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Enterobacteriaceae complicate the recovery of *Listeria monocytogenes* from food product enrichments using buffered *Listeria* enrichment broth

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Abstract

Buffered *Listeria* enrichment broth (BLEB) is the preferred enrichment formulation of the U.S. Food and Drug Administration (FDA) for the recovery of *Listeria monocytogenes*. BLEB permits the growth of all foodborne species of *Listeria* and several non-*Listeria* species including some members of the family *Enterobacteriaceae*. Five species of *Enterobacteriaceae* and four strains of non-pathogenic *Listeria* species were evaluated to determine which ones most suppressed to *L. monocytogenes* 48 h populations in BLEB; these were subsequently used in spiked-food enrichment competition studies. *L. monocytogenes* recovery was complicated by the simultaneous presence of both *Listeria innocua* and *Enterobacteriaceae* competitors. *L. innocua* was preferentially recovered from all spiked-food enrichments when using either Oxford or chromogenic agars. Matrix-free competition assays using non-pathogenic *Listeria* species and *Citrobacter braakii*, simultaneously, indicate the potential for *Enterobacteriaceae* competitors to contribute considerably to both *L. monocytogenes* population suppression and to large *Listeria* inter-species population differentials. In some instances, *Enterobacteriaceae* competitors suppressed *L. monocytogenes* populations more than the non-pathogenic *Listeria* species. The ability to recover *L. monocytogenes* from food matrices with complex microflora, including multiple species of *Listeria*, is an important issue for regulatory agencies since detection without recovery is considered a false positive test result.

Keywords:
regulatory microbiology, foodborne pathogen, real-time PCR, selective enrichment, microbial competition, *Listeria monocytogenes*, *Listeria innocua*, *Listeria welshimeri*

1. Introduction

Foodborne pathogens, such as *Listeria monocytogenes*, may be present at low levels (< 1 CFU/g); therefore, regulatory agencies use selective enrichment to ensure recovery of the target organism. The United States Food and Drug Administration (FDA) testing laboratories rely on a two-stage enrichment method, using buffered *Listeria* enrichment broth (BLEB) at 30 °C. The first stage is a four-hour incubation period under non-selective conditions followed by a 44-h incubation period after the addition of selective supplements. BLEB is essentially buffered Difco Trypticase™soy broth (BD Diagnostics, Inc.; Sparks, MD) with 0.6% yeast extract (TSBye) to which acriflavine-HCL, sodium nalidixate, and cycloheximide are added at final concentrations of 10, 40, and 50 mg/L, respectively. Although sodium nalidixate demonstrates broad activity against many Gram-negative bacteria, BLEB does not appear to demonstrate a level of selectivity necessary to restrict completely the growth of all non-target bacterial strains during selective enrichment [1]. The presence of some *Enterobacteriaceae* species can suppress the BLEB enrichment populations of *Listeria* species, including *L. monocytogenes* and may ultimately have a negative impact on detection and recovery of this organism. The potential for food background microorganisms to interfere with the recovery of *L. monocytogenes* due to the lack of selectivity of BLEB was recognized in the early 1990s [2–5]. However, these earlier studies looked only at the direct effects of non-*Listeria* competitors on *L. monocytogenes* recovery and did not consider the secondary effects that might occur when multiple *Listeria* species are present.

In addition to non-*Listeria* microbial growth, BLEB allows the growth of other foodborne species of *Listeria* including *L. monocytogenes*, *Listeria innocua*, *Listeria seeligeri*, and *Listeria welshimeri*. When multiple species of *Listeria* are present in the test sample it becomes increasingly difficult to recover and isolate *L. monocytogenes* particularly when differences in their rate of growth, under selective enrichment conditions, results in large inter-species population differentials [5, 6]. *Listeria*

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inter-species competition during selective enrichment was recognized in the mid-1990s [7–9]. Subsequent studies sought to determine the cause of competition [10–14] or to compare competition within spiked samples versus naturally contaminated samples [15].

Despite the interest that has been shown in *Listeria* inter-species competition there are questions that have not been addressed; this is evident by the fact that U.S. regulatory agencies have not been able to make any significant changes to their selective enrichment procedures to account for this issue. Although there are now several published reports on this subject, these studies have primarily focused on Fraser broth, one-half-strength Fraser broth, and UVM broth with less attention given to BLEB despite its current use by the FDA. The extent to which the natural background microflora of test samples affect the level of *Listeria* inter-species competition has not yet been resolved. This study evaluates the level of competition that occurs between select strains of *L. monocytogenes* and *Enterobacteriaceae* during selective enrichment using BLEB. This study also evaluates the extent to which *Enterobacteriaceae* affects *L. monocytogenes*/*L. innocua* competition in spiked-food enrichments. This current assessment includes both *L. monocytogenes*/*L. innocua* population differentials that result during the selective enrichment of spiked-food matrices and evaluates the effectiveness of chromogenic media to improve the recovery of *L. monocytogenes* when multiple species of *Listeria* are present. Finally, this study demonstrates the individual and combined contributions of *Enterobacteriaceae* and non-pathogenic *Listeria* competitors to the population suppression of *L. monocytogenes* during selective enrichment in BLEB.

2. Materials and Methods

2.1. Assessment of competitive fitness among Listeria strains

One hundred strains of *L. monocytogenes*, 55 stains of *L. innocua*, 14 strains of *L. seeligeri*, and 31 strains of *L. welshimeri* were screened for competitive fitness during selective enrichment in BLEB. The strains of *L. monocytogenes* were comprised primarily of serotypes 1/2a, 1/2b and 4b. The serotypes for the other species of *Listeria* were not determined. All strains were isolated from food or food processing environmental samples using FDA isolation procedures[17]. The strain of *C. braakii* (ARL-Cb-01) used in this study was isolated from papaya following 48 h incubation in selective BLEB at 30 °C and is the same strain reported earlier [1].

For inocula preparation, all cultures were statically incubated 18-24 h at 35 °C. Serial dilutions were prepared in Butterfield'phosphate buffer (BPB) [16] and all *Listeria* species inoculations (25 μL) were performed from the 10^{-6} dilution; *C. braakii* was inoculated (25μL) from the 10^{-5} dilution (approximately 1 log higher). Inoculum levels were verified using Difco Trypticase™soy agar with 0.6% yeast extract (TSAye) (BD Diagnostics; Sparks, MD). *C. braakii* was co-inoculated with each of the 200 strains of *Listeria* species into 25 mL of selective BLEB which was statically incubated for 48 h at 30 °C. Serial dilutions of the enrichments were prepared in BPB and plated onto Difco Oxford agar (BD Diagnostics; Sparks, MD). Oxford agar plates were incubated at 35 °C and enumerated at 24 h; the plates were re-examined at 48 h for the appearance of any slow forming colonies. The strain of *C. braakii* used in this study did not demonstrate growth on this medium. The number of strains that fell within defined population ranges (bin size = 1.0 log) were tabulated and reported as the post-enrichment population distribution.

