Published in final edited form as:

Food Microbiol. 2017 September ; 66: 40–47. doi:10.1016/j.fm.2017.03.021.

Comparison of *Listeria monocytogenes* recoveries from spiked mung bean sprouts by the enrichment methods of three regulatory agencies[☆]

Kaitlin E. Cauchon^a, Anthony D. Hitchins^{b,1}, and R. Derike Smiley^{a,*}

^aU.S. Food and Drug Administration, Office of Regulatory Affairs, Arkansas Regional Laboratory, 3900 NCTR Road, Building 26, Jefferson, AR, 72079, United States

^bU.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, 5001 Campus Drive, College Park, MD, 20740, United States

Abstract

Three selective enrichment methods, the United States Food and Drug Administration's (FDA method), the United States Department of Agriculture Food Safety Inspection Service's (USDA method), and the EN ISO 11290-1 standard method, were assessed for their suitability for recovery of *Listeria monocytogenes* from spiked mung bean sprouts. Three parameters were evaluated; the enrichment *L. monocytogenes* population from singly-spiked sprouts, the enrichment *L. monocytogenes* population from doubly-spiked (*L. monocytogenes* and *Listeria innocua*) sprouts, and the population differential resulting from the enrichment of doubly-spiked sprouts. Considerable *L. monocytogenes* inter-strain variation was observed. The mean enrichment *L. monocytogenes* populations for singly-spiked sprouts were 6.1 ± 1.2 , 4.9 ± 1.2 , and 6.9 ± 2.3 log CFU/mL for the FDA, USDA, and EN ISO 11290-1 methods, respectively. The mean *L. monocytogenes* populations for doubly-spiked sprouts were 4.7 ± 1.1 , 5.5 ± 1.3 , and 4.6 ± 1.4 log CFU/mL for the FDA, USDA, and ISO 11290-1 enrichment methods, respectively. The corresponding mean population differentials were 2.8 ± 1.1 , 3.3 ± 1.3 , and 3.6 ± 1.4 log CFU/mL for the same three enrichment methods, respectively. The presence of *L. innocua* and resident microorganisms on the sprouts negatively impacted final levels of *L. monocytogenes* with all three enrichment methods.

Keywords

Regulatory microbiology; *Listeria*; *Listeria innocua*; *Listeria monocytogenes*; Selective enrichment; Fraser broth; UVM broth; BLEB

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

^{*}Corresponding author. Ronald.Smiley@fda.hhs.gov (R.D. Smiley).

¹Retired.

1. Introduction

Sprouts are germinated seeds and are produced by first soaking the seed in water followed by prolonged incubation of up to one week at ambient temperature and high moisture levels; these conditions are favorable for growth of foodborne pathogens. Once harvested, the sprouts are packaged and usually refrigerated for transportation and retail sale. *Listeria monocytogenes* is a ubiquitous, pathogenic, soil borne microorganism capable of growth on sprouts during all stages of production including refrigerated storage. Because of the potential severity of the illness resulting from the consumption of this organism and because sprouts are frequently consumed raw, the United States Food and Drug Administration (FDA) maintains a “zero tolerance” level for *L. monocytogenes* contamination on sprouts.

Levels of *L. monocytogenes* on sprouts can be below threshold levels needed for direct detection and recovery so regulatory agencies rely on selective enrichment as the first step to detecting and recovering this organism. The three most popular regulatory *Listeria monocytogenes* enrichment methods are the FDA’s *Listeria* selective enrichment method, the United States Department of Agriculture Food Safety and Inspection Service’s *Listeria* selective enrichment method (USDA enrichment method), and the international standard method for *Listeria* detection (EN ISO 11290–1 enrichment method). Despite the use of selective agents in these enrichment methods, species-specific growth is not achieved. When more than one species of *Listeria* is present in the test sample each may respond differently to selective enrichment conditions (Petran and Swanson, 1993; Curiale and Lewus, 1994; Carnevali et al., 2010; Cornu et al., 2002; Ganou Besse et al., 2005; Keys et al., 2013; Dailey et al., 2015; Ganou Besse et al., 2016). The importance of this observation is that recovery of only *L. innocua* or any other non-pathogenic *Listeria* species does not preclude the presence of *L. monocytogenes*. In addition to supporting the growth of non-pathogenic species of *Listeria*, these selective enrichment broths also support growth of some strains of non-*Listeria* species (Hitchins and Tran, 1990; Tran et al., 1990; Dallas et al., 1991; Al-Zehara et al., 2011; Dailey et al., 2014; Keys et al., 2016). Growth of non-*Listeria* competitors during selective enrichment may prevent *L. monocytogenes* from reaching minimum threshold levels needed for detection (Dailey et al., 2014).

The objective of this study was to compare *Listeria* inter-species competition and to evaluate the effects of non-*Listeria* competitors during the selective enrichment of spiked mung bean sprouts using three enrichment methods favored by regulatory agencies; the FDA enrichment method, USDA enrichment method and EN ISO 11290–1 enrichment method. Mung bean sprouts were selected as the test matrix since they have naturally high microbial levels, the conditions used for sprout production are conducive to growth of foodborne pathogens, and because they are frequently consumed raw. Specific aims of this study in support of the overall objective included; determining the final populations of select strains of *L. monocytogenes* in mung bean sprout enrichments, determining the population differentials between select strains of *L. monocytogenes* and *L. innocua* in doubly-spiked mung bean sprout enrichments, and estimating the contribution of non-*Listeria* microbial competitors on the final populations of select strains of *L. monocytogenes* during selective enrichment.

