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1 **Sanitizing efficacy and antimicrobial mechanism of peracetic acid against**
2 **histamine-producing bacterium, *Morganella psychrotolerans***

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Abstract

In this study, we aimed to investigate the sanitizing effects of peracetic acid (PAA), and the underlying mechanism of its antimicrobial action against *Morganella psychrotolerans*. The exposure of *M. psychrotolerans* to 20 ppm PAA for 5 min led to its decrease below the detection level, indicating that PAA has a significant antibacterial effect against *M. psychrotolerans* *in vitro*. Moreover, the viable counts of *M. psychrotolerans* on saury (*Cololabis saira*) surface were showed a reduction of 1.40 and 2.23 log CFU/cm² upon treatment with 80 ppm PAA for 1 and 5 min, respectively. Further, PAA treatment delayed the growth of *M. psychrotolerans* on saury surface during storage at 4°C. Next, the antimicrobial mechanism of PAA against *M. psychrotolerans* cells was investigated, and the damage to the cell membrane and cell surface upon PAA treatment was observed using scanning electron microscopy (SEM) and epifluorescence microscopy. The chromosomal DNA and the protein profiles after PAA treatment were also analyzed. Form our results, we hypothesized that the bactericidal effect of PAA treatment was mainly attributed to damage the bacterial cell membrane. These results indicate that PAA may be an efficient disinfectant against *M. psychrotolerans* and has applications in seafood processing and storage.

Keywords

Morganella psychrotolerans; disinfection; peracetic acid; disinfectant effect; seafood safety; antimicrobial mechanism

1. Introduction

The consumption of seafood has increased considerably worldwide, over the past few decades, because of its rich nutritional composition (Seves *et al.*, 2016). However, histamine food poisoning is a major concern due to seafood consumption. This kind of food poisoning is mainly caused by the ingestion of seafood containing high levels of histamine (Bjornsdottir-Butler, McCarthy, Dunlap, & Benner, 2016). Histamine is generated from free histidine by the action of the enzyme bacterial decarboxylase. The psychrotrophic histamine-producing bacteria are of particular concern in the seafood industry. They can cause outbreaks of histamine poisoning at low temperatures (Emborg, 2006; Morii & Kasama, 2004). *M. psychrotolerans*, a pathogenic psychrotolerant histamine-producing bacterium, belongs to the family *Enterobacteriaceae* and is able to grow and produce toxic levels of histamine (Emborg, 2006; Emborg & Dalgaard, 2006). In previous studies, the link between the cases of histamine poisoning and seafood contaminated with *M. psychrotolerans* has been reported (Dalgaard, Emborg, Kjølby, Sørensen, & Ballin, 2008; Emborg & Dalgaard, 2006; Emborg, Laursen, & Dalgaard, 2005). In our previous studies, we revealed the presence of *M. psychrotolerans* in retail seafood distributed in Japan (Kato *et al.*, 2017) and also showed that *M. psychrotolerans* had high histamine-producing abilities (Wang, Yamaki, Kawai, & Yamazaki, 2020). Therefore, controlling *M. psychrotolerans* populations in seafood is a major challenge faced by the seafood industry.

54 In general, sanitizers can generally disinfect foodborne pathogens effectively and
55 affordably from the seafood surfaces, attributing to their high prevalence within the
56 food industry (Shen, Luo, Nou, Wang, & Millner, 2013; Rahman, Jin, & Oh, 2011).
57 Sodium hypochlorite (SH) is an effective disinfectant and is widely used to reduce
58 microbial counts. However, it has been shown that the improper use of chlorine can
59 produce carcinogenic by-products and residues, such as chloroform and
60 bromodichloromethane (Gil, Selma, López-Gálvez, & Allende, 2009; Reckhow,
61 Singer, & Malcolm, 1990). Therefore, it is necessary to find safer antimicrobial
62 agents for food sanitization. Peracetic acid (PAA) is a well-known sanitizer generally
63 used for processing water, fruits, and vegetables (Leggett *et al.*, 2016; Ho, Luzuriaga,
64 Rodde, Tang, & Phan, 2011; Hilgren & Salverda, 2000; Lambert, Johnston, & Simons,
65 1999; Rudd & Hopkinson, 1989). PAA by-products are safer than those produced
66 during SH treatment (Mora, Veijalainen, & Heinonen-Tanski, 2018; Dell'Erba,
67 Falsanisi, Liberti, Notarnicola, & Santoro, 2007). An earlier report shown that 15 ppm
68 PAA treatment resulted in the elimination of *Vibrio parahaemolyticus* cultured in
69 Luria-Bertani-3 % NaCl culture broth (Wong, Liao, Hsu, & Tang, 2018). Sheng, Shen,
70 & Zhu (2020) reported that 80 ppm PAA treatment for 2 min resulted in a 1.8 log
71 CFU/apple reduction in *Enterococcus faecium*. Moreover, 50 ppm PAA treatment for
72 240 s was shown to reduce the count of *Escherichia coli* below the detectable level (<
73 1 log CFU/g) on *pangasius* fillets (Thi *et al.*, 2015). However, there are limited

