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Carvacrol nanoemulsion combined with acid electrolysed water to inactivate bacteria, yeast *in vitro* and native microflora on shredded cabbages

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ABSTRACT

Carvacrol is an effective antimicrobial agent originated from essential oils, this natural antimicrobial agent has higher consumer acceptance compared to chemical agents. Due to the low solubility of carvacrol in water, carvacrol was delivered as a nanoemulsion. A carvacrol nanoemulsion contained 3.5% (w/w) oil phase (1% carvacrol and 2.5% corn oil, w/w) and 3.5% (w/w) Tween 80 was produced by ultrasonification at 10 min using 100% amplitude; the median particle size was 309 ± 19 nm. The nanoemulsion was shelf-life stable for 1 month without any significant changes in particle size. When applied against *Escherichia coli* ATCC 25922 and *Pichia pastoris* GS115 growth in nutrient broth, carvacrol nanoemulsion (0.5% w/w carvacrol) achieved 3 log reductions of microorganisms. When microorganisms were fixed and dried on stainless steel coupon surface, the carvacrol nanoemulsion treatment was more effective on *E. coli* than *P. pastoris* with about 5 and 0.3 log reduction of viable count, respectively. The native microflora on shredded cabbages was challenged by combining carvacrol nanoemulsion and acidic electrolysed water (AEW) that contained ≤ 4 mg/L free available chlorine (FAC). The treatment reduced about 0.5 log of aerobic mesophilic and psychrotropic bacteria counts and the antimicrobial activity of carvacrol nanoemulsion and AEW lasted up to 2 days. The results indicated that carvacrol nanoemulsion is promising in controlling the safety of fresh-cut vegetables.

1. Introduction

In recent years, there have been growing interest and potential of applying essential oils or the essential oil's active component in food as a natural antimicrobial agent (Chang, McLandsborough, & McClements, 2013). Essential oils possess broad spectrum antimicrobial activity, they also have the clean label status as they are generally recognised as safe (GRAS) (Chen, Davidson, & Zhong, 2014). Carvacrol is a phenolic compound that was proven to be a very active antimicrobial agent. It can disrupt the membrane of cell and mitochondria, causing damage of permeability barrier and leakages of ions, ATP, nucleic acids and amino acids (Donsì, Annunziata, Vincensi, & Ferrari, 2012).

Since the water solubility of carvacrol is as low as 0.11–0.83 g/L at 25 °C (Chen et al., 2014), it is difficult to directly apply carvacrol in food. An oil-in-water (O/W) nanoemulsion system can be used to deliver the active essential oils component. Nanoemulsion is a kinetically stable system which contains submicron size of dispersed particles and is mostly opaque (Ferreira et al., 2010). Nanoemulsion can provide protection to the active component against environmental stresses and increase the partition of the hydrophobic component to aqueous phase (Chang et al., 2013; Donsì et al., 2012), the small particle size of nanoemulsion also offers good physical stability and increased bioactivity (Donsì,

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Cuomo, Marchese, & Ferrari, 2014; McClements & Rao, 2011). The antimicrobial activity of carvacrol was found to be dependent on the composition of nanoemulsion (type of surfactant, concentration of oil phase, ratio of carvacrol to carrier oil, etc.), the particle size and the solubility of nanoemulsion, as well as the type of food matrix where the carvacrol nanoemulsion is applied (Chang et al., 2013; Donsì et al., 2012). Therefore, there is a compelling need to test and explore the antimicrobial efficacy of the carvacrol nanoemulsion on more varieties of food.

Acidic electrolysed water (AEW) can be applied on food and food contact surface, the active oxidising components and chlorines in AEW make it an effective sanitiser against foodborne pathogens including *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes* (Park, Alexander, Taylor, Costa, & Kang, 2008; Yang, Feirtag, & Diez-Gonzalez, 2013). When the free available chlorine concentration (FAC) of AEW is less than 5 mg/L, it complies with the regulation limit for treatment of drinking water and can be considered as safe (WHO, 2011), while up to 4 mg/L is permitted for organic food sanitisation. With the low surface tension property of nanoemulsion due to the incorporation of surfactants, the antimicrobial agents in nanoemulsion can have better contact onto the surface of fruits and vegetables that are mostly hydrophobic or non-uniform (Xiao et al., 2011). Previous reports by Zhang and Yang (2017) have studied the sanitising effect of electrolysed water with citric acid and H_2O_2. Zhang and Yang (2017) have also investigated the antimicrobial activity of the combined used of neutralised electrolysed water with ultrasonification. Liu, Tan, Yang, and Wang (2016) also explored the combination of low concentration AEW and mild heat to sanitise organic broccoli. It is, therefore, interesting to investigate the antimicrobial effect of combining the use of AEW and carvacrol nanoemulsion, especially on waxy fruits and vegetables.

Cabbage is a typical example of waxy vegatables. The cabbages are frequently subject to shredding and consumed raw as salad. This minimally processed product often has a shorter shelf-life than intact produce as the wounded tissues can undergo accelerated tissue softening and enzymatic browning and more prone to microbial contamination (Lin & Zhao, 2007). Organic cabbages have been studied due to the increasing demand of high quality organic produce, it is therefore important to ensure both chemical safety (Yu & Yang, 2017) and microbiological safety of organic produces (Zhang & Yang, 2017). Besides the food itself, food processing equipment could also be a carrier of spoilage microorganisms and foodborne pathogens. Hence, there is a need for sanitisation of food processing equipment surfaces during food processing and handling to ensure food safety.

The objectives of this study were to develop a stable carvacrol nanoemulsion and evaluate the antimicrobial activities of carvacrol nanoemulsion and AEW against *Escherichia coli* ATCC 25922 (*in vitro*) as a representative bacterium strain, *Pichia pastoris* GS115 (*in vitro*) as a representative yeast strain and the native microflora on shredded cabbages. The antimicrobial results could suggest suitable use of the carvacrol nanoemulsion. To the best of our knowledge, this is also the first study which combines the use of carvacrol nanoemulsion and AEW on food.

