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Combating Staphylococcus aureus and its methicillin resistance gene (mecA) with cold plasma

Xinyu Liao
Zhejiang University

Patrick J. Cullen
Technological University Dublin, patrick.j.cullen@tudublin.ie

Donghong Liu
Zhejiang University

See next page for additional authors

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Authors

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Xinyu Liao [a,b,c], P.J. Cullen [d,e], Donghong Liu [a,b,c], Aliyu Idris Muhammad [a,b,f], Shiguo Chen [a,b], Xingqian Ye [a,b], Jun Wang [g], Tian Ding [a,b,c,*]

[a] Department of Food Science and Nutrition, Zhejiang University, Hangzhou, Zhejiang 310058, China
[b] Key Laboratory for Agro-Products Postharvest Handling of Ministry of Agriculture, Zhejiang Key Laboratory for Agro-Food Processing, Hangzhou, Zhejiang 310058, China
[c] Fuli Institute of Food Science, Zhejiang University, Hangzhou, Zhejiang 310058, China
[d] BioPlasma Research Group, Dublin Institute of Technology, Dublin, Ireland
[e] Department of Chemical and Environmental Engineering, University of Nottingham, Nottingham NG7 2RD, United Kingdom
[f] Department of Agricultural and Environmental Engineering, Faculty of Engineering, Bayero University Kano, Nigeria
[g] College of Food Science and Engineering, Qingdao Agricultural University, Chengyang, Qingdao, China

HIGHLIGHTS

- Cold plasma was successfully applied for combating MRSA and *mecA* genes.
- A plasma dose of 2.3 kJ resulted in a 5-log reduction of MRSA.
- Degradation rates for *mecA* genes were slower than those found for MRSA.
- The extracellular *mecA* genes were more sensitive to plasma treatment.
- Removal of MRSA and *mecA* genes was more difficult in food wastewater effluents.

GRAPHICAL ABSTRACT
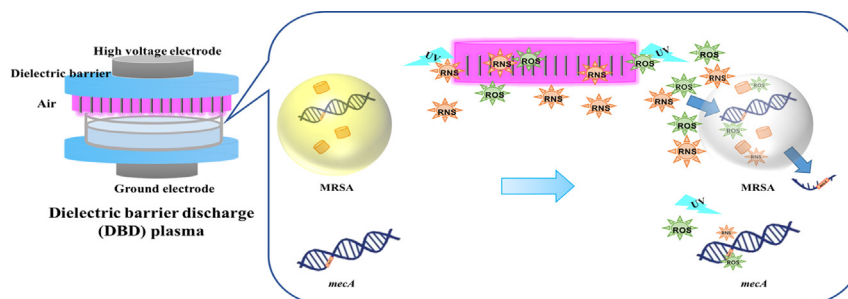
ARTICLE INFO

ABSTRACT

The increase in antibiotic resistance has become a global challenge to public health. In this study, an atmospheric cold plasma (ACP) system was applied for combating methicillin-resistant *Staphylococcus aureus* (MRSA) and its methicillin resistance gene (*mecA*) during food wastewater treatment. The plate count and flow cytometry methods were employed to estimate the damage in MRSA induced by plasma treatment. A quantitative real-time PCR (qPCR) method was used to assess the plasma-induced degradation of the *mecA* genes. The inactivation of MRSA and degradation of extracellular (e-) and intracellular (i-)*mecA* genes were investigated in phosphate buffered solution as a function of plasma exposure. A relatively low plasma influence of 0.12 kJ/cm² accounted for 5-log MRSA and 1.4-log e-*mecA* genes reduction, while only around 0.19-log degradation for i-*mecA* genes. As the plasma intensity was accumulated to 0.35 kJ/cm², the reduction of e- and i-*mecA* genes was increased to 2.6 and 0.8 logs, respectively. The degradation of i-*mecA* genes was much slower than that of e-*mecA* genes due to the protective effects of the outer envelopes or intracellular components against plasma. The matrix effect of wastewater effluents shielded both antibiotic resistance bacteria (ARB) and antibiotic resistance genes (ARGs) from plasma disinfection, which led to a lower degradation efficacy. Our results could support the development and optimization of plasma-based wastewater treatment.

* Corresponding author at: Department of Food Science and Nutrition, Zhejiang University, Hangzhou, Zhejiang 310058, China.
E-mail address: tding@zju.edu.cn (T. Ding).

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first identified two years after the introduction of methicillin in 1960 and has been considered as one of the most serious global health threats due to its capacity to cause nosocomial infections (Chambers and DeLeo, 2009; Emaneini et al., 2017). In the United States, methicillin resistance was reported to occupy 51.3% of *S. aureus* isolates in 2010 (CDC, 2013). In Europe, the ECDC reported that the percentage of MRSA in *S. aureus* strains reached 16.8% in 2015 (ECDC, 2016). In recent years, MRSA have been mainly associated with the infections in hospitals and the wider community which are generally referred to as hospital-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA). Numerous studies have reported on the isolation of MRSA from food-producing animals, their products and food-related environments, which are termed livestock-associated MRSA (LA-MRSA) (Narvaez-Bravo et al., 2016; Wendlandt et al., 2013). After the processing of soaking and washing animal products, these LA-MRSA could be subsequently transferred into food wastewater effluents, which can be released to the natural environments or human population silently. The antibiotic resistance gene (*mecA*) in MRSA, encoded by staphylococcal cassette chromosomes (SCCs), confers resistance to methicillin and other β-lactam antibiotics to MRSA (Malachowa and Deleo, 2010). Generally, *mecA* genes produce a penicillin-binding protein (PBP2a), which has a low affinity to all β-lactam antibiotics. The genomic island SCCmec in MRSA, a mobile genetic element, has been associated with the wide dissemination of methicillin resistance through the horizontal gene transfer (HGT) mechanisms, which include conjugation by cells to cells, transformation through uptake of mobile genetic elements and transduction mediated by bacteriophage (Juhas, 2015). Therefore, the existence and spread of the methicillin resistance gene *mecA* within environmental matrices accelerate the thriving of global antibiotic resistance, posing a significant threat to public health.

