A long-amplicon quantitative PCR assay with propidium monazide to enumerate viable *Listeria monocytogenes* after heat and desiccation treatments

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ABSTRACT

The objective of this study was to develop a qPCR method for specific enumeration of viable *Listeria monocytogenes* in food processing facilities and heat treated products. Primers specific for *L. monocytogenes* were designed to amplify a short (199 bp) or long (1561 bp) fragment of the listeriolysin (*hly*) gene. The short- and long-amplicon qPCR methods with and without propidium monoazide (PMA) treatment of the cells were tested for their ability to discriminate between viable (no heat) and heat-killed cells (90°C, 10 min). The PMA-qPCR methods were subsequently used to assess the survival of *L. monocytogenes* during desiccation (33% RH, 15°C) on stainless steel surfaces for ten days with and without prior biofilm formation. The long-amplicon qPCR method had a limit of quantification (LOQ) of 1.32 log CFU/reaction (efficiency 92%, $R^2=0.991$), while the LOQ for the short-amplicon qPCR method was 1.44 log CFU/reaction (efficiency 102%, $R^2=0.991$). PMA was essential for detection of viable cells, and the long-amplicon PMA-qPCR significantly ($p < 0.05$) reduced the signal from heat-killed cells compared to the short-amplicon method. *L. monocytogenes* survival during desiccation without biofilm formation was accurately enumerated with the long-amplicon PMA-qPCR method. However, when *L. monocytogenes* had formed biofilm prior to desiccation, the long-amplicon PMA-qPCR accurately measured the log fold inactivation but underestimated the number of viable cells even with use of an optimized DNA extraction method. This long-amplicon PMA-qPCR method can aid in the detection and enumeration of viable *L. monocytogenes* cells to further the understanding of its survival and persistence in food processing facilities. The developed method was demonstrated to

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35 work on both heat and desiccation treated cells and highlights the importance of amplicon
36 size in viability-qPCR.

37 **Keywords:** Amplicon length, viability, biofilm, stainless steel, PMA-qPCR

1. INTRODUCTION

The foodborne bacteria *Listeria monocytogenes* is repeatedly linked to food recalls and outbreaks of foodborne illness. *L. monocytogenes* causes the human invasive listeriosis, an infection with high case-fatality (20-30%) (Allerberger and Wagner, 2010). *L. monocytogenes* is very persistent once introduced into a food processing facility and with its ability to grow at low temperatures, contaminated ready-to-eat (RTE) food products such as sliced deli meats, soft cheeses and fresh produce are often involved in outbreaks (Cartwright et al, 2013; Todd and Notermans, 2011). There has been a steady increase in the number of listeriosis cases within EU over the last 10 years with an further increase in 2017 despite the *L. monocytogenes* content rarely exceeding the EU food safety limit (100 CFU/g) in routine surveillance of RTE foods (EFSA, 2018). A total of 2,480 confirmed cases of listeriosis and 255 related deaths (10%) were reported in the EU for 2017. Previous studies have shown that the presence of *L. monocytogenes* on food contact surfaces increases the risk of cross-contamination of foods (Keskinen et al., 2008; Midelet et al., 2006; Rodríguez and McLandsborough, 2007). Recent epidemiological evidence points to persistence of *L. monocytogenes* in food processing environments remaining the major source of RTE food contamination (EFSA BIOHAZ Panel, 2018). Therefore, rapid molecular methods, which specifically detect viable *L. monocytogenes*, remain of interest in the study and surveillance of the bacterium's survival in the food processing environment including the effect of sanitation strategies.

Compared to culture-based protocols, molecular-based techniques offer rapid detection, increased specificity and enable enumeration of viable but non-culturable cells (VBNC) (Ferrentino et al., 2015). However, DNA-based detection methods such as PCR

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may overestimate cell concentrations due to the presence of DNA from dead cells (Josephson et al., 1993). The introduction of the viability dye ethidium monoazide (EMA) and its successor propidium monoazide (PMA) was shown to reduce the signal from dead cells in a way that is not fully elucidated but thought to involve the penetration of the dyes through the leaky membranes of the dead cells followed by formation of covalent crosslinks with the intracellular DNA that then becomes unavailable for PCR amplification (Fittipaldi et al., 2012; Nocker et al., 2006). In theory, PMA cannot penetrate the membrane of 'viable' cells with intact membranes/cell walls and thus should enable accurate enumeration of viable bacteria when combined with qPCR (PMA-qPCR). Pan and Breidt (2007) showed linearity between viable *L. monocytogenes* cells enumerated using PMA-qPCR and plate counts as long as the difference between sanitizer-killed cells and viable cells was no more than 4 log CFU. However, another study with heat-killed *L. innocua* reported that the use of PMA-qPCR overestimated the number of viable cells in mixes of viable and heat-killed cells (Løvdaal et al., 2011). Same study also reported PMA-qPCR to overestimate the number of viable cells (false positive) in samples containing cells killed by heat or isopropanol. Several factors have been studied to improve the signal suppression from dead cells, including PMA concentration, incubation time, incubation temperature, light source for photoactivation, light exposure time, target gene sequence and length of amplicon (Fittipaldi et al., 2012).

Traditionally qPCR assays are based on short amplicons (75-150 bp) to ensure an optimal efficiency, short assay runs and enable the use of hydrolysis probes to increase the specificity of the method (Agilent Technologies, 2012; Nolan et al., 2013). However, early work on a viability end-point PCR assay with EMA showed that a short-amplicon

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(113 bp) assay was outperformed by a long-amplicon (894 bp) assay when used to test the viability of *L. monocytogenes* after heat treatment (Soejima et al., 2008). The usefulness of EMA may, however, be limited, as the dye is able to penetrate viable cells and hence caused an underestimation of the viable cell count of *L. monocytogenes* (Flekna et al., 2007; Nocker et al., 2006). The use of longer amplicons (899 bp and 1512 bp) in combination with the other viability dye, PMA, and qPCR was shown to be superior in suppression of the signal from heat-killed cells of *Campylobacter jejuni* compared to a 174 bp short-amplicon (Banihashemi et al., 2012). The same study also presented a long-amplicon PMA-qPCR assay for detection of heat-killed *Salmonella enterica*. Similar results were obtained for *Salmonella* in cooked ham and with heat-killed *Legionella pneumophila* in water (Ditommaso et al., 2015; Martin et al., 2013). However, to the best of our knowledge no studies have been published regarding the use of long-amplicon PMA-qPCR to detect viable *L. monocytogenes* cells in biofilms, or after desiccation or heat treatments.

