University of Massachusetts Amherst

ScholarWorks@UMass Amherst

April 2020

UTILIZATION OF EMULSION CHEMISTRIES FOR DELIVERY AND ANTIVIRAL APPLICATION OF CARVACROL

Hao-yuan Hsu
University of Massachusetts Amherst

Recommended Citation

UTILIZATION OF EMULSION CHEMISTRIES FOR DELIVERY AND ANTIVIRAL

APPLICATION OF CARVACROL

A Thesis Presented

By

HAO-YUAN HSU

Submitted to the Graduate School of the

University of Massachusetts Amherst in partial fulfillment

of the requirements for the degree of

MASTER OF SCIENCE

February 2020

Department of Food Science.

UTILIZATION OF EMULSION CHEMISTRIES FOR DELIVERY AND ANTIVIRAL

APPLICATION OF CARVACROL

A Thesis Presented

By

HAO-YUAN HSU

Approved as to style and content by:

_____

Matthew D. Moore, Chair

_____

Alissa A. Nolden, Member

_____

John Gibbons, Member

_____

Eric A. Decker, Department Head

Department of Food Science

ABSTRACT

UTILIZATION OF EMULSION CHEMISTRIES FOR DELIVERY AND ANTIVIRAL

APPLICATION OF CARVACROL

FEBUARY 2020

HAO-YUAN HSU, B.S., ASIA UNIVERSITY, TAIWAN

M.S., UNIVERSITY OF MASSACHUSETTS, AMHERST

Directed by: Professor Matthew D. Moore

Human norovirus (HuNoVs) are the most common enteric pathogen around the world that cause ~50% of foodborne illness of disease outbreaks annually. HuNoVs are the member of the *Caliciviridae* family, which consist of small (38 nm), unenveloped, single stranded RNA (ssRNA) viruses. Norovirus are divided into 5 genogroup (GI, GII, GIII, GIV, GV, GVI and GVII). The GI, GII, and GIV cause human illness, in addition, GII.4 genotype cause the most human disease. Due to HuNoVs are difficult cultured *in vitro*, the cultivable HuNoVs surrogates have been widely studied. Recently, some studies have been conducted with HuNoVs surrogates, for example bacteriophage MS2. MS2 is conservative surrogate for nonenveloped viruses which there is a close relationship to the behavior of HuNoVs, thus we can examine the infection control measures for HuNoVs. Despite plenty of treatment method been done on testing antiviral effect on bacteriophage MS2, for example UV inactivation, steam ultrasound and antimicrobial etc., plant-based nanoemulsion treatment has yet to be explored. Carvacrol is a major component of oregano essential oil and is responsible for their antimicrobial activity on the growth of various microorganism. In this study, carvacrol nanoemulsions were formed by using the spontaneous emulsification for

testing the nanoemulsion stability (14 days shelf life study on its droplet size and particle charge) and antimicrobial activity.

In carvacrol nanoemulsion 14 days shelf life test, the droplet size and particle charge stay stable at three different treatment environments (4°C, 20°C and 37°C). The results proved that nanoemulsion (was formed with surfactant agents and medium-chain triglycerides) is stable system that gives consistent droplet size and charge. Although, the low antimicrobial activity was investigated at carvacrol nanoemulsion, the strong antimicrobial effects have been found when carvacrol or carvacrol combined with ionic surfactant of treatment on MS2 and *Escherichia coli*. Taken together, in the wake of growing consumer demand for different "natural" products in a number of industries, our study broadly informs the development and study of functionalized carvacrol active compound that can not only provide beneficial health for human but can also examine antimicrobial efficacy of control measures for public health.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

REVIEW: DEVELOPMENTS IN INACTIVATION OF INFECTIOUS HUMAN NOROVIRUSES

1.1. Introduction

HuNoVs are the most common cause of epidemic and sporadic acute gastroenteritis around the world which are belong to the member of the *Caliciviridae* family. Noroviruses (NoVs) consist of small, 38 nm, nonenveloped single stranded RNA (ssRNA) viruses they are divided into 5 genogroups (GI, GII, GIII, GIV, GV, GVI and GVII); of which genogroups I, II and IV cause human illness. The genotypes are further divided from genogroup, and GII.4 causing the most HuNoVs disease (Moore et al., 2015). HuNoVs have a low infectious dose, as few as 18 viral particles, and it can be spread through fecal-oral-transmission, deposition on surfaces, and through airborne droplets of vomitus. Furthermore, HuNoVs can be easily spread through consumption of food, water and environmentally after deposition on surfaces. Therefore, identification and use of effective HuNoVs inactivation agents are crucially researched.

Although, in vitro cultivation techniques for HuNoVs have been reported (Ettayebi et al., 2016; Jones et al., 2014), these still difficult for utilizing on the study of HuNoVs inactivation. Therefore, reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is commonly used to quantify HuNoVs inactivation. However, viral reduction of RT-qPCR signal does not completely correspond to viral infectivity. Although, some techniques (porcine gastric mucin (PGM) binding assay) have been developed for use in conjunction with RT-qPCR (Manuel et al., 2018), the technique still cannot completely

present the entire RNA sequence for RT-qPCR. This is due to the fact that infectious or damaged protein may still attach with RT-qPCR when detect the antiviral effect. In addition to RT-qPCR, HuNoVs inactivation is also considered by using the close relationship of cultivable surrogate viruses; however, these surrogates have potential limitations in their translation to HuNoVs inactivation (Richards, 2012). Using these methods and treatments, numerous antimicrobial agents have been investigated; however, the identification of effective agents that are not consider for food grade supply. Additionally, numerous advances and investigation of HuNoVs inactivation agents have been reported in recent years. The purpose of this review is to present recent studies on HuNoVs inactivation that specifically by utilizing HuNoVs.

1.2. Human Norovirus Inactivation on Hands, Finger Pads or With Hand Sanitizer

The efficiency of handwashing with soap and water containing sterillium propan-1-ol 30% and mecetroniumetil sulfate sterillium, Viruguard hand disinfectants and Unicura hand soap were tested against NoVs GI.4 and GII.4 using finger pad tests (Tuladhar et al., 2015). The finger pads were contaminated with virus and dried before being applied to the treatments. Washing with soap and water removed genomic copies of noroviruses GI.4 (>6 $\log_{10}$), and GII.4 (4 $\log_{10}$) completely from all finger pads. Treating hands with propanol-based hand disinfectant showed low or no reduction to complete reduction with mean genomic copy reduction of NoVs GI.4 (>2.6 $\log_{10}$) and GII.4 (>3.3 $\log_{10}$) showed in Table 1.

In a recent study, two alcohol-based hand washes, quaternary ammonium compounds and chlorine dioxide were all ineffective at promoting virolysis of human norovirus (Nowak et al., 2011). However, it was found that NoVs GII.4 were sensitive (99.92% RNA digested) to a combination of heat and alkali condition (0.1M NaOH at 50°C) (Table 1). The authors examined the persistence of the NoV GII.4 by RT-qPCR for the amplification, for detecting

2

on inactivation effect.

Another study presented in 2010 has tested the effectiveness of sodium hypochlorite and ethanol (Liu et al., 2010) at different concentrations. The antibacterial hand sanitizer were observed for the inactivation of norovirus on finger pads. It was found that sodium hypochlorite has strong inactivation between 160 ppm and 1600 ppm after an exposure of 30s, a 5 $\log_{10}$ reduction was observed at sodium hypochlorite concentrations of 160 and 1,600 ppm on GI.1 (Table 1). At ethanol test, 3, 17, 31, 47, 62, and 95% concentrations were low antiviral efficacy (0.5 $\log_{10}$ reduction). Antibacterial liquid soap treatment gave a reduction of 0.67 to 1.20 log10 reduction and a water rinse only gave 0.58 to 1.58 $\log_{10}$ reduction. The alcohol-based hand sanitizer was low inactivation, reducing the norovirus genomic copies less than water alone, with only a 0.14 to 0.34 $\log_{10}$ reduction. The treatments in this study suggest that ethanol should not be used as an inactivation method.

