Verification of DuPont BAX Real-Time *Listeria monocytogenes* Detection Kit in Dark Chocolate Ice Cream

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Abstract

Listeria monocytogenes is a pathogen of great concern in the dairy industry and throughout the last several years has proven a deadly contaminate in ice cream specifically. In 2015, two major recalls of ice cream have occurred in the United States due to *Listeria monocytogenes* contamination. *Listeria* species are well adapted to persist within factory settings. Due to this adaptation, *Listeria* species are a concern not only in dairy manufacturing facilities but also in all ready-to-eat food manufacturing settings. Microbiological testing is performed and often identifies *Listeria,* specifically *Listeria monocytogenes,* on food contact surfaces and associated with raw ingredients. DuPont BAX has developed a new rapid *Listeria monocytogenes* Detection kit but has only performed validation on a few food matrices. This research is a verification study of the DuPont BAX Real-Time *Listeria monocytogenes* kit in dark chocolate ice cream. Verification on this detection kit could significantly speed up the time to a result for the frozen dairy industry on problematic food matrices.

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1. Introduction

A. Background

Listeria monocytogenes is a motile, Gram positive, rod-shaped bacteria. *Listeria monocytogenes* was first described in 1926 by E.G.D. Murray, but was not found to cause foodborne illness until 1981 (Ottesen, et. al., 2016). *Listeria monocytogenes* is an ubiquitous organism found in soil, water, and in decaying plant material that can survive for long periods of time on surfaces commonly found in food manufacturing environments. *Listeria* species are very hardy and can survive, even thrive, at refrigeration temperatures but are easily killed by heating (Buchanan, et. al. 2017).

Foods are considered the main vehicle of which *Listeria* spp. cause disease, with an estimated 99% of listeriosis cases in the United States being caused by contaminated food (Buchanan, et. al. 2017). *Listeria monocytogenes* is the etiologic agent of listeriosis, a serious infection that primarily affects the elderly, pregnant women, babies, and the immunocompromised. Symptoms of listeriosis include fever and muscle aches, with or without gastrointestinal problems or diarrhea. As the infection persists, Listeriosis can lead to septicemia, meningitis, and in pregnant women, spontaneous abortion or death of the fetus. Listeriosis has a mortality rate of about 24%. The Center for Disease Control (CDC) estimates that about 1600 people become ill and 240 people die per year in the United States due to listeriosis (CDC, 2017).

B. Statement of Problem

The Frozen dairy industry was not until recently a major cause for concern in the realm of food safety. Since the development of pasteurization, a process that involves heating to kill microbiological pathogens, in 1864 and its use in dairy beginning in the early 20th century, dairy and frozen dairy products have effectively been risk reduced. However, several major recalls in the last two years due to *Listeria* contamination have made it evident that no food is completely “safe” from foodborne

pathogens. Thus, rapid methods need to be developed to detect foodborne pathogens in these food matrices, with particular concern to *L. monocytogenes*.

Recent studies have focused on cold adaption of *L. monocytogenes*, which allows the bacteria to survive in frozen dairy products such as ice cream. Cold adapted *Listeria monocytogenes* survived 332 days while non-cold adapted cells survived 182 days (Mastronicolis, et. al., 2011). Interestingly, the researchers found that survival of the *L. monocytogenes* cells is influenced by the ingredients used in the ice cream (Mastronicolis, et. al., 2011). The researchers found that ice cream produced using corn syrup and sesame paste had a lower water activity which may have been a barrier to the viability of the cold-adapted *L. monocytogenes* cells. In contrast, ice cream produced with fructose did not prove anti-microbial at all (Mastronicolis, et. al., 2011).

In 2015, the first major outbreak in recent years of Listeriosis in the United States occurred as a result of ice cream contaminated with *L. monocytogenes* from Blue Bell Creameries. In total, 10 people were infected with *Listeria* related to Blue Bell branded products across four states, three of which died in Kansas. Using a national network known as PulseNet, the CDC was able to identify the outbreak and use pulse-field gel electrophoresis and whole genome sequencing to "DNA fingerprint" the strains of *L. monocytogenes*. The "DNA fingerprints" were then used to match strains isolated from patients who fell ill with strains isolated from Blue Bell ice cream samples. In April of 2015, after the CDC had linked the strains using whole genome sequencing, Blue Bell Creameries issued a voluntary recall of all products that were distributed in 23 states across the United States. The FDA performed inspections of three Blue Bell Creameries manufacturing facilities reporting that a breakdown of sanitation and cleaning procedures, repair and maintenance of the facility itself, and an overall failure to set controls to produce food in a safe manner as to prevent microbial contamination occurred at all three facilities (CDC Report, 2015).

On April 23, 2015, another ice cream manufacturer, Jeni's Splendid Ice Creams, issued a voluntary recall on all products due to a possible health risk from *L. monocytogenes*. The contamination was discovered as a result of random collection by the Nebraska Department of Agriculture. No human was reported ill due to the Jeni's Splendid Ice Cream contamination, but all stores and production facilities were closed at the time to investigate the contamination issue (Lowe, 2015). In August of 2016, the FDA inspected Jeni's Splendid Ice Cream production kitchen taking 75 environmental swabs as a follow-up to the previous year's recall. The FDA found two swabs that tested positive for *Listeria,* but no *L.monocytogenes* could be isolated from any product, therefore the FDA took no action. In total, *Listeria* issues have costed Jeni's Splendid Ice cream $2.7 million from the recall of 535,000 pounds of frozen dairy product (Malone, 2016).