2. Materials and methods

2.1. Bacterial strain selection and maintenance

The ten strains of *L. monocytogenes* used in the study were specifically selected based on their competitive performance in selective buffered *Listeria* enrichment broth (BLEB) with the competitor *Citrobacter braakii* (Keys et al., 2016). The primary selection criterion was their final enrichment population; the final strain selection included a wide range of competition sensitivities. The final populations of *L. monocytogenes*, expressed as log CFU/mL, following competitive growth with *C. braakii*, were: Lm002 = 5.4, Lm007 = 4.4, Lm031 = 4.4, Lm040 = 8.0, Lm043 = 7.0, Lm053 = 7.0, Lm056 = 5.1, Lm057 = 8.0, Lm064 = 6.0, Lm070 = 6.1 (Keys et al., 2016). The purpose of this strain selection process was to prevent the introduction of bias into the results by selecting only poorly competitive or only highly competitive *L. monocytogenes* strains.

The strains of *L. innocua* were also selected based on their final populations following selective enrichment of spiked mung bean sprouts. Ten strains of *L. innocua* were initially selected for screening based on how well they reportedly competed against *C. braakii* in BLEB enrichments; all ten strains had final populations $7.9 \log \text{ CFU/mL}$ (Keys et al., 2016). The ten *L. innocua* strains were screened in spiked mung bean sprouts using the FDA, USDA, and EN ISO 11290–1 enrichment methods. Strain Ln018 was selected because it achieved the highest population with the FDA enrichment method and Ln056 was selected because it had the highest population for both the USDA and EN ISO 11290–1 enrichment methods. The rationale for this selection process was to ensure that the most competitive strain of *L. innocua* was used for each specific enrichment method.

All of the *Listeria* strains used in this study were initially isolated and identified using the FDA *Listeria* species isolation method (Hitchins et al., 2016). In addition, 16S rDNA sequence analysis was used to further confirm species identity (Hellberg et al., 2013). Strain Lm002 (serotype 4b) was isolated from Mexican style soft cheese, strain Lm007 (serotype 1/2b) was isolated from butter, strain Lm031 (serotype 1/2a) was isolated from a commercially produced deli-style ham and cheese sandwich, strain Lm040 and Lm056 (both serotype 1/2a) were isolated from frozen smoked salmon, strain Lm043 (serotype 1/2a) was isolated from the environmental sampling of a deli sandwich manufacturing facility, strains Lm053 and Lm070 (both serotype 1/2a) were isolated from avocado, strain Lm057 (serotype 1/2a) was isolated from frozen blueberries, strain Lm064 (serotype 1/2b) was isolated from the environmental sampling of a soft cheese manufacturing facility. All strains were maintained cryoprotectively at -80°C and working stock cultures were maintained in motility agar deeps at room temperature.

2.2. Sprout inoculation

Mung bean sprouts were purchased at several local retailers and consisted of four brands. Although an effort was made to ensure that each brand was evenly represented, frequently brand selection was dictated by availability and so some brands were used more frequently than others. Analytical portions (25 g) of mung bean sprouts were placed into sterile 0.9 L Mason-style blender jars. The sprouts were then doubly-spiked with one of the ten strains of

L. monocytogenes and one of the two strains of *L. innocua*. Strain Ln018 was used for FDA method enrichments and strain Ln056 was used for USDA and EN ISO 11290-1 method enrichments. All ten *L. monocytogenes* strains were represented in all three enrichment methods. For comparison, enrichments were also performed with all 10 *L. monocytogenes* strains using singly-spiked mung bean sprouts. Individual cultures of each strain were prepared from working stocks and grown 18–24 h in non-selective BLEB at 35 °C. Serial dilutions were prepared in Butterfield's phosphate buffer (BPB) (Butterfield, 1932). The target inoculum levels were 1–5 CFU/mL which were verified by plating onto tryptic soy agar supplemented with 0.6% yeast extract (TSAye) with subsequent incubation of the plates at 35 °C for 24 h. The spiked mung bean sprouts were equilibrated for 24 h at 4 °C to simulate actual regulatory test samples that would normally be received following refrigerated shipment.

2.3. Selective enrichment of spiked mung bean sprouts

Listeria selective enrichment was performed on the spiked mung bean sprouts by the FDA (Hitchins et al., 2016), USDA (Anonymous, 2010), and EN ISO 11290-1 (Anonymous, 1996) methods. All incubations were performed statically. All enrichment methods were replicated three times for each of the ten *L. monocytogenes* strains. Difco™ brand (BD Diagnostics; Starks, MD) microbiological media were used unless otherwise stated. Selective agents, buffering salts, and other chemicals were purchased from Sigma-Aldrich Chemicals (St. Louis, MO) unless otherwise specified. Oxoid™ University of Vermont modified broth (UVM-1) (Remel, Inc.; Lenexa, KS), Oxoid™ Fraser broth, and Oxoid™ demi-Fraser broth were prepared following manufacturer's instructions.