In recent years, several disinfection methods have been proposed for the elimination of ARB and their ARGs in water or wastewater systems, such as ozone, ultraviolet (UV) irradiation, chlorination, Fenton's reagent and a combination of these strategies (Barancheshme and Munir, 2017; Czekalski et al., 2016; Sousa et al., 2017; Yoon et al., 2017). However, several studies have reported the insufficient removal of ARB and ARGs by some of these disinfection processes (Munir et al., 2011; Rizzo et al., 2013). Therefore, more efficient technologies should be developed to achieve inactivation of environmental contaminants-ARB and AGRs. Cold plasma is considered as a promising Advanced Oxidation Processing (AOP) in wastewater treatment. Plasma is a reactive cocktail consisting of various reactive oxygen and nitrogen species (e.g., hydrogen peroxides, hydroxyl, ozone, nitric oxide radicals), UV photons and charged particles (e.g., electrons, ions), which are responsible for microbial inactivation (Liao et al., 2017a; Liao et al., 2018a; Scholtz et al., 2011). A growing body of literature point toward the efficient inactivation capacity of cold plasma for a wide array of microorganisms (Alshraiedeh et al., 2013; Daeschlein et al., 2011; Liao et al., 2018b; Xiang et al., 2018; Scholtz et al., 2011). Additionally, several studies have reported on the application of cold plasma for the degradation of chemical contaminants, such as dyes and mycotoxins in wastewater effluents (Aggelopoulos, 2016; Benghanem, 2016; Kasih et al., 2017). The strong inactivation effect of cold plasma on various ARB, such as vancomycin-resistant *Enterococci* (VRE), MRSA, ampicillin and tetracycline-resistant *Pseudomonas aeruginosa*, and triazole antifungal-resistant *Candida albicans*, has been demonstrated (Guo et al., 2018; Kvam et al., 2012; Napp et al., 2016a, b). Most of the aforementioned investigations were performed with the simple media, such as buffer solutions, which were known to be different than that of actual food industry effluents.

In the present work, the inactivation mechanisms and degradation kinetics of MRSA and its antibiotic resistant gene-*mecA* by using cold plasma were investigated in a buffer system through conventional plate methods and flow cytometry combined with fluorescence technology. Then, the effect of a more complex food wastewater matrices on cold plasma-induced degradation of MRSA cells and *mecA* genes was explored.

2. Materials and methods

2.1. Bacterial preparation

In this study, a methicillin-resistant *S. aureus* (MRSA, ATCC 43300) was used. One single colony was transferred into 100 ml Luria-Bertani (LB) broth (Base Bio-Tech Co., Hangzhou, China) and incubated at 150 rpm and 37 °C for 24 h to reach the stationary phase. *S. aureus* cells were then harvested by centrifugation at 5000 rpm and 4 °C for 10 min and washed thrice by resuspension in sterile phosphate buffered solutions (PBS) at a pH of 7.0 ± 0.2. The final concentration of *S. aureus* was approximately 10^9 CFU/ml.

2.2. Wastewater model preparation

Models of dairy and meat wastewater were prepared according to the study of Patange et al. (2018). The synthetic dairy wastewater consisted of 2 g/l commercial milk powder, 0.2% (*w/v*) commercial milk fat, 0.01% (*w/v*) sodium hydroxide (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and sterile distilled water. The milk powder was composed of 32.6% proteins, 56.2% carbohydrates, 1.2% fat, 0.038% calcium, 0.55% sodium, and 0.038% magnesium. The synthetic meat wastewater system contained 1.95 g/l commercial meat extract powder (Sangon Biotech Co., Ltd., Shanghai, China), 0.2 g/l glycerol ($C_3H_8O_3$) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), 0.36 g/l ammonium chloride (NH_4Cl) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), 0.05 g/l sodium chloride (NaCl) (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) and sterile distilled water (Patange et al., 2018).

2.3. Cold plasma disinfection

A dielectric barrier discharge (DBD)-atmospheric cold plasma (ACP) system was built (Liao et al., 2017b), consisting of two circular quartz glass dielectric barriers with diameters of 90 mm (upper) and 150 mm (ground), two circular aluminum plate electrodes with a diameter of 50 mm, an aluminum petri dish with a diameter of 50 mm and a high-voltage power supply (CTP-2000K, Nanjing Suman Electronics Co., Ltd., Nanjing, China), which was set at a frequency of 10 kHz and an input voltage of 14 kV with a power of 2.94 W/cm². The gap between the upper barrier and the surface of wastewater was 2 mm, and atmospheric air was used as the working gas for the generation of plasma. The treatment periods ranged from 0 to 8 min, corresponding to the applied energy intensities of 0 to 1.41 kJ/cm².

2.4. Microbiological analysis

For microbiological analysis, the untreated and treated samples were serially diluted with sterile PBS and then spread over tryptone soya agar (TSA) (Base Bio-Tech Co., Hangzhou, China). After the incubation at 37 °C for 24 h, the survivors of *S. aureus* on the plates were determined.

2.5. Estimation of esterase activity

The *S. aureus* samples were incubated with Carboxyfluorescein diacetate (50 μM, cFDA, Sigma-Aldrich Corp., St. Louis, MO, USA) for 30 min at 37 °C. After centrifugation, the pellet cells were resuspended with sterile phosphate buffered solutions to remove excess cFDA. The stained samples were measured by flow cytometry (Beckman Coulter Inc., Miami, FL, USA).

Table 1
Primers used in the study.

Primer[a]	Sequences (5′-3′)	Primer size (bp)	Amplicon size (bp)	Annealing temperature (°C)	Reference
MecA-FP	GTTGTAGTTGT CGGGTTTGGT	21	1018	55.3	McKinney and Pruden (2012)
MecA-RP	GGTGGATAG CAGTACCTGA GC	21		57.8	

[a] FP = forward primer; RP = reverse primer.

2.6. Determination of intracellular ROS

The intracellular ROS was measured by a cellular assay probe, 2,7-dichlorofluorescin diactate (DCFH-DA; Beyotime Co., Ltd., Shanghai, China). Before plasma treatment, the *S. aureus* cells were incubated with DCFH-DA (10 μM) at 37 °C for 20 min. After treatment at various plasma doses, green fluorescence intensities were measured using flow cytometry (Beckman Coulter Inc., Maimi, FL, USA).

