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

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ABSTRACT

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

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1 Media and bacterial strains

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

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

2.2 Microplate preparation

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

2.3 Optimization of microplate immunocapture

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

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

PALCAM plates were incubated for 24 ± 2 h at 37 °C. The plates were then examined for typical *L. monocytogenes* growth, consisting of grey-green colonies with accompanied blackening of the agar. Once optimal microplate immunocapture conditions were determined using the PALCAM plates, the T1+T4 mixed culture as well as individual T1 and T4 cultures were tested in triplicate using the optimized procedure (Table 1).

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

Matrix	**# of fill cycles**	**Cycle hold time (min)**	***L. monocytogenes* antibody concentration (µg/mL)**	**Plate shaker speed (RPM)**	**Total time required (h)**	
					IC + PALCAM	**IC + qPCR**
TSBYE	3	15	1	10	24.8 ± 2	3.1
Skim milk	4	30	1	10	26 ± 2	4.3
Queso fresco cheese	4	45	1	10	27 ± 2	5.3

2.4 Preparation and microplate immunocapture of food samples

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

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

After microplate immunocapture, the wells of the microplate were scraped using an inoculating loop and streaked onto PALCAM agar or transferred to sterile Safe-lock microcentrifuge tubes containing 100 µl sterile water for DNA extraction. The PALCAM plates were incubated for 24 ± 2 h at 37 °C. The plates were then examined for typical *L. monocytogenes* growth. Once optimal microplate immunocapture conditions were determined using PALCAM plates, the milk and cheese samples were inoculated with the T1 + T4 mixed culture as well as individual T1 and T4 cultures and tested in triplicate using the optimized protocol (Table 1). Each inoculated sample was also enriched in buffered *Listeria* enrichment broth (BLEB) and plated in triplicate on PALCAM agar using the conventional culture method 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 10^0 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

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

Table 2 shows the results of triplicate testing of broth samples using the optimized conditions with *L. monocytogenes* T1, T4, and the mixed T1 + T4 culture. Overall, these conditions allowed for detection of the strains (individually or mixed) at a level of 10^0 CFU/mL when combined with selective plating on PALCAM or detection with real-time PCR. The results for all positive and negative controls were as expected. Microplate immunocapture combined with selective plating showed a slightly higher overall detection rate, with 35/36 detections (97.2%) on PALCAM across all inoculation levels compared to 33/36 detections (91.7%) with real-time PCR. Differences in the results occurred only at the lowest inoculation level (10^0 CFU/mL), with 8/9 detections (88.9%) on PALCAM and 6/9 detections (66.7%) with real-time PCR. Microplate immunocapture combined with selective plating or real-time PCR allowed for positive detections in 100% of replicates tested at inoculation levels of 10^2 to 10^6 CFU/mL. Real-time PCR was less consistent in detecting *L. monocytogenes* at the lowest inoculation level (10^0 CFU/mL), with detection in only 2 of the 3 replicates for the individual and mixed cultures. On 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	***L. monocytogenes* type**	**Rate of detection (no. positive samples/total no. samples)**											
		Broth (CFU/mL)				Milk (CFU/25 mL)				Cheese (CFU/25 g)			
		10^0	10^2	10^4	10^6	10^0	10^2	10^4	10^6	10^0	10^2	10^4	10^6
IC + PALCAM	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
IC + qPCR	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
Conventional culture	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

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

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

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

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

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

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

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

3.3 Microplate immunocapture with queso fresco samples

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

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

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

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

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

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

3.4 Mathematical explanation of immunocapture results

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

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

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

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

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

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

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

4. Conclusions

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

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

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