The objective of this study was to develop a *L. monocytogenes* specific long-amplicon PMA-qPCR assay to allow rapid enumeration of viable *L. monocytogenes* in the food processing environment. To simulate conditions faced by the bacterium in the food processing industry the assay was tested for its ability to enumerate viable *L. monocytogenes* cells after heat treatment and desiccation on stainless steel with and without prior biofilm formation.

2. MATERIALS AND METHODS

2.1 Bacterial strain and culture conditions

Three strains of *L. monocytogenes* were used in this study. *L. monocytogenes* EGD-e (Serotype 1/2a, (Murray et al., 1926)) was used in the initial development and validation of qPCR protocols. *L. monocytogenes* 568 (Serotype 1/2a, (Kalmokoff et al., 2001)), originally isolated from a shrimp processing plant and *L. monocytogenes* 08-5578 (Serotype 1/2a, (Gilmour et al., 2010)), originally isolated from a human blood sample in the 2008 Canadian listeriosis outbreak, were used in the desiccation survival experiments. Long-term stock cultures were stored in a 20% (v/v) glycerol peptone medium (TS/80, Technical Service Consultants Ltd, Heywood, UK) at -80°C. Routine culturing were done in Tryptic Soy Broth (TSB-glu) ((TSB, Merck, Darmstadt, Germany) supplemented with 1% (w/v) glucose (Fischer Scientific, Loughborough, UK)) or on Tryptic Soy Agar plates (TSA), composed of 30 g/l TSB and 15 g/l agar (Sigma-Aldrich, St. Louis, MO, USA).

2.2 Preparation of standardized cell suspensions

The *L. monocytogenes* strains were pre-cultured in ten ml TSB-glu overnight at room temperature (22-23°C) before cells were harvested by centrifugation at $2300 \times g$ for 5 min and resuspended in TSB-glu to an absorbance at 600 nm of 1 (NP80 NanoPhotometer, Implen, Westlake Village, CA, USA). The final cell concentrations were approximately 1×10^8 CFU/ml as determined by spot plating ($3 \times 20 \mu\text{l}$) of suitable ten-fold dilutions on TSA followed by enumeration after 48 h incubation at room temperature. In all desiccation experiments, the pre-culturing of *L. monocytogenes* strains was done in TSB-glu at 15°C for 48 h.

2.3 PMA treatment

Propidium monoazide (PMA, 20 mM, Biotium Inc., Hayward, CA, USA) was kept at -20°C and protected from light until use. Samples with *L. monocytogenes* cells harvested by centrifugation ($2300 \times g$ for 5 min) in 1.5 ml microcentrifuge tubes were resuspended in one ml peptone saline (PS, 1 g/l Peptone (Oxoid, Hampshire, UK), 8.5 g/l NaCl (Merck)) before addition of 2.5 µl PMA to a final concentration of 50 µM. This concentration was chosen, as it had previously been shown to be effective without being cytotoxic to live cells (Pan and Breidt, 2007; Martin et al., 2013). Each tube was then mixed by vortexing followed by incubation at 15°C for 30 min in the dark together with PMA free control samples. After incubation the tubes with and without PMA added were placed horizontally on ice (to prevent heating) and exposed to a 1000 W halogen lamp (Videolight 6, Kaiser Video, Germany) for 10 min at a distance of 20 cm. During the light exposure, the tubes were frequently turned to secure photoactivation and cross-linking of PMA to DNA (Nocker et al., 2006). Both PMA-treated and untreated control samples were centrifuged at $9,900 \times g$ for 3 min, before the supernatant was discarded and cell pellets frozen at -20 °C until DNA extraction.

2.4 DNA extraction

DNA from PMA-treated and non-PMA-treated pellets was extracted using the DNeasy PowerSoil Kit (Qiagen) as per the manufacturer's instructions. Extraction of DNA from desiccated cells was modified as described below (see section 2.8.3).

2.5 Quantitative PCR analysis

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Primers and probes (Table 1) for quantitative PCR (qPCR) were designed using the complete reference genome of *L. monocytogenes* EGD-e (GenBank Accession number NC_003210) (Glaser et al., 2001).

Table 1 Primers and probes used in qPCR and PMA-qPCR assays.

Gene target	Name	Sequence (5'-3') ^a	Location ^b	Size (bp)
<i>hly</i>	hly_short_F	5'- TACGCTAAAGAATGCAC TG-3'	1438 – 1456	199
	hly_short_R	5'- GTGTGTGTTAAGCGGTTT -3'	+29 – (+)46	
	hly_short_P	5'- Fam TAGCTTGGGAATGGTGG AGAACGG-BHQ-1-3'	1460 – 1483	
<i>hly</i>	hly_long_F	5'- TAAAGGGACAGCAGGAC TAG-3'	(-)162 – (-)143	1561
	hly_long_R	5'- ACGATGTGAAATGAGCT AGC-3'	1379 – 1399	

^aTaqMan probe labels: FAM, fluorescein; BHQ-1, Black Hole Quencher 1. ^bLocation is given in relation to the translation start site of the target gene (*hly*). This implies that hly_short_R is placed downstream (+) of *hly*, while hly_long_F is placed upstream (-) of the *hly* gene.

The *L. monocytogenes* *hly* gene was chosen as the gene target based on its length (1590 bp), which would increase the likelihood of designing *L. monocytogenes* species specific primers with a long (>1500 bp) amplicon. Primers and probes producing a short-amplicon (199 bp) were designed using CLC Workbench (Version 7.9.1, Qiagen Bioinformatics, Germantown, MD, USA). Primers and probes producing a long-amplicon (1561 bp) were designed using the online freeware tool OligoArchitectTM Primer and Probe Design (Sigma-Aldrich). Primer species specificity for *L. monocytogenes* was

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evaluated using the online NCBI tool Primer-Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) (Jian et al., 2012), while probe specificity was assessed using Blast-n (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). All primers and probes were purchased from TAG Copenhagen A/S (Frederiksberg, Denmark). The qPCR protocol for the primers and probe producing a short-amplicon was adapted from Huang et al. (2017) with the qPCR reaction being composed of: 7.7 µl of nuclease-free water, 12.5 µl of SsoAdvanced Universal Probes Supermix (Bio-Rad, Hercules, CA, USA)), 0.3 µl each of 10 µM forward and reverse primers, 0.2 µl of 10 µM TaqMan hydrolysis probe, and 4 µl of sample DNA. The long-amplicon qPCR reaction consisted of 7.5 µl of nuclease-free water, 12.5 µl of Type-it HRM master mix (contains EvaGreen Dye) (Qiagen, Hilden, Germany), 1.0 µl each of 10 µM forward and reverse primers, and 3 µl of sample DNA. The total volume in both qPCR reactions was 25 µl. qPCR were performed in optical tubes and caps (Agilent Technologies, Santa Clara, CA, USA) on a Stratagene Mx3000p qPCR System (Agilent Technologies) using the following conditions for both the amplicon lengths: initial denaturation for 10 min at 95°C, followed by 50 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min 45 s. Verification of primer specificity was further confirmed by checking for unspecific products by a melting curve analysis of the qPCR products between 50 - 95°C.

