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Efficacy of low concentration neutralised electrolysed water and ultrasound combination for inactivating *Escherichia coli* ATCC 25922, *Pichia pastoris* GS115 and *Aureobasidium pullulans* 2012 on stainless steel coupons

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ABSTRACT

The sanitising effect of low concentration neutralised electrolysed water (LCNEW, pH: 7.0, free available chlorine (FAC): 4 mg/L) combined with ultrasound (37 kHz, 80 W) on food contact surface was evaluated. Stainless steel coupon was chosen as attachment surface for *Escherichia coli* ATCC 25922, *Pichia pastoris* GS115 and *Aureobasidium pullulans* 2012, representing bacteria, yeast and mold, respectively. The results showed that although LCNEW itself could effectively reduce survival population of *E. coli* ATCC 25922, *P. pastoris* GS115 and low concentration *A. pullulans* 2012 in planktonic status, LCNEW combined with ultrasound showed more sanitising efficacy for air-dried cells on coupons, with swift drops: 2.2 and 3.1 log CFU/coupon reductions within 0.2 min for *E. coli* ATCC 25922 and *P. pastoris* GS115, respectively and 1.0 log CFU/coupon reductions within 0.1 min for *A. pullulans* 2012. Air-dried cells after treatment were studied by atomic force microscopy (AFM)/optical microscopy (OM) and protein leakage analyses further. All three strains showed visible cell damage after LCNEW and LCNEW combined with ultrasound treatment and 1.41 and 1.73 μg/mL of protein leakage were observed for *E. coli* ATCC 25922 and *P. pastoris* GS115, respectively after 3 min combination treatment, while 6.22 μg/mL of protein leakage for *A. pullulans* 2012 after 2 min combination treatment. For biofilms, LCNEW combined with ultrasound also significantly reduced the survival cells both on coupons and in suspension for all three strains. The results suggest that LCNEW combined with ultrasound is a promising approach to sanitise food equipment.

1. Introduction

Organic food has developed rapidly in recent years (Li et al., 2015; Yu & Yang, 2017). Due to its strict limitations of using pesticides and chemicals, organic food contains lower chemical contaminants than conventional counterparts. However, it is still susceptible to microbiological contamination due to using organic fertilisers (Maffei, Silveira, & Catanozi, 2013; Zhang & Yang, 2017). Therefore, proper sanitisation before consumption is a critical step to ensure organic food safety.

When many chemical sanitisers are banned or limited to be applied for organic food because of the strict regulations (NOP 5026, 2011; Zhang & Yang, 2017), electrolysed water (EW), which is produced by the electrolysis of a dilute sodium chloride solution, is gaining ever-increasing popularity in food processing due to its environmentally-friendly nature and strong sanitising effect (Hricova, Stephan, & Zweifel, 2008; Rahman, Ding, & Oh, 2010; Yang, Feirtag, & Diez-Gonzalez, 2013). Compared to acidic electrolysed water, neutralised electrolysed water (NEW) is milder and

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safer without significantly affecting foods' nutritional values and quality. Both EW reported showing effective bactericidal activity were usually with high concentration of HClO (Afari, Hung, King, & Hu, 2016; Fang, Cannon, & Hung, 2016; Hao, Li, Wan, & Liu, 2015; Jiménez-Pichardo et al., 2016). However, the National Organic Program (NOP) of US Department of Agriculture (USDA) regulates that free available chlorine (FAC) of organic food sanitisers after processing should not be more than 4 mg/L (NOP 5026, 2011; Zhang & Yang, 2017). Therefore, for organic food, another method needs to be used together with low concentration NEW (LCNEW) to get a desirable sanitising result.

Recently, ultrasound has been applied as a sanitising practice in food processing based on its strong physical and chemical energy on microorganisms, a result of intracellular cavitation (Huang et al., 2006; São José et al., 2014). According to previous reports, combined treatment of ultrasound with other chemical sanitisers could increase the bactericidal effectiveness than each used alone (Aday & Caner, 2014; Sánchez, Elizaquível, Aznar, & Selma, 2015; Zhou, Feng, & Luo, 2009). However, the synergistic effect reported from this combination mostly focused on food itself, few studies had paid much attention to sanitising food contact surface.

As microorganisms can attach to surface in two states: air-dried and biofilms, food product has a risk of microbial contamination from the processing environment. Once microorganisms attach to the surface, they have more resistance to antimicrobial agents than their planktonic counterparts (Abdallah, Benoliel, Drider, Dhulster, & Chihib, 2014; Maifreni et al., 2015; Olszewska, Zhao, & Doyle, 2016). Whereas a number of potential mechanisms of biofilms have been proposed to explain its resistance to sanitisers, studies related to the sanitising effect and distinct mechanism on microorganisms in the air-dried adhesion state are limited (Pan, Breidt, & Kathariou, 2006; Ryu & Beuchat, 2005).

Here we investigated a new sanitising method of using LCNEW (4 mg/L FAC) with or without ultrasound to determine its sanitising effect on *E. coli* ATCC 25922, *P. pastoris* GS115 and *A. pullulans* 2012 in three states: planktonic, air-dried adhesion on stainless steel coupons, and biofilms on stainless steel coupons. For air-dried cells on coupons, the sanitising kinetics, cell morphology imaged by atomic force microscopy (AFM) and optical microscopy (OM) and the intracellular protein leakage were studied further. The objective was to assess the effect of the combined sanitising treatment in a washing operation for decontamination of stainless steel coupons, simulating food processing environment, and to develop a practical and effective sanitising process for inactivating and detaching bacteria on the organic food contact surfaces.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Escherichia coli ATCC 25922 was obtained from the Food Science and Technology Programme, National University of Singapore, originally from ATCC. *Pichia pastoris* GS115 and *Aureobasidium pullulans* 2012 were obtained from College of Food Science and Technology, Nanjing Agricultural University, China, originally isolated from food environment. For *E. coli* ATCC 25922, the stock culture was transferred to 10 mL tryptic soy broth (TSB, Oxoid, Britain) to grow for 24 h at 37 °C. For *P. pastoris* GS115 and *A. pullulans* 2012, the stock culture were transferred to 10 mL malt extract broth (MEB, Oxoid, Britain) and incubated at 30 °C and 25 °C, respectively for 48 h. After 2 consecutive transfers of all three strains, *E. coli* ATCC 25922 was incubated on tryptic soy agar (TSA, Oxoid, Britain) at 37 °C for 24 h, whereas *P. pastoris* GS115 and *A. pullulans* 2012 were incubated on potato dextrose agar (PDA, Oxoid, Britain) for 48 h at 30 °C and 25 °C, respectively. All strains were subcultured again in respective broth for use.

Bacterial density was studied by spectrophotometric analysis and plate count method following a previous lab manual (Reynolds, 2011). All working cultures (ca. 9 log CFU/mL for *E. coli* ATCC 25922, ca. 8 log CFU/mL for *P. pastoris* GS115, ca. 6 log CFU/mL for *A. pullulans* 2012) were separately centrifuged (8200 × *g*, 10 min, 20 °C) (Eppendorf, Centrifuge 5804 R, Germany) and the harvested cells were washed twice in PBS (phosphate buffer saline, pH 7.2) and resuspended for following use.

2.2. Preparation of LCNEW

The electrolysed water was generated by electrolysis device (Hoshizaki, ROX-10WB3, Hoshizaki Singapore Pte Ltd, Singapore) with a continuous supply of dilute salt solution (0.9% NaCl in deionised water). The anode solution was modified by cathode solution until pH neutral (7.0 ± 0.1), which was measured by a pH meter (Thermo Orion pH meter, Waltham, MA, USA). The FAC of 4 mg/L was obtained by dilution with sterilised distilled water and was measured by chlorine test kit (Reflectoquant Chlorine test, Chlor-Test 0.5–10.0 mg/L Cl_2, Darmstadt, Germany). The oxidation reduction potential (ORP) was measured by ORP meter (HM Digital ORP-200, Culver City, CA, USA) immediately after preparation. The ORP of the LCNEW used in this study was 750 ± 15 mV.

