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Antimicrobial resistance and biofilm formation of *Escherichia coli* in a Vietnamese *Pangasius* fish processing facility

Phan Nguyen Trang [a,*], Tong Thi Anh Ngoc [a], Yoshimitsu Masuda [b], Ken-ichi Hohjoh [b], Takahisa Miyamoto [b]

[a] *Department of Food Technology, Institute of Food and Biotechnology, Can Tho University, Campus II, 3/2 Street, Ninh Kieu District, Can Tho, Viet Nam*

[b] *Division of Food Science and Biotechnology, Faculty of Agriculture, Kyushu University,744 Motooka, Nishi-ku, Fukuoka, 819-0395, Japan*

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ABSTRACT

This study aimed to investigate the occurrence, antibiotic resistance, and biofilm formation of *Escherichia coli* in the Vietnamese *Pangasius* fish processing facility. Among 144 samples including *Pangasius* fish, wash water, food contact surfaces, and personnel gloves, 18 *E. coli* isolates was detected and characterized. The *E. coli* was detected most frequently in wash water samples (22%, 8/36), followed by *Pangasius* fish (18%, 8/45). According to the antibiotic susceptibility test by the disc diffusion method, isolates showed the highest resistance against sulfamethoxazole/trimethoprim (45%), followed by tetracycline (39%), whereas all the *E. coli* isolates were susceptible to meropenem and fosfomycin. Notably, 39% of the isolates (7/18) were found to be multidrug resistant while no *E. coli* isolates were confirmed as extended-spectrum β-lactamase producers by the double-disk synergy test. The potency to form biofilm on the polystyrene surface of *E. coli* isolates indicated that 44% of the isolates (8/18) were classified as weak, 39% (7/18) as moderate, and 17% (3/18) as strong biofilm formers. Interestingly, multidrug resistant *E. coli* isolates were observed in moderate and strong biofilm producers. Additionally, either slightly acidic hypochlorous water with 40 mg/L of available chlorine or sodium hypochlorite with 100 mg/L of available chlorine exhibited a significant reduction in biofilm mass and biofilm cells of *E. coli* isolates. This study may provide helpful information about the actual state of *E. coli* isolates for effective control in the fish processing plant.

1. Introduction

Pangasius fish (*Pangasiandon hypophthalmus)* is a freshwater fish that is intensive fish farming by rearing primarily in ponds. Although implementing the food safety management system, the high contamination level of bacteria including *Escherichia coli*, *Salmonella* spp., *Vibrio cholerae,* and *Listeria monocytogenes* in the *Pangasius* fish processing plant has been reported by several studies [1–3]. Additionally, previous research emphasized that 50–60% of *E. coli* derived from *Pangasius* fillets originated from Vietnam were multidrug-resistant (MDR) strains [4–7]. The misuse and overuse of antibiotics in aquaculture, livestock, and humans might be contributed to the development and dissemination of antimicrobial resistance (AMR) in Vietnam in particular and over the world in general. Furthermore, the high prevalence of *E. coli* isolates produces extended-spectrum β-lactamase (ESBL) in the food chain

* Corresponding author.
E-mail address: pntrang@ctu.edu.vn (P.N. Trang).

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exposing a major threat to public health since fewer available antibiotics for the treatment of *Enterobacteriaceae* infection [8]. Thus, how to monitor the AMR in the food chain is urgent to provide safe and high-quality food for consumers.

Escherichia coli is normally found in the intestines of healthy humans and animals, some strains can cause intestinal infections which are primarily transmitted via the consumption of contaminated food [9]. The adherence ability to surfaces and biofilm formation for many bacteria including *E. coli* help them can survive up to ten years in the environment of food processing plants despite regular cleaning and sanitation treatment [10]. The biofilm cells exhibit different behavior compared to planktonic cells by increasing resistance to disinfectants [11,12]. Moreover, several studies indicated that the *E.coli* isolated from different sources had both high biofilm formation ability and antibiotics resistance [13–16]. As regards cleaning and disinfection procedure, sodium hypochlorite (NaOCl) has been used as the most common disinfectant in the food industry due to its effectiveness on various bacteria and low cost despite its harmful effects on human health and the environment [17]. Nevertheless, slightly acidic hypochlorous water (SAHW) has become an appropriate alternative substance for cleaning and disinfection procedures because of its excellent disinfection ability, quick on-site production, low cost, fewer health risks, and eco-friendly [18]. Especially, previous studies indicated a significant efficacy of SAHW on the biofilm of either *E.coli* isolates alone or mixed with other bacteria [19–21].

