Identification of a Universal Enrichment Broth for Simultaneous Enrichment of *Salmonell*a spp., *E. coli* O157:H7 and *Listeria monocytogenes* (CARTS: IR00085 and IR01132)

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ABSTRACT

Rapid detection of *Salmonella* spp., *Escherichia coli*, and *Listeria monocytogenes* in foods is important for the prevention of foodborne illnesses. In accordance with the FDA's Bacteriological Analytical Manual (BAM), different enrichment broths are used for culturing target pathogens which can also vary based on the food matrices tested. As any food sample may have multiple pathogens of concern, using different broths for each pathogen is labor intensive, time consuming and adds to the total cost of sample analysis. Multi-pathogen detection assays are thus desired and would benefit from the identification of a nutrient broth that can simultaneously enrich for multiple target pathogens. The objective of this study was to identify the best enrichment broth to simultaneously enrich *Salmonella* Typhimurium, *E. coli* O157:H7 (EHEC) and *Listeria monocytogenes.* Nine broths were tested for supporting growth of *Salmonella* spp., EHEC, and *Listeria monocytogenes* singly and in combination using varying inoculation ratios with detection of each pathogen confirmed by a unique multiplex qPCR assay. All target pathogens grew successfully in the candidate enrichment broths singly as determined through OD_{600nm} growth curve characterization and plate counts. Broths identified with the greatest potential to enrich these three pathogens individually include UPB, BMW and Buffered BMW. When the target pathogens were co-inoculated into candidate enrichment broths, all pathogens were detected by a multiplex qPCR assay at all time points and temperature conditions except for *Listeria monocytogenes* in TSB at 37C for 24h. Altering the inoculation ratios of *Salmonella* Typhimurium, EHEC, and *Listeria monocytogenes* (1:1:1, 10:1000:1, 1:10:1000, and 1000:1:10) resulted in varying Ct values of each pathogen that were relatively proportional to the initial cell number. Candidate universal enrichment broths that showed the most promise at varying inoculation ratios were BLEB, Buffered BMW, mBPW and mBPW+glucose based on their ability to successfully enrich all three pathogens simultaneously at all inoculation ratios. In general,

Listeria monocytogenes had higher Ct values than *Salmonella* Typhimurium and EHEC. This study supports the use of a universal enrichment broth combined with multiplex qPCR detection to reduce media requirements and analysis time resulting in a high-throughput, streamlined approach that will increase the capacity of public health laboratories to rapidly detect multiple pathogens.

INTRODUCTION

Foodborne bacterial pathogens have been the cause of high profile outbreaks in the last several years that have resulted in serious illnesses, hospitalizations and deaths. Scallan et al. (*25*) estimated that foodborne bacterial pathogens cause 3.6 million illnesses a year with *Salmonella* spp., *Listeria monocytogenes* and Enterohemorrhagic *E. coli* infections resulting in high hospitalization rates. Efforts to mitigate foodborne illness include routine testing of foods for these pathogens. Current microbiological methods utilized by FDA field labs for the testing of foods and environmental samples are culture based as outlined in the Bacteriological Analytical Manual (BAM). These methods vary by pathogen but typically involve two days of enrichment including both a non-selective pre-enrichment and a selective enrichment (*2, 12, 15*). Enrichment involves adding the food to a nutritional broth and incubating for 24-48 hours to allow for the target microbial cells to multiply. The need for non-selective bacterial pre-enrichment in food matrices is due to the likely low concentration of pathogens in food, sub-lethally injured bacterial cells from food processing and packaging, the high numbers of non-pathogenic background bacteria, and inhibitory effects of food components on molecular detection (*5*). Non-selective pre-enrichment broth is nutritionally rich and can increase the numbers of all microorganisms in the food matrix. After the resuscitation of injured bacterial cells and subsequent growth in the non-selective pre-enrichment broth, an aliquot is transferred to selective enrichment media which allows the microorganism of interest to grow but suppresses the growth of competitive background microflora. These enrichment steps are followed by culture isolation, biochemical testing and serological and/or genetic confirmation. While these methods are highly effective, they require a considerable amount of time, up to two weeks to confirm a positive finding, which can impede the progress of foodborne outbreak investigations, lead to additional illnesses, and be costly for industry when testing perishable foods waiting for distribution across the country.

Slight differences in the composition of media and enrichment broths can result in dramatically different growth characteristics of microbial pathogens. Media contain a variety of components depending on the target organism's nutritional and metabolic needs, but typically contain a carbohydrate and a protein source as well as buffering capacity to maintain a stable pH throughout the incubation time. Enrichment broths and media may also contain growth factors to promote specific growth of a target organism and selective agents to inhibit the growth of particular microorganisms. In recent years, a few studies have

evaluated and developed broths for multi-pathogen enrichment combined with multiplex PCR (*7, 14, 17, 19, 20, 27, 30*). In these studies, the broths used successfully enriched a limited number of pathogens but contained selective agents that would likely not be able to promote the growth of all the target pathogens routinely tested for in the FDA field labs.

The use of rapid molecular methods can reduce the time needed for foodborne bacterial pathogen detection. One of these rapid methods is the polymerase chain reaction (PCR) that amplifies a segment of the bacterial genome to detectable quantity. Real-time quantitative PCR (qPCR) assesses genome concentration within a sample that can be used to estimate bacterial concentration. Multiplex real-time quantitative PCR (multiplex qPCR) assays have been developed and applied for the detection of multiple foodborne pathogens simultaneously (*6, 26*); however, these rapid method endpoint assays still require an enrichment step to assure the target bacterial pathogens reach a sufficient cell density. To facilitate a multi-pathogen detection platform, a universal enrichment broth that would allow for the simultaneous enrichment of multiple foodborne pathogenic bacteria is necessary, as different enrichment broths as currently used require more resources, labor, and time, depending upon the food matrix or pathogens tested. The objective of this study is to identify a universal enrichment broth to simultaneously enrich *Salmonella* spp., Enterohemorrhagic *Escherichia coli* (EHEC) and *Listeria monocytogenes*, and to verify its performance by growth curve analysis and multiplex qPCR detection.

MATERIALS AND METHODS

Bacterial Cultures. *Salmonella* Typhimurium (isolated at PSFFL from frozen cooked mackerel), *Escherichia coli* O157:H7 (ATCC 43895) (EHEC), and *Listeria monocytogenes* (ATCC 19115) were used as target strains in this study. Bacterial isolates were recovered from frozen storage (-70˚C) and maintained on brain heart infusion (BHI) agar and broth. Cultures used for experiments were either from frozen stock or from fresh growth. Frozen cultures were prepared by freezing overnight enrichment cultures with 20% glycerol at -70˚C and keeping frozen until use. Fresh bacterial cultures were prepared by growing cultures overnight in BHI broth at 37˚C and using immediately to inoculate candidate universal enrichment broths. Culture inoculations for enrichment broths were prepared by serial dilutions in 0.85% NaCl solution. Inoculation concentrations were determined by plating serial dilutions on TSA or TSAYE.

