

Evaluation of *Listeria* Special Broth II (LSB II) for Recovery and Detection of Non-Stressed and Heat Stressed *Listeria* spp.



NQAC

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Abstract

Introduction: LSB II is Bio-Rad Laboratories' latest second-generation enrichment media that allows for detection of *Listeria* spp at 37°C at 18-24 hours post-incubation on the iQ-Check PCR platform. NQAC Dublin performed initial evaluations on this medium and is now pursuing full implementation of this method.

Purpose: The objectives of this study were to:

- Assess the performance of LSB II for detection of healthy and heat-stressed *L. monocytogenes* and *L. innocua* at minimum and maximum incubation times (18 and 24 hours) in validated and non-validated enrichment volumes (225ml and 1125ml, respectively).
- Perform implementation and (food) item verification via the estimated limit of detection at 50% (eLOD₅₀) of eight different ISO food categories per ISO 16140-3:2021 to claim a broad scope of foods.

Methods:

- Healthy and heat-stressed cultures (treated at 56°C for 15 minutes) were inoculated into 225 or 1125ml of prewarmed LSB II and incubated at 37°C for 18 and 24 hours. At both time points, aliquots were taken for enumeration onto RAPID[®] L.mono agar, and for PCR analysis on the iQ-Check *Listeria* spp. and *L. monocytogenes* II kits. Experiments were replicated three times on three different days, and statistical analyses were performed using Minitab's General Linear Model function.
- For implementation and (food) item verification, the eLOD₅₀ was conducted using protocol 1 in ISO 16140-3:2021. Healthy cultures of *L. monocytogenes* (5 strains), *L. innocua*, *L. welshimeri*, and *L. ivanovii* subsp. *londoniensis* were used for these studies.

Results:

- Both healthy cultures of *L. monocytogenes* and *L. innocua* grew to 6.81 and 6.89 log CFU/ml after 18hrs incubation, respectively, and greater than 9 log after 24hrs incubation in 225ml enrichments. In the 1125ml enrichment volume, concentrations of healthy cultures were around ≥ 1 log lower than the 225ml volume. Heat-stressed *L. monocytogenes* and *L. innocua* grew to around 3-4 log CFU/ml in both enrichment volumes after 18hrs incubation, and 6-7 logs after 24hrs. Over 3 replicates, *L. monocytogenes* and *L. innocua* were successfully detected as soon as 18 hrs of incubation in the smaller enrichment volume, however the larger enrichment volume required longer incubation times for successful detection. The effects of culture type, stress, enrichment volume, and incubation time were statistically significant ($p < 0.05$).
- The observed eLOD₅₀ for all eight food categories ranged from <0 to <1 and were determined to be acceptable for LSB II and the iQ-Check *Listeria* spp./*L. monocytogenes* PCR platform.

Significance: LSB II demonstrated great potential for rapid detection of *Listeria* for routine testing. NQAC Dublin will also be performing larger test portion (125g) validation in accordance with the draft guidelines set forth in ISO 16140-4:20/FDAM 1.

Materials & Methods

Organism Used	LSB II Study	Bacon	Beef Base	Butter	Cookie Dough	Egg Powder	Flour	Probiotic Infant Formula	Swab
<i>L. monocytogenes</i> ATCC 19392	✓								
<i>L. monocytogenes</i> ATCC 19116	✓								
<i>L. monocytogenes</i> ATCC 19111	✓								
<i>L. monocytogenes</i> GFP UV Biogel (FALSA)	✓								
<i>L. innocua</i> ATCC 33090	✓								
<i>L. innocua</i> ATCC 33090 HS	✓								
<i>L. innocua</i> ATCC 33090 HS	✓								
<i>L. innocua</i> ATCC 33090 HS	✓								
<i>L. innocua</i> ATCC 33090 HS	✓								

Table 1. Cultures used for each study.

Protocol for LSB II Growth Study Using Non-Stressed and Stressed Cells

Culture

- Inoculate 10ml of brain heart infusion broth (BHI) with 1 colony of *L. monocytogenes* 13932 or *L. innocua* 33090. Incubate at 37°C for 6 hours.
- Subculture 0.1ml of the 6-hour culture to 10ml of BHI broth. Incubate at 37°C for 18 hours. Use this stock for non-stressed inoculation and for the heat stress protocol.

