	Pasteurized Milk	275	273	191	264	619,579	219
	W7-2260	Pasteurized Milk	185	162	127	142	583,304	137
	A6-0209	Environment, Food or Food; Dairy	223	239	202	220	1,620,977	215
***Citrobacter* (EB)**	W4-0276	Pasteurized Milk	106	91	74	106	593,243	98
	A5-0438	Environment, Food or Food; Dairy	117	102	90	104	444,946	102
	A6-0107	Environment, Food or Food; Dairy	136	148	100	130	797,104	110
	A6-0122	Environment, Food or Food; Dairy	146	122	114	128	881,267	101
	A5-0459	Environment, Food or Food; Dairy	162	147	104	126	540,901	121
	A6-0116	Environment, Food or Food; Dairy	127	134	89	121	258,674	110
	J3-0001	Pasteurized Milk	132	144	103	137	489,196	129
	P4-0745	Pasteurized Milk	38	134	71	111	257,609	73
	W4-0256	Pasteurized Milk	126	161	126	142	810,133	120
	A6-0142	Environment, Food or Food; Dairy	160	163	135	116	341,886	136
	A6-0144	Environment, Food or Food; Dairy	143	135	126	160	428,854	108
	A6-0146	Environment, Food or Food; Dairy	137	126	110	154	464,464	118

	A6-0196	Environment, Food or Food; Dairy	148	138	139	134	283,204	153
	A6-0211	Environment, Food or Food; Dairy	106	67	76	0	917	71
***Cronobacter* (EB)**	F6-0048	Infant Formula	160	138	103	87	1,520,271	110
	F6-0042	Infant Formula	147	131	137	116	857,767	131
	F6-0032	Environment, Food	155	144	131	131	1,482,590	135
	F6-0037	Environment, Food	164	136	128	132	450,375	135
	F6-0038	Environment, Food	117	150	117	131	1,163,237	114
	F6-0034	Human, Clinical	18	19	11	12	296,312	6
	F6-0046	Infant Formula	160	145	109	137	1,282,641	120
	F6-0031	Infant Formula	159	137	88	132	1,005,054	108
	F6-0030	Infant Formula	95	91	69	79	317,081	0
	F6-0024	Infant Formula	66	81	76	76	811,105	79
	F6-0025	Environment, Food	152	144	124	135	942,924	112
	F6-0036	Environment, Food	175	130	128	130	1,401,649	121
	F6-0040	Environment, Food	128	110	98	100	1,233,350	104
	F4-0044	Environment, Food	127	113	122	99	826,945	145
***Enterobacter* (EB)**	A6-0025	Environment, Food or Food; Dairy	90	105	32	84	2,505	51
	J3-0010	Pasteurized Milk	178	156	151	160	765,252	163
	J3-0108	Pasteurized Milk	160	177	131	168	813,370	153
	P4-0777	Pasteurized Milk	61	96	52	65	34,463	60
	P4-0865	Pasteurized Milk	88	95	44	82	1,205	76
	P4-0933	Pasteurized Milk	112	135	74	112	17,849	100
	P4-0993	Pasteurized Milk	87	91	52	83	3,046	48
	R5-0458	Pasteurized Milk	132	130	59	124	9,168	97
	R5-0495	Pasteurized Milk	90	118	93	106	61,154	108
	R5-0535 (Lelliottia)	Pasteurized Milk	71	74	32	57	120	0
	A5-0465	Environment, Food or Food; Dairy	147	159	109	134	1,228,745	125
	A6-0155	Environment, Food or Food; Dairy	170	163	143	159	481,248	133
***Escherichia* (EB)**	A6-0028	Environment, Food or Food; Dairy	171	160	151	199	796,338	161
	A6-0054	Environment, Food or Food; Dairy	184	180	161	226	340,578	154