VIRUSES	INACTIVATION AGENT	RANGE OF CONCENTRATIONS OR TREATMENT TIME	QUANTIFICATION METHOD	RANGE OF REDUCTIONS	REFERENCE
GI.4, GII.4	Washing with soap and water	30 s	Real-time PCR	GI.4:>6 $\log_{10}$, GII.4: 4 $\log_{10}$	(Tuladhar et al., 2015)
GI.4 GII.4	Propanol-based hand disinfectant	30min	PCR units (PCRU)/mL	GI.4: >2.6 $\log_{10}$ GII.4: 3.3 $\log_{10}$	(Tuladhar et al., 2015)

GII.4	NaOH	0.1M at 50°C	RT-qPCR	99.92% RNA digested	(Nowak et al., 2011)
GI.1	Sodium hypochlorite	160 and 1600ppm for 30 s	Suspension assay	5 $\log_{10}$	(Liu et al., 2010)
GI.1	Ethanol	All concentration for 30s	Suspension assay	0.5 $\log_{10}$	(Liu et al., 2010)
GI.1	Antibacterial liquid soap	30s	RT-PCR	0.67 to 1.20 $\log_{10}$	(Liu et al., 2010)
GI.1	Water rinse	30s	American Society for Testing and Materials (ASTM)	0.58 to 1.58 $\log_{10}$	(Liu et al., 2010)
GI.1	Alcohol-based handwash	Containing 62% ethyl alcohol for 30s	ASTM	0.14 to 0.34 $\log_{10}$	(Liu et al., 2010)

Table 1. Inactivation of HuNoVs on hands, finger pads or with hand sanitizer

1.3. Human Norovirus Inactivation on Hard Surfaces

For a study conducted in 2017, the authors applied 7.5% hydrogen peroxide and a 0.2% chlorine dioxide-surfactant-based product using a fogging delivery system against NoVs GI.6 and GII.4 (Montazeri et al., 2017). At 12.4 ml/m^3 hydrogen peroxide, disinfectant achieved a 2.5 ± 0.1 and 2.7 ± 0.3 $\log_{10}$ reduction in NoV GI.6 and GII.4 genome copies within 5 min (Table 2). At the same disinfectant formulation concentration, 12.4 ml/m^3 chlorine dioxide-surfactant-based product resulted in 1.7 ± 0.2 and 0.6 $\log_{10}$ reduction in GI.6 and GII.4 within 10 min. However, increasing the disinfectant formulation concentration to 15.9 ml/m^3 negatively impacted its efficacy.

The efficiency (Table 2) of neutral electrolyzed water (NEW) was observed for the inactivation of GII.4 in suspension on stainless steel surfaces with and without an additional soil load (Norovirus et al., 2017). The degradation norovirus VP1 major capsid protein at 250 ppm around 5min, and increased virus particle aggregation at 150 ppm after 30min. Only the 250 ppm NEW concentration, without soil load, produced greater than a 5.4 $\log_{10}$ reduction in NoVs genome copy number. The contact time on surfaces to 5, 10 and 15 min reduced HuNoVs genomic copies by 0.5, 1.6 and 2.4 $\log_{10}$. Moreover, NEW at 250 ppm free available chlorine produced a 4.8 and 0.4 $\log_{10}$ reduction in norovirus genome copy number after 1 min in suspension and on stainless steel.

A blend of silver ions and citric acid (SDC) had an effect on HuNoVs GI.6 and GII.4 (Manuel et al., 2017). The suspension assays showed a 4 $\log_{10}$ reduction in RNA copy number within 5 min for both GI.6 and GII.4, along with a 2–3 $\log_{10}$ reduction in 30min (Table 2). The results showed no further additional $\log_{10}$ reduction when extend over than 5min. When incorporating a simulated soil load into the sample matrix significantly reduced formulation efficacy, ~2.5 $\log_{10}$ was achieved on both GI.6 and GII.4.

Fecal suspensions for a HuNoVs GII.4 or virus-like particles (VLPs) were exposed to copper alloys or stainless steel for 0, 60, 120, and 240min in a study conducted in 2015 (Manuel et al., 2015). When using RT-qPCR assays on stainless-steel, there was a 1.1 $\log_{10}$ reduction in RNA copy number after a time of 240min (Table 2). When exposed for 60 min, a 2–3 $\log_{10}$ reduction in RNA copy number was observed for surfaces containing 70% copper. The research also showed further evidence that although there was damage to the NoVs GII.4 capsid, HuNoVs remained stable on stainless steel surfaces for up to 240 min.

For a study in 2014, the authors applied different concentration of ethanol solutions, 70% and 90%, to test the reductions of NoVs GI.1, GI.5, GI.5 semi purified (SP) preparations, GII.13, and GII.13 SP preparations RNA levels after 1 min of exposure (Cromeans et al., 2014). The GI.1 RNA was reduced by as much as 1.1 $\log_{10}$ units on 90% of ethanol concentration, however, 70% was reduced by less than 1 $\log_{10}$ unit. The GI.5 SP RNA level was reduced by as much as 3.5 $\log_{10}$ units on both 70% and 90 % of ethanol concentration (Table 2). And GI.5 reach 2.0 $\log_{10}$ unit of reduction on 90% of ethanol concentration, whereas the GII.13 and GII.13 SP RNA levels were reduced <1 $\log_{10}$ unit. In the same article, the viruses were dried on stainless steel with fifty microliters of a chlorine solution at concentrations of 200 ppm or 1,000 ppm. Each was added to the virus for 5min. The GI.5 SP RNA level was reduced by <1 $\log_{10}$ unit after treatment with both 200 and 1,000 ppm chlorine. The GII.13 SP RNA level was reduced by <0.5 log10 unit at concentrations of 200 ppm or 1,000 ppm.

The qualities of HuNoVs attached to stainless steel disks was also observed in an article published in 2010. (Girard et al., 2010). The paper wanted to observe a technique for disinfecting NoVs using household disinfectants. The attachment of HuNoVs and murine norovirus (MNV) to stainless steel disks was tested against a range of pH and relative humidity (RH). The maximum attachment of 10^3 PFU was obtained after a contact time of 10 min. Interestingly, extending the contact time to 60 or 120min did not increase viral. A decrease in titer was more significant at low RH. When using household items for chemical treatments, sodium hypochlorite showed inactivation exceeding 3 $\log_{10}$ reduction for HuNoVs after a contact time of 10min (Table 2); however, only a 2 $\log_{10}$ reduction was obtained after 5 min. The study suggests that MNV was more sensitive than HuNoVs to chemical disinfectants. In the evaluation of disinfection efficacy, only sodium hypochlorite

was effective against NoVs.