Hazard Analysis and Critical Control Point (HACCP) programs become inherently important when working in dairy product production, specifically ice cream production. Since 2011 the CDC has documented 12 outbreaks of Listeriosis in the U.S., 7 of which involved dairy based foods (CDC, 2017). A study in 1996 found that 3-4% of all raw milk contains small numbers of *Listeria* species that could be killed by pasteurization of raw milk (Kozak, et. al., 1996). This means that the contamination of ice cream, as well as other dairy products, likely comes post-pasteurization. Recently it was reported that *L. monocytogenes* is impossible to permanently eradicate due to the ubiquitous nature of the organism (Buchanan, et. al., 2017). Further downstream of the manufacturing of the food, temperature abuse and fluctuations during transport and in retail setting must be addressed by any HACCP plan (Buchanan et. al., 2017). HACCP is a management system that is aimed to enhance food safety in food manufacturing operations. HACCP accesses all facets of the food production process to identify any biological, chemical, or physical factors that may render food unsafe. The HACCP program sets up critical control points in which the manufacturer can manage controllable hazards associated with

production of the food product. These critical control points are selected based on results of hazard analysis done by the manufacturer management team.

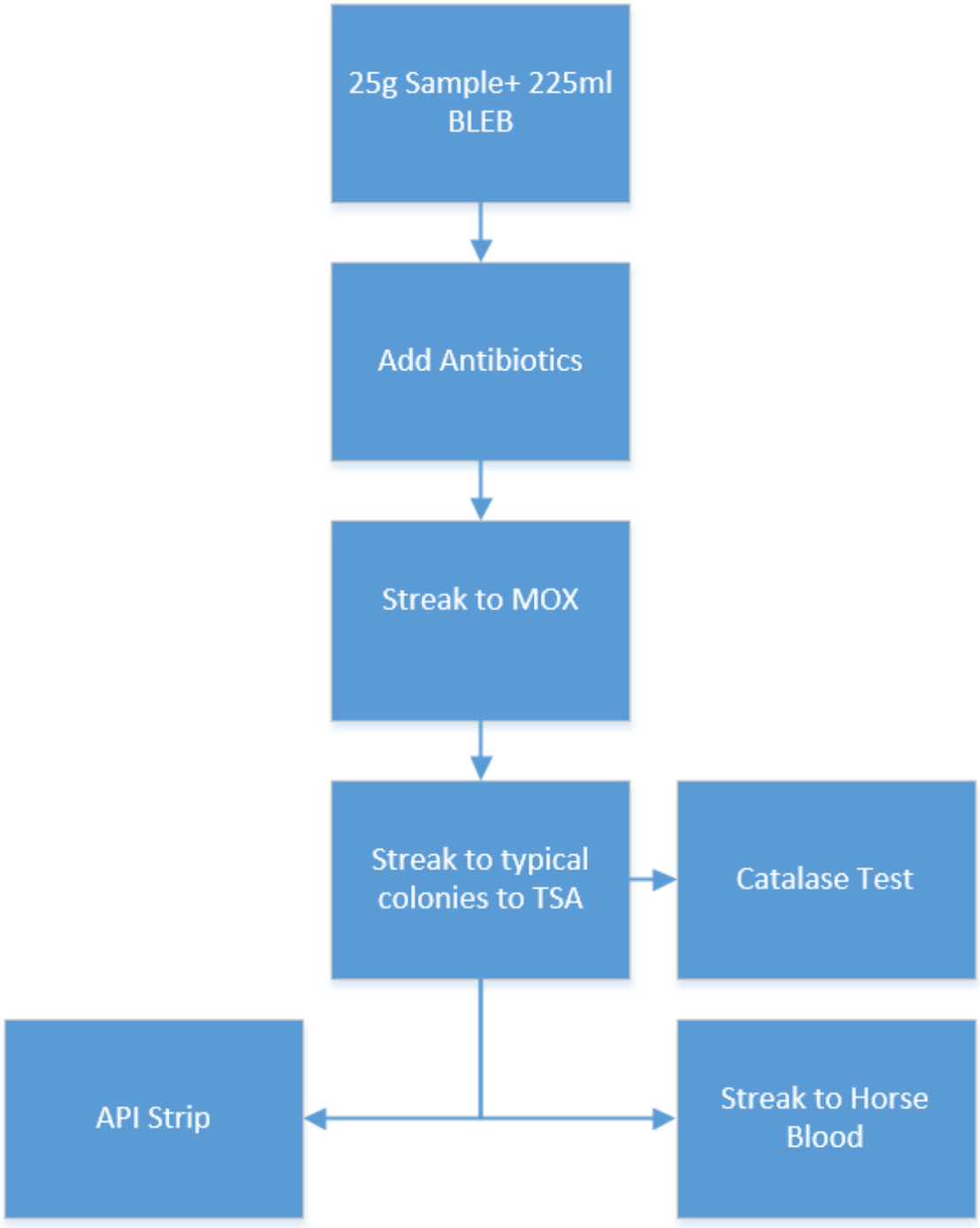
Implementation of a HACCP system in a chocolate ice cream plant was published in the Journal of Food and Drug Analysis (Lu, et. al., 2014). Specifically, this hazard analysis reported that the neutral pH profile of chocolate ice cream makes it a good growth medium for microorganisms. This places utmost importance on preventing microbial contamination when setting up critical control points for the manufacturing of chocolate ice cream. The critical control pasteurization, meaning controlling that the time and heat is long enough and hot enough to kill microorganisms, and freezing, making sure the food is cooled to a low enough temperature rapidly and thoroughly enough to not allow for growth of microorganisms and that the air in the freezer is not contaminated with bacteria. It was determined that by using these control points the manufacturing facility may mitigate the risk of microbial contamination. However, there remains a need for more rapid detection methods for pathogens, in order to ensure that these control points are serving their purpose in preventing contamination, particularly in difficult food matrices such as chocolate ice cream.

