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DEVELOPMENT OF THE LACTIC ACID PERMEABILIZER v-qPCR TECHNIQUE FOR *Salmonella* spp. DETECTION AND QUANTIFICATION

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Abstract

In the food industry, one of the biggest challenges is microbial contamination. Viability real-time PCR (v-qPCR) technology has made the detection and quantification of pathogens easier, and some issues remain with the accuracy of the results, especially in samples containing viable and non-viable cells. V-qPCR with propidium monoazide (PMA) and pre-treatment with permeability agents is one promising method to circumvent this issue. Therefore, the aim of the study was to improve the Salmonella spp. detection using a method with lactic acid (LA 5-10 mM) pre-treatment that permeabilized Gram-negative outer membrane. The signal reduction (dC_t = 13.97) after 10 mM LA pre-treatment was similar with the reduction in C_t signal (dC_t = 13.62) when only PMA treatment was applied to a suspension of non-viable cells. In the case of viable cells, the LA pre-treatment improved the v-qPCR detection. These preliminary studies provide useful information on the use of lactic acid, which has proven to be an effective, cheap and low-toxic permeabilizing agent.

Key words: *lactic acid, PMA, v-qPCR, pathogen detection, food safety application.*

INTRODUCTION

The pathogen *Salmonella* spp. can cause severe foodborne illness, so it is important to develop and improve detection technologies for this bacterium. The traditional detection approach based on cell enrichment (at least 10^4 cells per mL of *Salmonella* concentration) involves time-consuming steps to detect viable cells (Lee et al., 2015). In order to improve the *Salmonella* monitoring in food, this pathogenic microorganism must be detected quickly, accurately, quantitatively, and more efficiently, given short shelf life of food matrixes (Vichaibun & Kanchanaphum, 2020). Molecular techniques are increasingly acknowledged as beneficial substitutes of traditional microbiological methods based on their speed, sensitivity, and specificity. But DNA from both dead and living bacteria can be amplified using real-time PCR (qPCR) or polymerase chain reaction (PCR) (Masters et al., 1994; Wolffs et al., 2005; Li et al., 2013; Barbau-Piednoir et al., 2014). Therefore, viability qPCR (v-qPCR) is a promising method based on pre-treatment with photochromic dyes that bind to the DNA of dead or damaged cells, making it possible to specifically identify DNA only from living cells (Qin et al., 2020). The basic premise of the v-qPCR assay is that all alive microorganisms must have an intact cell membrane or be resistant to biological reagents, regardless of their metabolic state. Otherwise, these reagents enter the cells and will interact with DNA that can no longer be used as a template in qPCR experiments due to the photochemical reaction resulting from exposure to high-intensity light beam (Codony et al., 2023). Since these dyes only enter damaged or dead cells, the process depends on the integrity of the bacterial cells (Nocker et al., 2006). Ethidium monoazide (EMA) was initially shown to be selective in detecting living cells (Nogva et al., 2003; Rudi et al., 2005; Rudi et al., 2005). Later, propidium monoazide (PMA) has been proved to be more selective than ethidium monoazide (EMA) for

