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Detection by real-time PCR and conventional culture of *Salmonella* Typhimurium and *Listeria monocytogenes* adhered to stainless steel surfaces under dry conditions

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ABSTRACT

This study evaluated the capacity of real-time PCR and conventional culture methods to detect *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* adhered to stainless steel surfaces used as food contact surfaces. The adhesion of the microorganisms to the surfaces was performed under dry conditions to represent the stress to which these pathogens can be subjected in the food processing environment. The samples were analyzed with various pre-enrichment times: *S.* Typhymurium (0, 6, and 18 h) and *L. monocytogenes* (0, 6, and 25 h) and with procedures concentrating or not concentrating the samples after the pre-enrichments. The results showed that real-time PCR obtained increased capacity than the conventional method to detect a low number of both pathogens, and real-time PCR even detected samples without pre-enrichment. However, pre-enrichment is recommended to avoid the detection of false positives from dead cells during adhesion and to ensure the absence of false negatives due to low initial concentrations. The concentration of the adhering bacteria increased the frequency in the detection of positive results for *S.* Typhimurium, but this effect was not observed in the case of *L. monocytogenes*.

1. Introduction

The capacity of pathogenic bacteria to adhere to surfaces represents a potential risk because it may lead to bacterial transfer to food or subsequent adhesion and biofilm formation on surfaces that are more resistant to disinfection than free-living bacteria (Bae et al., 2012; González-Rivas et al., 2018). Even when the bacterial adhesion to surfaces is followed by adverse conditions for their growth, such as dryness, they can survive being protected under a biofilm structure, and therefore, be a source of contamination (Fuster-Valls et al., 2008; Lang et al., 2004). Previous works have indicated the relationship between the survival of pathogens on surfaces and cross-contamination of food in foodborne outbreaks (Kusumaningrum et al., 2003; Muhterem-Uyar et al., 2015). These outbreaks are associated with sanitation protocol failure, poor hygiene, and other environmental factors. In the food industry, stainless steel is widely used as food contact surfaces for its ease of cleaning. However, these surfaces also tend to become contaminated when hygienic conditions are not adequate, causing bacteria to persist on them (Truelstrup Hansen & Vogel, 2011).

Salmonella spp. and *Listeria monocytogenes* are two pathogens that can adhere to food contact materials and are involved in outbreaks associated with the ingestion of contaminated food. *Salmonella* spp. can survive in dry processing environments (Margas et al., 2014). It has been reported that this pathogen can survive on surfaces under dry conditions, and survival can even be prolonged at lower temperatures (Iibuchi et al., 2010). In the case of food production lines, when these objects are contaminated with *Salmonella* spp., this pathogen can remain on equipment and facility surfaces (Gounadaki et al., 2008). In the case of *L. monocytogenes*, it is a ubiquitous pathogen in the environment and resistant to diverse environmental conditions, representing a significant problem in the food industry because it causes numerous outbreaks throughout the world (Lepe, 2020; Mazaheri et al., 2021). It has been shown that *L. monocytogenes* can be transferred from food to surfaces and vice versa, possessing a substantial ability to survive desiccation, adhere to surfaces such as stainless steel, and form biofilms (Hingston et al., 2013; Truelstrup Hansen & Vogel, 2011).

Microbiological detection in food production and preparation environments guarantee the commercialization of safe food for consumption

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(Ríos-Castillo et al., 2021). Given that currently in the food industry, food production can be on a large scale with short times between production, an effective and rapid detection of bacterial pathogens on food contact surfaces and other surfaces involved in the production is necessary in order to take immediate hygienic and sanitary measures (Margas et al., 2014). The presence of *Salmonella* spp. and *L. monocytogenes* is based on standard microbiological culture methods that include pre-enrichment, selective enrichment, isolation, and microbiological characterization, all of which take several days. In this context, fast and accurate methods to identify pathogenic bacteria are of significant value in the food industry to shorten the analysis time. Real-time PCR is considered a fast and sensitive molecular method to detect and quantify bacterial pathogens. As in the case of conventional methods, an enrichment treatment can be combined with PCR to enhance assay sensitivity by ensuring the presence of viable pathogens because they often occur in very low numbers (Ferretti et al., 2001; Park et al., 2014). Although real-time PCR has been widely used for detecting pathogens in food, its effectiveness in determining their presence on surfaces is not particularly accurate. The objective of this study was to determine the capacity to detect *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* adhered to stainless steel surfaces under dry conditions by real-time PCR, comparing it with the conventional methodology recommended. The study also considered various pre-enrichment times and the concentration of the samples obtained from the surfaces.