2.7. Assessment of membrane-bound staphyloxanthin

The treated and untreated *S. aureus* samples were harvested by centrifugation at 5000 rpm and 4 °C for 10 min. The color of *S. aureus* cells on the bottom of centrifuge tubes was recorded.

2.8. Estimation of cell membrane potential

Membrane potential was measured with a BacLight™ Bacterial Membrane Potential Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). DiOC$_2$ (3) (3,3′-diethyloxacarbocyanine iodide) a molecular probe, alters from green to red fluorescence as the membrane potential increases. CCCP (carbonyl cyanide 3-chlorophenylhydrazone) can destroy the cell membrane potential by eliminating the membrane proton gradient. The sample treated with CCCP was designated as the control. Specifically, DiOC$_2$ (3) (3 mM, 10 μl) was added to 1 mL sample and mixed thoroughly. For the negative control, CCCP (500 μM, 10 μl) was transferred into the sample and mixed prior to the addition of DiOC$_2$ (3). The mixture was then incubated for 30 min at room temperature. The samples were then centrifuged and washed with sterile phosphate buffered solutions to remove excess DiOC$_2$ (3). The stained samples were then measured using a flow cytometer (Beckman Coulter Inc., Miami, FL, USA).
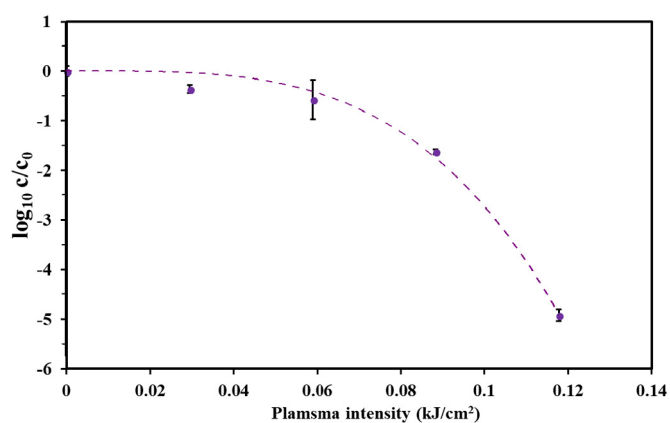

Fig. 1. The viability of methicillin-resistant *Staphylococcus aureus* (MRSA) during plasma treatment in phosphate buffered solutions (0.2 M) at pH 7.0. The dotted line represents the Weibull model fitted by the observed data.

Table 2
The inactivation kinetic parameters of MRSA and its *mecA* gene during plasma treatment in phosphate buffered solutions and wastewater effluent models.

Inactivation parameters	Medium	ARB or ARGs	First-order rate constant (k_{plasma}), cm^2/kJ for plasma exposure[a]	R^2 of the linear regression[a]
Culturability	PBS (pH 7.0)	MRSA	[b]	[b]
Gene degradation		e-*mecA*	6.70 ± 0.03	0.93 ± 0.05
		i-*mecA*	3.38 ± 0.18	0.96 ± 0.02
Culturability	Model dairy wastewater effluent	MRSA	8.70 ± 0.07	0.89 ± 0.01
Gene degradation		e-*mecA*	3.35 ± 0.01	0.94 ± 0.04
		i-*mecA*	2.49 ± 0.01	0.98 ± 0.01
Culturability	Model meat wastewater effluent	MRSA	10.99 ± 0.31	0.92 ± 0.01
Gene degradation		e-*mecA*	3.58 ± 0.02	0.98 ± 0.01
		i-*mecA*	2.87 ± 0.01	0.98 ± 0.01

[a] Values are the mean of triplicate measurements ± standard deviations (SDs).
[b] The degradation is a nonlinear fitting.

2.9. Measurement of cell membrane integrity

A LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to determine the microbial cell membrane integrity, with the fluorescent dyes of SYTO 9 (3.34 mM) and propidium iodide (PI, 20 mM) mixed in equal volumes. The untreated and treated *S. aureus* suspensions were then incubated with 3 μl of dye mixture at 37 °C for 15 min. The fluorescent analysis was performed with a Gallio flow cytometer (Beckman Coulter Inc., Miami FL, USA). After excitation with a laser light at 488 nm, SYTO 9 and PI emitted green fluorescence at 530 nm and red fluorescence at 630 nm, respectively. Each sample was collected with 10,000 events at a low flow rate of 400 to 600 events/s. Kaluza software (Beckman Coulter Inc., Miami FL, USA) was employed to analyze the collected flow cytometry data.

2.10. DNA extract and quantitative PCR (qPCR)

All DNA extractions were performed with a TIANamp Bacteria DNA Kit following the manufacturer's instructions (Tiangen Biotech CO., Ltd., Beijing, China). In this study, the qPCR analysis was performed with a QuantStudio® 3 Real-Time PCR systems (Thermo Fisher Scientific Inc., Waltham, MA, USA). Forget-Me-Not™ Evagreen® qPCR Master Mix (Biotium, Inc., Fremont, CA, USA) was used as the fluorescent dye for amplifying long amplicons. The primers and target amplicons are shown in Table 1. Each 20 μl PCR reaction mixture contained 10 μl of 2× EvaGreen qPCR Master Mix, 1 μl of forward and reverse primer (100 μM), 1 μl of DNA template, and 7 μl sterile DNase free H$_2$O. The qPCR procedure included 1 cycle of 2 min at 95 °C, 40 cycles of 5 s at 95 °C, 15 s at an annealing temperature of 50.3 °C, and 60 s at 72 °C, and then a melting curve from 65 to 95 °C. Standard curves for *mecA* genes were established with serial 10-fold dilutions of extracted DNA. The amplicon efficiency was 80.0 ± 1.4%. The R^2 measure of fit for the standard curves was 0.994 ± 0.005.

