Food Microbiology 79 (2019) 41–47

Contents lists available at ScienceDirect

Food Microbiology

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

# Comparative assessment of qPCR enumeration methods that discriminate between live and dead *Escherichia coli* O157:H7 on beef

Anna M. Laidlaw[a,b], Michael G. Gänzle[b,c], Xianqin Yang[a,*]

[a] Agriculture and Agri-Food Canada Lacombe Research and Development Centre, 6000 C & E Trail, Lacombe, Alberta, T4L 1W1, Canada
[b] Department of Agricultural, Food and Nutritional Science, University of Alberta, 4-10 Agriculture/Forestry Centre, Edmonton, AB, T6G 2P5, Canada
[c] Hubei University of Technology, College of Bioengineering and Food Science, Wuhan, PR China

## ARTICLE INFO

*Keywords:*
Propidium monoazide
Deoxycholate
qPCR
Detection
Beef
*Escherichia coli* O157:H7

## ABSTRACT

Quantitative Polymerase Chain Reaction (qPCR) is a molecular method commonly used to detect and quantify bacterial DNA on food but is limited by its inability to distinguish between live and dead cell DNA. To overcome this obstacle, propidium monoazide (PMA) alone or with deoxycholate (DC) was used to prevent dead cell detection in qPCR. qPCR methods were used to detect strains of *Escherichia coli* O157, which can cause infection in humans with an infectious dose of less than 10 cells. A 5 strain *E. coli* O157:H7 cocktail was inoculated onto beef steaks and treated with interventions used in meat facilities (lactic acid (5%), peroxyacetic acid (200 ppm) or hot water (80 °C for 10 s)). Treatment of PMA or PMA + DC was applied to samples followed by DNA extraction and quantification in qPCR. RNA was also quantified in addition to conventional plating. For lactic acid intervention, qPCR DNA quantification of *E. coli* O157:H7 yielded 6.59 ± 0.21 and 6.30 ± 0.11 log gene copy #/cm$^2$ for control and lactic acid samples, respectively and after treatment with PMA or PMA + DC this was further reduced to 6.31 ± 0.21 and 5.58 ± 0.38, respectively. This trend was also observed for peroxyacetic acid and hot water interventions. In comparison, RNA quantification yielded 7.65 ± 0.13 and 7.02 ± 0.38 log reverse transcript/cm$^2$ for rRNA control and lactic acid samples, respectively, and for plating (LB), 7.51 ± 0.06 and 6.86 ± 0.32 log CFU/cm$^2$, respectively. Our research determined that treatment of PMA + DC in conjunction with qPCR prevented dead cell DNA detection. However, it also killed cells injured from intervention that may have otherwise recovered. RNA quantification was more laborious and results had higher variability. Overall, quantification with conventional plating proved to be the most robust and reliable method for live EHEC detection on beef.

## 1. Introduction

Molecular detection methods have become a mainstay in assessment of microbiological composition and activity within microbial ecosystems, including food safety assessment, as they are faster and more selective than labor-intensive and time-consuming conventional plating methods (Ge and Meng, 2009). Quantitative polymerase chain reaction (qPCR) is one of the most commonly used methods of gene quantification, and is a validated method for pathogen detection in food (Health Canada, 2018; Wong and Medrano, 2005). However, qPCR is limited in that it does not discriminate between DNA from live and dead cells in a food sample, which may lead to false positive results and lead to unnecessary recalls of food products (Bae and Wuertz, 2009). Current methods for pathogen detection use an enrichment step to prevent detection of dead cells (Ge and Meng, 2009). Enrichment of samples also reduces the detection limit from > 1000 cells/g (qPCR only) to less than 1 cell per 400 g (qPCR after enrichment); however, an enrichment increases the time required for detection and does not allow quantitative results to be obtained.

Monoazide DNA binding dyes can enter dead cells with compromised membranes, intercalate with DNA bases and form covalent bonds upon exposure to light (Takahashi et al., 2017; Taskin et al., 2011). This modification of DNA prevents its amplification in qPCR. Ethidium monoazide (EMA) has previously been investigated for this purpose, but was found to cause significant DNA loss from viable cells after extraction (Flekna et al., 2007; Nocker et al., 2006a). Propidium monoazide (PMA) penetrates into cells with compromised membranes without affecting viable cells with intact membranes (Pan and Breidt, 2007). However, PMA is excluded from dead cells with intact membranes and therefore PMA-qPCR may overestimate the number of viable cells

---

[*] Corresponding author.
*E-mail address:* xianqin.yang@canada.ca (X. Yang).

https://doi.org/10.1016/j.fm.2018.11.002
Received 1 July 2018; Received in revised form 5 November 2018; Accepted 13 November 2018

(Løvdal et al., 2011; Nocker et al., 2007; Pacholewicz et al., 2013). To better select for live cells in qPCR, a sodium deoxycholate treatment prior to PMA application can enhance its penetration due to the emulsifier's ability to permeabilize intact membranes of dead cells (Yang et al., 2011). While *E. coli* is highly tolerant to bile salts, the effect of deoxycholate on injured cells is unknown. An alternative is quantification of RNA using qPCR as an indicator of cell viability as it degrades more rapidly upon cell death than DNA, with mRNA degrading more rapidly than rRNA (Keer and Birch, 2003). However, the low stability of RNA also increases the risk of degradation of the target molecule during sample preparation.

Ruminants including cattle are a major reservoir of enterohaemorrhagic *E. coli* (EHEC) (Croxen et al., 2013); accordingly, foodborne illness caused by EHEC has often been linked to the consumption of contaminated beef (Blagojevic et al., 2012; Rangel et al., 2005). Interventions implemented in beef packing plants to control EHEC on carcasses include lactic acid and peroxyacetic acid washes as well as hot water or steam application (Bacon et al., 2000). *E. coli* O157, the prototype serogroup of EHEC, is regulated in Canada and the US and current methods of detection of *E. coli* O157 in food include enrichment in conjunction with qPCR. However, qPCR does not differentiate between live cells and cells killed by the use of interventions, limiting its use when both live and dead cells are present. This investigation aimed to compare four different PCR-based detection methods to determine which method most accurately quantifies live cells of *E. coli* O157, in comparison to viable plate counts as a reference method for bacterial viability. Reverse-transcription (RT-)qPCR of mRNA and rRNA as well as PMA or PMA and deoxycholate treatment prior to DNA quantification in qPCR were evaluated. Quantification experiments were performed on beef steaks inoculated with a cocktail of *E. coli* O157:H7 strains, and treated with lactic acid, peroxyacetic acid or hot water as decontamination treatments.