The rising incidence of AMR in food animals including seafood may contribute to transmission to humans through food chains [22–24]. The risk can be expanded if pathogens are associated with consumption contaminated food. Therefore, the present study aims to evaluate the prevalence of potential pathogens using *E. coli* (non-fermenting sorbitol) in context with highlight its antimicrobial resistance profile and biofilm forming capacity of the isolates derived from the Pangasius fish processing facility. Additionally, the efficiency of slightly acidic hypochlorous water in comparison to sodium hypochlorite on the elimination of biofilm of *E. coli* was examined.

2. Materials and methods

2.1. Sampling

In this study, the sample were taken throughout the flowchart of production process at the *Pangasius* fish processing facility as described by Phan et al. [25]. In brief, the raw fish was manually cut the gill and then bled by dipping in a water sink at 35 °C for 30 min. Then the fish were manually filleted and washed by dipping in a water sink for 5 min at 20–25 °C at the step of washing 1. Thereafter the fillets were mechanically skinned and trimmed the subcutaneous fat and red muscle with a knife. The following step was washing 2 at which the fillets were washed by dipping in a water sink for 2 min at 16 °C before cooled by alternating layers of fish and flake ice within 4–6 h until freezing. The fillets were frozen at −18 °C, then glazed before weighted, packed labeled, and stored at −18 °C. A total of 144 samples of fish and environmental samples (i.e., wash water, swabs of gloves of workers, and food contact surfaces) were taken at different processing steps (16 critical sampling locations) at 3 different times and 3 visits (16×3 x $3 = 144$) based on the method of microbial assessment scheme [25,26].

2.2. Microbiological analysis

About 25 g of fish samples were suspended in 225 mL of Maximum Recovery Diluent (Merck, Darmstadt, Germany), then homogenized in a Stomacher® bag (Interscience, Ile-de-France, France). For the environmental samples, approximately 250 mL of the wash water in the stainless-steel sink (containing approximately 300L tap water per batch) was collected into sterile Stomacher® bags while the moistened swabbing was carried out with the dimensions of a 100 cm^2 area of the food processing surfaces and 25 cm^2 area of the personnel gloves. These samples were then further diluted, and 0.1 mL of the suspension was streaked on Coliform Agar Enhanced Selectivity (Merck, Darmstadt, Germany) and incubated for 18–24 h at 37 °C. Total of 58 presumptive colonies of *E. coli* with dark blue to violet color were confirmed by using the Nissui identification test EB-20 (Nissui, Tokyo, Japan). The EB-20 test was inoculated with *E. coli* culture and incubated at 37 °C for 18–20 h. The Nissui identification test EB 20 includes the following: hydrogen sulfide, menus of esculin, phenylalanine deaminase, indole, Voges-Proskauer, citrate, lysine decarboxylase, arginine dihydrolase, ornithine decarboxylase, *o*-nitrophenyl-β-D-galactopyranoside, urease, malonate, adonitol, inositol, raffinose, rhamnose, sucrose, mannose, sorbitol and arabinose.

2.3. The antimicrobial susceptibility testing

All 18 *E.coli* isolates derived from different samples and processing steps were subjected to antimicrobial susceptibility tests following the disc diffusion method as described by the Clinical and Laboratory Standards Institute [27]. The sensitivity of *E. coli* isolates was tested against fifteen different antibiotic discs including ampicillin 10 μg, cefotaxime 30 μg, ceftazidime 30 μg, cefoxitin 30 μg, meropenem 10 μg, gentamicin 10 μg, kanamycin 30 μg, streptomycin 10 μg, tetracycline 30 μg, chloramphenicol 30 μg, sulfamethoxazole/trimethoprim 23.75/1.25 μg, nalidixic acid 30 μg, ciprofloxacin 5 μg, fosfomycin 200 μg and colistin 10 μg (Abtek Ltd., the United Kingdom and Nam Khoa., Ltd., Vietnam). The diameter of the inhibitory zone was measured and interpreted according to the zone diameter breakpoints by CLSI, 2021. An isolate was considered MDR if it was resistant to ≥3 groups of antibiotics with different antibacterial mechanisms.