Heat Stress Protocol

- Serially dilute cultures to 10⁻² and transfer 1.2ml of this dilution to a microcentrifuge tube. Also prepare a water blank to measure temperature in the heating block.
- Place tubes in a heating block set to 56 ± 0.1°C. Place a thermocouple in the water blank.
- Once temperature reaches 55°C, begin timing for 15 minutes.
- After 15 minutes, immediately place tubes in an aluminum cooling block (stored at -20°C).
- Serially dilute cultures and plate onto non-selective (tryptic soy agar with 0.6% yeast extract) and selective (RAPID[®] L.mono agar) agars to determine counts and degree of injury. Incubate plates at 37°C for 48 hours.

LSB II Broth Study

- Dispense 100µl of the non-stressed 10⁻² dilution or 80µl of the heat stressed 10⁻⁴ dilution of each culture to either 225 or 1125ml of pre-warmed LSB II in stomacher bags. Incubate at 37°C.
- At 18- and 24-hours post-incubation, dispense 2ml of the enrichment to a false-bottom tube.
- Using 1ml of enrichment, serially dilute and plate RAPID[®] L.mono agar in duplicate. Incubate plates at 37°C for 48 hours.
- The remaining 1ml of enrichment will be used to run automated sample preparation on the iQ-Prep Check robot, followed by PCR analysis on both the iQ-Check *Listeria* spp and iQ-Check *Listeria monocytogenes* II PCR detection kits.
- Experiments were performed in triplicate.
- The General Linear Model function in Minitab 18 was used to determine if there were statistically significant differences between healthy vs stressed cell state, enrichment volumes, incubation times.

ISO Food Category	Food Type	Matrix Used	Challenging Matrix
Ready-to-eat, ready-to-reheat meat products	Cooked meat products	Precooked Bacon Pieces*	Yag, salt, fat, and curing additives
Multi-component foods or meat components	Ready-to-heat food: refrigerated	Beef Flavor Base*	Yag, salt
Multi-component milk and dairy products	Pasteurized milk-based products	Unsalted Butter*	Yag, fat that solidifies at room temperature
Multi-component foods or meat components	Composite foods with substantial raw ingredients	Chocolate Chip Cookie Dough*	No
Eggs and egg products (batter)	Dry	Dried Whole Egg Powder*	Yag, clumping
Dried cereals, fruits, nuts, seeds and vegetables	Flours	Bleached All-Purpose Flour*	Yag, high levels of background flora
Infant formula and infant cereals	Probiotic infant formula	Probiotic Infant Formula*	Yag, probiotics
Environmental samples (food or feed production)	Equipment or production environment	Environmental Sponge Swab*	Yag, potential PCR inhibitor in buffer, background flora from swabbing surface

* Food (item) verification implementation verification

Table 2. ISO food categories and matrices selected for estimated level of detection at 50% (eLOD₅₀) studies per ISO 16140-3:2021.

Protocol for eLOD₅₀ Verification of LSB II on the iQ-Check Platform for a Broad Range of Foods Scope per ISO 16140-3:2021

- Implementation verification – select one food item tested during the validation study that belongs within the scope of laboratory application of the user laboratory
- Food (item) verification – select 6 challenging (food) item from each (food) category listed within the scope of validation that is also a (food) category tested within the scope of laboratory application of the user laboratory

Culture

- Inoculate 10ml of BHI broth with 1 colony of desired culture (see Table 1). Incubate at 37°C for 18-24 hours.
- Serially dilute to obtain high, intermediate, and low spike levels, assuming an LOD₅₀ value of 0.5 (Table 3).

Sample Preparation

- Weigh 25g of matrix (Table 2) and spike with 1ml of either the high, intermediate, or low-level inoculum.
- Enrich with 225ml of pre-warmed LSB II. Stomach for 1 minute.
- Incubate samples at 37°C for 22-24 hours.

PCR Protocol

- Transfer 1ml of each enrichment to a false-bottom tube.
- Run automated sample preparation on the iQ-Prep Check robot.
- Run samples on the iQ-Check *Listeria* spp and iQ-Check *Listeria monocytogenes* II PCR detection kits.
- A minimum of one test portion at each inoculation will be confirmed by:
 - Listeria* spp. – streak sample to RAPID[®] *Listeria* spp and PALCAM agars. Look for blue-green and blue colonies, respectively.
 - L. monocytogenes* – streak sample to RAPID[®] L.mono and ALOA agars. Look for blue-green and blue-green colonies with halos, respectively.

