CENTER for PRODUCE SAFETY

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FINAL PROJECT REPORT

Project Title
Significance of sanitizers on induction of viable but non-cultivable (VBNC) foodborne bacteria and their survival and resuscitation in fresh produce

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Objectives

1. *Estimation of the microbial inactivation and the induction of VBNC state of foodborne pathogens in process wash water due to the action of commercial chemical sanitizers (sodium hypochlorite, calcium hypochlorite, chlorine dioxide and peroxyacetic acid) in the processing facilities of industry collaborators.*
2. *Establishment of the ability of foodborne bacteria in the VBNC state, present in the process wash water, to attach to the surface of fresh produce during washing.*
3. *Evaluation of the conditions needed for VBNC foodborne bacteria, attached to fresh produce, to survive and recover from VBNC to culturable state during storage and distribution mimicking the conditions of the cold chain.*
4. *Performance of challenge tests to assess the growth potential of L. monocytogenes in fresh produce under foreseeable conditions of transportation, distribution and storage using molecular techniques able to differentiate between viable-culturable (VC) and VBNC.*

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Abstract
Quantitative real-time polymerase chain reaction (qPCR), combined with two DNA amplificatory inhibitors (EMA and PMAxx), has been selected as the best method for the detection and quantification of cells in the VBNC state in process wash water (PWW). The use of these photoreactive DNA-dye combinations (EMA and PMAxx) yielded a more accurate estimation of the viable cells present in PWW than other methodologies, such as flow cytometry and PMAxx-qPCR. The validation of this methodology in an industrial setting allowed us to differentiate between dead and VBNC cells in chlorinated PWW. This method can be considered as a rapid and reliable one that can be recommended for the detection of VBNC in complex water matrixes such as those of the fresh produce industry. However, the complete discrimination between dead and VBNC cells was not achieved, which led to a slight overestimation of the percentage of VBNC, primarily due to the interfering compounds present in this type of water. In a second objective, the optimized methodology was used to investigate if current operational limits established in the fresh-cut processing industry effectively inactivated *L. monocytogenes* and *E. coli* O157:H7 in PWW, limiting the induction of VBNC cells. The results obtained showed that recommended operational limits for free chlorine (20–25 mg/L) were able to maintain the microbiological quality of PWW. *L. monocytogenes* and *E. coli* O157:H7 were fully inactivated in PWW treated with 20 mg/L free chlorine without the induction of VBNC cells. In the case of peroxyacetic acid (PAA), the authorized maximum concentration (80 mg/L) was not able to inactive cultivable cells in PWW. Chlorine dioxide (ClO_2) at the recommended dose (3 mg/L) reduced the levels of culturable pathogenic bacteria but induced the VBNC state of the remaining cells. Therefore, the sanitizers implemented in the fresh-cut industry lead to different inactivation effects in PWW, with chlorine being the most effective, followed by ClO_2. In contrast, PAA cannot be recommended to maintain the microbiological quality of PWW, as both cultivable and viable cells were recovered after 1-min contact time using 80 mg/L. When the capacity of VBNC *L. monocytogenes* inoculated in PWW to attach to fresh produce during washing was evaluated, it was observed that very few cells were able to adhere to the tissue. Additionally, although VBNC cells were able to resuscitate when present in PWW, resuscitation of *L. monocytogenes* cells in the fresh produce was rarely observed. On the other hand, results obtained for *E. coli* O157:H7 cells present in the VBNC state in the PWW treated with chlorine demonstrate that the attachment during washing or resuscitation to cultivable cells was not observed. Based on the results obtained at the lab scale, it can be concluded that injured *L. monocytogenes* cells present in fresh produce could be restored when subjected to optimal conditions. However, when industrial conditions were mimicked, bacterial cells showed a low resuscitation capacity. Therefore, there is a very low probability that VBNC *L. monocytogenes* cells present in fresh produce are able to resuscitate and multiply, which reduces the potential risk to public health.

Background
Washing is a critical process in the commercial operation of fresh-cut produce, as it is a major source of cross-contamination between different product batches. Commercial sanitizers are needed to maintain the microbial quality of process wash water (PWW) and prevent cross-contamination of the product. Sodium hypochlorite (chlorine), peroxyacetic acid (PAA) and chlorine dioxide (ClO_2) are the most commonly used commercial sanitizers. The residual concentrations of these sanitizers to maintain the microbial quality of PWW have been recommended or limited by scientific studies, guidelines and regulations as 20 mg/L for chlorine (Tudela et al., 2019), 80 mg/L for PAA (21CFR173.315) and 3 mg/L for ClO_2 (FDA, 2019). However, these are general recommendations that have been based on the inactivation studies by conventional plate counts. Recently, great efforts have been made to establish the operational limits in washing operations for different commercial sanitizers, including chlorine and different types of fresh-cut produce. The operational limits have been associated with

bacterial survival (Luo et al., 2018). Based on industrial-scale data, the minimum effective dose of each sanitizer depends on the type of fresh produce being washed. For chlorine, several authors have suggested that 10 mg/L of free chlorine could be the lowest effective concentration for most leafy greens, while other authors demonstrated that higher concentrations up to 20–25 mg/L of free chlorine were needed (Gombas et al., 2019; Tudela et al., 2019). The differences observed can be due to the inflow of microorganisms but also to the physiological state of the bacteria present in the PWW and the culturability ability.

Several studies have reported that many bacterial species, including foodborne pathogens, may enter into a temporary state of low metabolic activity, also called viable but non-cultivable (VBNC) state, when exposed to sanitizers. In this state, bacteria cells do not divide but maintain their intact cell membranes, have low metabolic activity, continue gene expression and have the ability to become culturable once resuscitated. However, they are unable to recuperate their ability to divide when plated on routine culture media. Therefore, the use of conventional plate count methods might lead to an overestimation of the efficacy of sanitizers and, consequently, bring in a safety issue. Several studies have proposed to detect and quantify VBNC cells in food and environmental samples, such as the use of DNA-dyes including PMA, PMAxx and PEMAX combined with qPCR. Recently, Gu et al. (2020) demonstrated that the use of PMA-qPCR allowed the detection of VBNC cells of several pathogens (e.g. *Escherichia coli*, *Salmonella* and *Listeria monocytogenes*) in PWW treated with free chlorine and PAA. This methodology enables the differentiation between viable and dead cells, avoiding the overestimation of results. In general, these photoreactive dyes are linked to dead bacteria with a compromised membrane, but if dead cells have an intact membrane, this might lead to an overestimation of the live cells (false-positive results). For free chlorine, some studies have observed that membrane damage is not the only event involved in bacterial inactivation.

On the other hand, several studies have reported that VBNC bacteria may resuscitate when optimal conditions are restored during processing, storage and distribution. Resuscitation of VBNC bacteria cells has been described in some bacterial species, including *E. coli* O157:H7 and *L. monocytogenes*. This term has been defined as the capability of VBNC cells to recover their metabolic activity and culturability. As far as we know, few studies have been focused on the capability of resuscitation of VBNC bacteria cells present in disinfected water with contradictory results. Therefore, more research was needed to determine if the operational limits established for some sanitizers to maintain the microbiological quality of PWW can inactivate bacteria cells or lead to the induction of the VBNC state. Additionally, the ability of VBNC cells potentially present in PWW to 1) attach to fresh produce during washing and 2) to resuscitate and multiply in fresh produce during storage under the conditions used by the industry were also evaluated.

Research Methods and Results

Plant material. Iceberg lettuce (*Lactuca sativa* L. var capitata) and onion (*Allium cepa* L.) were purchased from a local supermarket Murcia (Spain). Baby spinach (*Spinacia oleracea*) was obtained from a fresh-cut processing company (Murcia, Spain). Lettuce heads were cut into 6 mm pieces after outer leaves were manually removed. Onions were manually peeled and diced with a cutter (RG-100, AB). Leaves of baby spinach were used without additional processing. These fresh-cut products were confirmed first to be free of naturally occurring *L. monocytogenes*.

Process wash water. Process wash water (PWW) from washing shredded iceberg lettuce (*Lactuca sativa* L. *var capitata*), shredded cabbage (*Brassica oleracea* var. capitata f. rubra), diced onion (*Allium cepa* L.), and baby spinach (*Spinacia oleracea*) was generated in the lab mimicking industrial conditions previously described (Tudela et al., 2019). The content of organic matter in PWW was measured as chemical oxygen demand (COD) determined by the standard photometric method (APHA, 1998) using the Spectroquant NOVA 60 photometer

(Merck, Darmstadt, Germany). The concentrated wash water was stored at -20°C for a max. of 1 week until use. Then, each type of wash water was unfrozen and tap water was added to adjust COD to those found in washing tanks of commercial facilities of approx. 1000 mg/L, except for baby spinach that was of about 300 mg/L (Tudela et al., 2019).

Bacterial strains and cocktail preparation. PWW was inoculated using two different cocktails of *L. monocytogenes* and *E. coli* O157:H7. For *L. monocytogenes*, a cocktail of six strains isolated from leafy vegetables was used following the previously described protocol (Truchado et al., 2020a). For *E. coli* O157:H7, a cocktail of three strains (MB 3885 and CECT4972 and CECT5947) prepared as nalidixic-resistant (20 µg/mL nalidixic acid) was used. The *E. coli* strains were individually reconstituted in brain heart infusion broth (BHI, Scharlab, Barcelona, Spain) and consecutively subcultured twice in 10 mL of BHI, first at 37 °C for 24 h, and then at 37 °C for 16 h. After the second incubation, 1 mL of each strain was combined to obtain a six-strain cocktail of *L. monocytogenes* (10^9 CFU/mL) and a three-strain cocktail of *E. coli* O157:H7 (10^8 CFU/mL).

Sanitizers. For each sanitizer, the recommended concentration following optimal washing practices was considered (Tudela et al., 2019): 1) 20 mg/L for sodium hypochlorite (Chlorine; Industrias Gamer, Murcia, Spain); 2) 80 mg/L for PAA (PAA; Citrocide PC, Productos Citrosol S.A., Potríes, Spain); and 3) 3 mg/L for ClO_2 (AGRI DIS®, Servicios Técnicos de Canarias, Las Palmas de Gran Canaria, Spain). Temperature of PWW was always maintained between 4–5 ºC. For each sanitizer, different volumes were added to reach the threshold concentration selected in the different types of PWWs until the residual concentration was reached and remained stable. Concentration of chlorine was measured with the photometer Spectroquant NOVA 60 (Merck). PAA levels were measured by reflectometry using a Reflectoquant system (Merck). For ClO_2, the photometer Spectroquant NOVA 60 was used to measure the residual concentration. Additionally, the physicochemical characteristics of the PWW were monitored and pH, conductivity (EC), oxidation-reduction potential (ORP) and temperature (Tª) measured using a portable multimeter (Hach, Loveland, Colorado, USA), as previously described (Tudela et al., 2019).