2. Materials and methods

2.1. Materials

Food grade carvacrol (99%) and propylene glycol ($\geq$ 99.5%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Polyoxyethylene (20) monooleate (OmniPur® Tween 80®) was purchased from Merck (Darmstadt, Germany), corn oil (100%) was purchased from local supermarket (FairPrice, Singapore). Organic cabbage (*Brassica oleracea L. var. capitata*) was purchased from a local market (GreenCircle, Singapore). Microbiological media including tryptic soy broth (TSB), malt extract broth (MEB), tryptic soy agar (TSA), potato dextrose agar (PDA), standard plate count agar (PCA) and peptone were from Oxoid (Hampshire, UK).

2.2. Preparation of nanoemulsion

The total oil phase (3.5–10%, w/w) consisted of carvacrol and corn oil as carrier oil. A coarse emulsion was first produced by mixing oil phase and Tween 80 (3.5%, w/w) together followed by combining with deionised water using a high shear mixer (8000 rpm, 10 min). The coarse emulsion was homogenised by high pressure homogenisation (HPH) or ultrasonification (USF). HPH method was employed in screening of nanoemulsion formulation due to the ability to produce samples of large batch size within a short time. In HPH method, the coarse emulsion was passed through APV-2000 high pressure homogeniser (SPX FLOW, North Carolina, U.S.) at 1000/100 bar (first stage/second stage pressure) twice to form nanoemulsion. In USF method, the coarse emulsion was immersed with a sonotrode (3.4 cm diameter) attached to a 20 kHz sonicator with a power output of 1000 W (UIP1000, Hielscher, Germany) and the amplitude used was 100%. The maximum temperature of samples during sonication was 25 °C.

Blank nanoemulsion without carvacrol was used to screen suitable formulations (oil phase concentration, surfactant to oil ratio (SOR)) and processing time of USF. The optimised formula and method was selected to produce antimicrobial nanoemulsion by adding carvacrol (1%, w/w) to replace corn oil while retaining the total oil phase concentration. To prevent contamination of the carvacrol nanoemulsion (CRV) as much as possible, all the containers, water, apparatus used were sterilised by autoclaving at 121 °C for 15 min before use. The sonotrode was sanitised with 75% (v/v) ethanol before contact with the nanoemulsion.

2.3. Characterisation of nanoemulsion

The particle size distribution of nanoemulsion was determined using the Horiba laser scattering particle size distribution analyser (LA-950 V2, Horiba Ltd., Kyoto, Japan). The refractive index of 1.33 was used for all sample measurements. The volumetric distribution of particles was considered and the result was reported as D10, D50, D90, which were the size of particles (in nm) where 10%, 50%, 90% of the particles lied below each number, respectively. For stability testing, the nanoemulsion was stored for one month and the particle size was re-evaluated and compared to the particle size of freshly produced nanoemulsion (Pan, Chen, Davidson, & Zhong, 2014).

The turbidity of nanoemulsion was determined by diluting nanoemulsion with DI water in the ratio of 1:3 (v/v) and its absorbance was measured using UV-VIS spectrophotometer (UVmini-1240, Shimadzu (Asia Pacific) Pte. Ltd., Singapore) at 600 nm wavelength (Rao & McClements, 2011). The viscosity of nanoemulsion was determined using a Brookfield DV II+ viscometer (Brookfield Engineering, Middleboro, MA, USA) with No. 1 spindle at 150 rpm rotation at 25 °C. The surface tension was determined using du Noüy ring tensiometer with a ring having a circumference of 4 cm at 25 °C.

2.4. Antimicrobial activity against E. coli and P. pastoris

2.4.1. Bacterial and yeast strain

Escherichia coli ATCC 25922 was obtained from Dr. Hyun-Gyun Yuk of National University of Singapore, Food Science and Technology program, while *Pichia pastoris* GS115 was isolated by

Nanjing Agricultural University, China. The frozen culture (−80 °C) was activated in sterile TSB for *E. coli* or in sterile MEB for *P. pastoris*. The cultures were transferred three consecutive times at 37 °C for 24 h (*E. coli*) or 30 °C for 48 h (*P. pastoris*) to obtain sub-cultures. The sub-cultures were centrifuged (5000 × *g*, 4 °C, 10 min) and washed with sterile peptone water (0.1%, w/v), then re-combined and/or re-suspended into sterile peptone water to reach the appropriate concentration to use as working cultures (Zhao et al., 2017).

2.4.2. Preparation of treatment solutions

The acidic electrolysed water (AEW) was collected from the anode output of electrolysed water generator (Hoshizaki ROX-10WB3-EW, Smitech (Asia) Pte Ltd, Singapore). The AEW was diluted with sterile deionised water to achieve FAC concentration of 8 mg/L measured by a colourimetric chlorine test kit (Reflectoquant Chlorine test, Chlor-Test 0.5–10.0 mg/L Cl_2, Darmstadt, Germany). The pH was 3.91 ± 0.03 (Thermo Orion pH meter, Waltham, MA, USA). The nanoemulsion freshly produced by USF was used as stock nanoemulsion. The control nanoemulsion without carvacrol was labelled as NE (3.5% corn oil, w/w) while the carvacrol nanoemulsion was labelled as CRV (2.5% corn oil + 1% carvacrol, w/w). NE and CRV were diluted with sterile deionised water in 1:1 (v/v) to produce Ne and Crv. Thereafter, the 8 mg/L FAC AEW was mixed in 1:1 (v/v) with CRV (labelled as "CrvA"), the final FAC in the CrvA was therefore estimated to be ≤ 4 mg/L, given the possible degradation of FAC. The mixing was done immediately before the antimicrobial treatment to minimise the degradation of FAC. The detailed composition of all treatment groups (Ne, Crv, CrvA) were listed in Table 1.