Candidate Universal Enrichment Broths. Candidate universal enrichment broths used in this study are referenced in Table 1. All broths were used without selective agents except for SEL (*Salmonella*, *E. coli*, and *Listeria*) Broth. BMW broth was made with 6.0g yeast extract (Thermo Fisher Scientific, Waltham, MA) , 3.0g soytone (Becton Dickinson, Franklin Lakes, NJ), 25.0g heart infusion (Remel, San Diego,

CA), 0.27g monopotassium phosphate (Thermo Fisher Scientific), 10.46g MOPS (Thermo Fisher Scientific), 0.1g ferric ammonium sulfate (Sigma-Aldrich, St. Louis, MO) per 1L of water (Doran et al., 2013). Buffered BMW and mBPW+glucose broths are modifications of BMW and mBPW broths, respectively. Buffered BMW was made with 6.0g yeast extract, 3.0g soytone, 25.0g heart infusion, 3.0g monopotassium phosphate, 7.0g disodium phosphate (Thermo Fisher Scientific), 5.0g sodium chloride, 10.46g MOPS, and 0.1g ferric ammonium sulfate per 1L of water. mBPW+glucose was made by adding 2.5g glucose per 1L of mBPW.

Growth Curve Analysis with Bioscreen C. Candidate universal enrichment broths were inoculated with frozen culture stocks of each target bacterial pathogen individually. Inoculated broths were aliquoted into Honeycomb well 100-well plates (Growth Curves USA, Piscataway, NJ) at 400µL total volume, and plates were incubated in the Bioscreen C instrument (Growth Curves USA, Piscataway, NJ) at 37˚C for 24h with continuous shaking. Each candidate broth was inoculated with an initial population of 2.64 ± 0.46 CFU/well *Salmonella* Typhimurium, 3.2 ± 0.96 CFU/well EHEC, or 2.42 ± 0.07 CFU/well *Listeria monocytogenes*. OD_{600nm} measurements were taken every 20 min. After 24h, samples were removed and serial dilutions were plated on TSA (*Salmonella* Typhimurium and EHEC) and TSAYE (*Listeria monocytogenes*) to determine final concentrations (log CFU/mL). Growth curve analysis was performed using DMFit Excel Add in software program based on Baranyi and Roberts (*4*) for fitting curves where a linear phase is preceded and followed by a stationary phase (http://www.ifr.ac.uk/safety/DMfit/). From the OD_{600nm} readings, the potential maximum growth rate (μ_{max}) and lag phase duration (λ) were calculated. The potential maximum growth rate (μ_{max}) was calculated from the slope of the line where exponential growth occurs. Lag phase duration (λ) was determined from where this growth rate line bisects the horizontal axis.

Varying Enrichment Conditions on Bioscreen C. Candidate enrichment broths were inoculated with frozen culture stocks of each target bacterial pathogen individually. Inoculated broths were aliquoted into Honeycomb well 100-well plates, 400µL total volume, and plates were incubated in the Bioscreen C instrument at 30˚C or 37˚C for 18h or 24h during which the samples were shaken continuously. DNA was extracted post-enrichment from 200µL of broth by automated DNA Extraction using the Mag Max (Applied Biosystems, Foster City, CA) and the Qiagen BioSprint One-For-All Kit (Valencia, CA) following the manufacturer's directions.

Varying Inoculation Concentration Ratios. Candidate broths were inoculated with *Salmonella* Typhimurium, EHEC and *Listeria monocytogenes* in ratios of 1:1:1, 10:1000:1, 1:10:1000, 1000:1:10 (*Salmonella*: EHEC: *Listeria monocytogenes*). Average concentration at the 1:1:1 inoculation ratio was

11.9 ± 2.3, 7.5 ± 1.7 and 11.1 ± 0.1 CFU of *Salmonella* Typhimurium, EHEC and *Listeria monocytogenes,* respectively. The Wash, Spin, Boil method was used to extract DNA from the varying inoculation concentration cultures (*8, 9*). The enrichment sample (1mL) was centrifuged at 8000 rpm for 5 min and the supernatant was discarded. The pellet was resuspended in water (250 µL) and heated at 95-100˚C for 20 min. After heat treatment, tubes were transferred onto ice for 5 min before centrifuging at 8000rpm for 5 min. The supernatant containing the DNA was transferred to another labeled tube and used as the template for multiplex qPCR detection assays.

Multiplex qPCR. A multiplex qPCR assay to detect *Salmonella* spp., EHEC and *Listeria monocytogenes* using TaqMan probes as designed by Suo et al. (*26*) was performed. Primers and probes used are shown in Table 2 and were synthesized by Biosearch Technologies (Novato, CA). Amplification reactions were performed in a volume of 25 µL containing 1 µL DNA, 2.4 µL probe mix, 1 µL of each *Salmonella* primer (1 pm/µL), 1 µL of each *Listeria* primer (10 pm/µL), 3 µL of each *E. coli* primer (1 pm/µL), 5 µL of Multiplex PCR 5X Master Mix (5X Concentrate) (New England Biolabs, Ispwich, MA), 0.3 µL of OneTaq® Hot Start DNA Polymerase (5,000 U/mL) (New England Biolabs, Ispwich, MA), and 12.3 µL ddH_2O. Probe mix was prepared by mixing 10 µL, 1.6 µL, and 10 µL of each of *Salmonella* Typhimurium, EHEC, and *Listeria monocytogenes* probe (100 µM), respectively, with 226.2 µL of ddH_20. Multiplex PCR was performed on Smart Cycler II (Cepheid) at 50°C for 120s, 95°C for 600s, followed by 40 cycles each of 95°C, 15s and 60°C, 60s. A standard curve was constructed using DNA extracts of serial dilutions of each target pathogen from 10^9 to 10^3 CFU/mL (Figure 1).

Statistical Analysis. Experiments were performed in triplicate and data are represented as the mean ± the standard deviation from the mean. Statistical significance was determined from a t-test performed in Microsoft Excel and data were considered statistically significant if $p<0.05$.

