Food Control 62 (2016) 150–156

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Optimization and validation of a PMA qPCR method for *Escherichia coli* quantification in primary production

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ARTICLE INFO

Article history:
Received 22 July 2015
Received in revised form
13 October 2015
Accepted 14 October 2015
Available online 17 October 2015

Keywords:
Viable cells
Microbial sampling program
Indicator microorganisms
Cultivation-based techniques
Propidium monoazide

ABSTRACT

The microbial requirements defined in many quality assurance guidelines and standards of primary production demand the establishment of microbial sampling programs. Recently, a q-PCR *Escherichia coli* assay has been reported as a good method to quantify the presence of fecal indicator bacterial in groundwater samples. This study focuses on the optimization, validation and application of a qPCR method combined with propidium monoazide (PMA) treatment to exclude DNA from dead cells. A first screening consisting of six primer sets targeting single and multi-copy of *E. coli* were tested to evaluate the sensitivity of the assay. After that, four primer sets were selected, combined with PMA treatment and their capacity to distinguish viable cells when combined with a background of dead cells was assessed. A primer set targeting the 23S rRNA gene was 10-fold more sensitive than the rest of primers, enabling the detection of low concentrations of viable *E. coli* cells. This assay also exhibited good repeatability and reproducibility, which indicates the robustness of the method. Optimized and validated PMA-qPCR was used to enumerate *E. coli* in environmental samples including irrigation water and fresh produce. The results were compared with the levels quantified using qPCR and cultivation-based techniques. Counts of *E. coli* using plate count assay were significantly lower than the levels obtained by molecular techniques (PMA-qPCR and qPCR) in both irrigation water and fresh produce. *E. coli* PMA-qPCR enumeration method showed similar results as qPCR quantification, although the PMA-qPCR treatment seemed to a good alternative to distinguish between viable and dead cells. It can be concluded that the optimized PMA-qPCR assay can be used by the industry in microbial sampling programs, helping them with the implementation of Good Agricultural Practices (GAP).

1. Introduction

A food safety management program focused on primary production is the result of the implementation of available and relevant quality assurance guidelines and standards such as Codex Alimentarius, guidelines on good agricultural practices (GAP), hygiene legislation, and private standards (Kirezieva, Jacxsens, Uyttendaele, Van Boekel, & Luning, 2013). In the current European legal framework, microbiological criteria is not defined at the primary production stage, but growers must be able to demonstrate that their operations do not represent a food safety risk (EC, 2004). However, microbiological requirements have been defined in many quality assurance guidelines and standards, which make, the establishment of microbial sampling programs, necessary for growers (Allende & Monaghan, 2015).

Traditionally, sampling programs are based on the detection of specific pathogens and the enumeration of indicators of fecal contamination. However, the prohibitive cost and time required for pathogen detection, makes enumeration of microbial indicators a good strategy to characterize microbial contamination in the environment of field cultivation and fresh produce (Park et al., 2013).

Indicator microorganisms, such as *Escherichia coli*, are usually used by the industry and competent authorities to assess the implementation of Good Agricultural Practices (GAP). At primary production, *E. coli* have been identified as suitable hygiene criterion in leafy greens as its presence provides evidence of an increased likelihood of potential contamination by ecologically closely related pathogens (Castro-Ibáñez, Gil, Tudela, Ivanek, & Allende, 2015; EFSA, 2014; Holvoet, Sampers, Seynnaeve, Jacxsens, &

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http://dx.doi.org/10.1016/j.foodcont.2015.10.014
0956-7135/

Uyttendaele, 2015). Traditional culture techniques using pour and spread plate count methods are used to enumerate *E. coli* in water and produce samples. However, these cultivation-dependent assays may not represent the most efficient method to predict presence of bacterial pathogens. Ferguson et al., (2012) reported that a qPCR-based *E. coli* assay was the best indicator for the presence of bacterial pathogens in groundwater samples.