qPCR standard curves were built using DNA extracts from serial (ten-fold) dilutions of a standardized cell suspension of *L. monocytogenes* EDG-e. Volumes of 9 ml from each dilution (concentrations ranging from $\sim 10^1$ - 10^8 CFU/ml) were centrifuged for 10 min at $2,300 \times g$ before DNA was extracted from the pellet using the DNeasy

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PowerSoil Kit (Qiagen) and stored at -20°C. The diluted cultures were also enumerated by spot plating of appropriate dilutions in triplicates on TSA and counted after 48 h at 22-23°C. DNA extracted from the diluted standard cell suspensions were analysed by qPCR in triplicates as described above for both amplicon lengths. Quantification cycle threshold (C_q) values were analysed using the MxPro software (Version 4.10, Stratagene). The fluorescent baseline of the standard curves was set using the adaptive baseline tool integrated in MxPro. For other samples analysed with qPCR the fluorescent baseline were adjusted using the means of C_q of positive controls (from the standard curves). Each qPCR run included duplicates of positive and negative (no target DNA) controls. qPCR analysis of DNA from samples treated with PMA are referred to as PMA-qPCR, and used the same conditions as in qPCR analysis of samples prepared without PMA. The obtained standard curves (C_q values plotted versus the corresponding log CFU/reaction) for the short and long-amplicon assays had efficiencies of 102 and 92%, respectively, with R² values of 0.991 for both. The difference in the C_q values between replicates in the standard curves was less than 0.5.

qPCR amplification products were also analysed by agarose gel electrophoresis to confirm amplicon length. Here, 10 µl of amplification product were mixed with 2 µl 6× DNA-loading dye (New England Biolabs, Beverly, MA, USA) and analysed on a 1% (w/v) agarose gel (1 g SeaKem® LE Agarose, Lonza, Rockland, ME, USA), 100 ml Tris-acetate-EDTA (TAE, VWR, Solon, OH, USA) and 10 µl 10.000 × SYBR® Safe (Invitrogen, Eugene, OR, USA)) at a constant voltage of 80 V in TAE. The 100-bp Plus GeneRuler Ladder (Fischer Scientific) was used as a DNA marker. The agarose gel was visualized using blue light (~ 470 nm) in a Gel DOC 2000 (Bio-Rad).

2.6 Enumeration of viable and heat-killed *Listeria monocytogenes* cells

2.6.1 PMA treatment.

To prepare viable and heat-killed (dead) *L. monocytogenes* EGD-e cells eighteen sterile PCR tubes each containing 100 µl of standardized cell suspensions ($\sim 1 \times 10^8$ CFU/ml) of *L. monocytogenes* EGD-e were either incubated at 90°C for 15 min in a thermocycler (T3000 Thermocycler, Biometra, Göttingen, Germany) to heat-kill cells (9 tubes) or left at room temperature for 15 min (9 tubes, controls). Following treatment, 0.9 ml PS was added to each of the eighteen samples. Survivors were determined in three tubes from each treatment by spot plating appropriate dilutions on TSA and enumeration after incubation at 22-23°C for 48 h. Three tubes from each treatment were treated with PMA while the three remaining tubes were not, followed by pelleting of cells as described above and storage at -20°C until extraction of DNA using the DNeasy PowerSoil Kit (Qiagen). Samples were stored at -20°C until analyses using the short and long-amplicon qPCR protocols. The resulting Cq values were converted to log CFU/ml equivalents by using the standard curves and taking the dilutions into account.

2.6.2 Combined PMA and SDS treatment.

Penetration of PMA into dead cells with compromised cell membranes is instrumental to achieving a successful viability qPCR assay (Nocker et al., 2006). It was thus investigated whether the penetration of PMA into dead *L. monocytogenes* cells could be improved by a pretreatment with Sodium-Dodecyl-Sulphate (SDS, Sigma-Aldrich) as

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previously shown for *Escherichia coli* (Takahashi et al., 2017). Viable and heat-killed cell pellets were prepared and survivors were enumerated by plate counts as previously described. Duplicate samples of viable or dead cells were treated with all combinations of SDS and PMA (SDS- and PMA- (control), PMA+ SDS-, SDS+ PMA- or SDS+ PMA+). Briefly, four aliquots of cell pellets from viable and dead treatments were either resuspended in 1 ml of sterilized water with or without 250 µg/ml SDS. The samples were then incubated for 30 min at 37°C before adding 2.5 µl of PMA to two of the SDS treated and two of the untreated control samples. The remaining samples received no PMA. The ensuing incubation and light treatment, DNA extraction and qPCR analysis were performed as described above.

2.7 qPCR detection of viable cells in mixes of viable and heat-killed cells

L. monocytogenes EGD-e viable and heat-killed (dead) cells were prepared and treated with PMA followed by DNA extraction as described above. DNA extracted from viable *L. monocytogenes* EGD-e cells was ten-fold diluted in DNA extracted from heat-killed treated *L. monocytogenes* EGD-e cells in order to assess the influence of a background of dead cells on the enumeration of viable *Listeria monocytogenes* by PMA-qPCR. Four mixes (Table 2) of DNA were prepared: Ten µl of DNA from the viable cells was suspended in 90 µl nuclease-free water (I, 100% viable cells). Thirty µl DNA from the dead cells were mixed with 270 µl nuclease-free water. These working stocks were used to produce the following final mixes of DNA from viable/dead cells (%): 100/0 (I), 10/90 (II), 1/99 (III), 0.1/99.9 (IV). The mixes of DNA were analysed using both the short-amplicon and long-amplicon qPCR assays as previously described.

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Table 2 Mixing ratios of DNA from viable and heat-killed *Listeria monocytogenes* and their theoretical corresponding cell concentrations (CFU/ml).

