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Research Article

Reduction of *Raoultella ornithinolytica* TN5 Biofilm using Hot Water and Nanochitosan

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Abstract

The equipment surfaces in food processing industries have the potential to contaminate products. Bacteria on a surface are able to form a biofilm. This study aimed to determine the effect of a combination treatment using hot water immersion and nanochitosan on the reduction of *R. ornithinolytica*'s biofilm on stainless steel surfaces. *R. ornithinolytica* was applied to a stainless steel surface, incubated at 30°C for 48 hours, and tested for its reduction using hot water immersion treatment with different times. The viability of cells was determined using a swab and the total plate count method. A scanning electron microscope was used for qualitative observations of biofilm formed on stainless steel before and after sanitation. The result showed that 10 minutes of hot water immersion resulted in significant *R. ornithinolytica* biofilm reduction compared to 5 minutes of treatment ($p<0.05$). Furthermore, the combination treatment of 10 minutes of hot water with 15 minutes of nanochitosan (0.1%) immersion showed the highest percent reduction of *R. ornithinolytica* biofilm ($p<0.05$). The ability of the combination treatment to eliminate *R. ornithinolytica* biofilms is equivalent to or even better than sodium hypochlorite treatment.

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1. Introduction

The processing environment in fishery industries is a major source of bacterial proliferation. Research by Møretrø *et al.* (2016) showed that bacterial contamination levels of >3 log CFU/cm^2 were found in cutting areas, conveyors, filleting machines, and hard-to-reach equipment. There is a mixture of bacteria in biofilms adhering to the surface of seafood processing equipment in Thailand, with a composition of 30% Raoultella (*Raoultella ornithinolytica* and *R. terrigena*), 30% *Pseudomonas* spp., and 17% Serratia (Hoa, 2015; Tantasuttikul and Mahakarnchanakul, 2019). *R. ornithinolytica* is a histamine-producing bacterium that can cause food poisoning (Etani *et al.*, 2023). *R. ornithinolytica* is capable of producing histamine levels exceeding safety thresholds, posing risks of food poisoning (Hwang *et al.*, 2020). Research by Ballash *et al.* (2021) successfully isolated *R. ornithinolytica* from various types of fresh and processed meat products, indicating its potential as a foodborne pathogen. This bacterium grew rapidly in milkfish surimi from 5 log CFU/g to 9.1 log CFU/g after 24 hours and 9.4 log CFU/g after 48 hours of storage at 25°C, followed by an increase in histamine content from 0 mg/100 g to 67 mg/100 g and 129 mg/100 g, respectively. Higher growth was observed at 37°C where it reached 9.5 log CFU/g after 48 hours with a histamine level of 160 mg/100 g. Samples stored at 15°C also supported an increase in bacterial count to 8.9 log CFU after 48 hours, with a lower histamine content of 3 mg/100 g. However, growth was inhibited in samples stored at 4°C, with only 5.8 log CFU/g after 98 hours of storage. This result shows a correlation between bacterial count and the histamine level produced in samples (Hwang *et al.*, 2020). Lee *et al.* (2016) also reported that *R. ornithinolytica* is able to produce histamine around 276.6 ppm in TSBH medium.

The sanitation process for equipment plays an important role in reducing surface contaminants. The general stages of sanitation and those recommended are cleaning, rinsing, sanitizing, and drying (Ohman *et al.*, 2024). Physical treatments, such as hot water immersion, were effective in eliminating bacteria on the surface (Tompkins *et al.*, 2008). Disinfection aims to kill microorganisms, such as bacteria that can cause illness, using chemical agents to prevent contamination and spoilage of food products (Nocker *et al.*, 2021). Sanitation procedures cannot eliminate all existing bacteria. Schlegelová *et al.* (2010) reported the presence of bacteria from 10^6 to 10^7 log CFU/cm^2 on equipment surfaces after the sanitation process.

Chitosan is a natural material, a non-toxic, biodegradable, and biocompatible polymer (El-Naggar *et al.*, 2024). The ability of chitosan to interact with the compounds, pathogens, or even microorganisms is known as its bioactivity (Ihsan *et al.*, 2021). However, nanochitosan has higher antibacterial properties compared to chitosan, as its large surface area allows it to provide better penetration (Sektiaji *et al.*, 2022). Nanochitosan has been used as a biopreservative due to its strong antibacterial effect (Abdeltwab *et al.*, 2019). Saputra *et al.* (2022) reported that nanochitosan was able to inhibit various bacteria, such as *Bacillus subtilis* and *Escherichia coli.* The optimal concentration of nanochitosan to inhibit the growth of *Bacillus subtilis* is 5:1 (chitosan: sodium tripolyphosphate) (Saputra *et al.*, 2022). Furthermore, the results of the study showed that the highest inhibitory effect of nanochitosan against *Staphylococcus aureus* was at a concentration of 0.05%, with the inhibition zone diameters from the first to third day being 12.31 mm, 9.98 mm, and 20.46 mm, respectively (Magani *et al.*, 2020). Nanochitosan exhibited antibacterial activity against both Gram-negative and Gram-positive bacteria, with no significant differences observed between them (Pan *et al.*, 2019). Moreover, nanochitosan also exhibits a high ability to inhibit biofilm formation of *S. aureus* (Godoy *et al.*, 2025) and *Pseudomonas* sp. (Aguayo *et al.*, 2020). Nanochitosan solution was also applied as a biosanitizer to reduce *E. coli* and *Salmonella* typhimurium on lettuce by 1.63 and 1.16 log CFU/g, respectively, after 15 minutes of washing (Paomephan *et al.*, 2018).