scientific reports on the sanitizing effects and antimicrobial mechanisms of PAA on histamine-producing bacteria.

Therefore, in the present study, we evaluated the sanitization effect of PAA against *M. psychrotolerans* both *in vitro* and on saury (*Cololabis saira*) surface. The effect of PAA treatment on cell morphology, cell membranes, cellular protein, and chromosomal DNA was investigated to better understand the disinfection mechanism of PAA. Our study provides useful insight on the use of PAA as a disinfectant for seafood to prevent *M. psychrotolerans*-related histamine food poisoning.

2. Materials and methods

2.1. Cell culture conditions

M. psychrotolerans JCM 16473^T was cultured in tryptic soy broth (TSB, BD, Franklin Lakes, NJ) at 25 °C for 24 h. The cell cultures (approximately 10⁹ CFU/mL) were centrifuged at 6,000 × *g* for 10 min at 4 °C. The harvested cell pellets were washed twice with phosphate-buffered saline (0.01 M PBS, pH 7.2) and subjected to PAA treatments as described below.

2.2. Evaluation of antibacterial activity of PAA

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were assessed using a 96-well microplate. The washed bacterial cells were resuspended in 2-fold TSB at the inoculum concentration of approximately

5.2×10⁵ CFU/mL. In each plate, 0.1 mL of 2-fold TSB inoculated with *M. psychrotolerans* were mixed with 0.1 mL of PAA (Tec P-10, Adeka Clean Aid Co. Ltd. Tokyo, Japan), and incubated at 25 °C for 48 h. The MICs were determined by evaluating the lowest concentration of PAA with no bacterial growth observed by the visual turbidity (Chotigarpa *et al.*, 2018). The MBCs were determined by spreading the cells on tryptic soy agar (TSA, BD, Franklin Lakes, NJ) plates and incubating at 25 °C for 48 h after the inactivation step. Briefly, the 0.1 mL of the culture broth without the microorganism growth was inactivated using 0.9 ml of the inactivation buffer (Lectin 10 g/L, Polyoxyethylene (20) sorbitan monooleate 30 g/L, L-histidine 1 g/L, Tryptone 1 g/L, Na₂S₂O₃·5H₂O 20 g/L) and then inoculated the cells and plated on the TSA plate. The MBCs were determined by the lowest concentration of PAA that completely inhibited the *M. psychrotolerans* colony growth (Meireles *et al.*, 2015).

Next, the bacterial disinfection with PAA treatment was tested *in vitro*. The washed bacterial cells were resuspended in PBS at the inoculum size of approximately 10⁹ CFU/mL, and 9 mL of the bacterial suspension was mixed with 1 mL PAA (10, 20, 40, and 80 ppm). Sterile deionized water was used as the control. After treatment for 1, 3, or 5 min, 0.1 mL of treated suspension was immediately mixed with 0.9 mL of inactivation buffer. The neutralized samples were serially diluted (1:10) with PBS (pH 7.2) and 0.2 mL from each dilution was spread on agar plates consisting of TSA supplemented with 0.2 % sodium pyruvate. The *M. psychrotolerans* survivor colonies

were counted after incubation at 25 °C for 72 h.