2.3. Preparation of the stainless steel coupons

Stainless steel coupons (1 cm in diameter and 0.7 mm in thickness, type: 430, Muzeen and Blythe Co., Winnipeg, Canada) were used as attached surface. They were prepared according to a previous method with some modifications (Kim, Ryu, & Beuchat, 2007). Before each experiment, the coupons were sonicated in 80 °C 15% (v/v) phosphoric acid solution for 20 min and rinsed with distilled water, followed by sonicating in 80 °C 15% (v/v) alkali detergent solution for 20 min and rinsed with distilled water. Finally, the coupons were sonicated in 80 °C distilled water and rinsed. After dried at 50 °C, the coupons were sterilised by autoclaving at 121 °C for 15 min.

2.4. Treatment of planktonic cells with LCNEW

The cells concentration of the inoculums were 9, 8 and 6 log CFU/mL for *E. coli* ATCC 25922, *P. pastoris* GS115 and *A. pullulans* 2012, respectively. This was the concentration of their each stationary phase as prepared in 2.1. Besides, for *A. pullulans* 2012, lower concentration (5 log CFU/mL) was also studied. Each bacterial suspension (3 mL) was mixed thoroughly with 27 mL LCNEW (sterile deionised water (DW) as control) in sterile centrifuge tubes. Suspension (1 mL) was transferred to sterile centrifuge tubes at different treatment time (0–10 min), and then 9 mL neutralising buffer (containing 5 g/L sodium thiosulfate) was added (Deza, Araujo, & Garrido, 2003). After 5 min neutralising, all samples were serially 10-fold diluted in sterile PBS and 0.1 mL were plated on TSA for *E. coli* ATCC 25922 or on PDA for *P. pastoris* GS115 and *A. pullulans* 2012, incubated in the same conditions as mentioned in 2.1. The number of the living microorganisms was determined by viable count method.

2.5. Treatment of air-dried cells on the coupons

2.5.1. Air-dried cells on the coupons

Sterile stainless steel coupons were placed in sterile petri dishes and inoculated with 0.1 mL suspensions of 9 and 8 log CFU/mL, respectively for *E. coli* ATCC 25922 and *P. pastoris* GS115 as mentioned in 2.1, to reach ca. 8 and 7 log CFU/coupon, respectively.

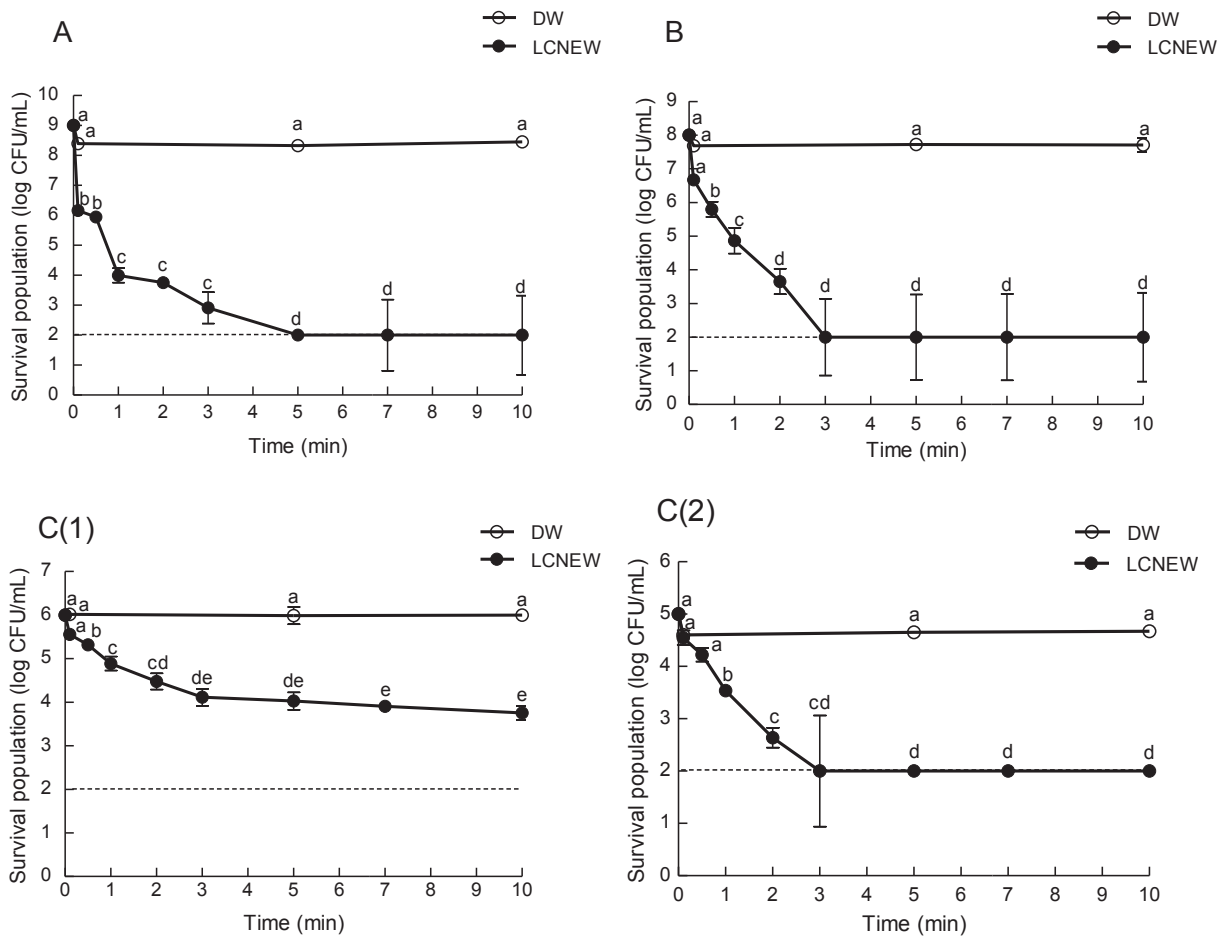

Fig. 1. Sanitising effect of low concentration neutralised electrolysed water (FAC: 4 mg/L) at different time on the planktonic cells. (A) *E. coli* ATCC 25922 with 9 log CFU/mL inoculum; (B) *P. pastoris* GS115 with 8 log CFU/mL inoculum; C(1) *A. pullulans* 2012 with 6 log CFU/mL inoculum; C(2) *A. pullulans* 2012 with 5 log CFU/mL inoculum. The detection limit was 2 log CFU/mL. Within each treatment, means with different letters are significantly different among different time points ($P < 0.05$). DW: deionised water; LCNEW, low concentration neutralised electrolysed water.

However, in this part, the inoculum concentration of *A. pullulans* 2012 was 7 log CFU/mL in order to detect the survival cells, reaching ca. 6 log CFU/coupon. All inoculated coupons were air-dried for 20 h in a laminar flow biosafety cabinet (Kim et al., 2007).

2.5.2. Treatment of air-dried cells

The inoculated coupons were immersed in sterile 15 mL centrifuge tubes (Dimensions: 17.0 mm × 120 mm, D × L) containing 10 mL LCNEW (sterile 10 mL DW as control). The initial direction of coupons in the centrifuge tubes was inoculated side up. For ultrasonic alone and combined method, the tubes were placed in ultrasonic tank (Elmasonic S 30 H, Siegen, Germany) with 37 kHz of ultrasonic frequency and 80 W of ultrasonic power.