Process wash water inoculation. PWW, previously treated until the threshold concentration of each sanitizer was reached, was inoculated with a six-strain *L. monocytogenes* cocktail (10^9–10^8 cfu/100 mL). Following the same procedure, the same experiments were repeated with PWW inoculated with a three-strain cocktail of nalidixic-resistant *E. coli* O157:H7 (10^9–10^8 cfu/100 mL). The final concentration of bacteria in each batch was about 10^7–10^6 cfu/100 mL. After 1 min contact time, samples of PWW were taken. Immediately, 0.3 M sodium thiosulfate pentahydrate was added to quench the residual concentrations. In the case of PAA, 0.3 M sodium thiosulfate supplemented with a solution of 7 g/L of catalase (Sigma-Aldrich, St. Louis, MO, USA) was added to neutralize any remainders of hydrogen peroxide. Then, three aliquots of 100 mL were taken to quantify the levels of culturable bacteria, viable bacteria and total bacteria.

Culturable bacteria in PWW. Levels of culturable *L. monocytogenes* and *E. coli* O157:H7 (cfu/mL) cells in PWW were confirmed by filtration through sterile cellulose nitrate filters (0.45 µm) using a vacuum filtration system (Sartorius, Göttingen, Germany). Different volumes of water (1, 10 and 100 mL) were filtered as needed. After filtration, the filters were placed in selective agar plates including ALOA/OCLA agar (Scharlab) and Choromocult agar (Merck) supplemented with nalidixic acid (50 µg/mL) to enumerate *L. monocytogenes* and *E. coli* O157:H7, respectively. Colonies were counted after 24 h of incubation at 37 °C.

Culturable bacteria in fresh produce. Fresh produce samples (16 g each) were homogenized for 1 min in 240 mL of 0.1% BPW using a Stomacher (IUL instruments, Barcelona, Spain). The homogenate was divided into three aliquots of 45 mL to determine the levels of culturable bacteria, total cells and viable bacterial cells. As previously indicated, levels

of culturable *L. monocytogenes* and *E. coli* O157:H7 (cfu/g) cells were determined using selective agar plates.

Total and viable bacteria. Levels of total bacteria were quantified using a qPCR. Forty-five mL of inoculated PWW of shredded lettuce, cabbage and diced onion and 10 mL of inoculated PWW of baby spinach were centrifuged (3000 *g*, 4 °C, 10 min). The supernatant was discarded and the remained pellet was kept at -20 ºC until DNA genomic extraction. Levels of viable bacteria were determined using a viable qPCR as previously described (Truchado et al., 2020b) in combination with two photoreactive dyes, ethidium monoazide (EMA; Biotium) and PMAxx™ (Biotium), an improved version of the PMA (propidium monoazide). Stained samples were exposed to the blue light PMA-Lite LED photolysis (Interchim, Montluçon, France) for 15 min. Bacteria cells were concentrated by centrifugation (9000 *g*, 4 °C, 10 min). The supernatant was discarded and the EMA+PMAxx treated pellet keep at -20 ºC until DNA genomic extraction.

DNA extraction and qPCR procedure. Genomic DNA was extracted using the MasterPureTM complete DNA and RNA Purification Kit (Epicentre, Madison, USA), according to the manufacturer's indications. The quality and concentration of DNA extracts were determined by spectrophotometric measurement at 260/280 nm and 260/230 nm using a NanoDrop®ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Quantitative real-time PCR (qPCR) and data analysis were performed using an ABI 7500 Sequence Detection System (ABI, Applied Biosystems, Madrid, Spain). For *L. monocytogenes* quantification, the qPCR procedure described in Truchado et al. (2020b) was followed. In the case of *E. coli* O157:H7, primers and probes, cycling parameters, and detection conditions were as previously described (Li and Chen, 2012). The standard curve for each pathogenic bacteria was also estimated as previously described (Truchado et al., 2016). The limit of detection (LOD) for *L. monocytogenes* was determined to be Ct=37 (23 cfu/ per reaction) and for *E. coli* O157:H7 CT=34 (39 cfu/per reaction). The theoretical limits for quantification *L. monocytogenes* in water and fresh produce resulted in 2.57 and 1.17 log cells/100 mL or gram, respectively. In the case of *E. coli* O157:H7, the theoretical limits of quantification resulted in 2.69 and 1.84 log cells/100 mL or gram in water and fresh produce, respectively. The samples with Ct values higher than LOQ were classified as non-determined, while Ct values lower than LOQ were classified as positive.

Suitability of different detection methods. To assay the suitability of detection methods to differentiate between dead and VBNC bacteria, bacterial suspensions containing different proportions of dead and VBCN cells were prepared as follows:

(i) Heat treatment: The *L. monocytogenes* cocktail was exposed at 85 ºC for 20 min using a laboratory standard heat block.

(ii) Sanitizing treatment: Sodium hypochlorite was added to the six-strain cocktail of *L. monocytogenes* (10^9 cfu/mL) until a residual of 10 mg/L free chlorine was measured to guarantee the complete inactivation of the cells. After a 1 min exposure time, 0.3 M of sodium thiosulfate pentahydrate (Scharlab) was added to quench the residual chlorine.

The cell inactivation after the treatments was confirmed by plating serial suspension dilutions of the treated *L. monocytogenes* cocktail in buffered peptone water (BPW, 2 g/L) (Oxoid; Basingstoke, UK) on Oxford agar (Scharlab) followed by incubation at 37 ºC for 24 h.

Quantitative Polymerase Chain Reaction (qPCR)

1. ***PMAxx qPCR.*** Separate flaks of PWW (50 mL) were inoculated with a cocktail of *L. monocytogenes* containing live (exponential phase), heat-treated and chlorine-treated cells to the desired concentration of approximately 10^4 cfu/mL. From each flask, 10 mL of PWW were centrifuged at 3000 *g* for 10 min at 4ºC. The supernatant was removed, and the pellet resuspended in PBS at a final volume of 1000 µL supplemented with PMAxx to obtain a final dye concentration of 50, 75 and 100 µM. After PMAxx addition,

the samples were incubated at 200 rpm in the dark at room temperature or 40 ºC for 10–60 min. Stained samples were subsequently exposed to the blue light PMA-Lite LED photolysis (Interchim) for 15 min. In parallel, 10 mL of inoculated PWW were taken from each flask to determine the level of total bacteria by qPCR. Bacteria cells were concentrated by centrifugation (4000 rpm, 4 °C, 10 min). The supernatant was discarded, and the untreated and PMAxx treated pellets were kept at -20 ºC until DNA genomic extraction.

2. ***EMA + PMAxx qPCR.*** Following the same procedure as previously described, three separate flasks of PWW (50 mL) were inoculated with a cocktail of live, heat-treated and chlorine-treated *L. monocytogenes* cells. Ten mL from each flask were centrifuged, the supernatants discarded, and the pellets were treated with a combination of 10 µM EMA and 75 µM PMAxx, incubated at room temperature and 40 ºC activated with the blue light PMA-Lite LED photolysis, as previously described. The total bacterial was also determined by qPCR, as described above.

Live/Dead Flow Cytometry Analysis

Cell viability was determined by flow cytometry (LSR Fortessa X-20 system) using the Live/Dead BacLight® bacterial viability kit (Invitrogen, Waltham, USA) that contains two nucleic acid stains with different ability to penetrate in bacteria cells: SYTO 9 and propidium iodine (PI). SYTO 9 is a cell-permeant green-fluorescent dye that enters both live and dead cells, while PI is a membrane-impermeant dye that penetrates only in damaged or dead cells and emits red fluorescence upon intercalation with double-stranded DNA. When both dyes are used simultaneously, SYTO 9 is replaced by PI due to its higher affinity to bind DNA, quenching SYTO 9 fluorescence signal (Stiefel et al., 2015). As a result, red signals from cells are considered as "dead", green signals as "alive" and the double staining is considered as an intermediate state of membrane-compromised cells. The staining procedure was performed according to the manufacturer's instructions. Flow cytometry was used for measuring the viability of *L. monocytogenes* cells inoculated in PWW after treatment with chlorine. Untreated inoculated PWW samples (50 mL) were used as controls and compared with chlorine-treated inoculated PWW with 10 mg/L of free chlorine. This treatment reproduces the conditions found in industrial washing tanks. One mL of each PWW was mixed with 3 µL of the mixture of both dyes (5 µM of SYTO 9 and 30 µM of PI) and incubated for 15 min at room temperature in the dark. The green fluorescence emission of live bacteria was detected at 520 nm in the FL1 channel of the cytometer, while the red fluorescence emission of compromised (double stained) or dead bacteria was detected at 630 nm on the FL4 channel. During data acquisition, all parameters were collected in the log mode, and data analysis was performed with the EC800 software version 1.3.6. (Sony Biotechnology Inc., Champaign, IL, USA). Forward and Side Scatter gates were established to exclude debris. Unstained and stained untreated live *L. monocytogenes* and dead *L. monocytogenes* cells were used as controls for gating the different regions and fluorescence adjustment.

Process wash water from an industrial setting. Samples of PWW were taken in an industrial processing line of shredded iceberg lettuce washed in large washing tanks of about 3000 L. A residual concentration of 10 mg/L of chlorine was set to maintain the microbiological quality of the water. The washing was performed by immersion of cut lettuce for 30 s in the chlorinated washing tank followed by a shower rinse with tap water for 30 s. The physicochemical parameters, such as pH, COD, temperature, ORP and free chlorine, and microbiological quality of PWW were monitored each hour over 4 hours, as previously described (Tudela et al., 2019) (**Table 1**). For microbiological analyses, three water samples (1 L each) per sampling point were taken. Samples were collected in a sterile bottle (1 L) containing sodium thiosulfate pentahydrate (Scharlab) to quench the residual chlorine and then transported to the CEBAS-CSIC lab under refrigerated conditions. Samples were stored at 4 ºC until the microbial determinations were prepared for the following day. Levels of culturable bacterial populations were performed by membrane filtration (0.45 µm) using a

vacuum filtration system as previously described (Truchado et al. 2020b). For total bacteria, prime concentrations, cycling parameters, and amplification and detection conditions were as previously described (Truchado et al., 2019). The limit of detection (LOD) was based on the cycle threshold (Ct) value of the last detectable standard. LOD was determined to be Ct = 34 (85 cfu per reaction) for total bacteria. The samples with Ct values higher than LOQ were classified as non-determined, while Ct values lower than LOQ were classified as positive.