2.4. Screening and confirmation of ESBL-producing E. coli isolates by phenotypic method

The initial screening test of all 18 *E. coli* isolates to produce ESBL was carried out by using both ceftazidime (CAZ, 30 μg) and cefotaxime (CTX, 30 μg) discs. If the zone of inhibition was ≤22 mm for CAZ and/or ≤27 mm for CTX, the isolate was considered as a potential ESBL producer as recommended by the CLSI, 2021. Afterward, ESBL producers were confirmed by the double-disc synergy test following the criteria established by CLSI. A ≥ 5 mm increase in zone diameter for either CAZ or CTX in combination with clavulanic acid (10 μg) versus its zone when tested alone confirms ESBL as the recommendation of CLSI, 2021.

2.5. Biofilm formation of E. coli isolates

Biofilm assays were performed on 96-well polystyrene microtiter plates with a flat bottom (Sanplatec Co., Ltd., Osaka, Japan) based on the methods of Miyamoto et al. [28]. In brief, *E. coli* strains were cultured overnight at 37 °C in Luria Broth (LB, Becton, Dickinson, and Company, Sparks, MD, USA). The culture was diluted with LB to attain an optical density (OD) at 660 nm of 0.7. Two hundred microliters were added to 96-well microtiter plates with flat bottoms (San-platec Co., Osaka, Japan) and incubation at 37 °C for 24 h without shaking. The biofilm mass was measured using the crystal violet staining method. After biofilm formation, the wells were washed twice using phosphate-buffered saline (PBS, 1.47 mM KH_2-PO_4, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 137 mM NaCl, pH 7.4) and dried at room temperature, then stained with 250 μL of 1% (*w/v*) crystal violet (Fujifilm, Osaka, Japan). After removing the unbound dye by washing with PBS, 300 μL of 99% ethanol was added to dissolve the crystal violet for 15 min before quantifying the absorbance at 595 nm with a microplate reader (Infinite F50, Tecan, Kanagawa, Japan). Based on their biofilm-forming ability, *E.coli* isolates were classified into four groups following the criteria i.e., strong ($4 \times OD_c < OD$), moderate ($2 \times OD_c < OD \leq 4 \times OD_c$), weak ($OD_c < OD \leq 2 \times OD_c$) and non-adherent ($OD \leq OD_c$) as described by Stepanović et al. [29].

2.6. The effect of SAHW or NaOCl on biofilm formed on the microtiter plate

SAHW containing 40 mg/L available chlorine, pH 5.5 was provided by Morinaga Milk Industry Co., Ltd. (Japan) whereas NaOCl containing 100 mg/L was prepared by diluting the 10% NaOCl solution (Sigma-Aldrich, Tokyo, Japan) with sterilized water. Following biofilm formation, supernatants were removed, wells were washed three times with sterile water, added with 250 μL SAWH or NaOCl, and kept at room temperature for 10 min. After the treatment, supernatants were carefully removed, and biofilm cells were recovered in PBS by scraping the surface of the well with a pipette tip. The recovered cell suspension was serially diluted with PBS, and viable counts were determined by plating on Tryptic Soy Agar (Becton and Dickinson, Franklin Lakes, USA) for enumeration of *E. coli* and expressed as log CFU/mL.

2.7. Statistics analysis

All experiments were performed in triplicates. Results are shown as mean values and standard deviations of the mean. Statistical significance was assessed using the student's t-test ($p < 0.05$) to determine statistically significant differences between the treatments and the control group.

3. Results and discussions

Consumption of contaminated food and/or improper sanitization condition could be a potential source of infection by antibiotic-resistant bacteria in humans. *E. coli* can be a reservoir for such transfers because of its diversity in animals, humans, and the environment. In this study, a total of 144 samples were collected from the fish, wash water, food contact surfaces, and hand/gloves of workers within the *Pangasius* processing plant. As shown in Table 1, the highest detection rate of *E. coli* was found in wash water (8/36, 22%), followed by fish (8/45,18%), food contact surfaces (2/36, 6%), and no detection in personnel hands/gloves sample. The overall contamination rate of *E.coli* was 12.5% which was relatively low as compared with the other previous studies, which reported up to 51% in India [30] or 45% in Malaysia [31]. Especially, Phan et al. [7] indicated the high prevalence of pathogenic *Listeria monocytogenes* strain in the wash water sample in the same *Pangasius* processing plant. The results of both the present and previous study suggested the washing step could be a high-risk step due to the high probability of transmission of bacteria from wash water into the fish products. Additionally, Ragert et al. [32], and Maffei et al. [33] reported that the bacterial contamination level in the wash water becomes higher as the accumulation of bacteria and organic matter after washing the products. Moreover, the frequency of change of

Table 1
Contamination of *E. coli* in the Vietnamese *Pangasius* fish processing plant.