## 2. Material and methods

### 2.1. Preparation of inoculum

Five Shiga toxin (stx) negative *E. coli* O157:H7 (02–0628, 02–1840, 02–0304, 02–0627, 00–3581; Luciano et al., 2011) were grown individually overnight in 9 mL of Luria-Bertani (LB; Oxoid, Mississauga, Ontario, Canada) broth in 15 mL glass tubes at 37 °C and 250 rpm. The next day, cells of each strain were harvested by centrifugation, cell pellets were washed twice with 9 mL of 0.1% (w/v) peptone water (Difco, Becton-Dickinson, Sparks, Maryland, USA) and then combined to create a 5 strain cocktail of *E. coli* O157:H7.

### 2.2. Primers

Quantification of DNA in qPCR was done with the species specific primers URL-301 (5′-TGT TAC GTC CTG TAG AAA GCC C-3′) and URR-432 (5′-AAA ACT GCC TGG CAC AGC AAT T-3′) that target *uidA* coding for β-glucuronidase in *E. coli* (Bej et al., 1991). For quantification of rRNA in reverse transcription qPCR (RT-qPCR) primers targeting partial 16S rRNA, HDAf (5′-ACT CCT ACG GGA GGC AGC AGT-3′) and HDAr (5′-GTA TTA CCG CGG CTG CTG GCA C-3′) were used (Walter et al., 2000; Wang et al., 2013). For quantification of mRNA in RT-qPCR, primers targeting glyceraldehyde-3-phosphate dehydrogenase were selected: gapAf (5′-GTT GAC CTG ACC GTT CGT CT-3′) and gapAr (5′-ACG TCA TCT TCG GTG TAG CC-3′). While not all primers were species specific, all primers amplified the respective target in *E. coli* and the beef samples were essentially free of other bacteria after decontamination of the surface.

### 2.3. Preparation of beef steaks for intervention and control samples

For each trial, fresh beef (eye of round) was purchased from a local grocery store. It was then boiled in water for 2 min to decontaminate surface. The cooked outer layer (2 mm) was removed with a sterile knife and then the clean beef cut was sliced into 2 cm thick steaks with surface area of 88 cm$^2$. One mL of the 5 strain cocktail of *E. coli* O157:H7, corresponding to an inoculum of $10^7$ to $10^8$ cfu/cm$^2$, was inoculated onto the top surface of a beef steak in a biosafety cabinet which was immediately spread evenly across the surface of the steak with a sterile plastic spreader. Steaks with inoculum were left to air-dry in the biosafety cabinet for 30 min to allow for attachment. Three trials were performed per intervention and for each trial, eight steaks were inoculated with the 5 strain *E. coli* O157:H7 cocktail: four for application of interventions (2 × 0 min and 2 × 30 min sampling) and four for inoculated control (no application of intervention; 2 × 0 min and 2 × 30 min sampling). The sampling times were selected to allow for a comparison of the effect of different interventions on cell viability, injury and recovery after immediate application and after a 30 min holding time. One steak was left uninoculated as a control sample per trial to confirm that the steak did not contain detectable bacteria or bacterial nucleic acids, where no intervention was applied. The control steak was immediately placed in 99 mL of 100 mM PBS buffer (includes buffering agents monopotassium phosphate and disodium phosphate) in a stomacher bag, manually massaged for 2 min and suspension plated on LB and Violet Red Bile (VRB, Oxoid) agars. Plates were incubated at 30 °C for 24 h but all counts were below the detection limit of 100 CFU/cm$^2$, corresponding to fewer than 2 colonies per plate. Samples of one mL were kept for DNA qPCR analysis; similar to plating, quantification yielded negative result denoted by either amplification at or above 35 Ct with species specific primers (*uidA*) for DNA qPCR or 10 Ct above last sample's Ct value for non-specific primers (HDA, gapA) used in both RNA RT-qPCR experiments.

### 2.4. Lactic acid or peroxyacetic acid intervention

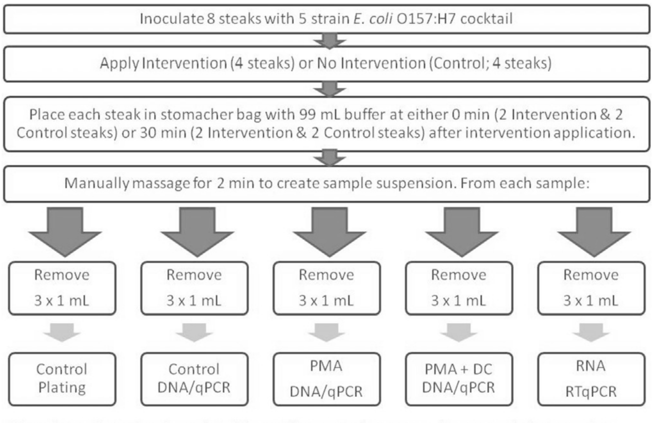
Prior to application of interventions, inoculated and control steaks were placed in separate aluminum trays (23 × 15 × 1.5 cm). A 5% (w/v) lactic acid (Sigma-Aldrich, Mississauga, Ontario, Canada) or 200 mg/L peroxyacetic acid (Inspexx, Ecolab Inc., St. Paul, Minnesota, USA) solution was prepared and connected to the equipment used for application of antimicrobials; this equipment has been previously described in detail (Youssef et al., 2012). The aluminum trays containing steaks were placed on the stainless steel conveyor belt that carried trays under a spray nozzle, exposing steaks to a 5% lactic acid spray or 200 mg/L peroxyacetic acid spray at a volume of 0.4 mL of fluid per cm$^2$ steak surface. The steaks were sampled immediately (0 min) or after 30 min of holding at room temperature.

### 2.5. Hot water intervention

To simulate spraying with hot water, which is used in beef processing as a pathogen intervention method, a 4 L beaker was filled with 2.5 L of sterile water and brought to 80 °C on a hot plate. Once the target temperature was reached, steaks were submerged individually for 10 s using sterile stainless steel tongs and immediately removed and sampled at 0 or 30 min.

### 2.6. Sampling of steaks after intervention

Each steak was placed in a sterile stomacher bag (VWR International, Radnor, Pennsylvania, USA) with 100 mL of buffer. PBS was used to neutralize lactic acid or hot water intervention samples and neutralizing buffer (includes buffering agent monopotassium phosphate and reducing agent sodium thiosulfate) was used for peroxyacetic acid intervention-treated samples to neutralize acidulant as well as quench oxidant. Steaks in buffer were then massaged manually in stomacher bags for 2 min. From sample suspension, 15 × 1 mL aliquots were transferred to separate 2 mL transparent Eppendorf tubes. Tubes were



Note: Leave 1 steak uninoculated for quality control purposes. Do not apply intervention; place in buffer, massage and only sample for control plating and control DNA/qPCR analysis.

