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# Concentration of *Listeria monocytogenes* in Skim Milk and Soft Cheese through Microplate Immunocapture

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1 **Concentration of *Listeria monocytogenes* in Skim Milk and Soft Cheese through Microplate**  
2 **Immunocapture**

3

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21 **ABSTRACT**

22 Microplate immunocapture is an inexpensive method for the concentration of foodborne  
23 pathogens using an antibody-coated microplate. The objective of this study was to determine the  
24 efficacy of microplate immunocapture as an alternative to traditional enrichment for  
25 concentrating *Listeria monocytogenes* to levels detectable with selective plating or real-time  
26 PCR. *L. monocytogenes* isolates serologically characterized as Type 1 (1/2a) and Type 4  
27 (untypeable) were grown overnight and diluted to  $10^0$  to  $10^6$  colony-forming units (CFU)/mL.  
28 The isolates were used to optimize microplate immunocapture in tryptic soy broth with 0.6%  
29 yeast extract (TSBYE), skim milk, and queso fresco samples. Following microplate  
30 immunocapture, the bacteria were streaked onto polymyxin-acriflavine-LiCl-ceftazidime-  
31 aesculin-mannitol (PALCAM) agar, followed by incubation at 37 °C for  $24 \pm 2$  h. The bacteria  
32 also underwent real-time polymerase chain reaction (PCR). The optimized microplate  
33 immunocapture method was tested in triplicate for its ability to capture *L. monocytogenes* in  
34 broth and food samples. Overall recovery rates for *L. monocytogenes* in food samples at cell  
35 populations of  $10^0$ ,  $10^2$ , and  $10^4$  CFU/25 g using microplate immunocapture with real-time PCR  
36 were 88.9%, 94.4%, and 100%, respectively. Recovery in these matrices using microplate  
37 immunocapture with selective plating was comparatively lower, at 0%, 44.4%, and 100%,  
38 respectively. Conventional culture method showed 100% detection at each inoculation level.  
39 Microplate immunocapture combined with real-time PCR shows high potential to reduce the  
40 time required for detection, with concentration of *L. monocytogenes* to detectable levels within  
41 1-4 h. The incorporation of a short enrichment step may improve recovery rates at low cell  
42 levels.

43 **Keywords:** Microplate immunocapture; cheese; milk; real-time PCR; *Listeria monocytogenes*

## 44 **1. Introduction**

45 *Listeria monocytogenes* is a facultative anaerobic bacterium that is especially problematic  
46 due to its ability to survive and grow at refrigerated conditions (FDA, 2012). This pathogen has  
47 the highest hospitalization rate (94.0%) and the third-highest death rate (15.9%) among  
48 foodborne pathogens in the United States (Scallan et al., 2011). Common symptoms caused by *L.*  
49 *monocytogenes* are fever, muscle aches, nausea, and vomiting (FDA, 2012). However, in more  
50 serious cases it can cause septicemia and meningitis, as well as induce stillbirth or miscarriage in  
51 pregnant women. Listeriosis is often linked to raw or ready-to-eat foods, such as fresh produce,  
52 unpasteurized milk, smoked fish, and deli meats. There are 13 known serotypes of *L.*  
53 *monocytogenes*, with strains of serotypes 1/2a, 1/2b, and 4b responsible for the majority of  
54 foodborne infections. The U.S. Food and Drug Administration (FDA) has a zero-tolerance policy  
55 for *L. monocytogenes* in ready-to-eat foods and it is consistently one of the most common  
56 pathogens associated with food recalls in the United States (FDA, 2018).

57 Dairy products, such as milk and cheeses, are a major cause of outbreaks linked to *L.*  
58 *monocytogenes* (CDC, 2017). For example, *L. monocytogenes* was among the top three  
59 pathogens linked to 90 foodborne outbreaks associated with cheese in the United States from  
60 1998 to 2011 and it was associated with 5 of the 6 deaths reported (Gould, Mungai, &  
61 Behravesh, 2014). Mexican-style cheese, including queso fresco, was the main type of cheese  
62 associated with illness from *L. monocytogenes* during this time period (Gould et al., 2014).  
63 Queso fresco is a soft, unaged cheese that is susceptible to *Listeria* survival and growth due to its  
64 relatively high moisture content and low acidity (Moreno-Enriquez et al., 2007).

65 Cultural methods for the isolation of *L. monocytogenes* involve a series of pre-enrichment  
66 and enrichment steps, followed by plating on selective/differential agar (Hitchens, Jinneman, &

67 Chen, 2016). This process is very time-consuming, usually requiring 2-4 days, not including the  
68 time required for confirmation of isolated colonies. Bacterial separation and concentration  
69 methods have the potential to reduce or possibly eliminate the need for pre-enrichment and  
70 enrichment steps, thereby significantly shortening the time required for isolation (Stevens &  
71 Jaykus, 2004). These techniques are also advantageous because they can be combined with rapid  
72 detection methods, such as polymerase chain reaction (PCR) or enzyme-linked immunosorbent  
73 assay (ELISA), further reducing the time to detection.