RESULTS

Individual Pathogen Enrichment in Candidate Universal Enrichment Broths. All three target pathogens grew successfully in all candidate universal enrichment broths individually as determined by plate counts shown in Figure 2. After incubation at 37˚C for 24h final populations *Salmonella* Typhimurium, EHEC, and *Listeria monocytogenes* were > 7 log CFU/mL (Figure 2). Table 3 shows the average lag phase (λ) and potential maximum growth rate (μ_{max}) for the three different pathogens after individual enrichment in each broth. The ideal enrichment broth has a high μ_{max} and a low λ which corresponds with a fast growth rate of the microorganism and a short resuscitation period, respectively. *Listeria monocytogenes* had significantly longer λ and a significantly slower μ_{max} as compared to *Salmonella* Typhimurium and EHEC for all broths respectively ($P < 0.05$) with the exception of mBPW broth. BMW broth supported a

significantly faster growth rate (greatest μ_{max}) of *Salmonella* Typhimurium and EHEC as compared to all candidate broths (P <0.05). The μ_{max} was significantly higher for *Listeria monocytogenes* in UPB as compared to all other candidate broths except for BLEB ($P < 0.05$).

The λ was significantly lower in UPB for *Salmonella* Typhimurium as compared to all other candidate broths ($P < 0.05$) (Table 3). UPB also had a significantly lower λ for EHEC enrichment as compared to Buffered BMW, mBPW, mBPW+glucose, SSL and the BAM broth mBPWp ($P < 0.05$). Buffered BMW broth had a significantly lower λ for *Listeria monocytogenes* compared to all candidate broths except for BMW ($P < 0.05$) (Table 3). Figure 3 shows the growth curves generated from OD_{600nm} data for *Salmonella* Typhimurium, EHEC, and *Listeria monocytogenes* in the broths with the ideal μ_{max} and λ for individual enrichment: UPB, BMW, and Buffered BMW.

Simultaneous Enrichment of Salmonella Typhimurium, EHEC *and Listeria monocytogenes in Candidate Universal Enrichment Broths at Varying Times and Temperatures.* The multiplex qPCR Ct values of the simultaneous enrichment of *Salmonella* Typhimurium, EHEC, and *Listeria monocytogenes* in varying enrichment conditions, time and temperature, is shown in Table 4. All three target pathogens were detected at all time points and temperature conditions with the exception of *Listeria monocytogenes* in TSB at 37˚C for 24h.

In UPB, no significant difference ($P \geq 0.05$) was observed for Ct values of *Salmonella* Typhimurium, *E. coli* O157:H7 and *Listeria monocytogenes* between 30˚C and 37˚C at both 18h and 24h. Ct values after 24h incubation in UPB were significantly less than values at 24h ($P < 0.05$) at 30˚C for both *Salmonella* Typhimurium and *Listeria monocytogenes*. At 37˚C, no significant differences in Ct values ($P \geq 0.05$) were observed for all three pathogens in BMW and Buffered BMW between 18h and 24h. At 30˚C, *Listeria monocytogenes* enrichment in BMW showed significantly lower Ct values at 24h as compared to 18h incubation ($P < 0.05$). After 18h incubation, no significant differences were observed between 30˚C and 37˚C for all individual target pathogens' enrichment in BMW and Buffered BMW broth. After 24h incubation in BMW and Buffered BMW, *Listeria monocytogenes* had significantly lower Ct values at 30˚C as compared to 37˚C ($P < 0.05$).

Simultaneous Enrichment of Salmonella Typhimurium, EHEC *and Listeria monocytogenes in Candidate Universal Enrichment Broths at Varying Inoculation Ratios.* Figure 4 shows the multiplex qPCR Ct values after enrichment at 37˚C for 22-24h when broths were inoculated with varying ratios of the initial inoculation concentrations of the three pathogens. Inoculation ratios included 1:1:1, 10:1000:1, 1:10:1000, and 1000:1:10 of *Salmonella* Typhimurium, EHEC, and *Listeria monocytogenes*, respectively. Inoculating concentrations in the 1:1:1 ratio were 11.85 ± 2.33, 7.53 ± 1.73, 11.1 ± 0.14 CFU/25mL

enrichment for *Salmonella* Typhimurium, EHEC, and *Listeria monocytogenes*, respectively (Figure 4A). After 24h of incubation all target pathogens were enriched to levels detectable by qPCR assay. *Listeria monocytogenes*' Ct values were significantly higher than *Salmonella* Typhimurium and EHEC in BMW, Buffered BMW, mBPW, mBPW+glucose, SEL, and SSL. When the ratio of initial inoculum concentration was 10:1000:1, all three target pathogens were detected (Figure 4B).

When the ratio of initial inoculum concentration was 1:10:1000 (S:E:L), *Salmonella* Typhimurium was not detected by multiplex qPCR assay in BMW, SSL, TSB and UPB (Figure 4C). *Listeria monocytogenes* Ct values were lower indicating a higher final concentration of bacterial cells when the *Listeria monocytogenes* inoculum was 100-1000X higher than its competitors. However, the Ct values were significantly lower than when all three pathogens were present at the 1:1:1 ratio. When the ratio of initial inoculum concentration was 1000:1:10 (S:E:L), EHEC was not detected by multiplex qPCR assay in SEL and TSB (Figure 4D).

DISCUSSION

Enrichment of multiple bacterial pathogens simultaneously is challenging due to the unique nutritional requirements of each pathogen as well as the inherent challenges with various food matrices. No single broth has been widely adopted for the enrichment of both gram-negative and gram-positive microorganisms in foods.

Candidate universal enrichment broths used in this study included broths routinely used in FDA field laboratories for the enrichment of foodborne pathogens as described in the Bacteriological Analytical Manual (BAM). The BAM broths used included Buffered Listeria Enrichment Broth base (BLEB), modified Buffered Peptone Water (mBPW), Tryptic Soy Broth (TSB) and Universal Pre-enrichment Broth (UPB) (Table 1). Published research broths for multi-pathogen enrichment were also investigated and included the *Salmonella*, EHEC and *Listeria monocytogenes* enrichment broth (SEL) which is a selective enrichment broth in which antibiotics including acriflavine, cycloheximide, fosfomycin and nalidixic acid are added to a buffered listeria enrichment buffer base (*19*). The broth base of SSL (*Salmonella* Enteritidis, *Staphylococcus aureus* and *Listeria monocytogenes*) without the addition of antibiotics was also considered as a candidate for multi-pathogen enrichment (30). Modifications of these broths as well as published research broths were considered including a broth named after the FDA location in which it was formulated "Bothell, Mod1 (CFSAN), WEAC" (BMW) for enriching select agents including *Yersinia pestis* (*11*). Two modified broths were considered including Buffered BMW broth and mBPW + glucose broth. Buffered BMW broth was composed of the BMW broth modified by

the additional buffering salts of mBPW broth. mBPW+glucose broth is the FDA BAM mBPW broth modified by the addition of 2.5% glucose.