Many studies have focused on the development and optimization of qPCR methods to detect and enumerate *E. coli* in environmental samples as a more sensitive, rapid and specific test that plate counts (Ahmed, Richardson, Sidhu, & Toze, 2012; Ferguson et al., 2012; Pitkänen et al., 2013). However, qPCR-based assays cannot differentiate between viable and dead bacterial cells as DNA of dead cell can persist in the environment, leading to a substantial overestimation of *E. coli* concentrations (Rudi Moen, Drømtorp, & Holck, 2005; Varma et al., 2009). To avoid false-positive results, a recently proposed strategy is the treatment of samples with propidium monoazide (PMA) before DNA extraction, allowing the differentiation between viable and dead cells. This technique is based on the ability of PMA to penetrate into the dead cells with compromised membrane integrity and in turn inhibit DNA amplification by PCR following light-induce cross-linking (Fittipaldi, Codony, Adrados, Camper, & Morató, 2011; Nocker & Camper, 2009; Nocker, Sossa-Fernandez, Burr, & Camper, 2007; Varma et al., 2009). The use of PMA has been successfully integrated with qPCR assays for the differentiation of viable and dead *E. coli* cells in different environmental samples, mostly in water samples (van Frankenhuyzen, Trevors, Flemming, Lee, & Habash, 2013; Gensberger et al., 2014; Kim, Gutiérrez-Cacciabue, Schriewer, Rajal, & Wuertz, 2014; Li et al., 2014). However, discrepancies regarding the most recommended primers and PMA concentrations make the selection of a PMA-qPCR method difficult based on the available literature.

Environmental samples from the primary production are complex matrices that may interfere with the efficacy of the PMA treatment. Factors such as the ratio between viable and dead bacterial cells, pH and salt concentrations as well as the natural presence of PMA inhibitors have been highlighted as potential inhibitors for the PMA treatment, DNA extraction and qPCR yield (van Frankenhuyzen, Trevors, Lee, Flemming, & Habash, 2011; Fittipaldi, Nocker, & Codony, 2012). Therefore, optimization and validation of previously developed PMA-qPCR methods as well as their suitability as monitoring systems in the primary production are essential before their application as routine tools in microbial sampling programs.

Thus, the aim of the present study was the optimization of a previously described PMA-qPCR assay for the quantification of viable generic *E. coli* cells in irrigation water and produce samples covering; (i) selection of specific primer sets for *E. coli* qPCR with TaqMan technology; (ii) characterization of the PMA-qPCR capacity to differentiate between viable and dead cells and (iii) validation of the assay in irrigation water and produce samples taken from a commercial-scale production field of leafy greens.

2. Material and methods

2.1. Bacterial strains and inoculum preparation

A seven-strain cocktail of *E. coli* (CECT 434, 471, 515, 516, 533 and LFMFP 679) was used in this study. Strains were obtained from the Spanish Type Culture Collection (CECT) (Valencia, Spain) and the Laboratory of Food Microbiology and Food Preservation (LFMP) (Ghent University, Belgium). Cultures were inoculated in Brain Heart Infusion (BHI) (Scharlau Chemie, Barcelona, Spain) and incubated without agitation at 37 °C for 24 h. Then, 2 ml of each strain were combined and washed twice by centrifugation (3500 g, 20 min) at 4 °C in 14 mL of 0.1% buffered peptone water (BPW; AES Chemunex, Marcy l'Etoile, France). The washed cell pellets were suspended in 2 mL of 0.1% BPW.

2.2. Sample collection

Water samples were collected at five sampling points from a stream bordering farmlands and urban areas in Murcia (Spain) at six different sampling times. A total volume of 3L was collected using sterile polypropylene plastic bottles. Iceberg lettuces were purchased from a local supermarket at the day of harvest. Samples were transported to the laboratory (within 30 min) and stored at 4 °C for maximum 16 h until further processing. Lettuce heads, which were confirmed to be free of *E. coli* by Chromocult agar (Oxoid, Hampshire, UK), were sprayed with irrigation water obtained from the urban stream (A) and a mixture of irrigation water and heat-treated irrigation water at a 1:1 ratio (B). Non-sprayed lettuce was used as negative control. After being sprayed with water, lettuce samples were incubated at room temperature for 16 h.

2.3. Cultivation-based E. coli quantification

Cultivation-based enumeration of *E. coli* in irrigation water and fresh lettuce samples was performed according to ISO 9308-1:2014. In the case of water samples, different volumes (10 mL and 100 mL) were filtered through 0.45 μm pore size nitrocellulose membranes (Sartorius, Madrid, Spain). For *E. coli* quantification, membranes were aseptically removed from the filter base and placed on Chromocult agar. For iceberg lettuces, external leaves were cut and samples (25 g each) were homogenized for 3 min in 225 mL of 0.1% BPW using a Stomacher (IUL instruments, Barcelona, Spain). Tenfold dilution series were made in 0.1% BPW and plated onto Chromocult agar. The plates were incubated at 37 °C for 24 h.