		<i>I</i>	<i>II</i>	<i>III</i>	<i>IV</i>	265
Viable	CFU/ml	10 ⁷	10 ⁶	10 ⁵	10 ⁴	
		100%	10%	1%	0.1%	
Heat-killed	CFU/ml	0	9×10 ⁶	9.9×10 ⁶	9.99×10 ⁷	
		0%	90%	99%	99.9%	

2.8 Discrimination of viable and dead *L. monocytogenes* during desiccation on stainless steel

L. monocytogenes 568 and 08-5578 were desiccated on stainless steel for up to ten days with and without prior biofilm formation. Survival of *L. monocytogenes* was enumerated using plate counts, qPCR and PMA-qPCR as described above. The desiccation of *L. monocytogenes* on stainless steel was done using a protocol adapted from Hingston et al. (2013).

2.8.1 Preparation of stainless steel coupons

The stainless steel (SS, Food grade AISI 316, type 4 finish, thickness 1 mm) was cut into coupons of 0.5 × 0.5 cm. Prior to use, coupons were boiled in 1% (w/v) SDS solution for 10 min, sonicated for 60 min with 50/60 kHz in a 1000 W sonication bath (Elmasonic S 120, Fisher Scientific) and immersed in 100% isopropanol (Sigma-Aldrich) to remove any remaining residues. SS coupons were then rinsed in distilled water, autoclaved and stored in 96% ethanol (VWR, Fontenay-sous-Bois, France). Before surface inoculation they were rinsed again in distilled water, autoclaved and dried in sterile petri dishes in a biosafety cabinet.

2.8.2 Inoculation and desiccation of non-biofilm and biofilm *L. monocytogenes* on stainless steel

Before inoculation of the SS coupons, a desiccation chamber (Mini 1 Desiccator Bohlender GmbH, Grünsfeld, Germany) was placed at 15°C with three open petri dishes filled with potassium acetate (CH₃COOK, Acros Organic, Geel, Belgium) in the bottom of the chamber the day prior to use. This ensured that the temperature and relative humidity (RH) inside the desiccation chamber was stabilized around 15°C and 33% RH to simulate conditions periodically faced by *Listeria monocytogenes* in the food processing environment (Hingston et al., 2017). To form a biofilm prior to desiccation of *L. monocytogenes* 568 and 08-5578 an additional desiccation chamber was placed at 15°C with three lid-less petri dishes in the bottom filled with distilled water to create an RH of 100%. The temperature and RH were monitored in both desiccation chambers using a built-in logger and a data logger (Tinytag, Gemini Data Loggers Ltd., Chichester, UK). Inoculation of SS coupons was done using standardized cell suspensions of *L. monocytogenes* 568 and 08-5578 pre-cultured in TSB-glu at 15°C for 48 h and resuspended in fresh TSB-glu as described above.

In non-biofilm desiccation experiments, 10 µl of *L. monocytogenes* 568 and 08-5578 ($\sim 1 \times 10^9$ CFU/ml) were spotted separately on individual SS coupons and placed in individual petri dishes without lids inside the desiccation chamber with 15°C and 33% RH for up to ten days.

In biofilm experiments, the SS coupons were inoculated with 10 µl of suspensions of *L. monocytogenes* 568 and 08-5578 (diluted to contain $\sim 10^4$ CFU/ml) and the petri dishes with lids were placed in the desiccation chamber with 100% RH at 15°C for 48-72

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hours to allow growth and biofilm formation before commencement of up to ten days of desiccation by transfer of the samples to a desiccation chamber held at 15°C and 33% RH.

2.8.3 Enumeration and discrimination of viable and dead *L. monocytogenes* on stainless steel coupons during desiccation

Seven SS coupons from each strain were sampled for enumeration on sampling days over the eight to ten day desiccation period to follow the survival of the two *L. monocytogenes* strains 568 and 08-5578. The SS coupons were placed in individual microcentrifuge tubes containing 1 ml of PS. Adhering cells were released from the SS coupons by sonication for 5 min with 50/60 kHz in a sonication bath (Elmasonic S 120) followed by vortexing for 20 s. This protocol for release of bacteria was adapted from the method originally developed by Leriche and Carpentier (1995). Three samples were serially diluted in PS and appropriate dilutions were spot plated on TSA plates. Colonies were enumerated after incubation for 48 h at room temperature and expressed as log CFU/cm². The content of the four remaining samples were transferred to four new microcentrifuge tubes, followed by the PMA treatment of two of these samples by addition of 2.5 µl PMA to a final concentration of 50 µM. Further treatment, isolation of cell pellets and freezing of cell pellets were done as described above.

DNA extraction of *L. monocytogenes* 568 and 08-5578 cell pellets from the non-biofilm desiccation experiments and the first biofilm desiccation experiment was done using the DNeasy PowerSoil Kit (Qiagen) and elution in 100 µl Elution Buffer (EB, Qiagen).

In order to optimize the DNA extraction from *L. monocytogenes* biofilm cells the DNeasy PowerBiofilm DNA extraction (Qiagen) was tested with and without a pretreatment cell lysis protocol. The DNA extraction from the biofilm desiccation experiments spanning ten days was done with this pretreatment to optimize the lysis of the Gram-positive *L. monocytogenes* biofilm cells before proceeding to the DNA extraction using the DNeasy PowerBiofilm Kit. The cell lysis protocol was adapted from an optional pretreatment described in the DNeasy Blood & Tissue Kit (Qiagen, 2006) with the following protocol: 180 µl of freshly prepared enzymatic lysis buffer (20 mM Tris-Cl pH 8.0, 2 mM sodium EDTA, 1.2% Triton X-100, 20 mg/ml lysozyme) was added directly to frozen cell pellets and incubated in a standard laboratory heat block for 20 min at 37°C with 550 rpm (ThermoMixer C, Eppendorf, Hamburg, Germany) The pretreatment was continued by addition of 25 µl Proteinase K (>600 mAU/ml) and vortexed 10 s before incubation in the heat block for 30 min at 56°C with 550 rpm. After incubation the DNA was extracted using the DNeasy PowerBiofilm Kit, where the protocol was followed from step two with the modification that the liquid from the pretreated samples was mixed with the first solution of the kit and transferred to the PowerBiofilm Bead tube. DNA was eluted in 100 µl EB as in the other DNA extractions.

DNA extracted with or without PMA treatment from cells from the desiccation experiments was analyzed with qPCR using the long-amplicon protocol as previously described. C_q values were converted to log CFU/cm². qPCR was done in duplicates with negative and positive controls.

