



Biofilm formation by *Vibrio parahaemolyticus* on different surfaces and its resistance to sodium hypochlorite

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ABSTRACT: *Vibrio parahaemolyticus* is an important pathogen for both fish industry and consumers. It forms biofilm which makes it difficult to eliminate this microorganism using sanitizers. This study aimed to assess biofilm formation on different surfaces and effect of biofilm on resistance to sanitizers. Eight isolates of biofilm-forming *V. parahaemolyticus* were tested for the ability to form biofilms on a number of surfaces including high density polyethylene, stainless steel, glass, exoskeleton of *Farfantepenaeus paulensis* (Pink Shrimp), and operculum of *Micropogonias furnieri* (Whitemouth Croaker). Efficiency of sanitizer sodium hypochlorite against the bacteria was evaluated in the biofilms formed on the surface of the materials used; out the eight strains analyzed four formed biofilm on different surfaces. The present study shows that there are variations between surfaces in terms of biofilm formation, with more than one bacterial strain being able to form biofilm on the surface of the operculum of *M. furnieri* and on high density polyethylene as well. One isolate formed biofilm on glass, and one isolate formed biofilm on stainless steel. Sanitizers reduced biofilm formation on all surfaces. Based on our findings, we concluded that *V. parahaemolyticus* isolates have different ability to form biofilm on different surfaces. No isolates formed biofilm on shrimp shells. Results of this study also showed that sodium hypochlorite at a concentration of 20 parts per million (20ppm) of Cl_2 , albeit not able to eliminate bacteria reported in biofilms, is still capable of reducing bacterial populations.

Key words: bacterial contamination, sanitizers, food safety, fish, shrimp.

Formação de biofilme por *Vibrio parahaemolyticus* em diferentes superfícies e resistência ao hipoclorito de sódio

RESUMO: *Vibrio parahaemolyticus* é uma bactéria patogênica importante tanto para a indústria como para os consumidores de pescados, uma vez que pode formar biofilme, dificultando a sua eliminação por sanitizantes. Este estudo teve como objetivo verificar a formação de biofilme em diferentes superfícies e o efeito do biofilme sobre a resistência a sanitizante. Oito isolados de *V. parahaemolyticus* formadores de biofilme foram testados quanto à capacidade de formar biofilme em superfícies de polietileno de alta densidade, aço inoxidável, vidro, exoesqueleto de *Farfantepenaeus paulensis* (Camarão-rosa) e opérculo de *Micropogonias furnieri* (Corvina). A eficiência do sanitizante hipoclorito de sódio foi avaliada frente às bactérias nos biofilmes formados sobre as superfícies dos materiais utilizados. Das oito cepas analisadas, quatro foram consideradas formadoras de biofilme em diferentes superfícies. Os resultados mostraram variação entre as superfícies, sendo que mais de uma cepa formou biofilme na superfície do opérculo de *M. furnieri* e do polietileno de alta densidade. Um isolado formou biofilme em vidro e um em aço inoxidável. Nenhum isolado formou biofilme na carapaça de camarão. O sanitizante reduziu a formação do biofilme em todas as superfícies. Conclui-se que os isolados de *V. parahaemolyticus* apresentam distinta capacidade de formar biofilme em diferentes superfícies e que o hipoclorito de sódio na concentração de 20 partes por milhão (20ppm) de Cl_2 , embora não elimine as bactérias que se encontram em biofilme, reduz a sua população.

Palavras-chave: contaminação bacteriana, sanitizantes, segurança alimentar, peixe, camarão.

INTRODUCTION

Fish has high nutritional value. It is composed of proteins, unsaturated lipids, vitamins, and minerals. However, their microbiota is closely linked to the microbiota of the water where they live which makes fish susceptible to contamination by several microorganisms (JAY, 2005) including some species of the genus *Vibrio*.

Bacteria of the genus *Vibrio* account for a significant number of cases of human infections caused by the consumption of raw or undercooked crustaceans (THOMPSON, 2004). There are three species of *Vibrio* that are pathogenic to humans: *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. *V. parahaemolyticus* is a halophilic bacterium reported mainly during summer months when water temperatures exceed 15°C (SU & LIU, 2007) in

coastal and estuarine waters (HEITMANN et al., 2005; OLIVER & KAPER, 2001).

V. parahaemolyticus may be reported in planktonic or sessile state, that is, within a microbial community, forming biofilm. Microbial biofilm is an association of bacterial cells that attach to biotic or abiotic surfaces, and are surrounded by a complex extracellular matrix (MCCARTER, 1999).