2.4. Molecular-based E. coli quantification

Water samples were taken from the surface water as previously described. Two 500 mL aliquots per sampling point were centrifuged at 3000 g for 20 min and supernatant was removed. In the case of iceberg lettuces, external leaves were cut and three samples (25 g each) were homogenized in 125 ml of sterile 0.1% BPW using a Stomacher at medium speed for 3 min. The homogenate of each sample was divided to obtain two pellets by centrifugation at 3000 g for 10 min. From each water or lettuce sample, one of the pellets was stored at −20 °C for genomic DNA extraction while the other pellet was resuspended in sterile water for PMA treatment.

2.5. PMA treatment for qPCR

Propidium monoazide (PMA) (Biotium Inc, Hayward, CA, USA) was dissolved in 20% dimethyl sulfoxide (DMSO); (Sigma–Aldrich, Ameresco, USA) as a 20 mM stock solution. Aliquots of 2 mM were transferred to light-impermeable 2 mL microtubes at kept at −20 °C in the dark. For PMA treatment cell suspensions were diluted to a cell density of 10^4 cells/mL in 0.1% BPW. Aliquots of 1 mL of the diluted cell suspensions, irrigation water and lettuce samples were transferred to clear transparent 2-mL microtubes. These aliquots were then centrifuged at 9000 *g* for 10 min and supernatants removed. Cell pellets were resuspended in 1 ml of sterile water with variable PMA concentrations (10 and 40 μM). PMA treated microtubes were shaken at 400 rpm for 5 min in darkness. The tubes laid on ice were then exposed to a 500-W halogen light source (GE lighting, Cleveland, USA) for 5 min. The

distance between microtubes and light source was 20 cm. After light exposure, the bacteria were harvested by centrifugation at 9000 g for 10 min.

To assay the suitability of PMA to distinguish between viable and dead cells, the above mentioned bacterial cocktail of seven *E. coli* strains was used. The cocktail was divided into two aliquots; one was exposed to 85 °C using a water bath for 15 min to obtain heat-killed bacteria while the other remained as a viable cell suspension. Then, the viable cell suspension was diluted to different concentrations (10^3, 10^5 and 10^7 cfu/mL) using 0.1% BPW. Cultivation-based enumeration of *E. coli* cells was carried out by plating on plate count agar (PCA; Oxoid, Basingstoke, UK). Viable and heat-killed cell suspensions were mixed to obtain three different mixtures containing ratios of viable:heat killed cells of 10^3:10^7, 10^5:10^7 and 10^7:10^7. Then bacterial suspensions were PMA treated as previously described. After PMA treatment, cells were pelleted at 9000 *g* for 10 min and stored at −20 °C until genomic DNA extraction.

2.6. DNA extraction

DNA from viable, heat-killed and mixed samples of viable and heat-killed cells was isolated using the PrepSEQ™ Rapid Spin sample preparation kit (Applied Biosystems, Madrid, Spain), following the manufacturer's instructions. Genomic DNA isolated from pure cultures was diluted in 100 μl DNAse free water. In the case of irrigation water and lettuce samples, genomic DNA was extracted using the MasterPure™ Complete DNA and RNA purification kit (Epicenter, Madison, USA) following the manufacturer's instructions. Briefly, pellets were lysed by enzymatic treatment with Proteinase K (50 μg/μL). DNA was precipitated with isopropanol and resuspended in 50 μL of TE Buffer (10 mM Tris–HCl [pH 7.5], 1 mM EDTA).

2.7. qPCR procedure

Several PCR primers were selected from the published literature. The list of selected primers and TaqMan probes is shown in Table 1. The procedure of qPCR was performed using an ABI 7500 Sequence Detection System (ABI, Applied Biosystems, Madrid, Spain). All primers and probes were commercially synthesized by Applied Biosystem. Amplification and detection were carried out in 96-well plates using KAPA PROBE FAST Universal qPCR Master mix kit (KapaBiosystems, Massachusetts, USA). Each reaction was run in triplicate containing 5 μL of DNA template Final concentrations of primers and probes as well as the applied cycling parameters are listed in Table 1. In all cases, a non-template control (NTC) was included using 5 μl of DNAse free water instead of the DNA template. Standard curves were made using known concentrations of genomic DNA isolated from *E. coli* CECT 515T. The *E. coli* concentration in the stock solution was verified by plating on PCA. The reproducibility of the qPCR assays was assessed by determining intra-assay repeatability and inter-assay reproducibility. The coefficient of variation (CV) was calculated using 10-fold serial dilution of genomic DNA isolated from the 7-strain *E. coli* cocktail. The CV was calculated based on the quantification cycle (Cq) value. For the reproducibility, three replicates were made per dilution at the day of experiment and the experiment was repeated on three separate days. To determine the qPCR limit of detection (LOD), standard curves of 10-fold serial dilution of DNA were examined in triplicate. The LOD was determined based on Cq of the last detectable standard. When NTC showed signal in amplification, the calculation of LOD was performed according to the formula Cq (LOD) = Cq (NTC) − 3 (Caraguel, Stryhn, Gagné, Dohoo, & Hammell, 2011; Gensberger et al., 2014). The samples with Cq values higher than Cq (LOD) were classified as non-determined and Cq values lower were classified as positive. Amplification efficiencies were calculated from the slope of the standard curve as $E = 10^{-1/s} - 1$, where *s* is the slope of the standard curves (Garrido et al., 2013).

