Appropriateness of the VIDAS *Listeria* Method (AOAC Official Method 999.06) for Screening Crab Meat Enrichments for the Presence of *Listeria monocytogenes*

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Abstract

As mandated by their ISO 17025 accreditation, U.S. FDA field laboratories require analytical methods to be validated for each regulated food matrix to ensure adequate performance. AOAC Official Method 999.06 (VIDAS *Listeria* Assay) is frequently used to screen regulatory food samples for the presence of *Listeria monocytogenes*. Crustacean seafoods were not included in the original validation study for this method. A comparative study, meeting independent laboratory validation (ILV) requirements, was performed to determine if the VIDAS *Listeria* (LIS) assay is appropriate for regulatory use as a method for screening crab meat enrichments for the presence of *L. monocytogenes*. Crab meat samples were spiked with *L. monocytogenes* (Strain ARL-Lm-012) at three different levels. Selective enrichment was performed on the spiked samples following the FDA BAM method. Testing for the presence of *L. monocytogenes* following enrichment was performed using the VIDAS LIS assay and using the FDA BAM reference method. The average post-enrichment *L. monocytogenes* population was less than 5.0 Log CFU/mL. Fractional detection was achieved for the VIDAS LIS at the low spiking level. Failure to detect *L. monocytogenes* was not the result of the samples being truly negative as all low-level spiked samples still tested positive by the reference method. The VIDAS LIS method was statistically less efficient than the FDA BAM method at detecting *L. monocytogenes* in crab meat enrichments.

1 Introduction

The FDA reference method for the detection and recovery of *L. monocytogenes* uses a two-day selective enrichment followed by streak plate recovery on selective/differential agar plates. The agar plates also require a two-day incubation before a “negative” determination can be made. The presence of colonies with typical phenotypic characteristics of *Listeria* require several additional days of analysis for confirmation. Because the *Listeria* isolation reference method is labor intensive and requires a minimum of four days before a “negative” result determination can be made, the FDA field laboratories routinely use rapid detection methods to screen enrichments for presumptive *Listeria*. This allows their organism recovery efforts to be focused on samples with a higher likelihood of *Listeria* contamination.

Because of the regulatory nature of their work and ISO 17025 accreditation specifications, all rapid microbiological detection methods used by the FDA field laboratories must meet the same high analytical performance standards as the laboratories’ reference (i.e. FDA BAM) microbiological methods. To ensure that rapid microbiological detection methods are appropriate for their intended use, standardized validation requirements must be met. There are multiple levels of validation based on the level of scrutiny required. The independent laboratory validation (ILV) can be used to examine previously untested matrices for a fully validated method; this process is often referred to as method-matrix extension (U.S. FDA, 2019). The ILV requires a minimum of three analyte (spiking) levels; one level is uninoculated, the second level results in fractional detection (either by the reference or alternate method), and the third level is usually one log higher than the fractional detection level. ILV studies require 20 replicates for the fractional detection level, and five replications each for the high spiking level and non-spiked levels. For refrigerated perishable foods, the SLV requires that the matrix be aged for 48-72 h following the addition of the target analyte. The matrix should have a background level of microbial competitors that is at least one log higher than the level of analyte needed for fractional detection. Other performance measurements such as inclusivity/exclusivity testing, multiple matrices, and testing by collaborating laboratories are not required for method-matrix extension of a previously validated method as these would have already been performed.

The Vitek Immuno-Diagnostic Assay System™ (VIDAS) is an automated enzyme-linked immuno-fluorescence assay (ELFA) system (bioMerieux USA; Durham, NC). The VIDAS microbial detection reagent strip contains eight wells. The sample is added to the first well. The solid phase receptacle (SPR), which is internally coated with a target specific antibody and which acts as a pipetting device, removes an aliquot of the sample from the sample well. The aliquot is transferred to the next well containing buffered diluent and the sample is cycled in and out of the SPR several times; during this step, the target microbial cells bind to the antibody labeled SPR. The remaining wells of the reagent test strip contain various binding and wash buffers, alkaline phosphatase conjugated antibody, substrate (4-methy umbelliferyl phosphate), and an optical cuvette to measure the activity of alkaline phosphatase. This type of antibody capture-detection is

often referred to as a sandwich ELFA since the target cell ends up sandwiched between two antibodies (the capture antibody and detection antibody).