After treatment for 5 min, each coupon was transferred into a 10 mL sterile PBS in a sterile centrifuge tube containing 0.2 g sterile glass beads (0.2 mm). The tube containing coupon, PBS and glass beads was vortexed at maximum speed (REAX top vortex shaker-AAR 2516, Heidolph, Germany) for 1 min to detach the adherent cells. After serially diluted, 0.1 mL of each dilution or undiluted samples were surface plated on agar (TSA for *E. coli* ATCC 25922, PDA for *P. pastoris* GS115 and *A. pullulans* 2012), and then cultivated at the given conditions. The survival cell populations on coupons before and after treatment were calculated (Chavant, Gaillard-Martinie, & Hébraud, 2004; Kim et al., 2007). To determine the

Table 1
Survival populations on coupon and in suspension when air-dried cells on coupons treated by DW, LCNEW, ultrasound and LCNEW combined with ultrasound.

Treatment	Survival populations on coupon (log CFU/coupon)			Survival populations in suspension (log CFU/mL)		
	E. coli	*P. pastoris*	*A. pullulans*	*E. coli*	*P. pastoris*	*A. pullulans*
Untreatment	6.87 ± 0.15^{a}	6.31 ± 0.22^{a}	5.04 ± 0.10^{a}	ND	ND	ND
DW	5.75 ± 0.23^{b}	5.42 ± 0.06^{b}	3.99 ± 0.11^{b}	5.55 ± 0.56^{a}	5.87 ± 0.03^{a}	3.44 ± 0.08^{a}
LCNEW*	4.56 ± 0.22^{c}	4.90 ± 0.61^{bc}	3.79 ± 0.21^{b}	ND	ND	ND
Ultrasound#	2.77 ± 0.29^{d}	4.33 ± 0.09^{c}	2.63 ± 0.21^{c}	5.75 ± 0.05^{b}	6.05 ± 0.05^{b}	3.73 ± 0.11^{b}
LCNEW* + ultrasound#	ND	ND	ND	ND	ND	ND

Within each column, means with different letters are significantly different among different treatments ($P < 0.05$). *: The FAC of LCNEW was 4 mg/L #: The frequency of ultrasound was 37 kHz, ultrasonic power effective was 80 W. ND: Not detectable. The survival population on coupon <2 log CFU/coupon. The survival population in suspension <2 log CFU/mL. DW, deionised water; LCNEW, low concentration neutralised electrolysed water.

microorganisms in the suspension after treatment, 1 mL of the suspension and 9 mL of neutralising buffer were added into sterile tubes for 5 min neutralisation. Following the same dilution and plating procedures given in 2.4, survival cells in suspension of three strains after different treatments were enumerated. In further, the kinetics of the LCNEW treatment and LCNEW combined with ultrasound treatment for cells air-dried on coupons was studied by the similar method with more treatment time points.

2.5.3. AFM and OM study

AFM is a powerful tool to study microbiological samples without being metal-coated or stained (Liu & Wang, 2010). After each treatment, the coupons were placed in a laminar flow biosafety cabinet and air-dried. The dried coupons were examined directly by AFM (TT-AFM, AFM workshop, Signal Hill, CA, USA) at ambient air and temperature (25 °C) using a Sensaprobe TM190-A-15 tip (Applied Nanostructures, Mountain View, CA, USA) at force constant: 25–95 N/m; resonance frequency: 145–230 KHz and a Z scanner: 0.2–0.4 Hz (Yang, 2014). For *P. pastoris* GS115 and *A. pullulans* 2012, OM was also used to get a quantification analysis as their sizes are much bigger than *E. coli* ATCC 25922. The inocula on glass slides were prepared by the similar method as described in 2.5.2. The images were analysed offline by using Gwyddion and imageJ software to get both qualitative and quantitative information (Chen et al., 2013; Collins, 2007; Sow & Yang, 2015).

2.5.4. The intracellular protein leakage of combination treatment

After combination treatment and neutralisation, the suspension was centrifuged (14,000*g*, 3 min, 4 °C). Supernatant (1 mL) was collected and added into a 5 mL Coomassie brilliant blue G-250 staining solution. After staining for 5 min, the absorption value at 595 nm was detected, and the leakage of proteins was detected using colorimetric assay by the Bradford method (Bradford, 1976).

2.6. Treatment of biofilms on the coupons

Preparation of biofilms was performed following previous methods with some modifications (Chen, Zhao, & Doyle, 2015; Kim et al., 2007; Ryu & Beuchat, 2005). Sterile stainless steel coupons were placed in sterile petri dishes containing 20 mL of the inoculation solution composed by 2 mL cell inoculums in sterile dH_2O (ca. 9 log CFU/mL for *E. coli* ATCC 25922, ca. 8 log CFU/mL for *P. pastoris* GS115, ca. 6 log CFU/mL for *A. pullulans* 2012) and 18 mL 10-fold diluted media (TSB for *E. coli* ATCC 25922, MEB for *P. pastoris* GS115 and *A. pullulans* 2012). Petri dishes were incubated at 4 °C for 24 h to facilitate attachment of cells, followed by 25 °C for 48 h. Then the coupons, after gently rinsed in a circular motion in 400 mL of sterile PBS for 15 s to remove the cells not firmly attached, were placed in sterile petri dishes containing 20 mL 10-fold diluted fresh media and incubated at 25 °C for 12 d to allow the formation of the biofilms. After the biofilms formation, coupons were washed in PBS