Protocol	Inoculation level of the test portion				Blank	Total number of replicates
	High level 9 × LOD ₅₀ / test portion	Intermediate level 3 × LOD ₅₀ / test portion	Low level 1 × LOD ₅₀ / test portion	3 cfu to 5 cfu / test portion		
1	1	4	4	-	1	10

Table 3. Inoculation protocol for estimated level of detection at 50% (eLOD₅₀) studies per ISO 16140-3:2021. Spike levels were calculated assuming an LOD₅₀ of 0.5 CFU per test portion.

Culture	Overnight Culture (log CFU/ml ± SD)	Non-stressed CFU in 100µl spike ± SD	After Heat Stress			
			Tryptic soy agar + 0.6% yeast extract (log CFU/ml ± SD)	RAPID [®] L.mono agar (log CFU/ml ± SD)	Degree of Injury (%) ± SD	Heat-stressed CFU in 80µl spike ± SD
<i>L. monocytogenes</i> 13932	9.07 ± 0.10	12.0 ± 2.6	8.47 ± 0.09	8.26 ± 0.06	38.52 ± 10.47	23.5 ± 5.0
<i>L. innocua</i> 33090	9.13 ± 0.08	13.7 ± 2.5	8.02 ± 0.06	7.79 ± 0.11	40.19 ± 10.12	8.6 ± 1.2

Table 4. Non-stressed and heat stressed (56° for 15 min) culture levels for LSB II broth studies.