74 Immunomagnetic separation is a widely used method for bacterial separation and  
75 concentration; however, it is relatively expensive due to the need for antibody-coated beads  
76 (Amagliani et al., 2006; Chen et al., 2017; Ma et al., 2014). Non-magnetic immunocapture is an  
77 inexpensive alternative that relies on the binding of antibodies to a solid plastic support (Arbault,  
78 Desroche, & Larose, 2014). This technique has been successfully used for the concentration of  
79 foodborne pathogens in a limited number of studies (Arbault, Larose, Desroche, & Nexidia,  
80 2014; Fakruddin, Hossain, & Ahmed, 2017; Molloy, Brydon, Porter, & Harris, 1995). For  
81 example, Arbault et al. (2014) were able to concentrate *Escherichia coli* O157:H7 from ground  
82 meat and raw milk cheese samples with an antibody-coated microplate. Using a combination of  
83 the microplate and a subculture step (3-5 h), *E. coli* was recovered at levels of  $10^5$  CFU as  
84 compared to  $10^3 - 10^4$  CFU with magnetic beads. In another study, microplate immunocapture  
85 was evaluated as a potential method for the concentration of *Vibrio cholera*, *Salmonella enterica*  
86 serovar Typhi, and *Shigella flexneri* from a variety of food samples (Fakruddin et al., 2017).  
87 Overall, the authors found that microplate immunocapture combined with PCR or selective  
88 plating allowed for improved recovery of the target pathogens from foods as compared to  
89 traditional culture methods.

90 PCR is a well-established technique for the rapid identification of foodborne pathogens  
91 and it is widely recognized for its specificity and sensitivity (Zhao, Lin, Wang, & Oh, 2014).  
92 Real-time PCR is advantageous over traditional PCR because it enables continuous monitoring  
93 of the results as the reaction proceeds and eliminates the need for post-PCR processing steps.  
94 There are numerous commercially available kits for the detection of *L. monocytogenes* using  
95 real-time PCR (Law, Ab Mutalib, Chan, & Lee, 2015) and a real-time PCR assay for detection of  
96 *L. monocytogenes* has been published in the FDA's Bacteriological Analytical Method (BAM)  
97 (FDA, 2015). Although PCR-based methods are susceptible to inhibition from compounds in the  
98 food matrix, concentration methods such as microplate immunocapture can help to overcome  
99 this by separating the target organism from the rest of the sample (Fakruddin et al., 2017;  
100 Stevens & Jaykus, 2004).

101 The specific aims of this study were to: (1) determine the ability of microplate  
102 immunocapture combined with selective plating or real-time PCR to detect *L. monocytogenes* in  
103 a pure broth solution within 1 workday (8 h), (2) optimize microplate immunocapture as a means  
104 of concentrating *L. monocytogenes* in milk and cheese samples for subsequent detection with  
105 selective plating or real-time PCR, and (3) determine the sensitivity and time to detection for  
106 microplate immunocapture combined with selective plating or real-time PCR.

## 107 **2. Materials and methods**

### 108 *2.1 Media and bacterial strains*

109 All media were obtained from Becton, Dickinson and Company [(BD) (Franklin Lakes,  
110 NJ)] unless otherwise stated. Two environmental isolates of *L. monocytogenes* were obtained  
111 from the U.S. Food and Drug Administration (FDA) Pacific Regional Laboratory Southwest  
112 (Irvine, CA). The isolates were serologically categorized as Type 1 (T1; serotype 1/2a) and Type

113 4 (T4; untypeable) by a combination of slide agglutination and multiplex PCR (Burall, Simpson,  
114 & Datta, 2011; Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004) using modifications  
115 described in Hellberg et al. (2013). The isolates were streaked to Tryptic Soy Agar (TSA) and  
116 incubated overnight at 37 °C, then transferred to tryptic soy broth with 0.6% yeast extract  
117 (TSBYE) and incubated overnight at 37 °C to concentrations of 10<sup>8</sup> CFU/mL. Concentration  
118 levels were determined by optical density (OD) measurement based on a logarithmic growth  
119 curve (not shown) and verified by plate count on TSA. Bacterial cultures from the T1 and T4  
120 isolates were grown separately. The cultures were then serially diluted to concentrations of 10<sup>6</sup>  
121 CFU/mL, 10<sup>4</sup> CFU/mL, 10<sup>2</sup> CFU/mL, and 10<sup>0</sup> CFU/mL in TSBYE. For T1 + T4 mixed culture  
122 testing, equivalent amounts of the T1 and T4 cultures (10<sup>8</sup> CFU/mL) were combined prior to  
123 carrying out serial dilutions.

