RESEARCH IN PROCESS

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

SUMMARY

Sanitizers are needed to maintain the microbial quality of process wash water and prevent cross-contamination of the product. The efficacy of sanitizers on bacterial inactivation is conventionally determined using standard plate count procedures. However, foodborne pathogens are able to develop stress resistance mechanisms that enable them to enter into a temporary state of low metabolic activity—called the viable but non-cultivable (VBNC) state—whereby bacteria are viable but are not able to grow on agar plates. This project focuses on the ability of sanitizers to induce the VBNC state of foodborne pathogens and the consequences of underestimating the number of viable bacteria in process wash water. The ability of foodborne bacteria in the VBNC state to attach and survive in fresh produce also will be evaluated. Data obtained will allow us to understand if sanitizers induce the VBNC stage and if these foodborne bacteria are able to survive and grow on fresh produce.

OBJECTIVES

1. Estimation of the microbial inactivation and the induction of VBNC state of foodborne pathogens in process wash water (PWW) 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 PWW, 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 *Listeria monocytogenes* in fresh produce under foreseeable conditions of transportation, distribution and storage using molecular techniques able to differentiate between viable-culturable (VC) and VBNC.

METHODS

The work performed during the first trimester focused on Objective 1 through two types of experiments:

- **Optimizing the protocols to quantify total, viable, VBNC, culturable, and dead bacteria in broth and process wash water (PWW)** based on: (1) quantitative PCR (qPCR) combined with membrane impermeant dye (propidium monoazide) (PMA-qPCR); (2) PMA-qPCR combined with deoxycholate treatment (DC-PMA-qPCR); (3) Flow cytometry using a Live/Dead bacterial viability kit and flow cytometry analysis (**Figure 1**); and (4) ATP analysis.
- **Assessing the efficacy of commercial sanitizers to inactivate foodborne bacteria present in the PWW and induce a VBNC state in bacteria cells**. Studies were performed in a commercial peeled-garlic facility of an industrial collaborator to evaluate two commercial sanitizers: sodium hypochlorite (chlorine) and peroxyacetic acid (PAA). PWW samples were taken after 30 min and 2 h from the beginning of the washing. The proportions of culturable, VBNC, and viable bacterial cells were determined.

RESULTS TO DATE

- **Optimization of the protocols**: Initial trials were performed using pure cultures of *Listeria monocytogenes* in BHI broth and PWW. The enumeration of cells at the different bacterial stages using PMA-qPCR, DC-PMA-qPCR and flow cytometry was not significantly different among the methods (**Figure 2**). However, the loads of viable cells obtained using ATP analysis were significantly lower compared with the rest of the methodologies. Experiments using inoculated PWW reflected the complexity of the matrix (e.g., background microbiota, solid particles), which caused interferences when using the flow cytometer. Based on the results, the PMA-qPCR and DC-PMA-qPCR methodologies were selected as the reference methods to be used for monitoring.
- **Commercial sanitizers**: Sodium hypochlorite (**Figure 3**) and PAA (**Figure 4**) significantly reduced the levels of culturable bacteria, but the number of viable and VBNC cells were significantly higher. These results showed that the use of culturable methods overestimates the antimicrobial efficacy of these sanitizers.

BENEFITS TO THE INDUSTRY

These results highlight the need to analyze bacterial viability and physiological state by using nongrowth-dependent methods to quantify VBNC cells instead of the inactivation of foodborne bacteria using plate count procedures. Selection of the appropriate methodology capable of distinguishing between the different physiological stages of the bacteria will allow the industry to monitor fresh produce washing and the efficacy expected from sanitizers. The selected methodologies tested in commercial operations emphasize the great benefit that the produce industry can obtain if the effectiveness of sanitizers can be better established. The project is timely because it will be able to corroborate previous recommendations of the optimum sanitizer dose of approximately 10 ppm of hypochlorous acid at optimum pH (6.5 to 7.0) to avoid cross-contamination during washing, not only based on culturable bacteria but also taking into account the viable cells that still may be pathogenic.