2.4.3. Inhibition of microorganism growth in broth

The inoculum (1 ml) was added to nutrient broth (8 ml) followed by the addition of treatment solution (1 ml) to test the ability of Crv and CrvA in inhibiting the growth of *E. coli* and *P. pastoris*. The inoculum concentration used was 10^7 CFU/ml and 10^6 CFU/ml for *E. coli* and *P. pastoris,* respectively. The TSB was incubated at 37 °C for 24 h while the MEB broth was incubated at 30 °C for 48 h. After the incubation, the broths were serially diluted with sterile peptone water for enumeration of viable counts which the level was expressed in log CFU/ml.

2.4.4. Inactivation of microorganisms dried on stainless-steel (SS) surface

The microorganisms were dried on SS surface according to the method of Yang et al. (2013) with modification. Microorganisms (25 μl) were inoculated as a spot on sterile SS coupon (1 cm diameter, 0.7 mm thickness) at approximated level of 8.4 log CFU/coupon and dried in laminar flow cabinet for 3 h. After drying, the inoculated coupons were immersed in treatment solution (12 ml) and agitated for 1 min. After treatment, the coupons were transferred into test tubes containing 10 ml sterile peptone water and 0.5 g sterile glass beads, vortexed vigorously for 1 min to dislodge microorganisms from the surface of the coupons, the viable microorganisms in the peptone water were enumerated and expressed as log CFU/coupon.

2.4.5. Enumeration

The viable *E. coli* and *P. pastoris* counts in the final diluted peptone water were determined through spread plating on TSA and PDA, respectively. The TSA plates were incubated at 37 °C for 24 h while PDA plates were incubated at 30 °C for 48 h.

2.5. Antimicrobial treatment on shredded cabbage

The shredded cabbages were prepared by, firstly, removing the two outermost layers and cores; followed by shredding into strips (1 cm width). The treatment solutions were prepared as in Table 1, AEW was prepared as in section 2.4.2. Shredded cabbages (25 g) were placed in the treatment solutions (500 ml) and were shaken for 1 min. After that, the cabbages was removed and dried for 1 h in the laminar flow cabinet. The samples were kept in Ziploc bags and stored in the fridge (7 °C ± 3 °C) for 2 days until the cabbages turned brown.

The microbiological analyses were performed on day 0 and day 2 according to the method of Chong, Lai, and Yang (2015) with slight modification. The shredded cabbages (25 g) were homogenised with peptone water (0.1%, 225 ml) for 1 min. The homogenates with appropriate dilutions were spread plated onto PCA and PDA. Aerobic mesophilic count was determined from PCA after incubation at 37 °C for 2 days while aerobic psychrotropic count was determined from PCA which was incubated at 4 °C for 10 days. Yeasts and moulds count were enumerated from PDA with 4 days of incubation at 25 °C.

2.6. Statistical analysis

The experiments were performed at duplicate and experimental data were analysed by IBM SPSS Statistics for Windows, version 20.0 (IBM Corp., Armonk, NY, USA). The results were reported as mean ± standard deviation. The differences in the results among different treatment groups were determined using analysis of variance (ANOVA) and Duncan's multiple range test. Comparisons with $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Screening of nanoemulsion formulation

In order to produce a stable nanoemulsion, the effect of total oil phase concentration and surfactant to oil ratio (SOR) were evaluated as shown in Fig. 1 and Table 2. The oil phase of blank nanoemulsion was corn oil, which is rich in long chain triacylglycerol and widely used in food production (McClements & Rao, 2011). When used as carrier oil for carvacrol, corn oil could retard Ostwald ripening (Ziani, Chang, McLandsborough, & McClements, 2011). Tween 80 was selected as surfactant, it was reported as a suitable non-ionic surfactant to produce carvacrol nanoemulsion (Chang et al., 2013), the surfactant stabilises the emulsion by steric stabilisation. The use of a high amount of synthetic surfactant is not desired due to regulatory, economic or sensory issues (Rao & McClements, 2011), thus the SOR level was kept below 1:1 which was different from other reported previous studies that SOR was as high as 2:1 (Chang & McClements, 2014; Qian & McClements, 2011;

Table 1
Compositions of treatment groups used for antimicrobial assay.

Composition/ % (w/w)	Treatment groups			
	Control	Ne	Crv	CrvA
Carvacrol	0.0	0.0	0.5	0.5
Corn oil	0.0	1.75	1.25	1.25
Tween 80	0.0	1.75	1.75	1.75
Deionised water	100.0	96.5	96.5	46.5
AEW	0.0	0.0	0.0	50
[a]pH	6.07 ± 0.11	4.95 ± 0.29	4.65 ± 0.17	4.13 ± 0.13

*The treatment nanoemulsions (Ne, Crv, CrvA) were produced by mixing the stock nanoemulsion (NE, CRV) with sterile deionised water or AEW in 1:1 (v/v). NE and CRV were the stock nanoemulsion with 3.5% corn oil and 2.5% corn oil + 1% carvacrol (w/w), respectively.
*Please refer to section 2.4.2 for the preparation of CrvA.
[a] The final pH of the treatment solutions was listed for the ease of comparison.