## 124 2.2 Microplate preparation

125 Polystyrene 96-well microtiter microplates separable into 8-well strips (Fisher Scientific,  
126 Waltham, MA) were prepared for the concentration of *L. monocytogenes* according to a protocol  
127 from Abcam (<http://www.abcam.com/protocols/sandwich-elisa-protocol-1>). Anti-*Listeria*  
128 Polyclonal Antibody, HRP conjugate PA1-73129 (Invitrogen, Carlsbad, CA) was diluted to 1-10  
129 µg/mL in carbonate-bicarbonate buffer. The diluted antibodies were adhered to the inner surface  
130 of the microplate by transferring 200 µL of the solution to each of the wells. The plates were  
131 then covered with plastic and held overnight (8-16 h) at 4 °C. The following day, the plates were  
132 rinsed with phosphate buffered saline solution (PBS), pH 7.4, blocked with a 5% skim milk/PBS  
133 solution, held at room temperature for 2 h, and then rinsed a final time with PBS. Following this  
134 process, the plates were used in microplate immunocapture, as described below, or stored at -  
135 20 °C until needed.



136 2.3 Optimization of microplate immunocapture

137 The antibody-coated microplates prepared above were first tested with mixed cultures of  
138 *L. monocytogenes* Types 1 and 4 in TSBYE to optimize the method in the absence of a food  
139 matrix. The T1 + T4 cultures were prepared as described above to allow for concentrations of  
140  $10^6$  CFU/mL,  $10^4$  CFU/mL,  $10^2$  CFU/mL, and  $10^0$  CFU/mL (Singh, Batish, & Grover, 2012). A  
141 blank sample containing TSBYE was run alongside each set of experiments as a negative culture  
142 control. Microplate immunocapture was carried out in a biosafety hood and optimized for the  
143 number of fill cycles (1-4), hold times (15-60 min), antibody concentration (1-10  $\mu$ g/mL), and  
144 use of a plate shaker (Bio Rad, Hercules, CA) at speeds of 10-120 RPM. For each fill cycle, 1.6  
145 mL of each inoculated broth or control sample were transferred to 8 wells of the antibody-coated  
146 plate, resulting in 200  $\mu$ L of sample per well. The sample was then incubated at room  
147 temperature for a specific period of time (i.e., hold time) before being discarded and replaced in  
148 the next fill cycle. With each fill cycle, an additional 1.6 mL of the sample (200  $\mu$ L per well) was  
149 added, resulting in a total volume of 6.4 mL per sample (800  $\mu$ L per well) when 4 fill cycles  
150 were carried out.

151 Following microplate immunocapture, all 8 wells were scraped for each sample using a  
152 disposable sterile inoculating loop. The loop was then streaked onto polymyxin-acriflavine-LiCl-  
153 ceftazidime-aesculin-mannitol (PALCAM) agar. Next, all 8 wells were scraped again for each  
154 sample using a second sterile inoculating loop. The second loop was then mixed with 100  $\mu$ l  
155 sterile water in a sterile Safe-lock microcentrifuge tube (Eppendorf, Hamburg, Germany) to  
156 release bacterial cells for DNA extraction, as described below. Positive culture controls were  
157 prepared using the  $10^8$  CFU/mL broth sample, which was streaked directly to PALCAM or  
158 transferred to a microcentrifuge tube for DNA extraction using a sterile disposable loop. The

159 PALCAM plates were incubated for  $24 \pm 2$  h at  $37^\circ\text{C}$ . The plates were then examined for typical  
 160 *L. monocytogenes* growth, consisting of grey-green colonies with accompanied blackening of the  
 161 agar. Once optimal microplate immunocapture conditions were determined using the PALCAM  
 162 plates, the T1+T4 mixed culture as well as individual T1 and T4 cultures were tested in triplicate  
 163 using the optimized procedure (Table 1).

164

165 **Table 1.** Optimized conditions for microplate immunocapture (IC) for each matrix. Total  
 166 working time is given for microplate immunocapture combined with either selective plating on  
 167 PALCAM or detection with real-time PCR (qPCR) and includes sample preparation time.