**Fig. 1.** Experimental flow chart per trial.

centrifuged at 10,000 × *g* for 5 min, supernatants discarded, cells washed in 1 mL peptone water, centrifuged and resuspended in 300 μL peptone water. Each tube was designated to one of 5 different sampling groups for subsequent treatment application, in triplicate: DNA extraction (control; no PMA or deoxycholate), PMA treatment and DNA extraction, PMA with deoxycholate treatment and DNA extraction, RNA extraction, plating (Fig. 1). Samples for RNA extraction were further washed with RNAprotect Bacteria Reagent (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions to stabilize and protect RNA from degradation, the resulting pellet was kept frozen (−20 °C) until use.

### 2.7. PMA and deoxycholate treatment

For PMA treatment of samples, a 20 mM stock solution of PMA (Biotium, Inc. Hayward, California, USA) was stored in dark at 4 °C until use. For each sample suspension (300 μL) in 2 mL Eppendorf tubes, 1.5 μL of 20 mM PMA was added and vortexed thoroughly. Tubes were placed on ice horizontally in the dark for 5 min followed by exposure to a 650-W halogen lamp for 2 min, held 20 cm above the tubes, while slowly rocking samples back and forth to ensure uniform exposure. Samples were then removed from light, centrifuged at 8000 × *g* for 3 min, the supernatant was discarded, and the cells were washed with peptone water. The cell pellet was stored at −20 °C until further analysis.

For samples treated with both PMA and deoxycholate, deoxycholate was applied to samples before PMA treatment using a protocol previously optimized by Yang et al. (2011). A 1% (w/v) solution of deoxycholate (Sigma-Aldrich) was stored at −20 °C until use. Sample suspensions (300 μL) in 2 mL Eppendorf tubes were centrifuged at 8000 × *g* for 3 min, and the supernatants were discarded. The pellets were resuspended with 300 μL 1% deoxycholate and incubated in a rocking water bath at 37 °C for 30 min. After incubation, the suspension was treated with PMA as described above.

### 2.8. Enumeration of E. coli O157:H7 on beef steaks

Viable cell counts were enumerated by surface plating on the day of treatment. Ten-fold dilutions were prepared in sterile 0.1% peptone water from original sample suspensions of beef steaks in stomacher bags with buffer, and 100 μL of dilutions estimated to provide 30–300 colonies per plate was used to manually plate onto LB and VRB agar. Plates were incubated at 30 °C for 24 h and enumerated next day. Uninjured cells (capable of reproduction without repair of cellular injury) were enumerated on VRB agar, a medium which prevents the growth of injured cells with compromised outer membrane due to the presence of bile salts (Gänzle and Vogel, 2001). All viable (live) cells (capable of reproduction, including cells that require repair of cellular injury prior to reproduction) were enumerated on LB agar, a non-selective medium (Scheusner et al., 1971).

### 2.9. DNA extraction and quantification in qPCR

DNA was extracted from samples (DNeasy blood and tissue kit; Qiagen) following instructions for Gram-negative bacteria with modifications. Cells suspended in buffer ATL (supplemented with proteinase K) were incubated for 2 h, and DNA was eluted from mini columns twice with 100 μL of butter AE. For quantification in qPCR, primers URL-301 and URR-432 were used to target *uidA* gene (Sigma-Aldrich). qPCR master mix was prepared as follows per reaction: 12.5 μL of SYBR green (Qiagen), 2.5 μL each forward and reverse primer, and 2.5 μL nuclease free water, with 5 μL template DNA added to 20 μL of master mix for a total volume of 25 μL per reaction. A standard curve was prepared from purified PCR product of *E. coli* O157:H7 (00–3581) DNA (QIAquick PCR Purification Kit; Qiagen), which was diluted to 100 mg/L and then further diluted in serial ten-fold dilutions. Six dilutions were used to generate a standard curve in the range of DNA concentrations in the samples. Nuclease free water served as negative control for all PCR reactions; uninoculated steaks served as negative control for native *E. coli* that may be already present on steak. A 7500 Fast Real-Time PCR system (Applied Biosystems) was used for quantification of DNA by one initial denaturation of template DNA and activation of Taq DNA polymerase at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 1 min, extension at 72 °C for 1 min. Quantification data was acquired from 7500 Software (version 2.0.5, Applied Biosystems) and analyzed. Melting curves of final products verified no formation of non-specific products or primer dimers. Each sample was quantified in duplicate per qPCR plate.

### 2.10. RNA extraction and quantification in RT-qPCR

RNA was extracted from samples (RNeasy Mini Kit; Qiagen) and reverse transcribed to cDNA following instructions stated in the Kit's manual (QuantiTect Reverse Transcription Kit; Qiagen). Two sets of primers were selected for RNA analysis, one targeting partial 16 S rRNA (HDAf and HDAr; Sigma-Aldrich) and the second targeting the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (gapAf and gapAr; Sigma-Aldrich). Stocks were prepared for each primer set with sterile nuclease free water. Master mix recipe per reaction was prepared as follows: 12.5 μL SYBR green (Qiagen), 0.5 μL each forward and reverse primers, 10.5 μL nuclease free water, and 1 μL sample cDNA. A standard curve was developed by extracting genomic DNA from an overnight culture of *E. coli* O157:H7 (00–3581) using Wizard Genomic DNA purification kit (Promega). Extracted genomic DNA was run in qPCR with respective primers (HDA or *gapA*) and the PCR product was purified according to purification kit instructions (QIAquick PCR Purification Kit, Qiagen) and diluted to 100 mg/L. For each primer set, this concentration was further diluted ten-fold (up to $10^{-8}$) to generate a standard curve. Negative controls included nuclease free water, samples after treatment with genomic DNA Wipeout but before reverse transcription, and isolation of RNA from uninoculated steaks. The Reverse Transcription qPCR (RT-qPCR) method was one initial denaturation of template DNA and activation of Taq DNA polymerase at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, primer annealing at 60 °C for 30 s, extension at 72 °C for 30 s. Data was acquired from 7500 software (version 2.0.5, Applied Biosystems) and analyzed. Melting curves of final products were used to verify that non-specific products or primer dimers did not form. Samples were run in triplicate reaction per plate.

### 2.11. Statistical analysis

Statistical Analysis Systems (SAS Institute, Inc., Cary, NC) was used to evaluate data by two way analysis of variance (ANOVA) followed by pair-wise comparison of means from different treatment groups with Bonferroni adjustment. A probability (*p*) of $< 0.05$ was considered statistically significant. Three independent trials were performed for each intervention, with triplicate samples per trial. Linear regressions were calculated in SigmaPlot 12.5.

## 3. Results

### 3.1. Viable plate counts and sublethal injury on treated and untreated beef

Cell counts for untreated, inoculated beef were on average 7.27 ± 0.15 log CFU/cm$^2$ (mean ± standard deviation); the difference in plate counts between LB and VRB, which represents the sub-lethally injured population, on average was 0.33 ± 0.18 log CFU/cm$^2$. After intervention with lactic acid or hot water, the difference in plate counts on LB and VRB increased, indicating that the number of injured cells had increased. However, the average proportion of injured cells was not increased by peroxyacetic acid treatment compared to the control (Table 1). Comparison of post-intervention counts to the controls indicated that the percentage of dead cells in samples was 68%, 45% and 90% for meat treated by lactic acid, peroxyacetic acid and hot water, respectively.