Types of samples	No. Of samples	No. Of *E. coli* positive samples (%)
Fish	45	8 (18)
Food contact surfaces	36	2 (6)
Wash water	36	8 (22)
Gloves of workers	27	0
Total	144	18 (12.5)

wash water, and the disinfectant concentration in the washing sinks have not often been examined in the actual processing plant. Thus, continuous control of the concentration of disinfectants and water quality in the washing step is not only a crucial factor to reduce initial contamination but also to prevent cross-contamination.

Antimicrobial resistance surveillance is important for monitoring community infections and for establishing treatment strategies. The results of antimicrobial susceptibility testing of all *E. coli* isolates are shown in Fig. 1. Among eighteen *E.coli* isolates, the highest rate of resistance was found in sulfamethoxazole/trimethoprim at 45%, followed by tetracycline at 39%, ampicillin at 33%, nalidixic acid at 27%, and streptomycin at 22%. Moreover, about 11% of the *E.coli* isolates exhibited resistance against ceftazidime, chloramphenicol, and ciprofloxacin, while nearly 6% of them showed resistance to cefotaxime, cefoxitin, and colistin. The high resistance of *E.coli* isolates against sulfamethoxazole/trimethoprim, tetracycline, and ampicillin was consistent with previous studies wherein a similar resistance profile of *E.coli* isolates was reported in food (i.e., fish, chicken meat, and seafood) and human in Vietnam [6,34–36]. These observations could be due to the usage of antibiotics based on empiric treatment usage in humans and animals or overuse in livestock feeding and aquaculture in Vietnam [37]. The antibiotic resistance pattern of *E. coli* isolates is listed in Table 2. About 39% (7/18 isolates) were found to be MDR since they exhibited resistance to at least 3 antibiotics with different modes of action. The result was relatively low as compared to 86% of MDR–*E.coli* derived from meat and seafood, 63% from chicken, 56% from human [8,38]. Remarkably, no *E.coli* isolates were confirmed as ESBL producers by the double-disk synergy test (data not shown). The results were in contrast to those from several studies that reported a high prevalence of ESBL-producing *E.coli* in food, human and environmental samples in Vietnam [32,39,40].

Biofilm acts as a barrier that protects microbes from antimicrobial drugs and disinfection treatments [41]. The biofilm formation of bacteria on food contact surfaces as a persistent source of food contamination threatens food safety. In this study, all eighteen *E.coli* isolates were evaluated for their potential to form biofilm on the polystyrene surface. Fig. 2 shows that 44% of *E. coli* isolates (8/18) were weak, 39% (7/18) were moderate and 17% (3/18) were strong biofilm formers. Furthermore, this study found that there was a relationship between biofilm formation and antibiotic resistance. Seven *E.coli* isolates which were MDR (Table 2), showed relatively strong biofilm formation (Fig. 2). Conversely, 4 *E.coli* isolates including 112E, 114E, 29E, and 31E were classified as weak biofilm formers (Fig. 2) and showed high sensitivity to all fifteen antibiotics tested. These obtained results are not consistent with some studies sine they reported antibiotic resistance *E. coli* isolated from chicken was observed in weak biofilm producers [42,43] whereas Kim et al. [44] and Safeena et al. [45] indicated that MDR *E. coli* isolates showed moderate biofilm forming ability in seafood and unpasteurized juice. It was supposed that antibiotic resistance was enhanced by biofilm formation since extracellular polymeric substances produced by biofilm-forming *E.coli* functions to prevent antibiotics from reaching their target in the cell [46]. However, the relationship between antimicrobial resistance and the capacity to form biofilm varies between strains. Therefore, it is needed to conduct additional research with a greater number of samples from different sources to clarify these relationships.

