Depuration and heat treatment to reduce pathogen levels in bivalve molluscs produced in Santa Catarina State, Brazil

Robson Ventura de Souza[1], Vanessa Moresco[2], Marilia Miotto[3], Doris Sobral Marques Souza[4], Carlos Campos[5] and Felipe Matarazzo Suplicy

Abstract – This review summarises the findings of studies, conducted in Santa Catarina State (SC), on the reduction of pathogens in molluscs via post-harvest treatments. Studies indicate that 48h depuration cycles can efficiently reduce the levels of *Salmonella* and naturally occurring *Vibrio* spp. in oysters, whereas viruses (hepatitis A virus and murine norovirus) require more than 48 hours to be completely inactivated. The tested depuration protocols were unable to eliminate the Protozoa *Cryptosporidium* and *Giardia*. The heat treatment protocol commonly adopted by local industries (steam cooking for 6 min at 100°C) can eliminate *Vibrio* spp. from mussels and murine norovirus from oysters. Despite some limitations, adopting these post-harvest treatments is an important strategy to improve the safety of mollusc consumption in SC.

Index terms: Oysters; Mussels; Post-harvesting treatment; Enteric virus.

Depuração e tratamento térmico para redução dos níveis de patógenos em moluscos bivalves produzidos em Santa Catarina, Brasil

Resumo – Esta revisão resume os achados de estudos realizados em Santa Catarina (SC) sobre redução de patógenos em moluscos por meio de tratamentos pós-colheita. Estudos indicam que os níveis de *Salmonella* e *Vibrio* spp. de ocorrência natural podem ser eficientemente reduzidos em ostras por meio de ciclos de depuração de 48h, enquanto vírus (hepatite A e norovírus murino) requerem mais de 48 horas para serem completamente inativados. Protozoários (*Cryptosporidium* e *Giardia*) não foram eliminados pelos protocolos de depuração testados. O protocolo de tratamento térmico adotado nas indústrias locais (cozimento a vapor durante 6 min a 100°C) é capaz de eliminar *Vibrio* spp. de mexilhões e norovírus murino de ostras. Apesar de algumas limitações, a adoção desses tratamentos pós-colheita é uma estratégia importante para melhorar a segurança dos moluscos em SC.

Termos para indexação: Ostras; Mexilhões; Tratamento pós-colheita; Vírus entéricos.

Introduction

Filter-feeding bivalve molluscs accumulate microorganisms, including human pathogenic bacteria and viruses when grown in sewage-polluted waters, and can present a significant health risk when consumed raw or lightly cooked (LEES, 2000; BUTT et al., 2004). To reduce the risk of human illnesses, many countries have implemented public health controls on the commercial production and/or wild gathering of bivalve molluscs (SOUZA et al., 2018). Essentially, these controls consist of monitoring faecal indicator organisms (FIOs) in molluscs and/or water and classifying production areas based on the results of this monitoring, which is followed, when required, by post-harvest treatments (depuration, relaying[6], and heat treatment) prior to sale for human consumption (FAO & WHO, 2018). Classifying production areas indicates their potential risk of contamination by pathogens. In 2012, Brazil enacted similar controls which require post-harvest treatments for molluscs from production areas with moderate levels of contamination.

Santa Catarina (SC) is the state which contributes the largest production of cultivated molluscs in Brazil. Mollusc production varied yearly over the last decade and has exceeded 20,000 tons of brown mussels (*Perna perna*) and 3,000 tons of Pacific oysters (*Crassostrea gigas*) per year (available at: www.infoagro.sc.gov.br). The total volume represents nearly 95% of the national production. The results from the official monitoring and classification programme conducted since 2012 (available at: www.cidasc.sc.gov.br) showed that molluscs from most marine farms in SC must be subjected to post-harvest treatments. This review summarises the findings of scientific