Nanochitosan is a potential biosanitizer to replace sodium hypochlorite, a widely used sanitizer in the food industry, particularly where safety, sustainability, and residue concerns are priorities. Sodium hypochlorite showed strong antibacterial activity to reduce *P. aeruginosa* biofilm on food contact surfaces (aluminium and stainless steel) (DeQueiroz and Day, 2007). Previous research from Roiska (2022) reported that 0.1% nanochitosan was able to reduce *R. ornithinolytica* biofilm by 94.82%, 99.55%, and 99.80% after treatment for 5, 10, and 15 minutes, respectively. However, these reductions are comparably lower than the effectiveness of 0.01% sodium hypochlorite, which was able to reduce biofilm by 99.67%, 99.86%, and 100% with the same immersion times. This result indicates the need for optimization of nanochitosan as an alternative sanitizer.

To enhance the effectiveness of nanochitosan as a sanitizer, a combination of physical and chemical treatments can be applied during the sanitation process. Physical treatment using hot water immersion can effectively remove exopolysaccharides in extracellular polymeric substance (EPS), thereby enhancing the exposure of the cleaning agent to the biofilm

(Kang *et al.*, 2021). Kharel *et al.* (2018) reported that hot water treatment at 80°C for 3, 4, and 5 minutes reduced *L. monocytogenes* by 4.93, 5.49, and almost 6 log CFU/g, respectively. Research by Nocker *et al.* (2021) indicated that temperatures of 70°C or 90°C resulted in a reduction in bacterial counts, with 90°C showing intact cell concentrations approaching the detection limit. This treatment can be used to sanitize small and hard-to-reach equipment (Bhagwat, 2019). The thermal is commonly used in the industry for sanitation processes (Karuppuchamy *et al.*, 2024). The physical and chemical treatments, as a combination, are expected to be more effective in reducing histamine-forming bacteria biofilm, such as *R. ornithinolytica*. Therefore, this study aims to observe the effect of a combination treatment of hot water and nanochitosan immersion on the reduction of *R. ornithinolytica*'s biofilm.

2. Materials and Methods

2.1 Materials

2.1.1 The equipment

The equipment used in this study included a hotplate (SP88857105; Thermo Scientific; USA), analytical balance (PX84; Ohaus; USA), electric pipette, vortex (VXMNFS; Ohaus; USA), waterbath (P30H; Elmasonic P; Germany), centrifuge (FC5515; Ohaus; Germany), incubator (IN110; Memmert; Germany), thin layer chromatography (TLC Silica gel 60 F254; Germany), stainless steel 304 2x2 cm^2, disposable petridish 90 mm x 15 mm (Onemed, Indonesia), sterile cotton swab (Onemed, Indonesia), spectrophotometer (G10S UV VIS; Thermo Scientific; China), Scanning Electron Microscope (JEOL JSM 6510LA), auto coater (JEOL JEC-3000FC) auto fine coater, pH meter (HI98107; Hanna; USA), blue tip (Axigen), particle size analyzer (SZ-100; Horiba; Japan), showcase chiller (r134A, RSA).

2.1.2 The materials

The materials used in this study were *R. ornithinolytica* TN5 (obtained from the Laboratory of Quality and Safety of Fishery Products, Department of Fisheries, Universitas Gadjah Mada), Tryptic Soy Agar (TSA; Merck; KGaA; Germany), Tryptic Soy Broth (TSB; Merck; KGaA, Germany), Tryptic Soy Broth with 1% L-Histidine (TSBH; Merck; KGaA, Germany), glycerol (Merck; KGaA; Germany), methanol (Merck; KGaA; Germany), alcohol 70% (Onemed, Indonesia), distilled water, L-histidine (Merck; KGaA; Germany), glacial acetic acid (Merck; KGaA; Germany), sodium hypochlorite 12%, chitosan powder (Bio Chitosan Indonesia), sodium tripolyphosphate (STPP; Sigma Aldrich; MQ; USA), disodium hydrogen phosphate dehydrate (Na_2HPO_4; Merck; KGaA, Germany), potassium dihydrogen phosphate (KH_2PO_4; Merck; KGaA, Germany), and sodium hypochlorite (NaCl; Sigma aldrich; Germany).

2.1.3 Ethical approval

This study did not involve any animal experiments; therefore, ethical approval was not required.