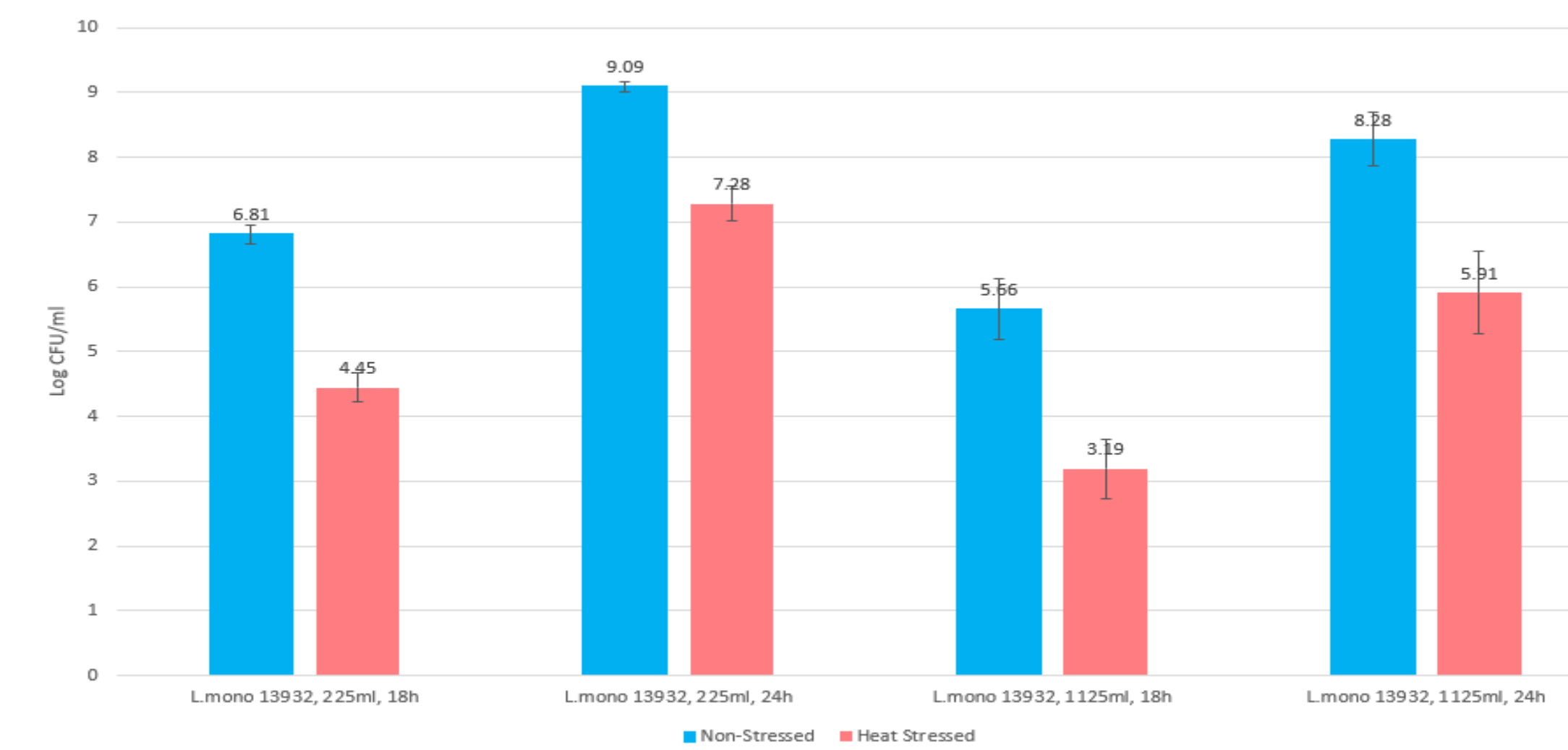


Figure 1. Enumeration of non-stressed and heat stressed *L. monocytogenes* 13932 in LSB II at two enrichment volumes and minimum/maximum (18 vs 24 hours) incubation at 37°C. Statistically significant differences ($p < 0.05$) were observed between healthy/stressed states, enrichment volume, and incubation time.