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[1] Veterinarian, Dr., Epagri/Centro de Desenvolvimento em Aquicultura e Pesca, CEP 88034-901, Florianópolis, SC, e-mail: robsonsouza@epagri.sc.gov.br

[2] Biologist, Dra., University of California Riverside/Division of Biomedical Sciences/School of Medicine, Riverside, CA 92521-0001, USA, e-mail: van.moresco@gmail.com

[3] Biochemical pharmacologist, Dra., Universidade Federal de Santa Catarina/Centro de Tecnologia de Alimentos/Departamento de Ciência e Tecnologia de Alimentos, Rodovia Admar Gonzaga, 1346, Itacorubi, Florianópolis, SC, 88034-001, e-mail: marilia.miotto@ufsc.br

[4] Biologist, Dra., Universidade Federal de Santa Catarina/Centro de Ciências Biológicas/Laboratório de Virologia Aplicada, CEP 88040-900, Florianópolis, SC, 88034-001, e-mail: doris.sobral@gmail.com

[5] Biologist, PhD, Cawthron Institute, 98 Halifax Street East Nelson 7010, Private Bag 2, Nelson 7042, New Zealand, e-mail: carlos.campos@cawthron.org.nz

[6] Relaying consists of transferring bivalve molluscs from polluted areas to areas with natural clean seawater for a period of time which can vary from a few days to several months, depending on the level of contamination.

studies on the efficiency of depuration and heat treatment in reducing pathogenic microorganisms under local conditions.

Bivalve depuration

Depuration consists of placing bivalve molluscs harvested from moderately polluted areas in tanks with clean seawater for a period of time (commonly at least 42 hours), thus enabling them to cleanse or purge themselves of microbiological contamination by continuing their normal filter-feeding and digestive processes (REES et al., 2010). Depuration is unsuitable for cleansing molluscs from heavily contaminated areas or areas affected by other types of contaminants (e.g., trace metals, pesticides, etc.) (LEE et al., 2008). Bivalve depuration experiments conducted in SC involved studies on the pathogens *Salmonella* Typhimurium (CORRÊA et al., 2007), *Vibrio parahaemolyticus* and *Vibrio vulnificus* (RAMOS et al., 2012), norovirus, hepatitis A virus (HAV) and human mastadenovirus (HAdV) (CORRÊA et al., 2012; SOUZA et al., 2013, PILOTTO et al., 2019), murine norovirus (MNoV) (SOUZA et al., 2013), and the Protozoa *Cryptosporidium* and *Giardia* (SOUZA et al., 2013).

Corrêa et al. (2007) investigated the reduction of *S.* Typhimurium contamination in artificially contaminated oysters using a commercial depuration system. They used a 1,000-L water tank with 2,400 oysters (*C. gigas)* placed in 19 perforated plastic baskets organized in four layers. A pump circulated water at a rate of 2,200L h-1. That study used a sterilizing system consisting of a 25W UV (254nm) low-pressure tube, a chlorine metering pump, and a sand filter. A refrigeration system maintained the water temperature at 19°C during all experiments. Prior to the start of depuration, *S.* Typhimurium counts in artificially contaminated oysters ranged from 15,000 to 20,000CFUg^{-1} (colony-forming units per gram) of digestive tissue. The water was disinfected by ultraviolet (UV) light, chlorine (1ppm), and both. The combined UV light and chlorine treatment resulted in no growth of *S.* Typhimurium in oyster samples within 12h. Both treatments with UV irradiation and chlorine reduced *S.* Typhimurium counts in oysters in all basket layers.