### 3.2. Quantification of *E. coli* O157:H7 on beef by qPCR after lactic acid intervention

To determine the method that most accurately quantifies viable *E. coli* O157:H7 on beef steak after lactic acid intervention, five different detection methods were compared. Fig. 2A and B correlate viable plate counts of *E. coli* O157:H7 after lactic acid intervention to the gene copy numbers obtained by quantification of microbial DNA or RNA. Control samples represent steaks that were inoculated but not treated; control quantification represents DNA quantification without PMA or PMA-DC. Lactic acid intervention reduced viable cell counts by about 1 log CFU/cm$^2$ (Table 1), however, this reduction was not significant. Interestingly, significant reduction in the log gene copy #/cm$^2$ of *E. coli* O157:H7 was determined by qPCR only in samples treated with PMA combined with deoxycholate. However, reductions observed at 0 and 30 min indicate that the combination treatment not only prevented amplification of DNA from cells killed by intervention, but it also prevented the amplification of DNA from sub-lethally injured cells when compared to plate count data. This explains why reductions were significant as compared to plating. Accordingly, the slope of the regression line correlating cell counts and gene copy numbers was $< 1.0$ for qPCR and PMA-qPCR quantification but $> 1$ for PMA-deoxycholate qPCR. Quantification with RT-qPCR targeting rRNA or mRNA provided comparable results (Fig. 2B).

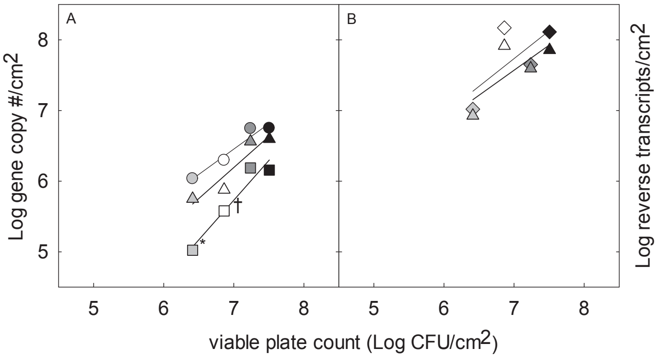


**Fig. 2.** (**A**) Correlation of cell counts on LB agar & qPCR DNA quantification of *E. coli* O157:H7 recovered from beef after lactic acid (LA) intervention & PMA or PMA + deoxycholate (DC) treatment. Circle, control; triangle, PMA; square, PMA + DC; black, control 0 min; white, LA 0 min; dark grey, control 30 min; light grey, LA 30 min. Treatments plotted at log CFU/cm$^2$ of respective control or LA sample at 0 or 30 min. Values are means of three trials with technical repeat (n = 3), standard error of means of LB ≤ 0.32; DNA ≤ 0.72. †Significantly different log gene copy #/cm$^2$ from Control at 0 & 30 min ($p < 0.05$). *Significantly different log gene copy #/cm$^2$ from Control, Control + PMA, Control + PMA + DC and LA at both 0 & 30 min ($p < 0.05$). Correlation coefficients r$^2$ of the linear regressions were 0.94 (control), 0.88 (PMA) and 0.94 (PMA + DC). (**B**) Correlation of cell counts on LB agar & RTqPCR RNA quantification of *E. coli* O157:H7 recovered from beef after LA intervention. Diamond, rRNA; triangle, mRNA; black, control 0 min; white, LA 0 min; dark grey, control 30 min; light grey, LA 30 min. Treatments plotted at log CFU/cm$^2$ of respective control or LA sample at 0 or 30 min. Values are means of three trials (n = 3) with technical repeat, standard error of means of rRNA ≤ 0.68; mRNA ≤ 0.65. Correlation coefficients r$^2$ of the linear regressions were 0.49 (rRNA) and 0.55 (mRNA).

### 3.3. Quantification of *E. coli* O157:H7 on beef by qPCR after peroxyacetic acid intervention

Similarly, after peroxyacetic acid intervention of steaks inoculated with *E. coli* O157:H7, five different detection methods were compared to determine the most accurate method to quantify viable cells. Fig. 3 A and B correlate quantification of *E. coli* O157:H7 on beef after peroxyacetic acid intervention. Enumeration on LB agar was compared to DNA or RNA quantification in qPCR or RT-qPCR, respectively. After peroxyacetic acid intervention, plate counts and the number of gene copies were not reduced at 0 or 30 min sampling times. The reduction of cell counts or of the copy number of nucleic acids after combination treatment of PMA with deoxycholate was also not significant. This is likely because this intervention did not cause the same degree of sub-

**Table 1**
Enumeration of *E. coli* O157:H7 (Log CFU/cm$^2$) Recovered on LB and VRB agars from Beef Steaks held for 30 min after Interventions of Lactic Acid, Peroxyacetic Acid and Hot Water and after Treatment of cells with PMA or PMA with Deoxycholate.

| Sample | Intervention | | | | | |
|---|---|---|---|---|---|---|
| | Lactic Acid | | Peroxyacetic Acid | | Hot Water | |
| | LB | VRB | LB | VRB | LB | VRB |
| Control | 7.24 ± 0.08 | 6.85 ± 0.27 | 7.00 ± 0.40 | 6.70 ± 0.70 | 7.35 ± 0.09$^a$ | 7.03 ± 0.18$^a$ |
| Intervention | 6.41 ± 0.14 | 5.27 ± 0.69 | 6.48 ± 0.13 | 6.08 ± 0.57 | 6.19 ± 0.54$^{ab}$ | 5.65 ± 0.62$^{ab}$ |
| Control + PMA | 6.65 ± 0.30 | 6.45 ± 0.17 | 6.98 ± 0.45 | 6.63 ± 0.79 | 7.57 ± 0.16$^a$ | 7.11 ± 0.46$^a$ |
| Intervention + PMA | 5.91 ± 0.54 | 5.44 ± 0.45 | 6.29 ± 0.61 | 5.75 ± 1.17 | 6.16 ± 0.64$^{ab}$ | 5.88 ± 0.77$^{ab}$ |
| Control + PMA + DC | 6.57 ± 0.09 | 6.15 ± 0.70 | 6.41 ± 0.16 | 6.22 ± 0.25 | 6.46 ± 0.22$^{ab}$ | 6.09 ± 0.48$^{ab}$ |
| Intervention + PMA + DC | 5.58 ± 0.47 | 4.85 ± 0.94 | 5.70 ± 0.41 | 5.62 ± 0.31 | 5.20 ± 0.72$^b$ | 4.85 ± 0.68$^b$ |

Values are mean ± standard deviation, n = 3. Values with superscripts are significantly different among values in the same column; those that do not share a common superscript are significantly different ($p < 0.05$).