2. Materials and methods

2.1. Bacterial strains and test surfaces

Salmonella enterica serovar Typhimurium (*S*. Typhimurium) (CCUG 29478) and *Listeria monocytogenes* (*L. monocytogenes*) (CCUG 15526) were the standard bacterial strains used for this study (Culture Collection of the University of Göteborg, Sweden). The strains, conserved on cryoballs at −20 °C, were resuspended in tryptone soy broth with 6 g/L of yeast extract (TSBYE; Becton Dickinson and Co., Le Pont De Claix, France) and incubated at 37 °C for 24 h. Afterwards, they were cultured on tryptone soy agar (TSA; Becton Dickinson and Co.) and incubated at 37 °C for 24 h. Once the incubation time had elapsed, isolated colonies of each microorganism were transferred to 10 mL of TSBYE and incubated at 37 °C for 18–24 h, obtaining bacterial cultures in the stationary phase. The cultures were centrifuged at 9800×*g* for 10 min and washed twice using saline solution (8.5 g/L NaCl). The resulting pellets were resuspended in 8.5 mL of saline-tryptone solution (8.5 g/L NaCl, 1.0 g/L tryptone, pH 7.0 ± 0.2) to obtain bacterial suspensions with an approximate concentration of 10^8 colony-forming units per milliliter (CFU/mL). The concentration of the bacterial suspensions was quantified by inoculating 20 μL in the center of stainless steel disc surfaces that were subsequently analyzed by direct epifluorescence microscopy (DEM, Olympus BX51/BX52; Olympus, Tokyo, Japan). A Live/Dead® BacLight™ Kit (L-13152, Molecular Probes, Inc., Oregon, USA) was used for microscopic viable cell counting. The kit contains two fluorescent nucleic acid-binding stains: SYTO 9 and propidium iodide. SYTO 9 penetrates the cells with intact and damaged cell membranes and dyes them green. On the other hand, propidium iodide penetrates only cells with damaged cell membranes, reducing the SYTO 9, and stains non-viable cells red. The kit was used by adding 20 μL of the stain to each disc surface with the corresponding cell concentrations. The surfaces were then left in the dark at room temperature for 15 min to allow the stains to penetrate the bacterial cells. Ten microscopic images were acquired to determine the viable cells in green color using the software analySIS Auto 3.2 (Soft Imaging System GmbH, Münster, Germany).

2.2. Characteristics of the surfaces and inoculation

The test surfaces were stainless steel discs type 304 grade 2B finish, 2 cm in diameter, and 1 mm thick. Surfaces were cleaned and sterilized at 121 °C for 15 min before their use in the tests. Once the concentration of the bacterial suspensions was determined by microscopy, the surfaces were inoculated in quadruple with 10, 100, and 1000 cells in volumes less than 50 μL. Three surfaces of each concentration were used for the study to obtain levels of contamination of 30, 300, and 3000 CFU/10 cm^2 and one was analyzed by microscopy, as it is described below in Section 2.3. An inoculum control for each inoculated concentration was carried out ten times in TSA plates.

2.3. Dry conditions

Immediately after the inoculation of surfaces, they were dried in a laminar flow cabinet at 37 °C for a time not exceeding 1 h to facilitate microbial adherence to the test surfaces. After the drying time elapsed, one of the four disc surfaces per concentration group was randomly chosen and analyzed by microscopy (Olympus BX51/BX52) using the Live/Dead® BacLight™ kit. A pre-enrichment step was applied to the three remaining inoculated disc surfaces.

2.4. Pre-enrichment

The remaining three disc surfaces of each concentration group were placed in parallel in flasks and in 50 mL conical tubes with 10 mL of pre-enrichment broth: TSBYE-T80 (TSBYE with 1 g/L of Tween 80) for *S.* Typhimurium and DFraser-T80 (Demi-Fraser Broth with 1 g/L of Tween 80) for *L. monocytogenes*. The flasks and tubes were allowed to rest for 5 min and then mixed by vortex for 1 min at maximum speed. The flasks and tubes with the discs were then left to rest for different pre-enrichment times: 0 h (immediately after mixing by vortex), 6 h, and 18 h for *S.* Typhimurium (incubated at 37 °C), and 0 h, 6 h, and 25 h for *L. monocytogenes* (incubated at 30 °C). Once the pre-enrichment times had elapsed, the flasks and tubes were mixed by vortex for 1 min, and the contents were recovered by either without concentrating or concentrating the microorganisms. The procedure followed to recover the target pathogens without concentrating the microorganisms consisted of taking the aliquots directly from the flasks after being mixed. The recovery procedure, concentrating the microorganisms, was performed from the tubes removing the discs previously. Then, the tubes were centrifuged at 12,000×*g* for 5 min. The supernatants were discarded, and the pellets were resuspended in 3 mL TSBYE-T80 broth for *S.* Typhimurium and 3.5 mL DFraser-T80 broth for *L. monocytogenes*. The discs from the tubes were recovered to determine the efficiency of bacterial detachment from the surfaces. For this stage, surfaces were washed with 10 mL of phosphate-buffered saline (PBS; 8.0 g/L sodium chloride, 0.2 g/L sodium potassium, 1.15 g/L bisodium phosphate, 0.2 g/L monopotassium phosphate; pH 7.3). Once washed, they were transferred to Petri dishes and cultured with TSA with 6 g/L yeast extract (TSAYE; Becton Dickinson and Co) at 37 °C for 24–48 h.