Matrix	# of fill cycles	Cycle hold time (min)	<i>L. monocytogenes</i> antibody concentration ( $\mu\text{g/mL}$ )	Plate shaker speed (RPM)	Total time required (h)	
					IC + PALCAM	IC + qPCR
TSBYE	3	15	1	10	$24.8 \pm 2$	3.1
Skim milk	4	30	1	10	$26 \pm 2$	4.3
Queso fresco cheese	4	45	1	10	$27 \pm 2$	5.3

168

#### 169 2.4 Preparation and microplate immunocapture of food samples

170 The microplate immunocapture method was next optimized with skim milk (BD)  
 171 rehydrated with sterile deionized water and queso fresco cheese purchased at a local grocery  
 172 store. Prior to use in the inoculation trials, the cheese samples were first confirmed negative for  
 173 the presence of *L. monocytogenes* using the conventional culture method described in the BAM,  
 174 Chapter 10 (Hitchens et al., 2016).

175 Milk and cheese samples (25 g) were inoculated with 1 mL of *L. monocytogenes* mixed  
176 T1 + T4 cultures prepared as described above, resulting in final concentrations in the food  
177 product of:  $10^6$  CFU/25 g,  $10^4$  CFU/25 g,  $10^2$  CFU/25 g, and  $10^0$  CFU/25 g. The samples were  
178 then allowed to sit at room temperature under a biosafety hood for 2 h (Singh et al., 2012). An  
179 un-inoculated sample was included in each trial as a negative control. The controls underwent  
180 the same microplate immunocapture treatment as the inoculated samples. Each 25 g sample was  
181 diluted with 225 mL TSBYE and then 1.6 mL of the mixture was transferred to 8 wells of the  
182 antibody-coated plate, resulting in 200  $\mu$ L of sample per well. A broth sample containing  $10^8$   
183 CFU/mL of *L. monocytogenes* T1 + T4 mixed culture was included in each trial as a positive  
184 control. Microplate immunocapture with milk and cheese samples was carried out under a  
185 biosafety hood at room temperature using an antibody concentration of 1  $\mu$ g/mL and a plate  
186 shaker speed of 10 RPM. The procedure was optimized for the number of fill cycles (2-4) and  
187 hold times (15-60 min).

188 After microplate immunocapture, the wells of the microplate were scraped using an  
189 inoculating loop and streaked onto PALCAM agar or transferred to sterile Safe-lock  
190 microcentrifuge tubes containing 100  $\mu$ l sterile water for DNA extraction. The PALCAM plates  
191 were incubated for  $24 \pm 2$  h at 37 °C. The plates were then examined for typical *L.*  
192 *monocytogenes* growth. Once optimal microplate immunocapture conditions were determined  
193 using PALCAM plates, the milk and cheese samples were inoculated with the T1 + T4 mixed  
194 culture as well as individual T1 and T4 cultures and tested in triplicate using the optimized  
195 protocol (Table 1). Each inoculated sample was also enriched in buffered *Listeria* enrichment  
196 broth (BLEB) and plated in triplicate on PALCAM agar using the conventional culture method  
197 described in the BAM, Chapter 10 (Hitchens et al., 2016).

## 198 2.5 Real-time polymerase chain reaction

199 DNA extraction was carried out by incubating samples in a dry heat block at 100 °C for  
200 10 min, followed by cooling on ice and then centrifugation at 12,000 x g for 5 min (Amagliani et  
201 al., 2006). The supernatant was transferred to a fresh microcentrifuge tube and stored at -20 °C  
202 until use in real-time PCR. Real-time PCR was carried out in a Rotor-Gene Q thermocycler  
203 (Qiagen, Hilden, Germany) using the *L. monocytogenes*-specific primers and probes detailed in  
204 FDA (2015). Each reaction tube contained 0.5 lyophilized OmniMix-HS beads (Takara Bio,  
205 Dalian, China), 0.625 µL each of 10 µM forward and reverse primers (0.25 µM final  
206 concentration), 0.25 µL of 10 µM probe (0.1 µM final concentration), 5 µL extracted template  
207 DNA, and sterile distilled water to bring the final reaction volume to 25 µL per sample. PCR  
208 cycling conditions started with an initial activation of 94 °C for 60 s, followed by 45 cycles of  
209 94 °C for 10 s and 60 °C for 45 s. Each real-time PCR run included a negative non-template  
210 control and three positive DNA controls originating from the bacterial culture: undiluted  
211 bacterial DNA and two tenfold serial dilutions of bacterial DNA (1:10 and 1:100). The results  
212 were analyzed using Rotor-Gene Q software and reported on a qualitative basis, where the  
213 presence of a cycle of quantitation (Cq) value indicated a positive sample.

