International Journal of Food Microbiology 295 (2019) 41–48

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Sanitising efficacy of lactic acid combined with low-concentration sodium hypochlorite on *Listeria innocua* in organic broccoli sprouts

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ARTICLE INFO

Keywords:
Sanitisation
Organic acid
Pathogen
Organic produce
Atomic force microscopy
Modelling

ABSTRACT

The sanitising effects of combined lactic acid (LA, 2%, v/v) and low-concentration sodium hypochlorite (SH, 4 mg/L) on *Listeria innocua* were investigated. The disinfectant kinetics and percentage of injured cells showed that the combined treatment resulted in dramatic inactivation of *L. innocua* after 60 s. The Weibull model with a high R^2 (1.00) and low root mean squared error (RMSE) (0.19) and Akaike Information Criterion (AIC) (−7.58) was verified as the most superior model to describe effects of the combination treatment. Protein leakage and the fluorescence intensity ratio revealed that LA might disrupt the cytoplasmic membrane rapidly and that SH could synergistically promote the sanitising effect. Atomic force microscopy (AFM) images showed notable morphological changes in the combination treated cells, which presented lower width (0.73 μm) and height (0.29 μm), and increased roughness (10.37 nm). Moreover, massive leakage of intracellular components indicated severe cell collapse. The antimicrobial effects on organic broccoli sprouts showed that the combined treatment resulted in reductions of 0.82, 1.51, and 1.77 log colony forming units (CFU)/g fresh weight (FW) for aerobic bacteria, yeasts and moulds, and inoculated *L. innocua* at day 0. In addition, there were no significant differences of sensory quality results between treatment and control groups and they were all greater than the acceptance limit. Thus, the disinfection treatments had no negative effects on the storage quality of the organic broccoli sprouts. The combined treatment could be a potential sanitising strategy for organic sprouts.

1. Introduction

The consumption of organic sprouting vegetables has increased dramatically in the last decades because of rising health and safety demands of consumers (Chen et al., 2019; Kim et al., 2016). Organic sprouting vegetables have attracted public attention because of their convenience and high nutritional value (Chen et al., 2018a). However, foodborne pathogen infection caused by contaminated sprouting vegetables is a major safety problem of fresh organic sprout consumption worldwide (Dechet et al., 2014; Olaimat and Holley, 2012). Thus, further sanitisation techniques for postharvest organic sprouting vegetables should be applied to enhance their safety.

Washing sanitisers have been widely applied to inactivate the pathogens in fruit and vegetables because of their effectiveness and low cost (Rahman et al., 2011). For example, 50–200 mg/L chlorine solutions (sodium hypochlorite) are used commercially to disinfect the fresh-cut produce (Adhikari et al., 2015; Pangloli and Hung, 2013). However, the National Organic Program (NOP) clarified that the residual chlorine levels in water contacted with organic produce must not exceed the maximum residual disinfection limit of the Safe Drinking Water Act, which is 4 mg/L (NOP, 2011). Therefore, an alternative sanitising strategy that meets the processing regulations of fresh organic sprouts should be developed to reduce microbial contamination.

Lactic acid is Generally Recognised as Safe (GRAS) in the food industry (Goñi et al., 2017), and its use in processing of organic fruits and vegetables was approved by the NOP (2011). In addition, previous reports confirmed the sanitising effect of lactic acid in contaminated alfalfa seeds, red chicory, cheese, meat, and poultry products (Lang et al., 2000; Mani-Lopez et al., 2012; Trevisani et al., 2017; Wemmenhove et al., 2018). Therefore, lactic acid could be a potential sanitising strategy for postharvest organic sprouting produce. *Listeria monocytogenes*, a pathogenic foodborne bacterium, is responsible for severe listeriosis (Liu et al., 2018). *L. monocytogenes* control in food is difficult because of its high adaptive ability and diverse food vehicles, including sprouting produce. *L. innocua* is an innocuous subspecies of *Listeria* that has high morphological, serological, and biochemical similarities to *L.*

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https://doi.org/10.1016/j.ijfoodmicro.2019.02.014
Received 13 July 2018; Received in revised form 15 February 2019; Accepted 18 February 2019
Available online 19 February 2019
0168-1605/

monocytogenes, making it an ideal surrogate for food safety research (Friedly et al., 2008).

In the present study, the sanitising effects on *L. innocua* by lactic acid (LA), sodium hypochlorite (SH; free available chlorine (FAC) 4 mg/L) and their combined treatment were investigated. The *in vitro* disinfection kinetics, model fitting test, protein leakage, intracellular reactive oxygen species (ROS) production, and cell morphological changes, as imaged using atomic force microscopy (AFM), were conducted to preliminarily clarify the sanitisation mechanism of each treatment. Furthermore, the sanitisation performance on broccoli sprouts and the effects of the treatments on sprout quality during 6 days of storage were tested.

2. Materials and methods

2.1. Bacterial strain and culture condition

L. innocua ATCC 33090 was obtained from the Food Science and Technology (FST) Programme, National University of Singapore (NUS), and stored in a 30% (v/v) glycerol solution at −80 °C. The strain was resuscitated by culturing in 10 mL of Tryptone Soya Broth (TSB, Oxoid, UK) and incubating at 37 °C overnight. Thereafter, the bacteria were adapted to 100 μg/mL of nalidixic acid (Sigma-Aldrich, USA) by stepwise increments over several rounds of transfers (Liu et al., 2017a). The working *L. innocua* suspension was prepared by inoculating the overnight culture 1:100 into fresh TSB and then incubating at 37 °C for about 18 h. The bacterial concentration was assayed using spectrophotometric analysis at 600 nm and the cell counts were determined by plate culture on Tryptone Soya Agar (TSA, Oxoid, UK). The working culture (approximately 8–9 log colony forming units (CFU)/mL) was separately centrifuged at 12,000 ×*g* for 10 min at 24 °C and the harvest cell pellet was washed with phosphate-buffered saline (PBS, pH 7.2) twice and resuspended for subsequent experiments (Kan et al., 2018; Zhao et al., 2017). To inoculate *L. innocua* on sprouts, the working culture was diluted to around 7 log CFU/mL. Harvested sprouts were submerged in the diluted bacterial suspension for 5 min. The inoculated sprouts were then air-dried at room temperature for 30 min and then subjected to different sanitising treatments (Liu et al., 2017a).