Candidate Universal Broths: Growth of Individual Pathogens

Before assessing multiplex enrichment, the efficacy of each candidate universal enrichment broth to support the growth of the target microorganisms individually without competition was established through characterizing the growth curve kinetics. To establish these growth parameters, growth curves were determined by optical density measurements using an automated growth curve analyzer. The Bioscreen C is an automated and convenient tool to measure bacterial growth by optical density. The first criterion to select an appropriate enrichment is a high μ_{max} correlating with fast growth. However, since it should not be assumed that a broth supporting a significantly faster μ_{max} is also the best broth to support resuscitation (*16*), the second criterion to select an appropriate enrichment broth is λ or lag phase duration. Lag phase duration (λ) corresponds to the time needed to enter exponential bacterial growth and corresponds to the time needed for bacterial resuscitation. When bacteria become sublethally injured due to stress, a longer lag phase ensues compared to a healthy cell due to time needed to repair (*28*). The ideal enrichment broth has a high μ_{max} and a low λ. In this study, cells inoculated into the enrichment broths for Bioscreen C automated growth curve analysis were frozen prior to inoculation and were therefore potentially stressed or sublethally injured (*29*). Target pathogens that experienced the exact same stress treatment showed significant differences in λ when they were inoculated into different broths (Table 3). UPB, BMW, and Buffered BMW showed most promise in this study as a universal enrichment broth based on the growth curves for the individual enrichment of *Salmonella* Typhimurium, EHEC and *Listeria monocytogenes* in candidate broths. The data also indicate that BMW is the best supporting enrichment broth for *Salmonella* Typhimurium and EHEC because μ_{max} was significantly greater as compared to all candidate broths. UPB was the best supporting enrichment broth for *Listeria monocytogenes* because μ_{max} was significantly greater as compared to all candidate broths except for BLEB. UPB was shown to be the best enrichment broth for *Salmonella* Typhimurium and EHEC as λ was significantly shorter as compared to all other candidate enrichment broths except for BLEB, BMW and TSB for EHEC enrichment. Buffered BMW was shown to be the best enrichment broth for *Listeria monocytogenes* as λ was significantly shorter as compared to all other candidate universal enrichment broths apart from BMW. The growth curves of *Salmonella* Typhimurium, EHEC and *Listeria monocytogenes* in the broths that show the most potential for use as a universal enrichment broth, UPB, Buffered BMW and BMW are shown in Figure 3.

Candidate Universal Broths: Growth of co-Inoculated Pathogens

Candidate universal enrichment broths were then evaluated for their ability to simultaneously enrich the three target pathogens, *Salmonella* Typhimurium, EHEC and *Listeria monocytogenes through* a unique multiplex qPCR assay. The multiplex qPCR assay used in this study was developed by researchers at the USDA (*26*) and has been tested for in food matrices including soft cheeses, fresh fruits, vegetables, and spices (*21, 22, 23, 24*). Ct values are inversely proportional to the amount of target DNA present in a sample. Lower Ct values correlate to a higher concentration of target DNA.

Varying enrichment conditions were assessed for simultaneous pathogen enrichment. These conditions included temperatures of 30˚C and 37˚C, and times of 18h and 24h. The experimental temperature and time conditions were chosen based on typical enrichment conditions for the three target pathogens as outlined in the FDA BAM. *Salmonella* enrichment is incubated at 35˚C for 24 ± 2h (*1*); EHEC enrichment is incubated at 37˚C for 4h, followed by 42˚C for 18-24h (*11*); *Listeria monocytogenes* enrichment is incubated at 30˚C for 24-48h (*14*). All three target pathogens were detected at all time points and temperature conditions except for *Listeria monocytogenes* in TSB at 37˚C for 24h (Table 4). Few significant differences were observed between time and temperature amongst the broths and target pathogens as noted in Table 4. Based on these results, ensuing enrichment experiments were conducted at 35-37˚C for 24h.

Candidate Universal Broths: Varying Ratios of Co-Inoculated Pathogens

To further assess the efficacy of candidate enrichment broths to simultaneously enrich multiple target pathogens, the target pathogens were inoculated at varying ratios into candidate enrichment broths. Inoculation ratios used in this study were the same inoculation ratios used by Kim and Bhunia (*17*) when evaluating SEL broth for multi-pathogen enrichment. At 1:1:1 ratio of *Salmonella* Typhimurium, EHEC and *Listeria monocytogenes* (S,E,L), all three target pathogens were successfully detected by the multiplex qPCR assay in all candidate enrichment broths, although, *Listeria monocytogenes* had significantly higher Ct values in BMW, Buffered BMW, mBPW, mBPW+glucose, SEL, and SSL as compared to *Salmonella* Typhimurium and EHEC (Figure 4A). When the target pathogens were inoculated at various ratios, the post-enrichment Ct values of each pathogen were relatively proportional to the initial cell number. When *Salmonella* Typhimurium was inoculated at the lowest concentration in the 1:10:1000 ratio (Figure 4C), *Salmonella* Typhimurium was not detected in BMW, SSL, TSB and UPB broth. At this ratio, where the initial *Listeria monocytogenes* inoculation is higher (1:10:1000), Ct values for *Listeria monocytogenes* are significantly lower than at other concentrations ($P<0.05$). When EHEC was lowest 1000:1:10 (Figure 4D), EHEC was not detected in SEL and TSB. Candidate universal

enrichment broths that showed the most promise at varying inoculation ratios were BLEB, Buffered BMW, mBPW and mBPW+glucose based on their ability to successfully enrich all three pathogens simultaneously at all inoculation ratios.

In general, enrichments of *Listeria monocytogenes* resulted in higher Ct values than *Salmonella* Typhimurium and EHEC which suggests that after 24h of growth, *Listeria monocytogenes* is present at a lower concentration than *Salmonella* and EHEC. *Listeria monocytogenes* is a slow-growing organism and a poor competitor (*13*). The growth of *Listeria monocytogenes* has been observed to be slow in mixed cultures and doesn't grow to as high of a concentration as co-inoculated strains (*13*). Low cell numbers for *Listeria monocytogenes* were also observed in SEL in multipathogen enrichment (*19*). In food matrices, the challenges of *Listeria monocytogenes* with *Salmonella* spp. and EHEC co-enrichment may be even greater due to bacterial cell damage and higher prevalence of non-target background microflora (1, 18).

Broths with the greatest potential to enrich these three pathogens include UPB, BMW and Buffered BMW. Ongoing research focuses on determining suitability of candidate enrichment broths to enrich all three target pathogen present simultaneously in FDA regulated food matrices including produce, spices, seafood, cheeses and pet foods.