## 214 3. Results and discussion

### 215 3.1 Microplate immunocapture with broth samples

216 The conditions for microplate immunocapture were successfully optimized using the  
217 mixed T1 + T4 culture of *Listeria monocytogenes* in TSBYE combined with plating on  
218 PALCAM agar. During optimization trials, it was found that the bacteria could be detected at a  
219 starting inoculation of 10<sup>0</sup> CFU/mL when 4 fill cycles were used with hold times of 1 h each,  
220 combined with the lowest antibody concentration tested (1 µg/mL). However, reducing either the

221 hold time or the number of fill cycles resulted in a reduction in the sensitivity of the method,  
222 with detection starting at  $10^2$  CFU/mL, even when the antibody concentration was increased to  
223  $10\ \mu\text{g/mL}$ . Interestingly, use of the plate shaker at speeds of 80-120 RPM did not reduce the  
224 number of fill cycles or the hold time required for detection at  $10^0$  CFU/mL. On the other hand,  
225 when the speed was reduced to 10-40 RPM, detection at  $10^0$  CFU/mL was possible using only 3  
226 fill cycles and hold times of 15 min each, combined with an antibody concentration of  $1\ \mu\text{g/mL}$ .  
227 This reduced the overall time required for concentration down to 75 min, as compared to 4 h in  
228 the absence of the plate shaker. Table 1 shows the optimal conditions determined for microplate  
229 immunocapture with broth.

230 Table 2 shows the results of triplicate testing of broth samples using the optimized  
231 conditions with *L. monocytogenes* T1, T4, and the mixed T1 + T4 culture. Overall, these  
232 conditions allowed for detection of the strains (individually or mixed) at a level of  $10^0$  CFU/mL  
233 when combined with selective plating on PALCAM or detection with real-time PCR. The results  
234 for all positive and negative controls were as expected. Microplate immunocapture combined  
235 with selective plating showed a slightly higher overall detection rate, with 35/36 detections  
236 (97.2%) on PALCAM across all inoculation levels compared to 33/36 detections (91.7%) with  
237 real-time PCR. Differences in the results occurred only at the lowest inoculation level ( $10^0$   
238 CFU/mL), with 8/9 detections (88.9%) on PALCAM and 6/9 detections (66.7%) with real-time  
239 PCR. Microplate immunocapture combined with selective plating or real-time PCR allowed for  
240 positive detections in 100% of replicates tested at inoculation levels of  $10^2$  to  $10^6$  CFU/mL. Real-  
241 time PCR was less consistent in detecting *L. monocytogenes* at the lowest inoculation level ( $10^0$   
242 CFU/mL), with detection in only 2 of the 3 replicates for the individual and mixed cultures. On  
243 the other hand, plating on PALCAM agar at the lowest inoculation level allowed for consistent

**Table 2.** Rates of *L. monocytogenes* detection for the immunocapture (IC) method combined with selective plating on PALCAM or real-time PCR (qPCR) at a range of cell concentrations. The results of detection in food samples using conventional culture with no IC are included for comparison.

Method	<i>L. monocytogenes</i> type	Rate of detection (no. positive samples/total no. samples)											
		Broth (CFU/mL)				Milk (CFU/25 mL)				Cheese (CFU/25 g)			
		10 <sup>0</sup>	10 <sup>2</sup>	10 <sup>4</sup>	10 <sup>6</sup>	10 <sup>0</sup>	10 <sup>2</sup>	10 <sup>4</sup>	10 <sup>6</sup>	10 <sup>0</sup>	10 <sup>2</sup>	10 <sup>4</sup>	10 <sup>6</sup>
<b>IC + PALCAM</b>	T1	3/3	3/3	3/3	3/3	0/3	2/3	3/3	3/3	0/3	0/3	3/3	3/3
	T4	2/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	0/3	0/3	3/3	3/3
	T1 + T4	3/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	0/3	0/3	3/3	3/3
	Total %	88.9	100	100	100	0	88.9	100	100	0	0	100	100
<b>IC + qPCR</b>	T1	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	T4	2/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	T1 + T4	2/3	3/3	3/3	3/3	2/3	2/3	3/3	3/3	2/3	3/3	3/3	3/3
	Total %	66.7	100	100	100	88.9	88.9	100	100	88.9	100	100	100
<b>Conventional culture</b>	T1	NA				3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	T4	NA				3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	T1 + T4	NA				3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
	Total %	NA				100	100	100	100	100	100	100	100

244 detection in all samples except one of the three T4 replicates. Combining the time for microplate  
245 immunocapture concentration with traditional plating, positive results can be determined in 24.8  
246 h  $\pm$  2 h while combining the concentration method with real-time PCR can allow for a positive  
247 detection in 3.1 h (Table 1). These times are substantially less compared to the traditional  
248 enrichment-based method, which takes at least 48  $\pm$  4 h for isolation of *L. monocytogenes*.3.2

#### 249 *Microplate immunocapture with skim milk samples*

250 The optimal conditions determined for the broth samples yielded no detection at the  
251 lowest inoculation levels ( $10^0 - 10^2$  CFU/25 mL) for skim milk inoculated with the T1 + T4  
252 mixed culture and plated on PALCAM agar. Therefore, further optimization was carried out for  
253 microplate immunocapture of *L. monocytogenes* in skim milk within the pre-determined range of  
254 parameters. This resulted in detection with PALCAM at a starting inoculation of  $10^2$  CFU/25 mL  
255 using the optimized run conditions (Table 1).