A number of studies mentioned the ability of many bacteria to form biofilm on different surfaces. MILAN et al. (2015) evaluated the biofilm-forming ability of two different isolates of *Salmonella enterica* subsp. *enterica* on high density polyethylene, stainless steel, and glass surfaces. These authors demonstrated that both isolates had the ability to attach to all the three surfaces tested. In another study carried out by QUATRIN et al. (2015), researchers evaluated the bacterial biofilm formation by *Pseudomonas aeruginosa* on metal, stainless steel, acrylic, glass, polyethylene terephthalate (PET), high density polyethylene (HDPE), and Teflon surfaces. This microorganism formed biofilms in different densities on all surfaces tested. These studies showed that isolates of each bacterial species has different biofilm formation ability on different surfaces.

According to FLACH et al. (2015), the organization of microorganisms in biofilms may provide protection against dehydration and resistance to sanitizers. This increased resistance results from the formation of a barrier consisting of an exopolysaccharide matrix which prevents or decreases contact between microbial biofilms and antimicrobial agents (SREY et al., 2013).

Resistance may occur due to the use of different sanitizers with similar mechanisms of action, which increases the risk of resistance development, particularly in biofilms (BRAOUDAKI & HILTON, 2004) or even resistance transmission between bacteria within the biofilm (BORGES et al., 2013). Due to all these factors, research should be carried out in order to broaden our knowledge on the resistance of *V. parahaemolyticus* against sanitizers in biofilms. Surfaces in the food industry are susceptible to bacterial adhesion and are, therefore, potential sources of contamination if microbial biofilms are formed (ROSSONI & GAYLARDE, 2000). If bacterial isolates from biofilms become more resistant, the concentration of product or contact time used during hygiene procedures may be insufficient to eliminate microorganisms from a contaminated surface (ANTONIOU & FRANK, 2005). Sodium hypochlorite induces changes in bacterial cell permeability and interferes with the enzymatic

processes of these microorganisms (EVANGELISTA, 2000). Biocidal and oxidizing activities of this compound increased with the formation of hypochlorous acid (HClO) in its undissociated form when in pure aqueous solution (EMMANUEL et al., 2004). The present study aimed to assess the formation of biofilm by *V. parahaemolyticus* on different surfaces as well as evaluating resistance of these microbial biofilms against sodium hypochlorite.

MATERIALS AND METHODS

We tested eight previously obtained isolates which were considered biofilm-forming strains by ROSA et al. (2017) as follows: one of *Micropogonias furnieri* (Whitemouth Croaker), four of *Mugil platanus* (Lebranche Mullet) and three of *Farfantepenaeus paulensis* (Pink Shrimp). These isolates were tested for their ability to form biofilm on different surfaces, according to the technique used by MILAN et al. (2015) with minor modifications in order to adapt this method for *V. parahaemolyticus*. We used high density polyethylene (HDPE) plastic coupons, stainless steel coupons, and 4cm² sterile glass vials with flat surfaces; 1cm² coupons of *F. paulensis* exoskeletons and *M. furnieri* opercula that were prepared according to CASTRO-ROSAS & ESCARTÍN (2002) were also used in this study. Exoskeletons and opercula were manually removed from fish specimens and cut into the appropriate size after being subjected to the following washing steps: (1) 30 seconds under running water. After washing, samples were shaken in order to remove any residual liquid and soft tissue; (2) 30 seconds in 70% alcohol to remove possible contaminants; (3) samples were then washed again under running water. After washing, these samples were stored at -20°C until use. Coupons were placed in Petri dishes containing 100ml of Alkaline Peptone Water (APW) with 1% NaCl (APW-1% NaCl, Himedia, Mumbai, India) and 2ml of culture from each isolate recovered in APW-1% NaCl for 24 hours. Plates were then incubated at 37°C. At each 48 hours of incubation, coupons were gently washed twice with APW-1% NaCl to remove unbound cells and were again placed in Petri dishes with 100ml APW-1% NaCl without the inoculum and incubated at 37°C. After five repetitions of this procedure, sterile swabs were scrubbed over the entire surface of each coupon and put in glass tubes containing 10mL APW-1% NaCl. Then serial dilutions were made for counting microorganisms in Plate Count Agar (Standard Methods Agar-SMA) (PCA-2% NaCl, Acumedia,

Lansing, Michigan, USA). An isolate unable to form biofilm as determined by ROSA et al. (2017) was used as a negative control. Isolates which counts were statistically different compared to those of the control isolate were considered competent biofilm formers.