REFERENCES

1. Al-Zeyara, S.A., B. Jarvis, and B.M. Mackey. 2011. The inhibitory effect of natural microflora of food on growth of *Listeria monocytogenes* in enrichment broths. Int. J. Food Microbiol. 145:98-115.
2. Andrews, W.H., H. Wang, A. Jacobson, and T. Hammack, *Bacteriological Analytical Manual, Chapter 5. Salmonella*. 2017.
3. Bailey, J.S. and N.A. Cox. 1992. Universal preenrichment broth for the simultaneous detection of Salmonella and Listeria in foods. *J. Food Prot*. 55:256-259.
4. Baranyi, J. and T.A. Roberts. 1994. A dynamic approach to predicting bacterial growth in food. *Int. J. Food Microbiol*. 23:277-294.
5. Brehm-Stecher, B., C. Young, L.A. Jaykus, and M.L. Tortorello. 2009. Sample Preparation: The Forgotten Beginning. *J. Food Prot*. 72:1774-1789.
6. Chen, J., J. Tang, J. Liu, Z. Cai, and X. Bai. 2012. Development and evaluation of a multiplex PCR for simultaneous detection of five foodborne pathogens. *J. Appl. Microbiol*. 112:823-830.
7. Chen, J., J. Tang, A.K. Bhunia, C. Tang, C. Wang, and S. Hui. 2015. Development of a multi-pathogen enrichment broth for simultaneous growth of five common foodborne pathogens. *J. Gen. Appl. Microbiol.* 61:224-231.
8. Cheng, C.M., K. Van, W. Lin, and R.M. Ruby. 2009. Interlaboratory validation of a real-time PCR 24-hour rapid method for detection of *Salmonella* in foods. *J. Food Prot*. 72:945-951.
9. Cheng, C.M., W. Lin, K.T. Van, L. Phan, N.N. Tran, and D. Farmer. 2008. Rapid detection of *Salmonella* in foods using real-time PCR. *J. Food Prot*. 71:2436-2441.
10. Domestic and Import Produce Assignments FY2014 DFP&G #14-05/14-06. "Sample Preparation for the Detection of *Salmonella* in Papaya Method G" pg. 50. http://inside.fda.gov:9003/downloads/ProgramsInitiatives/Food/FieldPrograms/UCM400671.pdf
11. Doran, T. Hanes, D., Weagent, S., Torosian, S., Burr, D., Yoshitomi, K., Jinneman, K., Penev, R., Adeyemo, O., Williams-Hill, D., and P. Morin. 2013. FERN *Yersinia pestis* Screening Method. FERN-MIC.0004.02.
12. Feng, P., S.D. Weagant, and K. Jinneman, *Bacteriological Analytical Manual, Chapter 4a. Diarrheagenic Escherichia coli*. 2017.
13. Gasanov, U., D. Hughes, and P.M. Hansbro. 2005. Methods for the isolation and identification of *Listeria* spp., and *Listeria monocytogenes*: a review. *FEMS Microbiol. Rev.* 29:851–875.
14. Gehring, A.G., D.M. Albin, A.K. Bhunia, H. Kim, S.A. Reed, and S. Tu. 2012. Mixed culture enrichment of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* and *Yersinia enterocolitica*. *Food Control*. 26:269-273.
15. Hitchins, A.D., K. Jinneman, and Y. Chen, *Bacteriological Analytical Manual, Chapter 10. Detection and Enumeration of Listeria monocytogenes*. 2017.
16. Jasson, V., A. Rajkovic, J. Debevere, and M. Uyttendaele. 2009. Kinetics of resuscitation and growth of *Listeria monocytogenes* as a tool to select appropriate enrichment conditions as a prior step to rapid detection methods. *Food Microbiol*. 26: 88-93.
17. Kanki, M., K. Seto, J. Sakata, T. Harada, and Y. Kumeda. 2009. Simultaneous enrichment of Shiga Toxin-producing *Escherichia coli* O157 and O26 and *Salmonella* in food samples using universal preenrichment broth. *J. Food Prot*. 72: 2065-2070.

18. Keys, A.L., R.C. Dailey, A.D. Hitchins, and D. Smiley. 2013. Postenrichment population differentials using buffered *Listeria* enrichment broth: Implications of the presence of *Listeria innocua* on *Listeria monocytogenes* in food test samples. J. Food Prot. 76:1854-1862.
19. Kim, H., and A.K. Bhunia. 2008. SEL, a selective enrichment broth for simultaneous growth of *Salmonella enterica, Escherichia coli* O157:H7, and *Listeria monocytogenes*. *Appl. Environ. Microbiol*. 74:4853-4866.
20. Kobayashi, H., J. Kobota, K. Fujihara, K. Honjoh, M. Iio, N. Fujiki, M. Nakabe, S. Oda, T. Satoyama, K. Takasu, H. Nakanishi, and T. Miyamoto. 2009. Simultaneous enrichment of *Salmonella* spp, *Escherichia coli* O157:H7, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes* by single broth and screening of the pathogens by multiples real-time PCR. *Food Sci. Technol. Res*. 15:427-438.
21. Sathyamoorthy, V., A. Datta, C. Lee, Y. He, J. Sadowski, B. Tall, and B. McCardell. 2012. A multiplex real-time PCR method for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157 and *Listeria monocytogenes* in soft cheeses. International Association for Food Protection Annual Meeting, Providence, RI. Poster Presentation P1-12.
22. Sathyamoorthy, V., A. Datta, L. Trach, Y. He, B. Tall, and B. McCardell. 2013. Simultaneous detection of *Salmonella* spp., *Escherichia coli* O157 and *Listeria monocytogenes* in a variety of cheeses and spinach using a multiplex real-time PCR method. International Association for Food Protection Annual Meeting, Charlotte, NC. Poster Presentation P3-36.
23. Sathyamoorthy, V., N. Noroozi, E. Park, Y. Kim, B. Tall, Y. He, S. Hwang, L. Trach, H. Chase, B. McCardell, and A. Datta. 2015. An improved method to simultaneously detect *Salmonella* Enteritidis, *Escherichia coli* O157 and *Listeria monocytogenes* in ground black pepper using multiplex real-time PCR. International Association for Food Protection Annual Meeting, Portland, OR. Poster Presentation P2-18.
24. Sathyamoorthy, V., L. Trach, Y. He, B. Tall, H. Chase, S. Hwang, B. Lee, B. McCardell, and A. Datta. 2014. A robust multiplex real-time PCR method for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157 and *Listeria monocytogenes* in fresh fruits and vegetables. International Association for Food Protection Annual Meeting, Indianapolis, IN. Poster Presentation P1-56.
25. Scallan, E., R.M. Hoekstra, F.J. Angulo, R.V. Tauxe, M.A. Widdowson, S.L. Roy, J.L. Jones, and P.M. Griffin. 2011. Foodborne illness acquired in the United States-Major pathogens. *Emerg. Infect. Diseases*.17:7-15.
26. Suo, B., Y. He, S. Tu, and X. Shi. 2010. A multiplex real-time polymerase chain reaction for simultaneous detection of *Salmonella* spp., *Escherichia coli* O157, and *Listeria monocytogenes* in meat products. *Foodborne Pathog. Dis.* 7:619-628.
27. Suo, Y., S. Gao, Y. Xie, Y. Liu, Y. Qu, T. Lin, and Z. Changyan. 2017. A multipathogen selective enrichment broth for simultaneous growth of *Salmonella enterica*, *Escherichia coli* O157:H7, and *Shigella flexneri*. *J. Food Safety*. e12388(9).
28. Van Houteghem N., F. Devlieghere, A. Rajkovic, S.M.O. Gomez, M. Uyttendaele, and J. Debevere. 2008. Effects of CO2 on the resuscitation of *Listeria monocytogenes* injured by various bactericidal treatments. *Int. J. Food Microbiol*. 123:67-73.
29. Wesche, A.M., J.B. Gurtler, B.P. Marks, and E.T. Ryser. 2009. Stress, sublethal injury, resuscitation and virulence of bacterial foodborne pathogens. *J. Food Prot*. 72:1121-1138.