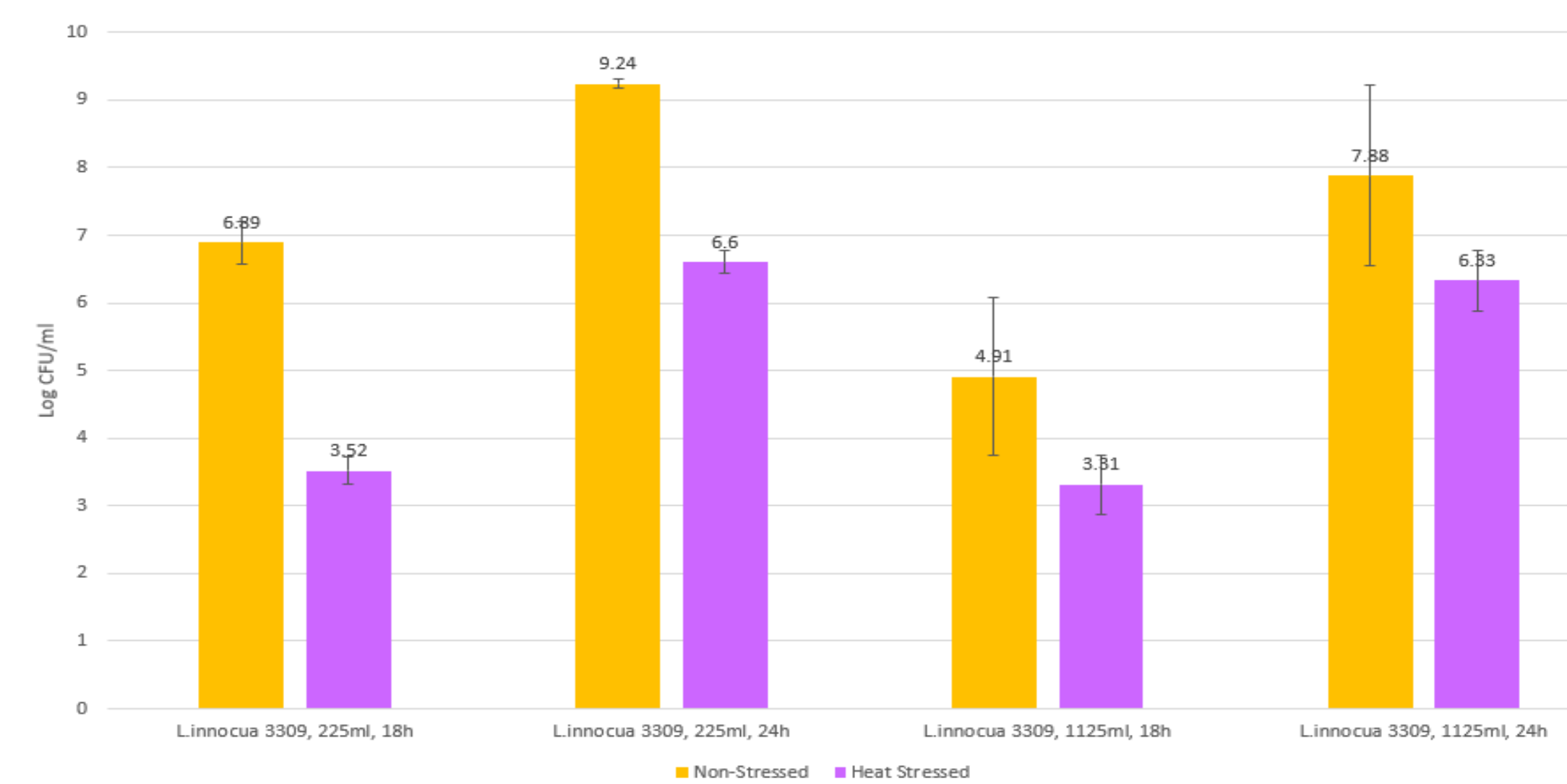


Figure 2. Enumeration of non-stressed and heat stressed *L. innocua* 33090 in LSB II at two enrichment volumes and minimum/maximum (18 vs 24 hours) incubation at 37°C. Statistically significant differences ($p < 0.05$) were observed between healthy/stressed states, enrichment volume, and incubation time.

Culture	LSB II Volume (ml)	Incubation Time	iQ-Check <i>Listeria</i> spp. (Cq)			iQ-Check <i>L. monocytogenes</i> II (Cq)		
			Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3
<i>L. monocytogenes</i> 13932	225	18h	25.29	22.37	26.73	27.56	24.37	29.22
		24h	19.74	16.84	21.05	21.73	18.65	22.58
	1125	18h	30.34	25.53	30.85	32.28	28.08	32.84
		24h	23.11	18.24	22.53	25.08	20.16	24.25
		18h	31.57	31.09	38.71	33.83	32.22	36.25
		24h	22.27	22.45	27.90	24.09	24.08	28.29
<i>L. innocua</i> 33090	225	18h	38.34	32.89	39.26	39.55	35.07	n/a
		24h	30.48	24.16	33.22	32.50	25.76	34.63
	1125	18h	26.38	22.28	25.16	n/a	n/a	n/a
		24h	19.64	16.07	18.35	n/a	n/a	n/a
		18h	37.36	25.02	29.03	n/a	n/a	n/a
		24h	28.46	16.45	20.09	n/a	n/a	n/a
<i>L. innocua</i> 33090 HS	225	18h	36.27	33.08	35.04	n/a	n/a	n/a
		24h	27.74	22.85	25.30	n/a	n/a	n/a
	1125	18h	36.26	34.00	36.83	n/a	n/a	n/a
		24h	28.97	23.97	27.15	n/a	n/a	n/a

HS – heat stressed
Red = Not Detected after 18h incubation
Green = Detected after 24h incubation
n/a = Not Detected

Table 5. iQ-Check *Listeria* spp. and iQ-Check *L. monocytogenes* II real-time PCR threshold values (Cq) for non-stressed and heat-stressed *L. monocytogenes* and *L. innocua* grown in LSB II at 37°C for 18 and 24 hours. One replicate of heat-stressed *L. monocytogenes* was not detected after 18 hours in the larger 1125ml enrichment but was successfully detected after 24 hours of incubation.

Results

Organism Used	Food Matrix	High Level 9 × LOD ₅₀ / Test Portion	Intermediate Level 3 × LOD ₅₀ / Test Portion	Low Level 1 × LOD ₅₀ / Test Portion
<i>L. monocytogenes</i> ATCC 13932	Precooked Bacon Pieces	3.2	1.3	0.5
<i>L. monocytogenes</i> ATCC 19116	Beef Flavor Base	2.9	1.2	0.4
<i>L. monocytogenes</i> ATCC 19111	Unsalted Butter	3.0	1.3	0.5
<i>L. monocytogenes</i> GFP UV- BioTAG FDA LSB10 (Microbiological)	Chocolate Chip Cookie Dough	3.1	1.2	0.6
<i>L. monocytogenes</i> NCTC 11994	Dried Whole Egg Powder	3.9	1.5	0.6
<i>L. innocua</i> ATCC 33090	Bleached All-Purpose Flour	3.4	1.3	0.6
<i>L. ivanovii</i> ATCC BAA-139	Probiotic Infant Formula	4.3	1.6	0.7
<i>L. welshimeri</i> ATCC 35897	Environmental Sponge Swab	3.2	1.2	0.5

Table 6. Spike levels for eLOD₅₀ (protocol 1) implementation and food (item) verification per ISO 16140-3:2021.