2.2. Bacterial strains

The seven strains of *L. monocytogenes* (ARL-Lm-007 (Lm-007), ARL-Lm-015 (Lm015), (ARL-Lm-031 (Lm031), ARL-Lm-054 (Lm054), ARL-Lm-062 (Lm062), ARL-Lm-065 (Lm-065), and ARL-Lm-087 (Lm087)) used in the remainder of the study were selected because they were determined to be the least competitive of the 100 strains initially screened. The strains were comprised of serotypes 1/2a (Lm031, Lm054, and Lm087) and 1/2b (Lm007, Lm015, Lm062, and Lm065). Two foodborne strains of *L. innocua* (ARL-Ln-024 (Ln024) and ARL-Ln-032 (Ln032)) and *L. welshimeri* (ARL-Lw-005 (Lw005) and ARL-Lw-008 (Lw008)) were also used in this study and were selected because they demonstrated the least level of sensitivity when grown competitively with *C. braakii* in BLEB. Five foodborne species of the family *Enterobacteriaceae* (*C. braakii* ARL-Cb-01, *Escherichia coli* ARL-Ec-01, *Enterobacter cloacae* ARL-Ecl-01, *Enterobacter aerogenes* ARL-Eae-01, and *Klebsiella pneumonia* ARL-Kp-01) were used as the background competitors. These strains were selected because they grew well in selective BLEB and because these same strains had been included in an earlier competition study [1]. All bacterial strains were isolated and confirmed to the species level using the FDA *Listeria* isolation method [17] and by 16S rRNA gene sequencing [18]. All strains were maintained cryoprotectively at -80 °C from their initial time of isolation and working stocks were maintained in BBL motility test medium (BD Diagnostics; Starks, MD) at room temperature.

2.3. Assessment of the most competitive Enterobacteriaceae species in BLEB

A subset of the seven *L. monocytogenes* strains (Lm031, Lm062 and Lm065) was used to determine which of the five *Enterobacteriaceae* species was most suppressive to the final population of *L. monocytogenes*. Strain Lm031 was selected because it had the highest enrichment population of the seven strains selected from the competitive fitness screen described in section 2.1. Strain Lm062 was included because it had one of the lowest enrichment populations and strain Lm065 was included because its enrichment population was intermediate to the other two strains. Each *L. monocytogenes* strain was individually paired with *C. braakii*, *E. coli*, *E. cloacae*, *E. aerogenes*, or *K. pneumoniae* for competition assays; no other *Listeria* species were present in the assay. Each strain was prepared by inoculating 9 mL of non-selective BLEB directly from the motility agar working stocks. All cultures were statically grown 18-24 h at 35 °C. Serial dilutions were prepared using BPB. The target inocula were 1-5 CFU/mL for all *L. monocytogenes* strains and 10-50 CFU/mL for all *Enterobacteriaceae*

Inoculation Levels		48 h Population (log CFU/mL)		Recovery Medium	
Competitive Grouping	CFU/mL	*L. innocua*	*L. monocytogenes*[1]	Oxford[2]	Chromogenic
C. braakii	16				
L. innocua[3]	1.8	7.7 ± 0.1	4.9 ± 0.2[a]	ND	ND
L. monocytogenes[4]	1.2				
E. coli	1.4				
L. innocua[3]	1.6	7.9 ± 0.1	4.7 ± 0.2[a]	0/3	1/3
L. monocytogenes[4]	1.0				
E. aerogenes	4.0				
L. innocua[3]	1.6	7.8 ± 0.1	5.0 ± 0.3[a]	0/3	1/3
L. monocytogenes[4]	1.0				
E. cloacae	2.5				
L. innocua[3]	1.6	7.8 ± 0.1	6.2 ± 0.1[b]	0/3	2/3
L. monocytogenes[4]	1.0				
K. pneumoniae	2.4				
L. innocua[3]	1.6	8.9 ± 0.1	5.3 ± 0.1[a]	0/3	0/3
L. monocytogenes[4]	1.0				

Table 1. The effects of *L. innocua* and select species of *Enterobacteriaceae* on recovery of *L. monocytogenes* from BLEB at 30 °C.
[1]Mean populations followed by the same superscript are not statistically different (P>0.05) as determined using a two way ANOVA with Tukey's mean separation test.
[2]10 colonies per plate (replication) tested
[3]strain ARL-Ln-032
[4]strain ARL-Lm-031
ND, not determined

strains and were verified by surface plating onto TSAye plates that were incubated at 35 °C for 24 h. All competition assays were performed using 25 mL of selective BLEB in 50 mL conical centrifuge tubes; selective BLEB had the selective agents already added at the time of inoculation. The inoculated tubes were statically incubated 48 h at 30 °C. The 48-h *L. monocytogenes* population was subsequently determined by surface plating onto Oxford agar plates that were incubated at 35 °C for 24 and 48 h. None of the *Enterobacteriaceae* study strains was capable of forming colonies on Oxford agar. Each experiment was performed in triplicate.

The data were analyzed using a two-way ANOVA with *L. monocytogenes* strain and *Enterobacteriaceae* species as fixed variables and *L. monocytogenes* population as the response variable. The statistical significance of both main effects and interactions was determined and mean comparisons were performed using the Tukey test as this test is the most conservative (i.e. least likely to result in a significant difference). The statistical assumption that the residual terms were normally distributed was verified using the Shapiro-Wilk normality test (P>0.05). Equal variance of the residual terms was determined by visually inspecting a scatterplot of the residuals for the absence of discernible patterns.