2.11. Statistical analysis

All measurements for this study were conducted in triplicate. The means and standard deviations (SDs) were determined from independent trials. Single-factor analysis of variance and Tukey's test were used for comparing the differences between the means by SPSS 20.0
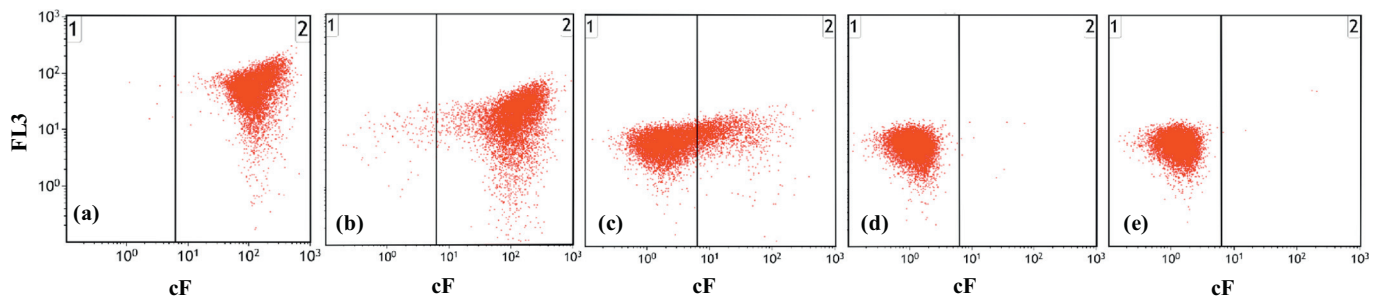
Fig. 2. Fluorescence dot plots for intracellular esterase activity changes during various plasma intensities of (a) 0, (b) 0.029, (c) 0.059, (d) 0.088, and (e) 0.12 kJ/cm^2 in phosphate buffered solutions (0.2 M) at pH 7.0.

(SPSS Inc., Chicago, IL, USA), and a $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Inactivation of MRSA by cold plasma

As Fig. 1 shows, the changes in the logarithmic concentration of the culturable MRSA cells in PBS (pH 7.0) are as a function of plasma intensity, which fitted well with a nonlinear Weibull model ($R^2 = 0.99$, $\log_{10} \frac{N_{I_{plasma}}}{N_0} = -\frac{1}{2.303} \left(\frac{I_{plasma}}{0.240} \right)^{3.58}$, I_{plasma} - the plasma intensity-kJ/cm^2). The concentration of MRSA cells was rapidly decreased by approximately 5 logs CFU/ml (i.e., a reduction rate > 99.999%) under the relatively low plasma exposure of 0.12 kJ/cm^2 (Table 2). The dose-dependent antimicrobial behavior of cold plasma against MRSA cells can be found in other studies (Cotter et al., 2011; Han et al., 2015; Maisch et al., 2012). For instance, Maisch et al. (2012) reported that as the plasma intensity was increased from 0.12 to 0.24 kJ/cm^2, the reduction level of MRSA (BAA-44) treated with a surface micro-discharge plasma increased from 99.9% to 99.999%. Therefore, cold plasma technology has considerable potential in deactivating MRSA. To investigate plasma-induced inactivation mechanisms for MRSA cells, the physiological states of MRSA cells were estimated in the subsequent sections.

3.2. Intracellular esterase activity and ROS accumulation

A nonfluorescent probe, carboxyfluorescein diacetate (cFDA), was used in this study to estimate the intracellular esterase activity for MRSA cells. After passing through viable cell membranes, cFDA is then converted into a membrane-impermeable green fluorescent dye, carboxyfluorescein (cF), by intracellular esterase (Li et al., 2016). As Fig. 2 shows, quadrant 2 represents the subpopulation of MRSA cells with completed esterase activity. As the plasma intensity was raised from 0

to 0.12 kJ/cm^2, the subpopulation of MRSA cells in quadrant 2 declined significantly ($P < 0.05$) from 99.66 to 0.04% due to the compromised esterase activity induced by plasma generated ROS. To estimate the intracellular ROS density of MRSA, a molecular probe of DCFH-DA was used in this study (Fig. 3). The nonpolar probe of DCFH-DA can pass through cell membranes and be converted to the nonfluorescent polar DCFH with the aid of intracellular esterase (Gomes et al., 2005). DCFH can then be oxidized by intracellular ROS into highly fluorescent dichlorofluorescein (DCF). As shown in Fig. 3, the fraction of the subpopulation stained with DCF (quadrant 2) increased from 0.52 to 18.38% when the plasma intensity increased from 0 to 0.12 kJ/cm^2. The ROS produced from the plasma can penetrate through the lipid layers of cell membranes and result in an accumulation of ROS in MRSA cells. These intracellular ROS could subsequently react with intracellular components (e.g., proteins and DNA), which might explain the above observation of the rapid inactivation of esterase activity in MRSA during plasma treatment. Similar trends for the enhancement of intracellular ROS level as a function of plasma treatment were obtained in other studies (Ali et al., 2014; Cheng et al., 2014; Guo et eal., 2018; Han et al., 2015). Han et al. (2015) also employed the same fluorescent probe of DCFH and found increasing intracellular ROS with a 5-min treatment of a high-voltage atmospheric cold plasma.