VIRUSES	INACTIVATION AGENT	RANGE OF CONCENTRATIONS OR TREATMENT TIME	QUANTIFICATION METHOD	RANGE OF REDUCTIONS	REFERENCE
GI.6 GII.4	Hydrogen peroxide on Stainless steel	5 min at 12.4 ml/m^3	RT-qPCR,	GI.6: 2.5 ± 0.1 log$_{10}$ GII.4: 2.7 ± 0.3 log$_{10}$	(Montazeri et al., 2017)
GI.4 GII.4	Chlorine dioxide on stainless steel embossing	10 min at 12.4 ml/m^3	RT-qPCR,	GI.6: 1.7 ± 0.2 log$_{10}$ GII.4: 0.6 log$_{10}$	(Montazeri et al., 2017)
GII.4	NEW on stainless steel	250 ppm after 1, 5, 10, 15, and 30 min	RT-qPCR	0.4, 0.5, 1.6, 2.4, and 5.0 log$_{10}$	(Norovirus et al., 2017)
GII.4	NEW 160 and 1600ppm on stainless steel	At 250 ppm free available chlorine for 1min	Suspension assay	4.8 log$_{10}$ on suspension 0.4 log$_{10}$ on stainless steel	(Norovirus et al., 2017)
GI.6 GII.4	SDCon stainless steel surfaces.	5 min 30 min	Suspension assay	4.0 log$_{10}$ and 2.0 to 3.0 log$_{10}$	(Manuel et al., 2017)
GII.4 VLPs	Stainless steel alloys contained >70% copper	60 min 240min	RT-qPCR	2 to 3 log$_{10}$ 1.1 log$_{10}$	(Manuel et al., 2015)

GI.1	90% of ethanol	1 min of exposure	RT-qPCR	1.1 $\log_{10}$	(Cromeans et al., 2014)
GI.5, GII.13 and GII.13 SP	90% of ethanol on stainless steel	1 min of exposure	RT-qPCR	GI.5: 2 $\log_{10}$ GII.13 and GII.13 SP: <1 $\log_{10}$	(Cromeans et al., 2014)
GI.5 SP	70 and 90% of ethanol on stainless steel	1 min of exposure	RT-qPCR	3.5 $\log_{10}$	(Cromeans et al., 2014)
GI.5 SP GII.13 SP	Chlorine solution on stainless steel	200 ppm or 1,000 ppm for 5 min	RT-qPCR	GI.5 SP: <1 $\log_{10}$ GII.13 SP: <0.5 $\log_{10}$	(Cromeans et al., 2014)
NoVs	Sodium hypochlorite on stainless steel	5 and 10 min	RT-PCR and plaque assay	5min: 2 $\log_{10}$ 10min: 3 $\log_{10}$	(GIRARD et al., 2010)

Table 2. Inactivation of HuNoVs on hard surface

1.4. Human Norovirus Inactivation in Solution

In 2017, peracetic acid (PAA) and monochloramine in both wastewater (WW) and phosphate buffer (PB) were tested for their ability to inactive HuNoVs GI and GII (Dunkin et al., 2017). A 3.3 $\log_{10}$ reductions of GI was found when treated with 15 mg/l at a dose of monochloramine after 120 min with enzymatic pretreatment (EPT) (Table 3). At a high dose of 10 mg/l PAA predicted reductions of GI were 3.3 with EPT. In PB, monochloramine and PAA exhibited similar effectiveness against GI and GII, both disinfectants were able to achieve approximately 3 $\log_{10}$ reduction. In WW,

monochloramine and PAA were more effective in treating GI. Monochloramine was able to achieve ~ 2 log_{10} reduction of GI, while PAA has only achieved less than 1 log10 reduction. However, GII in WW as for both disinfectants were unable to achieve even 0.5 log10 reduction.

One in 2016 looked at ethanol, sodium hypochlorite, hydrogen peroxide, quaternary ammonium compounds, and iodine using an anti NoV GII.4 monoclonal antibody conjugated immunomagnetic separation (IMS) combined with qRT-PCR (Ha et al., 2016). Ethanol was diluted between 10%-70% and had no disinfection effect against GII.4 as shown by the less than 1 log10 reduction (Table 3). Sodium hypochlorite at 200, 500, and 1000 ppm resulted in mean log10 reductions of 1.55, 1.85, and 2.45 (Table 3); however, 50 and 100 ppm sodium hypochlorite shown by the log10 reductions of less than 1. Alkyl dimethyl benzyl ammonium chloride (40%), containing quaternary ammonium of treatments at 200, 1,000, and 2000 ppm achieved log10 reductions of 0.06 ± 0.12, 0.19 ± 0.13, and 0.58 ± 0.33 In this study, 200 and 1000 ppm quaternary ammonium compounds had almost no effect, and 2000 ppm demonstrated a mean log10 reduction of less than 1 after 10 min of contact. Iodine (99.99% trace metal basis) was diluted with deionized sterile water to 25, 100, 250, and 500 ppm. A 0.30 ± 0.05, 0.41 ± 0.06, 0.57 ± 0.14, and 0.71 ± 0.13 log10 reduced of NoV GII.4 was found.

One study (Koromyslova et al., 2015) found that NoVs that authors treated VLPs with different concentrations of citrate buffer. Between 0.49 and 7.85 mM of citrate buffer, the VLPs appeared no effect compared with untreated VLPs. However, at 15.63 mM, a small number of the VLPs had slightly altered morphology, i.e., the outer spikes of the VLPs were surrounded by a new ring-like structure. Where at 62.50 mM of citrate buffer the

majority of VLPs showed the ring-like structure. The diameters of the VLPs were manually measured at 0, 0.95, 7.81, 62.50, and 125 mM of citrate buffer shown in Table 3. At 0, 1, and 7.81 mM of citrate buffer, the diameter of the VLPs were 42 to 44 nm, while at 62.50 and 125 mM of citrate buffer, the diameter of the VLPs were 46 to 49 nm.

In another study, it was found that antiviral activity on NoVs were generally < 0.5 $\log_{10}$ reduction for both GII.2 and GII.4 when using 50, 70, and 90% ethanol solutions tested at (Grace et al., 2013). The disinfection efficacy for sodium hypochlorite, authors tested at concentrations of 5, 75, 250, 500, and 1,000 ppm. The results showed no significant inactivation at $< 1,000$ ppm on GII.2 strain. However, for the GII.4, efficacy was only observed at the highest concentration tested, 1,000 ppm, there was a strong antiviral (4.5 $\log_{10}$) reduction in viral genome copy number (Table 3). The quaternary ammonium compound blend were ineffective at inactivating both strains, with < 0.5 log reductions at all concentrations on GII.2 and GII.4.

Different concentrations of 50%, 70%, 90%, of ethanol and isopropanol were tested for inactivation in an article published by Park in 2016 (Park et al., 2016). NoVs positive stool specimens (14 GI and 16 GII) and three stool samples of GI.1 (from human volunteers) were suspended in ethanol and isopropanol. The result showed that exposure to 70% and 90% ethanol reduced viral RNA titers of 9 and 13 of the 14 GI strains by > 1.8 $\log_{10}$ reduction shown in Table 3. The titers of 4 (3 GI.6 and 1 GI.7) of the GI strains were > 1.8 $\log_{10}$ reduction after exposure to 90% isopropanol, whereas no RNA reduction was observed for 50% ethanol, or for 50% and 70% isopropanol. Exposure to 90% alcohols achieved 0.9 $\log_{10}$ reduction of all 9 GII.4 strains. Overall, exposure to 70% and 90% ethanol and 90% isopropanol resulted with an average of 1.2 ± 1.1, 1.4 ± 0.9, and $1.0 \pm$

0.8 $\log_{10}$ reduction on RAN copies. After exposure to 50% and 70% ethanol, RNA titers of GII.4 Den Haag and GII.4 Sydney viruses were showed >1.9 $\log_{10}$ reduction whereas the titers for GII.4 New Orleans viruses were reduced by less than 0.5 $\log_{10}$ reduction. After exposure to 50% isopropanol, RNA titers of both GII.4 Den Haag viruses and 3 of the 4 GII.4 Sydney viruses were achieved > 1.0 $\log_{10}$ reduction, while RNA titers of GII.4 New Orleans were reduced by 0.5 log10 RNA copies/ml.