Despite having been evaluated by a multi-laboratory validation study and having AOAC International Official Method of Analysis status, the original study only included one seafood matrix, fish. The FDA field laboratories analyze a wide array of seafood matrices and method validation is required for each type of seafood encountered. The goal of this study was to evaluate the VIDAS LIS assay, under conditions which satisfy the requirements of an ILV, to determine if it was equivalent to the FDA BAM reference method for detecting *L. monocytogenes* from crab meat enrichments.

2 Materials and Methods

2.1 Strain Selection and Maintenance

Strain *L. monocytogenes* ARL-Lm-012 was used for this study; this strain is a serotype 1/2a and was isolated in 2007 from frozen crab claws during FDA field laboratory regulatory activities. ARL-Lm-012 was maintained cryogenically (-80 °C) in BBL Trypticase™ soy broth (TSB) (Thermo Fisher Scientific; Waltham, MA) with 20% glycerol. Working stock cultures were prepared as follows. Cells were propagated in non-selective BLEB for approximately 20-24 h at 35 ± 2 °C. The purity of the culture was confirmed by streak plate analysis using Trypticase soy agar supplemented with 0.6% yeast extract (TSAYE) with incubation at 35 ± 2 °C for approximately 24 h. A single well-isolated colony was selected and confirmed using the VIDAS LIS and by real-time PCR using the Applied Biosystems MicroSeq™ *Listeria monocytogenes* detection assay (Thermo Fisher Scientific). This colony was then used to prepare motility agar deeps which were stored at room temperature throughout the duration of the study (approximately 6 weeks).

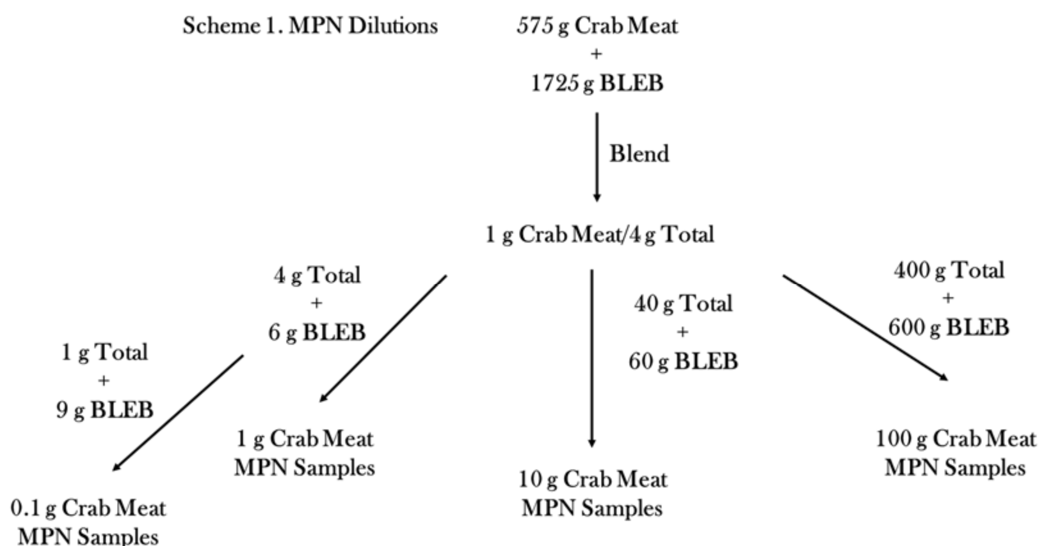
2.2 Inoculum Preparation and Matrix Spiking

