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

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\begin{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 \textit{Escherichia coli} ATCC 25922 and \textit{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 \textit{E. coli} than \textit{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 /C204 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.
\end{abstract}

\section{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,
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; Donsi 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 H2O2, 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 vegetables. 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 (≥ 99.5%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Polyoxymethylene (20) monooleate (OmniPure® 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, Hielser, 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., 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 Nouy 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 recombined and/or resuspended 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, Smititec (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 Cl₂, 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 CRV (2.5% corn oil labelled as NE (3.5% corn oil, w/w) while the carvacrol nanoemulsion was labelled as CRV (2.5% carvacrol, w/w) and CRV were diluted with sterile deionised water in 1:1 (v/v) to achieve FAC concentration of 3.5% corn oil and 2.5% corn oil, 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 10⁷ CFU/ml and 10⁶ 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 to achieve FAC concentration of 2.5% corn oil and 1.5% carvacrol (w/w), respectively.

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 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 psychrotrophic count was determined from PDA 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

<table>
<thead>
<tr>
<th>Composition/ % (w/w)</th>
<th>Treatment groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carvacrol</td>
<td>Control Ne Crv CrvA</td>
</tr>
<tr>
<td>Corn oil</td>
<td>0.0</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.0</td>
</tr>
<tr>
<td>Deionised water</td>
<td>100.0</td>
</tr>
<tr>
<td>AEW</td>
<td>0.0</td>
</tr>
<tr>
<td>pH</td>
<td>6.07 ± 0.11</td>
</tr>
</tbody>
</table>

*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.

* The final pH of the treatment solutions was listed for the ease of comparison.
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. When D50 decreased more than 20 times compared to the nanoemulsion produced by HPH method, smaller D10 value was similar to the nanoemulsion produced by HPH method. 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 nano-emulsion 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 nano-emulsion, 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 nanoemulsion before sonication (Table 3), 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).

After the base formulation and USF processing time had been confirmed, carvacrol nanoemulsion (CRV) was produced using ultrasonication for 10 min. The lower case letters indicate the results at initial (day 0) and end of storage (day 30), respectively. Table 3 lists 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 CPU/ml) and P. pastoris (6 log CPU/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-Grat, 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.

### 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 CPU/ml) and P. pastoris (6 log CPU/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-Grat, 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.
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 difference 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...
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 psychrotrophic bacteria from 4.88 ± 0.55 to 4.48 ± 0.32 log 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 show 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, psychrotrophic 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 psychrotrophic 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 psychrotrophic bacteria was observed. In contrast, the antimicrobial activity of Crv was lost after 2 days of storage. The mesophilic bacteria as well as yeast and moulds increased to the level before treatment, the total viable psychrotrophic 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 in control, the treatment of 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 psychrotrophic 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 cabbages 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 < 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 can 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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