It also has been stated that sodium hypochlorite, sodium hydroxide, sodium, ethanol, carbonate, potassium carbonate, potassium hydroxide and hydroxide can in active NoVs VLPs (Sato et al., 2016). The treatment on VLPs shown no change after 30s and 60s exposures to 200 ppm sodium hypochlorite, but were slightly deformed after exposure for 180s. VLPs were also slightly changed morphologically within 30s of exposure to 50% ethanol, but deformation after 60s with 60% ethanol. However, VLPs did not change morphologically after 180s of exposure to 12.5 mM carbonate but were slightly deformed after exposure to 25 mM for 10s. Deformation of VLPs was more marked after exposure to 25mM for 60s. Deformation and aggregation of VLPs were observed after exposure to sodium hydroxide and potassium hydroxide under specific conditions (Table 3). There were no significant differences between the morphology of particles treated with sodium and that of those treated with potassium hydroxide. VLPs were slightly deformed morphologically within 180s of exposure to 25mM hydroxide and within 10s of exposure to 50mM hydroxide.

Another study looked at the interactions of NoVs GII.4 with available chlorine (Illarruel-lopez, 2012). The results support the idea that the matrix effects have a significant effect on virus survival. GII.4 virolysis was measured using RNase pretreatment and RT-QPCR.

The 610ppm available chlorine at 10-13% concentration required to reduce GII.4 >4 $\log_{10}$ reduction in infectivity (Table 3).

Another study has observed sodium hypochlorite (Liu et al., 2015) at a concentration of 1600 ppm, produced complete inactivation of GI.1with an average of 4.84 $\log_{10}$ reduction and also completely inactivated GII.4 with an average 3.74 $\log_{10}$ reduction in 2min contact time. In contrast, 70% ethanol exhibited low antiviral activity, 0.81 and 0.14 $\log_{10}$ reduction for GI.1 and GII.4. Ammonium chloride exhibited no effect against either GI.1or GII.4. The disinfectant Oxivir-TB with 0.5% hydrogen peroxide exhibited 1.11 and 0.94 $\log_{10}$ reductions against GI.1and GII.4. Lysol with lactic acid, produced 2.29 $\log_{10}$ reduction in GI.1 and an average $\log_{10}$ reduction in GII.4 of 0.21. Exposure to the prototype disinfectant resulted in the greatest reductions of GI.1 (3.19 $\log_{10}$) and o GII.4 (1.38 $\log_{10}$) (Table 3).

VIRUSES	INACTIVATION AGENT	RANGE OF CONCENTRATIONS OR TREATMENT TIME	QUANTIFICATION METHOD	RANGE OF REDUCTIONS	REFERENCE
GI	Monochloram (15 mg-min/L)	120 min with enzymatic pretreatment (EPT)	RT-qPCR,	3.3$\log_{10}$	(Dunkin et al., 2017)
GI	Peracetic acid (PAA) (10 mg/L)	120 min with EPT	RT-qPCR,	3.3$\log_{10}$	(Dunkin et al., 2017)

GI GII	Monochloramine and PAA	120 min with 0.01M PB	RT-qPCR	3 $\log_{10}$	(Dunkin et al., 2017)
GI	Monochloramine and PAA	120 min with WW	RT-qPCR	Monochloramine: 2 $\log_{10}$ PAA: <1 $\log_{10}$	(Dunkin et al., 2017)
GII.4	Ethanol	Diluted to 10% to 70% 3000 ppm	IMS combined with qRT-PCR	<1 $\log_{10}$	(Ha et al., 2016)
GII.4	Sodium hypochlorite	200, 500, and 1000 ppm	IMS combined with qRT-PCR	1.55, 1.85, and 2.45 $\log_{10}$	(Ha et al., 2016)
GII.4	Alkyl dimethyl benzyl ammonium chloride (40%)	200, 1,000, and 2000 ppm	IMS combined with qRT-PCR	0.06 ± 0.12, 0.19 ± 0.13, and 0.58 ± 0.33 $\log_{10}$	(Ha et al., 2016)
GII.4	Iodine	25, 100, 250, and 500 ppm	IMS combined with qRT-PCR	0.30 ± 0.05, 0.41 ± 0.06, 0.57 ± 0.14, and 0.71 ± 0.13 $\log_{10}$	(Ha et al., 2016)
GII.4	Quaternary ammonium compounds	2000 ppm	IMS combined with qRT-PCR	<1 $\log_{10}$	(Ha et al., 2016)
VLPs.	Citrate buffer	0, 1, and 7.81 mM 30 min 62.50 and 125 mM 30 min	Electron microscopy and ELISA	VLPs from 42 to 44 nm VLP from 46 to 49 nm morphology	(Koromysl et al., 2015)

GII.2 GII.4	Ethanol solutions	50, 70, and 90%	RT-qPCR	<0.5 $\log_{10}$	(Grace et al., 2013)
GII.4	Sodium hypochlorite	1,000 ppm	RT-qPCR	4.5 $\log_{10}$	(Grace et al., 2013)
GII.2 GII.4	Quaternary ammonium compound	0.1, 1.0, and l0% concentrations	RT-qPCR	< 0 .5 $\log_{10}$	(Grace et al., 2013)
GI GI.6 GI.7	70% and 90% Ethanol and 90% isopropanol		RT-qPCR	> 1.8 $\log_{10}$	(Park et al., 2016).
GII.4	Ethanol and isopropanol	90%	RT-qPCR	0.9 $\log_{10}$	(Park et al., 2016).
GII.4	70% and 90% Ethanol and 90% isopropanol		RT-qPCR	70 and 90% ethanol:1.2 and 1.4 $\log_{10}$ 90% Isopropanol: 1.0 ± 0.8 $\log_{10}$	(Park et al., 2016).
GII.4 Den Haag and GII.4 Sydney	Ethanol	50% and 70%	RT-qPCR	>1.9 $\log_{10}$	(Park et al., 2016).
VLPs	200 ppm sodium hypochlorite	180s	TEM	Slightly deformed	(Sato et al., 2016)
VLPs	60% ethanol	60s	TEM	Deformation	(Sato et al., 2016)

VLPs	25mM carbonate	10s.	TEM	Slightly deformed	(Sato et al., 2016)
VLPs	25mM of sodium hydroxide and potassium hydroxide	180s	TEM	Slightly deformed	(Sato et al., 2016)
GII.4	10-13% Chlorine	610 ppm	Plaque assay and RT-qPCR	$>4 \log_{10}$	(Illarruel-lopez et al., 2012
GI.1 GII.4	Sodium hypochlorite	1600 ppm 2min	IMS /RT-qPCR	$4.84 \log_{10}$ $3.74 \log_{10}$	(Liu et al., 2015)
GI.1 GII.4	ethanol	70% ethanol 2-10min	IMS /RT-qPCR	$0.81 \log_{10}$ $0.14 \log_{10}$	(Liu et al., 2015)
GI.1 GII.4	Oxivir-TB with 0.5% hydrogen peroxide	5min	IMS /RT-qPCR	GI.1: 1.11 $\log_{10}$ GII.4:0.94 $\log_{10}$	(Liu et al., 2015)
GI.1 GII.4	Lysol lactic acid	5min	IMS /RT-qPCR	GI.1: 2.29 $\log_{10}$ GII.4: 0.21 $\log_{10}$	(Liu et al., 2015)
GI.1 GII.4	Prototype disinfectant using	4 to10min	IMS /RT-qPCR	GI.1: 3.19 $\log_{10}$ GII.4:1.38 log	(Liu et al., 2015)

Table 3. Inactivation of HuNoVs in solution

1.5. High Pressure Inactivation on Human Norovirus

High pressure processing (HPP) inactivation is commercial used to process

various kinds of foods mainly to increase their shelf life and enhance food safety by inactivating pathogenic bacteria. It is commercially used as a processing aid; for example, it has been used to facilitate oyster shucking. Commercially HPP-treated foods include those that have been involved in HuNoVs outbreaks, such as oysters, salsa, and guacamole (Li et al., 2015).