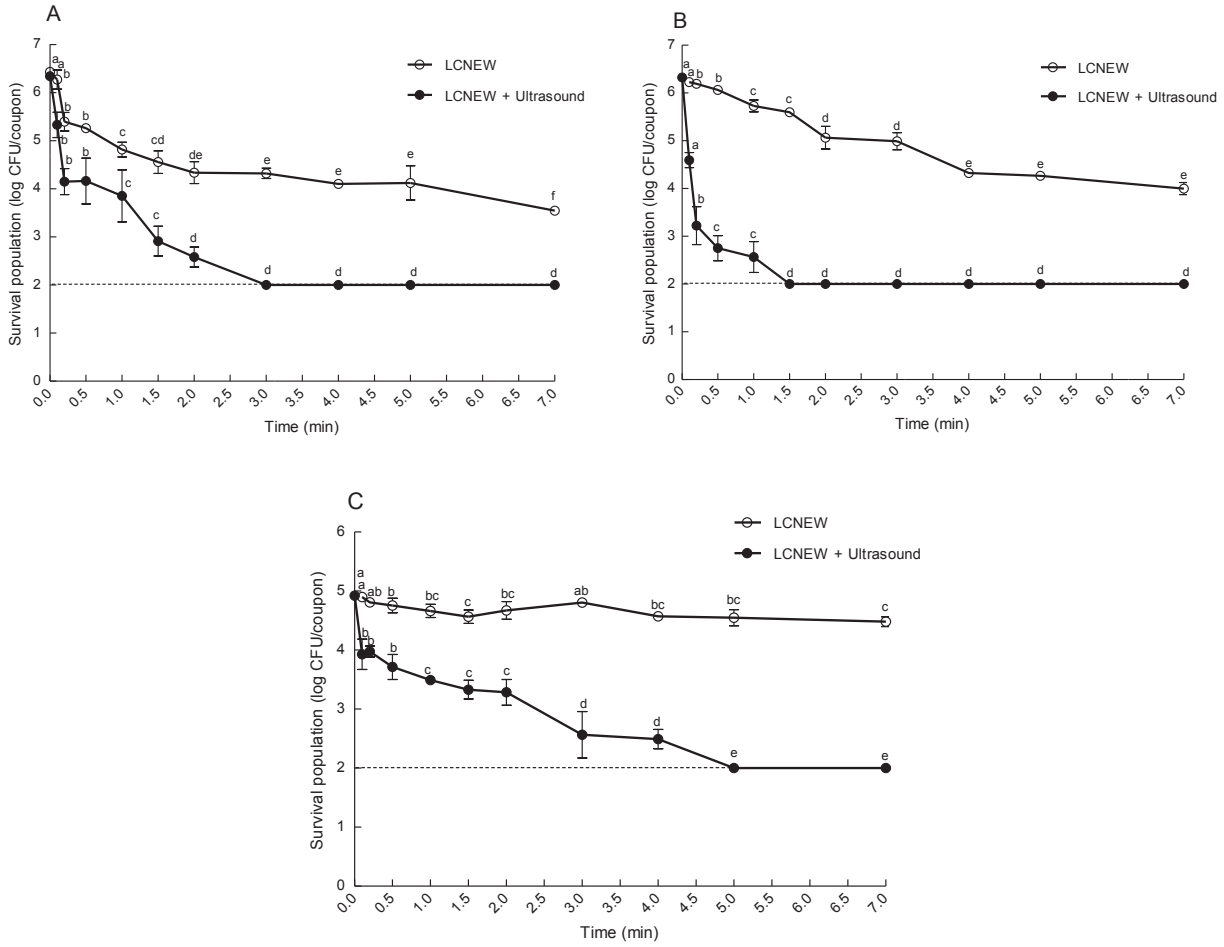

Fig. 2. Sanitising kinetics for bactericidal effect of LCNEW (FAC: 4 mg/L) and LCNEW combined with ultrasound (frequency: 37 kHz, ultrasonic power effective: 80 W) for air-dried cells on coupons (A) *E. coli* ATCC 25922 (6.34 log CFU/coupon); (B) *P. pastoris* GS115 (6.32 log CFU/coupon); (C) *A. pullulans* 2012 (4.92 log CFU/coupon). Within each series, means with different letters are significantly different among different time points ($P < 0.05$). The detection limit was 2 log CFU/coupon. LCNEW, low concentration neutralised electrolysed water.

Table 2
Survival populations in suspension when the air-dried cells on coupons were treated by LCNEW and LCNEW combined with ultrasound.

Treatment time (min)	*E.coli* ATCC 25922 (log CFU/mL)		*P. pastoris* GS115 (log CFU/mL)		*A. pullulans* 2012 (log CFU/mL)	
	LCNEW*	LCNEW*+Ultrasound#	LCNEW*	LCNEW*+Ultrasound#	LCNEW*	LCNEW*+Ultrasound#
0.1	4.79 ± 0.22^{aA}	4.61 ± 0.57^{aA}	4.26 ± 0.22^{abA}	4.69 ± 0.13^{aB}	ND	4.52 ± 0.11^{a}
0.2	5.26 ± 0.26^{aA}	4.06 ± 0.12^{bB}	4.65 ± 0.36^{aA}	4.22 ± 0.18^{bA}	ND	4.42 ± 0.08^{a}
0.5	4.23 ± 0.19^{bA}	3.73 ± 0.19^{bB}	3.99 ± 0.24^{abA}	3.18 ± 0.07^{cB}	ND	4.28 ± 0.10^{a}
1.0	3.52 ± 0.56^{bcA}	3.18 ± 0.24^{cA}	3.70 ± 0.83^{bcA}	2.39 ± 0.06^{dB}	ND	3.83 ± 0.41^{b}
1.5	2.90 ± 0.18^{cA}	2.53 ± 0.08^{dB}	3.00 ± 0.31^{cA}	1.84 ± 0.25^{eB}	ND	4.26 ± 0.12^{a}
2.0	2.67 ± 0.21^{dA}	2.10 ± 0.02^{dB}	2.97 ± 0.18^{cd}	ND	ND	3.63 ± 0.33^{bc}
3.0	ND	2.11 ± 0.44^{d}	2.28 ± 0.42^{e}	ND	ND	3.47 ± 0.25^{c}
4.0	2.47 ± 0.09^{dA}	1.48 ± 0.99^{eA}	2.55 ± 0.67^{de}	ND	ND	2.82 ± 0.57^{d}
5.0	ND	ND	1.87 ± 0.97^{e}	ND	ND	ND
7.0	ND	ND	ND	ND	ND	ND

Within each column, means with different lowercase letters are significantly different among different time points ($P < 0.05$). Within each row, means with different capital letters are significantly different among different treatments ($P < 0.05$). *: The FAC of LCNEW was 4 mg/L #: The frequency of ultrasound was 37 kHz; ultrasonic power effective was 80 W. ND: Not detectable. The survival population in suspension <2 log CFU/mL. LCNEW, low concentration neutralised electrolysed water.

again to remove loosely attached cells. The washed coupons were treated by different methods as mentioned in 2.5.2. After treatment, survival cells in each of the suspension and on the coupon were enumerated.

2.7. Statistical analysis

All experiments were performed in triplicate. In experiments evolving stainless steel coupons, three coupons were examined in each run. The results were expressed as mean ± standard deviation. ANOVA ($P < 0.05$) and Duncan's multiple-range test were used to determine the difference among different treatment groups, conducted by SAS software (version 9.2, Cary, NC, USA). For AFM and OM, a number of parallel images were analysed to gain representative and statistically valid results.

3. Results and discussion

3.1. Effect of LCNEW on planktonic cells

To determine the sanitising effect of LCNEW first, planktonic cells were used as experimental subject. Fig. 1 shows the sanitising effect of LCNEW at different time on the planktonic cells. As shown in Fig. 1A, LCNEW reduced about 2 log CFU/mL for *E. coli* ATCC 25922 within 0.1 min and its survival population was below detection limit after 5 min treatment. Moreover, the time for LCNEW to reduce *P. pastoris* GS115 below detection limit was as short as 3 min (Fig. 1B). However, as shown in Fig. 1C(1) and C(2), LCNEW failed to completely sanitise *A. pullulans* 2012 with high initial inoculum concentration (6 log CFU/mL) even within 10 min.

Abadias, Usall, Oliveira, Alegre, and Viñas (2008) reported that NEW containing 50 mg/L FAC resulted in >5 log CFU/mL reduction of four foodborne pathogens after 1 min treatment. Near neutral electrolysed water (pH = 5.8) with 21 mg/L FAC achieved > 5 log CFU/mL reductions for *E. coli* and *Staphylococcus aureus* after 90 s (Issa-Zacharia, Kamitani, Morita, & Iwasaki, 2010). In addition, NEW showed quicker bactericidal rate than AEW with the same FAC for *Aspergillus flavus*, which might be due to the OH• radical, an important bactericidal factor existed in higher content in NEW than AEW (Xiong, Liu, Liu, & Li, 2010).

The agents responsible for bactericidal effect in NEW can be attributed to its ORP, pH and the concentration of chlorine related substances, including Cl_2, HClO, and ClO^- (Hati et al., 2012; Park, Guo, Rahman, Ahn, & Oh, 2009; Rahman, Khan, & Oh, 2016). LCNEW was an effective method to control microorganism in pure cultures, and it had a broad spectrum antibacterial effect. More importantly, it was accessible for strict organic food sanitisation. However, attached cells would be likely to show more resistance to sanitisers. Therefore, the bactericidal effect of LCNEW and ultrasound was further investigated for the two attached cells, in air-dried attached and biofilm, respectively.

