XXV Congresso Brasileiro de Ciência e Tecnologia de Alimentos
Alimentação: a árvore que sustenta a vida
X CIGR Section IV International Technical Symposium
Food: the tree that sustains life
24 a 27 de outubro de 2016 • FAURGS • GRAMADO/RS

Development of a recommended protocol high pressure processing trials with pathogenic microorganisms for the prevention of cross-contamination

E.R. Griep[1], E.H.M. Walter [2], C. Moraru[1], A. Rosenthal[2]

1 - Department of Food Science – Cornell University – Zip Code: 14850 – Ithaca – NY – United States, Phone: (320)491-5723 – e-mail: (erg94@cornell.edu; cim24@cornell.edu);
2 - Embrapa Agroindustria de Alimentos – Zip Code: 23020-470 – Rio de Janeiro – RJ – Brazil, Phone: (21)36229620 – e-mail: (eduardo.walter@embrapa.br; amauri.rosenthal@embrapa.br)

ABSTRACT – High pressure processing is non-thermal process that can improve food safety and shelf life while maintaining important quality and sensory characteristics. While the process continues to grow within the commercial sector, different food matrices can impact the effectiveness of microbial inactivation, therefore continued research, especially with pathogenic microorganisms, can further improve the knowledge of this process as both a standalone and hurdle process. However, the use of pathogenic microorganisms within a pilot plant environment must be closely managed to prevent cross contamination and potential harborage of pathogenic bacteria within or around processing equipment. The development of a detailed biosafety procedure for these trials can decrease the risk of environmental contamination and can serve as important tools for both research pilot plants as well as commercial locations conducting process validation studies.

KEYWORDS: high pressure processing (HPP), pathogen trials, packaging, HPP validation

1. INTRODUCTION

High pressure processing (HPP) is a continuously growing processing method used within the food industry, either as one of multiple processing hurdles, or as a standalone processing method. When used at pressures of around 400-600 MPa, HPP has been shown to eliminate pathogenic and spoilage microorganisms thus improving the safety and shelf-life of HPP treated foods. Because this procedure is often carried out at temperatures below 45°C, products maintain both their nutritional and sensorial qualities, appealing to the rising consumer demand for fresh, minimally-processed foods. Commercially, HPP is often used for deli meats, vegetable products, seafood, juice, and dairy products (Balasubramaniam et al., 2008). Due to past recalls as a result of post-process *L. monocytogenese* contamination, fresh cheese is another category with a growing interest for HPP research due the numerous potential benefits (Tomasula et al., 2014).

Because of its use as an alternative to traditional thermal treatments such as pasteurization and sterilization, pilot-scale research on the inactivation of pathogenic bacteria by HPP in different food matrices is necessary to enable continued commercial expansion of this processing method. While microbial work often includes the study of non-pathogenic

strains as surrogates for the true pathogen, studies have shown variable pressure resistance between strains of the same genus such as *L. monocytogenese* (Simpson and Gilmour, 1997; Alpas et al., 1999; Tay et al., 2003). In addition, as a gram positive microorganism, *L. monocytogenese* is known to have one of the greatest pressure resistances (CFSAN, 2014). Therefore, often in inactivation studies the preferred method is to use the pathogenic microorganism. However, the use of pathogenic bacteria in both a laboratory and pilot plant environment add a human and environmental safety risk that must be managed. Since 1984, the Center for Disease Control and Prevention (CDC) has published and updated an extensive Biosafety in Microbiological and Biomedical laboratories (2009) manual. The purpose of this manual is to address these risks by making recommendations for the "safe handling and containment of infectious microorganisms and hazardous biological materials".

The objective of this project was to develop a standard operating procedure (SOP) and protocols based on the CDC guidelines to manage laboratory practices, equipment, personal protective equipment, and pilot plant practices to prevent the contamination of the equipment and surrounding environment with pathogenic microorganisms during HPP microbial inactivation trials. These procedures will be applied within microbiology lab and Pilot Plant 2 of the Embrapa Agroindústria de Alimentos campus. While the procedure will be applied initially to experiments on *L. monocytogenese* Scott A inactivation in fresh cheese, it is intended to be applicable to experiments involving any food product or target pathogen. The procedure also includes written sanitation guidelines in the case of suspected environmental contamination, as well as validation procedures to ensure the negative presence of *L. monocytogenese* within the processing environment. Finally, the procedure details the use and validation of a double packaging system, where a secondary package containing 70% ethanol surrounds the primary package of inoculated cheese in order to reduce the risk of environmental contamination by pathogenic bacteria during processing, should a leak in the primary packaging occur.