To assess the accuracy of the enumerations by PMA-qPCR compared to plate counts the accuracy factor (A_f) described by Ross (1996) was calculated:

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$$A_f = 10^{\frac{\sum \left| \log \left(\frac{\text{predicted}}{\text{observed}} \right) \right|}{n}}$$

Where n is the total number of data points. A value of 1 means there is complete agreement between the “predictions” (plate counts) and “observations” (qPCR results); whereas a value of 1.10 means that the predictions on average are 10% within the observations.

The qPCR, PMA-qPCR and plate count results were normalized ($\log(N_t/N_0)$) to allow comparison of the inactivation measured by the different methods (Fig. 5a) and to compare the inactivation determined by qPCR relative to the observations from plate counts (Fig. 5b).

In order to further optimize the enumeration by qPCR and PMA-qPCR a standard curve based on samples taken during biofilm formation was produced. Briefly, triplicate samples of plate counts and DNA were obtained by harvesting cells from SS coupons during 48 hours of biofilm formation as before. DNA from these cells was extracted using the optimized protocol with the lysis pretreatment and the DNeasy PowerBiofilm Kit and subjected to qPCR analyses.

2.9 Statistical analysis

All plate counts were done in triplicates while qPCR results were from analyses of at least duplicate samples. For enumeration of the viable and heat-killed cells, paired t-tests were performed on $\log(\text{CFU/ml})$ values to test for differences between plate counts, qPCR and PMA-qPCR results obtained from either the short or long-amplicon based qPCR protocols. Statistical analysis of the difference between the enumeration methods used for discrimination of viable and dead cells during desiccation of *L. monocytogenes*

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used the analysis of variance (ANOVA) on the normalized inactivation values to compare PMA-qPCR to the plate count inactivation values. This included a Tukey's HSD (honest significant difference) test at the 5% significance level. For all data $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Performance of qPCR assays

The original aim was to develop short and long-amplicon Taqman qPCR methods for specific detection of *L. monocytogenes*. However, in spite of trying different probe designs the long-amplicon qPCR assay with probe exhibited low efficiencies of $\leq 83\%$. Primers for the long-amplicon qPCR were instead designed to be species specific without a probe as confirmed by Primer-Blast and melt-curves of amplicons. The Primer-Blast and Blast-n analyses of the primers and probe showed that for the short-amplicon qPCR method, the probe was needed to attain species specificity. Non-specific products were not seen in the melt-curve analyses of either amplicon lengths and agarose gel electrophoresis confirmed this observation and the length of amplicons. The standard curves led to calculated efficiencies of 102 and 92% (slopes -3.3 and -3.5) for the short-amplicon with probe and the long-amplicon without probe qPCR assays, respectively, and R^2 values of 0.991 for both assays (Fig. S1 in the supplemental material). The limit of quantification (LOQ) for the qPCR assays was determined as the lowest concentration within the linear portion of the standard curve as recommended in the MIQE Guidelines (Bustin et al. 2009) and were 1.44 and 1.32 log CFU/reaction for the short-amplicon and long-amplicon qPCR assays, respectively, corresponding to 6886 CFU/ml and 6964

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CFU/ml. The limit of detection (LOD) was determined to be 0.32 log CFU/reaction (696 CFU/ml) for the short-amplicon and 0.44 log CFU/reaction (689 CFU/ml) for the long-amplicon qPCR assays.

3.2 Effect of amplicon size on discrimination between viable and dead cells pretreated with PMA

Plate counts before and after heat treatment (90°C for 15 min) of *L. monocytogenes* showed that the heat treatment was effective in killing the cells, i.e., the reduction was from 8.02 log CFU/ml to below the detection limit of 1.22 log CFU/ml. The treatment of heat-killed *L. monocytogenes* with 50 µM PMA prior to DNA extraction caused a significantly increase in Cq values (by 13 cycles and 18 cycles) for the short- or long-amplicon PMA-qPCR protocols compared to PMA-qPCR of viable cells. This corresponded to a reduction in viable cells of 4.2 log CFU/ml and 5.1 log CFU/ml for the short- and long-amplicon qPCR, respectively.

As shown in Fig. 1, the log CFU/ml result from long-amplicon PMA-qPCR assay fell below its LOD, whereas the signal suppression in the short-amplicon PMA-qPCR assay was incomplete leading to detection of a false positive log CFU/ml above the LOD. PMA (50 µM) treatment had no significant ($p > 0.05$) toxic effect on plate counts (data not shown). qPCR and PMA-qPCR with either amplicon size caused a slight but insignificant ($p > 0.05$) underestimation of the viable cell count.

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Figure 1. Effect of amplicon length and PMA treatment on qPCR detection of viable and heat-killed *L. monocytogenes*. Viable and heat-killed (dead) *L. monocytogenes* were enumerated by plate counts (□) and the short-amplicon (SA, 199 bp (▨)) and long-amplicon (LA, 1561 bp (■)) qPCR protocols with or without PMA. *Below the plate count limit of detection (LOD_{platecount} = 1.22 log CFU/ml.) The horizontal dashed lines correspond to the limits of quantification (LOQ) and detection (LOD) for the LA-qPCR of 3.84 and 2.84 log CFU/ml, respectively. Bars are averages of replicates (n=6) and error bars indicate standard deviation.

The membrane destabilization pretreatment with SDS prior to PMA treatment did not significantly ($p > 0.05$) enhance the effect of PMA in removing the false-positive DNA signal from heat-killed cells in long-amplicon PMA-qPCR (data not shown). Additionally, SDS appeared to have a toxic effect on viable *L. monocytogenes* cells and use of SDS treatment was not further investigated.

In the test of the ability of the short- and long-amplicon PMA-qPCR assays to enumerate viable *L. monocytogenes* in mixes of DNA from PMA treated dead and viable cells (Table 2), both amplicon lengths accurately quantified viable cells in the 100% viable cell mix (Fig. 2). However, when the short-amplicon was used to quantify the viable cells in samples containing increasing concentrations of DNA from dead PMA treated cells, a log fold reduction in the signal between each mix was not observed. In contrast, the long-amplicon qPCR quantified the concentration of viable cells resulting in values that were similar to the theoretically calculated plate counts for each mix (Table

2), leading to differences between the quantified cell concentrations in the 100% viable sample and the three viable/dead mixes of -1.03, -2.02, and -3.06 log CFU/ml (Fig. 2).

Figure 2. Effect of amplicon length on the ability of PMA-qPCR to detect viable cells in mixes of viable and heat-killed (dead) cells. DNA from viable cells was diluted in DNA from PMA treated dead cells and tested using PMA-qPCR. The horizontal dashed lines correspond to the LOQ and LOD for the PMA-qPCR of 3.84 and 2.84 log CFU/ml, respectively. Bars are averages of replicates (n=8) and error bars indicate standard deviation.