Non-selective BLEB (10 mL) was inoculated with strain ARL-Lm-012 directly from the working stock cultures. Cells were grown at 35 ± 2 °C for 20-24 h. Serial dilutions (final volume 10 mL) were prepared using Butterfield's phosphate buffer (Butterfield, 1932). A 2 mL aliquot from the 10^{-8} dilution was used to inoculate a 575 g portion of commercially prepared pasteurized crab meat which had been previously weighed into a sterile 1000 mL beaker. The target concentration for low level spiking was 0.5 to 1.5 CFU/ 25 g and selected to achieve 50 ± 25% positive test results for either the FDA BAM reference or VIDAS LIS alternate test methods. For high level spiking, a 2.0 mL aliquot from the 10^{-7} dilution was used to spike a 575 g test portion of crab meat. Spiked crab meat was held at 5 ± 2 °C for 72 h before initiating analysis.

2.3 Spiking Level Verification

The spiking level was initially estimated by plate count on buffered *Listeria* enrichment broth supplemented with 15% agar and sodium nalidixate (40 mg/L) and acriflavine-HCl (15 mg/L) (BLEBA). After the spiked crab meat was aged for 72 h at 5 °C, the pre-enrichment population of *L. monocytogenes* was re-determined using the five tube most probable number

(MPN) technique. Because samples were spiked at an estimated level of approximately 0.02-0.06 CFU/g, portion sizes of 100, 10, 1 and 0.1 g were required for MPN determination (Scheme 1). A volume equivalent to 1725 g of non-selective BLEB was added to the spiked 575 g sample resulting in 1 g sample per 4 g total weight (1/4 dilution). The sample was thoroughly blended at 500 rpm for two minutes using a Robot Coupe R602 commercial blender. To obtain 0.1 g samples, a 1 g sample was first prepared by diluting 4 g of the original blended sample with an additional 6 g of BLEB resulting in 0.1 g sample/g total. From that dilution, five 1 g (total) portions were transferred to tubes containing 9 g of BLEB. To prepare 1 g samples, five portions of 4 g of the original blended sample (1/4 dilution) were aliquoted into individual sterile test tubes. An additional 6 g of BLEB was added resulting in a final 1/10 dilution. To prepare 10 g samples, five portions of 40 g of the original blended sample were aliquoted into sterile blender jars. An additional 60 g of BLEB was added resulting in a final 1/10 dilution. Five individual portions of 400 g (100 g sample) were aliquoted into sterile Stomacher bags. An additional 600 g of BLEB was added to maintain a 1/10 sample to enrichment ratio. Following the addition of selective additives, the blender bags, jars and tubes were incubated for 48 h at 30 °C. The presence of *L. monocytogenes* in each tube/jar was determined by real-time PCR using the ABI MicroSeq *Listeria* detection assay.



2.4 Real-time PCR Confirmation of L. monocytogenes

Confirmation of *L. monocytogenes* in turbid MPN tubes was performed by real-time PCR using the MicroSeq *Listeria monocytogenes* detection assay (ThermoFisher). For this study, the PCR beads were first rehydrated with 28 µL of sterile nuclease-free water followed by the addition of 2 µL of purified DNA template. All DNA was prepared from 1.8 mL enrichment aliquots using the DNeasy UltraClean™ Microbial Kit (Qiagen) following the manufacturer's recommended protocol. Amplification was performed using an ABI 7500 Fast™ real-time thermal cycler

(ThermoFisher) using the manufacturer's recommended cycling parameters which were designed specifically for the MicroSeq *Listeria monocytogenes* detection assay.

2.5 Competitor Microorganisms

To verify that that the pasteurized crab meat possessed sufficient levels of naturally occurring competitive microorganisms, a microbial count on selective BLEBA plates was performed. BLEBA restricts colony formation to those microorganisms capable of growth under FDA selective enrichment conditions. A 25 g portion was blended (1/10) using Butterfield's phosphate diluent. Serial dilutions (1/10) were prepared and 100 µL from each dilution was surface plated onto prepared plates. Plates were incubated at 30 ± 2 °C for 48 h.