2.3. Analyses of the disinfectant effect of PAA on saury surfaces

Fresh sauries placed on ice were purchased from a local supermarket in Japan and immediately transported to the laboratory and stored at -70 °C. The whole saury was initially placed in tap water for 30-40 min until it was completely thawed and was then washed three times with 9-fold distilled water. Next, the saury was submerged in the *M. psychrotolerans* culture suspension (1:9 w/v, about 10⁹ CFU/mL) for 5 min. Then, the inoculated sauries were air-dried in a biological safety cabinet for 12 min (initial inoculum size: approximately 10⁶ CFU/cm²). Next, the samples were dipped into 9-fold (w/v) of 80 ppm PAA or 100 ppm SH (free available chlorine concentration; chlorine concentration assayed using the method regulated by the Ministry of Health, Labour and Welfare, 318, Japan) solutions for 1 or 5 min, respectively. Sterile deionized water was as control. Thereafter, the saury samples were immediately dipped into the deionized water (1:9, w/v), to remove the excess sanitizer. The saury surfaces were then swabbed with a 10 mL phosphate-buffered saline to perform the attached swab test (Kanto Chemical, Co., Inc, Tokyo, Japan).

For monitoring the growth of *M. psychrotolerans* on saury surfaces during storage, the guts and gills were removed from the sauries firstly removed after thawing and then washed several times with tap water. Thereafter, the sauries were washed three times using sterile deionized water, again. Washed sauries were inoculated with the

bacterial suspension. The inoculated sauries were dipped in 80 ppm PAA or 100ppm SH solutions for 5 min, and then were placed into a stomacher bag (Seward Ltd., Worthing, U.K.). The samples were stored at 4 °C. The *M. psychrotolerans* cells were counted at different time points by swabbing the surfaces. *M. psychrotolerans* cells were counted using the spreading method based on (Niven, Jeffery, & Corlett, 1981), based on growing the cells on Niven's agar supplemented with 0.2% sodium pyruvate at 25 °C for 72 h.

2.4. Scanning Electron Microscopy (SEM) observation

The morphology of *M. psychrotolerans* cells, that were treated with PAA, was observed with a scanning electron microscope (SEM). The harvested cells were centrifuged ($3,000 \times g$, 5 min, 4 °C) and washed three times with 0.2 M phosphate buffer (PB, pH 7.4). The cells were then fixed with 2% glutaraldehyde–PB (2 h, 25°C), washed, resuspended in the same PB, and placed on a Sempore ($\phi 0.6 \mu\text{m}$, JEOL, Tokyo, Japan) as a 100 μL droplet. After washing three times with PB, the samples were dehydrated in a graded ethanol solution (50% - 100%). The cells were washed with t-butyl alcohol (Wako Pure Chemicals Industries, Osaka, Japan) for 30 min and lyophilized. Finally, samples were then sputtered with Pt-Pd and observed using SEM (JSM-6010LA, JEOL, Tokyo, Japan).

2.5. Investigation of cell membrane permeability using fluorescent staining

Following disinfectant treatment, the cells were stained, using Bacteria Live/Dead Staining Kit (Promokine, PromoCell GmbH, Heidelberg, Germany). The DMAO (green fluorescence) and EtD-III (red fluorescence) dyes were mixed with 0.85% NaCl to obtain the dye mixture. Next, 1 μ L mixture dye was added to 100 μ L cell suspension, followed by dark incubation for 15 min at room temperature. Then, 5 μ L of the stained bacterial cells were placed on a glass slide and immediately visualized using the epifluorescence microscope (Olympus BX 51-34, Melville, USA). Fluorescence images were processed using the Image J software package (Schneider, Rasband, & Eliceiri, 2012).

2.6. Chromosomal DNA examination by agarose gel electrophoresis

The bacterial DNA was extracted from the PAA treated and control groups described in section 2.2, using the Genomic DNA Extraction Kit (Macherey-Nagel GmbH, Germany), according to the manufacturer's instructions. Extracted DNA was separated by agarose gel electrophoresis (1% agarose). After ethidium bromide staining, the agarose gel was digitalized, and the DNA bands were visualized using the UV Transilluminator STAGE-1000 (AMZ, Inc., Japan).