3.3 Enumeration of viable *Listeria monocytogenes* during desiccation on stainless steel

According to the plate counts, the survival of *L. monocytogenes* decreased steadily for both strains during desiccation for ten days on stainless steel at 15°C and 33% RH (Fig. 3). As it can be seen in Fig. 3 the effect of omitting the PMA treatment prior to DNA extraction resulted in a significant ($p < 0.05$) overestimation of the number of viable cells by the long-amplicon qPCR assay. This overestimation of surviving cells is evident from the first day and stable throughout the desiccation period. Without PMA

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the accuracy factor of the long-amplicon qPCR compared to the plate count was 1.18 and 1.26 for *L. monocytogenes* 08-5578 and 568, respectively.

Figure 3. Desiccation survival of *L. monocytogenes* 08-5578 (a) and 568 (b) on stainless steel coupons. Cells were pre-cultured at 15°C in TSB-glu and re-suspended in TSB-glu and desiccated (33% RH, 15°C) for 10 days. Cells were enumerated by plate counts (○) and the long-amplicon (1561 bp) qPCR protocol with (△) or without (■) PMA. Symbols are averages of replicates (n=2-4) and error bars indicate standard deviation.

Application of PMA eliminated the overestimation of viable cells as there was no significant difference ($p > 0.05$) between the PMA-qPCR and plate counts. The PMA-qPCR result was consistent for both strains (accuracy factors of 1.05 and 1.09, respectively) throughout the experiment except for day 10 in *L. monocytogenes* 568, where the PMA-qPCR overestimated the viable cell concentration. At day 10 both strains exhibited a remarkable drop in culturable survivors as estimated by plate counts, which was also seen with PMA-qPCR for *L. monocytogenes* 08-5578. The plate count quantified the overall reduction of culturable cells to 1.38 ± 0.11 log CFU/cm² for *L. monocytogenes* 08-5578 and 1.45 ± 0.11 log CFU/cm² for *L. monocytogenes* 568. In comparison, the reduction of viable cells measured by PMA-qPCR was 1.40 ± 0.09 log

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CFU/cm² for *L. monocytogenes* 08-5578 and 0.71 ± 0.06 log CFU/cm² for *L. monocytogenes* 568. In contrast, straight qPCR with the long-amplicon protocol failed to detect a reduction in survivors during desiccation.

3.4 Enumeration of viable *Listeria monocytogenes* biofilms during desiccation on stainless steel

The study of the performance of the long-amplicon PMA-qPCR assay in the detection of viable *L. monocytogenes* in pre-formed biofilms (48 hours at 15°C and 100% RH) being desiccated for eight days at 15°C and 33% RH was designed to probe the fate of *L. monocytogenes* left on insufficiently cleaned or soiled surfaces of stainless steel processing equipment in an environment, where RH varies due to non-continuous production activity. The plate count results showed that overall desiccation survival of *L. monocytogenes* 568 biofilm cells (Fig. 4a) were better than for the non-biofilm cells (Fig. 3b). The long-amplicon PMA-qPCR results from the first biofilm desiccation experiment using DNA from the DNeasy PowerSoil Kit, followed the decreasing trend in survivors but significantly ($p < 0.05$) underestimated the biofilm cell count by on average $2.21 \log \pm 0.26$ CFU/cm² compared to the plate count, as also evident in an accuracy factor of 1.48. As long-amplicon qPCR apparently detects listerial DNA from both viable and dead cells, this method was not expected to underestimate biofilm cell concentrations compared to plate counts as was observed with stable levels of ~ 5 - 5.5 log CFU/cm² (Fig. 4a). Consequently, it was hypothesized that the low cell estimates (i.e., low DNA concentrations) could be caused by insufficient DNA extraction from the biofilm cells. Use of the DNeasy PowerBiofilm Kit caused a significant ($p < 0.05$) improvement (data

not shown), but the underestimation persisted. Next, two different cell lysis treatments (see section 2.8.3) were tested to improve the DNA extraction from aggregated biofilm cells (Colagiorgi et al., 2016). Both lysis treatments reduced the underestimation of cell numbers by the qPCR (data not shown).

Figure 4. Survival of *L. monocytogenes* 568 biofilm cells during desiccation on stainless steel coupons, as enumerated by plate counts (○) and the long-amplicon qPCR protocol using DNA extracted either with (a) the DNeasy PowerSoil DNA Kit (▲, ■) or (b) a modified DNeasy PowerBiofilm Kit (△, ◻) with (△) or without (◻) PMA treatment prior to DNA extraction (averages of replicates (n=3) with standard deviation). Cells were spotted on stainless steel coupons and kept at 100% RH for 48-72 h to allow biofilm formation prior to desiccation (15°C, 33% RH, 8-10 days). Day 0 represents the beginning of desiccation.

Use of the best-performing cell lysis/DNA extraction protocol with the long-amplicon PMA-qPCR in a new biofilm desiccation experiment still resulted in a significant ($p < 0.05$) underestimation of the cell concentration by an average of 0.82 ± 0.31 log CFU/cm² cell compared to the plate count (Fig. 4b). The accuracy factor was, however, improved to 1.14. Moreover, when assessing the relative inactivation (log

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(N_t/N_0) in both biofilm experiments it became evident that long-amplicon PMA-qPCR correctly enumerated the relative reduction in viable cells during desiccation of the biofilm and agreed with the reduction observed by the plate count $\log(N_t/N_0)$ (Fig. 5a). In contrast, the cell concentrations measured by long-amplicon qPCR stayed constant leading to increasing differences relative to plate count results, which was not the case for long-amplicon PMA-qPCR (Fig. 5b).

Figure 5. Optimization of DNA preparation for the long-amplicon qPCR protocols to detect (a) the relative reduction ($\log(N_t/N_0)$) in viable cells counts and (b) the difference between qPCR results and plate counts during desiccation (15°C, 33% RH, 8-10 days) of biofilm cells of *L. monocytogenes* 568 on stainless steel. Survivors were enumerated by plate counts (○) and the long-amplicon qPCR protocol using DNA extracted either with (a) the DNeasy PowerSoil DNA Kit (▲, ■) or (b) a modified DNeasy PowerBiofilm Kit (Δ, □) with (Δ) or without (□) PMA treatment prior to DNA extraction (averages of replicates (n=3) with standard deviation).