Resuscitation procedure. The capacity of VBNC bacteria cells to resuscitate in PWW after disinfection with different sanitizes was evaluated in those experiments where culturable bacteria were not detected. After 1 min PWW containing the inoculated bacteria was in contact with the sanitizing agent, aliquots of 100 mL of each type of PWW were centrifuged at 3000 *g* for 10 min at 4 ºC. The supernatant was removed and the pellet resuspended in 1 mL of phosphate buffered saline (PBS) at pH=7. In the case of *L. monocytogenes*, resuspended cells were added to 9 mL of BHI broth and, in parallel, to 9 mL of half concentrate *Listeria* Fraser broth (Scharlab), both supplemented with 0.1% of sodium pyruvate (Jasson et al., 2009a; Pinto et al., 2011). Tubes were incubated for 20 h at 37 ºC. After that, a sample of 100 µL of half Fraser broth was transferred to 9 mL of Fraser supplemented with 0.1% sodium pyruvate and incubated for 24 h at 37 ºC. Samples were considered positive if the turbidity value exceeded 0.1 in the BHI, and if Listeria Fraser broth turned black. Positive samples were streak onto ALOA/OCLA (Oxoid). Two blue colonies showing inhibition halo were identified and confirmed by PCR using the target Listeriolysin O (hly) gene of *L. monocytogenes*. For *E. coli* O157:H7, the resuspended cells were also added to 9 mL of BHI broth supplemented with 0.1% of sodium pyruvate, and in 9 mL of modified buffered peptone water with pyruvate (mBPWp) (Jasson et al., 2009b). Samples were incubated for 24 h at 37 ºC and those showing turbidities were streaked onto CHROMagar O157 (Scharlab) and Cefixine-Tellurite SorbitoL MacConkey agar (CT-SMAC, Scharlab) media. Two pink colonies and two orange colonies were identified from CHROMagar O157 and CT-SMAC, respectively, and confirmed by Latex Confirmation (Oxoid).

Attachment assay. Residual concentrations of the selected sanitizers were maintained in the different types of PWW (4 L) until the inoculum was added. PWW was inoculated with the bacteria cocktail (10^9–10^8 cfu/100 mL) for *L. monocytogenes* and *E. coli* O157:H7. The final concentration of bacteria in each batch was about 10^7–10^6 cfu/100 mL. After 1 min contact time, fresh-cut iceberg lettuce (600 g) was immersed in the treated PWW. After 1 min contact time, the lettuce was removed from the washing tank and the water excess was eliminated using a manual centrifuge. To quench the sanitizer's residual concentrations, sodium thiosulfate pentahydrate (0.3M) was added to the buffered peptone water (BPW). Two repetitions per sanitizer and inoculum were carried out in a total of 4 individual experiments.

Packaging and storage. Lettuce (60 g), taken at random from each bach, was packed in individual bags (150 mm x 150 mm) of PET/PE film of 52 µm thickness (Amcor Flexibles, Bristol, UK) under a passive modified atmosphere. All bags were thermally sealed (Magneta 421, Audion Elektro BV, Netherlands) and stored for 0, 5, 10 and 15 days at 7 ºC, simulating commercial conditions of storage, distribution and commercialization.

Resuscitation procedure of bacteria attached to the surface of fresh produce. The capacity of VBNC bacteria cells attached to fresh produce during washing to resuscitate over the storage was evaluated. The tissue sample was mixed up 1:1 with tryptic soy broth (TSB). The enrichments were then incubated at 37 °C for 18 to 24 h. In the case of *L. monocytogenes*, 1 mL of enrichment was added to 9 mL of half concentrate Listeria Fraser broth (Scharlab), both supplemented with 0.1% of sodium pyruvate (Jasson et al., 2009a; Pinto et al., 2011). All tubes were incubated for 20 h at 37 ºC. After that, a sample of 100 µL of half Fraser broth was transferred to 9 mL of Fraser supplemented with 0.1 % sodium pyruvate and incubated for 24 h at 37 ºC. Samples were considered positive if Listeria Fraser broth turned black. Positive samples for *L. monocytogenes* or *E. coli* O157:H7 were confirmed following the protocol previously described.

Challenge test. Two different fresh-cut products (shredded lettuce and baby spinach) were inoculated with a cocktail of six strains of *L. monocytogenes* induced to VBNC state by the action of a sanitizer in each type of PWW. VBNC cells were generated using PWW (500 mL) treated with 3 mg/L of ClO_2 as specified previously in objective 1. A six-strain *L. monocytogenes* cocktail (10^9–10^8 cfu/100 mL) was added to PWW to reach a concentration of around 10^7 cfu/100 mL. VBNC cells were recovered from PWW by centrifugation at 3000 *g* 4ºC, 10 min. The supernatant was removed and the pellet was resupended in 500 mL of PBS. The levels of VBNC were verified using plate count and molecular techniques (qPCR and EMA+PMAxx-qPCR). The obtained VBNC suspension was used to inoculate shredded lettuce and baby spinach to reach a concentration of around 10^2–10^3 cfu/g of produce. For this purpose, 100 mL of VBNC suspension was sprayed homogeneously on the surface of fresh produce (400 g). After being sprayed with VBNC, samples were allowed to dry at room temperature for 15 min. Two repetitions per each fresh-cut produce were carried out in a total of 6 individual experiments.

Calculation of growth potential. The growth potential of VBNC was calculated as the difference between the median of the $\log_{10}$CFU/g at the end of the test and the median of the $\log_{10}$ CFU/g at the beginning of the test.

Outcomes and Accomplishments

Objective 1: Estimation of the microbial inactivation and the induction of VBNC state of foodborne pathogens in process wash water by commercial chemical sanitizers in washing lines of industry collaborators

The first activity was **to determine the viability *of L. monocytogenes* and *E. coli* O157:H7 in PWW and fresh produce by the optimization of the methodology**. In a first attempt, flow cytometry was used to determine the proportions of *L. monocytogenes* cells at different states (dead, viable and intermediate) comparing untreated and chlorine-treated PWW. Viable cells emitted green fluorescence, dead cells red fluorescence while intermediate state was dependent on the degree of the damaged bacterial membrane for live (SYTO 9) or dead (PI) cells. These double positive bacteria are defined as those cells that are compromised or are in an intermediate stage between live and dead (Stiefel et al., 2015). Preliminary experiments were performed to establish the appropriate level of fluorescence compensation and the visual interpretation of the quadrants (Q) for the viable and dead populations of *L. monocytogenes* cells. **Figure 1A** shows the green-fluorescent signal (FL1 at 520 nm) of the Q4 that corresponded with the alive inoculum. **Figure 1B** shows the red fluorescent signal (FL2 at 630 nm) of the Q2 that corresponded with the dead inoculum. When the double stain was applied (**Figure 1C**), the Q1 corresponded with the cells stained with PI (dead cells), the Q4 with the cells stained with SYTO9 (live cells), the Q2 with the double stained cells (membrane-comprised cells or VBNC) and the Q3 with the non-stained cells. *L. monocytogenes* levels obtained by plate count (log cfu/mL) were converted to percentages to compare data obtained using both flow cytometry and plate count methods. The inoculum levels recovered from PWW by plate count corresponded with the initial inoculum and was considered 100%. As expected, 100% of culturable cells were obtained in untreated PWW that changed to 99.9% of dead cells when determined by plate count and 65.7 % when determined by flow cytometry (**Table 2**). When looking at the cells in an intermediate stage, the percentage of VBNC cells in treated and untreated PWW was similar (7–8%).

Taking into account the complex composition of PWW, with high organic matter content including interfering compounds, the fluorescent dyes were not able to differentiate among the physiological stage of the different bacteria species. Based on results obtained, **flow cytometry was not a suitable methodology to reliably distinguish viable and dead cells in PWW**.

Secondly, **the viability of *L. monocytogenes* cells was determined using qPCR techniques.** Preliminary experiments performed to determine the optimal concentration of PMAxx showed that when 50 µM of PMAxx was added to PWW, the PCR signal of dead cells was not fully discriminative (data not shown). One reason could be that the presence of organic matter in PWW could interfere with the photoreactive DNA-dye, reducing its ability to bind to dead cells' DNA. Based on these results, two higher concentrations of PMAxx (75 and 100 µM) were tested. The quantitative PCR Ct values of heat-killed *L. monocytogenes* treated with 75 and 100 µM PMAxx concentrations were similar (**Figure 2A**). However, a toxicity effect was observed in the live cells when the higher concentration of dye was used (**Figure 2B**). Therefore, to avoid any side effects on the viability of the *L. monocytogenes* cells, the lowest concentration (75 µM) of PMAxx was selected for further analysis.

After this, lab-scale experiments were performed to study the mode of action by which chlorine killed *L. monocytogenes* cells and if the cell membrane integrity was or not affected. It has been accepted that chlorine and other chemical sanitizers usually inactivate the bacterial cell by disrupting the cytoplasmic membrane (Nocker et al., 2007). It is essential to determine the suitability of PMA to differentiate between live and dead cells when chlorine treatments are applied. In these experiments, PCR amplification of dead *L. monocytogenes* cells killed by heat-treatment was compared with those killed by chlorine. The initial conditions used for this comparison were those previously recommended to discriminate dead *L. monocytogenes* cells, including a 30-min dye incubation at 40 ºC (Nkuipou-Kenfack et al., 2013). As expected, *L. monocytogenes* DNA activated with PMAxx (75 µM) and isolated from heat-treated *L. monocytogenes* did not show amplification in the qPCR. However, the PCR signal of *L. monocytogenes* DNA activated with PMAxx (75 µM) obtained from chorine-treated cells was not completely inactivated, showing a cycle threshold value (Ct) below the limit of detection (LOD) (**Figure 3**). Based on these results, new experiments were performed under different conditions of time/temperature for the incubation of the DNA with the dye. However, none of the tested combinations improved the previous conditions (data not shown). The results indicated that there was an overestimation of the live cells because some dead cells could still be quantified (false-positive results) after the chlorine treatment, mostly due to the presence of an intact membrane. Based on these findings, PMAxx-qPCR method was not considered a suitable approach to differentiate between live and dead *L. monocytogenes* cells present in PWW treated with chlorine.

The alternative method studied was EMA combined with PMA, known as PEMAX reagent (a new commercial reagent that combines both EMA and PMA) to detect both membrane integrity and active metabolism (Codony, 2014). Some studies related to membrane integrity reported that the combination of EMA and PMA reduced the DNA signal from dead cells (intact and damaged membrane) and live cells with inactive membranes (Codony et al., 2015). This method is based on the EMA property, which accumulates in dormant cells that lack the metabolic ability to offset its uptake using active mechanisms such as efflux pumps. Concentrations of 75 µM of PMAxx and 10 µM of EMA followed by incubation at 40 ºC at two incubation times (40 and 60 min) and a 15-min light exposure were performed. The results showed that, independently of incubation time, the combination of the two photoreactive dyes (PMAxx and EMA) reduced the amplification of dead cells after chlorine treatment above the LOD (Ct value >37) (**Figure 4**). When the two incubation times were compared, a slight increase in the Ct values was observed when 60 min were applied versus 40 min (Ct value = 39 and 37, respectively). However, it should be considered that a long incubation time might have a negative effect on the viability of *L. monocytogenes* cells inoculated in PWW. The differences between the levels of *L. monocytogenes* enumerated by qPCR and qPCR EMA+PMA are shown in **Table 3**. Based on these results, an incubation time of 40 min was selected.