2.4. Effect of Enterobacteriaceae species on Listeria inter-species competition in BLEB

Seven strains of *L. monocytogenes* were individually paired with *L. innocua* (two strains) and *L. welshimeri* (two strains) and were inoculated into 50 mL conical centrifuge tubes containing 25 mL of selective BLEB as described in section 2.3. Each tube containing a unique *Listeria* species pairing was also inoculated with *C. braakii*. The inoculated BLEB tubes were statically incubated at 30 °C for 48 h. In order to estimate the individual populations of each species of *Listeria*, a total *Listeria* species count was first obtained. The BLEB enrichments were thoroughly mixed to re-suspend the cells and serial dilutions were prepared in BPB and were enumerated using Oxford agar plates. The population of *L. monocytogenes* was directly determined by quantitative real-time PCR (qPCR) using the Applied Biosystems MicroSeq®*Listeria* monocytogenes detection kit (Life Technologies, Inc.; Grand Island, NY) as previously described [6]. Briefly, 1 mL aliquots were removed from the 48-h enrichments and the cells were pelleted by centrifugation (10,000 x g for 5 min). Microbial genomic DNA was harvested using the UltraClean®Microbial DNA isolation kit (MoBio Laboratories, Inc.; Carlsbad, CA). Thirty microliters of the purified DNA was used for each PCR per the manufacturers instructions. All PCR assays were performed using an Applied Biosystems 7500 fast real-time thermocycler (Life Technologies, Inc.; Grand Island, NY). In order to make the assay quantitative, serial dilutions of prepared DNA originating from a known concentration of *L. monocytogenes* were included. Plots of cycle threshold (C_T) values versus cell concentration (log CFU/mL) were used for generating standard curves. The population of *L. innocua* or *L. welshimeri* was determined as the difference between the total *Listeria* species population determined using Oxford agar and the population of *L. monocytogenes* determined by qPCR. All experiments were performed in triplicate. The population differentials were calculated as the difference between the non-pathogenic Listeria levels (log CFU/mL) and the *L. monocytogenes* levels (log CFU/mL).

The quantitation of *L. monocytogenes* by qPCR, using the MicroSeq®*Listeria monocytogenes* detection kit, was directly compared to plate count enumeration in an earlier study [6]. It

Inoculation Levels		48 h Population (log CFU/mL)		Recovery Medium	
Competitive Grouping	CFU/g	*L. innocua*	*L. monocytogenes*[1]	Oxford[2]	Chromogenic
C. braakii	36				
L. innocua[3]	2.1	7.5 ± 0.1	3.6 ± 0.2[a]	0/3	0/3
L. monocytogenes[4]	1.5				
E. coli	18				
L. innocua[3]	3.8	7.7 ± 0.1	4.1 ± 0.2[a]	0/3	0/3
L. monocytogenes[4]	2.2				
E. aerogenes	28				
L. innocua[3]	3.8	7.7 ± 0.1	4.1 ± 0.4[a]	0/3	0/3
L. monocytogenes[4]	2.2				
E. cloacae	14				
L. innocua[3]	3.8	7.7 ± 0.1	3.9 ± 0.5[a]	0/3	0/3
L. monocytogenes[4]	2.2				
K. pneumoniae	14				
L. innocua[3]	3.8	9.4 ± 0.2	5.2 ± 0.4[b]	0/3	0/3
L. monocytogenes[4]	2.2				

Table 2. The effects of *L. innocua* and select species of *Enterobacteriaceae* on recovery of *L. monocytogenes* from spiked guacamole enrichments.
[1]Mean populations followed by the same superscript are not statistically different (P>0.05) as determined using a two way ANOVA with Tukey's mean separation test.
[2]10 colonies per plate (replication) tested
[3]strain ARL-Ln-032
[4]strain ARL-Lm-031

was shown that the presence of additional species of *Listeria* in the test sample did not interfere with the accurate quantitation of *L. monocytogenes*. In the current study, the MicroSeq®PCR assay was used to enumerate *L. monocytogenes* from spiked-food enrichments. The efficiency of the qPCR assay was estimated from the standard curves using the formula $(10^{(-1/slope)}-1)$. To verify the absence of any matrix-specific interference, the cycle threshold (C_T) values for the internal amplification control (IAC) reactions for the spiked-food enrichment samples and the control amplification reactions (i.e. standard curves) were compared by one-way ANOVA.

The *Listeria* inter-species population differential data were analyzed using a two-way analysis of variance (ANOVA), as described in section 2.3, with *L. monocytogenes* strain and *L. innocua* strain as fixed variables and population differential as the response variable. The effects of the other four species of *Enterobacteriaceae* on *Listeria* inter-species competition was also initially determined in the absence of food-matrix components using *L. monocytogenes* strain Lm031 and *L. innocua* strain Ln032. Strain Lm031 was selected because it appeared to be the least sensitive to the presence of *C. braakii*. All of the strains of non-pathogenic *Listeria* demonstrated approximately the same level of sensitivity to *C. braakii* so *L. innocua* strain Ln032 was selected at random. The competition assays were performed in the same manner that was used for evaluating *C. braakii*. In addition to determining the populations of *L. monocytogenes* and *L. innocua*, the recovery of the former by the streak plate method, using both Oxford and Rapid'L.mono™agars (Bio-Rad, Inc.; Hercules, CA), was attempted. After streaking for isolation (four quadrant streak), Oxford agar plates were incubated at 35 °C for 24 and 48 h and chromogenic agar plates were incubated at 37 °C for 24 and 48 h. For Oxford agar, which lacks the differentiating capability of chromogenic agar, 10 well-isolated colonies were selected and transferred to the surface of Trypticase™soy agar supplemented with 5% sheep blood (Remel Products, Thermo Fisher Scientific; Lexena, KS) to test for β-hemolysis. All hemolytic colonies were confirmed by real-time PCR using the MicroSEQ®*Listeria monocytogenes* real-time PCR test kit. Rapid'L.mono™plates were visually inspected at 24 and 48 h for the presence of blue colonies which are indicative of *L. monocytogenes*. Typical *L. monocytogenes* colonies, from chromogenic agar plates were also confirmed by real-time PCR. The data were analyzed using a two-way ANOVA, as described in section 2.3, with *Enterobacteriaceae* species and matrix type as fixed variables and *L. monocytogenes* population as the response variable.

2.5. *Effect of Enterobacteriaceae species on Listeria inter-species competition in spiked-food enrichments*

Analytical test portions (25 g) of guacamole, asadero-style cheese, and refrigerated crabmeat were placed in sterile 0.9 L Mason-style blender jars. Test samples were simultaneously inoculated with *C. braakii*, *L. innocua* strain Ln032, and *L. monocytogenes* strain Lm031, Lm062 or Lm065 to establish a *L. monocytogenes*/*L. innocua*/*C. braakii* ternary competition assay. All strains were prepared as described in section 2.3 and a similar inoculation procedure and levels were used. The inoculated test samples were equilibrated approximately 18-24 h at 5 °C prior to initiating the FDA *Listeria* selective enrichment procedure [17]. Following the 48-h incubation, each of the enrichments was mixed to re-suspend any sediment and the levels of *Listeria* species were determined as described in section 2.3. Aliquots (1 mL) were also removed from the enrichments and the levels of *L. monocytogenes* were specifically de-