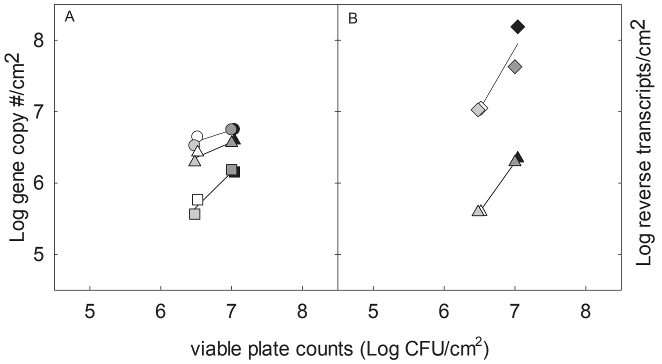


**Fig. 3.** (**A**) Correlation of cell counts on LB agar & qPCR DNA quantification of *E. coli* O157:H7 recovered from beef after peroxyacetic acid (PA) intervention and PMA or PMA with deoxycholate treatment. Circle, control; triangle, PMA; square, PMA with deoxycholate; black, control 0 min; white, PA 0 min; dark grey, control 30 min; light grey, PA 30 min. Treatments plotted at log CFU/$cm^2$ of respective control or PA sample at 0 or 30 min. Values are means of three trials with technical repeat (n = 3), standard error of means of LB ≤ 0.39; DNA ≤ 0.68. Correlation coefficients $r^2$ of the linear regressions were 0.83 (control), 0.87 (PMA) and 0.94 (PMA + DC). (**B**) Correlation of cell counts on LB agar & RTqPCR RNA quantification of *E. coli* O157:H7 recovered from beef after PA intervention. Diamond, rRNA; triangle, mRNA; black, control 0 min; white, PA 0 min; dark grey, control 30 min; light grey, PA 30 min. Treatments plotted at log CFU/$cm^2$ of respective control or PA sample at 0 or 30 min. Values are means of three trials with technical repeat (n = 3), standard error of means of rRNA ≤0.35; mRNA ≤1.12. Correlation coefficients $r^2$ of the linear regressions were 0.87 (rRNA) and 1.00 (mRNA).

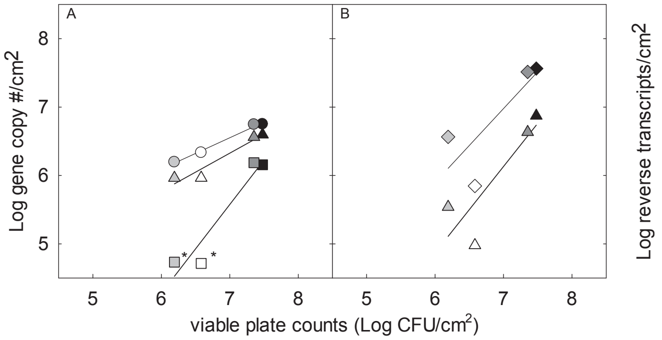


**Fig. 4.** (**A**) Correlation of cell counts on LB agar & qPCR DNA quantification of *E. coli* O157:H7 recovered from beef after hot water (HW) intervention & PMA or PMA with deoxycholate treatment. Circle, control; triangle, PMA; square, PMA with deoxycholate; black, control 0 min; white, HW 0 min; dark grey, control 30 min; light grey, HW 30 min. Treatments plotted at log CFU/$cm^2$ of respective control or HW sample. Values are means of three trials with technical repeat (n = 3), standard error of means of LB ≤ 0.54; DNA ≤ 0.93. *Significantly different log gene copy #/$cm^2$ from Control, Control + PMA, Control + PMA + DC, HW, HW + PMA at 0 and 30 min ($p < 0.05$). Correlation coefficients $r^2$ of the linear regressions were 0.99 (control), 0.93 (PMA) and 0.92 (PMA + DC). (**B**) Correlation of cell counts on LB agar & RTqPCR RNA quantification of *E. coli* O157:H7 recovered from beef after HW intervention. Diamond, rRNA; triangle, mRNA; black, control 0 min; white, HW 0 min; dark grey, control 30 min; light grey, HW 30 min. Treatments plotted at log CFU/$cm^2$ of respective control or HW sample at 0 or 30 min. Values are means of three trials with technical repeat (n = 3), standard error of means of rRNA ≤ 1.48; mRNA ≤ 1.20. Correlation coefficients $r^2$ of the linear regressions were 0.66 (rRNA) and 0.75 (mRNA).

lethal injury as the other two interventions.

### 3.4. *Quantification of E. coli O157:H7 on beef by qPCR after hot water intervention*

The same five different detection methods were compared to determine the most accurate method to quantify live cells after hot water intervention on beef steaks inoculated with *E. coli* O157:H7. The correlation of quantification of *E. coli* O157:H7 on beef after hot water intervention by means of enumeration on LB agar as compared to DNA or RNA quantification in qPCR or RT-qPCR is presented in Fig. 4 A and B, respectively. Enumeration by plating, qPCR or RT-qPCR determined that the numbers of viable *E. coli* O157:H7 on steaks at both 0 and 30 min were not significantly reduced by hot water intervention. However, PMA with deoxycholate treatment and qPCR quantification reduced the log gene copy #/$cm^2$ at 0 and 30 min. The significant reduction is likely attributable to the degree of sub-lethal injury that the intervention caused, and that this population of cells was sensitive to the combination of PMA with deoxycholate treatment, which then led to the prevention of amplification in qPCR. Accordingly, the slope of the regression line correlating plate counts to gene copy numbers was 1.26 for quantification with PMA-deoxycholate qPCR but only about 0.5 for quantification with qPCR and PMA-qPCR (Fig. 4A). Quantification with RT-qPCR targeting rRNA or mRNA provided comparable results with a slope of 1.1 and 1.3, respectively (Fig. 4B).

### 3.5. *E. coli O157:H7 recovered on LB and VRB from beef steaks at 30 min after intervention and after treatment of cells with PMA or PMA with deoxycholate*

qPCR quantification indicated that the combination of PMA and deoxycholate treatment permeabilized viable *E. coli* cells to PMA after intervention when compared to control and cell count enumeration data by plating. To determine whether treatment with PMA and PMA-DC affects viability of *E. coli*, the organism was enumerated after intervention treatments on beef steaks and PMA or PMA-deoxycholate treatment (Table 1). PMA did not affect the viability of *E. coli* (Table 1). Treatment with both PMA and deoxycholate reduced the viability of *E. coli*; this reduction was significant after treatment of *E. coli* that were previously exposed to hot water washes on beef. Taken together, these data suggest that PMA-deoxycholate may permeabilize and kill *E. coli* when cells were sublethally injured by intervention treatments.