2. MATERIALS AND METHODS

2.1. Development of biosafety procedure

The biosafety procedure was developed based off of the CDC Biosafety in Microbiological and Biomedical laboratories manual (2009), current documented microbiology lab practices for Embrapa Agroindústria de Alimentos, US FDA and Canadian Food Inspection agency packaging guidelines, as well as recommendations from HPP equipment manufacturer and in-person discussions with Embrapa technicians involved in the various procedural steps.

2.2. *L. monocytogenes* inactivation in whey by 70% ethanol

Whey from the fresh cheese making process was inoculated with *Listeria monocytogenes* Scott A. The contaminated whey was added to 70% ethanol in a 1:1 ratio to simulate experimental conditions if a leak occurred in the primary HPP package, subsequently allowing whey to leak into the secondary package containing 70% ethanol. The 1:1 ratio of whey to ethanol used in the experiment helped determined the volume of 70% ethanol that

should be added to the secondary package during actual HPP experiments with inoculated fresh cheese. Although fresh cheese will be the product used for *L. monocytogenes* inactivation by HPP, it is likely that should a leak occur, only the whey would leak from the package. Methods for determining disinfectant effectiveness are adapted from US EPA Standard Operating Procedure for Germicidal and Detergent Sanitizing Action of Disinfectants Test (2013).

2.2.1 Experimental Procedure: Stock culture of *Listeria monocytogenese* Scott A was inoculated into trypticase soy broth (TSB) and incubated at 37°C. After 24 hours, 1mL of culture was aseptically transferred to a fresh tube of TSB and incubated at 37°C again for 24 hours. The final inoculum was washed 3 times by centrifugation with 0.1% peptone water prior to use. 5mL of inoculum was added to 45mL of freshly prepared cheese whey to achieve a final target concentration of 10^7 CFU/mL in the contaminated whey.

In a flux laminar hood, 5 mL of 70% ethanol was added to sterilized 10mL test tubes (for triplicate analysis). 5 mL of 0.1% peptone was added to another test tube to serve as a control. 5 mL of contaminated whey was added to each tube, and serial dilutions were completed at 1, 5 and 10 minutes to determine disinfection effectiveness of the 70% ethanol. All platings were completed in duplicate on trypticase soy agar and incubated for 48 hours at 37°C. Plates were counted from 0-300 colonies. Log reduction was calculated as Log_{10}reduction = mean $\log_{10}$ control – mean $\log_{10}$ treated sample.

3. RESULTS AND DISCUSSION

The developed biosafety procedure includes recommendations for microbiology lab practice for experiment food product inoculation, packaging and inspection, package transfer, pilot plant practices, and sanitation. A list of materials as well as a detailed step-by-step procedure was included to serve as a useful tool during experiments that researchers may follow to avoid distractions or missing equipment and materials. This can decrease the risk of contamination that may occur if researchers must interrupt their experiment and search for materials within the lab or pilot plant. Additionally, by having documented step-by-step procedures for managing package leaks or suspected contamination within the pilot plant, researchers can act quickly to clean and sanitize the affected areas as soon as the risk is known. Because the Embrapa Agroindústria de Alimentos campus already maintains a fully established microbiology lab with experience in handling pathogens, few procedural changes were necessary in this area. The developed biosafety procedure serves more as a reminder to students and technicians of proper handling and management of pathogenic bacteria, especially in regards to strict separation of laboratory materials and areas used for pathogenic vs. non-pathogenic bacteria.

A critical step within the procedure is the inspection of sealed packages containing inoculated products. Products intended for HPP are packaged in semi-rigid or flexible packages which allow for the transmission of pressure from the pressurizing liquid to the product. Commercially, many high pressure processes are pre-fill, meaning the product is placed and sealed in its final packaging prior to the processing step. As a result, the package must resist volume change under pressure and maintain hermetic closure before and after processing (Brody, 2011). Heat seal strength is very important in this, because any imperfection or amount of delamination can compromise the safety and quality of the product.

This is equally important in research settings, especially when food products are inoculated with pathogenic bacteria. Consequently, the developed biosafety procedure includes a thorough visual inspection to look for evidence of wrinkles, folds, delamination, or product in the seal area, as is recommended by both the US Food and Drug Administration (CFSAN, 2001) and the Canadian Food Inspection Agency (2002). While other methods of inspection are available, visual examination should be expected to reveal most defects, especially if accompanied by light squeezing to verify seal strength. This will be completed on both the primary package containing the food sample, as well as the secondary outer packaging containing the 70% ethanol.