Inoculation Levels		48 h Population (log CFU/mL)		Recovery Medium	
Competitive Grouping	CFU/g	*L. innocua*	*L. monocytogenes*[1]	Oxford[2]	Chromogenic
C. braakii	46				
L. innocua[3]	1.8	5.7 ± 0.5	2.9 ± 0.1[a]	0/3	0/3
L. monocytogenes[4]	1.3				
E. coli	166				
L. innocua[3]	3.0	7.1 ± 0.1	1.9 ± 0.7[a]	0/3	0/3
L. monocytogene4[3]	2.2				
E. aerogenes	215				
L. innocua[3]	3.0	6.6 ± 0.1	2.6 ± 0.8[a]	0/3	0/3
L. monocytogenes[4]	2.2				
E. cloacae	125				
L. innocua[3]	3.0	6.7 ± 0.3	2.2 ± 0.5[a]	0/3	0/3
L. monocytogenes[4]	2.2				
K. pneumoniae	196				
L. innocua[3]	3.0	6.1 ± 0.9	2.6 ± 0.2[a]	0/3	0/3
L. monocytogenes[4]	2.2				

Table 3. Effects of *L. innocua* and select species of *Enterobacteriaceae* on recovery of *L. monocytogenes* from spiked asadero-style cheese enrichments.
[1]Mean populations followed by the same superscript are not statistically different (P>0.05) as determined using a two way ANOVA with Tukey's mean separation test.
[2]10 colonies per plate (replication) tested
[3]strain ARL-Ln-032
[4]strain ARL-Lm-031

termined by qPCR as described in section 2.4. The population of *L. innocua* was estimated as the difference between the total *Listeria* species population and the *L. monocytogenes* population. Population differentials were calculated as the population of *L. innocua* (log CFU/mL) minus the population of *L. monocytogenes* (log CFU/mL) and reported as Δlog CFU/mL.

The data were analyzed using a two-way ANOVA, as described in section 2.3, with *L. monocytogenes* strain and matrix as fixed variables and selective enrichment population as the response variable.

The effects of the other four *Enterobacteriaceae* species on *Listeria* inter-species competition during the selective enrichment of spiked-food matrices was also assessed using strains Lm031 and Ln032. The spiked-food enrichments were performed in the same manner as for *C. braakii*. The enrichment levels of Listeria species were determined by surface plating onto Oxford agar as described in section 2.3 and the levels of *L. monocytogenes* were determined by qPCR as described in section 2.4. The final population of *L. innocua* was estimated as the difference between the total *Listeria* species population and the *L. monocytogenes* population.

The data were analyzed using a two-way ANOVA, as described in section 2.3, with *Enterobacteriaceae* species and matrix as the two fixed variables and selective enrichment population as the response variable.

2.6. Effects of Enterobacteriaceae on recovery of L. monocytogenes from spiked-food enrichments

The recovery of *L. monocytogenes* by the streak plate method, using both Oxford and RapidL.mono™agars, was attempted on all of the spiked-food enrichments described in section 2.5. The isolation and confirmation of *L. monocytogenes* were performed as described in section 2.4.

3. Results and Discussion

3.1. Assessment of Listeria competitive fitness

The resulting distribution of the *Listeria* populations observed during competitive growth with *C. braakii* in selective BLEB at 30 °C is illustrated in Figure 1. No *Listeria* strain had a final enrichment population greater than 8.2 log CFU/mL. The lowest final enrichment population observed was 1.3 log CFU/mL and was observed for one strain of *L. seeligeri* and one strain of *L. welshimeri*. Overall there was a statistical difference (P<0.001) between the four species of *Listeria*. The selective enrichment populations for *L. innocua* (7.5 ± 0.9 log CFU/mL) and *L. welshimeri* (7.6 ± 1.2 log CFU/mL) were statistically similar (P>0.05) and both were statistically higher (P<0.05) than either *L. monocytogenes* (7.0 ± 1.1 log CFU/mL) or *L. seeligeri* (6.7 ± 1.6 log CFU/mL) (data not shown). The selective enrichment populations for *L. monocytogenes* and *L. seeligeri* were statistically similar (P>0.05). Based on the overall mean population value, *L. monocytogenes* appears to be more sensitive to competitive microorganisms during selective enrichment than *L. innocua* or *L. welshimeri* but less sensitive than *L. seeligeri*.

3.2. Assessment of the most competitive Enterobacteriaceae species in BLEB

Five species (one strain per species) of *Enterobacteriaceae* were evaluated, against three strains of *L. monocytogenes*, to determine which one was most capable of suppressing the population of *L. monocytogenes* in selective BLEB (Figure 2). The

Inoculation Levels		48 h Population (log CFU/mL)		Recovery Medium	
Competitive Grouping	CFU/g	*L. innocua*	*L. monocytogenes*[1]	Oxford[2]	Chromogenic
C. braakii	16				
L. innocua[3]	1.4	6.6 ± 0.1	3.3 ± 0.2[a]	0/3	0/3
L. monocytogenes[4]	0.9				
E. coli	1.2				
L. innocua[3]	1.9	6.4 ± 0.5	3.3 ± 0.1[a]	0/3	0/3
L. monocytogenes[4]	1.8				
E. aerogenes	2.8				
L. innocua[3]	1.9	6.6 ± 0.2	3.0 ± 0.2[a]	0/3	0/3
L. monocytogenes[4]	1.8				
E. cloacae	6.0				
L. innocua[3]	1.9	6.4 ± 0.2	2.9 ± 0.1[a]	0/3	0/3
L. monocytogenes[4]	1.8				
K. pneumoniae	6.0				
L. innocua[3]	1.9	6.5 ± 0.1	2.9 ± 0.2[a]	0/3	0/3
L. monocytogenes[4]	1.8				

Table 4. The effects of *L. innocua* and select species of *Enterobacteriaceae* on recovery of *L. monocytogenes* from spiked crab meat enrichments.
[1]Mean populations followed by the same superscript are not statistically different (P>0.05) as determined using a two way ANOVA with Tukey's mean separation test.
[2]10 colonies per plate (replication) tested
[3]strain ARL-Ln-032
[4]strain ARL-Lm-031

overall mean (n=9) *L. monocytogenes* population, for all three strains, was 4.1 ± 0.5 log CFU/mL when *C. braakii* was the competitor (data not shown) and was statistically (P<0.001) lower than all other *Enterobacteriaceae* species tested. The individual *L. monocytogenes* mean populations for strains Lm031, Lm062, and Lm065, following competitive growth with *C. braakii*, are shown in Figure 2 and all three were significantly different (P<0.001).