2.5. Count and detection of microorganisms

2.5.1. Plate count

To determine the total viable cell counts, a 1 mL aliquot of TSBYE-T80 and DFraser-T80 broths was cultured in duplicate in TSAYE and incubated at 37 °C for 24–48 h.

2.5.2. Conventional culture

The cultures for detecting and enumerating microorganisms were prepared according to ISO 6579-1:2017/AMD 1:2020 (ISO, 2020) for *S.* Typhimurium and ISO 11290-1:2017 (ISO, 2017) for *L. monocytogenes*. The procedure for *S.* Typhimurium involved transferring 0.1 mL aliquot of TSBYE-T80 to 10 mL of Rappaport-Vassiliadis selective enrichment broth (bioMérieux, Marcy-l'Étoile, France) and incubating at 42 °C for 24 h. Subsequently, loops of the enrichment broth were streaked on chromID *Salmonella* agar plates (bioMérieux, France) and incubated at

37 °C for 24–48 h. In the case of *L. monocytogenes*, 0.1 mL aliquot of DFraser-T80 was transferred to 10 mL of Fraser selective enrichment broth (bioMérieux, France) and incubated at 37 °C for 48 h. Loops of DFraser-T80 and Fraser broths were streaked onto PALCAM (bioMérieux, France) and ALOA (AES Laboratoire, Combourg, France) agar plates and incubated at 37 °C for 24–48 h.

2.5.3. Real-time PCR analysis

Real-time PCR analysis was performed with iQ-Check™ *Salmonella* and iQ-Check™ *L. monocytogenes* kits (Bio-Rad Laboratories, Inc; Hercules, CA, USA) according to the manufacturer's instructions. The procedure consisted of using aliquots of 1.0 mL of TSBYE-T80 for *S.* Typhimurium and 1.5 mL of DFraser-T80 for *L. monocytogenes*. Aliquots were transferred to Eppendorf tubes and centrifuged at 11,700×*g* for 5 min. Subsequently, the supernatants were removed, and the pellets were mixed with the lysis reagents (supplied with the iQ-Check kits): 200 μL for *S.* Typhimurium and 250 μL for *L. monocytogenes*. The obtained mixtures were vortexed for 30 s at full speed. Then, the tubes were placed in a water bath at 100 °C for 15 min, cooled at room temperature for 5 min, and again vortexed at maximum speed for 15 s. The tubes were again centrifuged at 11,700×*g* for 5 min. Then, the DNA was amplified by adding 45 μL of PCR mix that contained the amplification solutions and fluorescent probes to 5 μL of the supernatants on well plates. Thereafter, the well plates were sealed with optical caps and introduced into the thermocycler Chromo4™ RT-PCR Detection (Bio-Rad Laboratories, Inc). The thermocycler program steps were: 1st at 50 °C for 2 min, 2nd at 95 °C for 10 min, and 3rd (repeated 50 times) at 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 30 s. The obtained threshold cycle (Ct) values were analyzed using Opticon Monitor software v 3.1 (Bio-Rad Laboratories, Inc).

2.6. Statistical analysis

The tests were carried out in quadruple and repeated three times on different days (n = 12). Each test to compare the frequency of detection by the conventional method and real-time PCR was performed for each microorganism, recovery method, contamination levels, and pre-enrichment times. Viable cell counts in TSAYE were expressed in decimal logarithmic units (log). The results obtained by TSAYE by the microorganism recovery method without a concentration of the samples were compared using the Analysis of Variance and the subsequent post hoc Student-Newman-Keuls (SNK) test with a significance level of $\alpha = 0.05$. Ct values were presented as mean ± standard deviations. The relationship between Ct values and the viable cell count obtained by TSAYE was compared by regression analysis. Statistical analyses were performed using the SAS statistical program (version 9.1; SAS Institute, Inc., USA).