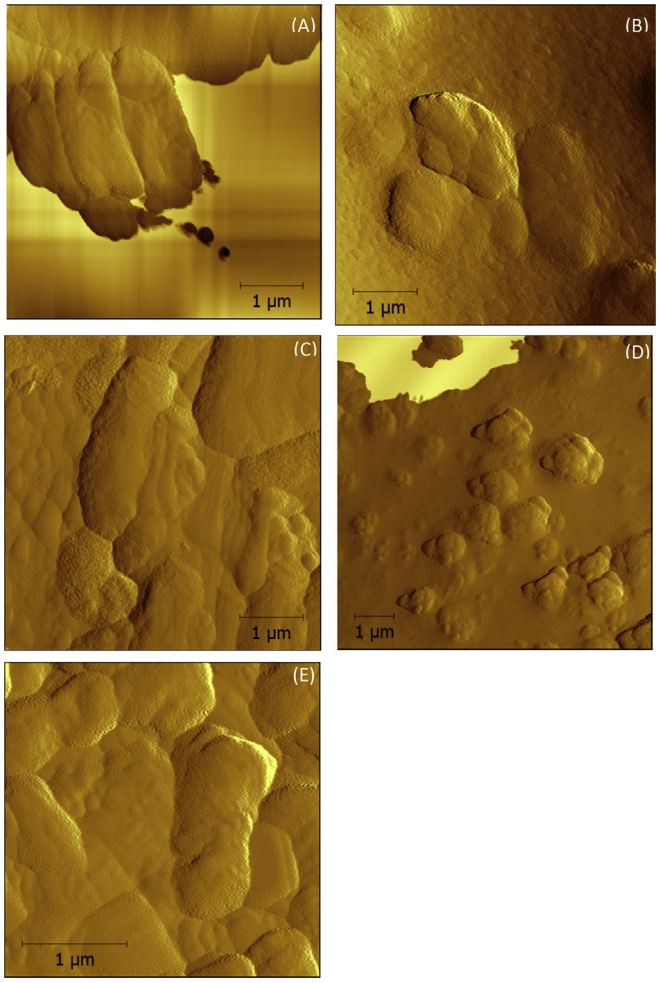

Fig. 3. AFM images for *E. coli* ATCC 25922 cells dried on coupons after different treatments. (A) Untreatment; (B) DW; (C) Ultrasound; (D) LCNEW; (E) LCNEW + Ultrasound. DW: deionised water; LCNEW, low concentration neutralised electrolysed water.

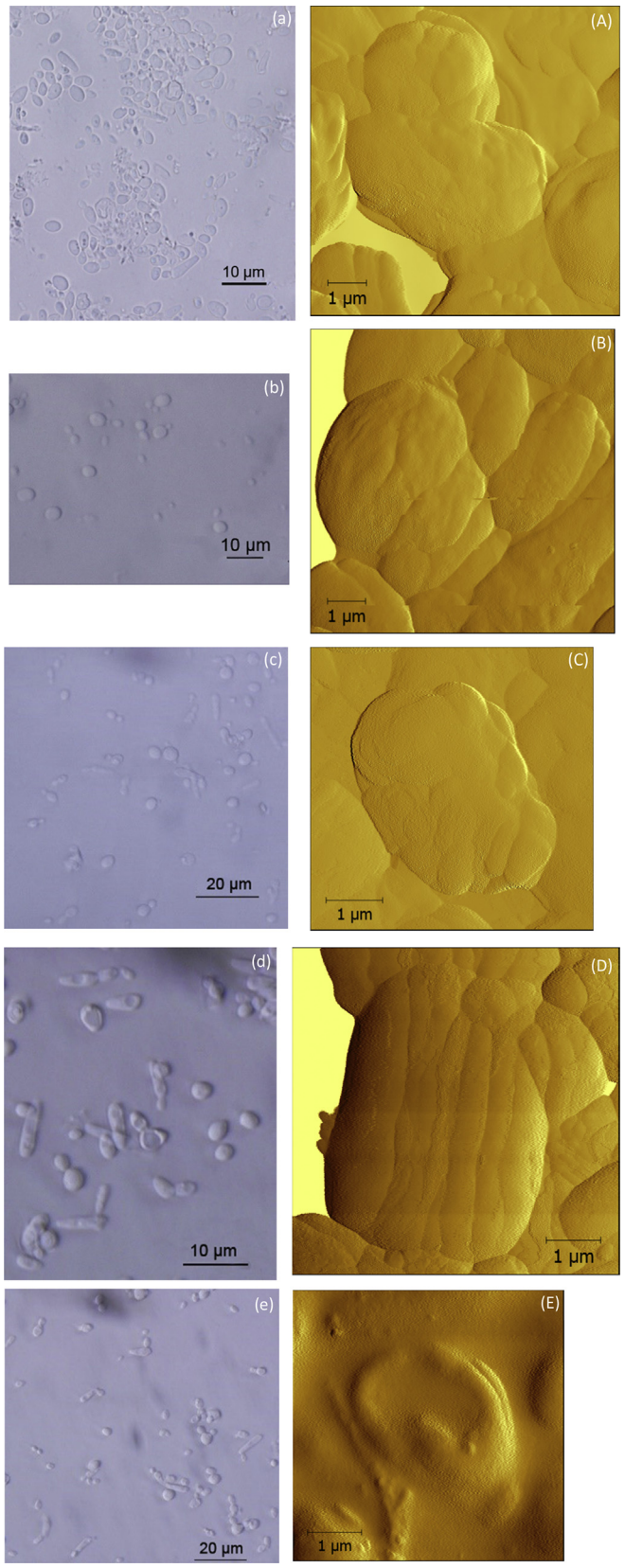

Fig. 4. OM images (first column) and AFM images (second column) for *P. pastoris* GS115 cells dried on coupons after different treatments. (Aa) Untreatment; (Bb) DW; (Cc) Ultrasound; (Dd) LCNEW; (Ee) LCNEW + Ultrasound. DW, deionised water; LCNEW, low concentration neutralised electrolysed water.

3.2. Treatment of air-dried cells on coupons

After 20 h drying, the number of each strain on coupons was decreased compared to their initial inocula concentrations (*E. coli* ATCC 25922: 6.87 log CFU/coupon; *P. pastoris* GS115: 6.31 log CFU/coupon; *A. pullulans* 2012: 5.04 log CFU/coupon). For the air-dried cells on coupons, DW reduced 1.12, 0.89 and 1.05 log CFU/coupon for *E. coli* ATCC 25922, *P. pastoris* GS115 and *A. pullulans* 2012, respectively (Table 1). However, the attached cells could not be readily accessible to LCNEW. For *E. coli* ATCC 25922, only another 1.2 log CFU/coupon reduction was achieved by LCNEW compared to DW, and it did not show significant effect for *P. pastoris* GS115 and *A. pullulans* 2012. Therefore, the air-dried attached cells were less susceptible to LCNEW than planktonic cells. The results were in agreement with previous reports that microorganisms in attached state were resistant to various sanitisers (Kim et al., 2007; Peta, Lindsay, Broezel, & von Holy, 2003). When NEW (50 mg/L FAC) was applied to microorganisms inoculated on vegetable, the reduction was much less than that in pure cultures (Abadias et al., 2008). The survival population of cells in suspension was also detected (Table 1). There were no live cells detected in suspension for LCNEW treatment combined with ultrasound or not, indicating the strong bactericidal effect of LCNEW on planktonic cells. The results were in good accordance with the effect shown in 3.1.