## 4. Discussion

This study compared five different detection methods for the quantification of viable *E. coli* O157 on beef, and after intervention treatments that are currently used in the meat industry. The application of lactic acid, peroxyacetic acid and hot water interventions to beef steaks created a heterogeneous population of viable, sub-lethally injured, and dead cells. These populations are highly suitable for testing detection methods for the ability to discriminate between live and dead bacterial cells. Lactic acid and hot water interventions were more lethal to *E. coli* O157:H7 on beef than peroxyacetic acid, which is consistent with previous studies (Castillo et al., 1998; Ellebracht et al., 2005; Gill and Bryant, 2000; King et al., 2005; Youssef et al., 2012). Experiments reported in this study were obtained with aseptic meat samples without background microbiota and primers that were not specific to *E. coli* O157. Equivalent results can be expected if primer sets with more specific targets are used to specifically detect *E. coli* O157 or EHEC in commercial meat samples.

Overall, the cell counts obtained by plating on LB agar were similar to RT-qPCR based quantification that targeted rRNA; mRNA copy numbers and DNA copy numbers were generally lower. This discrepancy reflects that mRNA is less stable than rRNA and that *E. coli* cells contain several thousand ribosomes (Keer and Birch, 2003). The decrease of viable cell counts after intervention treatments was reflected by a reduced copy number obtained by RT-qPCR. However, qPCR targeting DNA did not discriminate between viable and dead cells, though plating did (Sidari et al., 2011). Furthermore, rRNA provided a more robust estimation than mRNA, which is present at a lower percentage of total RNA and is more susceptible to degradation as compared to rRNA (Kushner, 2002).

Enumeration of viable cells by surface plating indicated that lactic acid was more lethal than peroxyacetic acid; this difference was not observed by RT-qPCR, demonstrating that conventional plating provided the most accurate estimate of viable cells. Hot water intervention resulted in a greater reduction in viable cells from the plate count quantification method and both RNA RT-qPCR quantification methods. Hot water washes acted immediately while lactic acid acted more slowly. The antimicrobial effect of lactic acid is based on penetration of cells and lowering intracellular pH (Alakomi et al., 2005) while heat leads to immediate disruption of membranes and irreversible denaturation of proteins (Lee and Kaletunc, 2002).

PMA treatment does not affect viability of cells (Elizaquivel et al., 2012; Nocker et al., 2006b, Yang et al., 2011; this study). PMA-qPCR, reduced the gene copy numbers detected in beef samples but the difference between qPCR and PMA-qPCR was small and remained unchanged by bactericidal intervention treatments (Figs. 2–4). A high number of dead cells may inhibit PMA crosslinking of DNA by light and thus reduce the correlation to viable plate counts (Løvdal et al., 2011; Pan and Breidt, 2007; Wagner et al., 2008; Yañez et al., 2011). To counter this, it has been suggested that incubating samples for a longer period of time and at higher temperatures to increase PMA penetration into dead cells (Nkuipou-kenfack et al., 2013).

Several past studies compare PMA-qPCR to quantification of live cells of foodborne pathogens by plate counts. Løvdal et al. (2011) applied lethal heat treatment of 80 °C for 15 min to *Listeria innocua* cells in broth and found large discrepancies between quantification methods: plating determined cells counts below the detection limit whereas PMA-qPCR quantified 8 log cells/mL Enumeration of *Campylobacter jejuni* on raw chicken carcasses after different slaughterhouse processing steps determined that PMA-qPCR consistently yielded up to a 1–1.5 log CFU/mL higher cell count than plating (Pacholewicz et al., 2013). After severe heat treatment of *C. jejuni* in chicken rinse at 95 °C for 5 min, PMA-qPCR and plating both indicated that cells counts were below the detection limit (Josefsen et al., 2010). Lastly, Ju et al. (2016) quantified live *E. coli* O157:H7 on lettuce. Plating indicated that *E. coli* O157:H7 did not survive on leaf surfaces and was reduced by $10^7$ cells within 96 h; however, RT-qPCR and PMA-qPCR indicated a reduction of $10^2$ and $10^3$ cells, respectively. Overall, a majority of studies indicate that PMA-qPCR can lead to false positive results. This conforms to the observation that the structurally related propidium iodide is excluded by a substantial proportion of dead cells after lethal interventions (Ulmer et al., 2000). Some studies defined PI or PMA excluding but non-culturable cells "viable but not culturable"; however, sublethally injured cells of *E. coli* are readily recovered by cultivation media containing pyruvate or catalase as scavengers for oxidative stress (Afari and Hunc, 2018; Mizunoe et al., 1999). We used LB as reference medium for detection of viable cells and thus did not account for those cells that require extended recovery times or addition of pyruvate or catalase to recover from injury.

The use of deoxycholate prior to PMA treatment was proposed to increase cell membrane permeability to permeabilize all dead cells (Yang et al., 2014). In suspensions where *E. coli* cells were killed after heat treatment with no viable cells remaining, treatment with PMA prior to qPCR significantly decreases amplification of dead cells in qPCR as compared to PMA alone (Yang et al., 2014). *E. coli* cells that were killed by 72 °C or less were not permeable to PMA but the addition of 1% deoxycholate treatment rendered a large portion of these cells permeable to the dye (Yang et al., 2011). After lactic acid intervention, membranes of dead and injured *E. coli* cells that were impermeable to PMA were rendered permeable to the dye after treatment with 1% deoxycholate treatment (Wang et al., 2014). Injured cells were resuscitated in nutrient rich broth for 2 h prior to deoxycholate treatment which restored injured cell membrane barrier properties. This could be a possible solution to injured cell sensitivity to PMA with deoxycholate treatment. However, since our study was performed in a more complex system rather than in broth or buffer, this could prove to be more complicated and warrants further investigation. Zhou et al. (2016) optimized the concentration of deoxycholate for use with *Cronobacter sakazakii* in powdered infant formula. The authors found that heat injured cells were not affected by a concentration of 0.08% deoxycholate and determined that at this concentration, the accuracy of viable cell detection when combined with PMA and qPCR was increased. Sodium dodecyl sulfate has also been used to aid in PMA penetration into dead cells and shows little impact on cell viability. While this could offer an alternative emulsifier to use with PMA to increase the accuracy of live cell detection in qPCR, it still requires further research in different food systems (Dong et al., 2018; Takahashi et al., 2017). Our work indicates that combination treatment of PMA with deoxycholate prevents amplification of DNA from sublethally injured cells, and could lead to false negative results.

The inability to discriminate between DNA in live and dead cells in qPCR is a problem that extends beyond *E. coli* O157:H7 in meat, this combination treatment could be a solution in other areas of research. This includes determination of viable probiotics in the gut or viable lactic acid bacteria or bifidobacteria in fermented foods (Cocolin et al., 2011; Garcia-Cayuela et al., 2009; Rantsiou and Cocolin, 2006; Villarreal et al., 2013). However, it is important to note that concentration of treatment should be adjusted to target microorganism, as Gram-positive microorganisms have different resistance to deoxycholate than Gram-negative microorganisms (Nkuipou-kenfack et al., 2013).