alive bacteria detection and quantification, due to the molecule's higher charge (Nocker et al., 2006; ElizaquÃvel et al., 2012; Thilakarathna et al., 2022). Several studies have demonstrated the effectiveness of v-qPCR in detecting and quantifying different microbial species, including foodborne pathogens. It has been noted that the v-qPCR method may have some practical limitations, even with improvements made to the assay, such as changes in the qPCR protocol, working time, or dye concentration used (Fittipaldi et al., 2012; Nkuipou-Kenfack et al., 2013). The complexity of the matrix (food, medical or environmental) which can block the signal from dead cells, leading to an overestimation of the number of live cells in samples, is one of the biggest obstacles. Another issue is related to the ability of some bacterial strains to avoid dye uptake. Therefore, *Salmonella enterica* serovar *typhimurium ATCC 53648* and *Listeria monocytogenes ATCC 49594* strains were identified by Nkuipou-Kenfack et al. (2013) using sodium deoxycholate permeabilizer (DOC) 0.01-0.3% (w/v) as pre-treatment, then treating cells with 10 μM PMA, for 5-30 minutes, at different temperature values (0°C, 20°C and 40°C). It was observed that only the detection of the *Salmonella* strain successfully benefited from DOC, as there was a signal drop more than 10. Furthermore, the investigation showed a strong correlation between the length and temperature of dye incubation and the effectiveness of PMA treatment (Nkuipou-Kenfack et al., 2013). Organic solvents such as acetone, methanol, and detergents have been proved to permeabilize bacterial cells (Jamur & Oliver, 2010). In another experiment, a multiplex PMA-qPCR assay with sarcosyl pre-treatment was successfully applied to detect *Legionella pneumophila*, *S. typhimurium*, and *S. aureus* from water samples (Li et al., 2015). The aim of the study was to test a new type of cell permeabilizer, which would allow the photoactive dye PMA to enter the *Salmonella* spp. cells more easily and thus improve the accuracy of the v-qPCR assay. Lactic acid (LA), the material suggested in this study, has the primary benefit of having far less toxicity when compared to the other permeabilizers used. It is also less expensive and simpler to handle.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Salmonella typhimurium ATCC 14028 strain from the Microorganisms Collection of the Laboratory of Applied Microbiology - Faculty of Biotechnology, was cultured for 24 h, at 37°C, on 20-25 mL Tryptone Soy Broth (TSB) medium (Neogen, USA). To measure the optical density at 600 nm (OD 600 nm) and to calculate the microbial load of the inoculum, an UV-1800 spectrophotometer (ChromTech, USA) was used. At the same time, decimal dilutions in sterile distilled water (10^{-1}-10^{-8}) were prepared to determine the number of colonies plated onto Tryptone Soy Agar (TSA) growth medium (Scharlau, Spain), expressed as CFU/mL.

Inactivation of bacterial cells by heat treatment

An amount of 10 mL of inoculum containing 10^8 bacterial cells/mL was incubated in glass tubes for 45 min at 80°C in a water bath (Memmert, Germany) to inactivate the cells. To test the loss of viability one milliliter of suspension was plated onto TSA medium for 24 h at 37°C.

Testing the effect of lactic acid (10-20 mM) on microbial vitality

A 30% (v/v) L-(+)-lactic acid stock solution (Sigma, USA) was used to prepare 10 to 20 mM LA solutions, pH=5-5.5. To test the sublethal effect of lactic acid on bacteria, two replicates of *Salmonella* spp. inoculum (3 mL) were combined with 3 mL of lactic acid solution at various concentrations between 10 to 20 mM. The control was represented by the mixture of 3 mL inoculum and 3 mL sterile distilled water. After 24 h at 37°C, the optical density was checked.

Testing the synergistic effect lactic acid - antibiotic CIP1

The Kirby-Bauer method was used to test the synergistic effect produced by the permeabilizer and antibiotic - ciprofloxacin 1 mcg - CIP1 (Sigma, USA). The antibiotic disks were soaked into permeabilizer solutions (LA 5-20 mM, EDTA 10 mM) and placed on the surface of the TSA medium inoculated with bacterial strain. After 24 h at 37°C incubation, the diameter of lysis zone was measured.

Pre-treatment with lactic acid (5 and 10 mM)

Aliquots (400 µL/each) of heat-killed cells and others with living cells (400 µL/each) were created in order to examine the qPCR signal reduction. They were subjected to bacterial outer membrane permeabilization treatment using 5 mM and 10 mM lactic acid and incubated under stirring conditions (150 rpm), for 30 min. at room temperature, before PMA treatment.

PMA treatment, photolysis and cell washing step

To prepare a stock solution of 20 mM PMA, 1 mg of PMA (Biotium, Hayward, CA, USA) was dissolved in 98 µL of sterile distilled water. The stock solution was kept at -20ºC, in the dark. PMA was used according to the manufacturer's instructions, in a 50 µM working concentration. Therefore, 1 µL of PMA stock was added into 400 µL aliquots for 10 minutes, and tubes were incubated at room temperature, 150 rpm, in the dark. A 1000-W halogen light source (Omnilux, Germany) was used to expose the sample tubes to light. It was positioned 20 cm away. The tubes were kept on ice during the photolysis process to prevent overheating and sporadic mixing. Following five minutes in the light, the cells were harvested using centrifugation at 12,000 rpm for two minutes. To ensure that any remaining traces of PMA solution would not impede the extraction of free DNA, the cells were twice washed with sterile distilled water. To isolate the DNA, the pellet was again suspended in 200 µL of distilled water. Control sample sets (400 µL aliquots with alive and others with heat-killed cells) were not treated with LA for set I, not treated with PMA for set II and without both LA and PMA treatment for set III.