2.8. Statistical analysis

Two experimental trials were carried out in independent days and three replicates were analyzed each time (n = 9) except for the experiments carried out for the validation of the method in irrigation water and fresh lettuce, where two experiments were done with two replicates (n = 6). For calculation and graphical presentation of the median and interquartile range (IQR) of microbial counts only positive samples (i.e., with values above the detection limit) were included. IBM SPSS Statistics 19 was used for statistical analysis. Except when stated otherwise, P values below 0.01 were considered statistically significant. Shapiro–Wilk test was performed to assess the normality of the data (P > 0.01).

3. Results

3.1. Primer selection

Primers were selected based on the minimum and maximum Cq obtained from amplification of genomic DNA isolated from viable

Table 1
Oligonucleotide primers and probes used in this study (qPCR and PMA-qPCR).

Code	Name	Gen	Sequence (5′–3′)	Concentration (nM)	qPCR cycling parameters	Reference
A	784F	uidA	GTGTGATATCTACCCGCTTCGC	900	10 min at 95 °C, 40 cycles of 15 s	Frahm and Obst, 2003
	866R		AGAACGGTTTGTGGTTAATCAGGA	300	at 95 °C, 60 s at 60 °C	
	EC807		(FAM)TCGGCATCCGGTCAGTGGCAGT(TAMRA)	200		
B	ECN1254F	23S rRNA	GCAAGGTGCACGGGAATATT	500	10 min at 95 °C, 40 cycles of 15 s	Gensberger et al., 2013
	ECN1328R		CAGGTGATCGGACGCGT	500	at 95 °C, 60 s at 62 °C	
	ECN1277P		(FAM)CGCCACTGGCGGAAGCAACG(TAMRA)	250		
C	rcEC23seq-1728F	23S rRNA	CATAAGCGTCGCTGCCG	1000	10 min at 95 °C, 40 cycles of 15 s	Ludwig & Schleifer, 2000
	rcEC23seq-1821R		AAAGAAAGCGTAATAGCTCACTGGTC	1000	at 95 °C, 60 s at 60 °C	
	rcEC23seq-1772T		(FAM)TACATCTTCCGCGCAGGCCGACT(TAMRA)	1000		
D	uid AF	uidA	CAACGAACTGAACTGGCAGA	1000	10 min at 95 °C, 40 cycles of 15 s	Chern et al., 2010
	uid AR		CATTACGCTGCGATGGAT	1000	at 95 °C, 60 s at 60 °C	
	uid AP		(FAM)CCCGCCGGGAATGGTGATTAC(TAMRA)	800		
E	Ec23Sf	23S rRNA	GAGCCTGAATCAGTGTGTGTG	800	10 min at 95 °C, 40 cycles of 30 s	Knappett et al., 2011
	Ec23Sr		ATTTTTGTGTACGGGGCTGT	800	at 95 °C, 45 s at 55 °C	
	EC23Srv1bhq		(FAM) CGCCTTTCCAGACGCTTCCAC(BHQ-1)	160		
F	23F	23S rRNA	GGTAGAGCACTGTTTTGGCA	800	10 min at 95 °C, 40 cycles of 15 s	Ahmed et al., 2012
	23R		TGTCTCCCGTGATAACTTTCTC	800	at 95 °C, 60 s at 60 °C	
	23P		(FAM)TCATCCCGACTTACCAACCCG (TAMRA)	160		

and heat-killed strains of *E. coli* after treatment with PMA (10 μM) (Table 2). Based on the minimum and maximum Cq, the primer sets A, B, D and F, showed the best DNA amplification and good differentiation between viable and heat-killed cells (Table 2). The primer set F exhibited the highest sensitivity (LOD 10 cfu) and therefore it was selected for further studies (Fig. 1).