Based on the high capacity of adherence to plastic surfaces and antimicrobial resistance, seven MDR *E. coli* isolates (including 92E, 93E, 80E, 75E, 123E, 42E and 4E) were selected and studied further for the efficacy of SAHW or NaOCl on the elimination of biofilm mass and viable biofilm cells. The results are shown in Fig. 3**A** and **B.** A significant decrease ($p < 0.05$) in biofilm mass and viable count of biofilm cells was observed after the treatment with either SAHW (40 mg/L available chlorine) or NaOCl (100 mg/L available chlorine). The results were consistent with previous findings which showed a 3–6 log CFU/ml reduction of biofilm cells by SAHW [47–50]. With a significant effect on the removal of 30% biomass, the result obtained in the present study was compatible with other reports that SAHW showed a great decrease in biomass on a variety of food contact surfaces [49,51]. Several findings have indicated that SAHW at lower available chlorine concentrations is a more effective disinfectant than the NaOCl and lesser residual chlorine in the treated area after disinfection [20], [52]-[53]. Because the antimicrobial activity of chlorine-based sanitizers could depend on the amount of HOCl at which SAHW contains a high amount of HOCl (92–98%) while NaOCl has only 5–10% at the same available

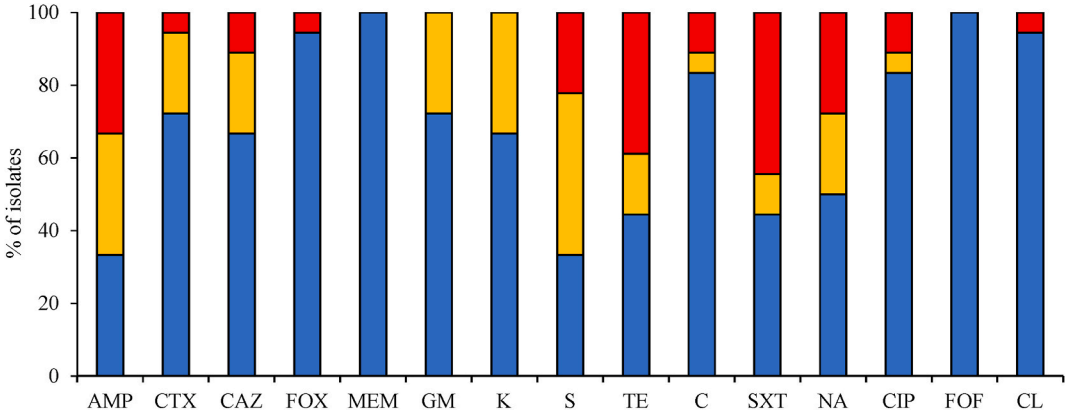

Fig. 1. Antibiotic resistance profiles of *E.coli* isolates to various antimicrobials. AMP: Ampicillin, CTX: Cefotaxime, CAZ: Ceftazidime, FOX: Cefoxitin, MEM: Meropenem, GM: Gentamicin, K: Kanamycin, S: Streptomycin, TE: Tetracycline, C: Chloramphenicol, SXT: Sulfamethoxazole/trimethoprim, NA: Nalidixic acid, CIP: Ciprofloxacin, FOF: Fosfomycin, CL: Colistin. The profile shows susceptible (■.), intermediate (■.), and resistance (■.).

Table 2
Antibiotics resistance pattern of *E. coli* isolates derived from a Vietnamese *Pangasius* processing plant.

Name of isolates	Resistance pattern[c]	Type of samples	Processing step	%
92E	AMP-CTX-*SS*-TE-*CC*-NA-SXT-CIP	Fish	Trimming	39[a]
93E	AMP-FOX-TE-SXT		Cooling	
80E	AMP-NA-CIP-CL		Filleting	
75E	AMP-TE-*C*-SXT		Packaging	
123E	AMP-*S*-SXT			
42E	*S*-TE-SXT	Wash water	Washing 1	
4E	*S*-TE-SXT	Food contact surfaces	Trimming	
49E	CTX- SXT	Wash water	Washing 2	39[b]
13E	AMP – NA	Food contact surfaces		
37E	TE-NA	Wash water	Bleeding	
16E	TE			
38E	AMP		Washing 1	
50E	SXT		Glazing	
110E	NA	Fish	Cooling	
29E	No resistance to tested antibiotics	Wash water	Bleeding	
31E				
112E		Fish	Cooling	
114E			Packaging	