256 Table 2 shows the results of triplicate testing of skim milk samples using the optimized  
257 conditions with *L. monocytogenes* T1, T4, and the mixed T1 + T4 culture. The results for all  
258 positive and negative controls were as expected. Overall, these conditions allowed for detection  
259 of the strains (individually or mixed) at a level of  $10^2$  CFU/25 mL when combined with selective  
260 plating on PALCAM and at a level of  $10^0$  CFU/25 mL when using real-time PCR. Microplate  
261 immunocapture combined with real-time PCR showed a greater overall detection rate, with  
262 34/36 detections (94.4%) across all inoculation levels, as compared to 26/36 detections (72.2%)  
263 using PALCAM. Similar to the broth results, differences between the two detection methods  
264 occurred only at the lower inoculation levels. At the  $10^0$  CFU/25 mL and  $10^2$  CFU/ 25 mL levels,  
265 16/18 detections (88.9%) were observed using real-time PCR with only 8/18 detections (44.4%)  
266 using PALCAM. However, both of these rates were lower than that obtained using the

267 conventional culture method, which showed 100% positive detections across all inoculation  
268 levels. A previous study on *V. cholerae*, *S. enterica* Typhi, and *S. flexneri* in meat and seafood  
269 samples reported overall detection rates of 56.0-65.3% for microplate immunocapture combined  
270 with selective plating and rates of 62.7-69.3% for microplate immunocapture combined with  
271 PCR (Fakruddin et al., 2017). These rates are based on the combined detections across all  
272 inoculation levels ( $10^1$  CFU/g to  $10^5$  CFU/g) for each pathogen tested. Similar to the current  
273 study, Yang, Qu, Wimbrow, Jiang, and Sun (2007) reported detection of *L. monocytogenes* in  
274 milk samples at the lowest inoculation level tested ( $10^2$  CFU/0.5 mL) when nanoparticle-based  
275 immunomagnetic separation was combined with real-time PCR.

276 As shown in Table 2, the use of microplate immunocapture combined with selective  
277 plating or real-time PCR allowed for positive detections in 100% of replicates tested at  
278 inoculation levels of  $10^4$  to  $10^6$  CFU/25 mL. At both the  $10^0$  and  $10^2$  CFU/25 mL levels, one of  
279 the three replicates of *L. monocytogenes* T1 + T4 was negative with PCR, however all other  
280 triplicate runs maintained 100% positive results. By comparison, a previous study utilizing  
281 immunomagnetic separation combined with PCR allowed for detection of 5 CFU/mL *L.*  
282 *monocytogenes* in 50% of milk samples and detection of 10 CFU/mL in 100% of milk samples  
283 (Amagliani et al., 2006). On the other hand, microplate immunocapture combined with  
284 PALCAM was unable to detect *L. monocytogenes* in any of the replicates tested at the lowest  
285 inoculation level.

286 As shown in Table 1, use of microplate immunocapture combined with selective plating  
287 can shorten the time for isolation of *L. monocytogenes* in milk to  $26 \pm 2$  h, while the use of  
288 microplate immunocapture combined with real-time PCR can reduce the time to detection to 4.0  
289 h. It is possible that the sensitivity of the method could be improved by increasing the number of



290 fill cycles or by combining the immunocapture assay with a short pre-enrichment period, while  
291 still allowing for a significantly shorter detection time than conventional methods.

### 292 3.3 Microplate immunocapture with queso fresco samples

293 Similar to the decrease in sensitivity observed for selective plating when moving from  
294 TSBYE to skim milk samples, the results with queso fresco cheese showed decreased sensitivity  
295 as compared to those with skim milk. When the optimized microplate immunocapture  
296 parameters for skim milk were applied to queso fresco, detection of *L. monocytogenes* with  
297 selective plating was only possible at the highest inoculation level ( $10^6$  CFU/25 g). Therefore,  
298 further optimization was carried out with queso fresco samples to improve the sensitivity of the  
299 method. The optimized conditions allowed for detection of *L. monocytogenes* with selective  
300 plating starting at an inoculation level of  $10^4$  CFU/25 g (Table 1).