The remaining overall mean populations (n=9 per strain) for *L. monocytogenes* were 6.8 ± 0.6, 6.5 ± 0.7, 6.6 ± 0.5, and 9.0 ± 0.2 log CFU/mL for *E. coli*, *E. aerogenes*, *E. cloacae*, and *K. pneumoniae* competitors, respectively (data not shown). The individual *L. monocytogenes* mean populations for strains Lm031, Lm062, and Lm065, following competitive growth with each of these competitors are shown in Figure 2. The populations for all three *L. monocytogenes* strains were statistically different when *E. coli*, *E. aerogenes*, and *E. cloacae* were used as the competitor. When *K. pneumoniae* was the background competitor the final *L. monocytogenes* population were statistically equivalent for all three *L. monocytogenes* strains; this suggests that this strain of *K. pneumoniae* exerts a minimal competitive effect on *L. monocytogenes* under these selective conditions.

3.3. Effect of Enterobacteriaceae on Listeria inter-species competition in BLEB

The efficiency of the qPCR assay was 1.05 ± 0.04 when calculated by $(10^{(-1/slope)}-1)$ which is equivalent to a slope of -3.2 ± 0.08, when C_T is plotted versus log CFU/mL (data not shown). The standard curve was constructed for each individual replication (N=24) and covered a range of 9.2 to 2.2 log CFU/mL (data not shown). The mean IAC C_T values for each matrix were 34.8 ± 0.3 for control (n=24), 33.6 ± 0.4 for guacamole (n=21), 34.5 ± 0.3 for asadero-style cheese (n=21), and 34.1 ± 0.3 for crabmeat (n=21) (data not shown). The IAC C_T values did not differ significantly (P>0.05) which is consistent with the absence of matrix-specific interferences in the final reaction.

It was shown in section 3.2 that *C. braakii* was the most competitive of the strains of *Enterobacteriaceae* tested and so it was the primary strain used for determining the effects of *Enterobacteriaceae* on *Listeria* inter-species competition in BLEB. The *Listeria* inter-species population differentials (Figure 3) ranged from 2.0 to 5.7 with an overall mean of 3.9 ± 1.0 log CFU/mL. ANOVA indicated statistically significant differences in the population differentials between the *L. monocytogenes* str-ains (P<-0.001) and between the non-pathogenic *Listeria* str-ains (P=0.004) and a statistically significant (P<0.001) interaction between *L. monocytogenes* and non-pathogenic *Listeria* strains. *L. monocytogenes* strain Lm031 demonstrated the overall lowest population differentials (mean of 2.8 ± 0.3 log CFU/mL) against all four strains of non-pathogenic *Listeria* tested (Figure 3). L. monocytogenes strain Lm015 demonstrated the largest population differentials (mean of 5.3 ± 0.2 log CFU/mL) with all four strains of non-pathogenic *Listeria* tested (Figure 3).

Although the ANOVA model indicated statistically significant differences between the non-pathogenic *Listeria*, individual mean comparisons only revealed statistical significance under one set of conditions. The mean population differentials for *L. welshimeri* strain Lw008 was significantly (P>0.05) larger (1 log) than for the other three strains of non-pathogenic *Listeria* when paired with *L. monocytogenes* strain Lm054. However, when the actual mean populations of the non-pathogenic *Lis-*

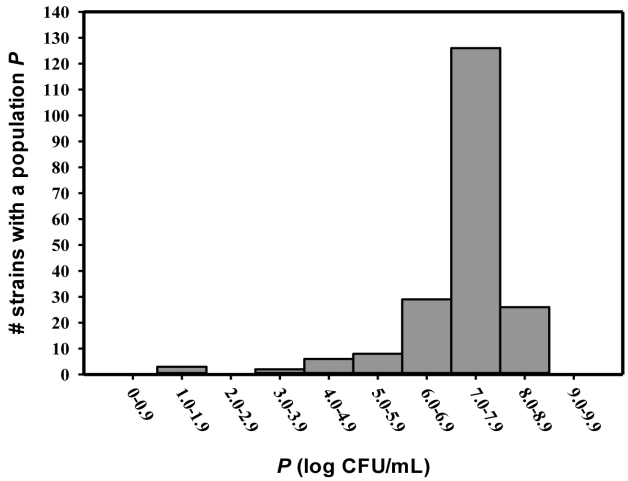

Figure 1. Observed distribution of the 48 h enrichment population (P) of *Listeria* species following competitive growth with *C. braakii* in selective BLEB at 30 °C. (Species include *L. monocytogenes* (n=100), *L. innocua* (n=55), *L. seeligeri* (n=14), and *L. welshimeri* (n=31). The maximum populations in the absence of competition would be around 10^9 CFU/mL. The heights of the vertical bars reflect the number of strains falling within a designated population range.)

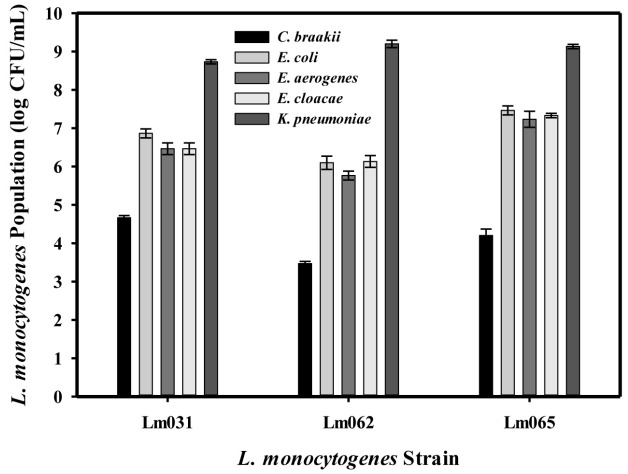

Figure 2. Effect of *Enterobacteriaceae* species on the population of *L. monocytogenes* in selective BLEB at 30 °C.

teria are compared, no statistical differences (P<0.05) are observed. The mean non-pathogenic *Listeria* populations when paired with *L. monocytogenes* strain Lm054 are 7.7 ± 0.1, 7.7 ± 0.1, 7.6 ± 0.1, and 7.7 ± 0.1 log CFU/mL for strains Ln024, Ln032, Lw005, and Lw008, respectively (data not shown). Thus, the only real significance appears to be between the *L. monocytogenes* strains. This is to be expected since the four non-pathogenic *Listeria* strains were selected based on their lack of sensitivity to the presence of *C. braakii* during growth in BLEB.