2.6 Sample Analysis

Sample analysis was initiated by removing the spiked crab meat from refrigerated storage and allowing it to temper at room temperature for approximately 1 h. A volume of 1725 g of non-selective BLEB was added to the spiked 575 g sample resulting in 1 g crab meat /4 g total. The sample was thoroughly blended at 500 rpm for two minutes using a Robot Coupe™ R602 commercial blender. The blended sample was dispensed in 100 g aliquots (25 g crab meat total) and an additional 150 g of BLEB was added yielding a final 1/10 dilution. The sample enrichments were incubated at 30 °C for four hours. Selective agents were added at the following final levels; sodium nalidixate 40 mg/L, acriflavine-HCl 15 mg/L, and cycloheximide 50 mg/L. Selective enrichment continued at 30 °C for an additional 44 h. At the end of the incubation period, sample enrichments were analyzed for the presence of *L. monocytogenes* by both the reference FDA BAM (Hitchins *et al.*, 2017) and the alternate AOAC Official Method 999.06 (Ganger et al., 2000) methods.

2.7 Post-enrichment L. monocytogenes Enumeration

The ability to detect *L. monocytogenes* using a rapid detection platform is dependent on its ability to reach minimum detection threshold populations. Two post-enrichment microbial counts, *L. monocytogenes* using both Oxford and PALCAM agars and competitor using BLEBA plates, were used to determine if the minimum detection threshold was met and assess the level of microbial competition experienced by *L. monocytogenes* during enrichment. Post-enrichment populations of *L. monocytogenes* and competitor microorganisms were compared between spiked enrichments testing positive (confirmed) by the VIDAS™ LIS method and those with a negative result by the same method.

2.8 Statistical Analysis

Because each sample enrichment was split and analyzed concurrently by both the FDA BAM and VIDAS LIS methods, the data can be considered a matched pair and thus appropriately analyzed using the McNemar X^2 test. The McNemar X^2 test was conducted using StatPlus™ v. 6

(Analystsoft, Inc.; Walnut, CA). Additionally, the sensitivity rate, specificity rate, false negative rate, and false positive rate were determined.

3 Results and Discussion

3.1 Spiking Levels

The initial spiking level estimate was lower than the MPN estimate for both the low and high spiking levels (Table 1). These inconsistencies may just be the result of differences between the two enumeration methods or there may have been some continued *L. monocytogenes* growth during the 72-h matrix tempering period since this organism is a known psychrotroph.

Table 1. Spiking levels used for ILV of crab meat enrichments for the VIDAS LIS immuno-diagnostic assay (AOAC OMA 999.06).

Spiking Level	Initial Spiking Estimate[a]	MPN Estimate[b]
None	NA[c]	NA
Low	0.08 CFU/g	0.31 (0.11-0.92)
High	0.85 CFU/g	5.4 (1.6-18.3)