2.2 Methods

2.2.1 Preparation of nanochitosan using the ionic gelation method

The preparation of 0.1% nanochitosan with the ionic gelation method refers to Nugraheni *et al.* (2019). Particle size and zeta potential of the nanochitosan were observed by Particle Size Analyzer (PSA) with the Dynamic Light Scattering (DLS) method to measure light scattering intensity (Anindya, 2018).

2.2.2 Preparation of sodium hypochlorite solution

The preparation of sodium hypochlorite solution is conducted by diluting 12% sodium hypochlorite with sterile distilled water and then homogenizing it for 10 minutes. The final concentration of free chlorine in the sodium hypochlorite solution used in this study is 0.004% (40 ppm) and was confirmed using a chlorine residue test with the comparator kit method (Handayani and Abdullah, 2016).

2.2.3 pH Measurement

pH measurement was conducted for each of the solutions used, including the chitosan-GI solution, sodium hypochlorite solution, acetic acid solution, sterile TSB medium, TSB medium with bacterial suspension, and crosslink solution. The pH measurement of each solution was done using a pH meter.

2.2.4 Bacterial isolate preparation

The preparation process for the isolate begins from a glycerol stock of 60% of *Raoultella ornithinolytica* TN5 bacterial culture. *R. ornithinolytica* TN5 used in this study is a histamine-producing strain, as confirmed in a previous study (Safitri, 2020). One inoculation loop of bacteria from the glycerol stock was streaked onto TSA petri dishes and incubated at 37°C for 24 hours to obtain single colonies. The single colonies were grown in 10 ml TSB medium using an inoculation loop and incubated for 24 hours at 37°C. After the incubation process, the bacterial isolate was homogenized using a vortex, and the inoculum was ready for use in the biofilm preparation step.

2.2.5 Biofilm preparation

The surface preparation used in this study was 2x2 cm^2 stainless steel 304 coupons. This type of coupon represents common equipment in fish processing industries. Prior to use, the coupons were marked on the unused side. Coupons were washed with detergent, followed by rinsing with distilled water to remove and clean off any residual dirt. Coupons were soaked in 70% ethanol for 10 minutes, rinsed again with sterile distilled water, dried, and sterilized in an autoclave at 121℃ for 15 minutes (da Silva Fernandes *et al.*, 2015). The prepared inoculum was measured, and the optical density (OD) of each bacterial suspension was adjusted to a final concentration of approximately 0.02 (600 nm) or equal to 10^6 log CFU/ml. Subsequently, 400 µl of the suspension was applied to the stainless steel coupon surface and incubated at 30℃ for 48 hours (Roiska, 2022). After 48 hours, coupons were rinsed with 3 mL of sterile PBS using a micropipette and left for 15 minutes until dried (Angarano *et al.*, 2020).

2.2.6 Calculation of biofilm

Remaining bacterial cells on the stainless steel coupon surface were quantified using the swab method (Raffaella *et al.*, 2017). A sterile cotton swab was dipped into 10 mL of sterile PBS solution and then used for swabbing the surface of the coupon. Next, the cotton swab was placed back into the sterile PBS solution and vortexed. The resulting bacterial suspension was diluted into several dilutions in sterile PBS and grown on TSA petri dishes, with two replicates for each dilution. The dishes were incubated at 30℃ for 24 hours, and the number of biofilm-forming bacteria was counted in Colony Forming Units (CFU). The number of biofilm-forming bacteria (CFU/cm^2) before and after sanitation treatments with sodium hypochlorite, nanochitosan, hot water immersion, and combination treatments was calculated based on ISO 18593 (2018):

$$Ns = (N \times F) / A \times D \quad \text{.....(i)}$$

Where:

Ns : Number of biofilm cells (CFU/$cm^{2)}$

N : Number of bacteria (CFU/mL)

F : Volume of solvent (mL)

A : Surfaces area of the coupon (cm^2)

D : Reciprocal of the dilution used

2.2.7 Biofilm reduction using hot water and nanochitosan immersion on stainless steel surfaces

The first treatment involved immersing the coupons with biofilm in hot water at 80℃ for 5 minutes and 10 minutes, within the recommended hot water sanitization range used in the food industry (71–85°C for 30 sec to 20 min) reviewed by Bhagwat (2019). This temperature ensures effective heat to eliminate *Raoultella* spp. and to prevent biofilm formation (Tantasuttikul and Mahakarnchanakul, 2019). The use of two exposure times enables evaluation of the minimal and extended exposure effects under realistic industrial conditions. A 90-minute interval was observed between each treatment to ensure proper completion. Following this, the optimal hot water immersion treatment was identified for further analysis. Then, combination treatments were conducted, which included a 10-minute exposure to hot water followed by immersion in 0.1% nanochitosan for 5 (K1 treatment), 10 (K2 treatment), and 15 minutes (K3 treatment). Additionally, a sodium hypochlorite treatment at 0.004% (40 ppm) was applied for 10 minutes, serving as a positive control. Sodium hypochlorite was selected as a disinfectant agent because it is commonly used in the fish processing industry. All coupons were allowed to rest for 10 minutes in a sterile petri dish before proceeding with biofilm quantification tests. The control used in this experiment was biofilms formed on stainless steel coupons without exposure to any sanitizing treatment. This untreated control serves as a baseline for developed biofilm and to compare the efficacy of various sanitizing treatments in reducing bacterial cell counts.