DNA extraction and qPCR amplification

In this step, the Quick-DNA Fungal/Bacterial Miniprep kit (Zymo Research, Germany) was used according to the manufacturer's instructions. Primer set Sc-8-F (5'–ATCGTGATACAGAACGCCG-3') and Sc-8-R (5'-TCTTCGTCATCCACCCAGA-3') was previously reported that target *Salmonella* spp. strains and the amplicon size is 83 bp (Yu et al., 2016). The qPCR assay was performed using a final volume of 25 µL/sample consisting of 12.5 µL Maxima SYBR Green/ROX qPCR Master mix (ThermoFisher, USA), 1 µL target DNA (up to 500 ng/reaction) 0.5 µL each forward and reverse primer (final concentration 0.3 µM/each) and 10.5 µL PCR-grade water. In order to quantify the microbes, quantitative PCR was carried out with the Rotor-Gene 6000 5plex HRM (Qiagen-Corbett Life Science, Australia). The three stages of the melting program were, depending on the product size (83 bp), denaturation at 95ºC for 10 min., followed by 40 cycles of 15 s denaturation at 95ºC, 30 s annealing at 60ºC, and 20 s elongation at 72ºC. Each time, a PCR-grade water solution (1 µL) was used in place of a DNA template as a negative control for the amplification process.

For signal reduction analysis after PMA and LA-PMA treatments, delta Ct (dCtPMA) for live and dead cells was calculated as shown:

$dC_{tALIVE\text{-}PMA} = C_{tALIVE,\ PMA} - C_{tALIVE,\ nonPMA}$

$dC_{tNON\text{-}VIABLE\text{-}PMA} = C_{tNON\text{-}VIABLE,\ PMA} - C_{tNON\text{-}VIABLE,\ nonPMA}$

$dC_{tALIVE\text{-}LA\text{-}PMA} = C_{tALIVE,\ LA\text{-}PMA} - C_{tALIVE,\ PMA}$

$dC_{tNON\text{-}VIABLE\text{-}LA\text{-}PMA} = C_{tNON\text{-}VIABLE,\ LA\text{-}PMA} - C_{tNON\text{-}VIABLE,\ PMA}$

Statistical analysis

The software IBM SPSS Statistics 23 was used for the statistical analysis. ANOVA was used for the analysis of variance at 95% significance (P = 0.05). For the qPCR analysis the average findings from two distinct tests with duplicates were analyzed. After plating, the counts were log converted, and the average and standard deviation were computed. Every experiment was run twice, with duplicates or triplicates.

RESULTS AND DISCUSSIONS

The sublethal effect of lactic acid on the strain *Salmonella typhimurium* ATCC 14028

The experiment was performed to determine the highest concentration of lactic acid at which the bacterial strains grow normally without being inhibited. Alakomi et al. (2000) have proved the direct correlation between the permeabilization potential of the lactic acid and its concentration (Alakomi et al., 2000). However, if the acidity of the substance ends

up damaging cell viability, then pre-treatment can create false negative results, which is not desirable. The results are shown in Figure 1 and proved that lactic acid concentration up to 10 mM do not significantly decrease the microbial viability.

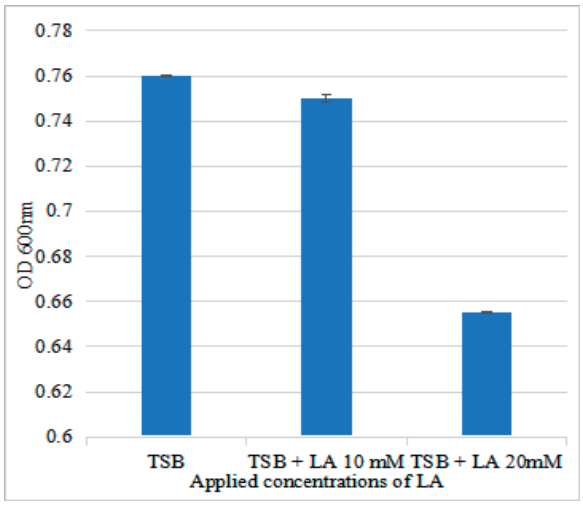

Figure 1. The sublethal effect of lactic acid (LA) on the growth of *Salmonella typhimurium* ATCC 14028 in tryptic soy broth (TSB)

The synergistic effect lactic acid - antibiotic on the strain *Salmonella typhimurium* ATCC 14028