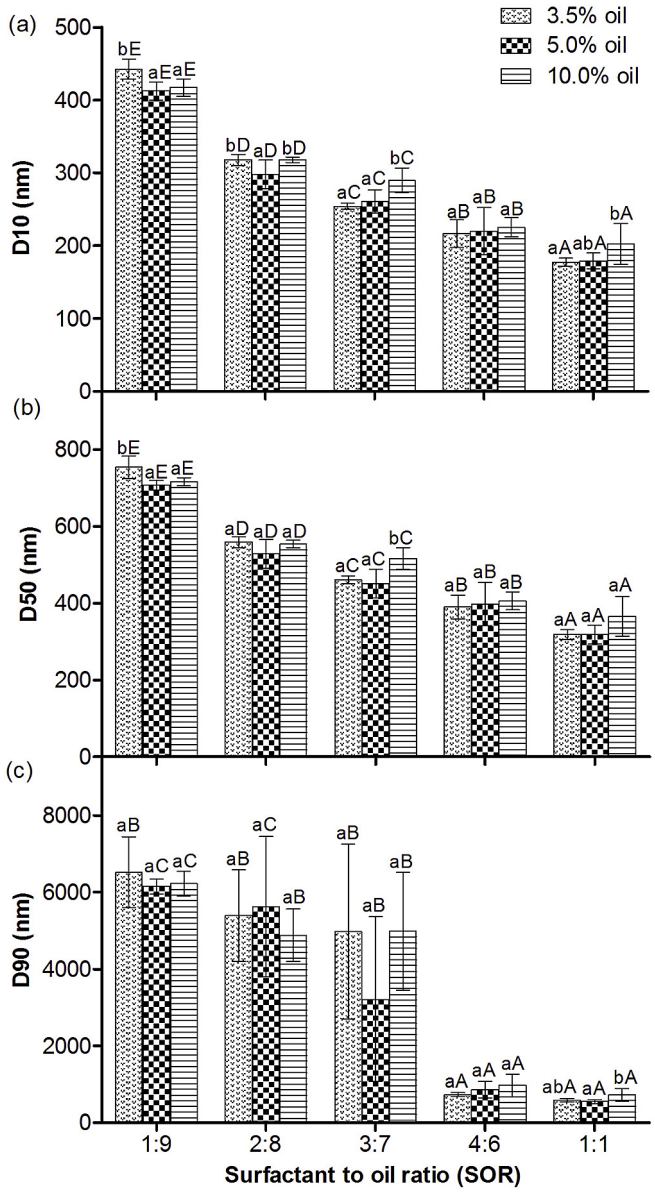

Fig. 1. Effects of surfactant to oil ratio (SOR) and oil concentration on the particle size (D10, D50, D90) of corn-oil-in water emulsion prepared by HPH. *Within same SOR, groups with different small case letters are significantly different from each other ($P < 0.05$); within same oil concentration, groups with different capital letters are significantly different from each other ($P < 0.05$).

Rao & McClements, 2011).

From Fig. 1, the increase in SOR significantly reduced the particle size, the smallest particle size (based on D50 & D10) was observed in nanoemulsion with SOR 1:1. When SOR increased above 4:6, it was observed that D90 decreased drastically. The smaller D90 value could indicate the narrower particle size range distribution of nanoemulsion. Comparing samples with same SOR, the differences in particle size due to different total oil phase concentration were not significant, the impact of total oil phase concentration were more significant on the turbidity (measured by absorbance at 600 nm) and viscosity (Table 2). It was expected that when turbidity increases with increasing oil phase concentration due to formation of more and larger oil droplets, the amount of light scattering increases. In contrast, as SOR increased, turbidity decreased as more surfactant particles are able to adsorb on the oil droplet and form smaller oil droplets with weaker light scattering ability (McClements & Rao, 2011).

Similar to the observation in this study, Rao and McClements (2011) have also reported that SOR has a major impact on the particle size and turbidity of sucrose monopalmitate-lemon oil nanoemulsion. On the other hand, Qian and McClements (2011) found that energy as high as 4000 bar to 14,000 bar (delivered by microfluidiser) are required to form a 5% (w/w) corn oil-in-water emulsion stabilised by 2% β-lactoglobulin with particle size less than 250 nm. Another report of Liang et al. (2012) found that when the HPH pressure was between 1000 and 1500 bar, more than 10 cycles of HPH were required to reduce nanoemulsion particle size (containing 2% peppermint oil, 10% medium chain triglyceride, and 12% waxy modified starch) to about 200 nm. The HPH in this study is limited to a maximum pressure of 1000 bar and not equipped with temperature control system for excessive cycles of HPH as the high energy could have increased the temperature of emulsion to more than 80 °C, thus it was not surprising that the particle size of nanoemulsion formed here were larger than 250 nm.

When increasing both SOR and total oil phase concentration viscosity increased significantly (Table 2). The high viscosity of dispersed phase has increased the amount of energy required to break down the particle to a smaller size thus it is not favourable (Qian & McClements, 2011). In contrast, increasing the viscosity of aqueous phase through addition of co-solvents such as glycerol may have helped to reduce the particle size (Qian & McClements, 2011). All nanoemulsion showed surface tension about 1.8 times lower than that of water (about 72 mN/m), a lower surface tension favors formation of oil droplet with smaller particle size by decreasing the Laplace pressure (Qian & McClements, 2011).

The base formulation of nanoemulsion was determined to have 3.5% oil, 3.5% Tween 80 (SOR 1:1), which has the smallest D50 particle size of 318 ± 13 nm, the turbidity and surface tension of this formulation also the lowest. Chang et al. (2013) have also found that SOR 1:1 is the most suitable ratio for producing carvacrol nanoemulsion using Tween 80 as surfactant.

3.2. Antimicrobial nanoemulsion produced by ultrasonification (USF)

After the base formulation was determined, the production of nanoemulsion using USF was explored. This switch in production method could provide better control of nanoemulsion sterility, as the USF probe can be sanitised prior to contacting the nanoemulsion, while the enclosed HPH system is difficult to clean and sanitise. To better fabricate the nanoemulsion, the effect of ultrasonic processing times on physical characteristics of nanoemulsion were investigated. The carvacrol nanoemulsion produced by HPH and USF were also compared (Table 3).

As seen in Table 3, increasing in ultrasonic processing time led to a decreasing trend of overall particle size, the effect was significant when sonication time increased from 0 to 5 min. After 5 min, D10 and D50 remained stable to pro-longed sonication and only D90 was reduced when sonication time increased to 10 min. The result was expected since longer sonication time leads to higher total energy input to the system for breaking up bigger droplets into smaller ones. A total sonication time of 10 min was found to reduce the particle size (D50) more than 20 times compared to the emulsion before sonication (Table 3), the particle size (D10, D50, D90) was similar to the nanoemulsion produced by HPH method. Hence, the USF processing time was set at 10 min for production of carvacrol nanoemulsion. The turbidity of nanoemulsion followed the trend of particle size which decreased significantly when ultrasonification time increased; the nanoemulsion was changed from milky white to more translucent.