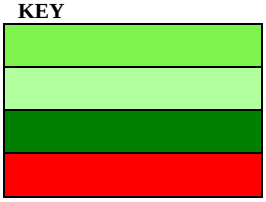
	A6-0037	Environment, Food or Food; Dairy	179	212	164	211	443,363	155
	A6-0059	Environment, Food or Food; Dairy	52	0	0	0	0	0
	A6-0088	Environment, Food or Food; Dairy	68	0	0	0	0	0
	W5-0777	Pasteurized Milk	126	135	123	136	442,812	128
	W5-0782	Pasteurized Milk	136	127	120	128	620,897	125
	W6-0367	Laboratory Heat Treated Raw Milk	155	156	135	132	417,584	121
	W7-0391	Raw Milk	170	172	145	137	826,530	175
	A6-0295	Environment, Food or Food; Dairy	207	195	193	222	1,672,646	213
	B1-0009	Unspecified	197	0	153	189	1,740,048	174
	B1-0010	Unspecified	150	0	117	144	405,956	120
Flavobacterium	R5-0497	Pasteurized Milk	6	0	0	0	0	0
	R5-0610	Pasteurized Milk	13	0	0	0	0	0
	W6-0716	Raw Milk	12	0	0	0	0	0
***Hafnia* (EB)**	A5-0001	Pasteurized Milk	282	260	224	286	432,731	233
	A5-0019	Pasteurized Milk	250	258	209	263	436,307	214
	J3-0049	Pasteurized Milk	157	159	147	176	260,005	141
	J3-0050	Pasteurized Milk	166	177	139	169	316,999	155
	P4-0747	Pasteurized Milk	214	217	146	238	282,632	172
	P4-0833	Pasteurized Milk	81	63	76	72	0	66
	P4-0805	Pasteurized Milk	222	212	168	224	374,963	198
	W4-0260	Pasteurized Milk	120	131	122	142	225,023	138
	W4-0268	Pasteurized Milk	204	213	146	228	436,920	184
	W4-0287	Pasteurized Milk	197	205	182	206	619,940	203
***Klebsiella* (EB)**	A6-0140	Environment, Food or Food; Dairy	109	101	84	93	969,163	80
	R7-0084	Pasteurized Milk	157	134	130	139	723,439	118
	W5-0288	Cheese	106	107	84	101	1,033,571	111
	W5-0290	Cheese	89	74	56	73	486,582	79
	W6-0554	Raw Milk	144	127	135	134	595,018	129
	W7-0463	Raw Milk	86	82	76	87	891,815	92
	C1-0024	Pasteurized Milk	131	106	104	119	697,887	119
	C1-0026	Pasteurized Milk	140	118	137	105	663,189	133
	W7-0500	Raw Milk	136	113	107	126	1,306,009	116
	W7-0630	Raw Milk	128	107	95	96	687,411	103

	A6-0154	Environment, Food or Food; Dairy	144	126	95	125	515,770	131
***Kluyvera* (EB)**	A6-0105	Environment, Food or Food; Dairy	89	71	80	66	112,979	90
	A6-0100	Environment, Food or Food; Dairy	154	128	48	133	445,596	104
	J3-0002	Pasteurized Milk	130	102	102	123	992,193	88
	J3-0083	Pasteurized Milk	247	248	320	296	639,753	380
	P4-0743	Pasteurized Milk	117	128	83	126	994,175	93
	P4-0827	Pasteurized Milk	162	175	126	179	1,036,159	125
	P4-0848	Pasteurized Milk	180	205	114	179	961,513	129
	W6-0858	Raw Milk	124	139	115	151	946,570	110
	W4-0248	Pasteurized Milk	103	119	69	112	985,609	79
***Leclercia* (EB)**	J3-0041	Pasteurized Milk	33	46	29	35	253,494	27
	J3-0042	Pasteurized Milk	35	48	29	55	253,288	44
***Pantoea* (EB)**	R5-0463	Pasteurized Milk	133	141	76	123	3,619	84
	R5-0559	Pasteurized Milk	121	233	155	228	18,473	0
	A5-0012	Pasteurized Chocolate Milk	194	303	197	333	41,827	0
	P4-0759	Pasteurized Milk	68	121	65	119	578	0
	P4-0760	Pasteurized Milk	71	97	68	101	638	0
	P4-0761	Pasteurized Milk	93	141	90	161	718	0
	P4-0762	Pasteurized Milk	91	165	93	151	688	0
	P4-0767	Pasteurized Milk	96	184	110	161	2,362	0
	P4-0768	Pasteurized Milk	102	93	65	87	478	0
***Plesiomonas* (EB)**	Y1-0254	Unspecified	39	0	12	0	0	0
***Proteus* (EB)**	A5-0110	Cheese	236	159	111	183	396,866	0
	A5-0127	Cheese	85	55	33	19	129,982	0
	R9-2925	Raw Milk	284	0	192	160	594,252	0
Pseudomonas	W5-0203	Cheese	8	16	0	0	0	0
	W7-0098	Pasteurized Milk	26	50	0	0	0	0
	R5-0202	Pasteurized Milk	42	43	0	0	0	0
	R5-0318	Pasteurized Milk	121	141	0	0	0	0
	A5-0017	Pasteurized Chocolate Milk	43	33	0	0	0	0
	C4-0037	Pasteurized Milk	22	24	0	0	0	0
	C4-0015	Pasteurized Milk	128	57	0	0	0	0
	F4-0169	Pasteurized Milk	57	0	0	0	0	0
	F4-0175	Pasteurized Milk	95	0	0	0	0	0
	F4-0290	Pasteurized	16	35	0	0	0	0