2.2. In vitro disinfection kinetics of each treatment

In a preliminary study, the organic broccoli sprouts were immersed in LA at different concentrations (0.5, 1, 2, and 4%, v/v) for different times (0.5, 1, 2, 4 min). The results showed that treatment with 4% (v/v) LA (1–4 min) and 2% LA (4 min) resulted in notable softening of the sprouts by finger touching. Interestingly, using 2% (v/v) LA (2 min), no obvious damage to the sprouts was observed. Thus, the immersion of sprouts in LA (2%) for 2 min was used as the working conditions. The microbial suspension (40 mL) prepared in Section 2.1 was concentrated by centrifugation and resuspended in 1 mL of sterile saline (8.5 g/L NaCl). The concentrated suspension was then mixed with 9 mL of SH (4 mg/L FAC final concentration), LA (2%, v/v), and the combined solution, respectively, in 15 mL sterile test tubes. The suspension mixed with 9 mL sterile deionised (DI) water was used as the control. The SH solution was prepared by dilution of sodium hypochlorite with sterilised DI water and assayed using a chlorine test kit (Reflectoquant Chlorine test, Chlor-Test 0.5–10.0 mg/L Cl_2, Darmstadt, Germany). During the disinfection process, the mixed suspension (1 mL) was transferred and immediately mixed with 2 mL neutralising buffer (5 g/L sodium thiosulfate in 0.1 M PBS, pH 7.5) at 0, 15, 30, 60, 120, and 180 s (Liu et al., 2017b). The neutralised samples were then serially diluted with neutralising buffer and 0.1 mL of the diluted samples was spread onto non-selective TSA plates and selective TSA plates with 3% (m/v) sodium chloride (TSA-SC). The colonies were counted after culturing for 72 h at 37 °C. The colonies on TSA were considered as uninjured and injured cells, while the colonies on TSA-SC were regarded as developing only from uninjured cells (Liu et al., 2018). The following equation was used to determine the percentage of sublethally injured cells:

$$[1 - (\text{count on TSA} - \text{SC/count on TSA})] \times 100$$

2.3. Mathematical model fitting of the antibacterial effect

The antibacterial effect (log CFU/mL reduction compared with the control group at each time point) of each treatment was described mathematically using three models as follows:

Linear model: $N = k \cdot t$, where N is the log CFU/mL reduction, t is the time in s and k is the rate of antibacterial effect in (log CFU/mL)/s.

Weibull model: $N = (t/t_1)^a$, where t_1 is the time needed to reduce 1 log CFU/mL and a is the shape parameter.

Gompertz model: $N = N_1 \exp\{-\exp[(k_1 e)(t_2 - t)/N_1 + 1]\}$, where N_1 is the antibacterial populations (log CFU/mL) after the maximum antibacterial time. k_1 is the maximum rate of antibacterial effect and t_2 is the lag phase duration.

The goodness of the model fitting was tested by R-square (R^2) and root mean squared Error (RMSE) using MATLAB R2013b (The Mathworks Inc., Natick, MA, USA). Furthermore, the Akaike Information Criterion (AIC) values were calculated to analyse overfitting: AIC = nln(SSE) + 2p, where n is the number of data points used, SSE is the sum of squares for error, and p is the parameter number used in each model (Ghate et al., 2017).

2.4. Protein leakage and intracellular ROS detection

Protein leakage and ROS production were determined according to the method of Liu et al. (2018) with some modifications. The mixed suspensions (1 mL) of *L. innocua* and different sanitisers from Section 2.2 were collected at different time points and immediately mixed with 2 mL neutralising buffer (5 g/L sodium thiosulfate in 0.1 M PBS, pH 7.5) to stop the disinfection process. The obtained neutralised suspensions of each treatment were centrifuged at 12,000 ×*g* for 10 min at 24 °C. The supernatant (20 μL) was mixed with 200 μL Coomassie brilliant blue G-250 solution in a 96-well plate. After incubation for 5 min, the absorbance of mixture was read at 595 nm and bovine serum albumin (BSA) was used as standard (20–100 μg/mL, $R^2 > 0.99$).

The intracellular ROS levels in *L. innocua* were tested using an oxidant-sensitive probe: 2′,7-dichlorodihydrofluorescein diacetate (H_2DCFDA). Briefly, the concentrated suspension (40 to 1 mL) prepared in Section 2.2 was incubated with H_2DCFDA (200 μM final concentration) at 37 °C for 30 min under dark conditions. Then, the bacterial cells were washed and resuspended in sterile saline, followed by the sanitisation treatments as in Section 2.2. The collected neutralised samples (200 μL) were transferred into 96-well plates and their fluorescence intensities were tested using a fluorescence plate reader (Spectrafluor Plus, Tecan, Durham, NC, USA) at excitation and emission wavelengths of 485 and 528 nm, respectively. The fluorescence intensity ratio was calculated as the ratio of the fluorescence of groups treated with sanitiser to the fluorescence of the control group (Cossu et al., 2017).