It is well known that Gram-negative bacteria are inherently harder to kill with antibiotics than Gram-positive bacteria as Gram-negative microbes have an outer membrane that is difficult to be penetrated by antibiotic molecules. Therefore, we tested the outer membrane-disrupting and synergistic effect of different concentrations of LA (5-20 mM) and antibiotic ciprofloxacin (CIP1). The results about additive effect antibiotic - permeabilizer, tested by Kirby-Bauer method are shown in Figure 2. Combination antibiotic and different concentrations of lactic acid and EDTA up to 10 mM were more effective and produced a higher lysis zone compared to ciprofloxacin, where the diameter of the lysis zone was 22±2.82 mm. The most effective was combination between CIP1 and LA 5 mM that produced a clear zone of 28±2.83 mm (P>0.05), proving the potential of lactic acid to be used as an antibiotic synergist in therapy. The chelating agent EDTA is a well known described synergist with a reported ability to potentiate antibiotics, therefore was used as a positive control in our experiments (Wesseling & Martin, 2022).

The effect of PMA treatment on qPCR amplification of *Salmonella typhimurium* ATCC 14028

First, we compared the qPCR results obtained with aliquots of 100% alive and others with heat-killed cells, without LA and PMA treatment (Table 1).

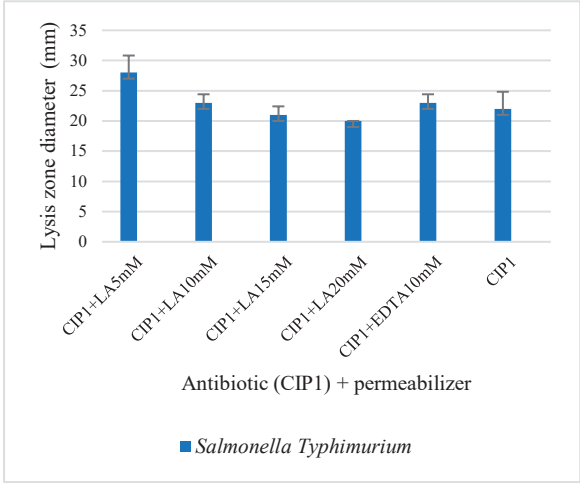

Figure 2. The synergistic effect antibiotic (CIP1) and permeabilizer (acid lactic LA 5-20 mM, EDTA 10 mM) on *Salmonella typhimurium* ATCC 14028

Table 1. Mean threshold values after different treatments with lactic acid (LA) and propidium monoazide (PMA) applied to *Salmonella* spp. suspensions of viable and non-viable cells

Treatment	C_t *viable*	C_t *non-viable*
-	9.99±0.10	**11.41±0.24**
LA 5 mM	ND	10.78±0.09
LA 10 mM	10.48±0.34	10.34±0.02
PMA 50 µM	13.41±0.28	**25.04±0.26**
	dC_t *viable*	dC_t *non-viable*
	3.42	13.62

ND- not determined

The mean threshold values (C_t) for non-viable and viable control samples were closer, 11.41±0.24 and 9.99±0.10, respectively. The results were not significantly different (P>0.05), proving that DNA from heat-killed cells were amplified (false positive result). Similar, control samples with 100% non-viable bacteria treated only with LA (5 mM, 10 mM) had mean C_t values of 10.78±0.09 and 10.34±0.02, respectively. At the same time, LA do not influence the DNA amplification from viable cells, mean threshold value for 10 mM

LA-treated cells being 10.48±0.34 compared to 9.99±0.10 for non-LA treated samples (Table 1). Another set of control samples were treated only with PMA, followed by photolysis and DNA amplification. Mean C_t values were significantly different (P>0.05), 25.04±0.26 for heat-killed and 13.41±0.28 for viable cells. These results proved that PMA efficiently penetrated the dead cells of *Salmonella* spp. and most of the DNA was not amplified after DNA isolation. The difference in the mean C_t values of PMA-treated and non-treated probes ($dC_{tNON\text{-}VIABLE\text{-}PMA}$) with non-viable cells is 13.62 suggesting a high qPCR signal reduction. It is considered that a signal reduction higher than 8 for non-viable cells indicates that 99.6% of DNA from these bacteria was removed (https://biotium.com/free-sample/). However, the DNA amplification from living cells was slightly influenced by the PMA treatment, as C_t values of PMA treated and untreated samples were similar and $dC_{tVIABLE\text{-}PMA}$ = 3.42 (Table 1). In another study with *Salmonella enterica* serovar *typhimurium* ATCC 53648 the signal reduction after 10 µM PMA treatment was related to the exposure time (5-30 min). The longest exposure time tested (30 minutes) produced the strongest signal reduction, with a difference of approximately 11 cycles between C_t values obtained from PMA-treated viable and non-viable samples (Nkuipou-Kenfack et al., 2013). In our experiment the difference between mean values obtained from PMA-treated viable and non-viable samples was 11.63 cycles, proving that PMA enter to all non-viable cells and inhibited the DNA amplification.