It was notable that ultrasound treatment reduced the cells on coupons more than 1 log CFU/coupon ($P < 0.01$) compared to DW group, and the value was as high as 2.98 log CFU/coupon for *E. coli* ATCC 25922 (Table 1). However, the population in suspension was about 0.2 log CFU/mL (equivalent to 2 log CFU/coupon) increased, which meant the total survival cells both on coupons and in suspensions had no significant difference between DW and ultrasound treatment, indicating that the ultrasound itself had little microbiocidal efficacy. However, when combining LCNEW, ultrasound could make HClO more easily to penetrate the cells after detaching them from coupons. In addition, the ultrasound could make better dispersion of HClO in the aqueous media, which was probably caused by localised heating and pressure (Piyasena, Mohareb, & McKellar, 2003). In another report, when the ultrasound was followed by NEW (5–10 mg/L FAC), the microorganism reduction on the lettuce increased from 2.32 to 3.18 log CFU/g compared to NEW individually (Forghani et al., 2013). In this current research, when the combination method was applied for sanitising stainless steel coupons, it not only shortened the bactericidal reaction time but also improved the sanitising effect.

3.3. The kinetics of the treatment for air-dried cells on coupons

In part 3.2, the sanitising effect only focused on one time point (5 min). There could be more information behind the sanitising process. Therefore, it was necessary to further study the kinetics of LCNEW treatment and LCNEW combined with ultrasound treatment for air-dried cells on coupons.

Fig. 2 shows the kinetics of LCNEW and LCNEW combined with ultrasound for air-dried cells on coupons. The value of *E. coli* ATCC 25922 reduced by 2 log CFU/coupon in 2 min under LCNEW treatment and by 2.2 log CFU/coupon in 0.2 min under combination treatment (Fig. 2A). For *P. pastoris* GS115 (Fig. 2B), the population reduced from initial 6.32 to 4.00 log CFU/coupon under LCNEW treatment. When ultrasound was combined, the time was as short as 1.5 min to reduce all cells on coupon below detection limit. However, as shown in Fig. 2C, LCNEW showed limited sanitising effect on *A. pullulans* 2012 dried on coupons, which could be due to the stronger acting force between cells and coupons and the cells' own strong resistance to LCNEW. However, in combination group, 1 log CFU/coupon reduction was reached within the first 0.1 min and

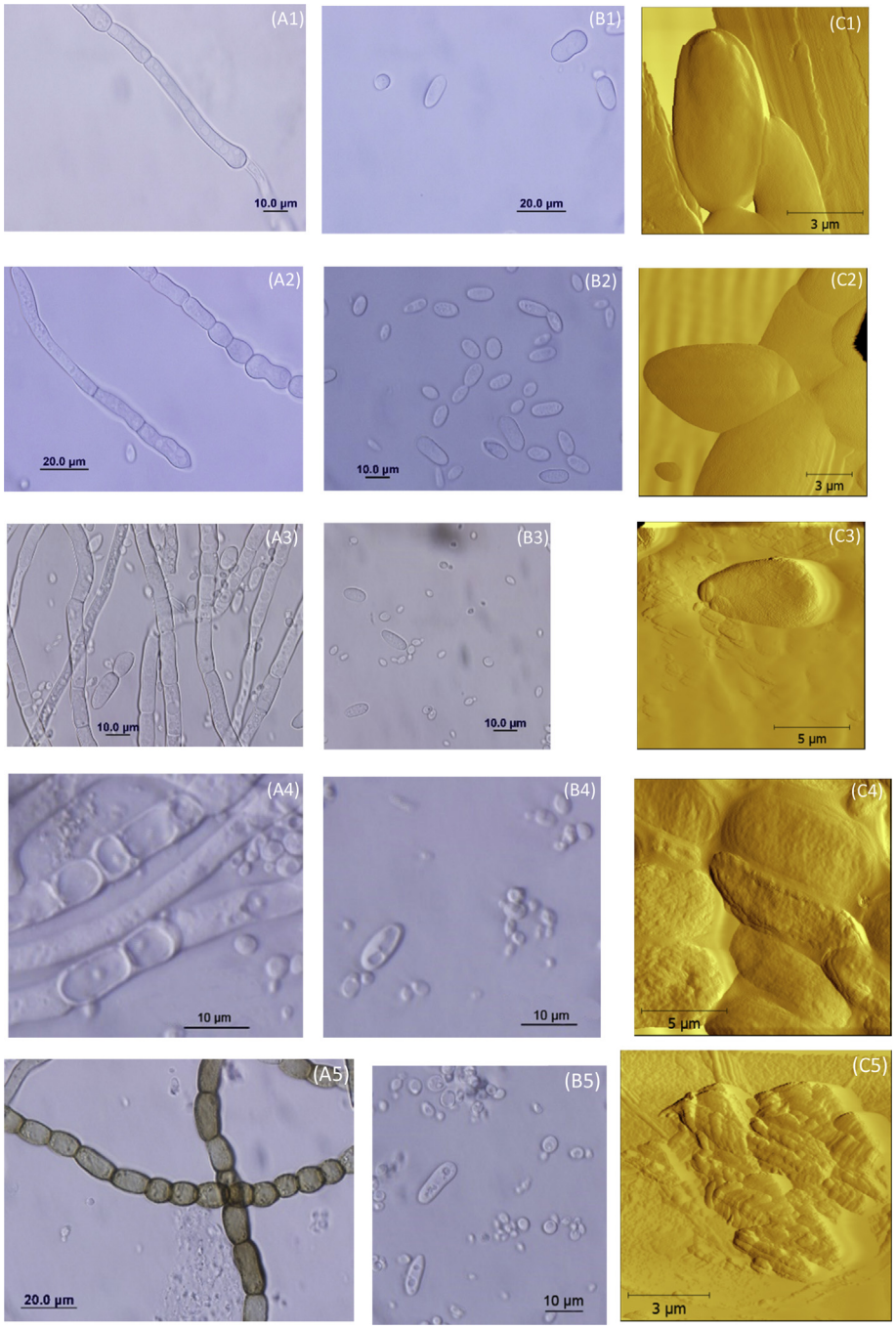

Fig. 5. OM images (first and second column) and AFM images (third column) for *A. pullulans* 2012 cells dried on coupons after different treatments. (A) Hyphae; (BC) Conidia; (A1B1C1) Untreatment; (A2B2C2) DW; (A3B3C3) Ultrasound; (A4B4C4) LCNEW; (A5B5C5) LCNEW + Ultrasound. DW, deionised water; LCNEW, low concentration neutralised electrolysed water.

at 5 min, its survival population was below detection limit.

The combination method showed much stronger sanitising effect for air-dried cells on coupons. However, if the cells were just transferred from the coupons to the solution, it could not completely avoid the microbial contamination on the food product. Therefore, it was necessary to further investigate the survival cells in suspension. Table 2 shows the survival populations in suspension when the air-dried cells on coupons were treated by LCNEW and LCNEW combined with ultrasound.

In Table 2, for *E. coli* ATCC 25922 and *P. pastoris* GS115, there were still some cells transferred to suspension spontaneously in LCNEW group, which could be caused by the loose interaction between the cells and the coupons. The interaction was likely to depend on the structure of the cell walls and the secretion of extracellular polymeric substance (Bang et al., 2014; Ryu & Beuchat, 2005). It should be more cells detached by ultrasound into suspension, but here we found less survival cells in suspension compared to LCNEW alone. The reason could be that when more cells were detached by ultrasound, more cells were killed by LCNEW in the suspension. Moreover, there were no survival cells detected for *A. pullulans* 2012 for the whole LCNEW treatment in the suspension, which was associated with that the cells on

Table 3
Effect of different treatments on the dimension of three strain microorganisms.