## 5. Conclusions

This study compared five different methods to determine the most accurate one for quantification of viable *E. coli* O157:H7 on beef steaks after intervention. PMA treatment with qPCR did not prevent DNA amplification from all dead cells in samples after intervention and therefore this treatment does not resolve the issue of false positive result in qPCR. However, it does prevent amplification of some dead cell DNA and therefore increases the accuracy of viable cell quantification compared to qPCR alone. In contrast, treatment of cells with PMA with deoxycholate prevented amplification of DNA from all dead cells but does so at the cost of some sub-lethally injured cells since deoxycholate supported PMA penetration into injured cells which may have otherwise recovered. This makes DNA inaccessible for qPCR quantification and would lead to false negative result. Both rRNA and mRNA quantification in qPCR had more variability and was not as sensitive for quantifying viable cells as plate count. While PMA with deoxycholate may not be suitable for pathogen detection in food where sublethal injury of cells is of concern, it could be used in other areas of research that aim to exclude all dead cells at the expense of injured ones. On the other hand, if research aims to amplify all viable cells including the injured cell population at the cost of also amplifying some dead cell DNA, PMA or rRNA with qPCR detection methods could be used. Overall, conventional plating provided the most sensitive and reliable quantification of viable *E. coli* O157:H7 on beef.

## Acknowledgements

The authors would like to thank Agriculture and Agri-Food Canada A-Base (1022) Funding for providing project support. Funding source had no involvement in study design, collection, analysis and interpretation of data, in writing of the report or in submission of the article.

## References

Afari, G.K., Hunc, Y.C., 2018. Detection and verification of the viable but nonculturable (VBNC) state of *Escherichia coli* O157:H7 and *Listeria monocytogenes* using flow cytometry and standard plating. J. Food Sci. https://doi.org/10.1111/1750-3841.

14203. (in press).

Alakomi, H., Skyttä, E., Saarela, M., Helander, I.M., 2005. Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. Appl. Environ. Microbiol. 66, 2000–2005.

Bae, S., Wuertz, S., 2009. Discrimination of viable and dead fecal *Bacteroidales* bacteria by quantitative PCR with propidium monoazide. Appl. Environ. Microbiol. 75, 2940–2944.

Bacon, R., Belk, K., Sofos, J., Clayton, R., Reagan, J., Smith, G., 2000. Microbial populations on animal hides and beef carcasses at different stages of slaughtering in plants employing multiple-seqiential interventions for decontamination. J. Food Protect. 63, 1080–1086.

Bej, A., DiCesare, J., Haff, L., Atlas, R., 1991. Detection of *Escherichia coli* and *Shigella* spp in water by using the polymerase chain-reaction and gene probes for *uid*. Appl. Environ. Microbiol. 57, 1013–1017.

Blagojevic, B., Antic, D., Ducic, M., Buncic, S., 2012. Visual cleanliness scores of cattle at slaughter and microbial loads on the hides and the carcases. Vet. Rec. 170 563–563.

Castillo, A., Lucia, L., Goodson, K., Savell, J., Acuff, G., 1998. Use of hot water for beef carcass decontamination. J. Food Protect. 61, 19–25.

Cocolin, L., Dolci, P., Rantsiou, K., 2011. Biodiversity and dynamics of meat fermentations: the contribution of molecular methods for a better comprehension of a complex ecosystem. Meat Sci. 89, 296–302.

Croxen, M.A., Law, R.J., Scholz, R., Keeney, K.M., Wlodarska, M., Finlay, B.B., 2013. Recent advances in understanding enteric pathogenic *Escherichia coli*. Clin. Microbiolol. Rev. 26, 822–880.

Dong, L., Liu, H., Meng, L., Xing, M., Wang, J., Wang, C., Chen, H., Zheng, N., 2018. Quantitative polymerase chain reaction coupled with sodium dodecyl sulfate and propidium monoazide for detection of viable *Staphylococcus aureus* in milk. J. Dairy Sci. 101, 1–8.

Elizaquível, P., Sánchez, G., Aznar, R., 2012. Quantitative detection of viable foodborne *E. coli* O157 : H7, *Listeria monocytogenes* and *Salmonella* in fresh-cut vegetables combining propidium monoazide and real-time PCR. Food Contr. 25, 704–708.

Ellebracht, J.W., King, D.A., Castillo, A., Lucia, L.M., Acuff, G.R., Harris, K.B., Savell, J.W., 2005. Evaluation of peroxyacetic acid as a potential pre-grinding treatment for control of *Escherichia coli* O157 : H7 and *Salmonella* Typhimurium on beef trimmings. Meat Sci. 70, 197–203.

Flekna, G., Polonca, S., Smulders, F.J.M., Mo, S.S., Hein, I., 2007. Insufficient differentiation of live and dead *Campylobacter jejuni* and *Listeria monocytogenes* cells by ethidium monoazide ( EMA ) compromises EMA/real-time PCR. Res. Microbiol. 158, 405–412.

Gänzle, M.G., Vogel, R.F., 2001. On-line fluorescence determination of pressure mediated outer membrane damage in *Escherichia coli*. Syst. Appl. Microbiol. 24, 477–485.

Garcia-Cayuela, T., Tabasco, R., Pela, C., Requena, T., 2009. Simultaneous detection and enumeration of viable lactic acid bacteria and bifidobacteria in fermented milk by using propidium monoazide and real-time PCR. Int. Dairy J. 19, 405–409.

Ge, B., Meng, J., 2009. Advanced technologies for pathogen and toxin detection in foods: current applications and future directions. J. Assoc. Lab. Autom. 14, 235–241.

Gill, C., Bryant, J., 2000. The effects on product of a hot water pasteurizing treatment applied routinely in a commercial beef carcass dressing process. Food Microbiol. 17, 495–504.

Health Canada, 2018. Laboratory procedures for the microbiological analysis of foods. Compendium Anal. Method. Vol. 3. http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3-eng.php, Accessed date: 4 May 2018.

Josefsen, M.H., Lo, C., Hansen, T.B., Christensen, L.S., Olsen, J.E., Hoorfar, J., 2010. Rapid quantification of viable *Campylobacter* bacteria on chicken carcasses, using real-time PCR and propidium monoazide treatment, as a tool for quantitative risk assessment. Appl. Environ. Microbiol. 76, 5097–5104.

Ju, W., Moyne, A.L., Marco, M.L., 2016. RNA-based detection does not accurately enumerate living Escherichia coli O157:H7 cells on plants. Front. Microbiol. 7 223-223.

Keer, J.T., Birch, L., 2003. Molecular methods for the assessment of bacterial viability. J. Microbiol. Methods 53, 175–183.