3.2. qPCR reproducibility

The reaction efficiencies were determined using 10-fold dilutions of genomic DNA isolated from a seven-strain cocktail of *E. coli* treated with PMA. The established detection limit of selected PMA-qPCR assay was determined at −10 cfu/rxn; accordingly the dilution 10^0 was removed from analysis. Amplification efficiencies were >97%, and correlation coefficient (r^2) >0.99 for all qPCR runs. The mean intra-assay and inter-assay CV values were 1.1 ± 0.4% and 3.9 ± 0.7%, which indicates a high reproducibility of the selected method (Table 3). The efficiency of the selected qPCR primer set (23F/23R/23P) to detect *E. coli* in water was previously reported by Ahmed et al. (2012). However, until now, the use of this primer set to amplify *E. coli* after PMA treatment has not been studied.

3.3. PMA-qPCR interference

The presence of high concentration of dead cells was evaluated in case it affected the accuracy of the PMA-qPCR method. Viable and heat-killed *E. coli* cells were mixed in different ratios of viable:heat-killed cells of 10^3:10^7, 10^5:10^7 and 10^7:10^7 and treated with PMA before qPCR quantification (Fig. 2). Viable cells were overestimated in 0.2 ± 0.0, 0.4 ± 0.1, and 0.1 ± 0.1 log units when 3, 5 and 7 log cfu/mL of viable *E. coli* cells, respectively, were quantified in the presence of 7 log cfu/mL of heat-killed *E. coli* cells. The obtained results indicated that DNA from viable cells was preferably amplified during the PMA-qPCR reaction, which confirms the low interferences between viable and dead cells.

Table 2
Maximum Cq values obtained for amplification of genomic DNA isolated from a seven-strain *E. coli* cocktail of viable and dead cells (4.4 log cfu/ml) using PMA-qPCR. Each value represents the mean ± standard deviations of three independent repetitions (n = 9).

Code	Primer sets	Viable	Dead
A	784F/866R/EC807	31.5 ± 2.6	38.5 ± 1.4
B	ECN 1254F/1328R/1277P	32.0 ± 2.8	38.8 ± 0.1
C	EC 1278F/1821R/772T	27.3 ± 0.0	31.1 ± 1.4
D	uidA/uidAR/uidAP	31.6 ± 0.8	39.1 ± 1.3
E	EC23sF/EC23Sr/EC23Srv1bhq	26.3 ± 2.9	33.5 ± 0.2
F	23F/23R/23P	25.2 ± 0.8	38.2 ± 0.1

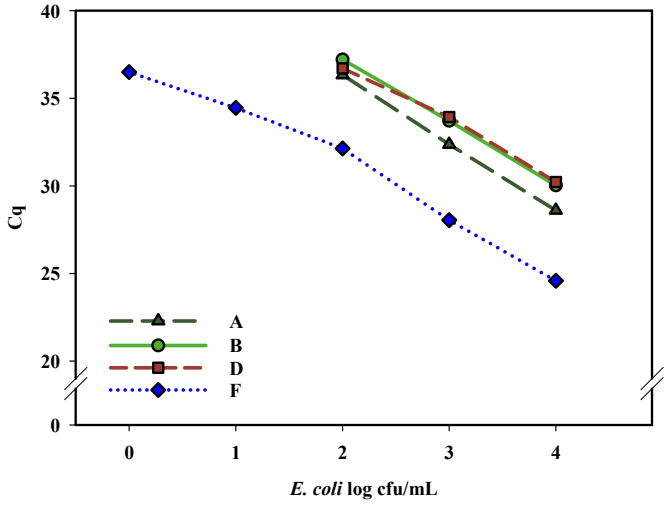

Fig. 1. Quantification cycle (Cq) values obtained during amplification of different dilutions of a seven-strain *Escherichia coli* cocktail using different primer sets (A: 784F/866R/Ec807; B: ECN 1254F/1328R/1277P; D: uidAF/uidAR/uidAP; F: 23F/23R/23P) for PMA-qPCR enumeration.

Table 3
Coefficient of variation (CV) for the PMA-qPCR assay F (23F/23R/23P; Ahmed et al., 2012) of different dilutions of a seven-strain *E. coli* cocktail. Each value represents the mean of three independent repetitions (n = 9).