[a]Direct plating onto BLEBA at the time of spiking

[b]MPN method following 72 h tempering at 5 °C

[c]Not Applicable

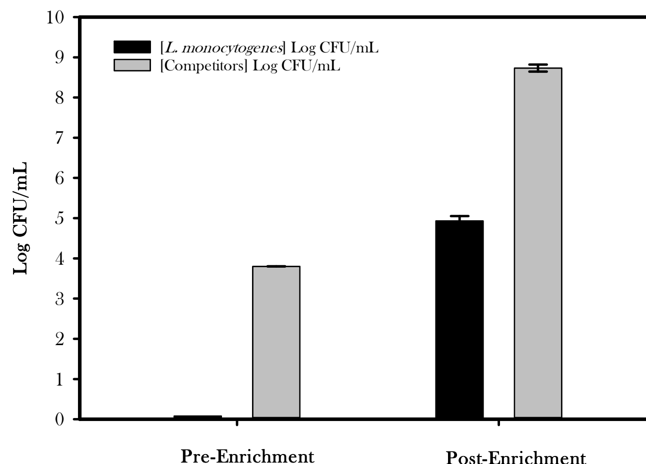
The only seafood included in the original validation study for the VIDAS LIS assay was finfish (Ganger et al., 2000). That study used inoculation levels of 0.04-0.2 CFU/g and 0.4-2.0 CFU/g for the low and high spiking levels, respectively. FDA method validation guidance requires that the low spiking level result in 50 ± 25% fractional detection for either the reference or test method, whichever is least sensitive (U.S. FDA, 2019). Their spiking level resulted in fractional detection (89%) with the VIDAS LIS assay. Our spiking level was similar, and we obtained fractional detection with the VIDAS LIS method that was within the FDA guidance specified limits (50 ± 25%).

3.2 Pre-Enrichment Microbial Competitor Levels

Pre-enrichment levels of microbial competitors, estimated by BLEBA plate count, are shown in Figure 1. The use of BLEBA restricts the counts to just those organisms capable of growth under the selective conditions described in the FDA BAM *Listeria* enrichment method. This provides a better estimate of the true microbial competitors compared to plating on a general non-selective medium which would include those organisms that are not capable of growth under the same conditions and which would not likely interfere with the growth and subsequent detection of the target, *L. monocytogenes*. The level of resident microbial competitors was approximately

3.7 Log CFU/g higher than the initial levels of *L. monocytogenes*, thus meeting the critical requirement for an ILV study without the need of artificial spiking of competitor organisms.

Figure 1. Comparison of Pre- and Post-Enrichment *L. monocytogenes* and Microbial Competitors Populations.



3.3 VIDAS LIS Spike Detection Results

The FDA BAM method was superior to the VIDAS LIS method for detecting *L. monocytogenes* in low-level spiked crab meat enrichments (Table 2). Of the 20 spiked samples, all tested positive by the FDA BAM; all presumptive positive samples were subsequently confirmed by real-time PCR as described in *Section 2.4* using a single well isolated colony from the PALCAM agar plate. Of the 20 confirmed positive low-spiked crab meat enrichments, only eight produced a positive result with the VIDAS LIS method.

Table 2. Contingency table for comparing matched, low spike level, crab meat enrichments analyzed concurrently by the FDA BAM reference and VIDAS LIS alternate test methods.

		VIDAS-LIS Method		
		Pos	Neg	Total
FDA BAM Method	Pos	8	12	20
	Neg	0	0	0
	Total	8	12	20

The McNemar's X^2 for the low-spike level analysis is 10.1 with an associated two-tailed P value of 0.0015 which is considered highly statistically significant. The McNemar's test value indicates that the proportion of matched samples testing positive by both methods is significantly different. The two methods should not be considered equivalent and the FDA BAM method is more appropriate

when analyzing crab meat samples. It should be noted that the two methods share the same selective enrichment procedure and differ only in the target detection step. For the FDA BAM reference method, the detection and recovery step are the same. For this study, PALCAM and Oxford selective/differential agars were used for the FDA BAM detection step; the FDA BAM method also gives the analyst the option of using one of several commercially available chromogenic agars. In this study, confirmation was performed using colonies selected from PALCAM agar as this medium appeared to be more selective than Oxford resulting in less non-target background organisms which confounded *L. monocytogenes* colony selection from the latter.

The sensitivity is the likelihood that the alternative test method (i.e. VIDAS LIS) will classify a test sample as positive, given that the test sample is a confirmed positive sample. The sensitivity rate for the VIDAS LIS method, calculated from the low-spike detection results (Table 2), is 40%. This can alternatively be expressed as the false negative rate which is 60%; the false negative rate is the number of test samples yielding a negative result by the VIDAS LIS method but a positive result by the FDA BAM method. The specificity is the likelihood that the alternative method (i.e. VIDAS LIS) will classify a sample as negative given that the sample is a confirmed negative sample. The specificity can be calculated from the data in Table 3.