Corrêa et al. (2012) investigated the reduction of HAV and HAdV-5 in artificially contaminated oysters using an apparently similar system to the one used in the previous study. Dimensions (tank volume), water temperature, number of animals, and pumping rate were the same as those in the 2007 study. Differences from that study were the absence of chlorine treatment, three layers of baskets (instead of four), and the type of UV light (18W [254nm] unit). Prior to depuration, the authors placed a dozen oysters for 3h in a tank containing an estimated concentration of 600 viral particles L^{-1} (5L of seawater seeded with 3 x 10^3 viral particles) of each type of virus. The oyster samples were tested using cell culture methods, as well as PCR and quantitative-PCR (qPCR). The molecular assays showed that the HAdV-5 genome was present in the oysters sampled in the beginning of the depuration experiment and over three subsequent time intervals (48, 72, and 96h), whereas the HAV genome was not detected after 96h of depuration. Viral viability tests (integrated cell culture-PCR and immunofluorescence assays) indicated that HAV was inactivated within 72h, whereas HAdV inactivation required 96h.

A subsequent study (RAMOS et al., 2012) investigated the reduction of *V. parahaemolyticus* and *V. vulnificus* in artificially contaminated oysters using different combinations of UV light and chlorinated seawater treatments. The authors contaminated the oysters with a mixture of five strains of *V. parahaemolyticus* or *V. vulnificus* at concentrations of 10^4 to 10^5 CFU mL^{-1}. Batches of 120 oysters were subjected to depuration, conducted in a 350-L depuration tank at room temperature. The depuration set up recirculated water at 420L h^{-1} for 48h and comprised a sand filter, centrifuge pump, chlorination tank, aeration system (Venturi tube), 3- and 5-mm polypropylene filters, an activated charcoal filter, a chlorine pump, and UV lamps. The authors tested three treatments: T1, untreated control; T2, UV light (two 16W lamps); and T3, UV light and chlorine (sodium hypochlorite at a final concentration of 1 to 2ppm). Initial concentrations of *V. parahaemolyticus* were 1.1 x 10^4, 6.6 x 10^4, and 2.0 x 10^5 (all MPN g^{-1}) for T1, T2, and T3. After 48h of depuration, these levels decreased by 2, 2.4, and 3.1 (all $\log_{10}$ MPN g^{-1}), respectively. The initial concentrations of *V. vulnificus* were 1.1 x 10^2, 6.3 x 10^2, and 5.0 x 10^2 (all MPN g^{-1}) and the reductions, after 48-hour depuration, were 1.4, 2.5, and 2.4 (all $\log_{10}$ MPN g^{-1}) for T1, T2, and T3, respectively.

Souza et al. (2013) evaluated norovirus, HAdV and protozoa *Cryptosporidium* and *Giardia* depuration dynamics in oysters using different UV doses. The authors contaminated the animals by translocating them to marine areas with different levels of contamination and harvesting them 14 days later. Some animals also underwent artificial contamination with HAdV type 2 (HAdV-2) and murine norovirus type 1 (MNoV-1). The depuration experiment was conducted on a batch of 540 oysters in a 300-L tank with a pump maintaining a flow rate of 1,800L h^{-1} and tested a control (no UV) and two treatments: T1 - UV reactors with a minimal dose of 16mJ cm^{-2} with 18W and T2 - minimal UV dose of 44mJ cm^{-2} with 36W. The room temperature during all the depuration procedures was 20±2°C. The authors detected HAdV 14 days after relaying in all the sites (6.2 x 10^5 to 4.4 x10^7 gc g^{-1}) and *Giardia* cysts in only one site. The authors observed HAdV genomes, *Giardia* cysts, and *Cryptosporidium* oocysts up to 168h of depuration, regardless of the UV treatment. In the artificially contaminated and depurated oysters, the authors detected genomes of HAdV up to 168h and of MNV-1 up to 96h of depuration. Depuration failed to eliminate the protozoa or degrade the HAdV genomes but managed to degrade the MNV-1 genomes.