2.7. SDS-PAGE

After disinfection, the bacterial cells were centrifuged to obtain the cell pellets. The pellets were washed and resuspended in PBS. The bacterial cells were disrupted with

the Zirconia Beads Kit (Zircon prep mini, Nippon Genetics Co.Ltd., Tokyo, Japan) and the Bead Beater (2,500 rpm, 30 min, CD-1000, EYELA, Tokyo, Japan). This was followed by centrifugation at $6,000 \times g$ for 10 min at 4 °C and the supernatants were collected. The protein concentrations of the supernatants were determined according to the Bradford method (Bradford, 1976). Loading samples were prepared according to the method described by Wang, Chang, Yang, & Cui, (2015). The supernatant (80 μ L, protein concentration of approximately 1.2 mg/mL) were mixed with the loading buffer (20 μ L), boiled for 5 min, cooled on ice, and centrifuged at $4,000 \times g$ for 5 min at 4°C. Finally, 20 μ L sample solutions were resolved by SDS-PAGE, and the gel was stained with Coomassie Brilliant R250 until the bands were clearly visible.

2.8. Statistical analysis

All experiments were performed in triplicate and the data was presented as the mean $\pm$ standard deviation. The data was analyzed by ANOVA and the least significant difference (LSD) was calculated for comparison of means. Statistical significance was evaluated based on ≤ 0.05 . All statistical analysis was performed using RStudio Desktop (RStudio Desktop, Inc., Boston, MA).

3. Results and discussion

3.1. Sanitizing effect on PAA against *M. psychrotolerans* in vitro

The MIC and MBC of PAA against *M. psychrotolerans* were 7.5 ppm and 12 ppm, respectively. Bridier, Briandet, Thomas, & Dubois-Brissonnet, (2011) have reported the MBC of PAA against *E. coli* PHL628 to be 7.4 ppm. Moreover, our results showed 10 ppm PAA treatment for 5 min significantly reduced the bacterial cell count from 8.92 to 2.00 log CFU/mL ($p \leq 0.05$). And, 20 ppm PAA treatment for 5 min reduced the populations of *M. psychrotolerans* to undetectable levels (< 1.7 log CFU/mL) (Table 1). Additionally, the antibacterial efficiency of PAA treatment increased with an increased its concentration. The counts of *M. psychrotolerans* also decreased to the undetectable levels upon 40 ppm PAA treatment for 1 min. In a previous study reported that the 80 ppm PAA treatment for 5 min was lethal for *E. coli* O157:H7 (Rodgers, Cash, Siddiq, & Ryser, 2004).

3.2. Sanitizing effect of PAA against *M. psychrotolerans* on saury surface

The antimicrobial effects of PAA and SH on *M. psychrotolerans* present on the saury surfaces are shown in Figure 1. The control group showed a 0.5 log CFU/cm² reduction of *M. psychrotolerans*. Treatments with 80 ppm PAA or 100 ppm SH for 1 min, showed 1.4 and 1.27 log CFU/cm² reductions, respectively. An increase in washing time improved the reduction in the *M. psychrotolerans* counts from the saury surfaces. Viable cell counts were reduced by 2.23 and 1.99 log CFU/cm² upon washing with 80 ppm PAA and 100 ppm SH for 5 min, respectively (Fig 1A). These results indicate that PAA as well as SH exhibits effective antimicrobial activity

against *M. psychrotolerans* on saury surface.

In addition, during the storage of saury samples at 4 °C, the viable counts of *M. psychrotolerans* in the control sample reached to 7.11 log CFU/cm² after 3 days, but the viable counts of *M. psychrotolerans* in PAA or SH treated samples reached to the same level (6.8 log CFU/cm²) after 5 days (Fig. 1B). In our previous study, we observed a large amount of histamine accumulation by *M. psychrotolerans* in broth, when the viable count of *M. psychrotolerans* reached 10⁷ CFU/ml (Wang, Yamaki, Kawai & Yamazaki, 2020; Kato *et al.*, 2017). Therefore, our results suggested that treatment with PAA was similar to SH during the fish washing process could extend the shelf life of stored fish.

In a previous study, it has been reported that soaking tomatoes and lettuce in 30 ppm PAA solution for 10 min significantly reduced the *E. coli* counts by 4.39 and 2.33 log CFU/g, respectively (Keeratipibul *et al.*, 2011). In this study, we reported the similar reduction in *M. psychrotolerans* counts on saury surfaces in response to 80 ppm PAA and 100 ppm SH treatments (Fig. 1). The 50-100 ppm SH solution is conventionally used to disinfect fish fillets (Thi *et al.*, 2013). Park *et al.* (2012) reported that PAA can potentially replace SH, as it was shown to be more effective at an equivalent concentration against *E. coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes*. Lee and Huang (2019) also demonstrated that PAA treatment generated much less disinfection-related by-products than SH treatment. Based on earlier studies and our results, we propose that PAA can be a better sanitizer for

seafood processing than SH.