3. Results and discussion

3.1. Detection of surface-adhered S. Typhimurium

The results for *S.* Typhimurium adhered to surfaces in dry conditions are shown in Table 1. When the contamination level was 30 CFU/10 cm^2, without pre-enrichment (0 h) and without concentrating or concentrating the samples, *S.* Typhimurium was not detected by the conventional ISO method (0/6), whereas real-time PCR detected 5 of 6 samples (5/6). At this same level of contamination, with a pre-enrichment time of 6 h, real-time PCR detected 2/6 without concentrating the samples, and the detection was total (6/6) after concentrating the samples. With 18 h of pre-enrichment, the detection was 4/6 by the conventional method and total (6/6) by real-time PCR using both recovery procedures. When the level of contamination was increased to 300 and 3000 CFU/10 cm^2, the detection of the conventional method was only total with the pre-enrichment times of 6 and 18 h using both recovery procedures. In the case of real-time PCR, the detection was total with all the pre-enrichment times (0, 6, and 18 h) for both procedures. According to the results, the detection limit without concentrating the samples for the real-time PCR was 300 CFU/10 cm^2 without pre-enrichment, and it was reduced to 30 CFU/10 cm^2 with 18 h of pre-enrichment. In the case of the procedure concentrating the samples, the real-time PCR detection limit was 300 CFU/10 cm^2 without pre-enrichment and 30 CFU/10 cm^2 with 6 h pre-enrichment. Although no differences were observed between the detection limits without pre-enrichment with both recovery procedures, it was possible to reduce the time to 6 h for detecting 30 CFU/10 cm^2 with the procedure concentrating the samples. Our results indicate that the detection of low *S.* Typhimurium contamination adhered to surfaces was more sensitive

Table 1
Comparison of conventional culture method (ISO 6579-1) and real-time PCR for detection of *S.* Typhimurium adhered under dry conditions to stainless steel with different levels of contamination, pre-enrichment times, and recovery methods.

Recovery method	Contamination level (CFU/10 cm^2)[a]	Pre-enrichment time (h)	TSAYE (log CFU/mL)[b]	Ct (Threshold cycle)[c]	Detection frequency[d]	
					ISO 6579-1	Real-time PCR
Without concentration	30	0	$0.05^{c} \pm 0.12$	38.50 ± 1.31	0/6	5/6
		6	$1.08^{b} \pm 1.05$	37.68 ± 0.57	3/6	2/6
		18	$8.85^{a} \pm 0.46$	15.21 ± 0.73	4/6	6/6
	300	0	$0.78^{c} \pm 0.31$	36.34 ± 0.66	2/6	6/6
		6	$3.68^{b} \pm 0.47$	30.73 ± 1.94	6/6	6/6
		18	$8.97^{a} \pm 0.24$	15.51 ± 0.49	6/6	6/6
	3000	0	$1.56^{c} \pm 0.30$	33.30 ± 0.36	4/6	6/6
		6	$4.37^{b} \pm 0.44$	28.25 ± 0.83	6/6	6/6
		18	$9.03^{a} \pm 0.20$	14.79 ± 0.69	6/6	6/6
Concentration	30	0		36.78 ± 2.29	0/6	5/6
		6		36.25 ± 1.56	6/6	6/6
		18		13.63 ± 0.45	4/6	6/6
	300	0		34.01 ± 2.96	3/6	6/6
		6		31.18 ± 1.13	6/6	6/6
		18		13.96 ± 0.33	6/6	6/6
	3000	0		32.45 ± 0.45	4/6	6/6
		6		29.91 ± 1.04	6/6	6/6
		18		13.86 ± 0.07	6/6	6/6

[a–c] Mean values with different lowercase letters are statistically different ($p < 0.05$).
[a] Total contamination level corresponding to three stainless steel discs.
[b] Mean values of cell count ± standard deviation; n = 6.
[c] Mean values of Ct ± standard deviation; n = 6.
[d] Number of positive samples/number of samples analyzed.

using real-time PCR than the conventional method. In addition, the results of the conventional culture method were inconsistently positive in samples without pre-enrichment for all levels of contamination, and a minimum enrichment period of 6 h was necessary for all samples with 300 CFU/10 cm^2 to give positive results. This observation agrees with other works, which described that PCR has the same or increased sensitivity compared to the culture method when tests were performed from environmental samples and food processing facilities (Hyeon et al., 2019; Nadin-Davis et al., 2019).

The *S.* Typhimurium count in TSAYE in the samples without concentration showed that even though this microorganism was subjected to stress through desiccation on the surfaces prior to pre-enrichment, it recovered when the level of contamination and the times of pre-enrichment increased ($p < 0.05$). Thus, the contamination level of 3000 CFU/10 cm^2 showed the highest counts in TSAYE: 1.56 log CFU/mL (0 h), 4.37 log CFU/mL (6 h), and 9.03 log CFU/mL (18 h). A high correlation was also observed between the Ct values obtained by real-time PCR and the viable cell counts in TSAYE ($R^2 = 0.9718$; $p < 0.0001$) (Fig. 1). The increase in *S.* Typhimurium count, while the Ct values were decreasing, indicated that the recovery with the pre-enrichment times was adequate because the Ct values were inversely proportional to higher amounts of target DNA in the sample (Pérez et al., 2013).