2.5. AFM analysis of morphological changes

An *L. innocua* suspension was prepared and sanitised as in Section 2.2. After disinfection for 2 min, the mixed suspension (20 μL) from each group was spread onto a mica sheet and immediately dried using an aurilave. The prepared sheet was then air-dried in a laminar flow cabinet for 4 h. The sheet for each treatment was then scanned using AFM in 4 h. A TT-atomic force microscope (AFM workshop, Signal Hill, CA, USA) equipped with a Sensaprobe TM190-A-15 tip (Applied Nanostructures, Mountain View, CA, USA) with resonance of 190 kHz and force constant of 45 N/m was applied to test the morphological changes of *L. innocua* under different treatments. The images were scanned by AFM under tapping mode with a scan rate of 0.3 Hz and 512 scan lines

(Zhang and Yang, 2017). The obtained images were analysed using the offline software Gwyddion (Chen et al., 2018b). The width, height, and root-mean-square (RMS) roughness of the bacterial cells were recorded. RMS roughness was calculated from individual regions ($0.2 \times 0.2\ \mu m^2$) on the central part of the cell surfaces and at least 20 measurements were carried out for data analysis.

2.6. Anti-microbial analysis on broccoli sprouts

Certificated organic broccoli seeds (*Brassica oleracea* var. *italica*) were purchased from a local supermarket in Singapore and the germination and sprouting of broccoli sprouts were conducted according to our previous study (Chen et al., 2018a). The broccoli seeds were immersed in 3% (v/v) SH solution for 5 min and washed by DI water several times. The seeds were then soaked in DI water overnight at room temperature. About 4 g of seeds were spread on a plastic seedling-raising plate (30 cm × 22 cm) and germinated at room temperature under darkness for 3 days. The plate was sanitised by 70% (v/v) ethanol before use. The germinated seeds were further sprouted for another 4 days under 16/8 h light/dark photoperiod at room temperature. The sterile DI water in the sprouting plates was refreshed daily. The organic broccoli sprouts were carefully harvested for subsequent microbial analysis.

To test the natural microbial population under different treatments during storage, the harvested sprouts were immersed in solutions of sterile DI water (control), SH (4 mg/L FAC), LA (2%), and the combination of SH and LA, respectively, for 2 min. The treated sprouts were then immersed in sterile DI water for 1 min to remove the residual treatment chemicals to avoid sprout softening caused by LA. The sprouts were stored under low temperature (4–6 °C) in the dark and sampled at day 0, 2, 4, and 6. The collected samples (1 g) in each group were then ground in 10 mL sterile saline (8.5 g/L NaCl) using a sterile mortar and pestle. Serial dilutions were conducted and 0.1 mL of the dilution was spread on plate count agar (PCA, Oxoid, UK) and incubated at 37 °C (48 h) to test for mesophilic aerobic microorganisms. For the yeast and mould counts, 0.1 mL of the dilution was spread on potato dextrose agar (PDA, Oxoid, UK) and incubated at 25 °C for 72 h. The results were expressed as log CFU/g fresh weight (FW) (Vong et al., 2018).

To determine the survival of populations of inoculated *L. innocua*, the sprouts were firstly inoculated by *L. innocua* as described in Section 2.1. The inoculated sprouts were then treated with different sanitising treatments in the same way as the natural microbial population test. The dilutions (0.1 mL) of different treatments at different sampling days were spread on TSA with nalidixic acid (100 μg/mL) and incubated at 37 °C for 48 h. The results were expressed as log CFU/g FW.

2.7. Qualitative sensory evaluation of broccoli sprouts during storage

The sprouts without *L. innocua* inoculation were used to conduct qualitative sensory evaluation. The harvested sprouts were treated by different sanitising treatments as mentioned in Section 2.6. The sprouts were further stored under low temperature (4–6 °C) in the dark and tested at day 0, 2, 4, and 6. On each test day, about 0.5 g of broccoli sprouts were assessed by trained testers (eight members aged from 25 to 30 years olds from the FST Programme, NUS). The evaluation was conducted immediately after sprouts were removed from storage condition. Sensory quality parameters including colour, texture, smell, and overall quality score were evaluated. The intensity of the sensory parameters was quantified on a scale from 1 to 5; colour was rated by 1 for yellowish florets, 3 for light green, and 5 for dark green, uniform colour. Texture was rated by 1 for very soft, 3 for rubbery, and 5 for crispy. Smell was rated by 1 for strong off-odour, 3 for slight but obvious off-odour, and 5 for no off-odour. The overall quality score was rated as 1 for dislike extremely, 3 for average like, and 5 for like extremely. The limit of accepted attributes of broccoli sprouts was 3, indicating that a score < 3 meant the end of shelf life (Moreira et al., 2011).

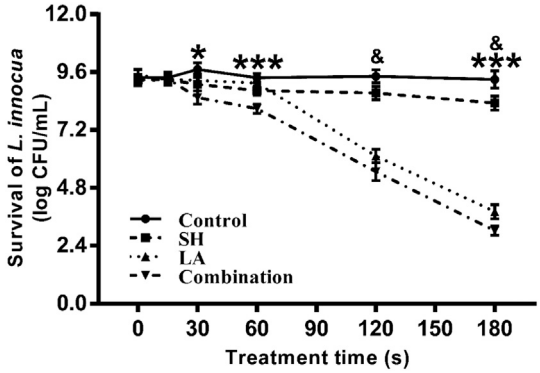

Fig. 1. *In vitro* survival curves of *L. innocua* ATCC 33090 under treatment by sodium hypochlorite (SH), lactic acid (LA), and their combination (Combination). Note: the marks * (Combination versus Control), *** (Combination versus LA), & (SH versus control) represent significant differences ($P \leq 0.05$). CFU, colony forming units.

2.8. Statistical analysis

Data were analysed statistically using analysis of variance (ANOVA), and means were compared using the least significant difference (LSD) method to assess the sanitisation effects of the treatments. In addition, differences with $P \leq 0.05$ were considered significant.