The combination of the two photoreactive dyes (PMAxx and EMA) reduced the amplification of dead cells after chlorine treatment. The selected methodology for the enumeration of viable *L. monocytogenes* using qPCR was the combination of 75 µM of PMAxx and 10 µM of EMA followed by incubation at 40 ºC, defined as qPCR EMA+PMA.

Validation of the detection and quantification method for VBNC cells. The detection method EMA+PMAxx qPCR selected was validated in PWW obtained in an industrial processing line where shredded lettuce was washed with chlorine. Based on the results obtained, no significant changes in the concentration of free chlorine and pH were observed during the sampling interval that was inspected to control the accumulation of bacteria in PWW, maintaining a total count below 3 log units (**Table 1**). When the levels of total bacteria present in PWW were quantified, the results obtained by quantitation-based methods were very different than those based on molecular-based techniques (both qPCR and EMA+PMAxx-qPCR). Several studies previously reported that plate count methods lead to underestimating the total bacterial levels in environmental samples, mostly due to the presence of VBNC cells (Truchado et al., 2019). The results obtained indicate that chlorine (10 mg/L) induced bacteria into a VBNC state (**Figure 5**). Only slight differences were observed between the sampling points examined after 1 and 3 h from the beginning of the washing.

This is the first study demonstrating the induction of the VBNC state in bacterial cells present in PWW when water was disinfected with chlorine in an industrial setting.

Once the induction of cells in the VBNC state in an industrial setting was demonstrated, the next activity was to demonstrate if pathogenic bacterial cells behave similarly under comparable conditions. For this objective, the efficacy of commercial sanitizers to inactivate foodborne bacteria present in PWW and the ability to develop a VBNC state was evaluated in different types of PWW, mimicking industrial conditions of processing lines of fresh produce. Four different fresh products were selected based on the differences in the physicochemical characteristics that conferred to PWWs, according to our previous results (CPS 2017CPS01 project). The selection of the minimum concentration used for each sanitizer was also based on previously reported data (CPS2017CPS01 project). The selected concentrations were: 1) sodium hypochlorite (free chlorine; 20 mg/L); 2) peroxyacetic acid (PAA; 80 mg/L); and 3) chlorine dioxide (ClO_2; 3 mg/L). The physicochemical characteristics of the different types of PWWs are listed in **Tables 4** and **5**. Two repetitions per type of PWW and inoculum were carried out in a total of 16 individual experiments. For free chlorine, the levels of culturable and VBNC *L. monocytogenes* and *E. coli* cells in PWW were below the detection limit (1 cfu/100 mL) after 1 min contact time, independently of the type of PWW (**Figure 6**). On the contrary, the recommended concentration of PAA (80 mg/L) was able to reduce the levels of culturable *L. monocytogenes* (approx. 4 unit log) and *E. coli* O157:H7 (approx. 5 unit log) inoculated in the four types of PWWs. However, the total inactivation was not achieved, leading to the induction of the VBNC state of the inoculated pathogens (**Figure 7**). When the recommended operational limit for ClO_2 (3 mg/L) was studied, it was observed that *L. monocytogenes* cells were more susceptible than *E. coli* O157:H7 cells to this sanitizer (**Figure 8**). Significant differences were also observed among the different types of PWWs. In the case of shredded lettuce, the concentration of 3 mg/L ClO_2 was able to inactivate *L. monocytogenes,* but it induced the presence of VBNC cells. However, in the case of diced onions, ClO_2 was more effective, and only dead cells of *L. monocytogenes* were recovered after the treatment. For *E. coli* O157:H7, the lowest concentration of culturable cells was observed when onion PWW was treated with ClO_2 (**Figure 8**), confirming the high efficacy of this disinfection treatment.

Free chlorine: Operational limit for free chlorine (20 mg/L) established by the fresh-cut processing industry was effective in inactivating *L. monocytogenes* and *E. coli* O157:H7 cells present in the different PWWs examined, including shredded lettuce, cabbage, diced onions and baby spinach.

PAA: A concentration of 80 mg/L of PAA was not able to inactivate pathogenic bacteria inoculated in any of the selected PWWs. Cultivable as well as VBNC cells were detected in all cases when PAA was used. Based on these results, PAA is not a suitable sanitizer to maintain the microbiological quality of PWW.

ClO_2: Significant differences were observed among the different types of PWW. In the case of shredded lettuce PWW, 3 mg/L ClO_2 was able to inactivate part of the inoculum, but the presence of VBNC cells was detected.

Additionally, the resuscitation capacity of VBNC *L. monocytogenes* and *E. coli* O157:H7 cells present in PWW was evaluated. Resuscitation was tested in PWW, where culturable bacteria were not detected (1 cfu/100 mL). This was the case of all PWWs treated with chlorine (20 mg/L) and PWW from shredded lettuce and diced onions treated with ClO_2 (3 mg/L). Results showed that none of the VBNC *L. monocytogenes* and *E. coli* O157:H7 cells were able to resuscitate when PWW was treated with 20–25 mg/L chlorine (**Table 6**). On the other hand, for ClO_2, resuscitation of VBNC *L. monocytogenes* cells present in PWW from shredded lettuce and diced onion was observed (**Table 6**).

Resuscitation of VBNC *L. monocytogenes* and *E. coli* O157:H7 cells in PWW treated with 20 mg/L of free chlorine was not observed. However, resuscitation of VBNC *L. monocytogenes* cells in PWW from shredded lettuce and diced onion was observed when 3 mg/L of ClO_2 was used.

Objectives 2 and 3: Establishment of the ability of foodborne bacteria in the VBNC state, present in the process wash water, to attach to the surface of fresh produce during washing and the evaluation of the conditions needed for VBNC foodborne bacteria attached to fresh produce, to survive and recover from VBNC to the culturable state during storage and distribution mimicking conditions of the cold chain.

Further studies were implemented to determine the attachment capacity of the VBNC cells to fresh produce during washing. For these studies, those cases where the combination of sanitizer/produce matrix lead to the induction of VBCN cells in PWW in the absence of culturable cells were selected. Previous results showed that 3 mg/L of ClO_2 was able to induce VBNC *L. monocytogenes* cells in PWW from shredded lettuce. This was not the case for free chlorine at the recommended operational limits (20 mg/L), as this concentration was able to inactivate all the cells inoculated in the PWW. However, lower concentrations of free chlorine (2.5 and 5 mg/L) tested were able to create a scenario where VBNC cells could be present in PWW. The physicochemical characteristics of PWW used in the different trials are listed in **Table 7**.

The results showed that when free chlorine was 2.5 mg/L, VBNC and culturable cells of *L. monocytogenes* and *E. coli* O157:H7 were recovered in PWW from shredded lettuce. However, when the concentration increased to 5 mg/L, only VBNC cells of *E. coli* O157:H7 were recovered. Therefore, 5 mg/L of free chlorine was selected as the concentration used in further experiments (**Figure 9**). **Table 8** shows the levels of total bacteria, VBNC and culturable *L. monocytogenes* and *E. coli* present in PWW of shredded lettuce after ClO_2 and chlorine treatments, respectively. The results confirmed that only VBNC bacteria cells were detected in PWW treated with ClO_2 and chlorine.

In the case of fresh produce, **Table 9** shows the results of the levels of VBNC, resuscitation capacity and culturable cells of *L. monocytogenes* attached to the surface of shredded lettuce after washing and during storage. After washing shredded lettuce in PWW treated with ClO_2 (day 0) viable and culturable cells were not detected in the tissue (**Table 9**). This indicates two options: 1) *L. monocytogenes* cells present in PWW in the VBNC state were not able to attach to the surface of fresh produce during washing; or 2) levels of VBNC cells attached to the tissue were below the detection limit (1.17 log cells/g). During storage, at least one replicate from each sampling day showed colonies after the resuscitation protocol. As indicated in **Table 9** for trial 1, *L. monocytogenes* cells attached to shredded lettuce were able to form a colony when using the resuscitation media after 0, 10 and 15 days of storage. On the other hand, after 15 days at 7 ºC, VBNC and cultivable cells of *L. monocytogenes* were detected in the fresh produce, showing that the cells were able to resuscitate. Therefore, it could be possible that very small amounts of VBNC cells were able to attach to the fresh produce during washing. However, we were not able to detect them by the selected molecular technique used for quantification. These results indicate that injured cells present in fresh produce could be restored when subjected to optimal conditions in lab-scale experiments. However, when industrial conditions were mimicked, we observed a lower resuscitation capacity.

Based on the low percentage of VBNC cells that were able to resuscitate in fresh produce, it could be concluded that the probability that VBNC cells present in fresh produce are able to resuscitate and multiply is very low, which reduces the potential risk to the public's health. On the other hand, results obtained for *E. coli* O157:H7 cells present in the VBNC state in PWW treated with chlorine could not demonstrate the attachment of VBNC cells to the fresh produce during washing and the resuscitation of VBNC cells to cultivable cells (data not shown).

Based on these results, VBNC cells present in the PWW could be able to attach to fresh produce. However, under commercial storage conditions the probability of resuscitation and multiplication of VBNC bacterial cells in the fresh produce is very low. This indicates that the presence of VBNC bacteria in PPW can be associated to a low risk for public health.

Objective 4: Performance of challenge tests to assess the growth potential of L. monocytogenes *in fresh produce under foreseeable conditions of transportation, distribution and storage using molecular techniques able to differentiate between VC and VBNC.*

Challenge tests were performed in two different commodities inoculated with a known amount of VBNC cells (10^2–10^3 cfu/g). The levels of dead cells, VBNC and culturable *L. monocytogenes* cells detected in different types of inoculated fresh produce during storage were investigated at different time points (0, 5,10 and 15 days) (**Figure 10**). During the storage time, the levels of inoculated VBNC cells did not significantly change and no culturable cells were recovered by plating (**Figure 10**). Results showed that VBNC cells artificially inoculated on fresh produce survive during storage (15 d at 7 ºC), but were not able to recover to the culturable state. The growth potential determined for inoculated VBNC cells indicated that the selected commodities included in this study did not support the growth of VBNC *L. monocytogenes* cells (**Table 10**).