The infective dose of *Salmonella* spp. to cause illness associated with food consumption is low. Salmonellosis has been reported to result from less than 10 cells/100 g in naturally contaminated cheese (Ratnam & March 1986). Surfaces are one of the most important sources from which bacterial pathogens contaminate food; hence, surface detection becomes important. The detection limits previously reported for *Salmonella* spp. by real-time PCR, although not on surfaces but on different foods and environmental samples, are comparable to our results. Malorny et al. (2004) studied *Salmonella* Enteritidis and detected <3 CFU/50 mL carcass rinse from whole chicken or 10 mL whole eggs with real-time PCR, preceded by a pre-enrichment step (18–24 h) in BPW, with the sensitivity and specificity of this method being 100% equivalent to the traditional culture-based detection method. In addition (Hyeon et al., 2019), noted that the detection limits of *Salmonella* spp. carried out by a combination of immunomagnetic separation, multiple displacement amplification, and real-time PCR with 0, 4 to 6, and 8 h enrichment with BPW were 10, 1, and 0.1 CFU/g, respectively.

As in our study, other works carried out on food and environmental samples have shown that although rapid methods are highly efficient in detecting *Salmonella* spp., pre-enrichment increases efficacy. This improvement particularly occurs when bacteria such as *Salmonella* spp. grow in a low concentration or in a sub-lethal state (Park et al., 2014; Taskila et al., 2012). Lin et al. (2004) observed that through a preculture step with selenite cystine broth for 8 h previous to the PCR assay, *Salmonella* spp. serovars from milk and raw chicken meat could be detected at concentrations as low as 1–9 CFU/g. Ferretti et al. (2001) demonstrated that PCR with 6 h non-selective enrichment using BPW detected *Salmonella* spp. in food at the level of 1 CFU/100 mL. In our study, with a concentration procedure by centrifugation after sampling, a low number of *Salmonella* spp. was detected using real-time PCR without pre-enrichment.

3.2. Detection of surface-adhered *L. monocytogenes*

The results of *L. monocytogenes* detection adhered to surfaces in dry conditions are provided in Table 2. With a contamination level of 30 CFU/10 cm^2, the conventional ISO method did not detect *L. monocytogenes* when the procedure was used without concentration of samples and with any of the pre-enrichments (0, 6, and 25 h). However, at 0 and 6 h of pre-enrichment and concentrating the samples, *L. monocytogenes* was detected for both in 1/6 samples (1/6). When the samples were evaluated by real-time PCR at this level of contamination, *L. monocytogenes* was detected at 0 (5/6), 6 (5/6), and 25 h pre-enrichment (4/6) without concentrating the samples; detection using real-time PCR was total (6/6) at 0 and 6 h when concentrating the samples. With a contamination level of 300 CFU/10 cm^2, the conventional method partially detected *L. monocytogenes* at 6 (1/6) and 25 h (3/6) without concentrating the samples and 2/6 at 25 h when concentrating the samples. In the case of detection by real-time PCR, the detection was complete with all pre-enrichment times, whether or not the samples were concentrated. When the level of contamination was increased to 3000 CFU/10 cm^2, the results by the conventional method showed that the detection was not total after 25 h pre-enrichment (5/6) without concentrating the samples. By concentrating the samples, *L. monocytogenes* was partially detected at 0 (2/6), 6 (1/6), and 25 h pre-

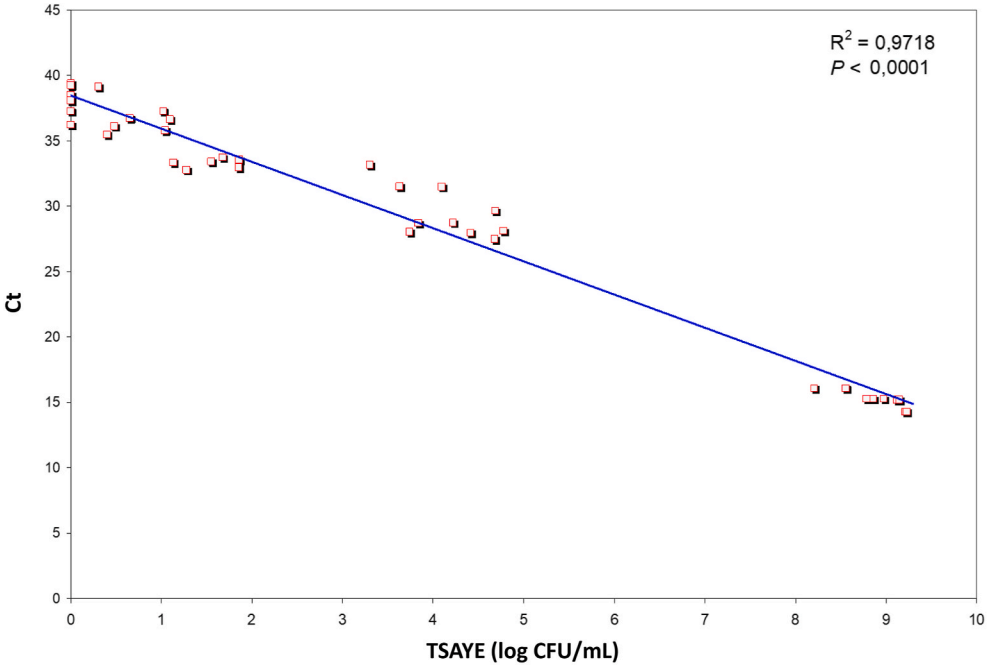

Fig. 1. Relationship between counts in TSAYE and Ct values obtained for *S.* Typhimurium adhered to stainless steel surfaces.