301 Table 2 shows the results of triplicate testing of queso fresco cheese samples using the  
302 optimized conditions with *L. monocytogenes* T1, T4, and the mixed T1 + T4 culture. All positive  
303 and negative control results were as expected. Overall, these conditions allowed for detection of  
304 the strains (individually or mixed) at a level of  $10^4$  CFU/25 g when combined with selective  
305 plating on PALCAM and at a level of  $10^0$  CFU/mL when using real-time PCR. As with the milk  
306 samples, microplate immunocapture combined with real-time PCR showed a higher overall  
307 detection rate, with 35/36 detections (97.2%) across all inoculation levels, as compared to 18/36  
308 detections (50.0%) with PALCAM. The differences in detection rates occurred at the lowest  
309 inoculation levels ( $10^0$  CFU/25 g and  $10^2$  CFU/25 g), with 17/18 detections (94.4%) for real-time  
310 PCR and 0/18 detections (0%) for PALCAM. In contrast, the conventional culture method  
311 showed 100% positive detection across all inoculation levels.

312 The overall detection rates for microplate immunocapture combined with real-time PCR  
313 for the cheese samples were higher than those reported by Fakruddin et al. (2017) for *V.*  
314 *cholerae*, *S. enterica* Typhi, and *S. flexneri* in meat and seafood samples (62.7-69.3%) inoculated  
315 at levels of  $10^1$  CFU/g to  $10^5$  CFU/g. However, the rate of detection determined in the current  
316 study (50%) using microplate immunocapture combined with selective plating for the cheese  
317 samples was slightly lower than the rates reported by Fakruddin et al. (2017) for meat and  
318 seafood samples (56.0-65.3%). Similar to the results obtained for microplate immunocapture  
319 combined with real-time PCR in the current study, Mao et al. (2016) reported detection of *L.*  
320 *monocytogenes* in lettuce at the lowest inoculation level tested ( $10^1$  CFU/g) using a combination  
321 of immunomagnetic separation and multiplex PCR. Likewise, Duodu, Mehmeti, Holst-Jensen,  
322 and Loncarevic (2009) used a combination of filtration, immunomagnetic separation, and real-  
323 time PCR to detect *L. monocytogenes* in smoked salmon at levels of  $10^1$  CFU/g.

324 As shown in Table 2, the use of microplate immunocapture combined with selective  
325 plating or real-time PCR allowed for positive detections in 100% of replicates tested at  
326 inoculation levels of  $10^4$  to  $10^6$  CFU/25 g. One of the three replicates of the T1+T4 mixed culture  
327 at the  $10^0$  CFU/25 g inoculation level was not detected by real-time PCR, but all other samples  
328 and replicates were detected by this method. Overall, the sensitivity in detecting *L.*  
329 *monocytogenes* decreases when moving from broth to skim milk and then to cheese for detection  
330 with selective plating but not for real-time PCR.

331 As shown in Table 1, use of microplate immunocapture combined with selective plating  
332 can reduce the time for isolation of *L. monocytogenes* in cheese samples to  $27 \pm 2$  h, while use of  
333 microplate immunocapture combined with real-time PCR can allow for detection of positive

334 samples within 4.5 h. As with the milk samples, the sensitivity of the method may be improved  
335 by increasing the number of fill cycles or by adding a short pre-enrichment period.

### 336 *3.4 Mathematical explanation of immunocapture results*

337 Microplate immunocapture coupled with real-time PCR or selective plating was capable  
338 of detecting *L. monocytogenes* isolates in 100% of food samples inoculated at  $10^4$  and  $10^6$   
339 CFU/25 g. However, these methods did not perform as well as conventional culture for the  
340 detection of *L. monocytogenes* at lower inoculation levels ( $10^0$  and  $10^2$  CFU/25 g). At these  
341 levels, microplate immunocapture showed a detection rate of 91.7% when coupled with real-time  
342 PCR and 22.2% when coupled with selective plating on PALCAM. In comparison, the  
343 conventional culture method showed consistent levels of sensitivity when moving from skim  
344 milk to cheese and had a 100% detection rate across all inoculation levels. These results are in  
345 agreement with the limit of detection reported in the BAM for *L. monocytogenes*, at <1 CFU per  
346 analytical unit (Hitchens et al., 2016).

347 Microplate immunocapture combined with selective plating allowed for detection of *L.*  
348 *monocytogenes* in cheese samples down to levels of  $10^4$  CFU/25 g. This detection limit can be  
349 explained mathematically, even when not considering incubation time following inoculation or  
350 hold times in the microplate. Inoculation started at 10,000 cells ( $10^4$  CFU/25 g) and 225 mL of  
351 TSBYE was added, resulting in a concentration of 10,000 cells in 250 mL. This equates to 40  
352 cells for every mL (40 CFU/mL). Considering that 4 fill cycles were used with the cheese  
353 samples (total volume of 6.4 mL), it is likely that detection would be possible with selective  
354 plating.