		Milk						
***Rahnella* (EB)**	W4-0273	Pasteurized Milk	101	117	69	76	684,937	79
	A5-0020	Pasteurized Milk	52	52	29	0	0	0
	J3-0059	Pasteurized Milk	56	41	144	0	15,936	144
	P4-0832	Pasteurized Milk	47	51	27	0	0	0
	P4-0879	Pasteurized Milk	52	73	40	0	0	49
	W5-0312	Pasteurized Milk	64	58	46	0	0	63
	W5-0313	Pasteurized Milk	72	59	45	0	0	50
	W5-0314	Pasteurized Milk	48	66	48	0	0	38
	W6-0365	Laboratory Heat Treated Raw Milk	109	87	48	0	0	0
	W6-0476	Laboratory Heat Treated Raw Milk	101	76	80	86	1,257	0
***Raoultella* (EB)**	A5-0481	Environment, Food or Food; Dairy	14	15	10	14	858,681	9
	A6-0118	Environment, Food or Food; Dairy	22	17	8	15	589,895	10
	A5-0482	Environment, Food or Food; Dairy	134	119	97	101	1,099,475	86
	A6-0098	Environment, Food or Food; Dairy	160	158	122	154	613,585	139
	J3-0072	Pasteurized Milk	118	122	95	113	842,244	108
	J3-0082	Pasteurized Milk	99	106	59	60	5,898	75
	J3-0090	Pasteurized Milk	118	127	90	141	677,219	116
	P4-0748	Pasteurized Milk	101	86	91	46	1,000,556	100
	P4-0976	Pasteurized Milk	205	176	67	182	16,483	87
	A5-0472	Pasteurized Milk	200	144	132	152	949,659	152
	W4-0272	Environment, Food or Food; Dairy	128	112	78	110	688,053	101
	W5-0289	Cheese	83	72	60	0	0	60
***Salmonella* (EB)**	A5-0214	Environment, Food or Food; Dairy	140	152	134	159	869,714	0
	A5-0218	Raw Milk	171	181	119	186	1,394,570	0
	A5-0285	Environment, Food or Food; Dairy	163	165	113	156	1,188,465	0
	A5-0272	Raw Milk	166	133	110	182	1,318,920	0
	A5-0288	Environment, Food or Food; Dairy	171	161	138	170	1,418,948	0
	A5-0290	Environment, Food or Food; Dairy	167	160	122	198	1,296,157	0

	A5-0295	Environment, Food or Food; Dairy	150	154	132	167	919,130	0
	A5-0299	Environment, Food or Food; Dairy	151	145	99	151	761,615	0
	A5-0306	Environment, Food or Food; Dairy	151	0	116	164	468,021	0
***Serratia* (EB)**	A5-0004	Pasteurized Milk	255	259	205	249	182,105	230
	A5-0021	Pasteurized Milk	232	235	203	222	249,029	234
	A5-0108	Cheese	209	171	173	177	34,257	163
	A5-0123	Cheese	217	174	170	195	25,524	191
	A5-0135	Cheese	203	168	163	195	41,752	164
	P4-0811	Pasteurized Milk	146	119	86	0	0	110
	P4-0836	Pasteurized Milk	135	134	132	142	10,504	118
	P4-0843	Pasteurized Milk	116	92	100	0	0	87
	P4-0889	Pasteurized Milk	111	98	82	0	0	65
	P4-0970	Pasteurized Milk	152	129	117	0	239	137
***Shigella* (EB)**	W5-0878	Raw Milk	155	0	119	122	496,697	122
	W6-0625	Raw Milk	175	0	143	184	240,208	140
	W6-0732	Raw Milk	260	202	166	183	815,558	165
	W7-0216	Raw Milk	191	146	141	176	1690753	166
	C7-1309	Unspecified	120	0	81	115	104,837	0
	C7-1310	Unspecified	62	0	48	49	200,183	0
	W6-0725	Raw Milk	153	133	138	133	1,006,981	143
Vibrio	A5-0095	Cheese	*TNTC*	0	0	0	0	0
***Yersinia* (EB)**	L6-0049	Pasteurized Milk	115	132	96	88	1,095	0
	R5-0595	Pasteurized Milk	110	0	72	104	0	0
	R5-0721	Pasteurized Milk	111	0	59	88	0	0
	R5-0724	Pasteurized Milk	116	0	65	100	0	0
	R5-0756	Pasteurized Milk	126	0	68	108	0	0
	R5-0762	Pasteurized Milk	95	0	57	124	0	0
	R5-0761	Pasteurized Milk	97	0	66	100	0	0
	R5-0722	Pasteurized Milk	87	0	64	96	0	0
	R5-0600	Pasteurized Milk	88	0	57	124	0	0
	R5-0725	Pasteurized Milk	112	0	77	100	0	0