Table 2
Comparison of conventional culture method (ISO 11290-1) and real-time PCR of *L. monocytogenes* detection adhered under dry conditions to stainless steel surfaces with different levels of contamination, pre-enrichment times, and recovery methods.

Recovery method	Contamination level (CFU/10 cm^2) [a]	Pre-enrichment time (h)	TSAYE (log CFU/mL) [b]	Ct (Threshold cycle) [c]	Detection frequency [d]	
					ISO 11290-1	Real-time PCR
Without concentration	30	0	< LD	40.27 ± 1.34	0/6	5/6
		6	< LD	39.99 ± 0.61	0/6	5/6
		25	< LD	40.06 ± 0.93	0/6	4/6
	300	0	0.23^b ± 0.36	36.24 ± 1.56	0/6	6/6
		6	0.39^b ± 0.61	36.96 ± 0.54	1/6	6/6
		25	2.48^a ± 0.2.8	31.42 ± 6.61	3/6	6/6
	3000	0	0.30^b ± 0.27	33.52 ± 0.66	0/6	6/6
		6	0.23^b ± 0.27	33.82 ± 1.07	0/6	6/6
		25	3.45^a ± 2.03	24.58 ± 1.88	5/6	6/6
Concentration	30	0		37.48 ± 1.91	1/6	6/6
		6		39.03 ± 1.47	1/6	6/6
		25		38.80 ± 0.78	0/6	5/6
	300	0		36.47 ± 0.79	0/6	6/6
		6		36.18 ± 0.91	0/6	6/6
		25		32.02 ± 6.03	2/6	6/6
	3000	0		31.89 ± 1.91	2/6	6/6
		6		33.27 ± 0.95	1/6	5/6
		25		31.69 ± 6.85	4/6	5/6

< LD: Lower than detection limits.
[a–b] Mean values with different lowercase letters are statistically different ($p < 0.05$).
[a] Total contamination level corresponding to three stainless steel discs.
[b] Mean values of cell count ± standard deviation; n = 6.
[c] Mean values of Ct ± standard deviation; n = 6.
[d] Number of positive samples/number of samples analyzed.

enrichment (4/6). Using real-time PCR, the detection was total with the three pre-enrichment times without concentrating the samples, and when concentrating the samples, the detection was total (6/6) at 0 h and 5/6 at 6 and 25 h.

According to the results, the real-time PCR detection limit for *L. monocytogenes* with the procedure without concentrating the samples and without pre-enrichment was 300 CFU/10 cm^2. When concentrating the samples, the detection limit was not established because inconsistent positive results were obtained. Furthermore, the results showed that the detection of *L. monocytogenes* by real-time PCR was more sensitive than by the conventional method (Table 2). These findings agree with Gattuso et al. (2014), who observed that real-time PCR showed higher performance in detecting *L. monocytogenes* compared to the standard method using reduced volumes of half-Fraser broth. The inconsistency in the positive results by the conventional method can be explained because the pre-enrichment broth (DFraser-T80) could have suppressed the recovery of cells that were sublethally injured during drying. The finding is also explained because when applying the sample without concentration procedure, the viable cell counts in TSAYE did not show differences at 0 and 6 h ($p > 0.05$) with contamination levels of 300 and 3000 CFU/10 cm^2, and the count only increased with a pre-enrichment time of 25 h. Besides, the real-time PCR results showed a low correlation