In a study conducted in 2012, the effectiveness of HPP is observed for its ability in disrupting the capsid of VLPs (Lou et al., 2012). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used, and the results showed that the integrity of the capsid structure was not disrupted when HuNoV VLPs were treated at 500 MPa for up to 30min. After pressurization for more than 45 min, the number of 38-nm particles observed was notably reduced shown in Table 4, while the 23-nm particles remained unaffected. The pressure was increased to 600 MPa at 4°C for 5 to 60 min, the results were essentially similar to those at 500 MPa. As the holding time increased to 60 min, the 38-nm VLPs disappeared, whereas the 23-nm VLPs were still intact. The pressure level was increased to 700 MPa, at 45 min, the 38-nm VLPs were undetectable (Table 4), but a considerable number of 23-nm particles were still present. At 800 MPa, the number of 38-nm VLPs was notably reduced after 15 min, and the 38-nm particles were undetectable after a 30-min treatment. The number of 23-nm particles also dramatically decreased after treatment at 800 MPa for 45 min. At 900 MPa, after a 1-min treatment, the number of 38-nm VLPs was significantly reduced, and after 2 min, no intact 38-nm VLPs were detected and the number of 23-nm VLPs was dramatically reduced

In 2011, it was also observed applying a high hydrostatic pressure (HHP) treatment can be used to inactive HuNoVs in HPP (Sanchez et al., 2011). Different time and pressure conditions were used on each sample, being 200, 300, 350, 400, 450, and 500 MPa for 15min at initial temperatures of 25°C and 45°C. All tested treatments reduced the numbers of HuNoV by < 0.5 $\log_{10}$ reduction as determined by RT-qPCR (Table 4); in other words, NoV was detected by RT-qPCR even after treatments at 500 MPa for 15 min. Similarly, the effect of HPP on NoVs in $CaCl_2$ resulted in inactivation no higher than 0.5 $\log_{10}$ reduction independently of treatment temperature.

In a more recent study from 2017, high hydrostatic pressure (HHP) was used on GII.4 and GI.1 when found on green onions and salsa (Sido et al., 2017). HHP inactivation studies were conducted at 100–600 MPa for 2 min at an initial temperature of 1°C to determine optimum HHP processing conditions. It was desired to achieve a ≥3 log reduction of the strains. To achieve >3 $\log_{10}$ reduction of GI.1, HHP treatment should be conducted at 600MPa and 500MPa for green onions and salsa respectively. To achieve >3 log reduction of GII.4, HHP treatment should be conducted at 500 MPa and 300MPa for green onions and salsa respectively. For green onions, HHP treatment could reduce GI.1 by >3.0 $\log_{10}$ at 600 MPa while >3.87 log reduction was achieved for GII.4 under the same condition (Table 4). The HHP treatment of 300 MPa reduced HuNoV GII.4 by 3.31 $\log_{10}$ reduction in salsa and 2.57 $\log_{10}$ reduction on green onions. Similar results were also found with salsa which showed 1.39 $\log_{10}$ reduction of HuNoV GI.1 and 3.52 $\log_{10}$ reduction of HuNoV GII.4 at 400 MPa. Food matrices also influenced HHP inactivation of GI.1 and GII.4, HuNoV showing higher

sensitivity to HHP treatment in salsa than on green onions.

Another group compared the results of HHP inactivation of a GI.1 and GII.4 strain using different PGM-MB/PCR (Li et al., 2015). HuNoV GII.4 and GI.1 were pressurized at 150 to 550, 50 to 400, 250 to 575, and 100 to 450 MPa by using an Avure PT-1 pressure unit. There was no virus inactivation at low pressure levels (50 to 200 MPa), and inactivation was found the pressure was increased above 550MPa. Results showed the HHP inactivation of GI.1 which showed the maximum of ~3 log reduction at 21°C natural PH under 550MPa and 21°C PH 4 under 550MPa. GII.4 had a maximum reduction of ~3.5 $\log_{10}$ units at both 4°C and 21°C natural PH under 250MPa (Table 4). However, increasing the pressure did not result in greater reduction. Both the GI.1 and the GII.4 strains were more sensitive to pressure at 4°C than at 21°C, along with neutral pH than at pH 4. It can be consulted that the GI.1 strain was more resistant to pressure than the GII.4 strain addressed Li in 2015.

In another study, it was found that HHP inactivation on HuNoV GI.1 and GII.4 strains under different pressure levels and temperatures were obtained using the direct RT-qPCR, PGM/PCR and PMA/ PCR assays (Li et al., 2017). HuNoV GI.1, and HuNoV GII.4 samples were HHP treated at 50–300, 250–550, and 100–400 MPa, respectively. HHP treatments were conducted at initial sample temperatures of 4 and 21 °C for 2 min using an Avure PT-1 pressure unit. Except for the HHP treatment of HuNoV GI.1 at 21 °C, direct RT-qPCR showed a <1.0 $\log_{10}$ reduction at all pressure level. At 500MPa pressure levels at 21°C, the PMA/PCR assay showed >2.5 $\log_{10}$ reduction of HuNoV GI.1 and ~1.7 $\log_{10}$

reduction at PGM/ PCR assay shown in Table 4. At 400MPa pressure levels at 21°C, the PMA/PCR assay and PGM/ PCR assay both showed >2.5 $\log_{10}$ reduction of HuNoV GI.1. For HuNoV GII.4, a maximum inactivation (~3.5 $\log_{10}$ reduction) was observed for both the PGM/PCR and PMA/PCR assays. The direct RT-qPCR showed much lower inactivation effect of HuNoV comparing to the other two assays. It could be logically concluded that the PGM/PCR and PMA/PCR assays were both better than the direct RT-qPCR assay.

VIRUSES	INACTIVATION AGENT	RANGE OF CONCENTRATIONS OR TREATMENT TIME	QUANTIFICATION METHOD	RANGE OF REDUCTIONS	REFERENCE
GI	HPP	500, 600MPa for 45 min. 700 and 800 for 30 min 900 MPa for 2min	SDS-PAGE	500, 600 MPa: notably reduced. 700 MPa: undetectable 800, 900MPa: undetectable.	(Lou et al., 2012)
GII	HPP	500, 600 and 700MPa for 45 min. 800 for 45 min 900 MPa for 2min	SDS-PAGE	500, 600 and 700 MPa: still intact 800 and 900 MPa: dramatically decreased	(Lou et al., 2012)

HuNoVs	HHP	200, 300, 350, 400, 450, and 500 MPa for 15min	RT-qPCR	<0.5 $\log_{10}$	(Sanchez et al., 2011)
GI.1 GII.4	HHP on green onions and salsa	600MPa and 500MPa (GI.1) 500 MPa and 300MPa (GII.4)	RT-qPCR	>3 $\log_{10}$ reduction	(Sido et al.,2017)
GI.1 GII.4	HHP	At 21°C with natural ph. and ph. 4 550MPa (GI.1) 250MPa (GII.4)	PGM/PCR PMA/PCR RT-qPCR	3.8 $\log_{10}$	(Li et al., 2015)
GI.1 GII.4	HHP	400 and 500MPa At 21°C	PGM/PCR PMA/PCR	GI.1: >2.5 log GII.4: ~3.5 $\log_{10}$	(Li, et al., 2017)

Table 4. High pressure inactivation of HuNoVs

1.6. Other Human Norovirus Inactivation Studies

Another study in 2017 (Li et al., 2017) compared the heat and HHP using HuNoV GI.1 strain and a GII.4 strains under different temperatures. The virus was evaluated using those different molecular assays by using the direct RT-qPCR, PGM/PCR, and PMA/PCR assays. For GI.1, the direct RT-qPCR assay showed no inactivation for all heat treatments at 60 to 90°C. The PGM/PCR assay and PMA/PCR assay showed different inactivation result. For example, for a 2min heat treatment at 90°C, PGM/PCR assay showed a 2.2 $\log_{10}$ reduction (Table 5) while PMA/PCR assay showed no inactivation (0-1.2 $\log_{10}$ reduction) from 60 to 80°C. The PGM/PCR assay showed a when increase of heat inactivation effect of GI.1 from 60 to 70°C followed by increase from 70 to 90°C (1.2 to 2.1 $\log_{10}$ reduction). As for HuNoV

GII.4, all three assays showed similar inactivation results, almost no inactivation of GII.4 from 60 to 80°C.