2.4. Matrix-free enrichments

Matrix-free enrichment studies were performed as previously reported (Keys et al., 2013, 2016; Dailey et al., 2014) and in a manner similar to that described in section 2.2. Briefly, 25 mL of enrichment broth were inoculated at a target level of 1–5 CFU/mL with each test strain(s). The primary incubation for the matrix-free enrichments was performed using 50 mL polystyrene, screw-capped, conical tubes. For the FDA enrichment method, selective additives were added following the initial four hours incubation as specified by the method (Hitchins et al., 2016). No other modifications were incorporated into any of the three methods.

2.5. Enumeration of *L. monocytogenes*, *L. innocua*, and total listeria

L. monocytogenes populations were estimated from enrichments of singly-spiked mung bean sprouts by surface plating onto Oxoid™ PALCAM agar (Remel, Inc.; Lenexa, KS) followed by incubation of the plates at 35 °C for 24 and 48 h. The enrichments were thoroughly mixed to re-suspend any sediment and serial dilutions were prepared using BPB.

L. monocytogenes populations were estimated from enrichments of doubly-spiked mung bean sprouts by quantitative real-time PCR (qPCR) using the Applied Biosystems MicroSeq™ *Listeria monocytogenes* detection kit (Life Technologies, Inc.; Grand Island, NY) as described previously (Keys et al., 2016; Dailey et al., 2015). Enumeration of *L. monocytogenes* by this qPCR method was shown to be statistically equivalent to plate

counting and was not affected by the presence of additional species of *Listeria* (Dailey et al., 2015). The UltraClean™ Microbial DNA isolation kit (Mo Bio Laboratories, Inc.; Carlsbad, CA) was used to prepare microbial genomic DNA from 1.2 mL aliquots taken from the enrichments. All reactions were prepared according to manufacturer's instructions and amplification was performed and monitored using an Applied Biosystems 7500 fast real-time thermocycler (Life Technologies, Inc.; Grand Island, NY). For accurate quantitation, it was necessary to include amplifications of serially diluted DNA from a preparation in which the population of *L. monocytogenes* had been established by plate count. A standard curve of cycle threshold (C_T) versus population (log CFU/mL) was then used to estimate unknown levels of *L. monocytogenes* from enrichments. The amplification efficiency of the qPCR assay was determined from the standard curve by formula $10^{(-1/\text{slope})}-1$. Since the microbial genomic DNA was prepared using a commercial DNA purification kit, the possibility of carryover of any matrix-specific PCR interference is low; however, to verify this, the C_T values for the internal amplification control (IAC) reactions for the enrichments of spiked mung bean sprouts and for the control amplification reactions (*i.e.*, standard curves) were compared using a non-paired *t*-test.

L. innocua populations were estimated from the enrichments of doubly-spiked mung bean sprouts as the difference between the total *Listeria* population determined by plate count and the *L. monocytogenes* population determined by qPCR (Keys et al., 2016; Dailey et al., 2015). The *Listeria* inter-species population differential was calculated as the difference between the *L. innocua* and the *L. monocytogenes* populations.

2.6. Recovery of *L. monocytogenes*

In order to determine if *L. innocua* masks the presence of *L. monocytogenes*, the recovery of the latter from the spiked enrichments was evaluated using a four-quadrant streak plate technique onto either Oxoid™ Oxford agar plates (Remel, Inc.; Lenexa, KS) or Rapid-*L.mono*™ agar plates (BioRad, Inc.; Hercules, CA). Rapid-*L.mono*™ agar plates were incubated at 35 °C for 24, 48, and 72 h. The plates were examined for the characteristic blue colony formation indicating the presence of *L. monocytogenes*. Oxford agar plates were incubated at 35 °C for 48 h. Ten colonies were then selected and transferred to tryptic soy agar supplemented with 5% sheep blood (BAP) (Remel, Inc.; Lenexa, KS) and screened for hemolytic activity. All *L. monocytogenes* strains included in this study were beta-hemolytic; conversely, all strains of *L. innocua* were non-beta-hemolytic. Identification of *L. monocytogenes* was only to the presumptive level; *i.e.*, blue colony formation on Rapid-*L.mono*™ agar plates or beta-hemolysis on BAP. To strengthen this approach, enrichments of non-spiked mung bean sprouts were also streaked onto Rapid-*L.mono*™ and Oxford agar plates to verify the absence of microbial species that might be misidentified as *L. monocytogenes*.

2.7. Enumeration of selective enrichment-resistant microorganisms

Selective BLEB, UVM-1, and Fraser broths were supplemented with 15 g/L Difco™ Bacto™ agar (BD Diagnostics; Sparks, MD) to convert the broths into their respective agars. Analytical test portions (25 g) of non-spiked mung bean sprouts were blended with 225 mL of BPB. Serial dilutions were surface plated onto BLEB, UVM-1, and Fraser agar plates.