3.3. SEM analysis

The sanitizing effect was determined by assessing the impact of PAA on cells morphology using SEM, The *M. psychrotolerans* cells exhibited a smooth and intact surface morphology in control groups (Fig. 2A). After 20 ppm PAA treatment for 5 min, the surface of *M. psychrotolerans* cells showed no clear differences compared to the control group (Fig. 2B). However, after a 5 min treatment with 80 ppm PAA, some bacterial cells became corrugated (Fig. 2C). This indicates that PAA treatment impacts the outer membrane of *M. psychrotolerans* cells and that the level of damage might depend on PAA concentration. Some studies revealed that the PAA treatment generates cell surface irregularities and partial grooves, with the damage becoming increasingly apparent as the sanitizer concentration is increased (Chino *et al.*, 2017; Ujimine *et al.*, 2017; Park *et al.*, 2013; Mustapha and Liewen, 1989). We did not observe any grave damage to *M. psychrotolerans* cell morphology after 20 ppm PAA treatment. Additionally, it has also been reported that the effectiveness of PAA treatment on bacterial cell morphology was significantly low below the concentration of 60 mg/L (Zhang *et al.*, 2019).

3.4. Evaluation of cell membrane integrity after sanitizing treatment

Bacterial cytoplasmic membrane permeability was evaluated by staining with

Bacterial Live/Dead Staining Kit. The kit provides two stains, DMAO and EtD-III. DMAO can stain both, live and dead bacteria, with intact and damaged cell membranes, while EtD-III can stain only dead bacteria with damaged cell membranes (Kaprelyants and Kell, 1992). *M. psychrotolerans* cells treated with water displayed green fluorescence, and the cells from the sanitizer-treated group displayed red fluorescence (Fig. 3). This indicates that cells lose their membrane integrity upon PAA treatment. However, the level of damage was unclear by staining the bacterial cells with this staining kit. Changes in bacterial cell membrane permeability and integrity might be attributed to various factors, such as inactivation of enzymes and alterations in membrane potential (Joux and Lebaron, 2000). SYTO 9 and PI staining of *Staphylococcus aureus* SA1 and *L. monocytogenes* cells, after 5000 ppm PAA treatment for 15 s also revealed cell membrane damage (Lee *et al.*, 2016). Zhang *et al.* (2018) reported that PAA disrupts the cell membrane integrity of inactivate microbes. These results were correspondent with our observations in figures 3.

3.5. Analyses of chromosomal DNA through agarose gel electrophoresis

Changes in bacterial chromosomal DNA upon PAA treatment were analyzed through agarose gel electrophoresis (Fig. 4). The DNA extracted from the control and the test group treated with PAA displayed a bright single band on the agarose gel. Additionally, an increase in PAA concentration showed no particular difference in the DNA band profiles. This result suggests that PAA kills *M. psychrotolerans* cells

without damaging the bacterial DNA. This finding confirms with the results of previous study that PAA exerted antimicrobial effects on *E. coli* TOP10 independent of affecting the plasmid DNA (Zhang *et al.*, 2019). Leggett *et al.* (2015) also reported no damage to the spore's DNA following PAA treatment. The PAA disinfection mechanism might differ from SH, that damages the bacterial DNA as a disinfectant (Fukuzaki, 2006). Ujimine *et al.* (2017) demonstrated that 3.1 ppm SH treatment damaged the molecular DNA in *S. aureus* cells and the DNA was undetectable on the agarose gel. However, there might be variations related to the differences in sanitizer efficacy, target microorganism, sanitizer concentration and treatment time (Zoellner *et al.*, 2018). Overall, our observations suggest that the bactericidal activity of PAA is not related to the damage to the bacterial chromosomal DNA.