Unit log	CV (%)	
	Intra-assay	Interassay
5	1.6	4.1
4	1.8	4.4
3	1.0	4.2
2	0.8	4.1
1	0.7	2.7

3.4. Selection of PMA concentration in environmental samples

PMA concentration of 10 and 40 μM have been described in the literature as optimal concentrations for inhibiting amplification of DNA from dead cells without affecting the quantification of live *E. coli* cells from different environmental samples (Gensberger, Sessitsch, & Kostić, 2013; Moyle, Harris, & Marco, 2013). Levels of *E. coli* quantified using PMA-qPCR with the two selected PMA concentrations did not show significant differences in irrigation water samples (Table 4). Therefore, the lower concentration (10 μM) of PMA was selected for further analysis.

3.5. PMA qPCR validation in irrigation water and fresh produce

Validation of the selected PMA-qPCR method was carried out in environmental samples of irrigation water and lettuce samples. In irrigation water significant differences in *E. coli* counts determined by cultivation-based method and molecular-based techniques

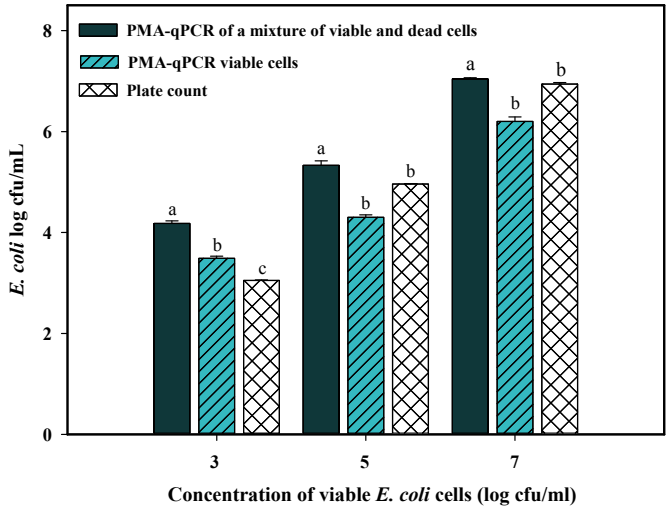

Fig. 2. *Escherichia coli* counts obtained using the optimized PMA-qPCR assay and cultivation-based techniques (plate count) of a seven-strain *E. coli* cocktail containing variable concentrations of a mixture of viable and heat-killed cells (7 log cfu/mL) and viable cells. Each bar represents the mean ± standard deviations of three independent repetitions (n = 9). Bars labeled with different letters indicate significant difference at $P < 0.01$.

Table 4
Escherichia coli counts in irrigation water using PMA-qPCR when variable concentrations (10 and 40 μM) of PMA were used.

PMA concentration	*E. coli* (log cfu/mL)	
	Median	IQR
40 μm	6.4	5.7–6.7
10 μm	6.4	5.7–6.7
0 μm	6.8	6.2–6.8

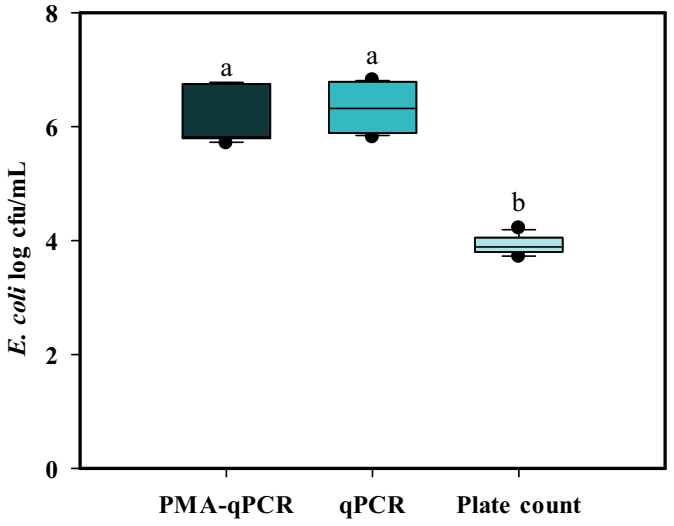

Fig. 3. *Escherichia coli* counts in irrigation water obtained using molecular based techniques (PMA-qPCR and qPCR) and cultivation-based techniques (plate count). Box plot represents three replicates of two independent assays (n = 6). The bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Whiskers show the greatest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile). Different letters indicate significant difference at P < 0.01.