KEY



Positive Result

EB Petrifilm Positive Result (Acid Production)

EB Petrifilm Positive Result (Acid and Gas Production)

Negative Result

Supplemental Table 3.1. Genus identification, isolate source, detection method counts, and test interpretation results for the 64 study isolates on Crystal Violet Tetrazolium Agar (CVTA), Violet Red Bile Glucose Agar (VRBGA; Becton Dickinson, Sparks, MD), *Enterobacteriaceae* (EB) Petrifilm (3M, St. Paul, MN), the D-Count (bioMérieux, Marcy-l'Étoile, France), and Coliform Petrifilm (3M)

Genus	FSL #	Isolate Source	cfu/g in Yogurt	CVTA Plate Count Average (cfu/g)	Coliform Petrifilm Count Average (cfu/g)	EB Petrifilm Count Average (cfu/g)	VRBGA Plate Count Average (cfu/g)	D-Count Average (Counts/mL)
Buttiauxella (Coliform)	W4-0259	Pasteurized Milk	449	120	< 10	< 10	40	1,038
	J3-0009	Pasteurized Milk	490	20	< 10	< 10	100	1,868
	J3-0007	Pasteurized Milk	495	80	< 10	< 10	< 20	5,533
Cedecea (Coliform)	A5-0474	Environment, Food or Food; Dairy	765	680	340	495	300	1,476,139
	A6-0007	Environment, Food or Food; Dairy	786	240	335	340	240	898,260
	A6-0012	Environment, Food or Food; Dairy	782	400	355	465	300	862,366
Citrobacter (Coliform)	A6-0107	Environment, Food or Food; Dairy	546	140	100	110	60	763,762
	A6-0122	Environment, Food or Food; Dairy	430	520	340	415	500	495,565
	J3-0001	Pasteurized Milk	582	780	385	500	520	402,345
Cronobacter (Coliform)	F6-0048	Infant Formula	910	80	80	70	40	732,128
	F6-0042	Infant Formula	852	120	130	125	80	689,476
	F6-0046	Infant Formula	977	220	315	325	140	865,044
Enterobacter (Coliform)	A6-0025	Environment, Food or Food; Dairy	502	100	105	115	80	20
	J3-0010	Pasteurized Milk	735	340	270	265	300	1,196,730
	R5-0495	Pasteurized Milk	589	560	365	390	480	485,891
Escherichia (Coliform)	A6-0028	Environment, Food or Food; Dairy	1,050	1,260	1,295	1,225	1,300	884,513
	A6-0054	Environment, Food or Food; Dairy	1,030	920	980	935	1,060	1,044,188
	W5-0782	Pasteurized Milk	749	1,060	1,070	940	1,280	1,711,970
Hafnia (Coliform)	A5-0019	Pasteurized Milk	899	2,520	2,445	2,485	2,860	1,046,195
	J3-0049	Pasteurized Milk	751	2,100	1,405	1,495	1,560	1,144,463
	W4-0268	Pasteurized Milk	870	2,260	1,830	1,825	2,140	1,154,120
Klebsiella (Coliform)	A6-0140	Environment, Food or Food; Dairy	413	1,220	680	820	920	501,080
	C1-0024	Pasteurized Milk	424	1,140	1,155	1,225	1,440	1,065,489
	R7-0084	Pasteurized Milk	550	1,320	1,370	1,325	1,400	949,640
Kluyvera (Coliform)	A6-0105	Environment, Food or Food; Dairy	329	420	250	300	220	131,149
	A6-0100	Environment, Food or Food; Dairy	889	1,120	675	670	620	65,095
	P4-0827	Pasteurized Milk	575	1,780	1,285	1,315	1,680	768,235
Leclercia (Coliform)	J3-0041	Pasteurized Milk	330	320	345	335	200	584,280
	J3-0042	Pasteurized Milk	364	560	325	370	180	533,495
Pantoea (Coliform)	R5-0463	Pasteurized Milk	702	880	930	955	760	110,749
Rahnella	P4-0879	Pasteurized Milk	1,120	200	115	170	140	0