3. Results and discussion

3.1. In vitro sanitising effect of different treatments on L. innocua

The *in vitro* disinfectant effects of *L. innocua* under four treatments are shown in Fig. 1. Cell concentrations in the control group were constantly maintained and ranged from 9.30 to 9.71 log CFU/mL (Fig. 1). SH (4 mg/L FAC) treatment significantly ($P \leq 0.05$) decreased the bacterial populations by 0.64 and 0.98 log CFU/mL compared with control group at 120 and 180 s, respectively (Fig. 1). Similarly, Zhao et al. (2017) reported that a low concentration of neutralised electrolysed water (pH: 7.0) with FAC at 4 mg/L effectively reduced the populations of *Escherichia coli*, *Pichia pastoris*, and *Aureobasidium pullulans* in planktonic status. Chlorine related substances, including Cl_2, HClO, and ClO^-, contribute to the sanitising effect (Zhao et al., 2017). For the LA (2%)-treated group, the bacterial populations were sharply reduced from 9.43 to 6.12 and from 9.30 to 3.61 at 120 and 180 s, respectively (Fig. 1). A previous *in vitro* study showed that LA at low concentration (0.5%) had effective antibacterial actions against *Salmonella*, *E. coli*, and *Listeria* after exposure for 6 h (Wang et al., 2015). Moreover, compared with the control group, notable reductions (1.16–6.27 log CFU/mL) of *L. innocua* were recorded for the combined treatment group. The combined use of antibacterial methods is an effective strategy to improve their sanitising abilities. For example, the combinations of electrolysed water with ultrasound, carvacrol nanoemulsion, or mild heat resulted in higher sanitising efficacies compared with the individual applications (Liu et al., 2017a; Sow et al., 2017; Zhao et al., 2017). In addition, our results showed that the combination of LA and SH with 4 mg/L FAC further promoted the disinfection effect.

Fig. S1 shows the percentage of cell injury for each group. The results showed that SH treatment induced a significantly ($P \leq 0.05$) higher percentage of *L. innocua* injury compared with that of control group at 120 (26.47%) and 180 s (42.68%). In addition, treatment with LA alone caused a continuous increase of the percentage of injured cells from 9.77 to 91.80%. The combined treatment resulted in more rapid sublethally injury of *L. innocua* and reached 94.49% at 180 s. The

Table 1
Fitting parameters of each treatment by linear, Weibull, and Gompertz models.

Treatment	Model	R^2	RMSE	SSE	AIC	k ($\times 10^{-2}$)[a]	t_1[a]	a[a]	N_1[a]	k_1 ($\times 10^{-2}$)[a]	t_2[a]
SH	Linear	0.93 c	0.11 d	0.06 d	−15.41 ef	0.60 c	–	–	–	–	–
	Weibull	0.96 b	0.03 e	0.03 d	−17.47 ef	–	174. 13 a	0.77 c	–	–	–
	Gompertz	0.94 c	0.13 d	0.05 d	−12.23 de	–	–	–	1.30 c	0.69 c	0.23 c
LA	Linear	0.89 d	0.78 a	3.02 a	8.60 a	2.74 b	–	–	–	–	–
	Weibull	0.98 ab	0.39 b	0.61 c	1.00 b	–	67.94 b	1.79 a	–	–	–
	Gompertz	1.00 a	0.08 de	0.03 d	−20.20 f	–	–	–	6.38 b	6.23 a	67.39 a
Combination	Linear	0.97 b	0.43 b	0.94 b	1.59 b	3.29 a	–	–	–	–	–
	Weibull	1.00 a	0.19 c	0.15 d	−7.58 d	–	45.62 c	1.35 b	–	–	–
	Gompertz	0.99 a	0.24 c	0.17 d	−4.66 c	–	–	–	10.85 a	4.63 b	38.83 b

Within the same column, mean values with different lowercase letters are significantly different from each other ($P \leq 0.05$).
Note: regression coefficient (R^2), Root Mean Squared Error (RMSE), Sum of Squares for Error (SSE), and Akaike Information Criterion (AIC). Symbol '–' indicates not available.
[a] The parameters of selected models. Linear model: $N = kt$; Weibull model: $N = (t/t_1)^a$; Gompertz model: $N = N_1\exp\{-\exp[(k_1e)(t_2 - t)/N_1 + 1]\}$.

results showed that the LA and combined treatments led to significant ($P \leq 0.05$) increases in the percentage of injured cells after 120 s. The results shown in Fig. 1 and Fig. S1 indicated that a certain accumulated level of sublethal damage might result in notable reduction of the survival *L. innocua* cells. The sublethally injury caused by the LA and combined treatments could result from damage to the membrane structure, DNA synthesis, or enzyme activities (Shi et al., 2017). Thus, the LA and combination treatments for 2 min could be potential disinfection methods for postharvest organic sprouts.

3.2. Model fitting of the antibacterial effects of different treatments

The model fitting curves for each treatment and the goodness-of-fit of each model are presented in Fig. S2 and Table 1, respectively. All the tested models (linear, Weibull, and Gompertz) exhibited relatively high R^2 values (0.89–1) in each treatment group (Table 1). Furthermore, the RMSE value is considered as the most simple and informative parameter of goodness-of-fit for linear and non-linear curves. Lower RMSE values represent better applicability of the fitted models (Scanlon et al., 2015). Based on the results of R^2 and RMSE, the Weibull model with higher R^2 (0.96) and lower RMSE (0.03) values exhibited a better fitting performance for the SH group (Table 1). The Gompertz model (R^2, 1.00; RMSE, 0.08) was more applicable to describe the antibacterial effect of the LA treatment. Furthermore, the Weibull and Gompertz models presented equal R^2 and RMSE values for the combination treatment. The overfitting of the models was further checked using the AIC test. AIC is considered a robust statistical method to compare models. It takes into account the sample size and parameter number, and a lower AIC value represents a better model (Ghate et al., 2017). No significant ($P \leq 0.05$) differences in the AIC values were observed among the three models in the SH group (Table 1). In the LA-treated group, the lowest AIC (−20.20) was recorded by Gompertz fitting, followed by Weibull (1.00), and linear (8.60) fittings. The results further confirmed that the Weibull and Gompertz were superior models to describe the sanitising kinetics of SH and LA treatment, respectively. Moreover, the Weibull model presented better fitting performance for the combination treatment when the AIC value was considered. The Weibull model could illustrate saturation or lag phase presented in a growth curve or inactivation curve (Ghate et al., 2017; Maresca and Ferrari, 2017). We observed a lag phase at the early stage (0–60 s) of the antibacterial curve for the three groups (Fig. S2). Thus, the compatibility of Weibull model and the antibacterial nature of the SH and combination treatments contributed to the success of model fitting. The Gompertz method is commonly used to describe the sigmoidal kinetics curves that have a lag time before the maximum inactivation rate and a tail (residual population) (Gil et al., 2011). Thus, the characteristics of the shoulder and tail phases shown in the curve of the LA treated group in Fig. S2 might result in the superior fitting of the Gompertz model.