VBNC cells artificially inoculated on fresh produce survived during storage but they rarely recovered to the culturable state.

Summary of Findings and Recommendations

- Taking into account the complex composition of PWW, with high organic matter content and interfering compounds, fluorescent dyes were not able to differentiate among the physiological stages of the different bacteria species. Based on the results obtained, the flow cytometry was not a suitable methodology to distinguish between viable and dead cells in PWW.
- The combination of two photoreactive dyes (PMAxx and EMA) reduced amplification of dead cells after chlorine treatment. Based on these results, the combination of 75 µM of PMAxx and 10 µM of EMA followed by incubation at 40 ºC was the methodology selected for enumeration of viable *L. monocytogenes* using qPCR. This method is defined as qPCR EMA+PMA.
- This is the first study demonstrating the induction of the VBNC state in bacterial cells present in PWW when water is disinfected with chlorine in an industrial setting.
- **Free chlorine**: Operational limits established by the fresh-cut processing industry for free chlorine (20 mg/L) are effective in inactivating *L. monocytogenes* and *E. coli* O157:H7 cells present in PWW in all the vegetable matrixes evaluated, including shredded lettuce, cabbage, diced onions and baby spinach.
- **PAA**: A concentration of 80 mg/L of PAA was not able to inactivate the pathogenic bacteria inoculated in any of the PWWs studied. Cultivable as well as VBNC cells were detected in all the cases when PAA was used. Based on these results, PAA is not a suitable sanitizer to maintain the microbiological quality of PWW.
- **ClO_2:** Depending on the type of PWW, ClO_2 showed different behavior. For instance, in the case of shredded lettuce PWW, 3 mg/L ClO_2 could inactivate part of the inoculum but induced the VBNC stage.
- Resuscitation of VBNC *L. monocytogenes* and *E. coli* O157:H7 cells present in PWW treated with 20 mg/L free chlorine was not observed. However, resuscitation of VBNC cells of *L. monocytogenes* present in PWW from shredded lettuce and diced onion was observed when 3 mg/L of ClO_2 was used.
- Based on these results, VBNC cells present in PWW could probably attach to fresh produce. However, under commercial storage conditions, the probability of resuscitation and multiplication of VBNC bacterial cells in fresh produce is very low. This indicates that the presence of VBNC bacteria in PWW can be associated with a low risk for public health.
- VBNC cells artificially inoculated on fresh produce survive during storage but, in most cases, they were not able to recover to the culturable state. Only in the case of diced onions, *L. monocytogenes* was able to resuscitate, showing levels between 1–2 logs cfu/g at the end of storage. More research should be performed to fully understand the specific characteristics of diced onions that allow the recovery of the injured *L monocytogenes* cells.

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APPENDICES

Publications and Presentations

Truchado, P., Gil, M.I., Larrosa, M., Allende, A. 2020. Detection and Quantification Methods for Viable but Non-culturable (VBNC) Cells in Process Wash Water of Fresh-Cut Produce: Industrial Validation. Frontiers in Microbiology, 11,673.

Truchado, P., Gil, M.I, Allende, A. 2021. Peroxyacetic acid and chlorine dioxide unlike chlorine induce viable but non-culturable (VBNC) stage of foodborne pathogens in wash water. Food Microbiology, *In press.*

Truchado, P., Gil, M.I, Allende, A. 2021. Are viable but non-cuturable (VBNC) cells induced by the use of sanitizers in process wash water able to attach to fresh produce during washing? Int. J. Food Microbiology. (In Preparation)

12th Annual CPS Research Symposium 2019, Austin TX. Significance of sanitizers on induction of viable but non-cultivable (VBNC) foodborne bacteria and their survival and resuscitation in fresh produce. Poster presentation, June 18th and 19th, 2019.

11th Annual CPS Research Symposium 2020, Webinar Series V. Significance of sanitizers on induction of viable but non-cultivable (VBNC) foodborne bacteria and their survival and resuscitation in fresh produce. Oral presentation, July 21st 2020.

Truchado, P., Gil, M.I, Allende, A. Significance of Viable but Non-Culturable Bacteria in the Fresh-Cut Produce Industry. 2021 European Symposium on Food Safety. Oral presentation. April 2021.

Budget Summary

CEBAS-CSIC	**Total**
Salaries and Wages	$129,274.32
Travel	$11,709.08
Materials and Supplies	$114,632.40
Services	$0
Other Direct Expenses	$3,443.74
Indirect Costs	$10,341.94
Subcontracts	$0
Total:	$269,401.49

Tables 1–10 and Figures 1–10

Table 1. Physicochemical and microbiological characteristics of process wash water (PWW) at different sampling times including free chlorine (10 mg/L), pH, chemical oxygen demand (COD), temperature (Tª), oxidation-reduction potential (ORP) and total counts (log cfu /100 mL).

Sampling time (h)	Free chlorine (mg/L)	pH	COD (mg/L)	Tª	ORP	Total count (log cfu/100 mL)
8:00	12.6	6.6	537 ± 11	4.0	818	2.4
10:00	14.6	6.5	503 ± 10	3.8	821	2.3
10:30	10.9	6.6	382 ± 20	3.8	807	2.6
11:00	9.0	6.5	469 ±19	4.3	783	2.5

Table 2. Percentage of viable, viable but non-culturable (VBNC) and dead cells in a six-strain cocktail of *Listeria monocytogenes* untreated and treated with chlorine (10 mg/L) determined by plate count and flow cytometry methods.

		Physiological Cell Stage		
Methodology	**Treatments**	**Viable**	**VBNC**	**Dead**
Plate count	Untreated	100.0 %		0.0 %
	Treated	0.0 %		99.9 %
Flow cytometry	Untreated	89.6 ± 2.3 %	7.8 ± 1.0 %	2.6 ± 1.2 %
	Treated	27.4 ± 3.3 %	6.9 ± 0.1 %	65.7 ± 3.3

Table 3. *Listeria monocytogenes* counts inoculated in process wash water detected by qPCR combined or not with EMA+PMAxx at two incubation times.

Time of incubation (min)	*L. monocytogenes* (log cfu/mL)	
	qPCR	**EMA+PMAxx-qPCR**
40	4.14 ± 0.10a	3.83 ± 0.11a
60	4.34 ± 0.02a	3.29 ± 0.06b

Values represent the mean ± standard deviations for three independent replicates.

Table 4. Physicochemical parameters of process wash water (PWW) inoculated with *Listeria monocytogenes,* including chemical oxygen demand (COD), pH, conductivity (EC), oxidation-reduction potential (ORP) and temperature (T^a).

Trial	Sanitizer	Process wash water	COD (mg L^{-1})	Sanitizer (mg L^{-1})	pH	EC (µS cm^{-1})	ORP (mV)	Temperature (º C)
1								
	Chlorine							
		Shredded lettuce	989	20	5.62	3040	794	5.5
		Shredded cabbage	935	21	5.66	2265	871	6.6
		Diced onion	992	21	5.54	2560	869	6.2
		Baby spinach	374	20	6.73	2060	780	4.7
	PAA							
		Shredded lettuce	1018	79	5.44	1135	403	6.3
		Shredded cabbage	1136	83	5.40	1023	419	5.7
		Diced onion	1092	81	5.36	969	379	5.2
		Baby spinach	370	98	5.74	1616	364	4.9
	ClO_2							
		Shredded lettuce	945	2	4.89	3080	674	5.3
		Shredded cabbage	1115	2	6.13	2780	707	5.2
		Diced onion	1140	2	5.50	2920	714	5.2
		Baby spinach	345	2	6.87	995	659	4.9
2								
	Chlorine							
		Shredded lettuce	1044	20	5.77	3210	776	5.3
		Shredded cabbage	972	19	5.73	1577	875	5.7
		Diced onion	1100	19	5.94	2735	812	5.2
		Baby spinach	282	22	6.72	1688	783	4.8
	PAA							
		Shredded lettuce	1020	89	5.25	1503	384	5.5
		Shredded cabbage	995	87	5.10	1369	383	5.5
		Diced onion	1060	86	5.29	1153	430	5.3
		Baby spinach	298	94	5.73	1362	356	4.9
	ClO_2							
		Shredded lettuce	1140	3	4.91	2698	683	5.7
		Shredded cabbage	980	2	5.99	2220	714	5.4
		Diced onion	1046	2	5.95	2390	718	5.4
		Baby spinach	288	2	7.23	1629	671	4.6

Table 5. Physicochemical parameters of process wash water (PWW) inoculated with *Escherichia coli,* chemical oxygen demand (COD), pH, conductivity (EC), oxidation-reduction potential (ORP) and temperature (Tª).

Trial	Sanitizer	Process wash water	COD (mg L^{-1})	Sanitizer (mg L^{-1})	pH	EC (µS cm^{-1})	ORP (mV)	Temperature (º C)
1								
	Chlorine							
		Shredded lettuce	1068	21.4	6.50	2590	819	4.1
		Shredded cabbage	1042	19.8	6.84	2310	804	4.1
		Diced onion	1030	20.2	6.68	2570	845	4.2
		Baby spinach	332	21.5	6.73	2170	793	4.1
	PAA							
		Shredded lettuce	1080	82.0	6.42	1828	381	4.2
		Shredded cabbage	1088	81.0	6.30	1671	381	4.6
		Diced onion	1066	85.6	6.00	1605	362	4.0
		Baby spinach	340	79.0	6.31	1787	362	5.3
	ClO_2							
		Shredded lettuce	960	1.8	5.88	1866	636	3.9
		Shredded cabbage	1014	2.68	6.99	1523	717	5.0
		Diced onion	1106	1.78	5.85	1842	685	4.0
		Baby spinach	278	1.93	6.39	1816	689	4.3
2								
	Chlorine							
		Shredded lettuce	1018	19.9	6.62	2500	792	4.1
		Shredded cabbage	1000	19.1	6.73	2613	829	4.2
		Diced onion	948	18.8	6.81	2795	829	4.3
		Baby spinach	308	22.9	6.79	1969	786	4.6
	PAA							
		Shredded lettuce	954	83.3	5.89	1495	394	4.1
		Shredded cabbage	996	79.7	6.13	1282	274	4.2
		Diced onion	1004	85.0	5.66	1389	386	3.9
		Baby spinach	294	83.5	6.04	1571	391	4.2
	ClO_2							
		Shredded lettuce	1000	2.3	6.99	1611	703	4.2
		Shredded cabbage	944	2.2	6.73	1621	705	4.1
		Diced onion	968	2.2	6.82	1526	698	4.3
		Baby spinach	328	2.2	6.49	1930	661	4.3

Table 6. Resuscitation capacity of VBNC *L. monocytogenes* and *E. coli* O157:H7 cells in process wash water (PWW) after 1 min exposure to chlorine (sodium hypochlorite) and chlorine dioxide (ClO_2).