(Coliform)	W5-0312	Pasteurized Milk	1,400	220	150	175	180	0
	W5-0314	Pasteurized Milk	1,640	140	110	140	160	0
Raoultella (Coliform)	A5-0482	Environment, Food or Food; Dairy	480	480	485	590	400	703,829
	A6-0098	Environment, Food or Food; Dairy	696	880	775	670	520	946,064
	J3-0090	Environment, Food or Food; Dairy	545	620	310	415	500	887,885
Serratia (Coliform)	A5-0004	Pasteurized Milk	808	1,060	765	725	1,160	546,817
	A5-0021	Pasteurized Milk	927	1,240	915	920	1,280	668,358
	P4-0836	Pasteurized Milk	665	440	445	435	600	58,851
Shigella (Coliform)	W6-0732	Raw Milk	1,010	1,660	1,200	1,105	1,540	1,057,286
	W7-0216	Raw Milk	697	1,240	1,185	1,140	1,060	1,395,650
	W6-0725	Raw Milk	565	840	820	910	980	1,309,500
Pantoea (Non-Coliform EB)	A5-0012	Pasteurized Chocolate Milk	1,030	820	645	645	620	14,711
	P4-0767	Pasteurized Milk	No Count Data	180	160	160	100	970
Plesiomonas (Non-Coliform EB)	Y1-0254	Unspecified	854	< 20	105	65	280	1,174,413
Proteus (Non-Coliform EB)	A5-0110	Cheese	No Count Data	2,440	1,900	1,755	2,380	968,617
	A5-0127	Cheese	No Count Data	580	670	675	760	572,921
	R9-2925	Raw Milk	2,180	2,500	1,620	1,890	2,280	936,835
Salmonella (Non-Coliform EB)	A5-0214	Dairy Plant Environment	731	180	25	80	160	1,791,313
	A5-0218	Raw Milk	722	80	< 10	70	60	1,941,707
	A5-0285	Dairy Plant Environment	775	100	80	70	120	1,705,712
Yersinia (Non-Coliform EB)	L6-0049	Pasteurized Milk	588	660	310	245	460	2,935
	R5-0761	Pasteurized Milk	547	820	80	205	620	541
	R5-0600	Pasteurized Milk	508	760	< 10	165	460	436
Acinetobacter (Non-EB Gram-Negative)	C4-0013	Pasteurized Milk	1,840	180	< 10	< 10	< 20	10
	C4-0087	Pasteurized Milk	3,600	40	< 10	< 10	< 20	0
Aeromonas (Non-EB Gram-Negative)	C4-0005	Pasteurized Milk	24,200	< 20	< 10	< 10	< 20	15
	R5-0758	Pasteurized Milk	23,000	< 20	< 10	< 10	< 20	5
Brevundimonas (Non-EB Gram-Negative)	C4-0016	Pasteurized Milk	7,590	< 20	< 10	< 10	< 20	10
	C4-0057	Pasteurized Milk	13,200	< 20	< 10	< 10	< 20	0
Flavobacterium (Non-EB Gram-Negative)	R5-0497	Pasteurized Milk	252,000	Brown film-like growth that did not present as countable colonies	< 10	< 10	< 20	5
	R5-0610	Pasteurized Milk	698,000	Brown film-like growth that did not present as countable colonies	< 10	< 10	< 20	0
Pseudomonas	R5-0318	Pasteurized Milk	12,400	540	< 10	< 10	< 20	15

(Non-EB Gram-Negative)	W7-0098	Pasteurized Milk	7,620	80	< 10	< 10	< 20	0

KEY

	Expected positive; Growth with typical colony characteristics on differential media or > 100 counts/mL on the D-Count
	Unexpected positive; Growth with typical colony characteristics on differential media or > 100 counts/mL on the D-Count
	Expected negative; No growth on differential media or < 100 counts/mL on the D-Count
	Unexpected negative; No growth on differential media or < 100 counts/mL on the D-Count
	Expected negative; Growth without typical colony characteristics on differential media or < 100 counts/mL on the D-Count
	Unexpected Negative; Growth without typical colony characteristics on differential media or < 100 counts/mL on the D-Count