3.6. SDS-PAGE analysis to investigate the bacterial proteins profiles

The cell protein profiles of *M. psychrotolerans* after PAA treatment are shown in Figure 5. The protein profile of *M. psychrotolerans* cells exhibited no major difference between the PAA-treated and control cells. However, there were some protein bands that appeared to be slightly faint with increased PAA concentration. In some studies, the mechanisms of PAA action is reported to include, degradation of proteins during treatment (Kerkaert *et al.*, 2011), enzyme oxidation and inactivation (Fraser, *et al.*, 1985) and impairment of lipoprotein cytoplasmic membrane permeability (Kitis, 2004). In this study, we demonstrated that PAA destroyed the cell

membrane integrity (Fig. 3), and did not affect the bacterial DNA integrity in the treated cells (Fig. 4). Therefore, the reduced amount of proteins after treating with PAA on the gel could be because of the damaged cell membrane proteins (Nakayama *et al.*, 2013). Also, depending on previous investigations, the difference in the cell protein profiles after PAA treatment might be attributed to alterations in some metabolic or protein synthesis pathways upon PAA treatment (Du, Liu, Cao, Zhao, & Huang, 2018; Liu *et al.*, 2018). Hence, further scientific studies are required to elucidate the underlying mechanism of PAA disinfectant function.

4. Conclusion

To the best of our knowledge, this is the first report to investigate the sanitizing efficacy of PAA against *M. psychrotolerans*, a histamine-producer responsible for seafood poisoning. We revealed that PAA could significantly reduction of *M. psychrotolerans*, both *in vitro* and on saury surfaces. And we also demonstrated that the antimicrobial mechanism of PAA was mainly related to damage the cell membranes. Our study provides evidence on the disinfectant efficiency of PAA and its potential application to improve seafood safety.

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Conflict of Interest

None declared.

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555 TABLE AND FIGURE LEGENDS

556

557 Table 1. Survival of *M. psychrotolerans* under treatment with PAA *in vitro*.

558

559 Fig. 1. (A) Reduction in bacteria cell counts of *M. psychrotolerans* on saury surfaces,
560 after washing with sanitizers for 1 or 5 min; (B) Effect of different sanitizer
561 treatments against *M. psychrotolerans* on saury surfaces during storage at 4 °C. White
562 bars, black bars, and gray bars represent control, 80 ppm PAA and 100 ppm SH,
563 respectively. Different lowercase letters are significantly different within same
564 treatment time group ($p < 0.05$).

565

566 Fig. 2. Scanning electron microscopic (SEM) observations of *M. psychrotolerans*
567 undergoing PAA and sodium hypochlorite (SH) treatments. A- Control; B- 20 ppm
568 PAA treated for 5 min; C- 80 ppm PAA treated for 5 min.

569

570 Fig. 3. Epifluorescence microscopic images of *M. psychrotolerans* cells, stained with
571 Live/Dead Kit after PAA treatments. A- Control; B- 20 ppm PAA treated 5 min; C- 80
572 ppm PAA treated 5 min.

573

574 Fig. 4. Chromosomal DNA of *M. psychrotolerans* after PAA treatment. M- Marker;

575 C- Control; P1- 20 ppm PAA treated 5 min; P2- 80 ppm PAA treated 5 min.

576

577 Fig. 5. SDS-PAGE profiles of protein in *M. psychrotolerans* cell proteins after PAA

578 treatment. M- Marker; C- Control; P1- 20 ppm PAA treated 5 min; P2- 80 ppm PAA

579 treated 5 min.

Table. 1 Wang et al.

Treatment	Treatment time (min)			
	0	1	3	5
Control	8.92 $\pm$ 0.04	8.93 $\pm$ 0.04 ^a	8.92 $\pm$ 0.03 ^a	8.90 $\pm$ 0.03 ^a
10 ppm	8.92 $\pm$ 0.04	4.59 $\pm$ 0.25 ^b	2.07 $\pm$ 0.22 ^b	2.00 $\pm$ 0.05 ^b
20 ppm	8.92 $\pm$ 0.04	1.87 $\pm$ 0.18 ^c	1.80 $\pm$ 0.17 ^b	ND
40 ppm	8.92 $\pm$ 0.04	ND	ND	ND
80 ppm	8.92 $\pm$ 0.04	ND	ND	ND

¹Different lowercase letters are significantly different within the same treatment time group (p < 0.05).

²ND:Below detection level: < 1.7 log CFU/mL.

Fig. 1 Wang et al.