2.2.8 Calculation of reduction percentage

The reduction percentage was calculated based on the ability of each cleaning agent to reduce bacterial biofilm on each coupon surface. This calculation was performed for each treatment applied. The reduction percentage was calculated using the formula referenced from Indonesian FDA (2011) as follows:

$$\text{Reduction Percentage} = (A-B) / A \times 100\% \quad \text{.....(ii)}$$

Where:

A: Number of biofilm-forming cells on the coupon without treatment (CFU/cm^2)

B: Number of biofilm-forming cells on the coupon with treatment (CFU/cm^2)

2.2.9 Qualitative assessment of biofilm

Qualitative assessment was performed using Scanning Electron Microscopy (SEM) to analyze the biofilm formed on the stainless steel surface before and after sanitation processes involving sodium hypo-

chlorite, hot water treatment, and a combination of hot water and nanochitosan immersion. Samples for SEM must be dry. Once dried, the samples are affixed to carbon tape on a specimen holder. The next step is to insert the sample into the SEM and create a vacuum for about 60 seconds. The sample is then bombarded with electrons at a specific probe level, allowing for observation of the surface topography of the tested sample.

2.3 Analysis Data

The research was conducted using a Completely Randomized Design (CRD) and analyzed using Analysis of Variance (ANOVA) to assess the effect of soaking time of nanochitosan in the combined treatment on the resistance of *R. ornithinolytica* on stainless steel surfaces. For significant data, Duncan's range test will be performed for further analysis. Additionally, a parametric T-test will be conducted to evaluate the effect of soaking time in hot water and to compare the effectiveness of sodium hypochlorite with each combination treatment. Data analysis will be carried out using IBM SPSS version 25.

3. Results and Discussion

3.1 Results

3.1.1 Characteristics of nanochitosan with the ionic gelation method

The nanochitosan synthesized using the ionic gelation method in this study showed an average particle size of 229.9±12.3 nm, as determined by dynamic light scattering (DLS). The polydispersity index (PDI) was 0.43, indicating a heterogeneous size distribution. The measured zeta potential was +32.6±1.3 mV, reflecting good stability of the nanoparticle suspension due to sufficient electrostatic repulsion.

3.1.2 The pH value of solutions

During the ionic gelation process, the pH of the acetic acid used to dissolve chitosan was 3.4, resulting in a nanochitosan suspension with a pH of 3.6. The sodium tripolyphosphate crosslinking solution had a pH of 9.0. For biofilm reduction testing, the sterile TSB medium used as a bacterial growth substrate had a pH of 6.2. The pH value of the solution used in nanochitosan synthesis via ionic gelation is important as it directly impacts particle formation and properties.

3.1.3 Biofilm reduction using hot water immersion

The result of the biofilm quantification of *R. ornithinolytica* TN5 is shown in Figure 1. The hot water immersion treatment for 5 and 10 minutes showed a significant difference ($p<0.05$) between treatments. The 10-minute hot water immersion resulted in a lower bacterial biofilm count compared to the 5-minute immersion. The 10 minutes of hot water immersion showed a remaining bacterial count of 3.22 log CFU/cm^2. In comparison, the untreated coupons had a bacterial count of 6.82 log CFU/cm^2. This suggests that immersing in hot water at 80°C for 10 minutes achieved a significant reduction effect of approximately 3 log-cycles. In this research, the treatment of 80°C hot water immersion for 10 minutes was selected for further combination treatment.

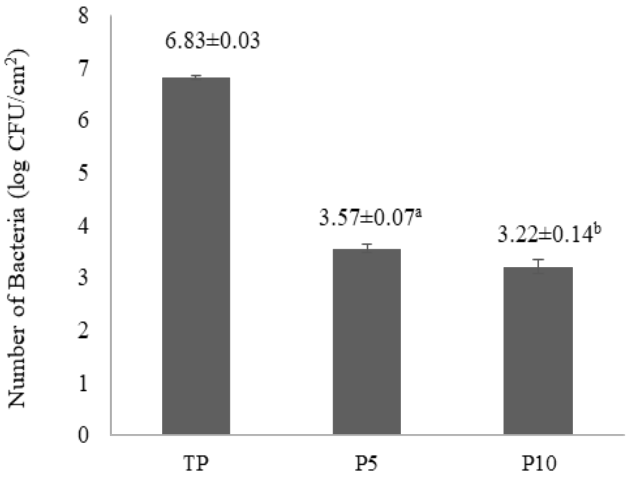

Figure 1. Effect of 80°C hot water immersion treatment on the reduction of *Raoultella ornithinolytica* TN5 cell count in the biofilm attached on stainless steel coupons. TP (no treatment), P5 (5 minutes of hot water immersion), P10 (10 minutes of hot water immersion). Different letters (a dan b) indicate a significant difference between treatments ($p< 0.05$).