The seven strains of *L. monocytogenes* and four strains of non-pathogenic *Listeria* were specifically selected based on their sensitivity and resistance to non-*Listeria* competitors, respectively. Statistical analysis of the BLEB enrichment populations used to generate the population differentials in Figure 3 indicate that the variability is between *L. monocytogenes* strains. The intention for using these three strains was to determine if the selective enrichment populations between *L. monocytogenes* and non-pathogenic species of *Listeria* can differ by a greater amount than previously reported for BLEB [5, 6]. Figure 3 supports this hypothesis. Earlier studies showed that non-*Listeria* microbial competitors could negatively influence the enrichment populations of *L. monocytogenes* but rarely prevented its recovery when no other species of *Listeria* was present in the test sample [1, 3, and 4]. These results extend those initial observations with detailed information on the extent to which *Enterobacteriaceae* affect the individual populations and the resulting inter-species population differentials when multiple species of *Listeria* are present in the test sample. The ternary competition assays show the combined effects of both non-pathogenic *Listeria* and *Enterobacteriaceae* competitors on the final populations and resulting inter-species population differentials of *L. monocytogenes*. To understand the contribution that each competitor makes, the individual effects of each have to be compared.

A direct comparison of the effects of *C. braakii* and non-pathogenic *Listeria* species on the matrix-free enrichment populations of three strains of *L. monocytogenes* is shown in Figure 4. In the absence of any competitors (Figure 4A), the final populations of *L. monocytogenes* were 9.0 ± 0.1, 9.3 ± 0.1, and 8.7 ± 0.6 log CFU/mL for strains Lm031, Lm062, and Lm065, re-

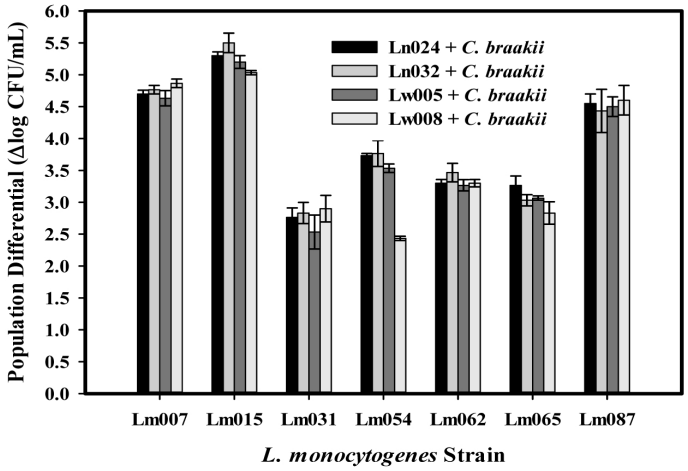

Figure 3. Population differentials between *L. monocytogenes* and other species of *Listeria* in selective BLEB at 30°C with *C. braakii* as the background competitor.

spectively. All three means were statistically similar (P>0.05). When C. braakii was present as the sole competitor, the resulting *L. monocytogenes* populations were 4.7 ± 0.1, 3.5 ± 0.1, and 4.2 ± 0.2 log CFU/mL for the same three strains, respectively. These three means were statistically different (P<0.001) from one another suggesting that different strains have different sensitivities to competition. The presence of non-pathogenic *Listeria* species, as the sole competitor, also resulted in a noticeable decrease in the overall populations of *L. monocytogenes* (Figure 4B). The estimated population means were 6.7 ± 1.5, 7.0 ± 1.4, and 6.6 ± 1.4 log CFU/mL for strains Lm031, Lm062, and Lm065, respectively. All three means were statistically similar (P>0.5).

Four strains of non-pathogenic *Listeria* species were individually tested, as the sole competitor, with each strain of *L. monocytogenes* (Figure 4B). *L. innocua* strain Ln024 had a minimal effect (≤0.2 log CFU/mL) on the matrix-free population of *L. monocytogenes* (Figure 4B). The other three non-pathogenic *Listeria* competitor strains (Ln032, Lw005, and Lw008) all resulted in statistically similar (P>0.05) reductions in the final population of *L. monocytogenes* strains Lm031 and Lm062 (Figure 4B). As for strain Lm065, significant (P>0.05) variation was observed with the Lm065/Lw005 pairing. The Lm065/Ln032 and Lm065/Lw008 pairings were not statistically different (P> 0.05). The mean reductions in the population of *L. monocytogenes*, due to these three specific competitors, were 3.1 ± 0.6, 3.0 ± 0.9, and 2.8 ± 0.9 log CFU/mL for Ln032, Lw005, and Lw008, respectively (Figure 4B). These results indicate that more variation is observed in the final *L. monocytogenes* populations when paired with *Enterobacteriaceae* as compared to being paired with non-pathogenic *Listeria* species. These results also indicate that *Enterobacteriaceae* can exert a more negative effect on *L. monocytogenes* populations and resulting inter-species population differentials than non-pathogenic *Listeria* species alone.

Both *C. braakii* and non-pathogenic *Listeria* species were combined for an *L. monocytogenes*/non-pathogenic *Listeria* species /*C. braakii* ternary competition assay resulting in overall mean *L. monocytogenes* populations (n = 12) of 5.0 ± 0.3, 4.3 ± 0.6, and 4.5 ± 0.2 log CFU/mL for Lm031, Lm062, and Lm065, respectively (Figure 4C). Four strains of non-pathogenic *Listeria* competitors were tested with each strain of *L. monocytogenes* (Figure 4C). The observed *L. monocytogenes* (n=3 strains) final populations, for each *L. innocua strain*, were 4.5 ± 0.5, 4.4 ± 0.4, 4.6 ± 0.5, and 4.8 ± 0.4 log CFU/mL for competitor strains Ln024, Ln032, Lw005, and Lw008, respectively. No significant differences (P>0.05) were observed. These results again confirm that *C. braakii* was more influential on the final *L. monocytogenes* population than was the non-pathogenic *Listeria* species.

The results in Figure 4 show the individual and combined contributions of *C. braakii* and non-pathogenic *Listeria* competitors to the population suppression of *L. monocytogenes* during matrix-free selective enrichment in BLEB. The individual contribution from *C. braakii* was generally consistent resulting in *L. monocytogenes* population suppression of 4.3 ± 0.1, 5.8 ± 0.1, and 4.5 ± 0.1 log CFU/mL for strains Lm031, Lm062, and Lm065, respectively (Figure 4A). The effects of non-pathogenic *Listeria* species competitors were more variable (Figure 4B). When *L. monocytogenes* strain Lm031 was paired with *L. welshimeri* strain Lw008 (Lm031/Lw008) the resulting final *L. monocytogenes* population was 4.7 ± 0.1 log CFU/mL, which corresponds to a population suppression of 3.7 ± 0.1 log CFU/mL. In contrast, when strain Lm031 was paired with *L. innocua* strain Ln024 (Lm031/Ln024), no suppression in the population of *L. monocytogenes* was observed (Figure 4B). No synergistic effect was observed when both *C. braakii* and non-pathogenic *Listeria* species were added and the observed *L. monocytogenes* population reduction mirrored the results of the strongest individual competitor (Figure 4C). For instance, when *C. braakii* was added to the Lm031/Lw008 pairing (Lm031/Lw008/*C. braakii*), the final *L. monocytogenes* population was 4.8 ± 04, which corresponds to a population reduction of 4.2 ± 0.4 log CFU/mL. This value is similar to that observed for the Lm031/*C. braakii* pairing; the final *L. monocytogenes* population was 4.7 ± 0.1 log CFU/mL and equates to a population reduction of 4.3 ± 0.1 log CFU/mL. Additionally, when *C. braakii* was added to the Lm031/Ln024 pairing (Lm031/Ln024/*C. braakii*), the resulting final population was 4.5 ± 0.5 log CFU/mL, which corresponds to a population suppression of 4.5 ± 0.5 log CFU/mL. Again,