King, D.A., Lucia, L.M., Castillo, A., Acuff, G.R., Harris, K.B., Savell, J.W., 2005. Evaluation of peroxyacetic acid as a post-chilling intervention for control of *Escherichia coli* O157 : H7 and *Salmonella* Typhimurium on beef carcass surfaces. Meat Sci. 69, 401–407.

Kushner, S., 2002. mRNA decay in *Escherichia coli* comes of age. J. Bacteriol. 184, 4658–4665.

Lee, J., Kaletunc, G., 2002. Evaluation of the heat inactivation of *Escherichia coli* and *Lactobacillus plantarum* by differential scanning calorimetry. Appl. Environ. Microbiol. 68, 5379–5386.

Løvdal, T., Befring, M., Björkblom, B., Møller, S.G., 2011. Propidium monoazide combined with real-time quantitative PCR underestimates heat-killed *Listeria innocua*. J. Microbiol. Methods 85, 164–169.

Luciano, F.B., Belland, J., Holley, R.A., 2011. Microbial and chemical origins of the bactericidal activity of thermally treated yellow mustard powder toward Escherichia coli O157:H7 during dry sausage ripening. Int. J. Food Microbiol. 145, 69–76.

Mizunoe, Y., Wai, S.N., Takade, A., Yoshida, S.I., 1999. Restoration of culturability of starvation-stressed and low-temperature-stressed *Escherichia coli* O157 cells by using $H_2O_2$-degrading compounds. Arch. Microbiol. 172, 63–67.

Nocker, A., Camper, A.K., 2006. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. Appl. Environ. Microbiol. 72, 1997–2004.

Nocker, A., Cheung, C., Camper, A.K., 2006. Comparison of propidium monoazide with ethidium monoazide for differentiation of live vs. dead bacteria by selective removal of DNA from dead cells. J. Microbiol. Methods 67, 310–320.

Nocker, A., Sossa, K.E., Camper, A.K., 2007. Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. J. Microbiol. Methods 70, 252–260.

Nkuipou-kenfack, E., Engel, H., Fakih, S., Nocker, A., 2013. Improving efficiency of viability-PCR for selective detection of live cells. J. Microbiol. Methods 93, 20–24.

Pacholewicz, E., Swart, A., Lipman, L.J., Wagenaar, J., Havelaar, A.H., Duim, B., 2013. Propidium monoazide does not fully inhibit the detection of dead *Campylobacter* on broiler chicken carcasses by qPCR. J. Microbiol. Methods 95, 32–38.

Pan, Y., Breidt, F., 2007. Enumeration of viable *Listeria monocytogenes* cells by real-time PCR with propidium monoazide and ethidium monoazide in the presence of dead cells. Appl. Environ. Microbiol. 73, 8028–8031.

Rangel, J.M., Sparling, P.H., Crowe, C., Griffin, P.M., Swerdlow, D.L., 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. Emerg. Infect. Dis. 11, 603–609.

Rantsiou, K., Cocolin, L., 2006. New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. Int. J. Food Microbiol. 108, 255–267.

Scheusner, S., Busta, F., Speck, M., 1971. Inhibition of injured *Escherichia coli* by several selective agents. Appl. Microbiol. 21, 46–49.

Sidari, R., Caridi, A., 2011. Methods for detecting Enterohaemorrhagic *Escherichia coli* in food. Food Rev. Int. 27, 134–153.

Takahashi, H., Gao, Y., Miya, S., Kuda, T., Kimura, B., 2017. Discrimination of live and dead cells of *Escherichia coli* using propidium monoazide after sodium dodecyl sulfate treatment. Food Contr. 71, 79–82.

Taskin, B., Gozen, A.G., Duran, M., 2011. Selective quantification of viable *Escherichia coli* bacteria in biosolids by quantitative PCR with propidium monoazide modification. Appl. Environ. Microbiol. 77, 4329–4335.

Ulmer, H.M., Gänzle, M.G., Vogel, R.F., 2000. Effects of high pressure on survival and metabolic activity of *Lactobacillus plantarum*. Appl. Environ. Microbiol. 66, 3966–3973.

Villarreal, M., Padilha, M., Diogo, A., Vieira, S., Franco, B., Martinez, R., Saad, S., 2013. Advantageous direct quantification of viable closely related probiotics in petit-suisse cheeses under in vitro gastrointestinal conditions by propidium monoazide - qPCR. PloS One 8, 1–11.

Wagner, A.O., Malin, C., Knapp, B.A., Illmer, P., 2008. Removal of free extracellular DNA from environmental samples by ethidium monoazide and propidium monoazide. Appl. Environ. Microbiol. 74, 2537–2539.

Walter, J., Tannock, G., Tisala-Timisiaarvi, A., Rodtong, S., Loach, D., Munroe, K., Alatossava, T., 2000. Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. Appl. Environ. Microbiol. 66, 297–303.

Wang, H., Gill, C., Yang, X., 2014. Development of a real-time PCR procedure for quantification of viable *Escherichia coli* in populations of *E. coli* exposed to lactic acid, and the acid tolerance of verotoxigenic *E. coli* (VTEC) from cattle hides. Food Contr. 43, 104–109.

Wang, Y., Ametaj, B., Ambrose, D., Gänzle, M., 2013. Characterisation of the bacterial microbiota of the vagina of dairy cows and isolation of pediocin-producing *Pediococcus acidilactici*. BMC Microbiol. 13, 19.

Wong, M.L., Medrano, J.F., 2005. Real-time PCR for mRNA quantitation. Biotechniques 1, 1–11 39.

Yáñez, M.A., Nocker, A., Soria-soria, E., Múrtula, R., Martínez, L., Catalán, V., 2011. Quantification of viable *Legionella pneumophila* cells using propidium monoazide combined with quantitative PCR. J. Microbiol. Methods 85, 124–130.

Yang, X., Badoni, M., Wang, H., Gill, C.O., 2014. Effects of mild and pasteurizing heat treatments on survival of generic and verotoxigenic *Escherichia coli* from beef enrichment cultures. Food Contr. 39, 100–104.

Yang, X., Badoni, M., Gill, C.O., 2011. Use of propidium monoazide and quantitative PCR for differentiation of viable *Escherichia coli* from *E. coli* killed by mild or pasteurizing heat treatments. Food Microbiol. 28, 1478–1482.

Youssef, M.K., Yang, X., Badoni, M., Gill, C.O., 2012. Effects of spray volume, type of surface tissue and inoculum level on the survival of *Escherichia coli* on beef sprayed with 5% lactic acid. Food Contr. 25, 717–722.

Zhou, B., Chen, B., Wu, X., Li, F., Yu, P., Aguilar, Z., Wei, H., Xu, H., 2016. A new application of a sodium deoxycholate-propidium monoazidequantitative PCR assay for rapid and sensitive detection of viable *Cronobacter sakazakii* in powdered infant formula. J. Dairy Sci. 99, 9550–9559.