3.1.4 Biofilm reduction using combination treatment

The initial biofilm count used in this study was 6.71 log CFU/cm^2. Based on Table 1, K1, K2, and K3 treatments show a reduction of 4-5 log cycles, while sodium hypochlorite reduced 3 log cycles. Overall, the combination treatment achieved a reduction effectiveness of more than 99.99%, significantly higher than sodium hypochlorite treatment. Based on Duncan's range test analysis, the combination treatment for K1 is significantly different from K2 and K3 ($p<0.05$). However, K2 and K3 are not significantly different. This research indicated that exposure times significantly affect the remaining bacterial biofilm count (Figure 2). A 10-minute immersion in nanochitosan showed an optimum effect of reduction. From

the reduction values of the three combination treatments (Table 1), it can be concluded that nanochitosan is effective in reducing the biofilm of *R. ornithinolytica* TN5 on stainless steel coupons.

3.1.5 Comparison of the effectiveness of sodium hypochlorite and nanochitosan in reducing biofilm of R. ornithinolytica on stainless steel surfaces

Based on Table 1, the reduction percentage achieved by the combination treatments (K1, K2, and K3) was higher than that of the 0.004% sodium hypochlorite treatment with a 10-minute immersion time. The sanitation treatment with sodium hypochlorite for 10 minutes resulted in a reduction percentage of 99.9731%, equivalent to 3 log-cycles. The reduction percentages for K1, K2, and K3 were 99.9961%, 99.9985%, and 99.9992%, respectively, equivalent to 4 to 5 log cycles. Based on Duncan's range test analysis, the sodium hypochlorite treatment with a 10-minute immersion time showed a significant difference with combination treatments (K1, K2, and K3). Therefore, it can be concluded that all combination treatments exhibit a higher reduction value compared to sodium hypochlorite.

Table 1. Reduction percentage of *R. ornithinolytica* biofilm on stainless steel coupons treated with different sanitizing treatment in hot water immersion-nanochitosan and sodium hypochlorite.

Treatment	Reduction (%)
Combination 1 (K1)	99.9961±0.0012^{b}
Combination 2 (K2)	99.9985±0.0003^{ab}
Combination 3 (K3)	99.9992±0.0000^{a}
0.004% Sodium hypochlorite	99.9826±0.0123^{c}

Description: 10 minutes hot water immersion and 5 minutes nanochitosan (K1), 10 minutes hot water immersion and 10 minutes nanochitosan (K2), 10 minutes hot water immersion and 15 minutes nanochitosan (K3). Different letters (a dan b) indicate a significant difference between treatments ($p < 0.05$).

3.1.6 Scanning electron microscope (SEM) imaging of biofilm before and after hot water immersion and combination treatment

SEM was used to represent the structure of the biofilm formed by *R. ornithinolytica* on the surface of stainless steel. Figure 3(K) shows that initially

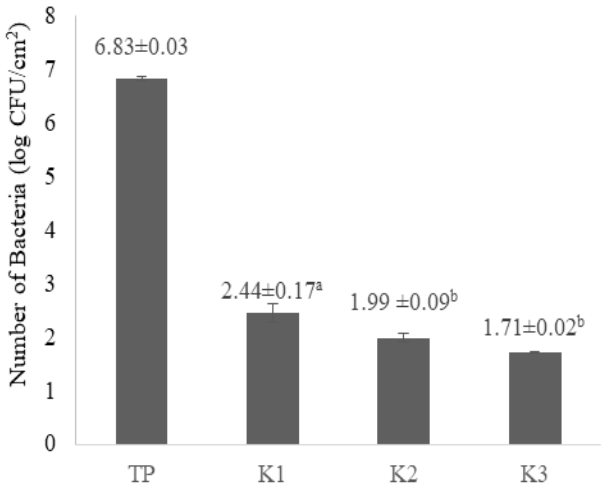

Figure 2. Combination treatment of 80°C hot water immersion and nanochitosan on the reduction of *R. ornithinolytica* TN5 cell count in the biofilm attached on stainless steel coupons. Combination 1 (K1) consists of a 10-minute hot water immersion followed by a 5-minute immersion in nanochitosan; Combination 2 (K2) involves a 10-minute hot water immersion followed by a 10-minute immersion in nanochitosan; and Combination 3 (K3) includes a 10-minute hot water immersion followed by a 15-minute immersion in nanochitosan. Different letters (a and b) indicate a significant difference between treatments ($p < 0.05$).

the coupon was colonized by *R. ornithinolytica*, exhibiting a dense bacterial presence, which may be due to the large number of microorganisms covered by EPS. The biofilm structure of a bacterium was af fected by the sanitation treatments. SEM images of biofilm after sanitation treatments (Figure 3A, B, and C) showed that EPS and bacterial cells were no longer clearly visible, although a small number of bacterial cells on the coupon treated with sodium hypochlorite were still detected.