One study applied sodium metasilicate and sodium hypochlorite to fresh vegetables (Ha, et al., 2017). The research evaluated the efficacy of a range concentrations of 50-1000 ppm NaOCl, for reducing the amounts of HuNoV GII.4 on lettuce, celery, and white cabbage. The reductions of GII.4 were 3.17, 3.06, and 3.27 $\log_{10}$ reduction for lettuce, celery, and cabbage, respectively, at 1000 ppm NaOCl, while a reduction of similar to 3 $\log_{10}$ reduction was obtained when the samples were treated with 100ppm NaOCl combined with 0.4% SMS pentahydrate (Table 5). Taken together, these results demonstrated that combined treatment with NaOCl and SMS pentahydrate was an efficient antimicrobial to reduce the concentration of NaOCl for HuNoV GII.4 contamination in fresh vegetables.

The stability and attachment to lettuce has also been observed. (Wang et al., 2012). The results have showed that after incubation for 30min at 56°C, HuNoVs has low effect but it changed significantly after 2 hours treatment, with a < 1.0 $\log_{10}$ reduction in both GII.12/HS200 and GII.4/HS194. In the ethanol treatment, strains were treated with two commonly used concentrations of ethanol, 60% and 70%, at room temperature for 5min, the results showed the RNA reduce with 1.51±0.15 and 1.37±0.32 $\log_{10}$ unites compared to water control. The resistance to chlorine treatment of HuNoVs showed that both virus' RNA became undetectable after sodium hypochlorite concentrations were increased to 200 mg/l.

In 2013, PGM-MB were used to inactive HuNoVs (Kingsley et al., 2014). The ability of HuNoV to bind to PGM-MBs was assessed after 1min treatments with effective concentrations of 33, 173, and 189 ppm of chlorine, respectively. As compared to the

untreated control, log_{10} reductions were 1.48 ± 0.42, 3.65 ± 0.41, and 4.14 ± 0.54, respectively. Initially, concentrations of 240 ppm chlorine dioxide were evaluated for 1 min. However limited inactivation (~0.33 log_{10}) was observed. Consequently, 240 ppm ClO_2 treatments were extended to up to 60 min. Results indicated that 10, 30, and 60 min treatments with 240 ppm ClO_2 gave 0.8 ± 0.24, 1.5 ± 0.42 and 2.8 ± 1.27 log_{10} reduction (Table 5), respectively. For 4% concentration of H_2O_2, exposing HuNoV for 1 min, a low log_{10} reduction (~0.1 log_{10}) was observed. The effect of trisodium phosphate (TSP) was also evaluated by mixing 5% TSP with HuNoV for 5, 15, and 30min treatments followed by PGM-MB and qRT-PCR assay. Results indicated that exposure to 5% TSP for 5min was reduced binding by 1.6 ± 0.58. log_{10}. When increased the contact time to 15 and 30min, did not result in substantially greater reductions.

In 2010, HuNoV was inoculated into chlorination and bench-scale free chlorine that performed for 0.1 and 0.5 mg l^{-1} concentrations, (Kitajima et al., 2010). At free chlorine concentrations 0.5 mg l^{-1}, a reduction in HuNoV from 1.10 to 3.64 log_{10} after contact time (5 to 30 min) using the direct RT-qPCR while at 0.1mg l^{-1} achieved less than 0.1 log_{10} reduction after 30 min contact time (Table 5). Viral RNA titer was almost constant regardless of the virus type. The results indicating similar persistence against free chlorine disinfection. Recent studies also demonstrated that MNV was more sensitive to free chlorine than other enteric viruses, and that HuNoV is not highly resistant to free chlorine disinfection.

VIRUSES	INACTIVATION AGENT	RANGE OF CONCENTRATIONS OR TREATMENT TIME	QUANTIFICATION METHOD	RANGE OF REDUCTIONS	REFERENCE
GI.1 GII.4,	Heat	2min at 90°C	PGM/PCR PMA/PCR	2.2 $\log_{10}$ (GI.1) No inactivation (GII.4)	(Li et al., 2017)
GII.4	NaOCl on lettuce, celery, and cabbage	1000 ppm	RT-qPCR	3.17, 3.06, and 3.27 $\log_{10}$	(Ha, et al., 2017)
GII.4	NaOCl and 0.4% SMS pentahydrate on lettuce, celery, and cabbage	100ppm	RRT-qPCR	3 $\log_{10}$	(Ha et al., 2017)
GII.4	Heat inactivation	Incubation at 56°C for 2 hours	RT-qPCR	< 1.0 log10	(Wang et al., 2012)
GI.1 GII.4	60% and 70%, ethanol	Room temperature for 5 min.	PGM-MB assay, RT-qPCR	1.51±0.15 and 1.37±0.32 log10	(Wang et al., 2012)
GI.1	240 ppm ClO_2	10, 30, and 60min	RT-qPCR, PGM/PCR	0.8 ± 0.24, 1.5 ± 0.42 and 2.8 ± 1.27 log10	(Kingsley et al., 2014)
GI.1	5% trisodium phosphate	5min	PGM-MB qRT-PCR	1.6 ± 0.58. $\log_{10}$	(Kingsley et al., 2014)

GI.1	4% H_2O_2	1min	PGM-MB qRT-PCR	~0.1 log10	(Kingsley et al.,2014)
HuNoV	Chlorine	0.1 mg l^{-1} 120 min 0.5 mg l^{-1} 5-30 min	qRT-PCR	0.1 mg l^{-1} 120 min: 3.84 log10 0.5 mg l^{-1} 5 to 30 min: 1.10 to 3.64 log10	(Kitajima et al., 2010)

Table 5 Other HuNoVs inactivation studies

1.7. Conclusion

HuNoV GI and GII type of cause outbreaks among the population with majority coming from GII.4. It is important to improve and understand methods of inactivate to prevent these outbreaks on food and otherwise. Although many researches have tested in vitro and in vivo cultivation methods, many methods still have limitation on HuNoVs viral reduction. As Inactivation of HuNoVs has been seen on a multitude study, for example, inactivation in solution, surfaces, high pressure and other inactivation; washing with soap shown the highest inactivation (>6 log10 reduction) in the review. Sodium hypochlorite (hand soap), NEW on hard surfaces also showed >4 log10 reduction on HuNoVs genome copies. Additionally, when inactivation in solution, 13% chlorine and 1000 ppm of 90% ethanol were achieved >4 log10 reduction. However, some of inactivation methods showed the limited viral inactivation. For example, 200-500 MPa pressure level after 15 min HHP treatment on HuNoV achieved <0.5 log_{10} reduction also a 0.5 log_{10} reduction of HuNoV GII.4 after exposure to 50% isopropanol. HuNoVs are resistant to quantification of different solutions is

important for identifying agents that may be of more practical value in an applied setting. Furthermore, the limitation of inactivation showed different sensitivity to HuNoV strains GI and GII should be conducted. In addition to HuNoV existing features as a near-perfect foodborne pathogen, we need to find more efficient method even in vitro and in vivo cultivation because HuNoVs are so difficult to study.