Listeria monocytogenes				
	Types of PWW			
Sanitizer	Shredded lettuce	Shredded cabbage	Diced onions	Baby spinach
Chlorine	-[a]	-	-	-
ClO_2	+[b]		+	
***Escherichia coli* O157:H7**				
	Types of PWW			
Sanitizer	Shredded lettuce	Shredded cabbage	Diced onions	Baby spinach
Chlorine	-	-	-	-

-[a], absence; +[b,] presence

Table 7. Physico-chemical characteristics of process wash water (PWW) obtained from washing shredded lettuce inoculated with *L. monocytogenes* or *E. coli* O157:H7 and treated with chlorine (sodium hypochlorite). Data include chemical oxygen demand (COD), pH, conductivity (EC), oxidation-reduction potential (ORP) and temperature (T).

Inoculated bacteria	Sanitizer (mg L^{-1})	Trial	COD (mg L^{-1})	pH	EC (µS cm^{-1})	ORP (mV)	Temperature (º C)
L. monocytogenes							
	2.6	1	1004	6.7	2290	734	4.6
	2.6	2	984	6.8	2320	695	4.5
	5.0	1	1175	6.6	3340	732	4.9
	5.0	2	984	6.7	2300	725	5.0
E. coli							
	2.8	1	982	6.4	2160	626	4.7
	2.6	2	966	6.7	2060	714	4.7
	5.3	1	1200	6.7	3620	727	4.9
	5.2	2	982	6.7	2390	774	5.3

Table 8. Physicochemical and microbial characteristics of process wash water (PWW) obtained from washing shredded lettuce inoculated with *L. monocytogenes* or *E. coli* O157:H7 and treated with chlorine dioxide or chlorine (sodium hypochlorite), respectively. Data include chemical oxygen demand (COD), pH, conductivity (EC), oxidation-reduction potential (ORP) and temperature (T).

					Listeria monocytogenes					
Trial	**Sanitizer**	**Sanitizer (mg L^{-1})**	**COD (mg L^{-1})**	**pH**	**EC (µS cm^{-1})**	**ORP (mV)**	**T^a (º C)**	**Total Cells Log (cells/g)**	**VBNC Log (cells/g)**	**Culturable Cells Log (cells/g)**
1	ClO_2	3.17	1016	6.14	842	687	9.7	8.15±0.15	7.29±0.02	0.00±0.00
2		3.04	998	6.29	1120	688	9.8	8.10±0.05	7.18±0.04	0.00±0.00
3		3.33	1070	6.56	1084	689	10.1	7.97±0.04	6.80±0.07	0.00±0.00
					***Escherichia coli* O157:H7**					
1	Chlorine	5.13	1016	5.36	-	874	11.3	6.42±0.00	3.14±0.16	0.00±0.00
2		5.19	1012	6.13	1205	783	9.6	6.37±0.01	4.68±0.07	0.00±0.00

Table 9. Viability and resuscitation capacity of *L. monocytogenes* adhering to shredded lettuce washed in PWW treated with 3 mg/L of chlorine dioxide (ClO_2) during 1 min and storage for 5, 10 and 15 days at 7 ºC.

Trial	Day	Replicate	Dead cells log cells/g	VBNC log (cells/g)	Resuscitation Capacity	Culturable cfu/g
1	**0**	**A**	5.06±0.20	-	-	-
		B	4.61±0.50	-	+	-
	5	**A**	5.15±0.05	-	-	-
		B	5.63±0.01	-	-	-
	10	**A**	4.98±0.17	-	+	-
		B	5.58±0.02	-	+	-
	15	**A**	5.21±0.13	4.34±0.15	+	0.62±0.01
		B	5.12±0.06	-	-	
2	**0**	**A**	5.45±0.08			
		B	5.46±0.05	-	-	-
	5	**A**	5.46±0.08	-	-	-
		B	5.37±0.05	-	-	-
	10	**A**	5.36±0.04	-	-	-
		B	5.56±0.05	-	-	-
	15	**A**	5.02±0.01	4.06±0.20	-	-
		B	6.23±0.10	4.26±2.50	-	-

Values are the mean and standard deviation of each repetition

Table 10. The growth potential of VBNC *L. monocytogenes* cells in different fresh produce stored for 15 days.

Fresh-produce	Growth potential VBNC cells	Growth potential Cultivable cells
Shredded lettuce	0.23	0.00
Baby spinach	0.18	0.00

Figure 1. Flow cytometry analysis showing a mixed culture of live and dead *Listeria monocytogenes* cells. The Q1 quadrant corresponds with the cells stained with propidium iodine red fluorescent signal (FL2 at 630 nm) (dead cells). The green-fluorescent signal (FL1 at 520 nm) of the Q4 corresponds with the live cells. The Q2 quadrant corresponds to the double stained cells (membrane-comprised cells or VBNC) and the Q3 with the non-stained cells.

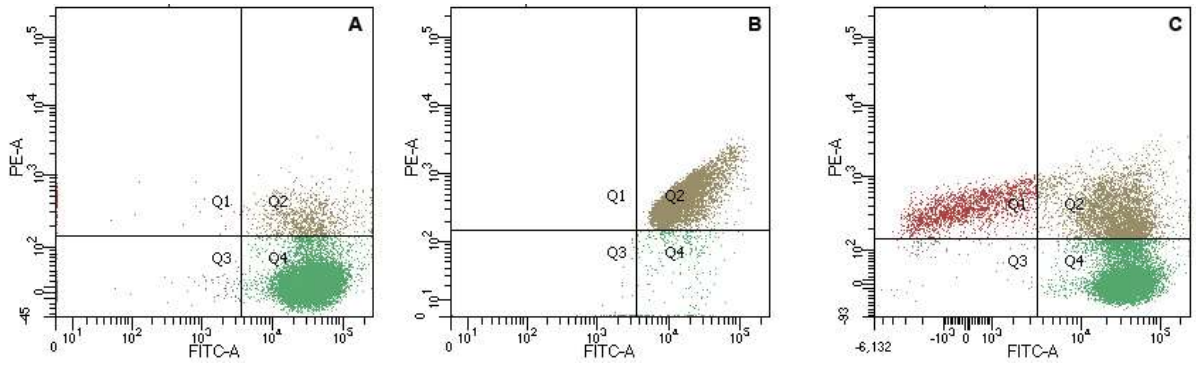

Figure 2. Cycle threshold (Ct) values obtained by PMAxx- qPCR at different concentrations with DNA extracted from live (A) or heat-killed (B) cells of a six-strain cocktail of *Listeria monocytogenes* inoculated in process wash water. The bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box represents the median. Whiskers show the highest values (defined as values more than 3/2 times the corresponding quartile). Different letters indicate significant differences at $P < 0.05$.

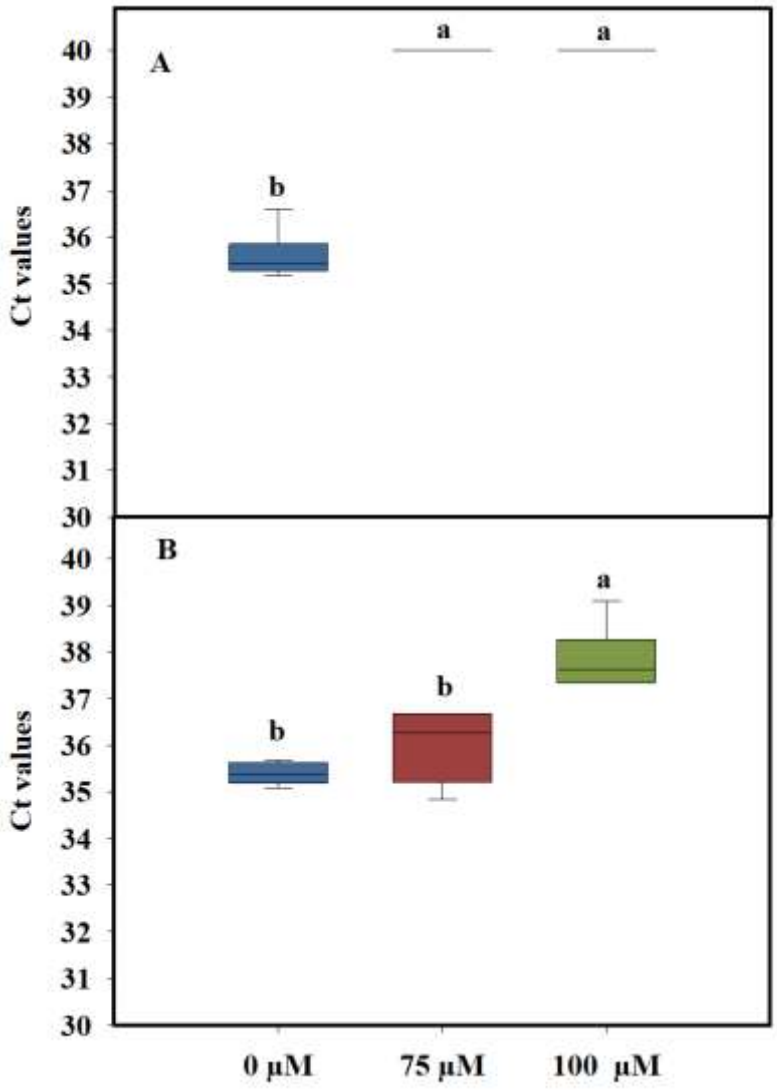

Figure 3. Cycle threshold (Ct) values obtained by PMAxx-qPCR with DNA extracted from cells of a six-strain cocktail of *Listeria monocytogenes* inoculated in heat-treated or chlorine-treated process wash water. Box plot represents three replicates of two independent assays (n=6). The bottom and top of the boxes represent the quartiles (25th and 75th percentile), with the line inside the box representing the median. Whiskers show the highest values (defined as values more than 3/2 times the corresponding quartile).