Table 2
Effect of surfactant-to-oil ratio (SOR) and oil concentration on turbidity, viscosity and surface tension of corn-oil-in-water emulsions prepared by HPH.

Oil phase (%, w/w)	SOR	Turbidity	Viscosity (cP)	Surface tension (mN/m)
3.5	1:9	3.15 ± 0.05^{hij}	4.80 ± 0.27^{a}	42.3 ± 0.6^{ab}
	2:8	2.94 ± 0.03^{gh}	4.91 ± 0.22^{a}	42.5 ± 1.3^{ab}
	3:7	2.24 ± 0.06^{f}	5.07 ± 0.11^{ab}	42.3 ± 0.9^{ab}
	4:6	1.20 ± 0.16^{c}	5.27 ± 0.12^{bc}	42.3 ± 1.2^{ab}
	1:1	0.59 ± 0.16^{a}	5.47 ± 0.09^{cd}	42.0 ± 0.6^{a}
5.0	1:9	3.24 ± 0.16^{ij}	4.97 ± 0.19^{a}	42.9 ± 0.5^{ab}
	2:8	3.00 ± 0.05^{hi}	5.31 ± 0.19^{bc}	42.8 ± 0.8^{ab}
	3:7	2.70 ± 0.09^{g}	5.48 ± 0.28^{cd}	44.3 ± 0.8^{cde}
	4:6	1.96 ± 0.41^{e}	5.73 ± 0.30^{de}	44.4 ± 0.7^{de}
	1:1	0.88 ± 0.36^{b}	6.12 ± 0.22^{f}	45.3 ± 0.5^{e}
10.0	1:9	3.38 ± 0.08^{j}	5.43 ± 0.44^{cd}	42.8 ± 0.3^{ab}
	2:8	3.25 ± 0.08^{j}	5.97 ± 0.21^{ef}	42.1 ± 0.6^{a}
	3:7	3.13 ± 0.04^{hij}	6.54 ± 0.33^{g}	42.3 ± 1.0^{ab}
	4:6	2.71 ± 0.06^{g}	7.18 ± 0.19^{h}	43.3 ± 1.0^{bc}
	1:1	1.69 ± 0.53^{d}	8.46 ± 0.35^{i}	44.1 ± 0.9^{cd}

*Within same column, groups with different small case letters are significantly different from each other ($P < 0.05$).

Table 3
Effect of sonication time on particle size, turbidity, viscosity and surface tension of nanoemulsion.

Sonication time/ min	Particle size/ nm			Turbidity	Viscosity (cP)	Surface tension (mN/m)
	D10	D50	D90			
#HPH	177 ± 6^{a}	318 ± 13^{a}	583 ± 46^{a}	0.59 ± 0.16^{b}	5.47 ± 0.09^{a}	42.0 ± 0.6^{b}
0	6711 ± 269^{c}	8561 ± 314^{c}	10895 ± 364^{d}	4.07 ± 1.80^{f}	5.52 ± 0.24^{a}	43.1 ± 0.8^{c}
1	613 ± 38^{b}	6001 ± 894^{b}	8399 ± 205^{c}	2.55 ± 0.04^{e}	5.45 ± 0.18^{a}	43.0 ± 0.7^{c}
5	192 ± 3^{a}	353 ± 4^{a}	796 ± 38^{b}	0.81 ± 0.03^{c}	5.57 ± 0.16^{a}	44.6 ± 0.6^{d}
^10i	167 ± 2^{a}	296 ± 5^{a}	564 ± 14^{a}	0.33 ± 0.02^{a}	5.51 ± 0.10^{a}	43.4 ± 0.5^{c}
^10e	176 ± 8^{a}	317 ± 20^{a}	645 ± 90^{ab}	–	–	–
^@CRV-i	176 ± 9^{a}	309 ± 19^{a}	515 ± 31^{a}	2.30 ± 0.20^{d}	5.51 ± 0.2^{a}	37.6 ± 0.6^{a}
^@CRV-e	171 ± 6^{a}	298 ± 13^{a}	502 ± 25^{a}	–	–	–

*Within same column, groups with different small case letters are significantly different from each other ($P < 0.05$).
#HPH indicates the blank nanoemulsion (3.5% corn oil, 3.5% Tween 80) that homogenised by high pressure homogeniser.
^The lower case letters "i" and "e" indicate the results at initial (day 0) and end of storage (day 30), respectively.
@CRV indicates the carvacrol nanoemulsion, the total oil phase consisted of 1% carvacrol + 2.5% corn oil; CRV was homogenised by ultrasonification for 10 min.

After the base formulation and USF processing time had been confirmed, carvacrol nanoemulsion (CRV) was produced using 10 min of sonification at 100% amplitude and the formulation contained 1% carvacrol, 2.5% corn oil, 3.5% Tween 80. According to Chang et al. (2013), the ratio of carrier oil (medium chain triglyceride) to carvacrol should be about 3:1 to 3:2 to give maximal stability and retain antimicrobial effect. From Table 3, the particle size of CRV was comparable to the blank nanoemulsion (NE). CRV nanoemulsion appeared significantly more turbid than the NE similar to observation of López-Mata López-Mata et al. (2013), due to different refractive index of carvacrol than corn oil. In addition, the surface tension of CRV was significantly lower than that of NE, the low surface tension is desirable in the application on waxy fruits and vegetable surface.

The nanoemulsion was stored for 30 days at 25 °C and the particle size was evaluated (Table 3), there was no significant change in the particle size between the freshly prepared emulsions and the stored emulsions. Also, no phase separation or creaming of the emulsions was observed after 1 month of storage. This indicated that the nanoemulsion is shelf-stable for at least 1 month.