The effect of LA and PMA treatment on qPCR amplification of *Salmonella typhimurium* ATCC 14028

To assess the impact of LA and PMA treatment on qPCR assay results, the "viable dCt" and "nonviable dCt" indices were calculated. The qPCR signal reductions after 5mM and 10 mM LA pre-treatment, followed by PMA treatment were analyzed (Figure 3). Delta C_t values are the difference between LA-PMA treated and LA-treated samples, for both viable and non-viable cells. Both PMA and LA-PMA treatments had non-viable dC_t values greater than 8, demonstrating that PMA efficiently penetrated the dead cells of *Salmonella* spp. and inhibited DNA amplification. No significant differences between dC_t non-viable for different treatments with 5 mM and 10 mM LA-PMA and PMA (P>0.05) were noted. Moreover, viable dCt value after 10 mM LA-PMA treatment was lower (2.325) than dCt viable after propidium monoazide application (3.425), suggesting that LA might improve the PMA-qPCR assay. For living cells threshold values in samples without PMA and samples treated with PMA should be similar and $dC_{tVIABLE\text{-}PMA}$ less than 3. Nkuipou-Kenfack et al. (2013) showed that co-incubation of cells with PMA and deoxycholate improved the qPCR detection for non-viable gram-negative bacteria *Salmonella enterica* serovar *typhimurium* ATCC 53648. Using 0.1% DOC the PMA-qPCR detection of non-viable cells improved with ~ 2 cycles compared to PMA-qPCR. DOC concentrations up to 0.3% do not influence the qPCR signal from viable cells (Nkuipou-Kenfack et al., 2013). The difference between this result and our work might be related to the strain used and experiment design. It has to be pointed out that in our study cell suspensions were not co-incubated with permeabilizer and PMA as in the previous study. The specificity of the qPCR amplification with *Sc-8-F/R* primer set was verified. Based on the dissociation curves result, non-specific products were not produced.

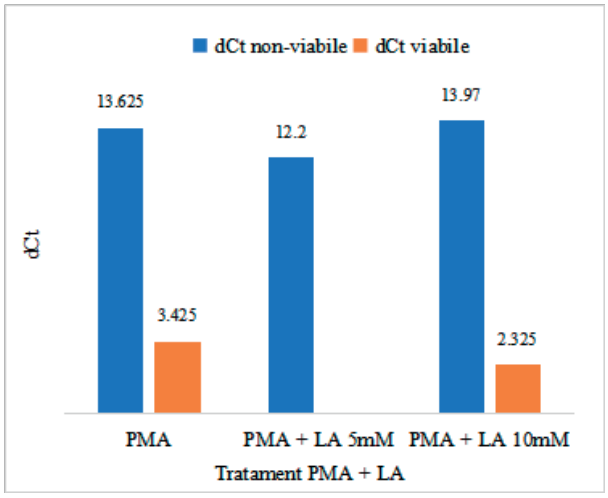

Figure 3. The signal reduction detected by qPCR after LA and PMA treatments applied to *Salmonella* spp. suspensions of viable and non-viable cells

CONCLUSIONS

This study aimed to contribute to the efforts to improve v-qPCR efficiency testing lactic acid, a new type of permeabilizing agent. The sublethal concentration of lactic acid enhanced the detection of viable Gram-negative bacteria ($dC_{tVIABLE\text{-}LA\text{-}PMA}$ = 2.32). However, the signal reduction ($dC_{tNON\text{-}VIABLE\text{-}LA\text{-}PMA}$ = 13.97) after 10 mM LA pre-treatment was similar with the reduction in C_t signal ($dC_{tNON\text{-}VIABLE\text{-}PMA}$ = 13.62) when only PMA treatment was applied to a suspension of non-viable cells. These preliminary studies provide useful information on the use of lactic acid, which has proven to be an effective, cheap and low-toxic enhancer for v-qPCR assay.

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