Treatment	*E.coli* ATCC 25922			*P. pastoris* GS115			*A. pullulans* 2012				
							Hyaline conidia			Hyaline vegetative hyphae	Dark brown hyphae
	Length (um)	Width (um)	Area (um2)	Length (um)	Width (um)	Area (um2)	Length (um)	Width (um)	Area (um2)	Width (um)	Width (um)
Untreatment	2.71 ± 0.23^{a}	1.15 ± 0.08^{b}	2.86 ± 0.69^{a}	4.55 ± 1.20^{ab}	2.39 ± 0.50^{d}	10.22 ± 3.17^{c}	13.95 ± 3.34^{a}	5.79 ± 1.29^{a}	70.17 ± 25.30^{a}	6.30 ± 2.40^{a}	NA
DW	2.63 ± 0.39^{a}	1.15 ± 0.14^{b}	2.71 ± 0.46^{a}	4.77 ± 0.73^{a}	3.31 ± 0.58^{a}	13.73 ± 2.85^{a}	10.19 ± 1.30^{c}	4.90 ± 0.65^{b}	53.96 ± 18.69^{b}	6.11 ± 1.58^{a}	NA
Ultrasound$^{\#}$	2.32 ± 0.42^{b}	1.01 ± 0.23^{c}	2.13 ± 0.57^{b}	4.47 ± 0.47^{ab}	3.02 ± 0.50^{b}	12.28 ± 2.22^{b}	11.87 ± 1.75^{b}	4.53 ± 0.50^{cd}	47.79 ± 10.59^{bc}	5.74 ± 1.03^{ab}	NA
LCNEW*	1.77 ± 0.29^{c}	1.28 ± 0.23^{a}	1.88 ± 0.48^{b}	4.43 ± 0.65^{b}	3.05 ± 0.49^{b}	11.55 ± 3.42^{b}	10.63 ± 1.96^{c}	4.35 ± 0.66^{d}	43.76 ± 14.22^{c}	5.34 ± 1.10^{b}	NA
LCNEW* + Ultrasound$^{\#}$	NA	NA	NA	4.48 ± 0.58^{ab}	2.79 ± 0.34^{c}	12.21 ± 2.95^{b}	12.22 ± 2.35^{b}	4.72 ± 1.08^{bc}	47.30 ± 14.93^{c}	5.44 ± 1.49^{b}	8.38 ± 2.92

Within each column, means with different letters are significantly different among different treatments ($P < 0.05$). *: The FAC of LCNEW was 4 mg/L #: The frequency of ultrasound was 37 kHz, ultrasonic power effective was 80 W. NA: Not available to get enough statistical samples. DW, deionised water; LCNEW, low concentration neutralised electrolysed water.

coupons were scarcely affected by LCNEW (Fig. 2C). However, in the combined treatment, the cells could be detached into suspension effectively by ultrasound, and then the LCNEW could attack more cells, resulting in more sanitising effect both for cells on coupon and in suspension.

3.4. AFM and OM analyses

To further understand the disinfection mechanism of changed populations by LCNEW and LCNEW combined with ultrasound, three microorganisms' nanostructural analyses were conducted using AFM, and for *P. pastoris* GS115 and *A. pullulans* 2012, OM was further applied to get quantification analysis (Figs. 3–5).

When the cells were treated by DW and ultrasound for 5 min, there were no visible damage for *E. coli* ATCC 25922 and *P. pastoris* GS115 cells, and the images respectively showed the typical rod-shaped and yeast-like elliptical morphology with smooth surfaces (Fig. 3A–C; Fig. 4Aa, 4Bb, 4Cc). However, after sanitised by LCNEW with or without ultrasound for 5 min, there was obvious damage on the cells. The cellular structure became disordered, and the surface became irregular and experienced some wrinkling, with breaches in the wall. The small substances would be the leakage of intracellular contents of the cells caused by the change of cellular membrane permeability and destruction of microbiological protective barriers (Fig. 3D and E; Fig. 4Dd, Ee).

In Zeng's report, transmission electronic microscopy (TEM) was used to investigate the effects of electrolysed water with 12.4 mg/L FAC on bacterial ultrastructure. After treated for 5 min, the cell wall was seriously damaged, leading to leakage of cytoplasm (Zeng et al., 2010). The changes to cell envelope and cytoplasm caused by NEW were also observed in another study (Feliciano, Lee, & Pascall, 2012). These reports supported our current results.

For *A. pullulans* 2012, conidia and hyphae are two major morphologies. Although in the above section, LCNEW showed no significant effect on *A. pullulans* 2012 populations on coupons, it could make some damage to its cell morphology. When treated by DW or ultrasound for 5 min, there were no obvious morphology changes for both conidia and hyphae compared to the untreatment group (Fig. 5A1, B1, C1, A2, B2, C2, A3, B3, C3). However, in the last two treatment groups, the hyphae membrane was destroyed with some notches and thus change colour to dark brown (Fig. 5A4, A5). The conidia structure became disordered, and the surface became wrinkled with breaches (Fig. 5B4, C4, B5, C5). According to a previous report, the antifungal effect made by chloride sanitisers included preventing conidia germination and slowing hyphal elongation, which was similar to the results here (Basaran, 2011).

Dimensions of three strains after corresponding treatments were quantified (Table 3). For each strain and each condition, the replica number was 90–110 with a purpose of making the statistical result representative. For *E. coli* ATCC 25922, after treated by LCNEW, its morphology became more spherical and the cell bodies became smaller, which might be due to the serious leakage of cytoplasm and aggregation of the cytoplasmic components (Tetsuaki, Tomoko, & Takashi, 2006). Although ultrasound alone could not effectively reduce the bacterial population, it changed cell size at non-lethal level by cavitation. This explanation could also be used in the ultrasound group of latter two strains. As for *P. pastoris* GS115, the reason why DW made its size larger might be due to OM examination method (see 2.5.3). As the examination process was undertaken in a liquid form, not like AFM in the dry state, *P. pastoris* GS115 might absorb water during the examination period, making its size larger. Since DW is the yardstick by which we measure the effect of LCNEW, the last two treatment methods