The three selected models exhibited generally good fitting performances with relatively high R^2 values (Table 1); therefore, the model parameters of each model under different treatments were further calculated to analyse the detailed information of the sanitising kinetics. The general antibacterial rate (k) in linear model indicated the overall inactivation efficiency of each treatment (Ghate et al., 2017). The combination treatment exhibited the highest antibacterial rate (k), 3.29×10^{-2} (log CFU/mL)/s, followed by LA [2.74×10^{-2} (log CFU/mL)/s], and SH [0.60×10^{-2} (log CFU/mL)/s] treatments (Table 1). The results indicated stacking of the disinfectant effects of SH and LA treatments in the combined treatment. Parameters of the Weibull model, t_1 and a, represented the time needed to reduce 1 log CFU/mL and the shape characteristic, respectively (Huang, 2009). The t_1 values of SH, LA, and the combination treatment were 174.13, 67.94, and 45.62 s, respectively. The results for t_1 were in accord with the linear antibacterial rate (k). Furthermore, the highest shape parameter a (1.79) was recorded in the LA group. The Combination and SH treatments showed relatively lower a values, 1.35 and 0.77, respectively. The results for the a value indicated the concave-upward sanitising curves of LA and combination treatment and the concave-downward curve of SH treatment (Koyama et al., 2017; Luo and Oh, 2016). Parameter N_1 in the Gompertz model indicates the antibacterial population after maximum time. The highest N_1 value (10.85 log CFU/mL) was recorded by the combination treatment. Moreover, N_1 values of 6.38 and 1.30 log CFU/mL were presented in LA and SH treated groups, respectively. Furthermore, Gompertz fitting of the LA group showed a significantly ($P \leq 0.05$) higher maximum rate of antibacterial effect (k_1, 6.23 (log CFU/mL)/s) and lag phase duration (t_2, 67.39 s) compared with the other two treatments (Table 1). The highest maximum rate of the antibacterial effect measured in the LA treatment group might result from the longest shoulder lag time (Gil et al., 2011).

3.3. The protein leakage and intracellular ROS production

Fig. 2 shows the protein leakage and intracellular produced ROS in each treatment group. Compared with that in the control group, the protein concentrations in the sanitised groups increased by different degrees. SH treatment increased the protein leakage from 4.80 to 8.94 μg/mL. Furthermore, LA treatment induced significantly ($P \leq 0.05$) higher protein concentrations compared with those of the SH treated group at 30 (9.27 μg/mL) and 180 s (10.40 μg/mL). The protein content in combined treatment group increased to 10.60 μg/mL in 30 s and was maintained at around 11 μg/mL for the remaining treatment time (Fig. 2a). We also observed relatively rapid increases in protein leakage during first 60 s in the LA and combined treatment groups. Based on the data shown in Fig. 1 and the protein leakage, we concluded that LA and combination treatments led to severe structural damage of *L. innocua* in a relatively short time (60 s). The leakage of cytoplasmic proteins indicated conformational changes to the outer membrane of *L. innocua* (Liu et al., 2018). Previous studies also showed

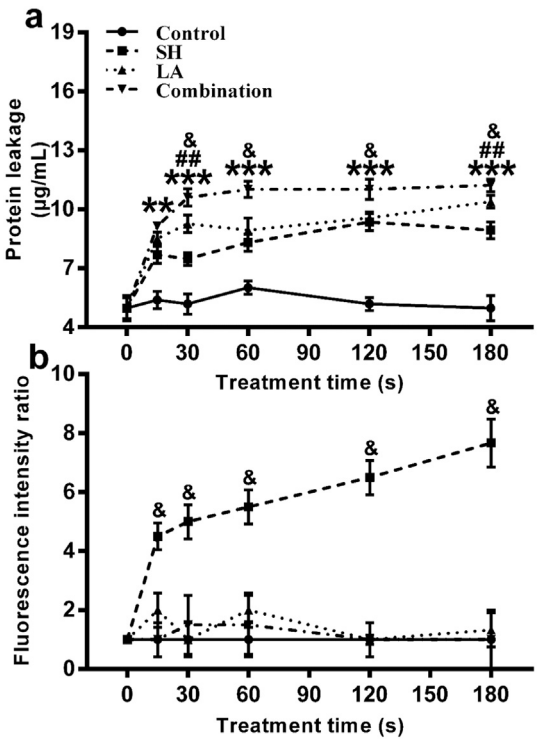

Fig. 2. Effect of different sanitising treatments on protein leakage (a) and the fluorescence intensity ratio (b) of *L. innocua*. Note: the marks ** (Combination versus SH), *** (Combination versus LA), ## (LA versus SH), & (SH versus control) present significant differences ($P \leq 0.05$). Sodium hypochlorite (SH), lactic acid (LA), and their combination (Combination).

that a weak organic acid inactivated bacteria by increasing the permeability of membrane structures (Mani-Lopez et al., 2012; Wang et al., 2013). Moreover, LA may mainly contribute to the sanitising effect of the combined treatment and SH at low concentrations played an auxiliary role.