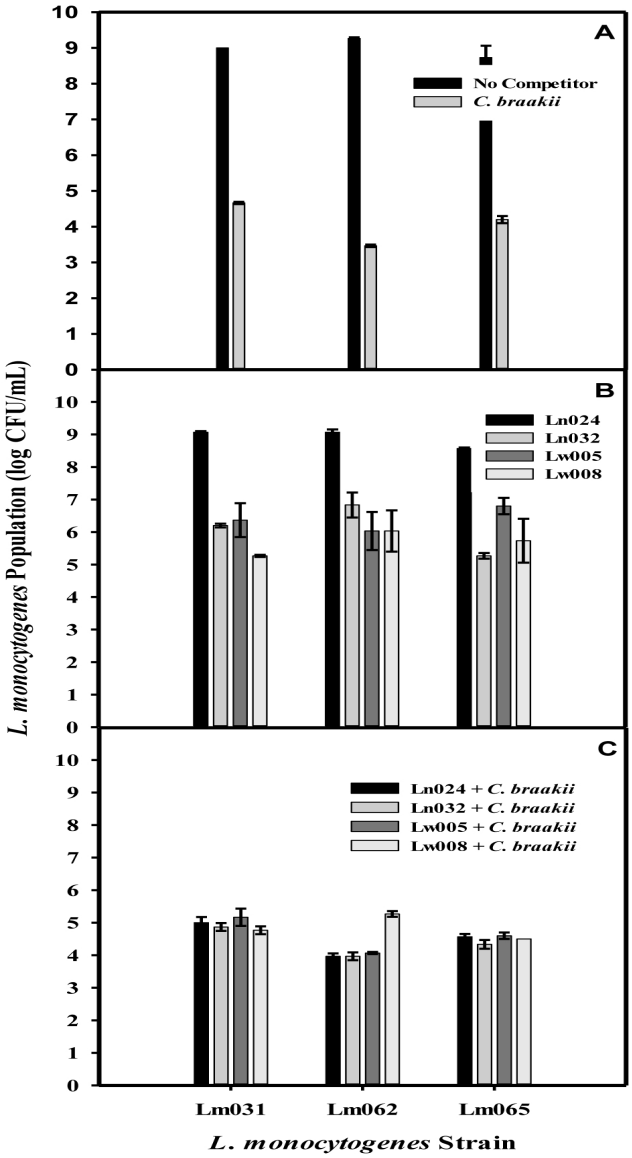

Figure 4. Population of *L. monocytogenes* during competitive growth with *L. innocua* or *L. welshimeri* and with or without *C. braakii* as the background competitor in selective BLEB at 30 °C. (Panel A: populations of *L. monocytogenes* in the absence of competitors or with *C. braakii* only. Panel B: populations of *L. monocytogenes* in the presence of non-pathogenic *Listeria* competitors. Panel C: populations of *L. monocytogenes* in the presence of both non-pathogenic *Listeria* and *C. braakii*.

this value is similar to that obtained for the Lm031/*C. braakii* pairing.

Although it was established in section 3.2 that *C. braakii* was the most competitive of the strains of tested, the effects of the other four strains of *Enterobacteriaceae* on *L. monocytogenes* population suppression were investigated using strains Ln032 and Lm031. The mean *L. monocytogenes* matrix-free enrichment populations were statistically similar (P>0.05) between 3 of the 4 species of *Enterobacteriaceae* tested in ternary species competition assays (Table 1). Only the final *L. monocytogenes* population resulting during competition with *E. cloacae* was statistically different. Overall, the *L. monocytogenes* populations ranged from 4.4 to 6.2 log CFU/mL. The mean populations are listed in Table 1. Both non-pathogenic *Listeria* species and *Enterobacteriaceae* competitors similarly contributed to *L. monocytogenes* population suppression for 3 of the 4 *Enterobacteriaceae* species tested. The decrease in the mean *L. monocytogenes* populations for the Lm031/Ln032 pairing (Figure 4B) compared to the absence of any competitor (Figure 3A) was 2.8 log CFU/mL. Decreases in the final *L. monocytogenes* populations for Lm031/*Enterobacteriaceae* pairings (Figure 2) compared to the absence of any competitor (Figure 4A) were 2.1, 2.5, 2.6, and 0.3 log CFU/mL for *E. coli*, *E. aerogenes*, *E. cloacae*, and *K. pneumoniae*, respectively. A synergistic effect on the *L. monocytogenes* population suppression was observed between *L. innocua* strain Ln032 and three of the *Enterobacteriaceae* competitors. When both competitors were present, the *L. monocytogenes* population reductions were 4.3 ± 0.2, 4.0 ± 0.3, 2.8 ± 0.1, and 3.7 ± 0.1 log CFU/mL for *E. coli*, *E. aerogenes*, *E. cloacae*, and *K. pneumoniae*, respectively (Table 1).

The *Listeria* inter-species population differentials, that resulted during the ternary species competition assays, were 3.2 ± 0.2, 2.8 ± 0.3, 1.6 ± 0.1, and 3.6 ± 0.1 log CFU/mL for

E. coli, *E. aerogenes*, *E. cloacae*, and *K. pneumoniae*, respectively (Table 1). These large, *Enterobacteriaceae* induced *Listeria* inter-species population differentials prevented the recovery of *L. monocytogenes* from any of the enrichments when Oxford agar, a non-species-differentiating medium, was used (Table 1). When Rapid'L.mono™agar was used for recovery, *L. monocytogenes* was recovered from 4 of the 12 total enrichments. In two instances, one where *E. coli* was the background competitor and the other where *E. aerogenes* was the background competitor, *L. monocytogenes* recovery was the result of the presence of a single colony with the remaining colonies being typical for *L. innocua*. The use of a chromogenic medium allowed *L. monocytogenes* to be recovered from 2 of 3 enrichments in which *E. cloacae* was the background competitor. The ability to recover *L. monocytogenes* using chromogenic media was not surprising since the *L. innocua* and *L. monocytogenes* population differentials were low (mean=1.6 ± 0.1 log CFU/mL) in these enrichments.