A study by Pilotto et al. (2019) investigated the effect of UV light in inactivating HAdV-2 and MNV-1 in depurated oysters. The authors used two depuration units with the same specifications as those used by Souza et al. (2013), one with a UV reactor (36W) and another tank without a reactor. The authors placed 50 artificially contaminated oysters in each tank, dividing them into two plastic baskets

maintained under controlled seawater temperature (19°C). Virus infectivity tests were performed by plaque assay and the results, reported as plaque forming units per millilitre (PFU ml^{-1}). The authors did not specify virus levels at the beginning of their experiments. After 24h, UV light treatment completely inactivated HAdV-2. The authors found no infectious virus in the tank without UV light after 48h but detected infectious MNV-1 in both treatments (with and without UV) after 120h of depuration, decreasing by only 1.2 log_{10} during this period.

Thermal inactivation of pathogens

We only reviewed studies which focused on heat treatment to eliminate the FIOs/pathogens accumulated by the molluscs in farms or wild harvesting areas. Results on the effect of different processing and packaging techniques on the shelf life of products based, for instance, on mesophile and psychrotroph counts are not included.

Ramos et al. (2011) assessed the microbiological quality of pre-cooked and refrigerated seafood marketed in SC. They determined *E. coli*, coagulase-positive staphylococci, and *Salmonella* spp. levels in 12 mussel and 12 clam samples purchased in fish markets in Santa Catarina Island from June to September 2008. The authors reported maximum *E. coli* counts of 24,000MPN $100g^{-1}$ and 2,300MPN 100 g^{-1} (data transformed from MPN/g to MPN/100 g) in *P. perna* mussels and clams *Anomalocardia brasiliana*, respectively. *Salmonella* spp. was absent in all samples (25g samples). In total, five clam samples exceeded the national legal limit (ANVISA, 2019) for coagulase-positive staphylococci (10^3CFU g^{-1}). Lima et al. (2017) evaluated the microbiological characteristics of *P. perna* mussel samples before and after the heat treatment commonly used by the industry in SC. The samples were cooked under steam at 100°C for 6 min in a vertical autoclave and cooled by immersion in a tank containing water and ice at 10°C for 10 min. The authors noticed that heat treatment eliminated *Vibrio* spp. (which had been detected in fresh mussels), without recontamination after processing.

Souza et al. (2018) evaluated the viral infectivity of HAdV-2 in *A. brasiliana* clams using ICC-et-RT-qPCR after heat treatment. The authors immersed the animals in boiling water over different time periods (0, 1, 1.5, 3, and 5min), finding that the temperature inside their digestive tissues remained <80°C over time. After 5 min of cooking, HAdV-2 levels decreased by 90% (1log_{10}) but were still detectable. The study concluded that consumers can be exposed to infectious human viruses if clams are only slightly cooked.

Pilotto et al. (2019) investigated HAdV-2 and MNV-1 stability in steamed oysters (*C. gigas*). The oysters were kept in boiling water until their valves opened (<6 min). Cooking times ranged from 2 min 5 s to 6 min 15s and their average internal temperature ranged from 83°C to 93.25°C. The minimum internal temperature measured in the oyster tissues immediately after shell opening was 83°C at 3.5 min. The plaque assay revealed no infectious HAdV-2 and MNV-1 inside oyster tissues after cooking, but PCR still detected MNV-1 genome copies after heating, which were probably derived from non-infectious virus particles.

Discussion

While mussels and clams are traditionally sold as cooked products in SC, Brazil has an important market for live oysters. Considering that a significant proportion of the bivalve molluscs produced in SC must be subjected to post-harvesting treatments, depuration can be a viable option to reduce the microbiological risks of such products. Research findings on depuration in SC indicate that this process can efficiently reduce the levels of the most prevalent bacterial pathogens, such as *Salmonella* and *Vibrio* (SOUZA et al., 2022), in oysters. For instance, the process eliminated *S.* Typhimurium within 12h and reduced *V. parahaemolyticus* and *V. vulnificus* by at least 2.4log^{10} MPN g^{-1} in 48h.