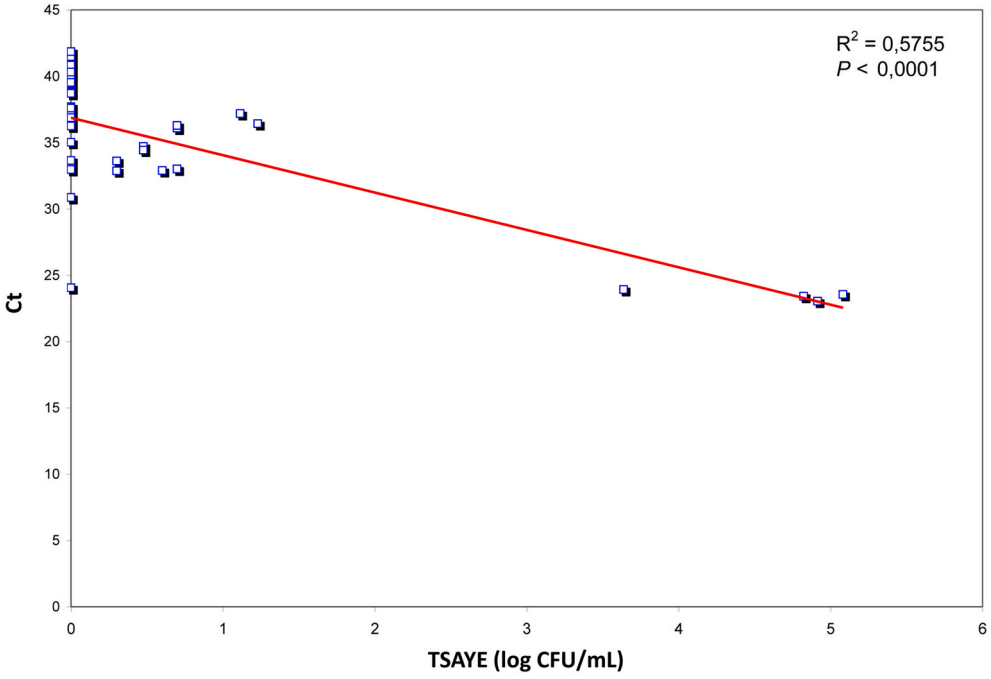

Fig. 2. Relationship between counts in TSAYE and Ct values obtained for *L. monocytogenes* adhered to stainless steel surfaces.

between Ct values and viable cell counts in TSAYE ($R^2 = 0.5755$, $p < 0.0001$). In this sense, the samples using 0 and 6 h of pre-enrichment showed a higher dispersion compared to the results obtained after 25 h, where the values were observed mainly related to the viable cell counts (Fig. 2). This observation demonstrates that the pre-enrichment medium is essential, significantly influencing the sensitivity of detection methods and increasing the probability of recovering injured cells (Nam et al., 2005).

Although the infective dose of *L. monocytogenes* is considered high (10^3 CFU), the initial contamination with low numbers of this pathogen can be potentially dangerous due to its ability to grow at low temperatures (Bhagwat, 2003; Walker et al., 1990). Hence, it is important that the detection limit of rapid methods fulfills this characteristic for practical application purposes in food processing plants. Liming and Bhagwat (2004) indicated a detection limit of *L. monocytogenes* of 4–7 CFU/25 g of mixed salad, with 25 h enrichment time and using the iQ-Check protocol; however, the inoculated cells were not subjected to any stress process, and the samples were processed within 10 min after inoculation. Rodríguez-Lázaro et al. (2005) observed that real-time PCR with a previous procedure that involved filtration and DNA purification could detect 10 CFU/g L. *monocytogenes* from smoked salmon, showing the high accuracy of real-time PCR compared to the standard plate count method. In another study carried out in 11 laboratories, 10 CFU/25 g *L. monocytogenes* were detected in artificially contaminated cheese samples using real-time PCR with enrichment and DNA extraction in 27 h, demonstrating that this procedure was more sensitive than the ISO reference method (Gianfranceschi et al., 2014). Oravcová et al. (2007), using a two-step enrichment involving a 24-h incubation in half-Fraser broth followed by a 6-h subculture in Frasger broth, detected 1 CFU/g with real-time PCR from 61 food samples contaminated at a level of 100 CFU per 25 g. Moreover, in the same work, real-time PCR detected *L. monocytogenes* in all samples; by contrast, the conventional ISO method only detected 58 positive samples.

3.3. S. Typhimurium and L. monocytogenes detection under dry conditions

For both microorganisms, real-time PCR showed a better detection limit of *S.* Typhimurium and L. *monocytogenes* under dry conditions compared to the conventional culture method. Similar results were observed by Mafu et al. (2009), who indicated that real-time PCR was more sensitive and faster than the standard microbiological method for detecting *Salmonella* spp., *L. monocytogenes*, and *Escherichia coli* O157: H7 when at least 10^3 CFU/mL bacteria were present on surfaces of wood, polypropylene, and stainless steel using a single enrichment medium. Under natural conditions, bacteria can be found in various states of stress and with sublethal damage, whereas when grown in pure cultures under laboratory conditions, viability may not necessarily be affected and therefore, microbiological results may be overestimated. This fact was considered in the present study, applying dry conditions during bacterial adhesion to represent real growth conditions. The maximum time required for drying was 1 h at 37 °C. When the Live/Dead® kit was applied to verify the vitality of the bacteria after the drying process, it was observed that most of the cells were dead or damaged. When comparing the TSAYE counts of the microorganisms inoculated on the surfaces, before and after desiccation, a significant reduction ($p < 0.05$) slightly greater than one log unit was observed at all levels of contamination (30, 300, and 3000 cells) for *S.* Typhimurium and L. *monocytogenes* (data not shown). In addition, the efficiency of the procedure to verify the bacterial recovery adhered to the test surfaces when the samples were concentrated, performed with washes with PBS and seeding in TSAYE, did not show counts at 0 and 6 h of pre-enrichment for either microorganism. However, counts of 3 log CFU/mL at 18 h for *S.* Typhimurium and 0.47 log CFU/mL at 25 h for *L. monocytogenes* were obtained. These results could be explained due to the increase in the number of microorganisms with a longer pre-enrichment time and that washing with 10 mL of PBS could not recover. Furthermore, in our study, the time required for adhesion was not exceeding 1 h. In this sense, Lang et al. (2004) observed that *Salmonella* spp. and *L. monocytogenes* could adhere to tomato surfaces in 1 h at 20 °C under dry conditions. In another study, carried out on fruit skin, 1 h after *Salmonella* inoculation, extensive fixation structures were formed that helped to irreversibly anchor the pathogen to the surface (Mathew et al., 2018).