The efficiency of the sanitizer sodium hypochlorite (solution containing 20ppm of Cl_2) was evaluated against bacteria in biofilms formed on the surface of the different materials used according to the technique described by MILAN et al. (2015) with some modifications. The same procedure described above was repeated for the biofilm-forming isolates and biofilm-forming materials. After the last wash, the biofilm coupons were immersed in vials containing the sanitizer for 10 minutes. Once the established contact time was reached, the coupons were immersed in a neutralizing solution (0.1M $\text{Na}_2\text{S}_2\text{O}_3$) for 30 seconds. After washing with APW-1% NaCl, a sterile swab was scrubbed on the surface of each coupon and counting was performed in PCW-2% NaCl. Biofilms formed by the same isolates were used as controls and counted in PCW-2% NaCl before coming into contact with the sanitizer.

All experiments were repeated three times independently with new bacterial cultures and new coupons. In order to assess biofilm formation on different surfaces and bacterial resistance to sanitizers, analysis of variance of *V. parahaemolyticus* counts was performed. Results were evaluated by Tukey's test ($P < 0.05$) using the software Statistix® (2003).

RESULTS AND DISCUSSION

In the present study, four out of the eight isolates analyzed were considered biofilm formers on

different surfaces (Table 1). Our results showed that there are variations in biofilm formation between the surfaces tested. More than one isolate formed biofilm on the surface of the operculum of *M. furnieri* and on a high density polyethylene (HDPE) surface. Over time, plastic surfaces may become rough. Cracks may form as a result of the wear and tear of these surfaces which may in turn harbor residues that protect bacteria; and therefore, favor the formation of biofilms (SHI & ZHU, 2009). This highlights the importance of our findings in such material. HAN et al. (2016) tested the ability of different isolates of *V. parahaemolyticus* to form biofilm in shrimp and crab shells. The authors observed that these isolates were able to form biofilm on these surfaces. Their results differ from the results obtained in our study since none of the isolates that we tested formed a biofilm in shrimp shells. However, we observed that *V. parahaemolyticus* is able to form biofilm in opercula, which may hinder the elimination of the microorganism from the surface of fish. This makes this microorganism a potential source of contamination for other fish, utensils, and equipment both in vessels and in the fish industry. ABDALLAH et al. (2009) and HAN et al. (2016) carried out studies in which they observed that isolates of *V. parahaemolyticus* are able to form biofilm in glass and stainless steel, respectively. Similar findings were obtained in our study with some isolates on both surfaces. When analyzing the results of our study as well as comparing these results with those obtained in the afore mentioned studies, we noticed that there is a great variation among isolates regarding their ability to form biofilm on different surfaces. This variation may occur due to the bacterial extracellular matrix. This matrix may vary even within the same

Table 1 - Counts and assessment of biofilm formation by *V. parahaemolyticus* on different surfaces (Log/cm^2).

Isolate	Glass (SD)	High density polyethylene (SD)	Stainless steel (SD)	Operculum (SD)	Carapace (SD)
A	5.2(0.7)[-]	5.2(1.2)[-]	5.9(0.5)[+]	5.4(1.6)[-]	6.2(0.5)[-]
B	5.4(0.3)[-]	5.9(1.1)[+]	5.8(1.0)[-]	5.3(0.9)[-]	6.2(0.6)[-]
C	5.3(0.6)[-]	6.0(0.2)[+]	5.2(0.8)[-]	5.7(0.5)[+]	6.7(0.7)[-]
D	5.3(0.2)[-]	4.9(0.3)[-]	5.0(0.1)[-]	5.1(0.3)[-]	5.4(0.1)[-]
E	5.8(0.6)[-]	4.5(0.1)[-]	5.4(1.2)[-]	5.6(0.5)[-]	6.0(0.9)[-]
F	5.9(0.6)[+]	5.7(0.0)[-]	5.7(0.1)[-]	5.9(0.5)[+]	5.8(0.4)[-]
G	5.2(0.3)[-]	5.0(0.6)[-]	5.2(0.5)[-]	5.4(0.1)[-]	5.8(0.4)[-]
H	5.2(0.5)[-]	5.7(0.2)[-]	5.7(0.2)[-]	5.7(0.9)[-]	5.1(0.6)[-]
Control[-]	4.5(0.6)	4.3(0.4)	4.6(0.6)	4.3(0.2)	5.5(0.06)

SD = standard deviation; [-] without biofilm formation; [+] with biofilm formation.