Plates were incubated at 30 °C for 48 h for BLEB and UVM-1 agar plates and at 35 °C for Fraser agar plates. The number of colony forming units (CFU) was then determined.

2.8. Statistical analysis

Mean comparison analyses involving a single fixed variable with only two levels were performed using the two-tailed Student's t-test. Statistical analyses involving a single fixed variable with three or more levels were performed using the one-way analysis of variance (ANOVA) test with SigmaPlot™ version 12.0 software (Systat Software, Inc.; San Jose, CA). Statistical analyses involving two fixed variables were performed using a two-way ANOVA. Mean comparisons were performed using Tukey's mean separation test. The statistical assumptions of normal distribution and equal variance were checked using Shapiro-Wilkes' normality test ($P > 0.05$) and Levene's test of equal variance ($P > 0.05$), respectively.

3. Results and discussion

3.1. Post-enrichment levels of *L. monocytogenes*

3.1.1. Singly-spiked mung bean sprouts—Post-enrichment levels of *L. monocytogenes* are shown in Figs. 1–3 for the FDA, USDA, and EN ISO 11290-1 *Listeria* selective enrichment methods, respectively. For comparison, the final levels of *L. monocytogenes* in matrix-free enrichments are also shown in these same figures. The mean final population from the matrix-free enrichments confirms that the selective conditions used in the FDA enrichment method (Fig. 1) were not detrimental to the growth of the *L. monocytogenes* test strains. However, the mean populations observed for the USDA (Fig. 2) and the EN ISO 11290–1 (Fig. 3) enrichment methods were approximately 1.2 and 1.6 log CFU/mL lower, respectively, and indicate that the selective conditions used in these two enrichment methods are slightly detrimental to the growth of some strains of *L. monocytogenes*.

The final populations of *L. monocytogenes* were reduced between singly-spiked mung bean sprout and matrix-free enrichments and were likely due to competition resulting from the presence of naturally occurring microorganisms that are resistant to the selective conditions used since no other competitors were added. This was observed for all three *Listeria* selective enrichment methods. The reduced *L. monocytogenes* populations of spiked mung bean sprout enrichments compared to matrix-free enrichments are consistent with the effects of simple microbial competition resulting from the presence of sprout-resident microorganisms resistant to the selective conditions used in these enrichment methods. The rapid growth of these non-target organisms likely leads to depletion of one or more key nutrients that result in the inability of the media to support further exponential growth of all organisms present; thus, *L. monocytogenes* is prevented from reaching similar levels seen during matrix-free growth. It should be noted that it is not possible to directly discern any potential *L. monocytogenes* growth limiting properties that are inherent to the matrix itself; this would require an equivalent sterilized matrix in which the sterilization process did not appreciably alter the physiochemical properties of the matrix. However, it is possible to indirectly assess the influence of the matrix on *L. monocytogenes* enrichment populations.

In section 2.1 the enrichment populations following competitive growth with *C. braakii* with the 10 strains of *L. monocytogenes* used in this study are reported. These populations were originally reported in Keys et al. (2016) and were used as the basis of strain selection in this study. The mean population of those 10 strains following competitive growth with *C. braakii* in the absence of any matrix is 6.1 ± 1.3 (1 s.d., $n = 10$) log CFU/mL with a range of 4.4–8.0 log CFU/mL. The mean population of these same 10 *L. monocytogenes* strains following selective enrichment of spiked mung bean sprouts was 6.1 ± 1.2 (1 s.d., $n = 30$) log CFU/mL with a range of 4.7–7.8 log CFU/mL. These two means are in excellent agreement which is consistent with the absence of matrix-specific growth inhibition.

When the three enrichment methods were compared, a statistically significant ($P < 0.001$) interaction between strain and method was noted in the model. The EN ISO 11290–1 enrichment method yielded significantly ($P < 0.05$) higher mean *L. monocytogenes* populations for four of the ten test strains (Lm002, Lm007, Lm053, and Lm070). The FDA enrichment method yielded a significantly ($P < 0.05$) higher mean *L. monocytogenes* population for one of the ten test strains (Lm056). The EN ISO 11290–1 and FDA enrichment methods yielded statistically equivalent ($P > 0.05$) final *L. monocytogenes* populations for strains Lm040, Lm043, Lm057, and Lm064. The USDA enrichment method did not yield any mean *L. monocytogenes* strain populations that were statistically higher compared to the other two enrichment methods. All three enrichment methods were statistically equivalent ($P > 0.05$) when strain Lm031 was tested.