C. Review of Current Methods

The testing of microbiological pathogens in frozen dairy products fall under the regulation of the Food and Drug Administration (FDA). Chapter 10 in the FDA's Bacteriological Analytical Manual (FDA-BAM), entitled Detection and Enumeration of *Listeria monocytogenes* in Foods, hereinafter referred to as the Reference Method, is the current procedure used in the industry to test food products for *Listeria* contamination. The Reference Method involves culturing the microbe from foods using differential and selective media. The Reference Method uses Buffered *Listeria* Enrichment Broth (BLEB) as the enrichment media to which the sample is homogenized and incubated at 30˚C for 4 hours to allow the cells to repair before selection, then antibiotics acriflavin, sodium nalidixate and cylcoheximide are

added to the enrichment to inhibit growth of other food associated bacterium and incubated for another 48 hours at 30˚C. From the enrichment process, samples are streaked to MOX plates, a common differential and selective agar for *Listeria* species. Typical colonies are selected after incubation from the MOX plates. Typical colonies are gun-metal gray in color, small with a distinct dimple and produce an esculin reaction, turning the Modified Oxford Agar (MOX agar) black in the area in which they grow. The typical colonies are streaked to TSA plates to grow up for 24 hours. A catalase test is then performed by selecting several well isolated colonies using a cotton swab and hydrogen peroxide is added to the swab to test for the enzyme catalase which would cause the swab to form bubbles. All species of *Listeria* are catalase positive. All suspect colonies are then transferred to horse blood agar to test for hemolysis. Three types of hemolysis exist: alpha, gamma, and beta, but only colonies exhibiting beta and gamma are carried through the rest of the confirmation. Next, biochemical confirmation is done to confirm the species of the isolates. Biochemical confirmation can be done in a number of ways according to the FDA, however, Analytical Profile Index (API) strips produced by BioMerieux were used for rapid biochemical confirmation in this validation. (FDA, 2017).

Figure 1: Flowchart of Reference Method.



RapidChek *Listeria* species Test Kit is another platform in which frozen dairy products can be tested for *Listeria*. The RapidChek *Listeria* species Test Kit will only test for *Listeria* species and further

confirmation must be done after the screen test to determine if *L. monocytogenes* is the species present in the sample. The RapidChek kit uses Lateral Flow technology to determine the presence of *Listeria* in a sample. This immunoassay uses an antibody that is specific to *Listeria* on a line on the test strip membrane, this is the test line. A second antibody specific to the detection of *Listeria* is labelled with colloidal gold and is contained in the reagent pad upstream of the test line. As the sample, post-enrichment, moves up the pad by capillary action, the gold antibody binds to *Listeria* and moves with the sample to the test line where it binds to the other antibody in the test line forming an antibody-*Listeria* complex. When the antibody-*Listeria* complex is formed at the test line it causes the line to turn a red color if *Listeria* is present. A control line is placed upstream of the test line which turns red when the gold antibodies found downstream of the strip flow through it (see Figure 2). If both the control and test lines turn red on the RapidChek strip the sample can be interpreted as positive for *Listeria.* However, following a positive result through the RapidChek method, a FDA-BAM confirmation must take place. Confirmation begins from the second half of the Reference Method, one MOX plate is streaked and incubated, catalase and hemolysis are tested, followed by biochemical confirmation (SDIX, 2010).

Another immunoassay that is widely used is BioMereiux's VIDAS platform. The VIDAS or VITEK Immuno Diagnostic Assay Systems, detects antigens of *Listeria* or *Listeria monocytogenes* using an Enzyme-Linked Fluorescent Assay method performed in an automated system. There are several different test kits available on the VIDAS platform including *Listeria* species or *Listeria monocytogenes*. The VIDAS platform uses a strip of different wells, one of which contains antibodies that bind to antigens on *Listeria.* The antibodies are attached to a fluorescent marker that is cleaved during the last step of the process in the instrument and read to calculate a Relative Fluorescence Value (RFV). The RFV of the sample is divided by the RFV of the background to calculate the test value which is compared to the threshold, set by DuPont, to interpret a result of positive or negative. Similar to the RapidCheck

platform, the VIDAS results must also be confirmed through the FDA-BAM method of confirmation (BioMerieux, 2010).