3.3. Antimicrobial activity of carvacrol nanoemulsion against E. coli and P. pastoris

All nanoemulsion was diluted 2-fold for application, the composition of treatment groups (Ne, Crv and CrvA) are listed in Table 1. From Fig. 2a, when the treatment solution (1 ml) of Crv was added into 9 ml of inoculated broth, the growth of *E. coli* and *P. pastoris* were reduced by 3 log CFU/ml compared to control (deionised water). When Crv was combined with AEW (CrvA), the log reduction was enhanced by an additional 0.4 log and 0.6 logs for *E. coli* and *P. pastoris*, respectively. The addition of blank nanoemulsion (Ne) alone also reduced about 0.3 log of both microorganisms. When comparing initial inoculation level of *E. coli* (7 log CFU/ml) and *P. pastoris* (6 log CFU/ml), there were higher reduction of viable counts for *P. pastoris* (> 2 log reduction) than *E. coli* (< 1 log reduction) in Crv and CrvA treatment groups.

To preserve the antimicrobial properties of nanoemulsion, Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, and Martín-Belloso (2014) recommended microfluidisation over ultrasonification as processing method. Due to the high heat generated during ultrasonification process (100% amplitude, 180 s, 47 °C), a lemongrass oil nanoemulsion showed about 23.5 times degradation of antimicrobial activity with only 0.3 log CFU/ml of reduction of *E. coli in vitro* (Salvia-Trujillo et al., 2014). However, the antimicrobial activity of the carvacrol nanoemulsion produced in our experiment by ultrasonification (100% amplitude, 10 min, 25 °C) was well preserved with log reduction of at least 3 log, suggesting that the processing temperature and heat stability of the active component can be more important than the processing method.

It is reported that at least 0.0625% (w/w) to more than 1% (w/w) of carvacrol was required to inhibit at least 0.5 log of acid-resistant spoilage yeasts (Chang et al., 2013). Also, Donsì et al. (2012) have found that 0.1% (w/w) carvacrol is required to inactivate *E. coli* ATCC 26 and *Lactobacillus delbrueckii*. Our results suggested a lower inhibition concentration 0.05% (w/w) of carvacrol in broth could be used to inhibit the growth of *E. coli* ATCC 25922 and *P. pastoris* GS115 of about 3 log reduction.

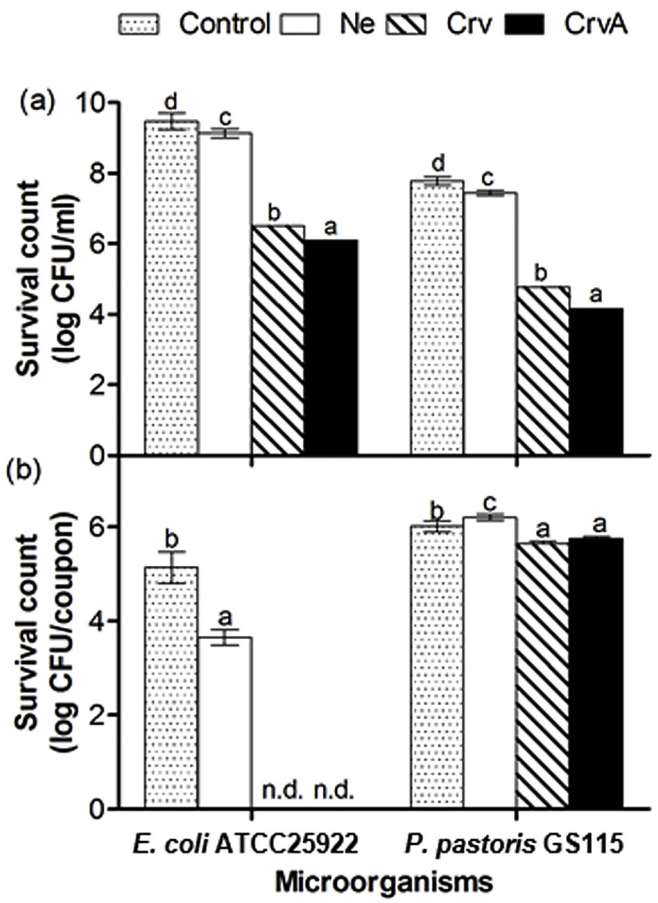

Fig. 2. Antimicrobial activity of treatment solutions against *E. coli* and *P. pastoris* (a) growth in broth (b) dried on stainless steel (SS) coupon surface. *Within same type of microorganism, treatment groups with different small case letters are significantly different from each other ($P < 0.05$).*The initial survival counts of *E. coli* and *P. pastoris* after drying on the SS coupon surface were 6.25 ± 0.23 and 6.57 ± 0.21 log CFU/coupon, respectively. *n.d. indicated the micro-organism were not detected, the detection limit was 2 log CFU/ml.

Microorganisms that attached and dried on surface possess greater resistance to antimicrobial agent than planktonic cells (Zhao et al., 2017). About 8.4 log CFU/coupon of *E. coli* and *P. pastoris* were inoculated on stainless steel (SS) surface. After drying, the level of *E. coli* and *P. pastoris* was reduced to 6.25 ± 0.23 and 6.57 ± 0.21 log CFU/coupon, respectively. As shown in Fig. 2b, control treatment by deionised water mildly reduced *E. coli* and *P. pastoris* adhering on SS surface, the remaining viable counts of *E. coli* and *P. pastoris* were 5.14 ± 0.33 and 6.01 ± 0.12 log CFU/coupon. *E. coli* was susceptible to the blank nanoemulsion (Ne), the viable count was reduced by 2.6 log compared to the initial level after drying. Treatments of Crv and CrvA reduced the viable *E. coli* below detection limit (2 log CFU/coupon), the reduction of *E. coli* by Crv and CrvA was at least 4.14 log compared to the initial level after drying. *P. pastoris* showed higher resistance than *E. coli*, the blank Ne treatment was even less effective than the control treatment, when subject to the antimicrobial treatment of Crv and CrvA, the survival count of *E. coli* was as high as 5.65 ± 0.05 and 5.75 ± 0.05 log CFU/coupon, respectively.