CHAPTER 2

GENERATION OF POSITIVELY CHARGED CARVACROL NANOEMULSIONS AND THEIR SHELF LIFE

2.1. Abstract

Due to the low solubility of carvacrol in water, carvacrol must be delivered as a nanoemulsion. In this study, we applied a low energy method (spontaneous emulsification) to generate nanoemulsions containing carvacrol, medium chain triglyceride (MCT) and surfactant. The most optimal carvacrol nanoemulsion contained 10% (v/v) organic phase (0.33 to 1.0% carvacrol, 4.67 to 4.0% MCT, and 5.0% Tween 80, v/v) and 90% aqueous phase (RNase-DNase-Free water with 0.02% CTAB), and was produced at room temperature by spontaneous emulsification. In order to enhance delivery application, we applied the Cethyltrimethylammonium bromide (CTAB) to make the nanoemulsion contain a positive charge, thus increasing the delivery of the carvacrol nanoemulsion to negatively charged biological molecules of interest. The droplet size was decreased (from d ≈ 200 to d ≈ 95 nm) and the mean Zeta-potential stay stable (mean value from 11.0 to 13.0 mV) when carvacrol concentration was increased from 0.33 to 1.0% (and MCT was decreased from 4.67 to 4.0% v/v). For 14 days shelf life study, 0.5% carvacrol nanoemulsions droplet sizes and Zeta-potential were examined at three different temperatures reflecting different potential applications (37°C, 20°C and 4°C). The mean droplet size and Zeta-potential were stable at three different temperatures for the duration of the test. The results of this study inform the design and utilization of spontaneously formed,

positively charged carvacrol nanoemulsions and the extent of their stability in a number of different applications.

2.2. Introduction

Nanoemulsions are emulsions with droplet size on the order of 1 to 100 nm. A typical nanoemulsion contains oil, surfactant and water. In order to prepare a stable and smaller droplet size (d ≈ 100nm) of nanoemulsion, the droplet size, stability and solubility were found to be dependent on the composition of carvacrol nanoemulsion (type of surfactant, concentration of oil phase, ratio of carvacrol to carrier oil, etc.) as well as the type of food matrix where the carvacrol nanoemulsion is applied (Chang et al., 2013; Donsì et al., 2012).

Compared to oil-in-water emulsion, much more work can be done to understand the behavior and stability of spontaneously formed nanoemulsions containing a number of natural bioactive compounds in the light of increasing consumer demand for "natural" products. The growing demand for the use of natural additives has produced a substantial increase in the number of studies based on natural extracts such as carvacrol or its main compounds in the last decade. Carvacrol are categorized as Generally Recognized as Safe (GRAS), and are therefore potential alternatives to chemical additives (Sanchez et al., 2015; Mason et al., 2006).

The food industries have paid attention to the natural alternatives to assure food safety and quality. Oregano is a natural food additive which bioactive components are beneficial as flavoring or seasoning agents in some of the most accepted cuisines around the world. Additionally, oregano oil is attributed to

antioxidant effect from their major components, carvacrol and thymol, and it is the result of various possible mechanisms: free-radical scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen- quenching capacity (Shan et al., 2005).

Carvacrol are considered to present no risk to the health of consumers and have been registered by U.S. Food and Drug Administration and generally recognized as safe components (Burt et al., 2004). Additionally, carvacrol and thymol are the main antimicrobial and antioxidant monoterpene phenolic compounds that constitute 78–85% of oregano (Govaris *et al.,* 2010). Its components are potential natural food antimicrobials, which can meet the increasing demands of fresh and chemical-additive-free food products from more health-conscious consumers and legal authorities (Smith-Palmer et al., 1998). Moreover, the anti-inflammatory potential of essential oils containing carvacrol and itself have been investigated in details in various models of inflammation (Hotta et al., 2010; Lima et al., 2013). Furthermore, another group (Lima et al., 2013) also demonstrated that carvacrol exerts presets anti-inflammatory activity on a typical mice inflammation model.

Over the past decade or more, the research focus has been on preparing nanoemulsions through various methods, broadly classified into two primary categories: high-energy and low-energy methods. High-energy methods utilize mechanical devices that are capable of disrupting and intermingling the oil and aqueous phases into tiny oil droplets dispersed in water. Low-energy methods mainly rely on the spontaneous formation of droplets at the interface between oil

and water phases and depend strongly on the nature of any surface-active molecules present, e.g., their solubility and molecular geometry (Chang et al. 2013). Low energy approaches may have advantages over high-energy approaches for certain applications: they are often more effective at producing desired droplets, they have lower equipment and energy required, and they are simpler to implement (Chang et al., 2013).

By contrast, nanoemulsions do not form spontaneously; an external shear must be applied to separate larger droplets into smaller droplet. In this study, we examine the potential of using the spontaneous emulsification method (low-energy method) for producing carvacrol nanoemulsions. In general, this method involves pouring an organic phase (containing oil and surfactant) into an aqueous phase, which leads to the spontaneous formation of desired droplets due to rapid diffusion of the surfactant from the oil phase into the aqueous phase (Anton et al., 2009). The movement of the hydrophilic surfactant from the oil phase to the aqueous phase after mixing leads to the spontaneous formation of desired oil droplets at the oil−water boundary. This method allows nanoemulsions to be produced at room temperature using simple stirring rather than expensive homogenization equipment (Chang et al., 2013).

Nanoemulsions are kinetically stable, but given sufficient time, will separate into different phases. The different destabilization mechanisms of nanoemulsions are primarily flocculation, coalescence, and Ostwald ripening. In flocculation, droplets come closer to each other because of attractive interactions and move as a single entity. In contrast, in coalescence, the droplets merge into each other

to become a bigger droplet. Ostwald ripening occurs due to the difference in chemical potential of solute within droplets of different sizes. The chemical potential of the dispersed phase provides the driving force for mass transfer from the smaller to the larger droplets. Thus, the smaller droplets become smaller and the larger droplets grow (Gupta et al., 2016).

To make stable nanoemulsions, we can apply MCT to stabilize nanoemulsions due to its highly nonpolar nature. In this study, MCT was beneficial not only for the spontaneous formation of carvacrol nanoemulsions but also for ensuring their shelf life stability test, to avoid Ostwald ripening and coalescence inhibitor (Chang et al., 2013). The addition of nonpolar triglyceride oils (such as MCT) may therefore have decreased the coalescence rate by decreasing the polarity and increasing the interfacial tension. An alternative approach to enhancing the long-term stability of nanoemulsions would be to store the antimicrobial as an organic phase containing carvacrol and carrier oil (MCT) and then add this organic phase to an aqueous product when needed (Chang et al., 2013).

The addition of surfactant is critical for the creation of small sized droplets as it decreases the interfacial tension i.e., the surface energy per unit area, between the oil and water phases of the emulsion (Gupta et al., 2016). Carvacrol oil-in-water nanoemulsions can also stabilized by a nonionic surfactant (Tween 80). Tween 80 dissolved in sterile deionized distilled water will be prepared to determine the optimum interfacial composition to obtain small stable droplets with high antimicrobial efficacy. The decrease in droplet size is because of the accumulation of surfactant molecules at the interface which leads to increase in

the interfacial area and decrease of the interfacial energy (Tadros et al., 2004).

Cethyltrimethylammonium bromide (CTAB) is a cationic surfactant that self-assembles as micelles, and other structures and phases depending on the concentration and solvent characteristics. CTAB is appropriate for extraction of biomolecules, since its cationic micelles are stable over a wide range of pH (J̈onsson et al., 1998). Moreover, addition of a cationic surfactant to emulsions can further enhance functionality and delivery to negatively charged target molecules; for instance, it can enhance antimicrobial activity against bacteria by better delivering antimicrobial to the negatively charged cell surface (Ziani *et al.*, 2011).

2.3. Materials and Methods

2.3.1. Carvacrol Nanoemulsion Materials

Purified carvacrol (>98%) was purchased from Sigma-Aldrich (St. Louis, MO). Organic MCT oil was purchased from Nature's Way (Green Bay, WI). Polyoxyethylene-80 (Tween 80) and Hexadecyltrimethylammonium bromide (CTAB) were purchased from (Markham, ON). Sterile RNase-DNase-Free water and Phosphate-buffered saline (PBS) were purchased from Thermo Fisher (Waltham, MA).