(both qPCR and PMA-qPCR) were observed. In all the cases, counts of cultivable *E. coli* were significantly lower that levels of viable *E. coli* determined by qPCR and PMA-qPCR, approximately 2.28 ± 0.6 and 1.9 ± 0.6 log units, respectively (Fig. 3). However, no significant differences were observed between the *E. coli* levels quantified by qPCR and PMA-qPCR. The selected PMA-qPCR assay was also validated in fresh lettuce. The median count of *E. coli* enumerated in lettuce samples using cultivation-based assay was 3.23 log cfu/g (IQR 3.3–3.1). *E. coli* levels quantified by PMA-qPCR were significantly higher than those estimated by the plate count method as previously observed for irrigation water (Fig. 4A). When irrigation water from the urban stream was used, enumeration results of *E. coli* in lettuce samples did not show significant differences between qPCR and PMA-qPCR. However, when iceberg lettuce was sprayed with water containing irrigation water and heat-treated irrigation water, which increased the levels of dead cells, significant differences were observed between the two selected PCR techniques (qPCR and PMA-qPCR) (Fig. 4B). DNA isolated from non-irrigated lettuce samples did not show amplification.

4. Discussion

Progressively more growers are requested to implement microbial monitoring programs to compile with the microbial requirements defined in quality assurance guidelines and standards (Uyttendaele et al., 2015). Microbial analyses have been traditionally based in cultivation-based techniques for determination of microbial quality parameters such as *E. coli* (Gensberger et al., 2014). However, recent studies reported that more rapid and specific technologies, such as qPCR, seem to be a more suitable method to evaluate the microbial quality of environmental samples (Ferguson et al., 2012). Some studies have reported PMA-qPCR methods to quantify *E. coli* in environmental samples, predominantly in water (van Frankenhuyzen et al., 2013; Gensberger et al., 2013; Gensberger et al., 2014; Kim et al., 2014; Li et al., 2014; Varma et al., 2009). However, available literature reports the use of different primer sets and PMA concentration, which complicates the selection of the most suitable method. Versatility of the method is also important, making the validation in different sample types, such as irrigation water and fresh produce, necessary. Therefore, this study focused on the evaluation of previously reported PMA-qPCR methods for the enumeration of *E. coli* to select the most suitable one.

Many different primer sets have been reported for enumeration of *E. coli* using qPCR (Chern, Siefring, Paar, Doolittle, & Haugland, 2010; Gensberger et al., 2014; Maheuxa et al., 2009). Some authors suggested the utilization of specific marker genes for *E. coli*, the *uid*A gene, which encodes the β-glucoronidase enzyme (Gensberger et al., 2013; Takahashi et al., 2009), while others recommended to target the 23S rRNA, as a more advantageous approach for water samples where numbers of microorganisms are low (Ahmed et al., 2012). Gensberger et al. (2014) reported that the use of the DNA-intercalating dye technology for detecting *E. coli* by

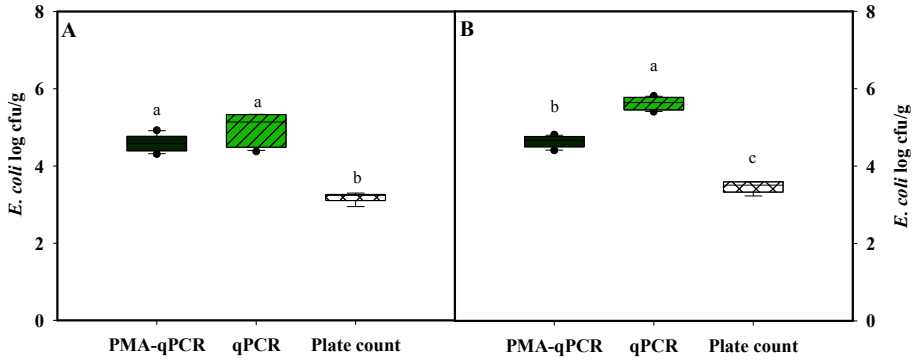

Fig. 4. *Escherichia coli* counts in lettuce samples after sprayed with irrigation water obtained from the urban stream (A) and a mixture of irrigation water and heat-treated irrigation water at a 1:1 ratio (B) using molecular based techniques (PMA-qPCR and qPCR) and cultivation-based techniques (plate count). Box plot represents three replicates of two independent assays (n = 6). The bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Whiskers show the greatest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile). Different letters indicate significant difference at P < 0.01.