Zhao et al. (2017) treated air-dried *E. coli* and *P. pastoris* with neutral electrolysed water (4 mg/L FAC), with a longer treatment time of 5 min, the *E. coli* and *P. pastoris* were reduced by 2.31 and 1.41 log, respectively. However, in our experiment, the main antimicrobial effect observed was contributed by carvacrol or the nanoemulsion (for *E. coli* only), since there was no significant different between the level of survival microorganisms treated by Crv and CrvA. It is likely that the FAC in AEW degraded upon mixing with nanoemulsion, as the FAC is susceptible to organic substances. The actual FAC concentration remaining in the samples hence could be lower than 4 mg/L. Carvacrol was found to be effective against wine spoilage yeasts with a minimum inhibitory concentration (MIC) of 1.6–12.8% (Chavan & Tupe, 2014). For pathogenic bacteria including *E. coli*, *Staphylococcus aureus*, *L. monocytogenes* and *Salmonella* Typhimurium, the MIC of carvacrol was much lower at about 0.0175%–0.5% (Burt, 2004). This was consistent with the greater log reduction observed in *E. coli* than *P. pastoris* after treatment.

3.4. Antimicrobial effect of nanoemulsion on shredded-cabbages

The antimicrobial effect of the treatment groups on shredded cabbages is shown in Fig. 3. After shredding of cabbages, the total

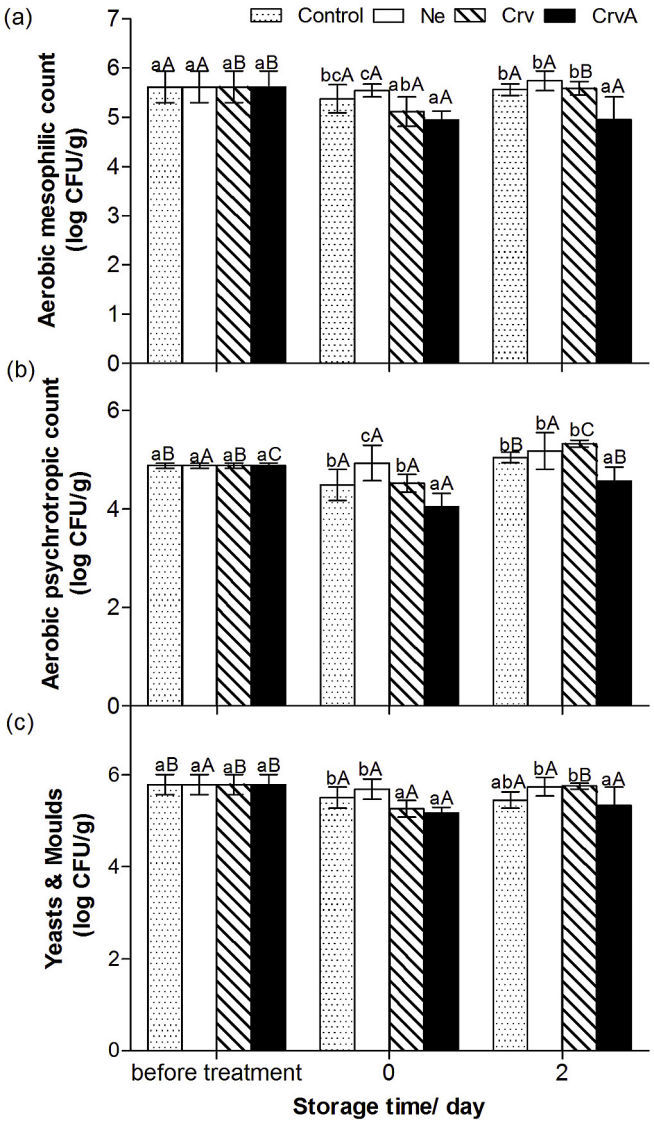

Fig. 3. Total viable counts of (a) aerobic mesophilic bacteria (b) aerobic psychrotropic bacteria and (c) yeasts and moulds on shredded cabbages before, after treatment (day 0) and storage up to 2 days at 7 ± 2 °C. *Within same time frame, groups with different small case letters are significantly different from each other ($P < 0.05$); within same treatment groups, groups with different capital letters are significantly different from each other ($P < 0.05$). *The detailed composition of Ne, Crv and CrvA were defined in Table 1.

viable mesophilic bacteria were as high as 5.61 ± 0.32 log CFU/g, this exceed the Singapore regulation limit of 5.0 log CFU/g of aerobic bacteria for a ready-to-eat food (AVA, 2006). After washing the shredded cabbages in sterile deionised water for 1 min, reduction of psychrotropic bacteria from 4.88 ± 0.55 to 4.48 ± 0.32 CFU/g and yeasts and moulds counts from 5.79 ± 0.22 to 5.50 ± 0.23 log CFU/g were observed in control group. However, the mesophilic bacteria of control group were not significant from the level before treatment. This indicated that the usual practice of washing cabbages with deionised water was not sufficient to produce ready-to-eat shredded cabbages. Also, the blank nanoemulsion alone (Ne) did not showed any antimicrobial effect on shredded cabbages.

The antimicrobial effect was observed on cabbages treated by carvacrol nanoemulsion (Crv) and the combined use of Crv and AEW (CrvA), with a stronger and more lasting antimicrobial effect observed with CrvA. On day 0 (after treatment), compared to control, the treatment of CrvA significantly decreased viable counts of mesophilic bacteria, psychrotropic bacteria and yeast and moulds to 4.94 ± 0.18, 4.05 ± 0.27 and 5.17 ± 0.12 log CFU/g, respectively (Fig. 3). Without AEW, Crv treatment alone only achieved reduction of yeast and moulds compared to control on day 0, but no reduction of mesophilic and psychrotropic bacteria. The antimicrobial effects were followed up to day 2, the microorganism levels on cabbages treated by CrvA remained lower than control, although a slight increase in psychrotropic bacteria was observed. In contrast, the antimicrobial activity of Crv was loss after 2 days of storage, the mesophilic bacteria as well as yeast and moulds increased to the level before treatment, the total viable psychrotropic counts even higher than the cabbages before treatment.