30. Yu, G.Y., H. Wu, Y.Y. Liu, S.L. Li, X.Q. Yang, and X.L. Xiao. 2010. A multipathogen selective enrichment broth for simultaneous growth of *Salmonella enterica* serovar Enteritidis, *Staphylococcus aureus*, and *Listeria monocytogenes*. *Can. J. Microbiol*. 56:585-597.

TABLES

Table 1. Candidate broths for simultaneous enrichment of *Salmonella* Typhimurium, EHEC, and *Listeria monocytogenes*. Except for SEL, no selective supplements were added.

Broth	Formulation Reference
BLEB base	*15*
BMW	*11*
Buffered BMW	BMW broth modified by the addition of buffering salts
mBPW	*10*
mBPW+glucose	mBPW broth modified by the addition of 2.5% glucose
SEL	*19*
SSL base	*30*
TSB	*2*
UPB	*2, 3*

Table 2. Primers and probes for multiplex quantitative real-time PCR for simultaneous detection of *Salmonella*, EHEC, and *Listeria monocytogenes (26)*.

Pathogen	Gene Target	Sequence (5' → 3')
Salmonella spp.	*invA*	F: GTTGAGGATGTTATTCGCAAAGG R: GGAGGCTTCCGGGTCAAG Probe: CAL Flour Orange 560- CCGTCAGACCTCTGGCAGTACCTTCCTC
EHEC	*rfbE*	F: TGTTCCAACACTGACATATATAGCATCA R: TGCCAAGTTTCATTATCTGAATCAA Probe: Quasar 670- ATGCTATAAAATACACAGGAGCCACCCCCA
Listeria monocytogenes	*hlyA*	F: ACTGAAGCAAAGGATCGATCTG R: TTTTCGATTGGCGTCTTAGGA Probe: FAM-CACCACCAGCATCTCCGCCTGC

Table 3. Growth curve characterization of *Salmonella* Typhimurium, EHEC and *Listeria monocytogenes* enrichment in candidate universal enrichment broths. Individual pathogens were inoculated from frozen culture and incubated for 24h at 37˚C.

	***Salmonella* Typhimurium**		**EHEC**		***Listeria monocytogenes***	
	Lag Phase (λ) hours	Potential Maximum Growth Rate (μ_{max})	Lag Phase (λ) hours	Potential Maximum Growth Rate (μ_{max})	Lag Phase (λ) hours	Potential Maximum Growth Rate (μ_{max})
BLEB	9.23 ± 0.43	0.238 ± 0.012	8.88 ± 0.39	0.384 ± 0.008	12.65 ± 1.18	0.111 ± 0.031
BMW	8.90 ± 0.14	0.404 ± 0.008	7.47 ± 0.12	0.568 ± 0.022	11.18 ± 0.32	0.081 ± 0.001
Buffered BMW	9.42 ± 0.12	0.239 ± 0.019	8.45 ± 0.40	0.363 ± 0.048	10.49 ± 0.17	0.064 ± 0.004
mBPW	9.52 ± 0.74	0.037 ± 0.001	10.81 ± 1.33	0.063 ± 0.021	12.27 ± 0.23	0.042 ± 0.001
mBPW+ glucose	11.06 ± 0.54	0.305 ± 0.025	9.59 ± 0.47	0.328 ± 0.022	15.90 ± 0.47	0.028 ± 0.004
SSL	10.82 ± 0.73	0.295 ± 0.017	9.10 ± 0.11	0.344 ± 0.189	17.37 ± 0.19	0.111 ± 0.005
TSB	9.55 ± 0.53	0.257 ± 0.010	7.28 ± 0.12	0.231 ± 0.005	12.11 ± 0.42	0.054 ± 0.002
UPB	7.32 ± 0.25	0.077 ± 0.001	7.26 ± 0.52	0.197 ± 0.003	12.80 ± 0.23	0.118 ± 0.005
BAM Broth	9.31 ± 0.28 (LB)	0.038 ± 0.003 (LB)	8.64 ± 0.28 (mBPWp)	0.321 ± 0.007 (mBPWp)	12.65 ± 1.18 (LEB)	0.111 ± 0.031 (LEB)

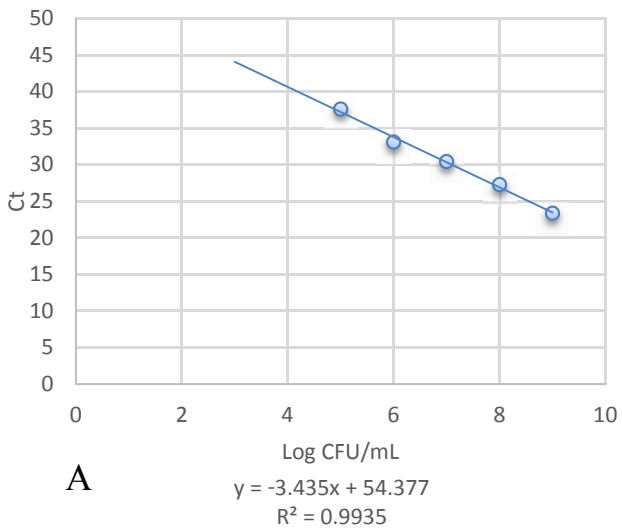
Table 4. Simultaneous enrichment of *Salmonella* Typhimurium, *E. coli* O157:H7 and *Listeria monocytogenes* in candidate enrichment broths after 18h or 24h incubation at 37°C and 30°C. After enrichment, DNA was extracted from broth and final concentrations of each target pathogen were determined by multiplex qPCR; Ct values are shown.