In the *L. monocytogenese* inactivation by 70% ethanol experiments, the control had a concentration of 6.82 log (Table 1) and was fully inactivated after 5 minutes of contact with ethanol. Only 1 plate out of all dilutions completed in triplicate after 1 minute of contact was found to have a single colony of growth. This colony was collected and streaked onto modified oxford agar and incubated for 48 hours at 37°C. The black discoloration of the agar indicated that the colony was likely the *L. monocytogenese* Scott A from the control inoculum, but further biochemical confirmation tests were not completed. These results are not unexpected given the effectiveness and common use of 70% ethanol as a disinfectant in laboratory settings, however if not enough ethanol is used it is possible that a leak in the primary package may dilute the ethanol to below its effective concentration (Morton, 1950; Best et al., 1990; Carballo and Araújo, 2012) These data indicate that a volume of 70% ethanol in the secondary package equal to that of the liquid in the primary package should be sufficient to eliminate the risk of environmental contamination by *L. monocytogenese* in the rare event of a leaked package. Further experiments can be completed to determine the effectiveness of this packaging strategy with HPP juice products.

Table 1: Reduction of *L. monocytogenese* Scott A by 1:1 ratio of 70% ethanol:inoculated whey

Initial Concentration (CFU/mL)	Concentration at 1 minute (CFU/mL)	Concentration at 5 minutes (CFU/mL)	Concentration at 10 minutes (CFU/mL)
6.65E+06	1.67E+01	0	0
Log Initial Concentration	Log reduction at 1 minute	Log reduction at 5 minutes	Log reduction at 10 minutes
6.82	5.60	6.82	6.82

Cleaning and sanitizing procedures in the event of suspected environmental contamination are critical for the pilot plant environment not only for researcher safety, but also to prevent the harboring of *L. monocytogenese* and growth of biofilms, which then have the potential to cause cross-contamination of any additional experiments conducted in the same area. One of the main components of any HPP unit is the pressurizing fluid. Commercially, water or another food-grade liquid are generally used, with the ability to be filtered and re-used if the unit is equipped to do so (Balasubramaniam et al., 2008). However, in pilot scale HPP units, it is not uncommon for 70% ethanol to be used as the pressurizing fluid, as is the case for Embrapa Agroindústria de Alimentos. This is advantageous because it greatly decreases the risk of environmental contamination even in the case of both the primary

and secondary package leakage. Prior to running any experiments, the concentration of ethanol used as a pressurizing fluid will be verified with a hydrometer. At the end of any experiment which has used pathogenic bacteria, or in the case of a leaked package, the recommended sanitizing procedure within the HPP unit is to simply refresh the unit with new 70% ethanol. After doing so, the unit will be run for 3 cycles at 450 MPa for 10 minutes to ensure wetted parts are exposed to the fresh ethanol solution. Because any potential contamination would still be in the planktonic form rather than in a biofilm, the ethanol alone should be sufficient in eliminating any vegetative bacteria. With the addition of pressure treatments, the chance is extremely low of remaining vegetative bacteria within the unit. Should suspected contamination occur in the pilot plant environment, the area will be disinfected with a 2.5% solution of sodium hypochlorite for 15 minutes, as is currently an alternate disinfection used within the Embrapa Agroindústria de Alimentos microbiology laboratory. As a safeguard, environmental swabbing for *L. monocytogenes* will be completed per FDA procedure (CFSAN, 2015) within the HPP unit as well as environmental locations deemed necessary.

4. CONCLUSIONS

The presence of documented procedures for pathogenic trails in a pilot plant can reduce the risk of environmental contamination, especially when accompanied by sanitation and environmental validation. When conducting HPP experiments with pathogenic microorganisms, important points to consider include packaging seal inspection and sanitation procedures. The use of a double packaging strategy can also be utilized to prevent contamination in the event of a package leak caused by HPP. By working closely with researchers involved in all steps of the process, a comprehensive SOP can be developed that adequately reduces foods safety without the addition of unnecessary or burdening tasks.

5. ACKNOWLEDGEMENTS

Emily Griep would like to thank the USDA National Needs Fellowship program (award 2013-38420-20519) which allowed the collaboration with Embrapa Agroindústria de Alimentos researchers.

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