3.4. Effect of Enterobacteriaceae on Listeria inter-species competition and recovery of L. monocytogenes from spiked-food enrichments

C. braakii suppressed the levels of *L. monocytogenes* to a greater extent than it suppressed the levels of non-pathogenic *Listeria* species resulting in the large population differentials seen in Figure 2. To determine if this would ultimately hinder *L. monocytogenes* recovery from food matrix enrichments, *C. braakii*, *L. innocua* strain Ln032, and *L. monocytogenes* strains Lm031, Lm062, or Lm065 were used to evaluate *Listeria* inter-species competition in spiked guacamole, asadero-style cheese, and refrigerated crab meat enrichments. In spiked guacamole enrichments, *Listeria* inter-species population differentials of 3.9 ± 0.2, 5.4 ± 0.3, and 4.0 ± 0.5 log CFU/mL were observed for strains Lm031, Lm062, and Lm065, respectively (data not shown). In spiked cheese enrichments, *Listeria* inter-species population differentials of 2.8± 0.5, 2.0 ± 0.4, and 2.3± 0.3 log CFU/mL were observed for the same three *L. monocytogenes* strains, respectively (data not shown). Finally, for spiked crab meat enrichments, the *Listeria* inter-species population differentials were 3.3 ± 0.2, 4.3 ± 0.2, and 2.3 ± 0.2 log CFU/mL for strains Lm031, Lm062, and Lm065, respectively (data not shown).

L. monocytogenes was not recovered by the streak plate method using either Oxford or RapidL.mono™agars from any of the three spiked food enrichments. *L. innocua* was recovered from all matrices and from all replications with both media. Although the use of chromogenic media has become favored in recent years because of its ability to distinguish *L. monocytogenes* from other species of *Listeria*, it has limitations. Species-differentiating media are more useful when the populations of target and non-target organisms are within 1-2 orders of magnitude. Species-differentiating media can still be useful for detection when the population differentials are large; however, this frequently requires prolonged incubation (48-72 h) followed by re-isolation of any regions of the plate demonstrating the differentiating reaction. The mean population differentials between *L. monocytogenes* and *L. innocua* ranged from 2-5 log CFU/mL which would require 100 to 100,000 isolated colonies to ensure recovery of the former. *L. monocytogenes* can be recovered from BLEB-based enrichments when the inter-species population differential is approximately 2 log CFU/mL [5, 6]; however, as the differentials become larger recovery becomes increasingly difficult.

The effects of the remaining four of the five strains of *Enterobacteriaceae* on the *Listeria* inter-species population differentials and subsequent recovery of *L. monocytogenes* strain Lm031 from spiked- food enrichments are also shown in Tables 2, 3, and 4. For spiked guacamole enrichments (Table 2), *Listeria* inter-species population differentials of 3.6 ± 0.2, 3.6 ± 0.4, 3.8 ± 0.5, and 4.2 ± 0.4 log CFU/mL were observed for *E. coli*, *E. aerogenes*, *E. cloacae*, and *K. pneumoniae* competitors, respectively. For spiked cheese enrichments (Table 3), the corresponding *Listeria* inter-species population differentials were 5.2 ± 0.7, 4.0 ± 0.8, 4.5 ± 0.5, and 3.5 ± 0.9 log CFU/mL for the same competitors, respectively. Finally, for crab meat enrichments (Table 4), *Listeria* inter-species population differentials of 3.1 ± 0.5, 3.6 ± 0.2, 3.5 ± 0.2, and 3.6 ± 0.2 log CFU/mL were observed for the same *Enterobacteriaceae* strains, respectively. Typically, the *Listeria* inter-species population differentials that resulted during the selective enrichment of spiked foods (Tables 2, 3 and 4) were greater than the population differentials observed in the absence of any food matrices (i.e. pure culture) (Table 1). Additionally, the final populations of both *L. innocua* and *L. monocytogenes* were usually lower in spiked food enrichments (Tables 2, 3 and 4) compared to the absence of matrix material (Table 1). A plausible explanation to account for these observations is that the food matrices were not sterile and there are likely to be additional competitive microorganisms exerting a negative influence on the growth of *Listeria* species.

As with the matrix-free experiments, *L. monocytogenes* could not be recovered from any of the spiked food enrichments by the streak plate method when using either Oxford or chromogenic agars. *L. innocua* was recovered from all spiked food enrichments. This was consistent with what was expected based on the large *L. monocytogenes*/*L. innocua* post-enrichment population differentials. These results show that *L. monocytogenes* population suppression and resulting large *Listeria* inter-species population differentials are not restricted just to the presence of *C. braakii*; additional species of *Enterobacteriaceae* can also negatively affect selective enrichment and organism recovery efforts.

3.5. Study Summary

It is generally accepted that when two or more species of *Listeria* are present during the selective enrichment of a food product, the non-pathogenic species is preferentially recovered over *L. monocytogenes* [5, 7, 8, 13, 15, 19]. Food matrices can have natural microflora that may also exert a negative effect on *L. monocytogenes* during selective enrichment. The effects of *Enterobacteriaceae* on the suppression of *L. monocytogenes* populations, the *Listeria* inter-species population differentials, and on the recovery of *L. monocytogenes* from BLEB selective enrichments were investigated in the current study. It

was shown that the presence of select strains of *Enterobacteriaceae* can facilitate the formation of large (>5 logs/mL) *Listeria* inter-species population differentials during selective enrichment in BLEB at 30 °C. It was also shown in this study that the presence of both *Enterobacteriaceae* and non-pathogenic species of *Listeria* prevented the recovery of *L. monocytogenes* from spiked food enrichments even when a *Listeria* species-differentiating chromogenic medium was used. The complications arising from *Listeria* inter-species competition is of great interest to regulatory agencies where recovery, not just detection, is the ultimate goal in microbial surveillance and food safety testing. The current selective enrichment formulation used by the FDA allows the growth of some non-*Listeria* species which suppresses *L. monocytogenes* populations and ultimately reduces detection sensitivity. This study also shows that non-*Listeria* species competitors can contribute directly to inter-species population differentials when more than one species of *Listeria* is present. Continued inquiry into this problem is necessary if meaningful changes to the selective enrichment procedure are to occur.

4. Declaration of Conflicting Interest

This work is the opinion of the authors and not that of the U.S. Food & Drug Administration. Reference to commercial technologies does not imply endorsement nor does omission of similar technologies imply criticism by the U.S. FDA.

5. Acknowledgment

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6. Article information

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