	***Salmonella* Typhimurium**	**EHEC**	***Listeria monocytogenes***	***Salmonella* Typhimurium**	**EHEC**	***Listeria monocytogenes***
	18h incubation @ 37°C			***24h incubation @ 37°C***		
BLEB	28.77 ± 0.47	27.55 ± 0.29*	29.38 ± 0.62	23.68 ± 2.38[a]	22.22 ± 1.89*	32.89 ± 3.73[a]
BMW	30.76 ± 1.44	27.63 ± 0.21	30.69 ± 0.56	20.33 ± 1.38	22.17 ± 2.92	34.48 ± 0.32[a]
Buffered BMW	25.34 ± 4.54	27.24 ± 0.44	32.94 ± 1.24	24.26 ± 1.91	21.36 ± 1.64	34.15 ± 3.19[a]
mBPW	27.46 ± 0.34	30.4 ± 0.46	33.79 ± 0.34*	24.8 ± 3.57	20.85 ± 1.57	32.57 ± 2.65*
mBPW+glucose	25.69 ± 0.90	28.84 ± 0.87	33.38 ± 0.45[a]	23.42 ± 2.29	23.12 ± 2.07[a]	33.40 ± 1.68
SEL	30.94 ± 2.10[a]	30.08 ± 1.33[a]	32.66 ± 2.99	24.05 ± 2.29	23.38 ± 2.20[a]	23.57 ± 2.68
SSL	28.01 ± 0.62[a]	28.09 ± 0.33	35.75 ± 0.01	26.49 ± 5.20	21.81 ± 3.16	35.99 ± 0.53
TSB	32.88 ± 0.64*	27.25 ± 0.43	30.87 ± 0.90	22.59 ± 1.46*	23.29 ± 2.30	ND
UPB	28.79 ± 0.86	27.93 ± 0.89	31.72 ± 0.56	23.47 ± 2.88	21.13 ± 1.60	36.76 ± 1.14
	18h incubation @ 30°C			***24h incubation @ 30°C***		
BLEB	28.86 ± 0.45*	27.38 ± 0.19	29.28 ± 0.05*	25.67 ± 0.38*[a]	27.09 ± 0.52	26.99 ± 0.32*[a]
BMW	30.97 ± 1.27	28.37 ± 0.10	29.86 ± 0.52*	28.7 ± 0.47	27.16 ± 0.43	27.84 ± 0.27*[a]
Buffered BMW	27.16 ± 0.81	28.08 ± 0.69	28.4 ± 1.18	26.58 ± 0.71	26.54 ± 0.24	26.56 ± 0.39[a]
mBPW	26.71 ± 0.42	30.04 ± 0.49	32.47 ± 0.82	16.48 ± 1.20	28.32 ± 2.15	29.66 ± 2.16
mBPW+glucose	27.23 ± 0.42	29.28 ± 0.37	32.08 ± 0.58[a]	27.28 ± 0.84	27.7 ± 0.55[a]	31.06 ± 0.89
SEL	25.02 ± 1.26[a]	22.94 ± 0.45*[a]	27.85 ± 0.28	24.64 ± 0.50	26.53 ± 0.10*[a]	29.65 ± 0.93
SSL	29.35 ± 0.18*[a]	29.73 ± 0.01	32.24 ± 0.29*	26.93 ± 0.50*	28.52 ± 0.22	29.85 ± 0.38*
TSB	34.07 ± 0.46*	27.88 ± 0.69	31.16 ± 0.10*	27.78 ± 2.21*	26.06 ± 1.32	27.49 ± 0.18*
UPB	30.87 ± 1.14*	28.74 ± 0.83	30.34 ± 0.99*	28.31 ± 2.00*	28.4 ± 0.15	29.23 ± 0.98*

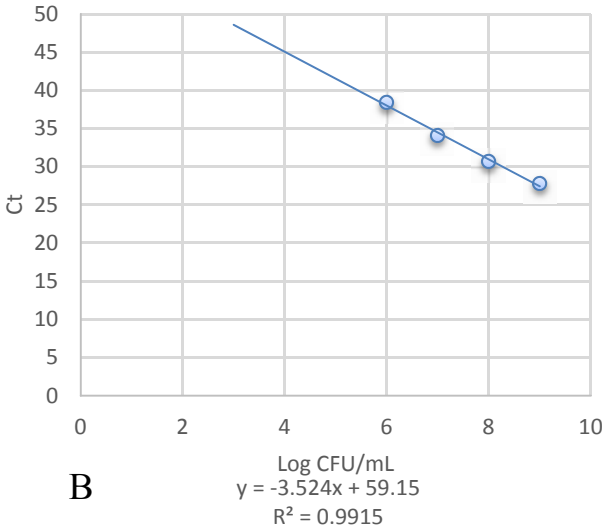
(*) Ct values in the same row and corresponding to the Ct values of *Salmonella* Typhimurium, *E. coli* O157:H7 and *Listeria monocytogenes* at a given time point are significantly different ($p<0.05$). ([a]) Ct values in the same column and corresponding to the Ct values of *Salmonella* Typhimurium, *E. coli* O157:H7 and *Listeria monocytogenes* at a given temperature are significantly different ($p<0.05$). ND indicates the target sequence was not detected.

FIGURES

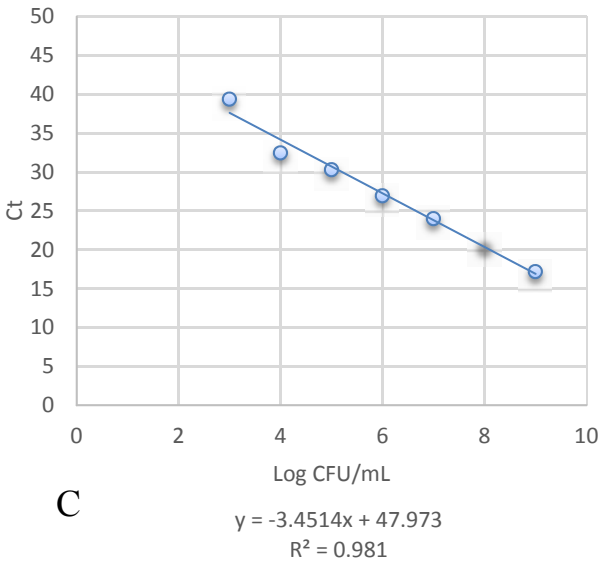
Figure 1. Multiplex qPCR Standard Curves were constructed using DNA extracts of serial dilutions of each target pathogen from 10^9 to 10^3 CFU/mL. *Salmonella* Typhimurirum (A), EHEC (B) and *Listeria monocytogenes* (C).



A



B



C

Figure 2. Log CFU/mL (average ± standard deviation) of *Salmonella* Typhimurium (■) , EHEC (▨) , and *Listeria monocytogenes* (■) after 24h enrichment at 37˚C in Bioscreen C.