Intracellular ROS was tested using the H_2DCFDA probe method (Fig. 2b). The results showed no notable differences in fluorescence intensity ratio among the control, LA, and combined treatments, with the ratio remaining constant at around 1. However, SH treatment resulted in a continuous increase in ROS production. The fluorescence intensity ratio increased from 1 to 7.67 in 180 s. The results of Fig. 2 indicated that different sanitising mechanisms might contribute to the disinfection effect of each treatment. SH at a low concentration of FAC (4 mg/L) showed a relatively slow sanitising effect and might inactivate the bacterial cells by inducing oxidative damage, which would further affect intracellular metabolism and membrane permeability (Feliziani et al., 2016; Wang et al., 2010). By contrast, LA at 2% (v/v) might disrupt the cell membrane of *L. innocua* in a short time, eventually leading to cell rupture (Wang et al., 2015). For the combination treatment, cell susceptibility to LA could be increased by the added SH, resulting in a synergistically promoted sanitising effect.

3.4. Morphological changes assessed using AFM

AFM provides a new strategy to investigate 3-dimensional information for cell surface structures at nanoscale resolution (Liu and Yang, 2018). The obtained images showed that bacterial cells in the control group adhered to each other. Furthermore, a smooth cell surface and integrated cell structure were observed (Fig. 3). Aggregated cells were also observed in the SH-treated group. However, the cell surface became wrinkled and irregular. The bacterial cells under the LA and combined treatments presented a relatively decentralised, non-adherent distribution. This might be because extracellular polymeric

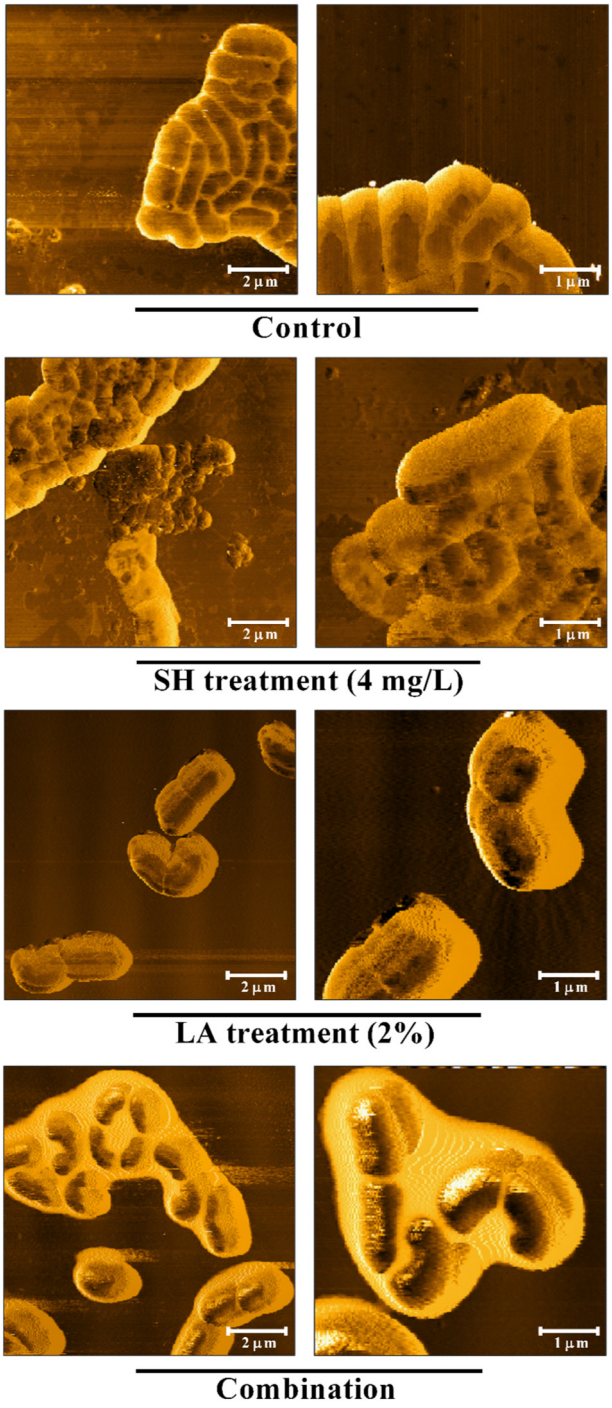

Fig. 3. Atomic force microscopy (AFM) images of *L. innocua* cells under different disinfectant treatments. Sodium hypochlorite (SH), lactic acid (LA), and their combination (Combination).

substances, such as polysaccharides, were disturbed along with the membrane disruption caused by the LA and combination treatments (Colagiorgi et al., 2016). Moreover, the cell surface of the LA-treated bacteria was rough and some residues around the cell bodies might be the leaked intracellular components of *L. innocua*. In the combination treatment, the treated cells showed dramatic morphological changes. Irregular cell bodies represented the breakdown and cracking of the bacteria. Furthermore, abundant suspected cell leakage was observed.

Further data analysis conducted using Gwyddion software revealed the detailed information of the *L. innocua* cells in each group (Table 2). There was no significant ($P \leq 0.05$) difference in cell width among the

Table 2
Changes of width, height, and root mean squared (RMS) roughness under different treatments.

Treatment	Width (μm)	Height (μm)	RMS roughness (nm)
Control	1.10 a	0.38 a	5.78 d
SH	0.98 a	0.31 b	6.70 c
LA	1.02 a	0.29 c	7.91 b
Combination	0.73 b	0.29 c	10.37 a

Within the same column, values with different lowercase letters are significantly different from each other ($P \leq 0.05$).

control, SH, and LA treated groups. However, the combination treatment resulted in a lower width of 0.73 μm. Compared with the cell height in the control group (0.38 μm), the SH, LA and combined treatments led to significantly ($P \leq 0.05$) lower heights of 0.31, 0.29, and 0.29 μm, respectively. RMS roughness was quantitatively analysed to assess the surface morphology of *L. innocua*. A relatively low RMS value was calculated for the control group (5.78 nm). This increased to 6.70 nm under SH treatment and 7.91 nm under LA treatment. The highest RMS value (10.37 nm) was recorded in the combination treatment group.