3.3. Membrane-bound staphyloxanthin destruction

As observed from Fig. 4, the color of the untreated MRSA cell pellets exhibits golden or yellow, which is imparted by staphyloxanthin, a membrane-bound triterpenoid carotenoid pigment (Liu et al., 2005). Staphyloxanthin has been documented to be associated with protection of S. aureus against oxidative stress through scavenging free radicals, especially ROS, due to its conjugated double bonds (Clauditz et al., 2006). Therefore, staphyloxanthin has been considered as a potential target for antimicrobial therapies. In this study, as the plasma influence was increased to 0.12 kJ/cm^2, the color of MRSA cell pellets gradually faded
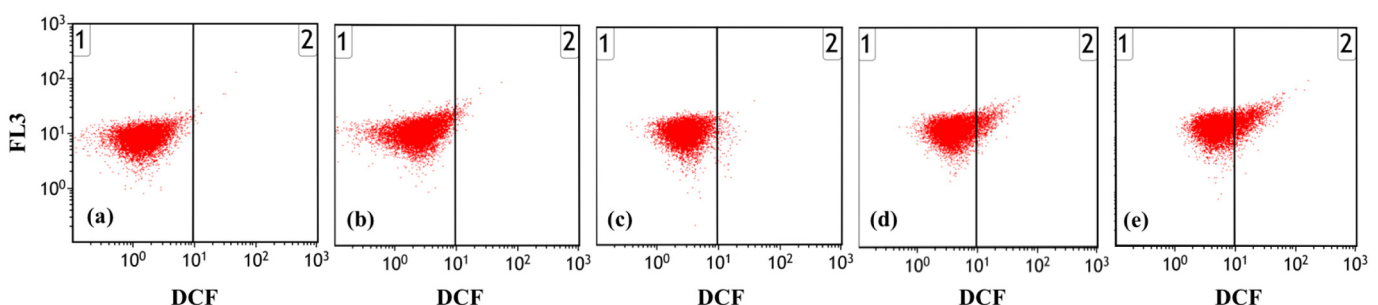

Fig. 3. The intracellular ROS in MRSA during various plasma intensities of (a) 0, (b) 0.029, (c) 0.059, (d) 0.088, and (e) 0.12 kJ/cm^2 in phosphate buffered solutions (0.2 M) at pH 7.0.

Fig. 4. Elimination of MRSA pigmentation during various plasma intensities of (a) 0, (b) 0.029, (c) 0.059, (d) 0.088, and (e) 0.12 kJ/cm^2 in phosphate buffer solutions (0.2 M) at pH 7.0.
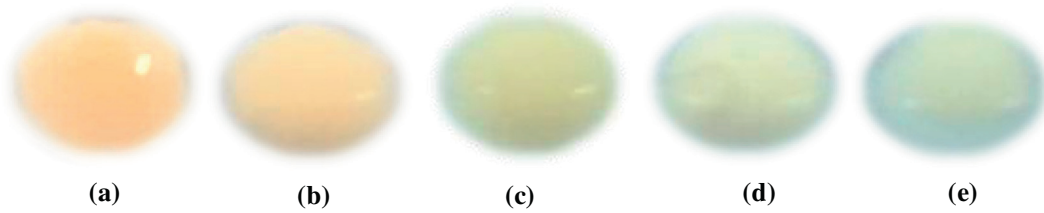
and turned from a golden-yellow color into white (Fig. 4). Staphyloxanthin is located within the cell membranes, making it easy to attack and oxidize by numerous reactive species produced from plasma. To the best of our knowledge, no information is available on the reaction of staphyloxanthin with cold plasma. However, there were some attempts to investigate the effect of cold plasma on other pigments. In a study by Ramazzina et al. (2015), the pigment of chlorophyll in kiwifruit slices was observed to decrease significantly after an atmospheric cold plasma treatment. The authors hypothesized that free radicals produced during the plasma treatments were attributed to the processing of pigments' oxidation. However, the exact products from the reaction between the pigment and the plasma species remain unclear.

3.4. Membrane potentials and membrane integrity

Microbial membranes that are associated with energy production and cellular viability, play a significant role in maintaining the normal physiological activity of microorganisms (Zhang et al., 2016). In this study, the changes in the membrane potential and membrane integrity of MRSA induced by plasma exposure were assessed by flow cytometry combined with a fluorescence technique. A membrane potential indicator dye (DiOC$_2$ (3)) was used to measure the microbial membrane potential. The membrane potentials of MRSA cells treated with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) are fully depolarized as the control. A higher red/green ratio of 1.31 for untreated MRSA cells revealed the normal cell membrane potential (Fig. 5(A)). Interestingly, the membrane potential of MRSA with 10-s cold plasma exposure significantly ($P < 0.05$) increased to a red/green ratio of 1.49. One possible explanation for this observation is that a relatively low cold plasma influence might induce the response of MRSA to oxidative stress through alteration of cell membrane potentials. Once the plasma intensity was enhanced to 0.12 kJ/cm^2, the cell membrane potentials of MRSA declined to a red/green ratio of 0.98, close to depolarization. As for the cell membrane integrity, a double-staining procedure was employed for this study, which included two kinds of nucleic acid fluorescent probes SYTO 9 and PI. The PI probe is limited to entering microbial cells with a compromised cell membrane, while the SYTO 9 probe can pass through intact cell membranes. It can be observed from Fig. 5 (B) that quadrant 1 is composed of the subpopulation of MRSA with intact cell membranes. Under a plasma intensity lower than 0.088 kJ/cm^2, the proportion of cells with intact membranes did not change
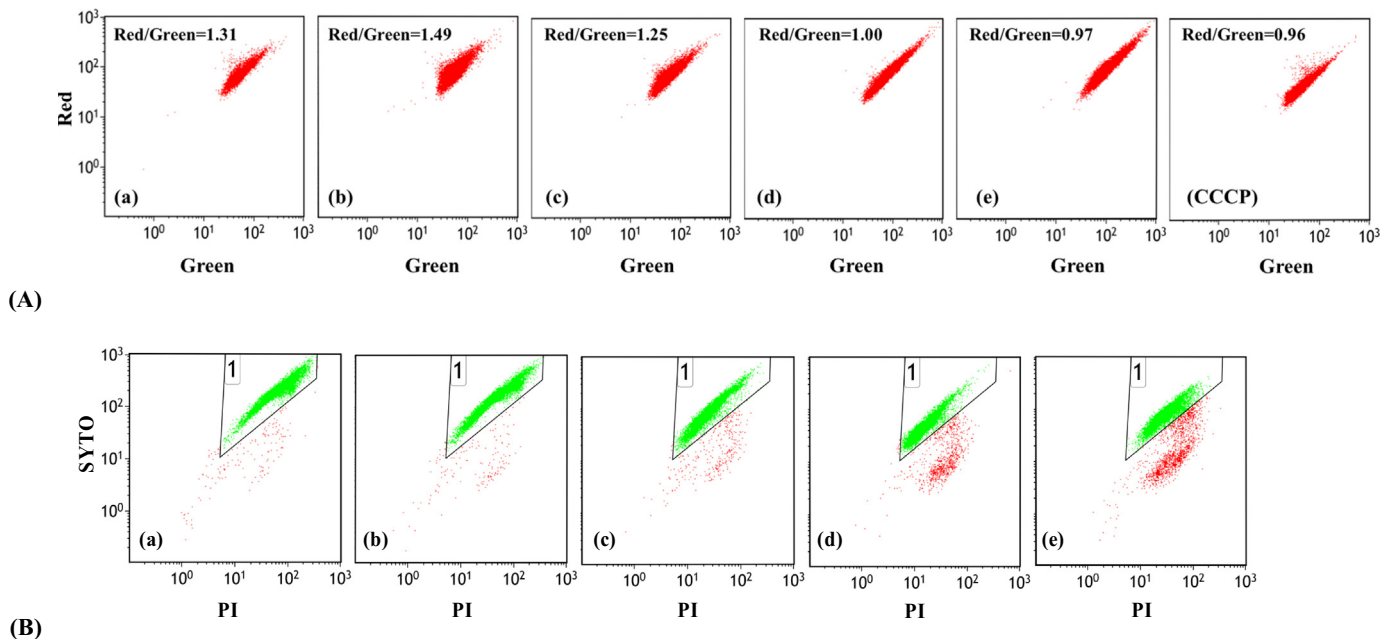