As shown in Figure 3, all treatments effectively reduced the *R. ornithinolytica* biofilm on the stainless steel coupons compared to the control. This aligned with previous calculations of bacterial counts on biofilm, which showed a reduction of more than 99.9% for all treatments (Table 1). However, SEM analysis revealed no prominent difference in biofilm structure across all treatment groups, while CFU counts showed that a substantial number of viable cells were still present in 10 minutes of hot water treatment (3.22 log CFU/cm^2), combination treatment of 10 minutes of hot water followed by 5 minutes nanochitosan immersion (2.44 log CFU/cm^2), and sodium hypochlorite treatment (2.97 log CFU/cm^2).

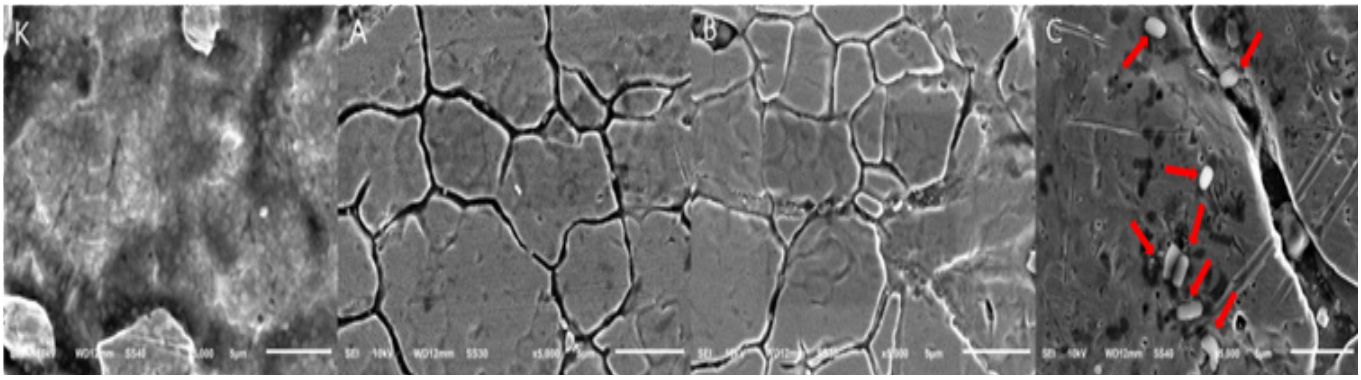

Figure 3. SEM images (x5000) of biofilm before and after different sanitizing treatments. Coupon A refers to the coupon after 10 min heat treatment; coupon B refers to the coupon after combination treatment of 10 min heat and 5 min nanochitosan treatment; and coupon C refers to the coupon after 10 min sodium hypochlorite treatment. The control coupon (K) represented biofilm without treatment. Red arrows indicate remaining cells.

3.2 Discussion

The sanitation process is essential in the fish processing industry. Surfaces that are not properly sanitized may leave biofilm residues that may develop and contaminate products. Therefore, the sanitation process needs to be carefully planned and designed to be optimal. The negative effects of cleaning agents used in sanitation should also be considered. According to the research presented, combination treatments exhibit reduction activities comparable to those of sodium hypochlorite. The sanitization process with hot water is considered simple and cost-effective, and is generally effective against many types of microorganisms; it is non-corrosive and can reach hard-to-access surfaces (Schmidt, 2012).

This study shows that hot water immersion at 80°C effectively reduces the number of bacteria in biofilm. Bacteria within biofilms reside in a self-produced matrix called extracellular polymeric substances (EPS). EPS is made up of polysaccharides, proteins, nucleic acids, and lipids that support structure, enable surface attachment, and bind cells in a cohesive 3D network. It also protects bacteria from various environmental threats (Flemming and Wingender, 2010). Hot water breaks down exopolysaccharides, a major component of EPS, thereby helping cleaning agents to directly contact bacterial cells in biofilm (Kang *et al.*, 2021). This is supported by research from Susilowati *et al.* (2011), which indicated that hot water immersion at 70 to 80°C led to gelatinization of exopolysaccharide and the formation of colloidal gels with long polysaccharide chains consisting of mono-, di-, and oligosaccharide units. The temperature rise during hot water immersion leads to dehydration and pyrolysis of the exopolysaccharide (Kanamarlapudi and Muddada, 2017). Research by Kanamarlapudi and Muddada (2017) indicated a decrease in the initial weight of exopolysaccharide and loss of moisture due to heating treatments at 50 to 99°C.