The DuPont Method for the Detection of *Listeria monocytogenes* by end point PCR using the BAX Standard Assay is yet another screening platform used often for the testing of frozen dairy products. The DuPont BAX Standard Assay uses a polymerase chain reaction (PCR) platform meaning it amplifies specific fragments of DNA in the automated Q7 instrument to determine whether *Listeria* is present in a sample. Control DNA from the test kit or DNA from the test sample is combined with DNA polymerase, nucleotides and *Listeria* or *Listeria monocytogenes* sequence-specific primers. This mixture goes through heating and cooling steps to denature the DNA into single stranded DNA then is rapidly cooled to allow the primers to anneal. The DNA polymerase then uses the nucleotides to create two copies of the target DNA fragment. The process is repeated many times amplifying the DNA exponentially. During this process an intercalating fluorescent dye in the PCR tablet binds with the double stranded DNA. Following amplification, the BAX system enters a detection phase. This detection phase begins by taking an initial reading of fluorescent signal. The temperature is then raised incrementally causing the DNA to separate into single strands, thereby releasing the fluorescent dye and lowering the fluorescent signal. The change in fluorescence is plotted into a graph called a melting curve which is interpreted by the instrument to give a positive or a negative result. Similar to the VIDAS system, DuPont BAX has several different test kits that can test for either *Listeria* species or *Listeria monocytogenes*, but all positives again must be confirmed through the FDA-BAM confirmation (DuPont, 2011).

DuPont BAX also has another platform known as DuPont Method for Detection of *Listeria monocytogenes* by Real-Time PCR. Similar to the method discussed above, the Real-Time method uses the Q7 instrument and the BAX system, but it is a different test kit that uses probes bound to short nucleotide sequences. The probes only bind to certain fragments of DNA and when bound they

fluoresce. As opposed to the DuPont BAX Standard Assay, the Real-Time Assay's melting curve is plotted as the reactions occur. Like all screen platforms, positives must be confirmed via FDA-BAM confirmation method (DuPont, 2014).

Several considerations need to be made before choosing a method to test samples, including cost, sensitivity and turn-around-time or how long the method takes. Cost is the most variable because several factors go into cost including supplies and if the testing is done in house or sent to a third party lab to perform. The Reference Method would be the most cost effective method due to the use of widely available media, a relatively cheap rapid biochemical testing kit, and no expensive instruments. The next most cost effective method would be the RapidChek method which does require a purchased kit, but again requires no instrumentation. Both VIDAS and DuPont PCR-BAX require both test kits and instrumentation, making them the most expensive methods. Sensitivity is another major issue that needs to be addressed when determining which method to use for sample testing. Choosing a method with low sensitivity could lead to false positives or negatives, which could end up costing companies millions of dollars in lost product due to the test and hold process. In broad terms, the screen tests (VIDAS and DuPont PCR-BAX) would be more sensitive because there is less chance for human error to play a role in the result. In theory, streaking and interpretation errors could easily be made in the Reference method that could lead to a false result. In general terms, DNA based systems like the DuPont PCR-BAX Standard Assay and Real-Time Assay, would be more sensitive than antibody systems like the RapidChek and the VIDAS, because DNA is the most specific to any organism and cross-reactors can be an issue with antibody based systems. Cross-reactors are other organisms that have similar enough antigens that can bind to the antibodies and give false readings. Turn-around-time is usually the major deciding factor in the food industry when it comes to selecting methods for testing. The old adage "Time is Money" plays a large part in this when food manufacturers decide testing because usually food is held after production until the lot is tested for pathogens before it is sent out to customers. In terms of turn-

around-time, the Reference Method is by far the least efficient, taking a total of 8 days to confirm the presence of *Listeria monocytogenes*. This lengthy, time-consuming process is the reason why most of the screen methods were developed. The VIDAS, RapidChek, and the DuPont PCR-BAX Standard Assay methods take a total of 6 days to confirm the presence of *Listeria monocytogenes*. However, the DuPont PCR-BAX Real-Time Assay will confirm positive results in 5 days, giving manufacturers a result one full day faster than all other methods.

D. Purpose of Project

DuPont BAX has recently produced a new Real-Time BAX method of identifying *L.monocytogenes* which speeds up the testing time of foods to 26 hours, thus allowing for the rapid testing of the product throughout production. However, the DuPont Real-Time BAX method is not yet validated. Currently, eight different molecular methods are validated for detection of *L. monocytogenes* in ice cream, most of which are only validated for vanilla ice cream. The problem with only validating a method in vanilla, a bland flavor without many inhibitory ingredients, is that problems that can occur in other flavors will not be detected. This is particularly true for flavors such as chocolate. As food research, development, and marketing increase, the flavors of food will become more robust and complex, adding to the problem of method validation. In this project I will verify DuPont BAX Real-Time PCR Assay for *L. monocytogenes* Method in dark chocolate ice cream to determine if it is statistically equivalent to the reference FDA-BAM *L. monocytogenes* cultural method.