Table 3. Contingency table for comparing matched, non-spiked, crab meat enrichments analyzed concurrently by the FDA BAM reference and VIDAS LIS alternate test methods.

		VIDAS-LIS Method		
		Pos	Neg	Total
FDA BAM Method	Pos	0	0	0
	Neg	0	5	5
	Total	0	5	5

The specificity can be further defined as the total number of samples confirmed negative by both the reference and alternate test methods divided by the number of samples confirmed negative by the reference method only. The FDA guidance (U.S. FDA, 2019) for conducting an ILV study indicates that only five non-spiked samples are required to confirm the specificity which is typically established during the single laboratory validation (SLV) study phase by the originating laboratory. The calculated specificity from the data in Table 3 is 1.0 (or 100%). Alternatively, specificity can be expressed as the false negative rate (1-specificity) which would be zero.

FDA validation study guidance (U.S. FDA, 2019) requires the high spiking level to be 1 log higher than the low spiking level. The *L. monocytogenes* detection results for the high spiking level are shown in Table 4. At the higher spike level, the FDA BAM method demonstrated a 100% detection efficiency whereas the VIDAS LIS method exhibited one confirmed detection failure. FDA validation guidance (U.S. FDA, 2019) does not address the reason for performing high spike

level analyses. Meaningful comparative statistical analysis cannot be performed on data where all samples test positive by both methods. Multiple target levels for qualitative method validation have traditionally been used to ensure that at least one level resulted in fractional detection (either by the reference or alternate method). Additionally, multiple target levels have been used to establish a 50% endpoint, with associated confidence intervals, for the alternate method; typically, at least five target levels are needed to accomplish this. However, calculating a 50% endpoint is not critical when an alternate method is being directly compared to a reference method in a matched pair study and when only a determination of whether the two methods are equivalent is needed.

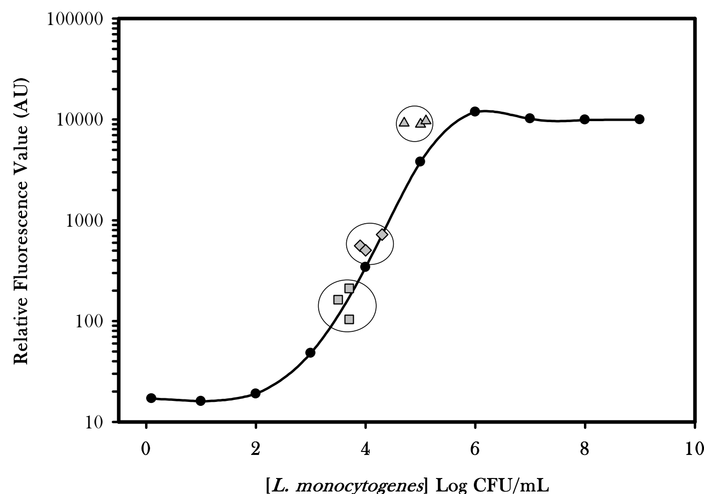
Table 4. Contingency table for comparing matched, high spike level, crab meat enrichments analyzed concurrently by the FDA BAM reference and VIDAS LIS alternate test methods.

		VIDAS-LIS Method		
		Pos	Neg	Total
FDA BAM Method	Pos	9	1	10
	Neg	0	0	0
	Total	9	1	10