The AFM results demonstrated that the cell integrity was preserved after SH treatment, although visual and statistical assessments showed the modified characteristics of the cell membrane surface. These results were consistent with a previous report in which certain levels of FAC inactivated bacterial cells by altering membrane permeability; however, the cells may have remained intact (Wang et al., 2010; Zhang and Hu, 2013; Zhang et al., 2018). Moreover, LA treatment resulted in the leakage of intracellular components, decreased cell height, and increased roughness in *L. innocua* cells. Thus, LA might enter the bacterial cells, damage the cytoplasmic membrane, and further modify the surface characteristics and cytoskeleton structure (Wang et al., 2014). Furthermore, the notable morphological and statistical changes of *L. innocua* cells treated with the combined treatment indicated that the disinfectant effect of LA was promoted by combining it with SH. The AFM results provided visual evidence that confirmed the hypothetical sanitising mechanisms of the different groups.

3.5. Microbial profile in broccoli sprouts under different treatments

The environment of sprouts production makes them be an ideal medium for microbial growth. Previous studies reported high populations of total plate counts, which could reach to 10^8–10^9 CFU/g, in sprouts (Gabriel et al., 2007; Martínez-Villaluenga et al., 2008). Furthermore, sprouting produce can be contaminated in each part of the production process, including sprouting, harvesting, postharvest handling, transportation, and storage (Baenas et al., 2017; Juck et al., 2012). Therefore, the changes in microbial populations (aerobic bacteria, yeasts, and moulds, and inoculated *L. innocua*) in stored broccoli sprouts were monitored after different sanitising treatments (Fig. 4). The results showed that compared with the control group (6.58 log CFU/g FW), the SH, LA, and combined treatments significantly decreased the aerobic bacteria counts by 0.44, 0.77 and 0.82 log CFU/g FW, respectively, at day 0 (Fig. 4a). With increasing periods of low temperature storage, the aerobic bacteria populations in four groups increased constantly. At day 6, the highest aerobic bacteria count was recorded in the control group (7.30 log CFU/g FW), followed by SH treatment (7.04 log CFU/g FW), LA treatment (6.98 log CFU/g FW), and combination treatment (6.68 log CFU/g FW).

Similar changing trends were observed for yeast and mould populations in broccoli sprouts. The cell counts in the control, SH, LA, and combination treatment groups were 6.72, 6.09, 5.94, and 5.21 log CFU/g FW, respectively at day 0. After storage for 6 days, yeasts and moulds in the control group had increased to 8.05 log CFU/g FW. Lower cell counts were measured in the SH and LA treated groups: 7.76 and

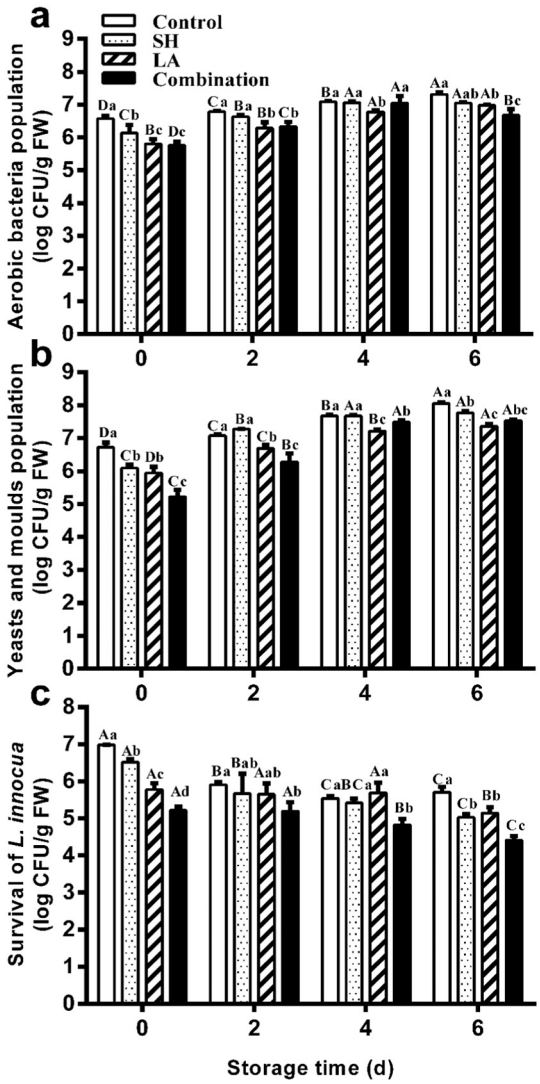

Fig. 4. Inactivation effect of sanitising treatments on aerobic bacteria (a), yeasts and moulds (b), and inoculated *L. innocua* (c) in organic broccoli sprouts during storage. Note: Within the same day, mean values with different lowercase letters are significantly different from each other ($P \leq 0.05$); within the same treatment, mean values with different capital letters are significantly different from each other ($P \leq 0.05$). Sodium hypochlorite (SH), lactic acid (LA), and their combination (Combination); CFU, colony forming units.

7.36 log CFU/g FW, respectively. By contrast, the inoculated *L. innocua* in each group showed decreased populations during the storage. Moreover, the combined treatment resulted in the greatest reduction in inoculated *L. innocua* by 1.77 and 1.19 log CFU/g FW at day 0 and 6, respectively. It was noted that the antibacterial effect of the combination treatment on *L. innocua* inoculated on broccoli sprouts (0 day) was decreased compared with that in suspension cells (120 s). Similar results were also reported previously (Rodgers et al., 2004). This might be because the food matrix weakens the sanitising effect of the sanitisers (Vandekinderen et al., 2009). Nevertheless, the sanitising treatments, especially the combined treatment, presented suitable disinfection effects.