The construction of a qPCR standard curve using biofilm samples did not reduce the gap between plate counts and the cell concentrations calculated using the long-amplicon PMA-qPCR standard curve derived from planktonic cells (data not shown).

4. DISCUSSION

4.1 Effect of amplicon size on discrimination between viable and dead cells pretreated with PMA

PMA treatment of *L. monocytogenes* samples prior to DNA extraction coupled with qPCR (PMA-qPCR) proved to be a valid method for enumeration of viable cells. When preparations of dead cells were analyzed by PMA-qPCR, the long-amplicon assay significantly improved the suppression of the false-positive signal from the dead cells. These findings were in line with observations from other PMA-qPCR studies, where shorter and longer amplicon assays were compared for enumeration of viable *Campylobacter*, *Salmonella* and *Legionella* (Banihashemi et al. 2012; Martin et al. 2013; Ditommaso et al. 2015). While the present study is to the best of our knowledge the first to develop a long-amplicon PMA-qPCR assay that is specific for *L. monocytogenes*, Soejima et al. (2008) showed in an EMA end-point PCR assay that a longer amplicon (894 bp) improved detection of survivors after heat-treatment of *L. monocytogenes* compared to a short-amplicon (113 bp) assay.

The signal detected from the heat-killed cells by the long-amplicon PMA-qPCR method fell below the LOD, whereas the signal in the short-amplicon PMA-qPCR remained above LOD (Fig 1). Some studies interpret a qPCR signal below LOD as a complete suppression of the ‘dead signal’ and thus depict bars in figures with the measured cell concentration as zero for these observations (Banihashemi et al., 2012; Ditommaso et al., 2015). Others show the obtained Cq value or the increase in Cq without explicit information about the LOD (Martin et al., 2013; Thanh et al., 2017). This

563 makes direct comparisons between studies regarding the complete and incomplete
564 suppression of the ‘dead signal’ difficult.

565 When DNA from different concentrations of viable cells was quantified in a
566 background of DNA from high concentrations of dead cells, the long-amplicon PMA-
567 qPCR assay accurately enumerated the viable cells, whereas the short-amplicon PMA-
568 qPCR assay failed to differentiate between viable and dead cells (Fig. 2). The inability of
569 the short-amplicon PMA-qPCR assay to accurately enumerate the viable cell
570 concentrations below 6 log CFU/ml is consistent with the findings of Løvdal et al.
571 (2011), where a short-amplicon (101 bp) PMA-qPCR assay was able to enumerate viable
572 cells in mixes of viable and heat-killed cells of *Listeria innocua*, but only when the viable
573 cell concentration was ≥ 6 log CFU/ml. Similar observations have been done for
574 *Salmonella*, where a short-amplicon (95 bp) could not accurately enumerate the viable
575 cells in a background of DNA from 10^8 CFU/g dead cells, if the concentration of viable
576 *Salmonella* was ≤ 6 log CFU/g (Martin et al., 2013). In agreement with the finding from
577 the present study, increasing the amplicon size from 95 to 417 bp enabled accurate
578 enumeration of viable cells in concentrations $\geq 10^3$ CFU/g in a background of dead
579 *Salmonella* (Martin et al., 2013).

580 The reason for improved performance of longer amplicons in PMA-qPCR and
581 EMA-qPCR assays in the discrimination of viable and dead cells is not known. It is
582 thought that PMA and EMA suppresses the qPCR signal from cells with a compromised
583 membrane by introducing random cross-links between PMA or EMA and cellular DNA,
584 which limits amplification of cellular DNA in a way that is not fully elucidated (Fittipaldi
585 et al., 2012; Nocker et al., 2006). The longer sequences used in long-amplicon PMA-

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qPCR assays logically increase the likelihood of PMA cross-links and the prevention of a qPCR signal. Soejima et al. (2011) showed a positive correlation between the signal suppression from dead cells and the amplicon length in EMA-qPCR in a study with varying amplicon lengths ranging from 110 to 2840 bp. Our results for *L. monocytogenes* and similar findings in other foodborne pathogens underpin the importance of using longer amplicons in viability PMA-qPCR assays in contrast to traditional guidelines for qPCR which recommends short-amplicons (75-300 bp, Nolan et al. 2013; Agilent Technologies 2012).

Attempts to further improve the long-amplicon PMA-qPCR method by implementation of a SDS pre-treatment step as suggested by Takahashi et al. (2017) failed for the *L. monocytogenes* assay. It may be that this method originally developed for the Gram-negative *Escherichia coli* cannot be used for Gram-positive bacteria, likely due to the observed toxic effect of SDS on *L. monocytogenes*.

4.2 Long-amplicon PMA-qPCR as a method to discriminate between viable and dead *Listeria monocytogenes* during desiccation on stainless steel

Listeria monocytogenes is capable of surviving up to at least 90 days on stainless steel, which is a widely used material in the food industry (Vogel et al., 2010). To test the ability of the assay to accurately enumerate viable *L. monocytogenes* in a food processing facility (e.g., tables, slicers, conveyor belts, drains, etc.), the assay was tested on cells being desiccated on stainless steel. Enumeration of survivors during ten days of desiccation by long-amplicon PMA-qPCR and plate counts was not significantly ($p > 0.05$) different, although the long amplicon PMA-qPCR assay tended to overestimate

viable cells compared to the plate counts (Fig. 3). The slight overestimation of viable cells may possibly be explained by the presence of VBNC cells as also implied by Overney et al. (2017), who using a short amplicon (113 bp) PMA-qPCR assay observed a higher number of viable cells compared to culturable cells after 24 hours of desiccation on stainless steel in different media. This observation may perhaps also be explained by the amplicon length, as the present study observed a greater discrepancy between plate counts and short-amplicon PMA-qPCR results (Fig. S2) than were the case for the long-amplicon results when enumerating desiccation survivors. To determine if the minor overestimation of viable cells by the long-amplicon PMA-qPCR assay during the desiccation experiment is due to the presence of VBNC *L. monocytogenes* evidence from other experimental approaches would be needed such as metabolic activity tests, ongoing mRNA synthesis, CTC counts or flow cytometry (Besnard et al., 2000; Lindbäck et al., 2010; Winkelströter and De Martinis, 2015). In a study of the efficiency of SC-CO₂ pasteurization treatment to inactivate *L. monocytogenes* spiked on dry cured ham, flow cytometry coupled (FCM) with SYBR-Green I and short amplicon (101 bp) PMA-qPCR was used to enumerate membrane intact (viable) cells (Ferrentino et al., 2015). Both methods indicated high amounts of VBNC cells as the reduction ($\log N_t/N_0$) of “viable” cells compared to plate counts was much lower with short amplicon PMA-qPCR and FCM indicating the presence of approximately 4 and 2 log VBNC cells, respectively. Thus our measurement of an average of 0.35 log CFU/cm² VBNC cells by the long-amplicon PMA-qPCR assay in our study, is markedly lower and may perhaps be explained by the improved suppression of signal from dead cells obtained due to the increased amplicon length.