Fig. 5. Fluorescence dot plots for cell membrane potentials (A) and membrane integrity (B) changes during various plasma intensities of (a) 0, (b) 0.029, (c) 0.059, (d) 0.088, and (e) 0.12 kJ/cm^2 in phosphate buffer solutions (0.2 M) at pH 7.0.
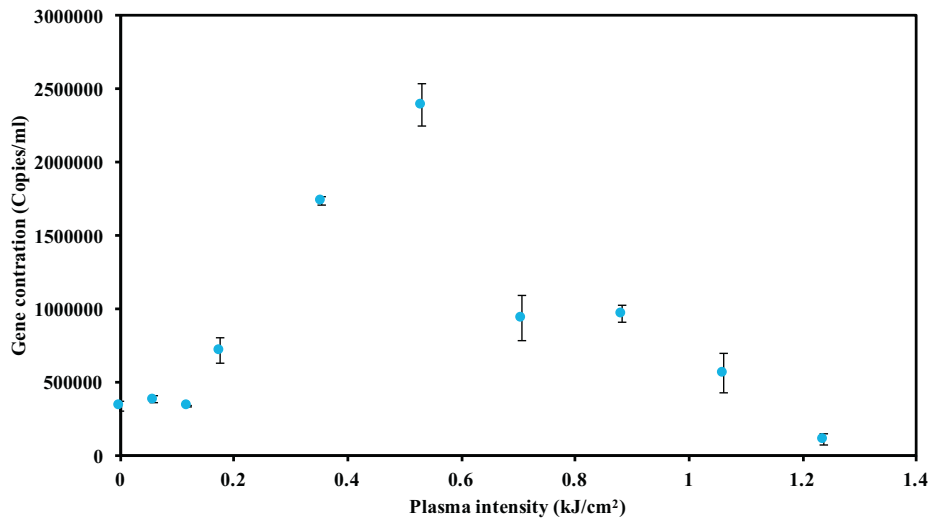
Fig. 6. The methicillin resistance genes-*mecA* leaked into phosphate buffered solutions (0.2 M) at pH 7.0 during the plasma treatments at 0 to 1.23 kJ/cm^2.

significantly ($P > 0.05$), but decreased rapidly to 92.3 and 84.4% after exposure to a plasma influence of 0.088 and 0.12 kJ/cm^2, respectively. As mentioned above, plasma exposure at 0.12 kJ/cm^2 caused over 90% loss in intracellular esterase activity, while the same applied plasma intensity resulted in approximately 14% of MRSA cells with damages in cell membranes. These differences might be attributed to the characteristics in the outer structures of MRSA, including the antioxidant pigments and their more rigid and tight structure. Similarly, in the study conducted by Han et al. (2015), the intracellular damages in *S. aureus* were observed to be more serious and obvious than the damages of the cell envelope after a high-voltage atmospheric cold plasma treatment.

3.5. Leakage of the mecA gene in medium

According to the above discussion, the loss of culturability occurred first for MRSA, followed by the inactivation of intracellular esterase activity, then damage to the cell membrane potential and integrity, and finally damage to DNA. It is worth noting that the plasma-induced damage to the cell membranes may likely result in the probable leakage of ARGs to the surrounding environment even though MRSA cells have lost their viability. Fig. 6 shows the leakage of ARGs in the medium from plasma treatment. With a plasma dose lower than 0.35 kJ/cm^2, the *mecA* level leaking from the MRSA cells into the medium increased rapidly from 338,431 to 1,737,672 copies/ml. With the HGT mechanisms, these free *mecA* genes might be disseminated among the microbial community, which could endow some antibiotic sensitive microorganisms with antibiotic resistant abilities or assist antibiotic resistance microorganisms to develop resistance toward new antibiotics. As the plasma intensities were increased to over 0.53 kJ/cm^2, the degradation of *mecA* in the medium was faster than the leakage of *mecA* from MRSA, thus, the concentrations of *mecA* in the medium declined rapidly. In most studies on the application of disinfection methods for abasement of ARGs, the possibility for the leakage of ARGs is generally
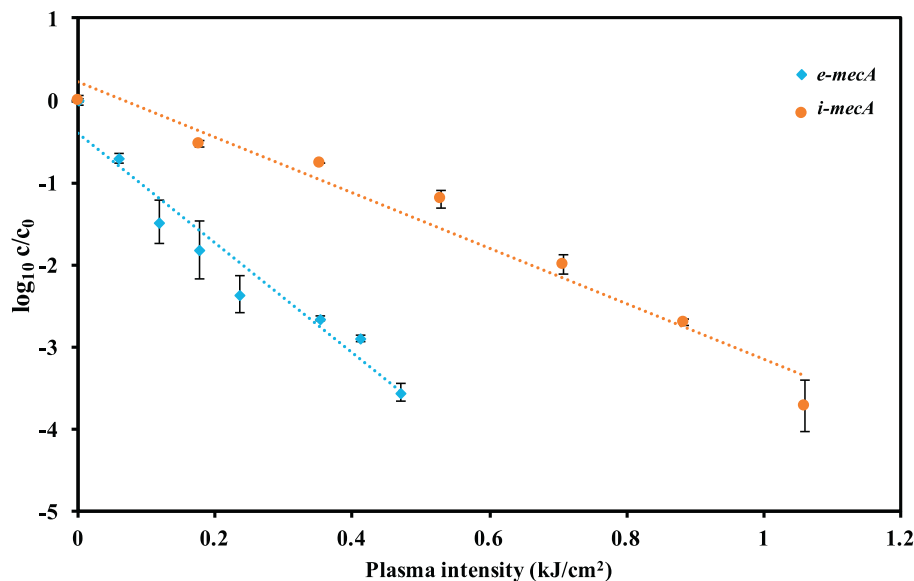