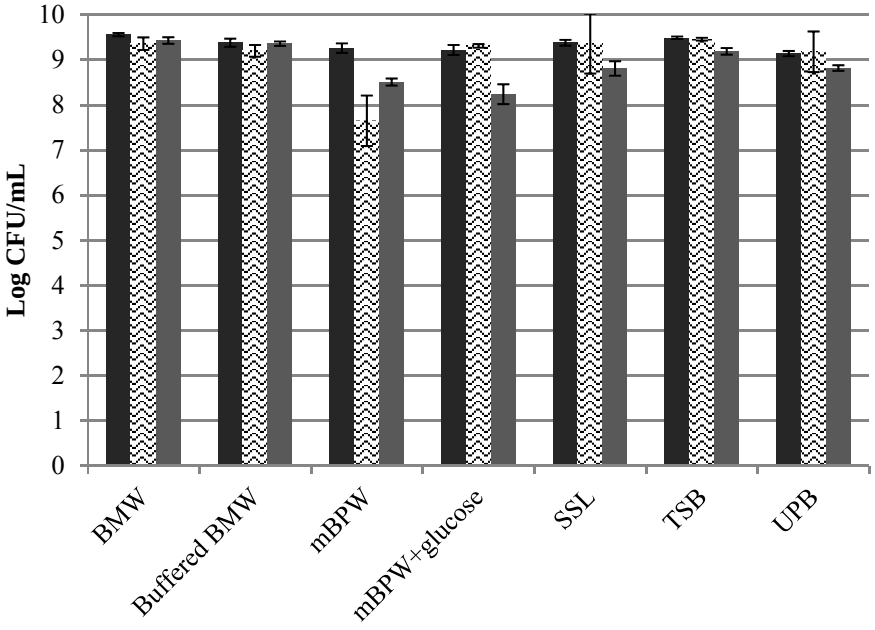


Figure 3. Individual growth curves (OD_{600nm}) of *Salmonella* Typhimurium (◆), EHEC (■) and *Listeria monocytogenes* (▲) in UPB (A), BMW (B), and Buffered BMW (C).

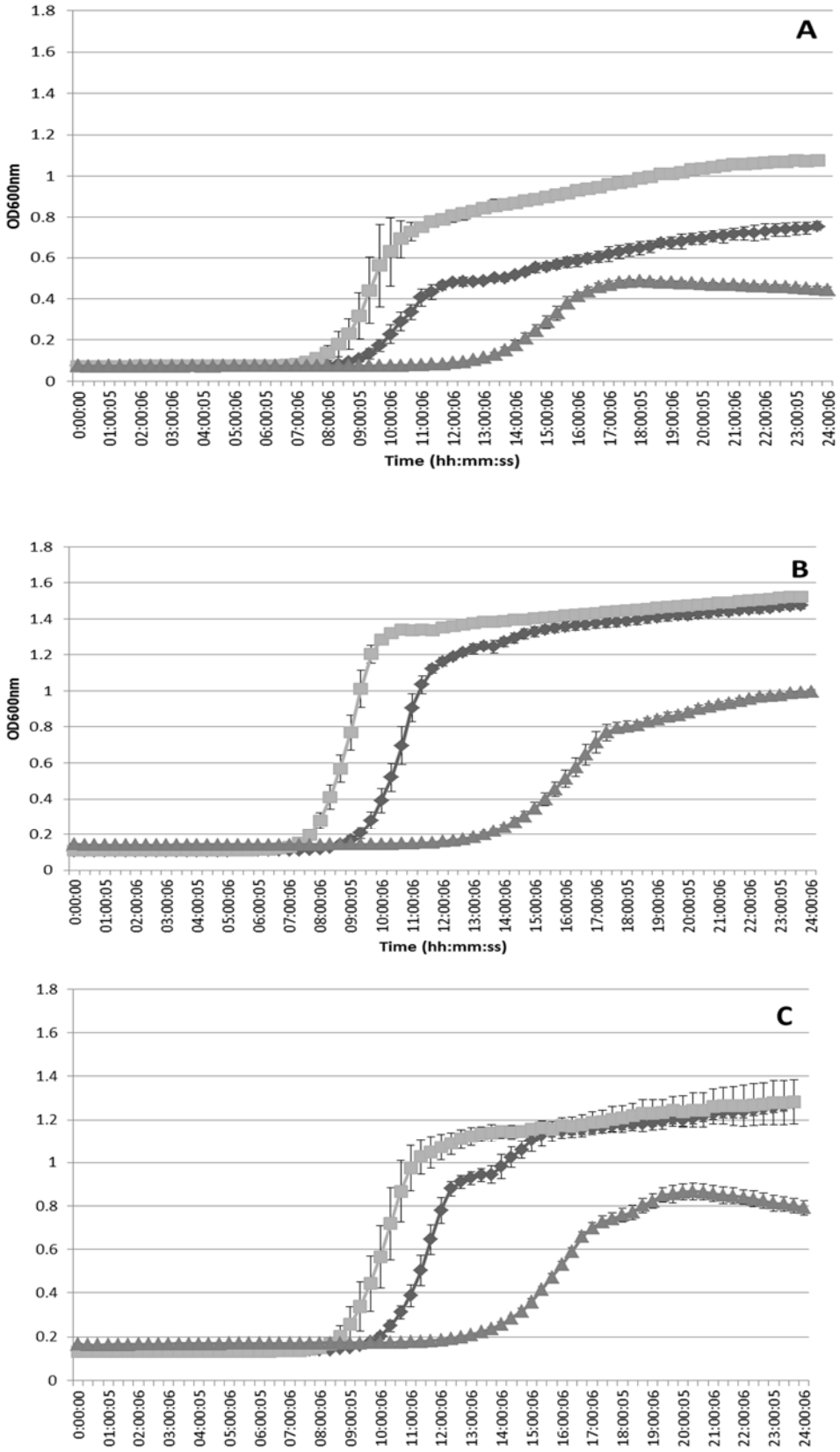


Figure 4. Simultaneous enrichment of *Salmonella* Typhiumurium (■), EHEC (▨), and *Listeria monocytogenes* (■) in 25mL of the candidate enrichment broths at 35˚C for 22-24h. Pathogens were inoculated into broths at varying ratios 1:1:1 (A), 10:1000:1 (B), 1:10:1000 (C), and 1000:1:10 (D) of *Salmonella* Typhiumurium, EHEC and *Listeria monocytogenes*, respectively. At the 1:1:1 ratio, 11.85 ±2.33 CFU, 7.53 ± 1.73 CFU and 11.1 ± 0.14 CFU of *Salmonella* Typhiumurium, EHEC, and *Listeria monocytogenes* from fresh culture were inoculated into 25mL of each candidate enrichment broths, respectively. (*) indicates no Ct value detected. N =3.