RT-PCR showed some difficulties in distinguish clearly and assign melt peaks. In the current study, among the tested sets, two primers targeting the gen *uid*A and two targeting the 23S rRNA gen were preliminary selected because they showed the best Cq values for amplifying viable and heat-killed cells from *E. coli* mixture cultures. However, targeting the sequence encoding in the 23S rRNA gen was 10-fold more sensitive than targeting the *uidA* gene sequence, enabling detection of lower concentrations of viable *E. coli* cells. This effect can be explained by the fact that uidA is a single copy gene, whereas average copy number of 23S rRNA genes in *E. coli* is 7 (https://rrndb.umms.med.umich.edu/). This primer set also showed a very good repeatability and reproducibility, which indicates the robustness of the method. Although qPCR-based *E. coli* assays have been reported to be more suitable for enumeration of fecal indicator bacteria than cultivation-based techniques (Ferguson et al., 2012), they may overestimate *E. coli* loads due to the DNA amplification from both viable and dead cells (Rudi, Moen, Drømtorp, & Holck, 2005; Levy-Booth et al., 2007). In the present study, enumeration of *E. coli* concentrations using qPCR assay yielded values around 1.4 orders of magnitude higher than that obtained by cultivation-based techniques. This is in agreement with previously reported results which detected similar differences between the use of qPCR assays and cultivation-based methods due to the quantification of DNA from dead cells (Ahmed et al., 2012; van Frankenhuyzen et al., 2013). Thus, differences observed between qPCR and cultivation-based methods were initially attributed to dead cells, but it could be also due to the presence of viable but none cultivable (VBNC) cells. In fact, the quantification of *E. coli* carried out using qPCR and PMA-qPCR techniques were very similar. Irrigation water is exposed to many environmental factors which might stress the bacterial cells, leading to a loss of bacterial cultivability while viability remains unaltered (Heim, Lleo, Bonato, Guzman, & Canepari, 2002). Turbidity of the water has been reported that might interference with the photoactivation step of PMA, reducing the efficiency of the treatment (van Frankenhuyzen et al., 2011). However, the low turbidity (Nephelometric Turbidity Units (NTU) = 8) of the irrigation water tested in this study makes this hypothesis difficult to sustain as higher NTU values have been reported to interfere with the PMA photocativation (Gedalanga & Olson, 2009; Luo, Lin, & Guo, 2010). Therefore, further studies are needed to determine if VBNC organisms play a role in this discrepancy.

Some studies demonstrated that viable and dead cell mixtures containing high density of dead cells reduce the performance of PMA treatment (Contreras, Urrutia, Sossa, & Nocker, 2011; Elizaquivel, Sánchez, & Aznar, 2012; Løvdal, Hovda, Björkblom, & Møller. 2011; Pan & Breidt, 2007). In fact, PMA differentiation capacity lies approximately in a ratio of viable and dead cells of 10^3:10^7 (Pan & Breidt, 2007; Løvdal, Hovda, Björkblom, & Møller, 2011). This could be partially due to the presence of "ghost" bacteria, which have an intact cell-wall/membrane but are metabolically inactive, leading to the inhibition of PMA effect (Nocker & Camper, 2009). Results obtained in the present study showed that the selected primer set at a PMA concentration of 10 μM can be used to detect low concentrations of viable *E. coli* cells when mixed with heat-killed bacteria without overestimation.

In order to determine the versatility of the selected method to monitor the microbial safety in primary production, enumeration of *E. coli* was carried out in both, irrigation water and produce samples. Results showed that the selected PMA-qPCR methodology was able to detect viable *E. coli* cells in complex matrices, such as irrigation water and lettuce with a mixed background of native microbiota (Rastogi et al., 2012; Staley et al., 2013). In the case of lettuce samples, the number of viable *E. coli* cells quantified by the PMA-qPCR methodology was 2 log units higher than those recovered by cultivation-based techniques. As far as we know, this is the first time that viable *E. coli* cells were detected in irrigation water and on fresh product using the same PMA-qPCR method.

Based on the obtained results it can be concluded that the described PMA-qPCR assay represents a suitable technique for the selective detection and quantification of viable *E. coli* cells in irrigation water and produce samples. The use of this methodology may yield a more accurate estimation of *E. coli* loads than traditional cultivation-based methods. This may be a suitable monitoring system to be routinely used by the fresh produce industry for the quantification of indicator microorganisms of fecal contamination, such as *E. coli* without interferences of native microbiota.

Acknowledgments

Authors are thankful for the financial support from the Center for Produce Safety (Grant Agreement 2015-374) and the MINECO (Grant Agreement AGL2013-48529-R). Support provided by the COST ACTION FA1202 BacFoodNet through the concession of a Short Term Scientific Mission aimed to promote collaboration between the two research institutions is also appreciated. The authors also wish to acknowledge technical advice received from Eva T. Gensberger.

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