The results of virus depuration experiments undertaken in SC show that enteric viruses persist longer in oysters than bacteria; agreeing with many other studies worldwide. When discussing virus reduction in depuration experiments, it is important to bear in mind that some studies evaluated depuration efficiency based solely on the detection of virus genomes (e.g., PCR and qPCR), whereas others also investigated virus viability using cell culture techniques (Plaque Assay and ICC-RT-qPCR). Virus genomes can last longer than viable viruses. Therefore, the presence of genome does not necessarily indicate the presence of viruses capable of causing an infection. To date, most studies worldwide on viral depuration efficiency have focused on HNoV and/or surrogates (e.g., the feline calicivirus, F-specific RNA bacteriophages, and MNoV1) since these viruses persist longer in oysters than other enteric viruses and are a main cause of mollusc-related illnesses. These studies have used molecular PCR-based techniques to detect and quantify genome copies since there is still no standard cell culture method to determine HNoV infectivity (MCLEOD et al., 2017).

The studies conducted in SC which tested for HAV and HAdV in molluscs differed in their testing protocols for viral analyses: Souza et al. (2013) only used a molecular technique (qPCR) to detect and quantify genome copies whereas Corrêa et al. (2012) and Pilotto et al. (2019) used both qPCR and cell culture techniques (plaque assay and ICC-RT-qPCR) to evaluate viral infectivity. Corrêa et al. (2012) observed that infectious HAdV-5 were not detected in oysters after 72h of depuration, whereas they consistently found the virus genome up to 96h if depuration. That study used HAV as RNA virus controls and failed to detect this virus after 48 and 72h (infectious virus and genome copies, respectively) of depuration, suggesting its faster elimination than HAdV-5. The study of Souza et al. (2013) detected HAdV-2 genome in molluscs until 168h of UV-depuration. The most recent study (PILOTTO et al., 2019) found contrasting results, suggesting that infectious HAdV-2 in oysters can be completely inactivated after 12h when UV light is used to sterilise the water in depuration tanks. These studies indicated that, although HAdV (types 2 and 5) genomes can persist for days under depuration,

infectious particles decay before 24h in UV-treated depuration tanks.

It is important to bear in mind that no results show the efficiency of depuration in non-experimental conditions in SC. However, the characteristics of the depuration tanks, such as flow rates and UV dose, generally follow international best practices (LEE et al., 2008). In total, four of the reviewed studies artificially contaminated animals with laboratory-cultivated pathogens (ABREU CORRÊA et al., 2007; CORRÊA et al., 2012; RAMOS et al., 2012; PILOTTO et al., 2019) and only one exposed oysters to environments with different degrees of contamination over time. For other types of microorganisms, such as *E. coli*, the use of molluscs contaminated with artificially prepared cultures is considered inadequate to test depuration efficacy. Studies on the removal of bacteria during depuration using bivalves artificially seeded with bacterial cultures tend to show greater removal than studies using naturally contaminated shellfish (LEE et al., 2008).

In addition to studies on the reduction of pathogens reviewed in this article, other studies focused exclusively on reducing FIO levels during the depuration of *P. perna* mussels (SUPLICY, 1998), *C. gigas* oysters (BOBERMIN, 2013), and *A. brasiliana* clams (LAGREZE et al., 2022). These report significant reductions within 48h. None of the reviewed studies tested the reduction of pathogen levels in conjunction with FIOs to provide information on potential exposure of local consumers to pathogen contamination via products meeting the end-product bacterial standard and this is an important knowledge gap. Furthermore, the literature lacks studies on the effectiveness of relaying as a treatment option for contaminated molluscs in the natural marine environment in SC.