[a] Number of isolates resistant to three or more antibiotics over the total number of isolates.
[b] Number of isolates resistant to two or fewer antibiotics over the total number of isolates.
[c] AMP: Ampicillin, CTX: Cefotaxime, CAZ: Ceftazidime, FOX: Cefoxitin, MEM: Meropenem, GM: Gentamicin, KM: Kanamycin, S: Streptomycin, TE: Tetracycline, C: Chloramphenicol, SXT: Sulfamethoxazole/trimethoprim, NA: Nalidixic acid, CIP: Ciprofloxacin, FOF: Fosfomycin, CL: Colistin.

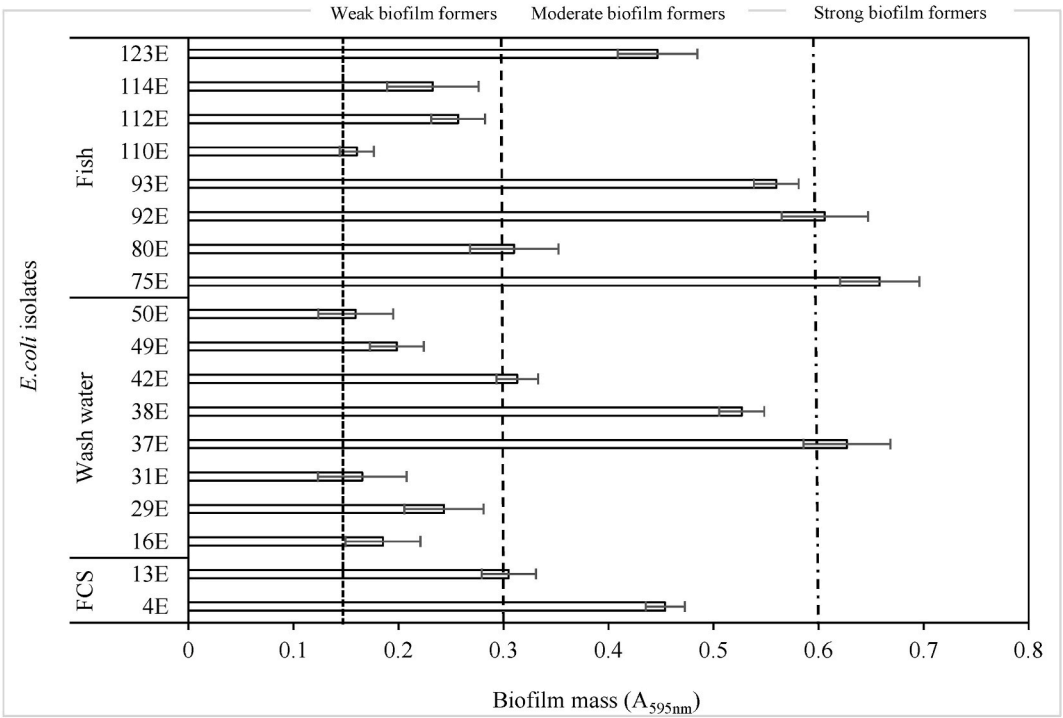

Fig. 2. Biofilm formation of eighteen *E.coli* isolates onto the polystyrene plate as determined by crystal violet assay. The biofilm mass $A_{595nm} \geqq 0.15$; 0.3; and 0.6 with dashed line indicated to weak, moderate, and strong biofilm formers, respectively. Error bars show the standard deviation of the mean (n = 3).

chlorine concentration [54]. In the *Pangasius* fish processing plant, NaOCl with an available chlorine concentration of 50–200 ppm has been used to decontaminate the fillets at the washing step and applied to cleaning and disinfection of various equipment and environments regardless drawbacks to human health and environments. This leads to the need to look for an alternative promising disinfectant to NaOCl on-site production. Therefore, much works remains to be done to increase SAHW applicability in the actual practices in the near future.