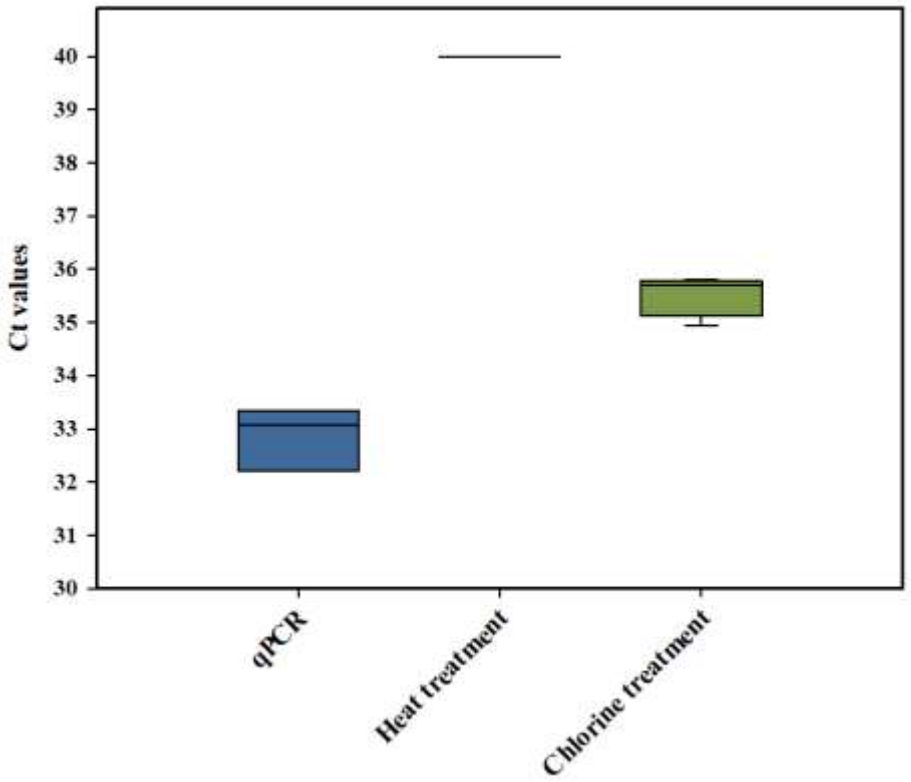

Figure 4. Effect of EMA+PMAxx incubation time on cycle threshold values obtained in qPCR with DNA extracted from dead cells of a six-strains cocktail of *L. monocytogenes* inoculated in heat-treated or chlorine-treated process wash water. Times of incubation were 40 min (A) and 60 min (B). Different letters denote significant differences (P<0.05). 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 highest values excluding outliers and dots represent outliers (defined as values more than 3/2 times the corresponding quartile).

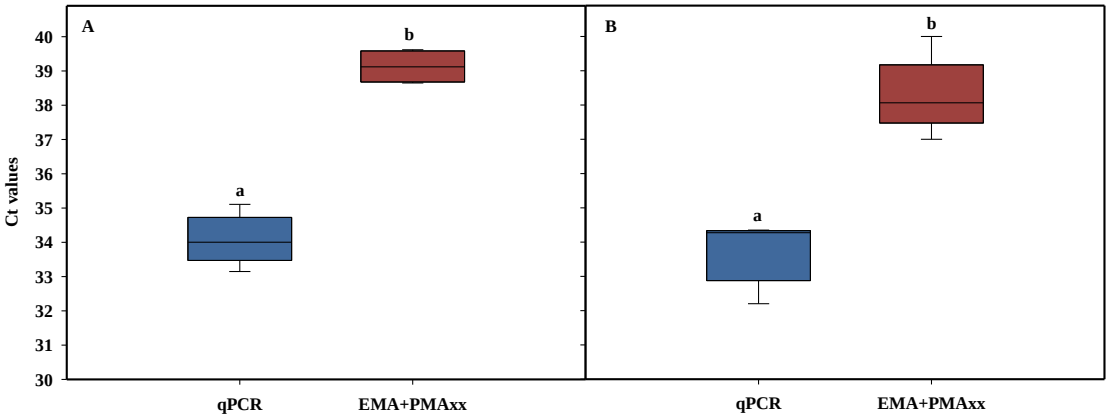

Figure 5 Populations of total bacteria (log cfu or cells/100 mL) in chlorine-treated process wash water from an industrial processing line of shredded lettuce after 2 h and 3 h from the beginning of the working day, corresponding to time 1 and time 3, respectively. Levels of culturable bacteria were obtained by plate count, levels of total bacterial by qPCR and viable bacteria by EMA+PMAxx-qPCR. Levels of VBNC were calculated by the differences between viable and culturable bacteria and dead cells by the differences between total and viable bacteria. Bars represent the mean and the standard deviation.

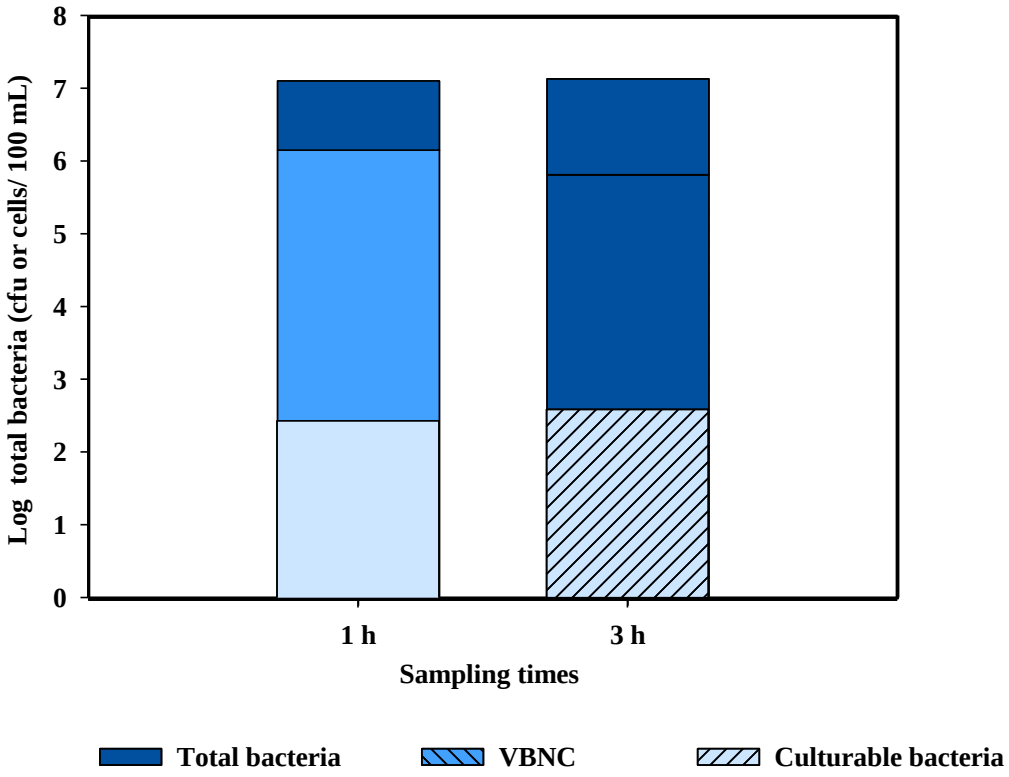

Figure 6. Viability of *L. monocytogenes* (**A**) and *E. coli* O157:H (**B**) (log cfu or cells/100 mL) in process wash water (PWW) of shredded lettuce, shredded cabbage, diced onions and baby spinach treated with 20 mg/L of chlorine (sodium hypochlorite) for 1 min. Bars represent the mean and the standard deviation (n=4).

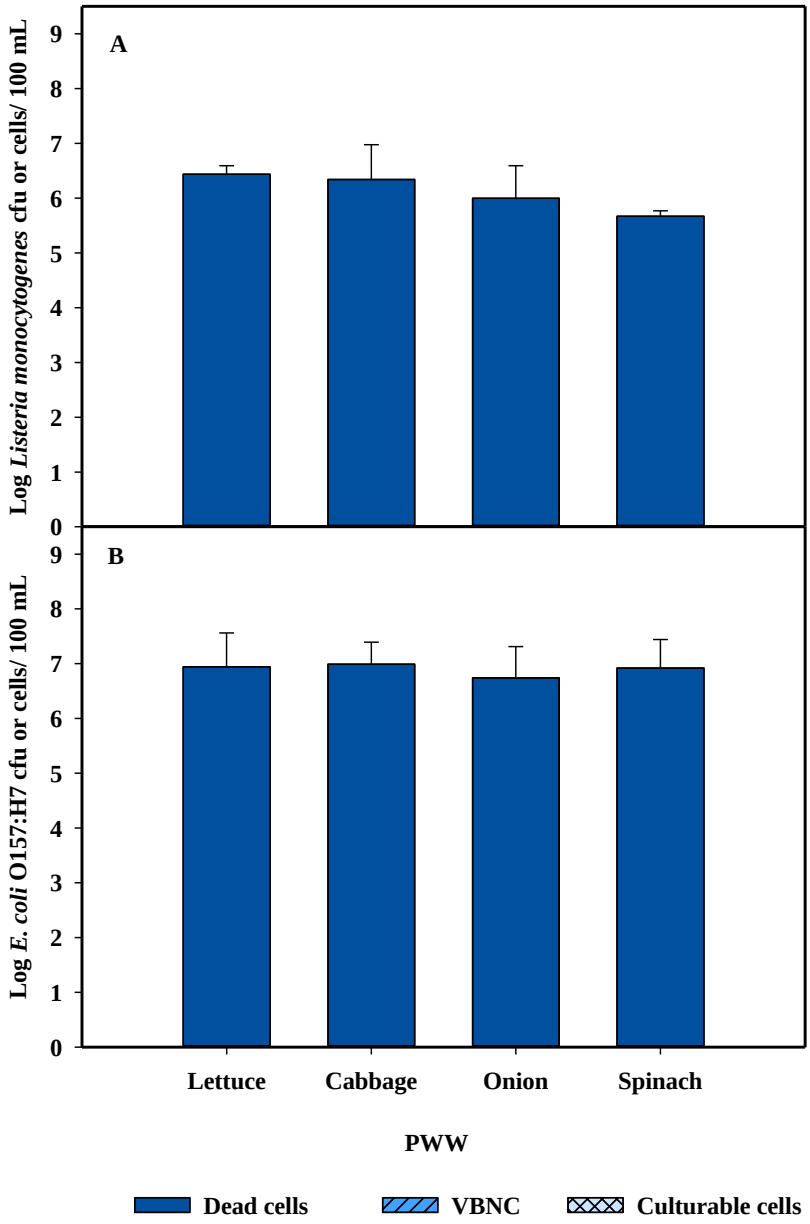

Figure 7. Viability of *L. monocytogenes* (**A**) and *E. coli* O157:H7 (**B**) (log cfu or cells/100 mL) in process wash water (PWW) of shredded lettuce, shredded cabbage, diced onions and baby spinach treated with 80 mg/L of peroxyacetic acid (PAA) for 1 min. Bars represent the mean and the standard deviation (n=4)