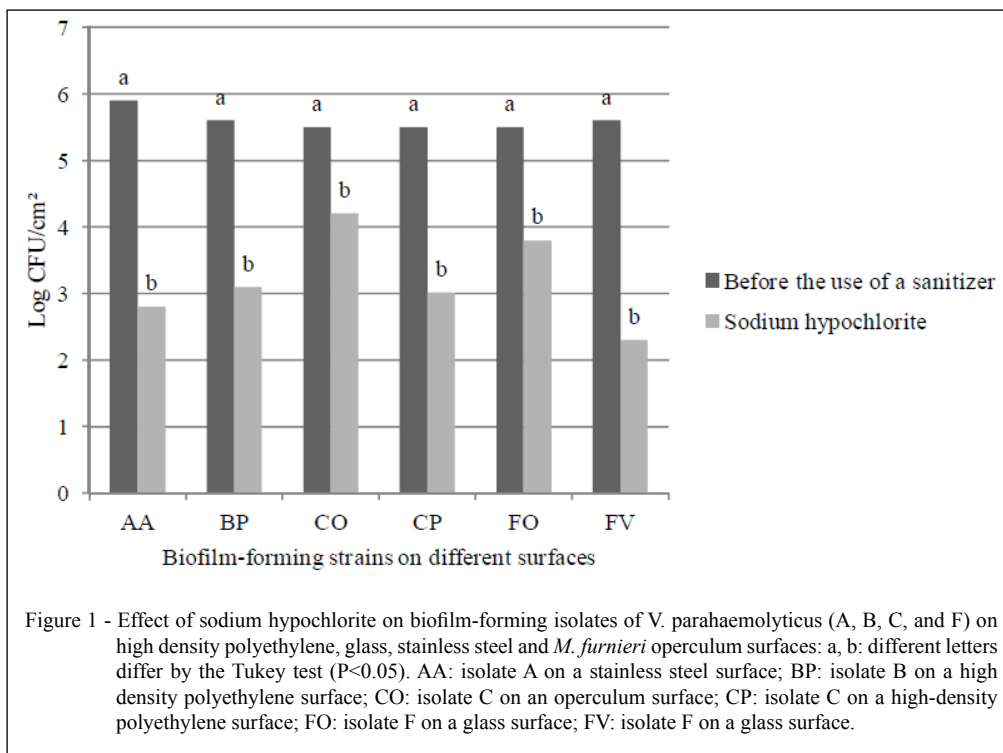
bacterial species as previously mentioned. It is the matrix that forms biofilms, which components include bacterial cells, exopolysaccharides, proteins, nucleic acids, glycoproteins, phospholipids, debris, and inorganic matter (SUTHERLAND, 2001). Results of the present study show that we should not generalize about the genus and species of this bacterium. Differences between the isolates of *V. parahaemolyticus* according to each surface tested showed that they may present different features regarding the ability to form biofilm.

To evaluate the efficiency of the sanitizer, the surfaces in which the isolates formed biofilm were immersed in flasks containing sodium hypochlorite. The analysis of variance showed effect only for the use of the sanitizer which reduced the bacterial population in the biofilms from all surfaces tested (Figure 1). However, this sanitizer was unable to eliminate the microorganisms. This result raises concern since the solution containing 20ppm sodium hypochlorite for 10 minutes that was used in our study is commonly used in the fish industry. To obtain the effective elimination of *V. parahaemolyticus* in biofilms it would be necessary to increase the exposure time or the concentration of the sanitizer in the solution.

BELTRAME et al. (2015) and BELTRAME et al. (2016) tested the efficiency of sodium hypochlorite against biofilms formed by *Listeria monocytogenes* and *Escherichia coli* in coupons made of high density polyethylene. These authors noted that the use of a solution containing 10ppm of sodium hypochlorite for 10 minutes fully removed *E. coli* from these coupons. In contrast, *L. monocytogenes* was fully removed from these coupons only when the concentration of 40ppm of this sanitizer was used. Based on the results of our study, we may infer that each bacterium from a biofilm reacts differently when coming into contact with a sanitizer. According to MEYER (2003), sodium hypochlorite acts not only on bacteria in a planktonic state but also acts in biofilms by removing exopolysaccharides from surfaces which makes difficult for new bacteria to attach (SINDE & CARBALLO, 2000).

CONCLUSION

Some isolates of *V. parahaemolyticus* are able to form biofilm a number of surfaces including glass, high-density polyethylene (HDPE), stainless steel, and operculum of *M. furnieri*. Isolates of *V.*



parahaemolyticus display a singular ability to form biofilm on different surfaces.

Sodium hypochlorite solutions at the concentration of 20ppm of Cl₂ is able to reduce the bacterial population of *V. parahaemolyticus* in biofilm but fails to eliminate the bacteria. These findings serve as an eye alert for those involved in the fish industry fish as microbial biofilms may form on a number of different surfaces. Hygiene and sanitation procedures may be reevaluated.

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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