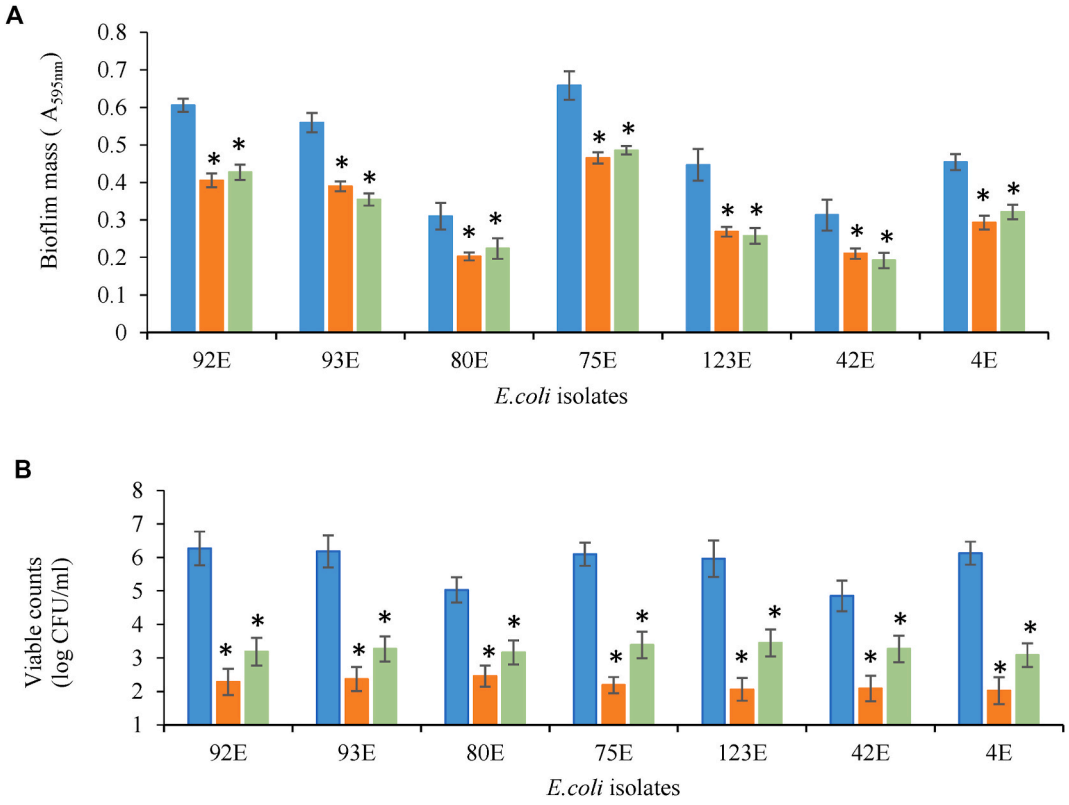

Fig. 3. A and BEffects of SAHW and NaOCl on biofilm mass and viable biofilm cells of 48-h biofilm of *E. coli* in the microplate plate. *E. coli* biofilm was treated with PBS (■.), SAHW (40 mg/L available chlorine■) , and NaOCl (100 mg/L available chlorine■). *$p < 0.05$ compared to the control for each *E. coli* isolate. Error bars show the standard deviation of the mean (n = 3).

4. Conclusions

This present study provided useful information on the antimicrobial resistance profile of *E. coli* in both fish and environmental samples in the Vietnamese *Pangasius* fish processing plant. The prevalence of MDR *E. coli* highlighted the importance of continuous monitoring of this bacterium in food processing. Moreover, it should be outlined that the majority of MDR strains and strong biofilm producers are from fish source. To clarify the mechanism for the relationship between biofilm formation and antibiotic resistance of *E. coli* isolates, further research is necessary. Specifically, implementing additional studies with a greater number of samples from different sources not only come from the fish processing plants but also in the farming ponds to elucidate these correlations. Furthermore, the characteristics of *E. coli* regarding to virulence potential, phylogenetics group and genetic diversity should be investigated in the next studies.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy restrictions.

CRediT authorship contribution statement

Phan Nguyen Trang: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing – original draft, Writing – review & editing, Investigation. **Tong Thi Anh Ngoc:** Funding acquisition, Project administration, Supervision, Writing – review & editing. **Yoshimitsu Masuda:** Formal analysis, Methodology, Resources. **Ken-ichi Hohjoh:** Formal analysis, Methodology, Resources. **Takahisa Miyamoto:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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