Implementation verification	(Food) Item							
	Bacon pieces	Beef base	Butter	Egg	Flour	Swab	Yag	Yag
Determined low inoculum level LIL (cfu/test portion):	0.5	0.5	0.4	0.6	0.5	0.6	0.6	0.7
Determination of eLOD ₅₀ using protocol 1	Results per inoculum level (number of positive (food) item test portions per inoculum level: enter 0, 1, 2, 3 or 4 in each cell)							
Inoculum level of the test portions	Cookie Dough	Bacon pieces	Beef base	Butter	Egg	Flour	Swab	Yag
High inoculum	1	1	1	1	1	1	1	1
Intermediate inoculum	4	4	3	4	1	4	4	4
Low inoculum	3	4	3	4	2	4	4	4
Blank (uninoculated)	0	0	0	0	0	0	0	0
eLOD ₅₀ (cfu/test portion)	<0.5 × LIL	<1.0 × LIL	<0.7 × LIL	<0.5 × LIL	<2.8 × LIL	<1.0 × LIL	<1.0 × LIL	<1.0 × LIL
Determined eLOD ₅₀ (cfu/test portion)	0.2	<1	0.2	0.4	0.2	<1	<1	<1
Acceptability limits (protocol 1)	The eLOD ₅₀ shall not be > 4 × LOD ₅₀ observed in the validation study							
Published validation data of the method. If no validation data is available, assume an LOD ₅₀ of 1 cfu/test portion	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Observed eLOD ₅₀ (cfu/test portion)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Acceptable eLOD ₅₀ (cfu/test portion) = 4 × LOD ₅₀	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Acceptability limit evaluation	Determined eLOD ₅₀ ≤ Acceptable eLOD ₅₀							
	Accepted	Accepted	Accepted	Accepted	Accepted	Accepted	Accepted	Accepted

Figure 3. Estimated limit of detection at 50% (eLOD₅₀) of *Listeria* spp. in eight different food categories on the iQ-Check *Listeria* spp kit.

Implementation verification	(Food) Item				
	Cookie Dough	Bacon pieces	Beef Base	Butter	Swab
Determined low inoculum level LIL (cfu/test portion):	0.5	0.5	0.4	0.6	0.6
Determination of eLOD ₅₀ using protocol 1	Results per inoculum level (number of positive (food) item test portions per inoculum level: enter 0, 1, 2, 3 or 4 in each cell)				
Inoculum level of the test portions	Cookie Dough	Bacon pieces	Beef Base	Butter	Swab
High inoculum	1	1	1	1	1
Intermediate inoculum	4	4	4	3	4
Low inoculum	3	4	3	4	4
Blank (uninoculated)	0	0	0	0	0
eLOD ₅₀ (cfu/test portion)	<0.5 × LIL	<1.0 × LIL	<0.5 × LIL	<0.7 × LIL	<1.0 × LIL
Determined eLOD ₅₀ (cfu/test portion)	0.3	<1	0.2	0.4	<1
Acceptability limits (protocol 1)	The eLOD ₅₀ shall not be > 4 × LOD ₅₀ observed in the validation study				
Published validation data of the method. If no validation data is available, assume an LOD ₅₀ of 1 cfu/test portion	1.0	1.0	1.0	1.0	1.0
Observed eLOD ₅₀ (cfu/test portion)	4.0	4.0	4.0	4.0	4.0
Acceptable eLOD ₅₀ (cfu/test portion) = 4 × LOD ₅₀	4.0	4.0	4.0	4.0	4.0
Acceptability limit evaluation	Determined eLOD ₅₀ ≤ Acceptable eLOD ₅₀				
	Accepted	Accepted	Accepted	Accepted	Accepted

Figure 4. Estimated limit of detection at 50% (eLOD₅₀) of *Listeria monocytogenes* in five different food categories on the iQ-Check *Listeria monocytogenes* II kit.

Discussion/Conclusion

- At the time of the initial LSB II evaluation, the incub