This study demonstrated a 3-log reduction of bacteria in biofilms after hot water treatment, which is comparable to the findings of Ricker *et al.* (2018), which demonstrated that exposure to 80°C for 5 minutes resulted in a reduction of *Pseudomonas aeruginosa* to the lower limit of quantification (2 log-cycles). Hot water immersion at temperatures of 70°C to 80°C for 20 and 30 seconds led to a reduction of about 1 log CFU/ml in *Campylobacter* and *Salmonella* on broiler carcasses (Beterams *et al.*, 2024). Kang *et al.* (2021) found that *E. coli* O157:H7 biofilm on a stainless steel coupon with an initial count of 5.90 log CFU/cm^2 showed a 4.17 log reduction when subjected to hot water immersion at 70°C for 15 sec. The result from this research indicates that the 10 minutes of hot water immersion resulted in a lower bacterial biofilm count compared to the 5-minute immersion. However, increasing exposure time in the application to obtain a higher effect requires careful consideration. A study by Hua *et al.* (2021) on *Listeria innocua* biofilms revealed that 100°C saturated steam had a quick bactericidal effect, but the inactivation rate declined with increasing time. A rapid 5.5-log CFU/coupon reduction of 1-day-old *L. innocua* on a stainless steel surface was achieved within 6 sec, and increasing the exposure time to 90 sec only resulted in another 2-log CFU/coupon reduction until an undetectable level (0.3 log CFU/coupon). Therefore, prolonged hot water exposure could increase energy cost, water usage, and process time, which reduces overall efficiency.

Combining heat treatment with nanochitosan improved the removal of bacterial biofilms during sanitation. Nanochitosan has a broad spectrum of antibacterial activity; however, Chandrasekaran *et al.* (2020) showed several factors affect the antibacterial

properties of nanochitosan, including bacterial species, zeta potential, particle size, growth curves, pH, concentration, and degree of acetylation. Chitosan has polycationic properties in acidic media with a pKa of 6.3. This condition allows chitosan to interact with negatively charged solutions such as TPP as a crosslinker (Bhumkar and Pokharkar, 2006). The non-toxic tripolyphosphate interacts with chitosan through electrostatic forces, resulting in the formation of ionic crosslinks (Kurniawidi *et al.,* 2022). In this study, a 5:2 ratio of chitosan solution to crosslinker was used. TPP is added using a dripping method to prevent the rapid solidification of chitosan, which could lead to the formation of clumping particles (Rahayu and Khabibi, 2016). In addition, pH is also a crucial factor in the production of nanochitosan, as it has an important role in the formation of particles and polydispersity in chitosan solution (Van Bavel *et al.,* 2023). A study by Nallamuthu *et al.* (2015) reported that when the pH of the suspension increased due to the addition of NaOH, it caused a decrease in the zeta potential of nanochitosan from +30mV to -5mV, while increasing the particle size from 170 nm to 1800 nm. In general, the particle size will increase rapidly from pH 1 to 3.5 and decrease from pH 3.5 to 5.5. Meanwhile, in terms of zeta potential, pH 1 to 4 shows an increasing zeta potential value and will gradually decrease from pH 4 to 5.5 (Warsito and Agustiani, 2021). The pH obtained in the nanochitosan-GI sample (3.6) and acetic acid solution (3.4) are consistent with the study by Adlu (2022). Furthermore, the pH value of the crosslinker solution is close to that studied by Nugraheni *et al.* (2019). These results supported the reproducibility of the method to achieve the nanoparticle size of chitosan as reported in the previous section. Particles sized between 10 and 1000 nm can be classified as nanoparticles (Nagpal *et al.,* 2010). According to Murdock *et al.* (2008), a zeta potential value greater than +30 mV or less than -30 mV indicates a stable dispersion.

Nanochitosans are derived from chitosan, a natural material that is biodegradable and non-toxic, which undergoes several processes and stages. Hardiningtyas *et al.* (2022) showed that nanochitosan has antibacterial activity of 93.44%, equivalent to 1 log cycle. The antibacterial mechanism of nanochitosan may involve electrostatic interactions between positively charged amino groups from nanochitosan and the negatively charged cell membrane. This interaction results in disrupting the cell surface, leading to modifications in membrane permeability that trigger osmotic imbalance and depletion of intracellular substances, which can cause cell damage or even death (Chandrasekaran *et al.,* 2020). In this study, a combination treatment of hot water immersion and nanochitosan showed a reduction of up to 4 to 5 log cycles. An extended immersion duration allows nanochitosan to interact for a longer time with bacterial cells, thereby enhancing its antibacterial efficacy (Chandrasekaran *et al.,* 2020). Reduction in biofilm count is also related to the particle size of nanochitosan used in this research (229.9±12.3 nm). Sarwar *et al.* (2014) reported that the particle size of nanochitosan negatively correlated with its antibacterial activity. Nanochitosan of 196 nm demonstrated higher antibacterial activity with better permeation through bacterial cell membranes compared to 598 and 872 nm. Additionally, research by Nugraheni *et al.* (2019) reported that ionic gelation of nanochitosan with a particle size of 252.72 nm exhibited inhibitory activity against *B. subtilis, E. coli, S. aureus*, and *Vibrio parahaemolyticus*. Nanochitosan with a particle size of <300 nm showed better antimicrobial properties against *L. monocytogenes* (Pereira *et al.,* 2023). A study by Hassan *et al.* (2016) shows that nanochitosan has effective inhibitory activity against the histamine-forming bacteria group, specifically *K. pneumoniae*, at concentrations greater than 60 µg/ml with an inhibition percentage of over 80%. In line with this research, the treatment of hot water followed by nanochitosan immersion also showed effective results.