Fig. 7. Changes in the logarithmic relative concentration of the *mecA* genes during plasma treatment of phosphate buffered solutions (0.2 M) containing extracted DNA (extracellular *mecA*) (♦) and MRSA (intracellular *mecA*) (●) at pH 7.0.
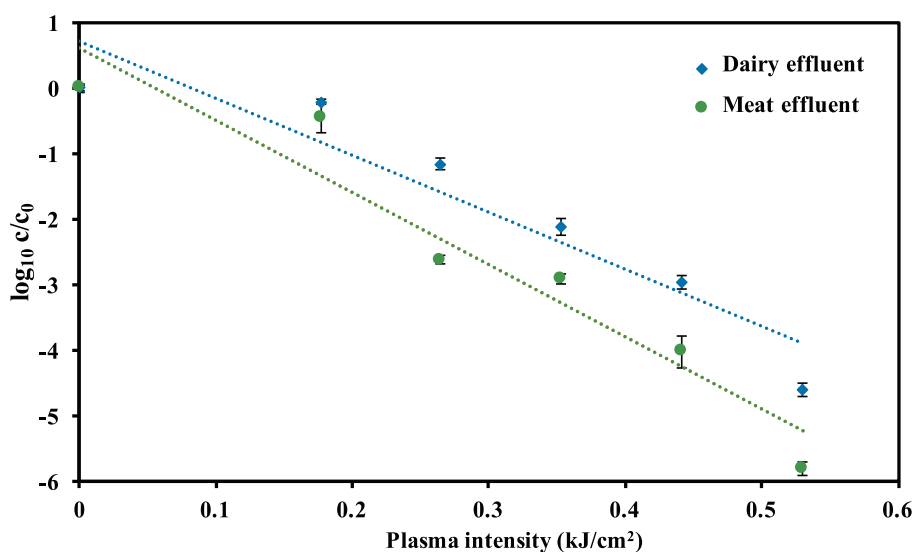
Fig. 8. The inactivation of MRSA during plasma treatment in dairy (◆) and meat (●) wastewater effluents.

ignored and not estimated. To assess the feasibility of a disinfection strategy, controlling the leakage of ARGs with a proper plasma intensity should be taken into consideration.

3.6. The degradation kinetics of intracellular and extracellular mecA in phosphate buffered solutions

As Fig. 7 shows, the changes in the logarithmic concentration of intracellular (i-) and extracellular (e-)*mecA* genes were a function of plasma intensities. The concentration of e-*mecA* genes decreased due to plasma-induced DNA damage. As Table 2 shows, the degradation of i-*mecA* genes (i.e., k_{plasma} = 3.38 ± 0.18 cm²/kJ) was much slower than that of e-*mecA* genes (i.e., k_{plasma} = 6.70 ± 0.03 cm²/kJ). For example, the degradation of 1 log i-*mecA* required plasma treatment of over 0.53 kJ/cm², while only 0.12 kJ/cm² could result in >1 log abatement of e-*mecA*. On the one hand, the outer cell walls and membranes of MRSA cells acted as barriers to shield intracellular DNA from reactive species in the plasma. On the other hand, DNA-binding proteins (DPs) and DNA repair mechanisms in MRSA might also be involved in protecting i-*mecA* genes from the damages induced by cold plasma. MrgA is a DNA-binding protein in *S. aureus* cells, which plays a critical role in protecting bacterial genes against oxidative damage through nucleoid condensation (Gaupp et al., 2012). The staphylococcal DNA repair mechanisms for genetic integrity mainly include excision and recombination repairs. The excision repair mechanism aims to repair base excision and nucleotide excision in DNA during the period of oxidative stress. As for recombination repair, this mechanism is used to repair the strand breakage induced by the oxidative damage to sugar in the DNA.

3.7. Degradation of MRSA, i- and e-mecA in food wastewater effluents

Fig. 8 presents the changes in cell viability of MRSA as a function of cold plasma exposure in model dairy and meat wastewater effluents. The degradation of ARB in the wastewater system (i.e., k_{plasma} = 8.70 ± 0.07 cm²/kJ for dairy effluent; k_{plasma} = 10.99 ± 0.31 cm²/kJ for meat effluent) is much slower than that in phosphate buffered solutions. When applied plasma intensity was <0.12 kJ/cm², no significant reduction of MRSA was found in wastewater system, while it was enough for inactivating >5-log subpopulation in phosphate buffer system. Additionally, MRSA cells in dairy effluent were found to be more resistant to cold plasma than those in meat effluent ($P < 0.05$). For

example, a plasma intensity of 10.4 kJ resulted in a reduction level of 4.6 logs CFU/ml in the dairy effluent medium, while a 5.8-log CFU/ml reduction in meat effluent medium was recorded. The differences in bacterial inactivation efficiencies in dairy and meat effluents were also observed during a study by Patange et al. (2018), in which the inactivation levels of *E. coli*, *E. faecalis* and *C. perfringens* in meat effluent induced by a DBD-ACP system were much higher than those in the dairy effluent. This observation might be explained by the different compositions (e.g., fat, proteins) of these two kinds of food industry effluents.