3.1.2. Doubly-spiked mung bean sprouts—Quantifiable levels for all ten strains of *L. monocytogenes* were observed in FDA, USDA, and EN ISO 11290-1 method enrichments of doubly-spiked mung bean sprouts. The final populations of *L. monocytogenes* are shown in Figs. 1–3 for the same three enrichment methods, respectively. When the three enrichment methods were compared, a statistically significant ($P < 0.002$) interaction between strain and method was noted in the model. All three selective enrichment methods yielded statistically equivalent ($P > 0.05$) *L. monocytogenes* populations for six of the ten *L. monocytogenes* test strains (Lm002, Lm007, Lm031, Lm040, Lm 053, and Lm064). The mean population of strain Lm043 was highest with the USDA enrichment method; however, the difference was only significant when compared to the EN ISO 11290–1 enrichment method. The mean populations for strain Lm056 achieved with the USDA and FDA enrichment methods were statistically equivalent ($P > 0.05$) and both were significantly ($P < 0.05$) higher than the EN ISO 11290–1 enrichment method. For strain Lm057, the mean *L. monocytogenes* population was highest with the USDA enrichment method; however, the difference was only significant when compared to the FDA enrichment method. Finally for strain Lm070, the mean *L. monocytogenes* populations were all significantly different ($P < 0.05$) with the USDA enrichment method achieving the highest mean level.

The mean populations of *L. innocua* were 7.6 ± 0.2 , 8.7 ± 0.2 , and 8.2 ± 0.4 log CFU/mL in FDA, USDA, and EN ISO 11290-1 method enrichments of doubly-spiked mung bean sprouts (data not shown). The *L. innocua* populations were statistically higher ($P < 0.05$) than *L. monocytogenes* with all three enrichment methods. The mean population differentials by strain are shown in Fig. 4. Considerable inter-strain variation was observed for *L. monocytogenes* and was expected as these strains were specifically selected to reflect

a wide variation in sensitivity to microbial competition during enrichment as reported by Keys et al. (2016). For the FDA enrichment method, the population differentials (denoted by the symbol) ranged from 0.6 to 5.0 log CFU/mL with an overall mean of ($n = 30$; 10 strains $\times$ 3 replications) of 2.8 ± 1.1 log CFU/mL (data not shown). For the USDA enrichment method, the population differentials ranged from 1.7 to 6.2 log CFU/mL with an overall mean of 3.3 ± 1.3 log CFU/mL (data not shown). For the EN ISO 11290–1 enrichment method, the population differentials ranged from 1.6 to 6.6 log CFU/mL with a mean of 3.6 ± 1.4 log CFU/mL (data not shown). A statistically significant ($P < 0.006$) interaction between strain and enrichment method was observed in the model. The mean population differentials were statistically equivalent ($P > 0.05$) between all three enrichment methods for five of the test strains (Lm007, Lm040, Lm053, Lm057, and Lm064). For strain Lm002, the FDA enrichment method resulted in the lowest mean population differential and was significantly ($P < 0.05$) less than the USDA enrichment method; the FDA and EN ISO 11290–1 enrichment methods were statistically equivalent ($P > 0.05$) for strain Lm002. For strains Lm031 and Lm056, the mean population differential was significantly ($P < 0.05$) lower for the FDA enrichment method compared to both of the other enrichment methods. For strain Lm043, the FDA enrichment method resulted in the lowest mean population differential but the difference between means was only significant ($P < 0.05$) when compared with the EN ISO 11290–1 enrichment method. For strain Lm070, the lowest mean population differential was observed with the USDA enrichment method and was significantly ($P < 0.05$) lower than the FDA enrichment method; the USDA and ISO 11290–1 enrichment methods were statistically equivalent ($P > 0.05$).

3.2. Non-listeria competitor effects on *L. monocytogenes* populations

3.2.1. Competitor effects on *L. monocytogenes* populations during FDA method enrichment—The enrichment population of *L. monocytogenes* can be influenced by the presence of both *L. innocua* and non-target microorganisms that are capable of growth under selective conditions and that also reside on the product being testing (Keys et al., 2016; Dailey et al., 2014). In order to determine the significance of the contribution of each, the *L. monocytogenes* populations following double spiking (*L. monocytogenes*/*L. innocua*) and single spiking (*L. monocytogenes*) are compared for both matrix-free enrichments and enrichments of mung bean sprouts. For the FDA enrichment method, the presence of *L. innocua* resulted in an approximately 1.7 log CFU/mL reduction in the mean *L. monocytogenes* population when no other competitors were present (*i.e.*, doubly-spiked versus singly-spiked matrix-free enrichments) (Fig. 1); this difference was statistically significant ($P < 0.05$). The mean ($n = 30$; 10 strains $\times$ 3 replications) population of *L. monocytogenes* was reduced by approximately 3.2 log CFU/mL when enrichments of singly-spiked mung bean sprouts are compared to singly-spiked matrix-free enrichments (Fig. 1). This could be interpreted as one or more mung bean sprout-resident microorganism(s) is more competitive during FDA method enrichments compared to *L. innocua* strain Ln018. However, this level of population suppression is likely due to the actual levels of microorganisms present on mung bean sprouts that were resistant to the selective conditions; the initial level of these resistant resident microorganisms was determined to be 6.3 ± 0.1 log CFU/mL (data not shown). Given that the initial level of *L. innocua* in doubly-spiked, matrix-free enrichments was <0.7 log CFU/mL and the level of