P. perna mussels and *A. brasiliana* clams are traditionally sold and eaten cooked in SC. This can be positive from a consumer safety viewpoint because it mitigates the risks of contamination from any pathogens acquired during primary production. However, light cooking practices (steaming, searing) do not necessarily provide the required temperature/time combination for efficient pathogen inactivation (BIOHAZ, 2015). The reviewed studies suggested that the heat treatment protocol commonly used by the industry in SC (steam cooking during 6 min at 100°C) potentially eliminated naturally occurring *Vibrio* spp. from produced mussels and eliminated HAdV-2 and MNV-1 from oyster samples. On the other hand, evidence suggests that 5 min of immersion in boiling water fails to completely eliminate HAdV from artificially contaminated clams. No studies were found on HAV inactivation in *P. perna* mussels or *A. brasiliana* clams under heat treatment. Overseas studies have evaluated this for cultivated mussels (CROCI et al., 1999; HEWITT & GREENING, 2006; BIOHAZ, 2015; MESSENS et al., 2018). Croci et al. (1999) tested different protocols (60°C for 30 min, 80°C for 10 min, and immersion at 100°C for 1 min) which were insufficient to inactivate all the viruses in the mussels (*Mytilus galloprovincialis*). The authors found no viable HAV only when they prolonged the treatment at 100°C for 2 min. Hewitt & Greening also failed to inactivate all HAV from mussels (*Perna canaliculus*) steamed for 180s (mean internal temperature of 63°C), finding no viable HAV only when immersed mussels in boiling water for 180s (mean internal temperature of 92°C).

In a comprehensive review of thermal processing of live bivalve molluscs for controlling viruses, Messens et al. (2018) used a HAV thermal inactivation model to predict HAV inactivation in molluscs under three time–temperature profiles complying with the European '90°C for 90s' criterion prescribed by Regulation EC 853/2004. They predicted mean HAV reductions of 1.67, 2.92, and 4.13log plaque-forming units PFU g^{-1}, depending on the analysed profile. Messens et al. show that the '90°C for 90 seconds' criterion may lead to significantly different HAV inactivation depending on the commercial process design. This evinces the need to develop thermal inactivation models for the mollusc species cultivated in SC and for locally prevalent pathogens to enable the definition of adequate heat treatment protocols. This is important since heat treatments which follow too conservative protocols may change the organoleptic characteristics and, therefore, the commercial value of the products, whereas less stringent protocols could result in unsafe products and greater risks to consumers.

In the study by Ramos et al. (2011), which assessed the microbiological quality of pre-cooked and refrigerated seafood marketed in SC, maximum *E. coli* in both mussels and clams were higher than the end-product standard internationally recommended for live and raw bivalve molluscs (STANDARD FOR LIVE AND RAW BIVALVE MOLLUSCS CODEX STAN 292-2008). The authors did not specify if the samples were obtained from products processed in approved establishments. Therefore, their results should not be considered conclusive evidence that the molluscs sold in SC markets contain high FIO levels, including batches subjected to heat treatment. It is known that a significant proportion of the locally traded molluscs is processed in non-approved facilities and sold illegally. Such products pose risks to consumers due to inadequate hygiene and safety conditions. Future investigations on the microbiological quality of molluscs should consider this question. Nevertheless, Ramos et al. (2011) provides evidence that molluscs subjected to heat treatment can have poor microbiological quality and that processing conditions also determined quality.

Conclusion

Laboratory experiments indicate that depuration can efficiently reduce the levels of some of the most prevalent bacterial pathogens in oysters. *S.* Typhimurium can be eliminated within 12h and *V. parahaemolyticus* and *V. vulnificus* can be reduced by as much as 2 log^{10} MPN g^{-1} in a two-day depuration cycle. On the other hand, HAV can take more than 48 hours to be inactivated and the only study which analysed a NoV surrogate failed to detect virus inactivation after 120h of depuration. This treatment was inefficient in eliminating protozoa from oysters. The heat treatment protocol commonly used by the industry in SC (steam cooking during 6 min at 100°C) can eliminate naturally occurring *Vibrio* spp. from mussels and HAdV-2 and MNV-1 from oysters. The HAV inactivation

dynamics under heat treatment for the species cultivated in SC (mussels *P. perna* and clams *A. brasiliana*) is an important knowledge gap.

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