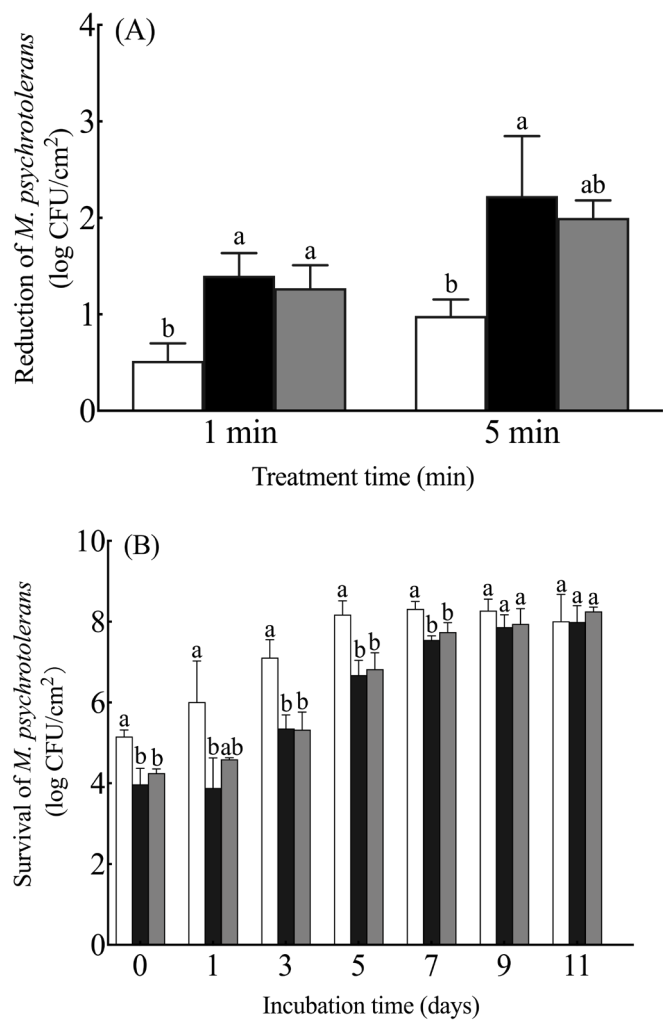


Fig. 2 Wang et al.

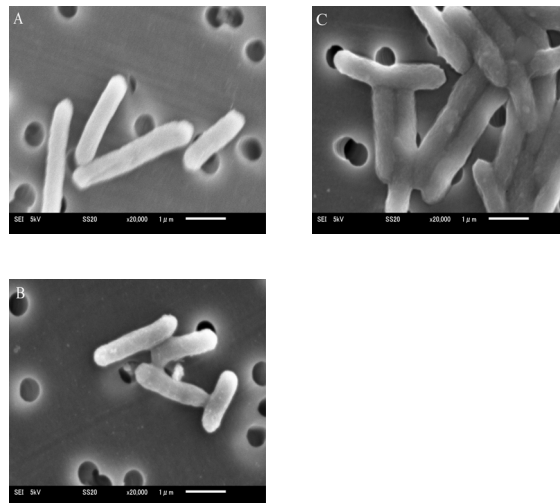


Fig. 3 Wang et al.

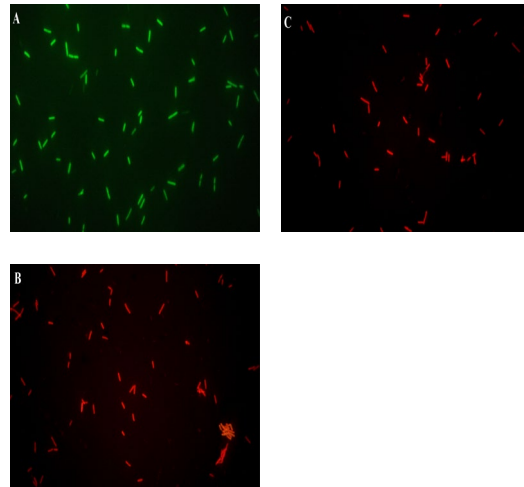


Fig. 4 Wang et al.

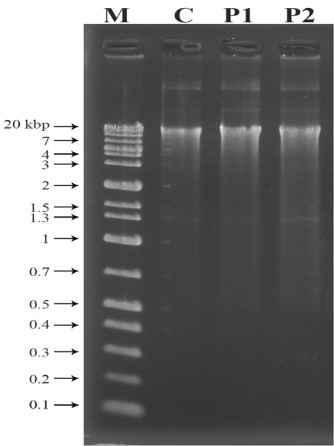


Fig. 5 Wang et al.