4.3 Limitations of long-amplicon PMA-qPCR as a method for direct enumeration of viable *Listeria monocytogenes* biofilm cells during desiccation on stainless steel.

The long-amplicon PMA-qPCR assay was also tested on *L. monocytogenes* 08-5578 and 568 biofilms being desiccated on stainless steel. The first protocol yielded a significant ($p < 0.05$) underestimation of both viable and total cells as measured by long-amplicon PMA-qPCR and qPCR, respectively, compared to plate counts (Fig. 4a). The PMA-qPCR underestimated the viable cells in the desiccated biofilm by an average of $2.21 \log \pm 0.26 \text{ CFU/cm}^2$ which was in contrast to the non-biofilm experiments where there was an insignificant ($p > 0.05$) overestimation. Winkelströter and De Martinis (2015) also reported an underestimation of 2 log CFU/ml when attempting to enumerate biofilm cells of *L. monocytogenes* using EMA-qPCR. They explained this observation as having been caused by the cytotoxicity of EMA. PMA has, however, been shown not to be cytotoxic to *L. monocytogenes* at effective concentrations (50 μM) (Pan and Breidt, 2007) and should not be responsible for the observed PMA-qPCR underestimation of viable *L. monocytogenes* biofilm cells during desiccation. In view of the current understanding of the mechanism of PMA and the presence of extracellular DNA (eDNA) in the biofilm matrix of *L. monocytogenes* one might actually have expected that the viable biofilm cells would be overestimated if PMA were to bind to eDNA instead of entering dead cells (Colagiorgi et al., 2016). As the underestimation of viable cells occurred in both the qPCR and PMA-qPCR results, it is more likely to be explained by factors relating to biological differences between planktonic cultures and biofilms of *L. monocytogenes*. Indeed, optimization of the cell lysis and the use of a DNA extraction kit

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commercially designed for biofilm cells (DNeasy PowerBiofilm kit) reduced the underestimation in both assays. The underestimation of viable cells by long-amplicon PMA-qPCR was reduced from 2.21 log CFU/cm² to 0.82 log CFU/cm² as compared to plate counts (Fig. 4b). The long-amplicon PMA-qPCR enumeration was still significantly ($p < 0.05$) lower than the plate counts. However, when looking at the inactivation during desiccation in relation to the starting concentration ($\log N_t/N_0$) as determined by either qPCR or PMA-qPCR, it became evident that the long-amplicon PMA-qPCR accurately enumerated the loss of viable cells regardless of the DNA extraction method, while the long-amplicon qPCR assay did not (Fig. 5b). This means that the long-amplicon PMA-qPCR method developed in the present study accurately measured the relative inactivation of *L. monocytogenes* biofilm cells during the desiccation treatment but could not be used to estimate the “exact” viable cell concentrations.

Interestingly, the underestimation in the PMA-qPCR assay seemed to be related to a lower cellular DNA content or DNA extraction efficiency from *L. monocytogenes* biofilm cells being desiccated. In a study of qPCR detection of *L. monocytogenes* biofilms (without viability dyes) four different DNA extraction methods were tested by Guilbaud et al. (2005) on both adherent and non-adherent cells and found to performed differently on the two types of cells. More effort will have to be made in order to optimize the DNA extraction from biofilm cells if an accurately enumeration of viable *L. monocytogenes* is desired.

Comparison of qPCR standard curves made from *L. monocytogenes* planktonic and wet biofilm cells grown on stainless steel revealed no significant ($p > 0.05$) differences, meaning that PCR inhibitors in the biofilm seemed less likely to have been

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responsible for the underestimation by both the long-amplicon qPCR and PMA-qPCR assays.

In general the results from the present study add to the mounting evidence that increasing the amplicon length in PMA-qPCR improves the suppression of the signal from dead cells and the ability to discriminate between viable and dead cells. One drawback of long-amplicon PMA-qPCR is the increased runtime, which is, however, negligible compared to the time it takes to use culture dependent methods. A new version of PMA called PMAxx has been marketed by Biotium, which in *Salmonella* has shown to improve suppression of the dead signal (Biotium, 2013). The number of studies using PMAxx is, however, still limited and has mainly been used in studies with viruses. Also of great interest is the recently developed dye PEMAX, marketed by GenIUL, which takes advantage of two photoreactive dyes and has shown to effectively suppress the signal from dead *Salmonella enterica* in 33 food samples using a 262 bp amplicon (Thanh et al., 2017). The PEMAX double dye method is based on the finding that use of a combination of EMA and PMA suppresses the qPCR signal from dead cells with intact membranes but no ATP turnover (Codony et al., 2015). Other compounds such as palladium (Pd-qPCR) and platinum (Pt-qPCR) have shown to be a competitive and cheaper choice for viability-qPCR with very comparable results to PMA-qPCR and thus further studies should compare PMA and PMAxx with these alternative compounds.

Also, use of the long-amplicon qPCR method for the evaluation of other inactivation methods (e.g., isopropanol, non-thermal plasma, thermal lysis, autoclaving, UV-C irradiation, peracetic acid, freezing/thawing, air-drying), should be explored. Different inactivation methods were recently shown to affect the efficiency and accuracy

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of short-amplicon PMA-qPCR and EMA-qPCR to detect viable *Campylobacter* (Vondrakova et al., 2018).

In conclusion, the application of a long-amplicon PMA-qPCR assay to assess the survival of *L. monocytogenes* after heat treatment and desiccation with and without prior biofilm formation is promising and could be helpful in studies and surveillance of the survival and inactivation of *L. monocytogenes* in food processing environments.

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Figure S1. Standard curves for the *hly* long- and short-amplicon qPCR.

Figure S2. Desiccation survival of *L. monocytogenes* 08-5578 (a) and 568 (b) on stainless steel coupons. Cells were pre-cultured at 15°C in TSB-glu and re-suspended in TSB-glu and desiccated (33% RH, 15°C) for 10 days. Cells were enumerated by plate counts (○) and the short-amplicon (SA, 199 bp) qPCR protocol with (△) or without (■) PMA. Symbols are averages of duplicates and error bars indicate standard deviation.