2. Project Overview

A. Target Audience

President Barack Obama signed into law the Food Safety Modernization Act (FSMA) on January 4, 2011, recognizing the foodborne illness burden in the United States taking a stand with the food industry to lessen it. FSMA sets the foundation to improve the food safety culture and awareness in the

United States and puts a focus on broad prevention mandates and accountability of the food industry. Preventative Controls for Human Food rule requires compliance for some food manufacturing businesses starting in September 2016. This rule requires a hazard analysis to be done, monitoring of the food safety process in which food is produced, as well as verification that the control activities in place are implemented and effective. Many parts of this rule indirectly requires food sample testing. For example, hazard analysis requires food manufactures to decide whether pathogens, such as *L.monocytogenes*, are a potential hazard for the facility. Testing the food for *L. monocytogenes* can be a verification that their process for control of *Listeria monocytogenes* is working. If it is not working, work surface testing can come into play more to determine where the organism is harbored in the facility and to determine whether more testing needs to be done throughout the production process to ensure the safety of the food produced. The intended audience for this manuscript can therefore be anyone interested in food safety in the food manufacturing industry or anyone generally interested in method verification.

B. Methodology

Ice cream falls under regulations of Food and Drug Administration (FDA). Regulation regarding ice cream can be found in the Code of Federal Regulations (CFR) Title 21, Volume 2, Part 135: Frozen Desserts. This section of the CFR describes the requirements that constitutes what food ice cream is and the manner in which it must be labeled. The CFR also establishes the FDA as the regulatory body that governs the production of ice cream. Because the FDA governs production of ice cream, manufacturers are required to use methods recognized by FDA. Thus, it is necessary to validate the DuPont BAX Real-

Time *Listeria monocytogenes* Detection Kit against the FDA-BAM cultural reference method, the current method recognized by the FDA.

AOAC International is a globally recognized, non-profit, third party organization that develops microbiological and chemical standards and analytical methods to promote trade and facilitate public health and safety. AOAC International has set standards for matrix validation of new methods to prove them equivocal to reference methods that are accepted by AOAC. AOAC requires a validation study to evaluate the following: inclusivity, exclusivity, and probability of detection (AOAC, 2012). As this study is simply a matrix verification, evaluation of inclusivity and exclusivity is not required. The target concentration of the strain of *L. monocytogenes* is 100 times the LOD_{50} (limit of detection) of the candidate method. The AOAC replicate requirement is 20 fractional positives and 5 uncontaminated control samples. When preparing the contaminated samples, the ice cream will be thawed, inoculated, mixed, and re-frozen.

Dark chocolate ice cream was selected to be validated for the Real-Time PCR-BAX method to test the possibility of the dark pigment inhibiting the DuPont's Q7 instrument from reading the fluorescence and creating a melt curve. DuPont has developed their PCR-BAX kits to run on the Q7 instrument that serves as a thermocycler to facilitate the PCR reaction and reads the fluorescent signal at the end of each PCR cycle to plot amplification. Dark chocolate ice cream poses a possible risk of inhibiting the Q7 instrument from being able to read the fluorescent signal due to the dark matter in the sample used in the reaction. From previous background work done with similar ice cream samples it was discovered that on a comparable *Listeria monocytogenes* kit, the ice cream had to be filtered before running on the Q7 for the instrument to read the reaction. However, in this matrix verification the ice cream sample was ran on the instrument without filtration.

Prior to full validation of the Real-Time BAX Method for Identifying *L. monocytogenes* in the dark chocolate ice cream matrix, a preliminary study must be completed to determine if any of the ingredients in the test ice cream inhibit the PCR reaction. Nine dark chocolate ice cream samples were spiked with 10 colony forming units (CFU) of *L. monocytogenes*. Different dilutions of enrichment broth were used In order to ensure that possible inhibitory factors did not mask a positive result. Three of these spiked samples were enriched with 225mL of 24E *Listeria* enrichment broth (a 1:10 dilution), three spiked samples were enriched with 475mL of 24E *Listeria* enrichment broth (a 1:20 dilution), and three spiked samples were enriched with 2475mL of 24E *Listeria* enrichment broth (a 1:100 dilution). All of the spiked samples were incubated at 35˚C for 24 hours, pulled, and ran on the Q7 BAX instrument using the Real-Time *L. monocytogenes* Detection Kit following the provided protocol. The spiked samples were then re-incubated for another 24 hours and pulled again to be ran on the Q7 BAX instrument using the Real-Time *L. monocytogenes* Detection Kit, which allows for either a 24- or 48-hour incubation. Due to the relatively slow growth rate of *L. monocytogenes* it was important to test whether the bacteria would need an extra 24 hours in the incubator to meet the threshold set in the BAX program. This preliminary study determined the optimal conditions necessary for the full verification

Growth and Preparation

The first step in matrix validation of the DuPont BAX Real-Time *L. monocytogenes* Detection Kit was preparation of the inoculum. The *L. monocyotgenes* inoculum that was used is the American Type Culture Collection number 19115. One isolated colony was suspended in brain heart infusion broth (BHI) and incubated overnight at 30˚C, this culture was at a concentration of $1x10^9$ cfu/mL. The next day, the overnight *L. monocytogenes* culture was diluted to $1x10^2$ cfu/mL in BHI. To achieve fractional

recovery forty 25 gram samples of dark chocolate ice cream were spiked and ten 25 gram samples of dark chocolate ice cream were reserved as negative controls. *L. monocytogenes* enumeration was then performed by plating 0.1 mL of the cellular suspension in triplicate onto standard methods plate count agar and incubated overnight at 35˚C. The spiked samples were held at -10°C for 24 hours to cold stress the target *Listeria* cells prior to the enrichment process.