BLEB-resistant resident competitor microorganisms in enrichments of singly-spiked mung bean sprouts were >6 log CFU/g, it is reasonable to assume that nutrient depletion and subsequent termination of exponential phase growth would occur much sooner in the latter situation. This would result in lower *L. monocytogenes* populations. The addition of *L. innocua* in doubly spiked mung bean sprouts further decreased the *L. monocytogenes* enrichment populations (Fig. 1). This may initially seem surprising since the level of *L. innocua* that was added was small compared to the levels of naturally occurring competitors (0.7 versus 6 log CFU/g). However, the mean ($n = 30$) final population of *L. innocua* strain Ln018 was 8.2 ± 0.5 log CFU/mL in enrichments of doubly-spiked mung bean sprouts (data not shown) indicating that this strain is capable of reaching relatively high levels against the same microbial background that impedes *L. monocytogenes* growth. Thus, it is reasonable that the addition of strain Ln018 would measurably contribute to the *L. monocytogenes* population reduction despite the high levels of microbial competitors already present. The *L. monocytogenes* population suppression observed in FDA method enrichments of mung bean sprouts reflect the collective effects of all species capable of growth under these specific selective conditions. Individual comparison studies with microorganisms isolated from mung bean sprouts are still needed to assess which competitor species are most influential in limiting final *L. monocytogenes* populations.

3.2.2. Competitor contributions on *L. monocytogenes* populations during

USDA-method enrichments—The final mean level of *L. monocytogenes* in doubly-spiked matrix-free enrichments was significantly ($P < 0.05$) lower compared to singly-spiked matrix-free enrichments (Fig. 2) consistent with the original observation of Petran and Swanson (1993) whose work on *Listeria* inter-species competition also utilized the USDA enrichment method. The mean final population of *L. monocytogenes* in enrichments of singly-spiked and doubly-spiked mung bean sprouts and doubly-spiked matrix-free enrichments were all statistically similar ($P > 0.05$) and all were significantly lower than that observed for singly-spiked matrix-free enrichments (Fig. 2). Both *L. innocua* strain Ln056 and the competitor microorganism(s) originating from the mung bean sprouts appeared equally dominant during the enrichment; the *L. monocytogenes* population suppression effects from either are similar. The population suppression when both *L. innocua* and non-*Listeria* species competitors were present (*i.e.*, doubly-spiked mung bean sprouts) were not additive. Addition of *L. innocua* did not further reduce the final levels of *L. monocytogenes* in USDA method enrichments of doubly-spiked mung bean sprouts; this differs from what was observed for the FDA enrichment method (Fig. 1 and section 3.2.1). To understand why this may have occurred, it is important to note the initial levels of competitors present on the sprouts prior to the start of the enrichment. The levels of added *L. innocua* (<0.7 log CFU/g sprouts) were similar between the FDA and USDA enrichment methods. The levels of mung bean sprout-associated non-*Listeria* species competitors capable of growth in UVM-1 and Fraser broths were considerably lower than that observed for the FDA enrichment method. The levels of microorganisms capable of forming colonies on UVM-1 agar and Fraser agar plates were typically below the level of detection (<2 log CFU/g) while those capable of forming colonies on BLEB agar plates were approximately 6 log CFU/g. Because the initial levels of *L. innocua* and non-*Listeria* competitors were similar they could have contributed

similarly to the *L. monocytogenes* population suppression during enrichment by the USDA method.

3.2.3. Competitor contributions on *L. monocytogenes* populations during EN ISO 11290-1 method enrichments—The *L. monocytogenes* populations from doubly-spiked (*L. monocytogenes*/*L. innocua*) and singly-spiked (*L. monocytogenes*) matrix-free and mung bean sprout enrichments are compared in Fig. 3. The final mean level of *L. monocytogenes* in doubly-spiked matrix-free enrichments was significantly ($P < 0.05$) lower compared to singly-spiked matrix-free enrichments (Fig. 3). This indicates that the test strain of *L. innocua* is capable of out-competing the test strains of *L. monocytogenes* during selective enrichment by the EN ISO 11290–1 enrichment method and is consistent with previously published results using this enrichment method (Ganou Besse et al., 2005; Carnevalheira et al., 2010; Zitz et al., 2011). The final mean level of *L. monocytogenes* in singly-spiked mung bean sprout enrichments was statistically similar ($P > 0.05$) to singly-spiked matrix-free enrichments (Fig. 3). This suggests that one or more microorganisms present in mung bean sprouts have a competitive growth advantage over *L. monocytogenes* that is similar to that of *L. innocua* strain Ln056. The extent of *L. monocytogenes* population suppression when *L. innocua* and non-*Listeria* species competitors were present together was larger than the level of suppression with either alone. The *L. monocytogenes* mean population in doubly-spiked mung bean sprout enrichments was statistically lower ($P < 0.05$) compared to doubly-spiked matrix-free enrichments and singly-spiked mung bean sprout enrichments (Fig. 3). For the EN ISO 11290–1 enrichment method, the enhanced *L. monocytogenes* population suppression observed in the doubly-spiked mung bean sprout enrichments may simply be the result of the overall increase in the initial levels of competitors due to the addition of *L. innocua*. The initial levels of competitors were less than the limit of detection ($<2 \log \text{CFU/g}$) and the level of *L. innocua* that was added was approximately $0.7 \log \text{CFU/g}$. The increase in the total initial levels of competitors resulting from spiking mung bean sprouts could account for the observed additive effects on *L. monocytogenes* population suppression.