This study indicates that nanochitosan may be considered a promising substitute for sodium hypochlorite in the food industry sanitation. Generally, sodium hypochlorite is known as effective in inactivating pathogenic bacteria through two main mechanisms: damaging cell permeability and disrupting nucleic acids and enzymes within the bacteria (Said, 2018). Single-species biofilms can be easily inactivated with sodium hypochlorite at 30 ppm, while dual-species biofilms require sodium hypochlorite concentrations of up to 80 ppm (Behnke *et al.,* 2011). Research by Arifani *et al.* (2017) reported a significant reduction in *P. aeruginosa* biofilm, with the optimal sodium hypochlorite concentration being 30 ppm. This research shows that 40 ppm hypochlorite treatment resulted in a 3-log reduction of bacterial biofilm, while combination treatments of hot water and nanochitosan achieved a 4-5 log reduction of *R. ornithinolytica* biofilm. The reduction of biofilm was confirmed by visualization using SEM images that show no prominent number of cells remained on the surfaces across all treatments. This inconsistency between SEM images and CFU counts suggests a potential limitation of SEM, as it captures only a small, localized area and may not accurately represent the entire surface, especially if bacteria are unevenly distributed. Another limitation in using SEM for qualitative bacterial analysis was extensive preparation, such as fixation, dehydration, and coating, that might damage the biofilm and produce artifacts (Achinas *et al*., 2020).

In this study, the incubation time required to grow bacteria on agar media after hot water immersion or combination treatment is up to two days. This might occur due to exposure to physical and chemical processes that can damage microorganisms without completely killing them. This phase is commonly referred to as "stress" or cell injury (Wesche *et al.,* 2009). The generation time depends on the level of environmental stress and sublethal damage experienced by the bacteria. Cells have a limited repertoire of responses to injury, which depends on the type of cell and the nature of the injury. The responses can be classified as adaptation, degeneration, and death (Miller and Zachary, 2017). In general, the primary biochemical mechanisms of cell injury include ATP depletion, cell membrane permeabilization, disruption of biochemical pathways, and DNA damage (Miller and Zachary, 2017). For example, research by Busta (1976) indicated that heat-injured *Staphylococcus* bacteria could release potassium, amino acids, and proteins. The loss of intracellular compounds indicates damage to the cell membrane, which can inhibit the growth and replication of those cells. Most injured cells, including the structures of the cytoplasmic membrane, may become susceptible to various antimicrobial agents. *R. ornithinolytica* is a histamine-producing bacterium (Kanki *et al.,* 2002). Alya'ainun *et al.* (2021) show the histamine levels of *R. ornithinolytica* in TFIB medium range from 620 to 1078 ppm after incubation for 6 hours. The strain that was used in this research was classified as "strong" biofilm producers (Safitri, 2020). When biofilms are formed on the food-contact surfaces, bacteria become more tolerant to cleaning agents and sanitizers, which raises the risk of ongoing contamination and elevated histamine levels in food products, particularly seafood. This research demonstrates that integrating advanced treatments like nanochitosan with physical methods such as hot water may significantly improve the effectiveness of sanitation processes in the seafood industry and reduce the risk of cross-contamination. A synergistic effect of this combination offers a more effective strategy to eliminate histamine-producing bacteria and their biofilms from food contact surfaces, thereby reducing histamine risk in fish products and improving overall seafood safety.

4. Conclusion

This study demonstrated that the combination treatment of hot water immersion followed by nanochitosan treatment significantly reduced *R. ornithinolytica* biofilm on stainless steel surfaces with greater efficacy compared to sodium hypochlorite treatment alone. The synergistic effect of thermal degradation on biofilm matrix and the antimicrobial properties of nanochitosan through cell membrane disruption contributed to the reduction in viable bacterial counts and biofilm structure. Findings of this research suggest that this combination may potentially serve as an effective and safer alternative to sodium hypochlorite-based sanitation in the seafood industry. However, the use of hot water for huge applications requires further analysis and review regarding temperature control mechanisms and the energy required. Further research is recommended to evaluate the efficacy of the treatment against multispecies biofilms representative of food industry contaminants. Other techniques to better assess cell viability in biofilm and EPS integrity are also suggested.

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Authors' Contributions

All authors contributed to the final manuscript. The contribution of each author as follow, KFF; collected the data, drafted the manuscript and designed the figures. IDP, PSN, and MMPP; devised the main conceptual ideas and critical revision of the article. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

Declaration of Artificial Intelligence (AI)

The author(s) affirm that no artificial intelligence (AI) tools, services, or technologies were employed in the creation, editing, or refinement of this manuscript. All content presented is the result of the independent intellectual efforts of the author(s), ensuring originality and integrity.

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