Enrichment of *Listeria* in Sample

Of the 25 gram samples, 20 were enriched with 24E LEB Complete (Real-Time PCR method samples) and 20 of the 25 gram samples were enriched with pre-warmed Buffered *Listeria* Enrichment Broth (BLEB) without antibiotics (Reference Method Samples). In addition, five of the negative control bags were enriched with 24E LEB Complete and five of the negative control bags were enriched with BLEB broth without antibiotics. All samples were then homogenized for two minutes in a stomacher.

***Listeria* Detection**

The Real-Time PCR samples were incubated at 35±1˚C for 24 hours and the Reference Method samples incubated at 30±1˚C for 24 hours. Per the FDA-BAM Chapter 10 Detection and Enumeration of *L. monocytogenes* in Food method, the antibiotics Acriflavin HCl, Nalidixic Acid, and Cyloheximide were added 4 hours into incubation of the Reference Method samples.

After the 24 hour incubation, the Real-Time PCR samples were removed from the incubator and 5µL of the enriched sample was transferred into a cluster tube containing 200µL of Real-Time *Listeria* lysis reagent provided in the Real-Time *L. monocytogenes* Detection kit, which contains protease and lysis buffer (proprietary to DuPont). The transfer step was repeated for all Real-Time PCR samples. Lysis was then performed by heating the cluster tubes at 55˚C for 30 minutes, followed by incubation at 95˚C for 10 minutes. The tubes containing the samples were then chilled at 2-8˚C for at least 5 minutes. The BAX System Q7 instrument was then initialized and set up for Real-Time using *L. monocytogenes* as the

target. PCR tubes, containing the PCR tablets from the Real-Time *L. monocytogenes* Detection kit, were arranged in a chilled cooling block and 30µL of the appropriate lysate added to each PCR tube. The PCR tablets are included in each DuPont PCR-BAX kit and contain the primers or probes necessary for the PCR reaction, nucleotides and DNA polymerase. The full process was then run on the BAX System Q7 all positives were culturally confirmed in accordance with the FDA-BAM Chapter 10 Detection and Enumeration of *L. monocytogenes*.

Following the 24 and 48 hour incubation, the Reference Method enriched samples were diluted and streaked onto MOX plates and incubated at 35˚C for 24 or 48 hour. At this point, both Reference Method samples as well as screened-positive Real-Time PCR samples, were streaked to MOX plates and incubated. Up to five typical *Listeria* colonies from each sample were transferred to a TSA plate. All TSA plates were incubated at 35˚C for 24 hours. A few colonies from each TSA plate were removed with a cotton swab and tested for catalase by the addition of hydrogen peroxide and detection of bubble formation, which would indicate the presence of catalase. A Gram stain was performed on all samples to determine if they contained short, Gram positive rods, which would indicate the presence of *Listeria*. All suspect *Listeria* colonies were streaked to horse blood agar plates and incubated at 35±2˚C for 24-48 hours to test for hemolytic activity. If beta-lysis was observed after incubation, an API strip was inoculated to biochemically determine whether each isolate was *L. monocytogenes*.

3. Discussion

A. Results

After performing the Reference Method, 15 of the 20 samples were positive for *Listeria* at the first differential step, with two failing confirmation (Table 1). All samples that failed confirmation displayed alpha hemolysis on the horse blood plates. After performing the Real-Time BAX method, 14 of the 20 samples were positive, with two failing confirmation (Table 1). Samples failing confirmation did

not display esculin reactions on the MOX plates. Figures 3 and 4 show a typical Melt Curve for a negative result and a positive result respectively. Control samples of the dark chocolate ice cream that were not spiked with *L.monocytogenes* were also ran on both the Reference method and Real-Time PCR-BAX method to ensure that there were no false positives or negatives. The Negative control samples resulted in a negative result using the BAX system meaning that the system could read through the dark pigment of the ice cream, if it could not "indeterminate" would have been the result. Figure 1 and 2 show the difference between a negative and positive melting point graph. Chi square analysis was done resulting in a p value of 0.940754, and therefore the difference is not significant at $p < 0.05$.

Table 1: Cultural FDA-BAM Reference Method vs. DuPont RT PCR-BAX for Detected *Listeria monocytogenes* in Dark Chocolate Ice Cream.

Method	# of Spiked Samples	# of Initial Positives	# of Confirmed Positives
Cultural FDA-BAM	20	15	13
RT PCR-BAX	20	14	12

Figure 3: Example of a Negative Result Melting Point Graph (Unprocessed Fluorescent Intensity vs. Temperature).