3.3. Detection/recovery of *L. monocytogenes* from doubly-spiked enrichments

When Oxford agar plates were used, no *L. monocytogenes* was recovered from FDA method enrichments. *L. monocytogenes* was recovered from two of 30 USDA method enrichments and from two of 30 EN ISO 11290-1 method enrichments. When Rapid-*L.mono*TM agar plates were used, *L. monocytogenes* was recovered from nine of 30 FDA method enrichments, three of 30 USDA method enrichments, and five of 30 EN ISO 11290-1 method enrichments. Rapid-*L.mono*TM agar was also used for detection. Detection differs from recovery in that it includes both well isolated presumptive *L. monocytogenes* colonies and presumptive colonies that form in areas of confluent growth which would require additional purification prior to moving forward to confirmatory testing. Presumptive *L. monocytogenes* was detected, using Rapid-*L.mono*TM agar plates, in 19 of 30 FDA method enrichments, 15 of 30 USDA method enrichments, and 12 of 30 EN ISO 11290-1 method enrichments.

4. Summary

Food matrices may harbor microorganisms capable of growth under the selective enrichment conditions used for propagating *L. monocytogenes* to levels needed for detection and recovery. The presence of these non-*Listeria* species competitors can impede growth of *L. monocytogenes* through simple competition resulting in the depletion of one or more key nutrients. Once the medium can no longer sustain exponential growth the populations of all microorganisms, including the species targeted for detection/recovery, cease progressing. Diminished enrichment populations can impede organism detection resulting in false negative test outcomes. Recovery of *L. monocytogenes* can be further complicated by the presence of additional non-pathogenic species of *Listeria* if the predominant species at the end of the enrichment period is one of the latter. For this study, ten strains of *L. monocytogenes* were specifically selected based upon their previously observed competitiveness during selective enrichment. These ten strains were then used to evaluate the appropriateness of three selective enrichment methods used by various food regulatory agencies worldwide to recover *L. monocytogenes* from spiked mung bean sprouts. No one enrichment method emerged as the most appropriate when all three comparison criteria were considered. The EN ISO 11290–1 enrichment method resulted in the highest overall mean population of *L. monocytogenes* but only when it was the sole *Listeria* species present. When two species of *Listeria* were present the USDA enrichment method yielded the highest overall mean *L. monocytogenes* population. However, the lowest mean *Listeria* inter-species population differential was observed with the FDA enrichment method. When coupled with chromogenic agar detection, the FDA enrichment method had the best recovery of *L. monocytogenes* from mung bean sprout enrichments having more than one *Listeria* species; this is likely attributable to the observation that this enrichment method had the lowest inter-species population differentials.

Acknowledgments

The authors would like to thank Brooke Whitney from the FDA Office of Food and Veterinary Medicine for reviewing this work and for providing insightful suggestions. The authors acknowledge the FDA Office of Regulatory Affairs and the Arkansas Regional Laboratory for their support of the study.

References

- Al-Zehara SA, Jarvis B, Mackey BM. The inhibitory effect of natural microflora of food on growth of *Listeria monocytogenes* in enrichment broth. *Int J Food Microbiol.* 2011; 145:98–105. [PubMed: 21176988]
- Anonymous. ISO 11290-1 Microbiology of Food and Animal Feeding Stuffs-Horizontal Method for Detection and Enumeration of *Listeria Monocytogenes*-Part 1: Detection Method. International Organization for Standardization; Geneva, Switzerland: 1996.
- Anonymous. Isolation and identification of *Listeria monocytogenes* from red meat, poultry, and egg product and environmental samples. United States Department of Agriculture-Food Safety and Inspection Service Microbiology Laboratory Guidebook; 2010. <http://www.fsis.usda.gov/wps/wcm/connect/1710bee8-76b9-4e6c-92fc-fdc290dbfa92/MLG-8.pdf?MOD=AJPERES> (Accessed 14 July 2016)
- Butterfield CT. The selection of a dilution water for bacterial examinations. *J Bacteriol.* 1932; 23:355–368. [PubMed: 16559560]
- Carvalho A, Eusebio C, Silva J, Gibbs P, Teixeira P. Influence of *Listeria innocua* on the growth of *Listeria monocytogenes*. *Food Control.* 2010; 21:1492–1496.