3.4 Post-Enrichment Microbial Populations

For antibody-based detection assays, the level of fluorescence is typically proportional to the level of target analyte present in the sample. If the population of the target organism does not meet the minimum fluorescence threshold level for the detection step, then the target could go undetected. The relationship between post-enrichment *L. monocytogenes* population and the relative fluorescence value (RFV) measured during the VIDAS LIS assay is shown in Figure 2. When plotted on a logarithmic scale, the data is approximately linear from a *L. monocytogenes* population of 3 Log CFU/mL to 5 Log CFU/mL after which the RFVs plateau. The minimum threshold RFV, to trigger an automatic "positive" result, appears to be around 3.8 Log CFU/mL. The difference in post-enrichment *L. monocytogenes* populations for VIDAS negative (grey squares) and positive (grey diamonds) low-spiked samples was only 0.5 Log CFU/mL; 3.6 ± 0.1 versus 4.1 ± 0.2, respectively. The post-enrichment populations of the high spiked crab meat samples (grey triangles) were only slightly higher than the low spiked samples yielding positive VIDAS LIS results; 4.8 ± 0.3 versus 4.1 ± 0.2, respectively. However, the high-level spiked samples had much higher RFVs compared to the low-level spiked samples (Figure 2) which is due to the sensitivity of the VIDAS assay (i.e. steepness of the curve) to post-enrichment *L. monocytogenes* populations.

Figure 2. VIDAS relative fluorescence values for high and low level *L. monocytogenes* spiked crab meat enrichments. (Grey squares = VIDAS Neg, low spiked samples; Grey diamonds = VIDAS Pos, low spiked samples; Grey triangles = VIDAS Pos, high spiked samples).



3.5 Microbial Competition and Strain Selection

Knowing the post-enrichment levels of both target organism and microbial competitors (Figure 1) allows for a better understanding of why the VIDAS LIS method was less efficient at *L. monocytogenes* detection compared to the FDA BAM reference method. Although not directly assessed in this study, both microbial competition and *L. monocytogenes* strain are influential on the outcome. The levels of non-target microbial competitors were considerably higher (3.9 Log CFU/mL) than the levels of *L. monocytogenes* at the start of selective enrichment (Figure 1). These competitors may not necessarily directly inhibit *L. monocytogenes* growth (e.g. through the production of antibiotic-like compounds); however, they do indirectly inhibit the latter's growth through nutrient depletion and by altering the growth environment through the excretion of metabolic waste products. The severity of the inhibition will depend on the differences in the growth rates between the target and competitor organisms (Dailey et al., 2014). Growth rate can also vary by *L. monocytogenes* strain, further complicating the interpretation of method comparison studies. Thus, it is possible that a different outcome could result from the use of a different strain of *L. monocytogenes*. ILVs require the use of only one strain of the target microorganism (U.S. FDA, 2019) and there are no firm guidelines or requirements for strain selection. Strain ARL-Lm-012 was selected for this study because it had previously been isolated from crab meat. This strain had also been included in a competitive fitness study that compared its post-enrichment population to over 200 additional *Listeria* strains grown in the presence of a single non-*Listeria* (*Citrobacter braakii*) competitor (Keys et al., 2016). In the presence of the single competitor organism, strain ARL-Lm-012 achieved a final FDA BAM enrichment population of 6.1 Log CFU/mL (Keys et al., 2016). Strain ARL-Lm-012 demonstrates the ability to reach minimum threshold populations to elicit a positive detection response with the VIDAS LIS assay

while at the same time demonstrating some sensitivity to the presence of non-*Listeria* competitors. Other *L. monocytogenes* strains that are too weak or too robust may not accurately reflect the attributes of most strains encountered from food matrices/systems and could result in misleadingly high negative or positive detection rates.

4 Study Summary

Alternate foodborne pathogen detection technologies and methods, used by the FDA, require validation for each individual matrix type for which the method is used. The initial validation study for AOAC OMA 999.06, the VIDAS *Listeria* spp. assay, included only one seafood, fin-fish. In order to expand the number of validated seafoods, an ILV study was performed on pasteurized crab meat. The performance of AOAC OMA 999.06 was directly compared to the FDA BAM method for *Listeria* spp. detection/recovery. This study indicated that the former method was statistically less efficient at detecting *L. monocytogenes* in crab meat enrichments. The FDA BAM *Listeria* method is the more appropriate method for analyzing crab meat and should be used by the field laboratories going forward.

5 Acknowledgements

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6 Disclaimer

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