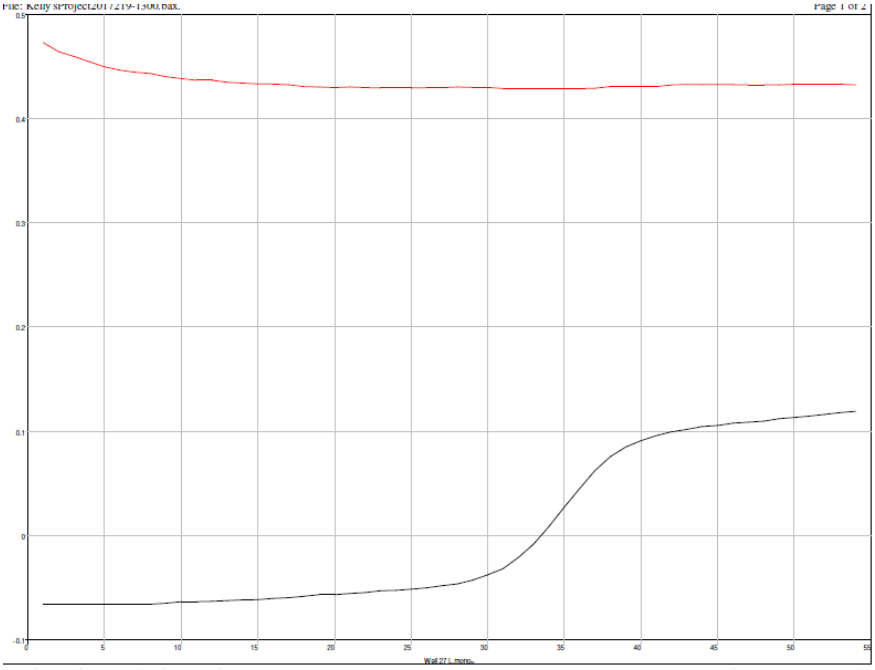
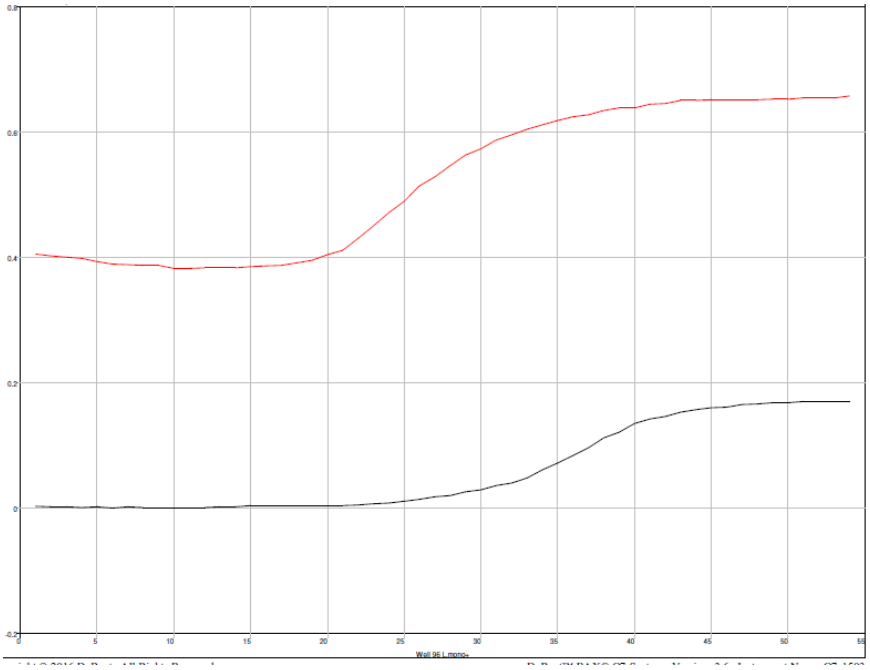


Figure 4: Example of a Positive Result Melting Point Graph (Unprocessed Fluorescent Intensity vs. Temperature).



B. Implementations

Per AOAC International, matrix validation must prove equivalent to the reference method with fractionally positive spiked samples. Based on the data gathered above the DuPont Real-Time PCR-BAX *L. monocytogenes* Detection Kit is equivalent to the FDA-BAM Chapter 10 Method for Detection of *L. monocytogenes*. While the FDA-BAM method did result in one more positive than the RT PCR-BAX method, this difference is likely because the samples had to be enriched with different media, which may alter the growth of *L. monocytogenes* resulting in uneven samples between the two methods. It would be quite interesting to study the differences in media, the BLEB used in the Reference Method compared to the LEB in the Real-Time PCR-BAX method, however the ingredients in the LEB are proprietary to DuPont. Further work could be done in verifying more flavors of ice cream on the DuPont Real-Time PCR-BAX *L. monocytogenes* Detection kit because each different flavor could pose a challenge for the kit given the varying ingredients. However, with the validation that the kit is equivalent to the FDA-BAM Reference method in both dark chocolate and vanilla ice cream matrices, it is a good

indication that the kit will be proficient in detecting *L. monocytogenes* in most flavors of ice cream. These results will be of great value to the frozen dairy industry as it shortens the test and hold process for the detection of *L. monocytogenes* by up to 22 hours. With the recent surge in recall due to *L. monocytogenes* contamination in the frozen dairy industry, the industry is under a microscope in the public eye and the need for more sensitive and rapid testing for the detection of *L. monocytogenes* is at an all-time high. DuPont's Real-Time PCR-BAX *L. monocytogenes* Detection Kit is available and validated to now fill the need for sensitive and rapid testing of frozen dairy.