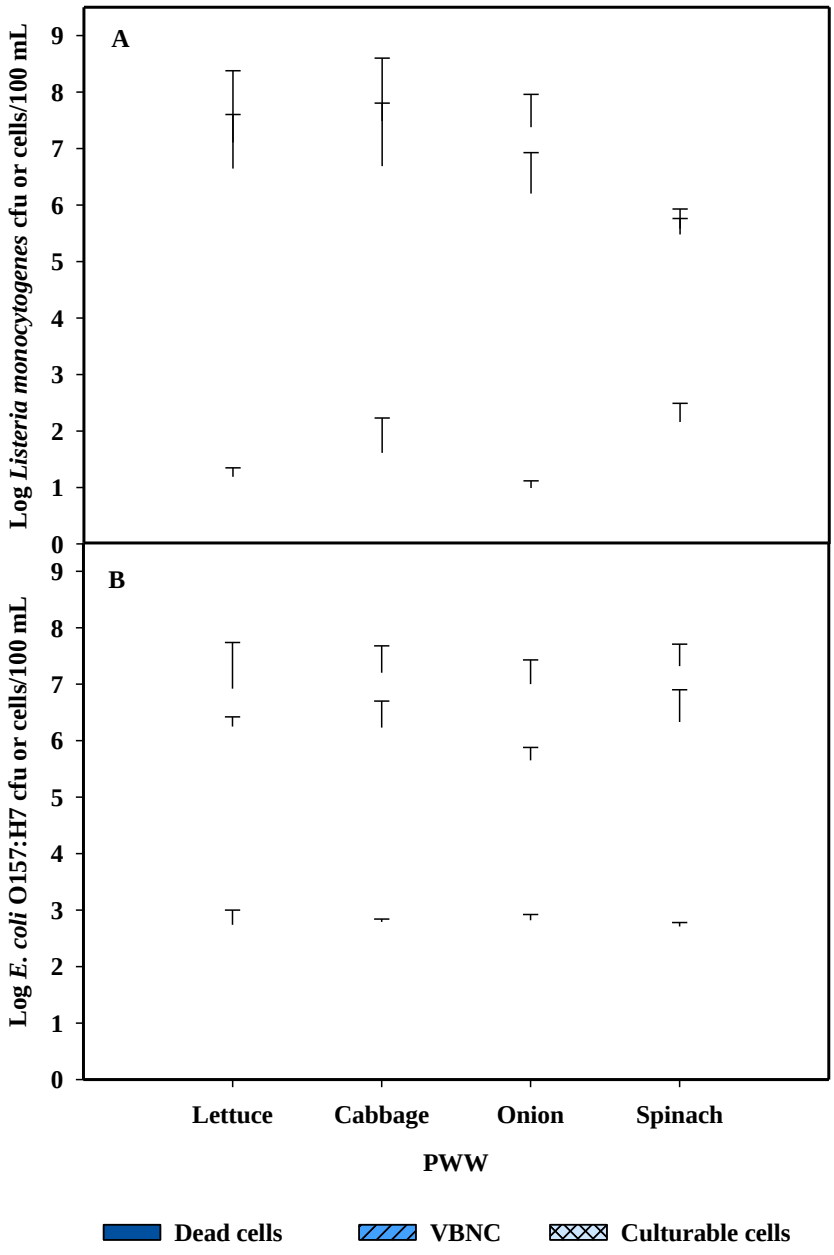

Figure 8. Viability of *L. monocytogenes* (**A**) and *E. coli* O157:H7 (**B**) (log cfu or cells/100 mL) in process wash water (PWW) of shredded lettuce, shredded cabbage, diced onions and baby spinach treated with 3 mg/L of chlorine dioxide (ClO_2) for 1 min.
Bars represent the mean and the standard deviation (n=4)

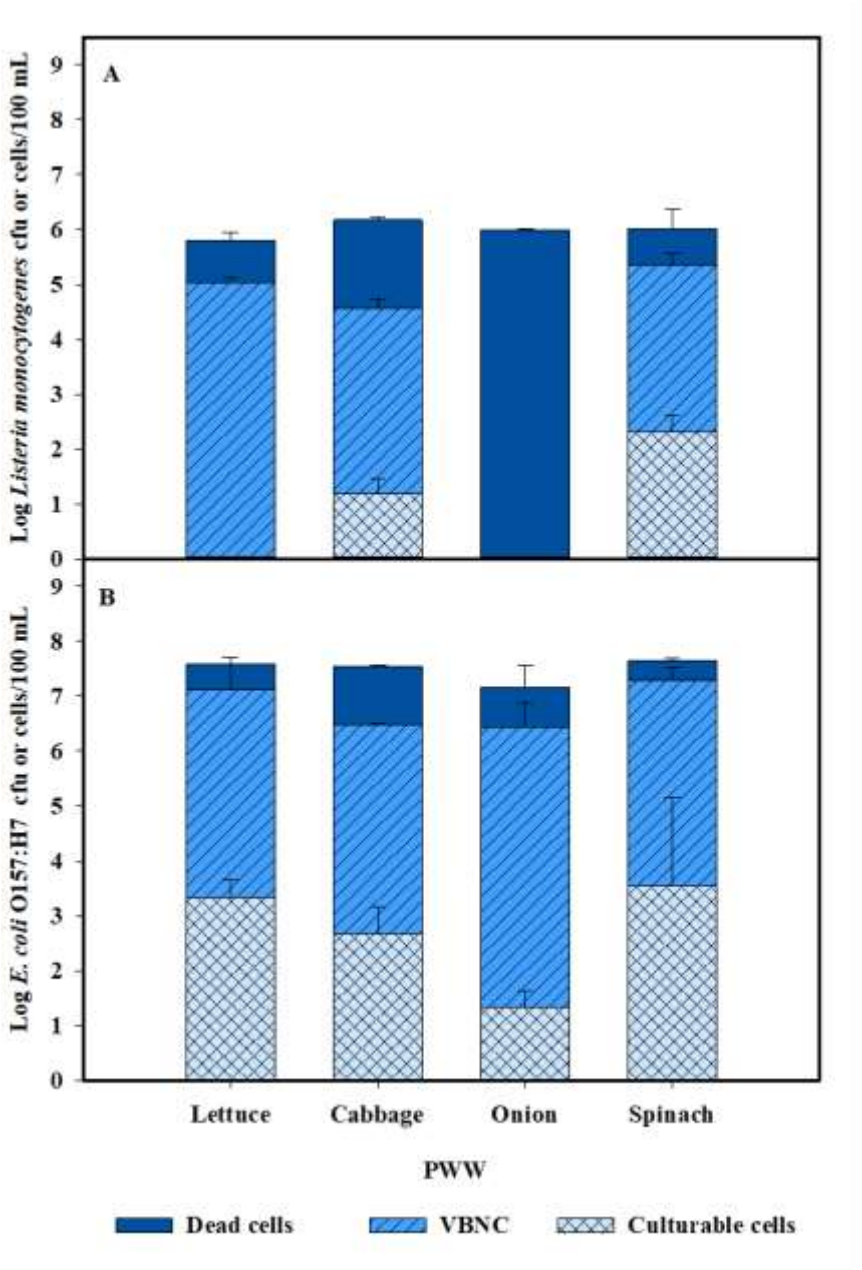

Figure 9. Levels of *L. monocytogenes* (**A**) and *E. coli* O157:H (**B**) (log cfu or cells/100 mL) in PWW of shredded lettuce treated with 2.5 and 5 mg/L of chlorine (sodium hypochlorite). Levels of culturable bacteria were obtained by plate count, levels of total bacterial by qPCR and viable bacteria by EMA+PMAxx-qPCR. Levels of VBNC was calculated by the differences between viable and culturable bacteria, and dead cells by the differences between total and viable bacteria. Bars represent the mean and the standard deviation (n=4)

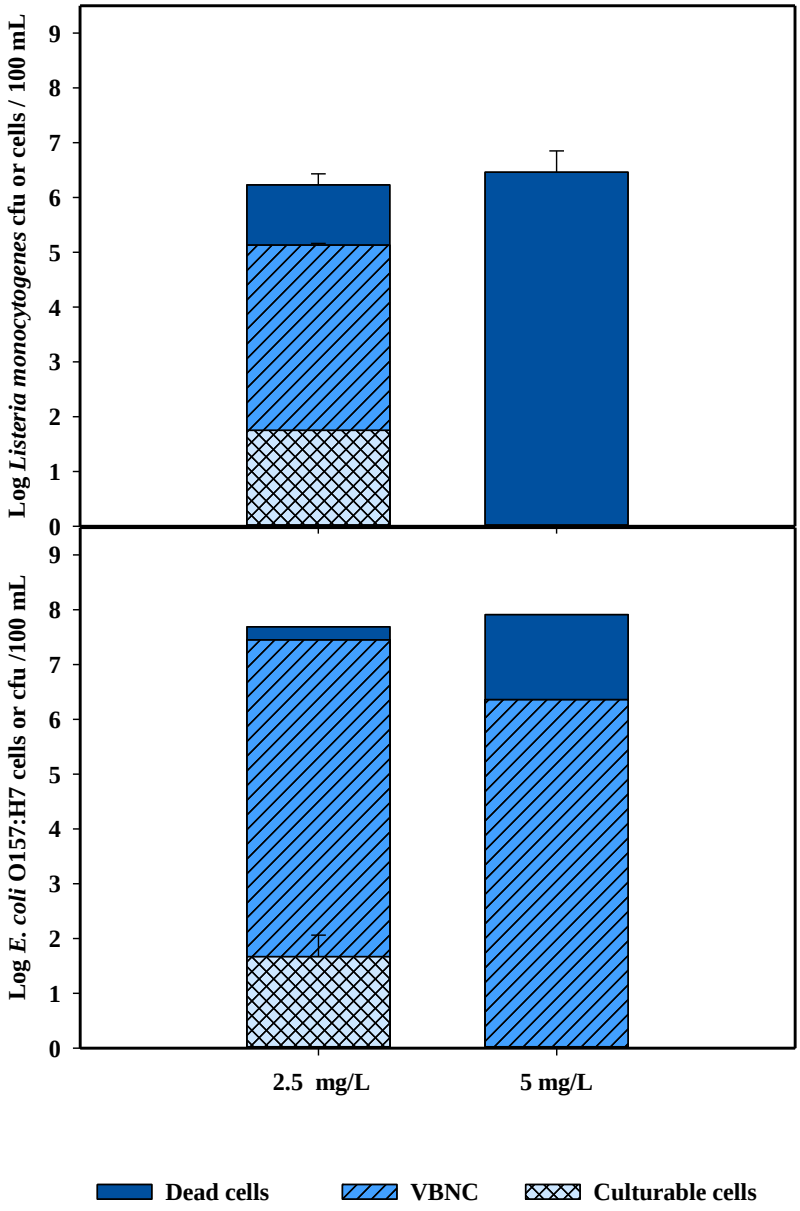

Figure 10. Levels of VBNC *L. monocytogenes* inoculated in shredded lettuce (A), and baby spinach (C). Bars are mean and standard deviation of each experiment (n= 2). . Bars represent the mean and the standard deviation (n=4).