355 Detection of *L. monocytogenes* was possible in the majority (89%) of milk samples  
356 inoculated at  $10^2$  CFU/25 g. Theoretically, after the addition of 225 mL TSBYE, this inoculation

357 level should have contained 100 cells in 250 mL (0.4 CFU/mL). The use of 4 fill cycles at 1.6  
358 mL each would have resulted in exposure of the microplate wells to 2.56 cells. However, this  
359 does not take into account the 2 h sample incubation following inoculation or the microplate hold  
360 times in TSBYE broth. The hold times for skim milk were 30 min for a total of 2 h after all 4 fill  
361 cycles were completed. During this time, the bacteria would have likely continued to grow both  
362 within the microplate wells and in the bag containing the inoculated sample. Given the  
363 generation time for *L. monocytogenes* is approximately 1-2 h in growth medium or skim milk at  
364 room temperature, the final concentration of cells in the sample could have reached 1.6-6.4  
365 CFU/mL (Kato, 1989; Petran & Zottola, 1989; Rosenow & Marth, 1987). The cheese samples  
366 were not capable of positive results at this level most likely due to food matrix interference. Even  
367 considering hold times and multiple fill cycles, small particles of cheese were seen in the  
368 microplate and were unavoidable, making capturing such low concentrations of *L.*  
369 *monocytogenes* cells difficult.

370         Microplate immunocapture combined with real-time PCR showed detection of *L.*  
371 *monocytogenes* down to  $10^0$  CFU/25 g in food samples. Theoretically, after the addition of 225  
372 mL TSBYE, this inoculation level would be expected to contain 1 cell in 250 mL (0.004  
373 CFU/mL). The use of 4 fill cycles at 1.6 mL each would have resulted in exposure of the  
374 microplate wells to 0.026 cells. However, this does not take into account the 2 h incubation  
375 following inoculation or the hold times in TSBYE broth. Hold times were 30 min for skim milk  
376 and 45 min for cheese, resulting in a total of 2-3 h after all fill cycles were completed. Based on  
377 the generation times stated above, the concentration of *L. monocytogenes* in samples could have  
378 reached  $1.6 \times 10^{-2}$  CFU/mL to  $1.3 \times 10^{-1}$  CFU/mL after the final hold time. Although these levels  
379 were not detectable with selective plating, real-time PCR does not require viable or even

380 complete cells for detection, but rather it shows the presence of specific DNA fragments from  
381 lysed cells.

382         Though non-viable cells of *L. monocytogenes* are not considered pathogenic, detecting  
383 them using microplate immunocapture coupled with real-time PCR may prove beneficial in  
384 showing the presence of *Listeria* in a statistical sample batch, which is exceptionally important in  
385 foods eaten raw. Thus, the present method may have greater potential beyond viable cell  
386 detection and is yet another data point on the validity of using microplate immunocapture along  
387 with the studies conducted by Fakruddin et al. (2017), Arbault et al. (2014), and Molloy et al.  
388 (1995).

#### 389 **4. Conclusions**

390         Overall, this study showed that recovery of *L. monocytogenes* at cell levels of  $10^0$   
391 CFU/25 g could be achieved at much higher rates in milk and cheese samples using microplate  
392 immunocapture combined with real-time PCR detection as compared to microplate  
393 immunocapture combined with selective plating. The overall recovery rates for *L.*  
394 *monocytogenes* in these matrices (milk and cheese) at cell populations of  $10^0$ ,  $10^2$ , and  $10^4$   
395 CFU/25 g using microplate immunocapture with real-time PCR detection were 88.9%, 94.4%,  
396 and 100%, respectively. Recovery using microplate immunocapture combined with selective  
397 plating was comparatively lower, at 0%, 44.4%, and 100%, respectively. The complexity of the  
398 matrix impacted *L. monocytogenes* recoveries using selective plating, with procedures becoming  
399 increasingly less effective as the food matrix became more complex. However, this trend was not  
400 observed with real-time PCR, which actually showed the greatest detection rates for the most  
401 complex matrix (cheese). Although microplate immunocapture combined with real-time PCR  
402 shows promise as a rapid means for concentrating and detecting *L. monocytogenes*, the recovery

403 rate at low initial cell populations was not equivalent to that obtained with the conventional  
404 culture method. Therefore, future studies should investigate the incorporation of a short  
405 enrichment period and/or additional optimization of the microplate immunocapture method.  
406 Additionally, the optimized method should undergo inclusivity testing with a panel of *L.*  
407 *monocytogenes* isolates as well as testing to ensure that the presence of other *Listeria* spp. does  
408 not interfere with the ability of the assay to capture *L. monocytogenes*.

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