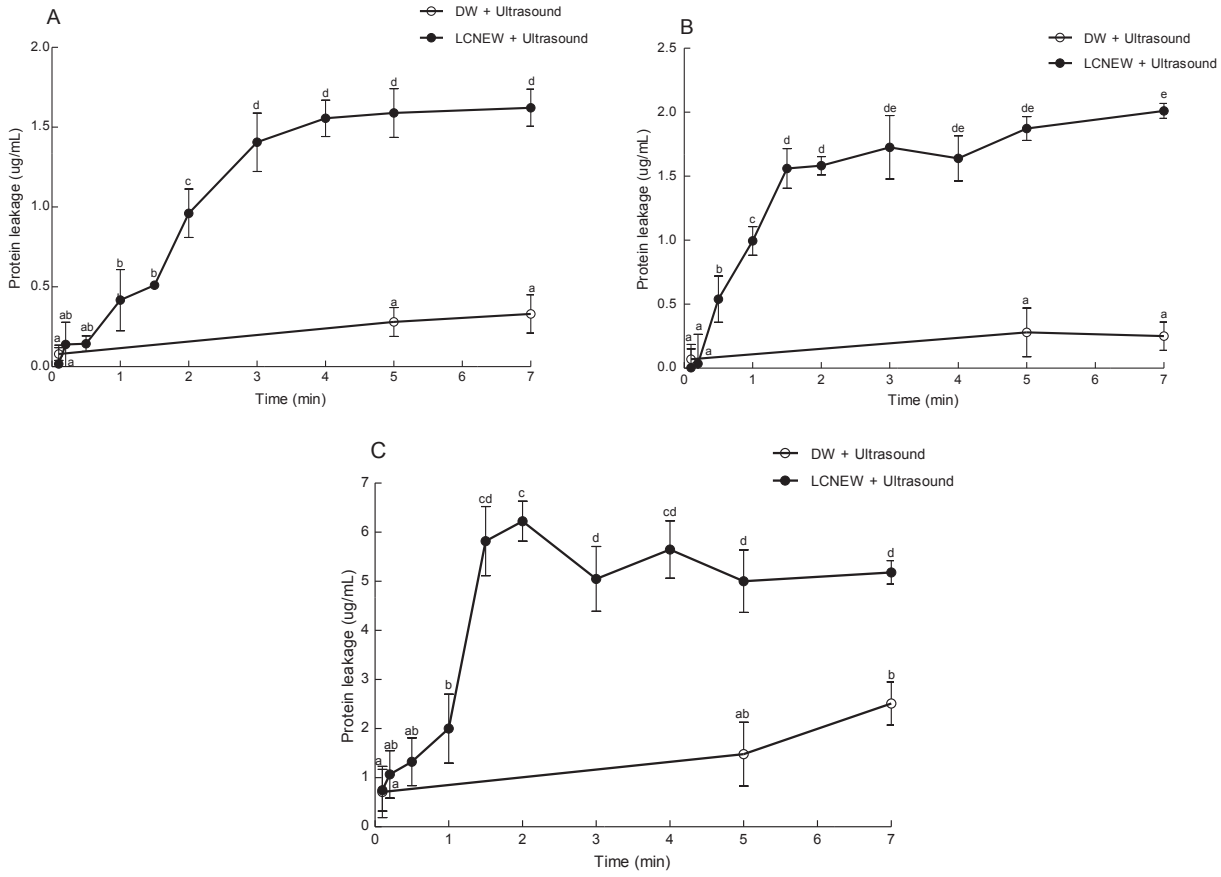

Fig. 6. Protein leakage in suspension after air-dried coupons treated by ultrasound (frequency: 37 kHz, ultrasonic power effective: 80 W) and LCNEW (FAC: 4 mg/L) combined with ultrasound. (A) *E. coli* ATCC 25922 (theoretically 8 log CFU/coupon); (B) *P. pastoris* GS115 (theoretically 7 log CFU/coupon); (C) *A. pullulans* 2012 (theoretically 6 log CFU/coupon). Within each series, means with different letters are significantly different among different time points ($P < 0.05$). DW: deionised water; LCNEW, low concentration neutralised electrolysed water.

did make some differences compared to DW group, resulting from damaged cell membrane and leakage of cytoplasm. As for *A. pullulans* 2012, the conidia were more sensitive to each treatment than the hyphae, which was supported by a previous report (Basaran, 2011). Though no significant change of the hyphae sizes among different groups, the change of conidia sizes really existed, especially under LCNEW treatment. Interestingly, the conidia became smaller under DW treatment, which might be caused by changes of osmotic pressures of environment during OM examination.

3.5. The intracellular protein leakage of combination treatment

The protein leakage could be used to investigate the damage of the cell membranes caused by the sanitising (Tang et al., 2011). As shown in Fig. 6, for *E. coli* ATCC 25922 and *P. pastoris* GS115, the protein concentrations in suspension increased to 1.41 and 1.73 μg/mL, respectively after 3 min, and then stayed relatively stable (Fig. 6A, B). However, for *A. pullulans* 2012, its protein leakage rapidly increased to 6.22 μg/mL after 2 min treatment but dropped to 5.18 μg/mL at 7 min (Fig. 6C). The slight protein decrease could be attributed to two reasons. One could be protein denaturalisation after long-time treatment. The other could be further reactions with the reactive oxygen or nascent oxygen produced from hypochlorite in LCNEW (Zeng et al., 2010). The absence of the decrease stage for the former two strains could be due to the lower protein concentration in suspension. For the ultrasound group, the protein leakage was much less. Therefore, the main protein leakage in the

Table 4
Survival populations on coupon and in suspension when biofilms on coupons treated by DW, LCNEW, ultrasound and LCNEW combined with ultrasound.

Treatment	Survival populations on coupon (log CFU/coupon)			Survival populations in suspension (log CFU/mL)		
	E. coli	*P. pastoris*	*A. pullulans*	*E. coli*	*P. pastoris*	*A. pullulans*
Untreatment	7.21 ± 0.12^{a}	6.04 ± 0.25^{a}	4.83 ± 0.31^{a}	ND	ND	ND
DW	6.83 ± 0.25^{b}	5.42 ± 0.28^{b}	3.84 ± 0.06^{b}	5.24 ± 0.56^{a}	3.85 ± 0.42^{a}	2.78 ± 0.11^{a}
LCNEW*	4.65 ± 0.35^{c}	4.46 ± 0.37^{c}	2.50 ± 0.05^{c}	ND	ND	ND
Ultrasound#	4.89 ± 0.04^{c}	3.70 ± 0.08^{d}	3.03 ± 0.31^{c}	6.18 ± 0.08^{b}	4.92 ± 0.12^{b}	3.65 ± 0.48^{b}
LCNEW* + ultrasound#	ND	ND	ND	ND	ND	ND

Within each column, means with different letters are significantly different among different treatments ($P < 0.05$). *: The FAC of LCNEW was 4 mg/L #: The frequency of ultrasound was 37 kHz, ultrasonic power effective was 80 W. ND: Not detectable. The survival population on coupon <2 log CFU/coupon. The survival population in suspension <2 log CFU/mL. DW, deionised water; LCNEW, low concentration neutralised electrolysed water.

combination group might be caused by the attack of the active substances (such as Cl_2, HClO, ClO^-) in LCNEW on microorganism membranes and walls.

3.6. Biofilms on coupons

After the biofilms formation, the cells population was 7.21, 6.04 and 4.83 log CFU/coupon for *E. coli* ATCC 25922, *P. pastoris* GS115 and *A. pullulans* 2012, respectively. The sanitising efficacy for biofilms is shown in Table 4. For DW, the cells on coupon decreased by 0.38, 0.62, 0.99 log CFU/coupon for *E. coli* ATCC 25922, *P. pastoris* GS115, *A. pullulans* 2012 ($P < 0.05$) respectively, which were less than the results (1.12, 0.89, 1.05 log CFU/coupon reductions, respectively) for cells air-dried on coupon (Table 1), indicating that the interaction between cells and coupons in biofilms was much stronger than that in air-dried adhesion. More sanitising effects (0.96–2.18 log CFU/coupon reductions) were found by LCNEW compared to DW control ($P < 0.05$). For combined method, the results were in accordance with the air-dried cells. Ultrasound detached the cells and made better dispersion of HClO. The survival population of cells in suspension is also shown in Table 4. The ultrasound treatment reduced the cell population on coupons but increased it in suspension, which verified again it did not have much sanitising effect but detached the cells. For LCNEW and LCNEW combined with ultrasound groups, there were no detectable survival cells. The results were in good accordance with the treatment on planktonic cells and air-dried cells (Fig. 1 and Table 1). In general, the LCNEW combined with ultrasound effectively sanitised the biofilms on coupons for all three microorganisms.

4. Conclusion

Low concentration neutralised electrolysed water (4 mg/L FAC) showed microbiocidal efficacy for planktonic cells in a broad-spectrum range (*E. coli* ATCC 25922, *P. pastoris* GS115 and low concentration *A. pullulans* 2012). For air-dried cells and biofilms attached on coupons, LCNEW combined with ultrasound showed the best sanitising efficacy, with a significant reduction of survival cells both on coupons and in suspension within 5 min. Through AFM/OM and protein leakage studies, the morphologies of three air-dried strains showed visible change under LCNEW combined with ultrasound treatment. Therefore, this combination method showed potential to remove microbial contamination on the surfaces of food processing equipment, and other related substrates. LCNEW in combination with ultrasound could be developed as a practical and effective short-time food processing intervention for food industry.

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