Fig. 9 shows the changes in the logarithmic relative concentration of the *mecA* gene as a function of plasma intensity during the treatment of the wastewater effluents. Firstly, the degradation for i-*mecA* (i.e., k_{plasma} = 2.49 ± 0.01 cm²/kJ for dairy effluent; k_{plasma} = 2.87 ± 0.01 cm²/kJ for meat effluent) was much slower than that of e-*mecA* (i.e., k_{plasma} = 3.35 ± 0.01 cm²/kJ for dairy effluent; k_{plasma} = 3.58 ± 0.02 cm²/kJ for meat effluent), which exhibited a similar trend to the phosphate buffer solution. Secondly, compared with the phosphate buffered system, a higher dose of plasma was required for the efficient degradation of both e- and i-*mecA* in both the dairy and meat effluents. The organic materials in the dairy and meat effluents, such as proteins and lipids, act as scavengers to quench radicals produced from the plasma, especially OH•. As a result, MRSA cells and the resistant gene *mecA* were protected to a greater extent from the attack of reactive species in the plasma. Such a matrix effect has also been reported to shield both ARB and ARGs from other disinfection methods (Czekalski et al., 2016; McKinney and Pruden, 2012). Therefore, the applied intensity of plasma for application in actual wastewater systems should be carefully evaluated.

This study demonstrated the potential of a DBD-ACP system to eliminate both MRSA and the *mecA* gene in phosphate buffer solutions and food wastewater effluents in order to reduce the risk for the propagation of antibiotic resistance in the environment. During plasma exposure, the oxidative damages to the intracellular components and exterior structure of MRSA were observed. Importantly, the intracellular damages to the esterase activity was primarily responsible for MRSA cells losing their viability. In this study, the outer structures of MRSA cells seem to be less vulnerable to cold plasma than the intracellular esterase activity due to the advantage of thicker cell walls and membranes. Furthermore, this study found that the degradation of *mecA* genes occurred after the loss of MRSA cells' viability. Therefore, a plasma intensity for valid inactivation of MRSA cells is not enough for efficient abasement of *mecA* genes. With regard to the buffered solution, a plasma intensity of 0.12 kJ/cm² was sufficient to eliminate over
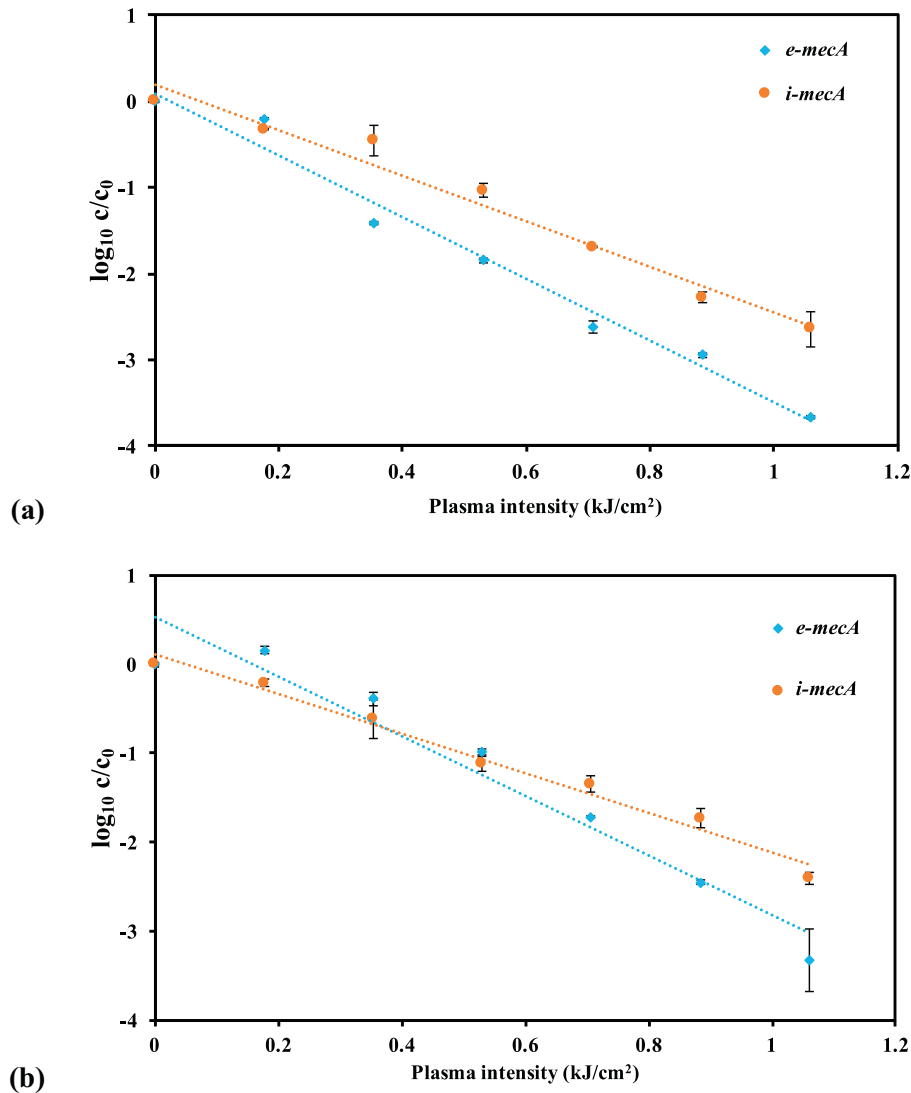
Fig. 9. Changes in the logarithmic relative concentration of the *mecA* genes during plasma treatment of dairy (a) and meat (b) wastewater effluents containing extracted DNA (extracellular *mecA*) (♦) and MRSA (intracellular *mecA*) (●).

99.999% of MRSA cells. Likewise, >0.53 kJ/cm² of plasma intensity was required for 90% degradation of *mecA* genes. Due to the shielding effects of matrices, the required plasma dose for the elimination of *mecA* genes in food industry wastewater effluents was significantly higher than that in the relatively simple phosphate buffered system. Further investigations are required to explore the impact of various factors on plasma effectiveness for the removal of MRSA and its methicillin resistance gene – *mecA* in various water and wastewater systems.

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