- Cornu M, Kalmokoff M, Flandrois JP. Modelling the competitive growth of *Listeria monocytogenes* and *Listeria innocua* in enrichment broths. *Int J Food Microbiol.* 2002; 73:261–274. [PubMed: 11934034]
- Curiale MS, Lewus C. Detection of *Listeria monocytogenes* in samples containing *Listeria innocua*. *J Food Prot.* 1994; 57:1048–1051.
- Dallas HL, Tran TT, Poindexter CE, Hitchins AD. Competition of food bacteria with *Listeria monocytogenes* during enrichment culture. *J Food Saf.* 1991; 11:293–301.
- Dailey RC, Martin KG, Smiley RD. The effects of competition from non-pathogenic foodborne bacteria during the selective enrichment of *Listeria monocytogenes* using buffered *Listeria* enrichment broth. *Food Microbiol.* 2014; 44:173–179. [PubMed: 25084660]
- Dailey RC, Welch LJ, Hitchins AD, Smiley RD. Effect of *Listeria seeligeri* or *Listeria welshimeri* on *Listeria monocytogenes* detection in and recovery from buffered *Listeria* enrichment broth. *Food Microbiol.* 2015; 46:528–534. [PubMed: 25475325]
- Ganou Besse N, Audinet N, Kerouanton A, Colin P, Kalmokoff M. Evolution of *Listeria* populations in food samples undergoing enrichment culturing. *Int J Food Microbiol.* 2005; 104:123–134. [PubMed: 15975676]
- Ganou Besse N, Favret S, Desreumaux J, Decourseulles Brasseur E, Kalmokoff M. Evaluation of reduction of Fraser incubation by 24 h in the EN ISO 11290-1 standard on detection and diversity of *Listeria* species. *Int J Food Microbiol.* 2016; 224:16–21. [PubMed: 26913375]
- Hellberg RS, Martin KG, Keys AL, Haney CJ, Shen Y, Smiley RD. 16S rRNA partial gene sequencing for the differentiation and molecular subtyping of *Listeria* species. *Food Microbiol.* 2013; 36:231–240. [PubMed: 24010602]
- Hitchins AD, Jinneman K, Chen Y. Detection and Enumeration of *Listeria Monocytogenes* in Foods. 2016. Ch. 10 in U.S. FDA BAM Available at: <http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm> (Accessed 15 August 2016)
- Hitchins AD, Tran T. Initial cell concentration and selective media effects on isolation of *Listeria monocytogenes* from enrichment cultures of inoculated foods. *J Food Prot.* 1990; 53:502–504.
- Keys AL, Dailey RC, Hitchins AD, Smiley RD. Post-enrichment population differentials using buffered *Listeria* enrichment broth: implications of the presence of *Listeria innocua* on *Listeria monocytogenes* in food test samples. *J Food Prot.* 2013; 76:1854–1862. [PubMed: 24215687]
- Keys AL, Hitchins AD, Smiley RD. *Enterobacteriaceae* complicate the recovery of *Listeria monocytogenes* from food product enrichments using buffered *Listeria* enrichment broth. *J Reg Sci.* 2016; 4:10–20.
- Petran RL, Swanson KMJ. Simultaneous growth of *Listeria monocytogenes* and *List. innocua* *J Food Prot.* 1993; 56:616–618.
- Tran TT, Stephenson P, Hitchins AD. The effect of aerobic mesophilic microfloral levels on the isolation of inoculated *Listeria monocytogenes* strain LM82 from selected foods. *J Food Saf.* 1990; 10:267–275.
- Zitz U, Zunabovic M, Domig KJ, Wilrich PT, Kneifel W. Reduced detectability of *Listeria monocytogenes* in the presence of *List. innocua* *J Food Prot.* 2011; 74:1282–1287. [PubMed: 21819654]

Fig. 1.

Effects of matrix-resident microflora and *L. innocua* strain Ln018 on populations of *L. monocytogenes* from FDA method enrichments of spiked mung bean sprouts. (Open bars = mung bean sprout enrichments, grey bars = matrix-free enrichments. Letters at the base of the bars denote statistical significance ($P < 0.05$) as determined by Tukey's mean comparison test. Error bars reflect the standard deviation of 10 strains $\times$ 3 replications.).

Fig. 2. Effects of matrix-resident microflora and *L. innocua* strain Ln056 on populations of *L. monocytogenes* from USDA method enrichments of spiked mung bean sprouts. (Open bars = mung bean sprout enrichments, grey bars = matrix-free enrichments. Letters at the base of the bars denote statistical significance ($P < 0.05$) as determined by Tukey's mean comparison test. Error bars reflect the standard deviation of $10 \text{ strains} \times 3 \text{ replications}$.).

Fig. 3.

Effects of matrix-resident microflora and *L. innocua* strain Ln056 on populations of *L. monocytogenes* from EN ISO 11290-1 method enrichments of spiked mung bean sprouts. (Open bars = mung bean sprout enrichments, grey bars = matrix-free enrichments. Letters at the base of the bars denote statistical significance ($P < 0.05$) as determined by Tukey's mean comparison test. Error bars reflect the standard deviation of 10 strains $\times$ 3 replications.).

Fig. 4.

Listeria inter-species population differentials following selective enrichment of doubly-spiked mung bean sprouts. (Panel A = *L. monocytogenes* strains for which the FDA enrichment method yielded significantly ($P < 0.05$) lower population differentials, Panel B = *L. monocytogenes* strains for which all three enrichment methods yielded statistically equivalent ($P > 0.05$) population differentials, Panel C = *L. monocytogenes* strains for which the USDA enrichment method yielded significantly ($P < 0.05$) lower population differentials. Error bars reflect the standard error associated with three replications. Lower case letters at

the base of the bars indicate statistical significance ($P < 0.05$) as determined by Tukey's mean separation test. Because a significant interaction was noted between enrichment method and strain, all statistical comparisons are limited to within strain and comparisons between strains are not supported.).