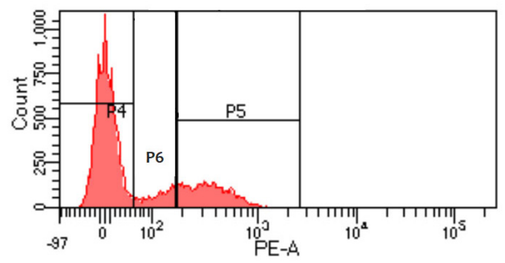

Figure 1. Histogram from a mix of viable (50%) and dead (50%) cells of a 3-strain cocktail of *Listeria monocytogenes* overnight culture, obtained using the Live/Dead flow cytometry analysis. The x-axis shows the intensity of the red fluorescence. The vertical lines separate viable bacterial cells (P4) from damaged cells (P6) and dead cells (P5). The cytometer used for the analysis was a LSR-Fortessa X-20.

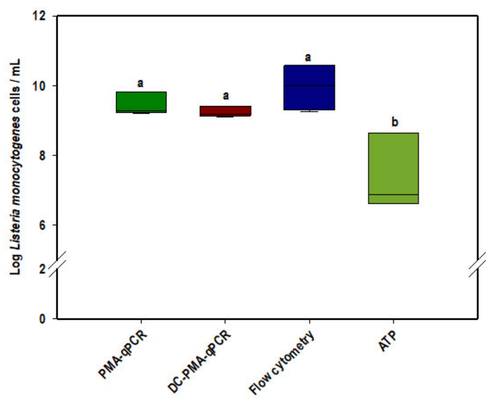

Figure 2. Comparison between the enumerations of viable cells obtained using different methodologies, including PMA-qPCR, DC-PMA-qPCR, flow cytometry, and ATP analysis, in a 3-strain cocktail of *Listeria monocytogenes* overnight culture in broth. Data obtained with the flow cytometer was transformed to log cells/mL based on the enumeration of total bacteria obtained using qPCR. Different letters denote significant differences ($P < 0.05$).

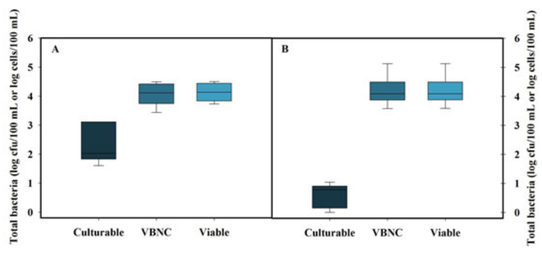

Figure 3. Populations of total bacteria (log cfu or cells/100 mL) in process wash water treated with sodium hypochlorite used for washing peeled garlic, after 0.5 h (A) and 2 h (B) from the beginning of the washing. Levels of culturable bacteria were obtained by plate count, levels of viable bacteria by PMA-qPCR, and VBNC by the differences between viable and culturable bacteria.

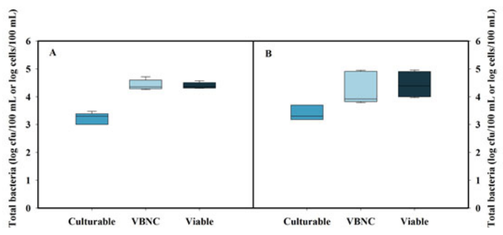

Figure 4. Populations of total bacteria (log cfu or cells/100 mL) in process wash water treated with peroxyacetic acid used for washing peeled garlic after 0.5 h (A) and 2 h (B) from the beginning of the washing. Levels of culturable bacteria were obtained by plate count, levels of viable bacteria by PMA-qPCR, and VBNC by the differences between viable and culturable bacteria.

Reference: FreshPlaza.com (23 January 2019). China: Demand surges for fresh garlic cloves in the European market. https://www.freshplaza.com/article/9064688/china-demand-surges-for-fresh-garlic-cloves-in-the-european-market/. Accessed May 2019.

Figure 5. Peeled garlic processing plant (FreshPlaza.com, 2019)

CONTACT

Ana Allende
CEBAS-CSIC, Spain
T: +34 968396200 Ext. 6377
E: aallende@cebas.csic.es

AUTHORS

Ana Allende
Mabel Gil (Co-PI)
Pilar Truchado (Co-PI)

ACKNOWLEDGEMENTS

Support provided by the Dr. J.A. Tudela and technicians involved in the project (Macarena Moreno, Silvia Andújar, Natalia Hernández and Jennifer Cascales) is greatly appreciated.
The collaboration and guidance of the industrial partners Primaflor SAT, Florette, STC S.A. and Citrosol is also recognized.

LENGTH OF FUNDING

January 1, 2019 – December 31, 2020

CENTER *for* PRODUCE SAFETY