As a summary, the results indicated that the antimicrobial activity of Crv on fresh-cut cabbage was only observed on day 0, CrvA has stronger antimicrobial activity that can reduce the level of aerobic bacteria below 5 log CFU/g and the effect can last for 2 days. The antimicrobial effect of CrvA was slightly stronger against mesophilic and psychrotropic bacteria than yeasts and moulds, which was similar to the results of antimicrobial testing against *E. coli* and *P. pastoris* that dried on SS coupon.

It was observed that the colour of Crv and CrvA samples changed from green to brownish-white on day 2 onwards, which may suggest structural damage to the cabbage. It was reported previously that turnip-cabbages leaves could be dried and discoloured due to phytotoxic effect of *Nepeta cataria* and *Rosmarinus officinalis* oils (Pavela, 2006), while carvacrol was one of the major compounds found in *Nepeta cataria* (Li, 2000). Hence, there is concern of phytotoxic effect due to introduction of carvacrol on cabbages, which might limit the use of this active ingredient on cabbages. On the contrary, an oregano oil (0.05%, v/v) nanoemulsion with carvacrol as active component was reported to be very effective on controlling *E. coli* O157:H7, *Salmonella* Typhimurium and *L.monocytogenes* on fresh cut lettuce, the reduction level was more than 2 log reduction up to 72 h of storage (Bhargava, Conti, da Rocha, & Zhang, 2015). The concentration of carvacrol or the susceptibility of the microorganism might contribute to the differences of the results.

Previous reports of Zhang and Yang (2017) did not find antimicrobial effect from electrolysed water of 4 mg/L FAC on the native microflora on fresh-cut lettuce, a higher concentration of FAC to 75 mg/L in AEW was required to observe log reduction on native microflora on shredded turnip (Tan et al., 2015). As suggested earlier in the results of antimicrobial treatment against dried cells on SS surface, it is possible that the FAC in AEW degraded during mixing with nanoemulsion, as well as contact with shredded cabbages, rendered the FAC even lower than $\leq$ 4 mg/L, this suggests that the antimicrobial effect observed from CrvA might be due to synergistic effect between AEW and carvacrol nanoemulsion. The slightly lower pH of CrvA (4.13 ± 0.13) than Crv (4.65 ± 0.17) might contribute to the slight increase in antimicrobial effect. This is because the susceptibility of microorganisms to essential oils component was found to increase with decreasing pH, possibly due to the increase hydrophobicity and enhance lipid dissolving power targeting cell membrane of bacteria (Burt, 2004; Gutierrez, Barry-Ryan, & Bourke, 2008). The antimicrobial mechanism of carvacrol nanoemulsion and AEW could be further explored.

Compared to the antimicrobial testing *in vitro* (section 3.3), the antimicrobial activity of Crv and CrvA reduced. The actual uptake of carvacrol onto cabbages was not investigated but would likely to be lower than the amount introduced. In addition, antimicrobial activity of essential oils component could be decreased in complex food system compared to simple microbial growth media (Chen et al., 2014; Burt, 2004). For example, the minimum bactericidal concentration of carvacrol against 6 log CFU/ml of *L. monocytogenes* in broth was 0.03% (w/v), while the higher amount of carvacrol (0.25%, w/v) showed decreasing antilisterial activity from more than 5.3 log reduction in skim milk to −2.0 log reduction in full fat milk (Chen et al., 2014). Proteins and fats could hinder the antimicrobial effect of hydrophobic essential oil component by binding with it, since cabbages are generally low in fat and protein, other possible reason is the lower water content of food that delay the progress of antibacterial agents to the bacteria cell (Burt, 2004).

It is possible to increase antimicrobial activity of carvacrol nanoemulsion on shredded cabbages by prolong the treatment time, as shown by Donsì et al. (2014) who studied infusion of carvacrol nanoemulsion (0.5–2%, w/w) in zucchini and cooked sausage inoculated with *E. coli* ATCC 26, the log reduction of microbial level increased from 0.8 to 1.6 times with increasing treatment time from 1 to 5 min. In addition, adjustment of carrier oil-to-carvacrol ratio (Chang et al., 2013), increment of available concentration of carvacrol (Donsì et al., 2012), as well as reduction of particle size of nanoemulsion (Donsì et al., 2014) might also help to increase the antimicrobial activity of carvacrol nanoemulsion. For direct application of nanoemulsion on ready-to-eat food, the optimisation of nanoemulsion formulation via reducing oil and surfactant level, the selection of food grade surfactant such as sucrose esters and β-lactoglobulin (Qian & McClements, 2011; Rao & McClements, 2011), as well as application in other food matrix as an antimicrobial additive could be further explored. Sensory and consumer acceptance must also be considered for the actual food application, especially as the essential oil component is highly volatile, which could influence odour and flavour of food.

4. Conclusion

It is feasible to produce nanoemulsion by using high pressure homogeniser and ultrasonification with comparable results. The final carvacrol nanoemulsion consisted of 3.5% Tween 80, 2.5% corn oil and 1% carvacrol, the particle size (D50) was 309 ± 19 nm and it was stable for one-month storage at 25 °C. The antimicrobial activity of carvacrol nanoemulsion was proven from the results of inhibition of *E. coli* and *P. pastoris* to grow in nutrient broth at concentration as low as 0.05% (w/w) in broth. The carvacrol nanoemulsion (0.5%, w/w) could also be used to sanitise stainless steel (SS) surface by effectively inactivated microorganisms dried on SS coupon surface, higher antimicrobial activity was observed against *E. coli* then *P. pastoris* on SS surface. AEW can be used together with carvacrol nanoemulsion, the antimicrobial activity was the strongest and able to reduce the aerobic bacteria count on shredded cabbages below the level safe for consumption, this antimicrobial activity was last for 2 days.

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