Salmonella spp. and *Listeria monocytogenes* can survive under various stress conditions, such as desiccation (Lang et al., 2004; Ríos-Castillo et al., 2020). The fact that both studied pathogens could remain viable on dry surfaces represents a potential recontamination risk. The capacity of *Salmonella* spp. to adhere and form biofilms at room temperature is of particular interest to the food industry. This pathogen even has the ability to survive on food contact surfaces after long periods of time under dry conditions (Gruzdev et al., 2011; Margas et al., 2014). Therefore, regular and adequate cleaning of surfaces against *Salmonella* spp. is necessary (Humphrey, 2004; Ripolles-Avila et al., 2020). In the case of *L. monocytogenes*, this microorganism can easily enter food-processing environments, adhere to surfaces, and multiply even at low temperatures, leading to an increased risk of cross-contamination from surfaces to food (Carpentier & Cerf, 2011; Muhterem-Uyar et al., 2015).

Today, standard pathogen detection methods can determine the presence of a low number of viable cells after pre- and selective enrichment steps (Chapela et al., 2015). However, when pathogens are subjected to stressful conditions, the detection methods' sensitivities can be interfered with and, in turn, be ineffective (Myint et al., 2006). The ability of real-time PCR to detect DNA from dead bacteria as false positives is considered a disadvantage (de Boer et al., 2015; Wolffs et al., 2005). However, false-positive PCR signals can also occur due to the presence of a high initial number of the pathogen and the time that elapses between bacterial death and analysis (Wolffs et al., 2005; Young et al., 2007). In this sense, if DNA degradation is rapid, it minimizes the risk of a false positive; however, when a partial release of DNA occurs or is protected by dead cells, the risk increases (Wolffs et al., 2005). Therefore, it is essential to ensure optimal enrichment to guarantee bacterial recovery and growth during PCR detection analyses (Myint et al., 2006). In addition, PCR is more sensitive than conventional methods because microorganisms can be presented in a viable but non-culturable state (VBNC), and the growth of target cells can be inhibited by the presence of other bacteria during enrichment (Patel et al., 2006). The PCR detection of cells in the VBNC state is an advantage because certain microorganisms have the capacity to repair the damage caused by environmental factors and remain in a potentially infectious VBNC state (Fakruddin et al., 2013; Jackson et al., 2009). Food processing can lead to bacterial stress and result in damaged or dead bacteria; therefore, the detection in these states indicates the previous presence or even the permanence of bacteria in a VBNC state, which is of concern (Ceuppens et al., 2014; de Boer et al., 2015). Alessandria et al. (2010) observed that real-time PCR detected more positive samples of *L. monocytogenes* than a modified ISO method in samples of cheese brines and environmental samples from a dairy processing plant, where bacteria have to respond to several stresses, such as disinfectants, starvation, and dried conditions, and therefore, these bacteria could be in a VBNC state. In our study, the detection of pathogens was carried out on clean surfaces; therefore, we consider it necessary to perform additional studies to evaluate the detection limit of these pathogens on surfaces with interfering substances that simulate the presence of food residues in the food industry.

4. Conclusions

Real-time PCR provided a better detection limit than the ISO conventional culture method of a low level of contamination of *S.* Typhymurium and L. *monocytogenes* adhered in dry conditions to stainless steel

surfaces, and it was possible to detect microorganisms by real-time PCR in samples without pre-enrichment. Pre-enrichment is recommended to avoid false positives derived from dead cells and to ensure the absence of false negatives due to a very low initial cell concentration. This study presents a recovery procedure concentrating the samples for real-time PCR analysis as an alternative to the conventional culture method for detecting a low amount of bacterial contamination. Comparing the evaluated microorganisms, the recovery by concentration of the samples after bacterial adhesion increased the frequency of positive results for *S.* Typhimurium by real-time PCR, but the same effect was not observed for *L. monocytogenes*.

CRediT authorship contribution statement

Abel Guillermo Ríos-Castillo: Data curation, Formal analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing. **Carolina Ripolles-Avila:** Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – review & editing. **José Juan Rodríguez-Jerez:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Declarations of competing interest

The authors declare no conflict of interest related to this manuscript. José Juan Rodríguez-Jerez as corresponding author and in the name of all authors.

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