C. Recommendations

Following the above success of DuPont Real-Time PCR-BAX Method for the Detection of *L. monocytogenes*, DuPont has decided to send out a technical bulletin approving the verification of dark chocolate ice cream using this procedure. However, more studies are recommended if a frozen dairy product has a different base other than dark chocolate or vanilla ice cream. As flavors become more varied, different ingredients may play roles in inhibiting either the PCR reaction or prevent the BAX system from reading the melting curves properly. Matrix verification similar to the procedure done here are sufficient per AOAC International to verify a food to a method that has already gone through complete verification.

4. Acknowledgements

I would like to acknowledge the assistance of the DuPont Technical Team, specifically Casey Simmons and Stacey Stolenberg, as well as DuPont for providing all supplies used in the study. I would also like to acknowledge Food Safety Net Services for allowing me to perform the verification within the Food Safety Net Services Columbus, Ohio location. Finally, I would like to thank Corinne Haines for her help in editing this manuscript. I would also like to acknowledge that DuPont Diagnostics is now owned by Hygiena.

5. References

CDC. 2017. Centers for Disease Control and Prevention. Centers for Disease Control and Prevention. Centers for Disease Control and Prevention. Date Accessed 4/01/17. https://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/

AOAC International. 2012. AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces. AOAC International. Date Accessed: 3/15/17. http://www.aoac.org/aoac_prod_imis/AOAC_Docs/StandardsDevelopment/AOAC_Validation_Guidelines_for_Food_Microbiology-Prepub_version.pdf

Center for Food Safety and Applied Nutrition. Hazard Analysis Critical Control Point (HACCP) - Dairy Grade A Voluntary HACCP. U S Food and Drug Administration Home Page. Center for Food Safety and Applied Nutrition. Date Accessed: 03/15/17. https://www.fda.gov/food/guidanceregulation/haccp/ucm2007982.htm

DuPont. 2012. BAX System- Ready Reference for Real-Time PCR Assays. BAX System- Ready Reference for Real-Time PCR Assays Pamphlet. DuPont. DuPont Wilmington, DE.

DuPont. 2011. DuPont BAX System User Guide. Part Number 049. 10-0212 v3.0. DuPont Wilmington, DE.

SDIX. 2010. SDIX RapidChek *Listeria* species Food System Kit User Guide. Part Number 3090063 Version 3.0. SDIX Newark, DE.

BioMerieux. 2009-2010. BioMerieux VIDAS *Listeria monocytogenes* II. Product Insert 11600. BioMerieux Durham, NC.

United States Food and Drug Administration. 2016. Chapter 10 Detection and Enumeration of *Listeria monocytogenes* in Foods, Bacteriological Analytical Manual, 8th edition. FDA Rockville, MD. Date Accessed: 01/03/17. https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm

Lowe, John. Jeni's Splendid Ice Creams. Response to Form 483 Issued April 30 2015. United States Food and Drug Administration. Columbus, Ohio. https://www.fda.gov/ucm/groups/fdagov-public/@fdagov-afda-orgs/documents/document/ucm451241.pdf

Malone, J.D. 2016. FDA: *Listeria* found in Jeni's production kitchen. *The Columbus Dispatch*. The Columbus Dispatch.

Center for Food Safety and Applied Nutrition. FDA Food Safety Modernization Act (FSMA). *US Food and Drug Administration Home Page*. Center for Food Safety and Applied Nutrition.

Kozak, J., T. Balmer, R. Byrne, and K. Fisher. 1996. Prevalence of *Listeria monocytogenes* in foods: Incidence in dairy products. *Food Control* 7:215–221.

Lu, J., X.-H. Pua, C.-T. Liu, C.-L. Chang, and K.-C. Cheng. 2014. The implementation of HACCP management system in a chocolate ice cream plant. *Journal of Food and Drug Analysis* 22:391–398.

DuPont. 2014. Method Comparsion: BAX System Real-Time PCR Assay for *L. monocytogenes* vs. Reference Methods for *Listeria monocytogenes*. DuPont Study Report. Wilmington, DE.

Mastronicolis, S. K., I. Diakogiannis, A. Berberi, P. Bisbiroulas, C. Soukoulis, and C. Tzia. 2011. Effect of cold adaptation on the survival of *Listeria monocytogenes* in ice-cream formulations during long-term frozen storage. *Annals of Microbiology* 61:931–937.

Buchanan, R. L., L. G. Gorris, M. M. Hayman, T. C. Jackson, and R. C. Whiting. 2017. A review of *Listeria monocytogenes*: An update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Control* 75:1–13.

Ottesen, A., P. Ramachandran, E. Reed, J. R. White, N. Hasan, P. Subramanian, G. Ryan, K. Jarvis, C. Grim, N. Daquiqan, D. Hanes, M. Allard, R. Colwell, E. Brown, and Y. Chen. 2016. Enrichment dynamics of *Listeria monocytogenes* and the associated microbiome from naturally contaminated ice cream linked to a listeriosis outbreak. *BMC Microbiology* 16.

Centers for Disease Control. *Listeria* (Listeriosis). Date Accessed 3/15/17. https://www.cdc.gov/listeria/