3.6. Qualitative sensory evaluation of postharvest broccoli sprouts

Broccoli sprouts are treated and consumed as fresh vegetable products. This kind of micro green should be stored under low temperature and consumed in a short time. In addition, the processing steps should not affect the quality of these organic products. Sensory qualities such

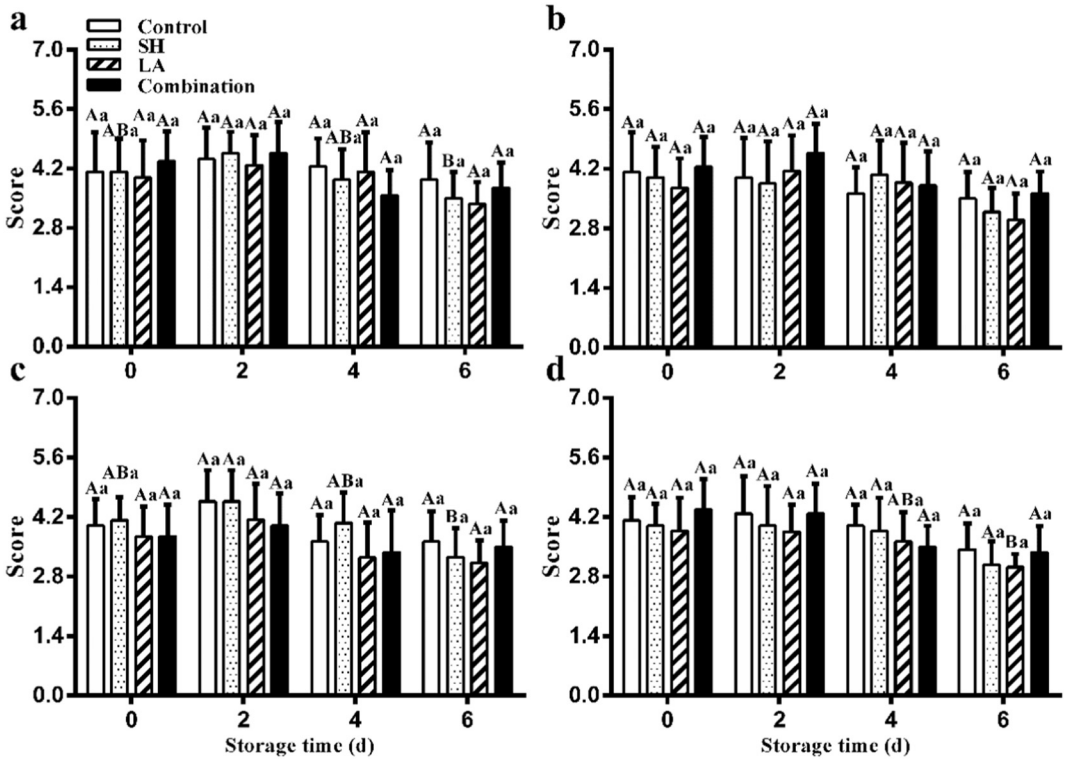

Fig. 5. Sensory evaluation of broccoli sprouts under different sanitising treatments during storage: (a) colour, (b) texture, (c) smell, and (d) overall quality score. Note: Within the same day, mean values with different lowercase letters are significantly different from each other ($P \leq 0.05$); within the same treatment, mean values with different capital letters are significantly different from each other ($P \leq 0.05$). Sodium hypochlorite (SH), lactic acid (LA), and their combination (Combination).

as colour, texture, and smell are crucial factors for the acceptance of consumers (Kwon et al., 2013). The results of the qualitative sensory evaluation are presented in Fig. 5. During the 6 days of storage of sprouts under different sanitising treatments, the colour and smell were only slightly affected by SH treatment at day 6. The score for colour decreased from 4.13 (day 0) to 3.50 and the smell score was reduced from 4.13 (day 0) to 3.25 (day 6) (Fig. 5a, c). Moreover, no significant ($P \leq 0.05$) difference of texture scores was observed among the treated groups during storage (Fig. 5b). The overall quality scores of four groups were maintained at similar levels at the same storage day (Fig. 5d). The scores of all parameters were > 3, which was the acceptance limit (Moreira et al., 2011). Thus, we concluded that the different sanitising treatments did not significantly affect the quality of the broccoli sprouts.

4. Conclusion

The sanitising effects and mechanism of SH (4 mg/L FAC final concentration), LA (2%, v/v), and combined treatments on *L. innocua* were examined. The combination treatment presented best sanitising performance according to the in vitro disinfectant kinetics. The accumulated sublethal damage resulted in the rapid decrease in surviving populations of *L. innocua* cells after 60 s. Modelling of the antibacterial effect of the different treatments showed that the Weibull model was the best model to describe the combination treatment. The combination treatment required the lowest killing time (t_1) of 45.62 s to reduce 1 log CFU/mL of *L. innocua*. Further analysis of protein leakage and fluorescence intensity ratios revealed that the sanitising mechanism of each treatment was different. SH might inactivate the bacterial cells by inducing oxidative damage and further increasing membrane permeability. The cell destruction caused by LA might result from the rapid damage to the cytoplasmic membrane and the SH could synergistically promote the sanitising effect of LA. AFM images provided a visual understanding of the disinfectant process and helped to confirm the hypothesis. The application of the treatments on organic broccoli sprouts demonstrated their relative applicability as disinfectants, especially for the inoculated *L. innocua* under the combined treatment. In addition, the sensory qualities including colour, texture, smell and overall quality were not compromised after sanitising treatments, indicating that the storage qualities of organic broccoli sprouts were not negatively affected by these processing methods. Thus, the combination of LA (2%, v/v) and low concentration SH (4 mg/L FAC final concentration) could be a potential sanitising strategy for organic sprouting produce.

Acknowledgement

This study was funded by the Singapore Ministry of Education Academic Research Fund Tier 1 (R-143-000-A40-114), projects 31371851 supported by NSFC, Natural Science Foundation of Jiangsu Province (BK20181184) and an industry grant supported by Changzhou Qihui Management & Consulting Co., Ltd. (R-143-000-616-597).

Conflict of interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with this manuscript. We have no financial and personal relationships with other people or organisations that can inappropriately influence our work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.02.014.

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