2.3.2. Nanoemulsion Preparation

Initially, to make carvacrol nanoemulsions, we first prepared aqueous phase and lipid phase separately. Organic phases were prepared by mixing different concentration (0.33, 0.5 or 1.0% v/v) of purified carvacrol, different concentrations of MCT (4.67, 4.5 or 4% v/v) and 5.0% Tween 80 were added to make a total organic phase of 10% (v/v) by using a stir bar for 15min at

31

room temperature. The aqueous phase used to prepare the nanoemulsions consisted of 0.02% of CTAB dispersed in 50ml of nuclease-free distilled water at room temperature. The organic phase (10% v/v) was added then mixed with aqueous phase (90% v/v) by using a manual dispenser (Repeater® M4, Eppendorf, Hauppauge, NY). All components were mixed for 30 min at 25°C.

2.3.3. Nanoemulsion Shelf Life Test

For the purposes of shelf life testing, a 0.5% carvacrol nanoemulsion was evaluated, by generation of two nanoemulsions sealed in 100ml conical flasks and then separately stored at 4°C, 20°C and 37°C for 14 days. We separately tested each nanoemulsion sample (from 4°C, 20°C and 37°C incubators and in each condition has two samples) at days 1, 3, 7 and 14

2.3.4. Droplet Size and Zeta-Potential Measurement

The particle size and zeta-potential of the nanoemulsions were determined using (Zetasizer Nano ZS, Malvern Instruments, Worcestershire, UK). Samples were diluted to an oil droplet concentration of 5.0% (v/v) using the same buffer (nuclease-free distilled water) as the original sample to eliminate multiple scattering effects. A measured refractive index value of 1.456 for the 0.5% carvacrol mixed with 4.5% MCT was used. The refractive index of the mixed oil phase was calculated by the mass fraction on a Refractometer (Bausch & Lomb, Rochester, New York). The droplet size and Zeta-potential were used to represent the mean particle diameter and surface potential of the lipid droplets. After 60s of equilibrium, each sample was scanned three times and the average was recorded. The Z-Average and Zeta-potential were used to represent the mean particle diameter and surface potential of the lipid

droplets.

2.3.5. Statistical Analysis

Nanoemulsion preparation experiments, Z-averages, and Zeta-potential measurements were performed in triplicate on freshly prepared samples. Nanoemulsion stability to shelf life test were tested from two samples at different temperature environment by following manufacturer instructions at 60 seconds. The results were then reported as averages and standard deviations of these measurements.

2.4. Results

2.4.1. Z-Average and Zeta-Potential of Oil Phase Composition on Nanoemulsion Formation

The mean droplet diameter decreased from 220.8 nm to 112.3 and 95.5 nm when the carvacrol concentration was increased from 0.33 to 0.5 and 1.0% (Table 6) after combination with 4.67, 4.5 and 4.0% MCT and 5.0% Tween 80 and 90% aqueous phase, respectively. The mean Zeta-potential stayed relatively stable, with mean values from (+)11.0-13.0 for the above variations in conditions.

Carvacrol oil %	MCT %	Tween 80 %	Aqueous phase %	Mean droplet diameter (nm)	Mean Zeta-potential (mV)
0.33	4.7	5.0	90.0	220.8	11.3
0.5	4.5	5.0	90.0	112.3	13.0
1.0	4.0	5.0	90.0	95.5	11.0

Table 6. Effect of mean droplet diameter and mean Zeta-potential of carvacrol oil

nanoemulsions fabricated combining with differing MCT concentration.

2.4.2. Storage Stability of 0.5% Carvacrol Nanoemulsions

We examined the influence of storage time on the stability of 0.5% carvacrol nanoemulsions that were found to be stable to visible creaming over 14 days at three different temperature (4°C, 20°C and 37°C). These systems consisted of an oil phase (10% v/v) of 5% oil (0.5% carvacrol and 4.5% MCT), 5% surfactant (Tween 80), and 90% aqueous phase (included nuclease-free distilled water and 0.02%CTAB). Initially, these systems had different mean diameters due to the influence of oil phase composition on the efficiency of nanoemulsion formation. When 0.5% carvacrol nanoemulsion storage at 37°C (body temperature for potential nutritional applications), the mean diameter (Fig. 1) were relatively stable, ranging from 130.4-294.9 nm over 14 days. At 20°C (room temperature condition), mean diameter (Fig. 1) were also relatively stable, ranging from 221.7-295.5 nm over 14 days. The mean diameter (Fig. 1) were also relatively stable at 4°C, ranging from 215.7-277.8 nm over 14 days as similar results to 37°C and 20°C

The mean Zeta-potential (Fig. 2) were stable, the ranging from (+)12.1-17.9 mV over 14 days at 37°C. At 20°C and 4°C, the Zeta-Potential results were similar to storage at 37°C, ranging from (+)14-18.7 mV over 14 days.
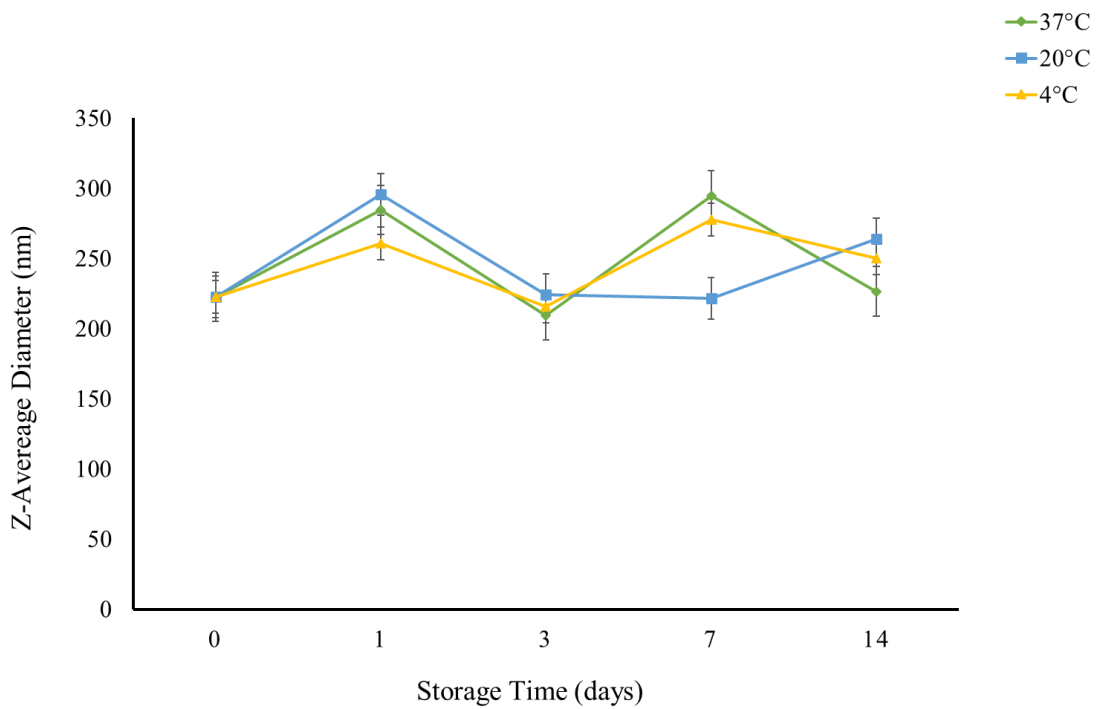
Fig. 1. 0.5% nanoemulsion shelf-life study on Z-Average. Z-Average stays stable in mean particle diameter of selected nanoemulsions during 14 days of storage at three different temperature. Nanoemulsions were prepared using 5% oil (carvacrol + MCT of varying ratios), 5% surfactant (Tween 80), and 90% aqueous phase (included deionized distilled water and 0.02%CTAB) at a stirring speed of 700 rpm at ambient temperature (25 °C).
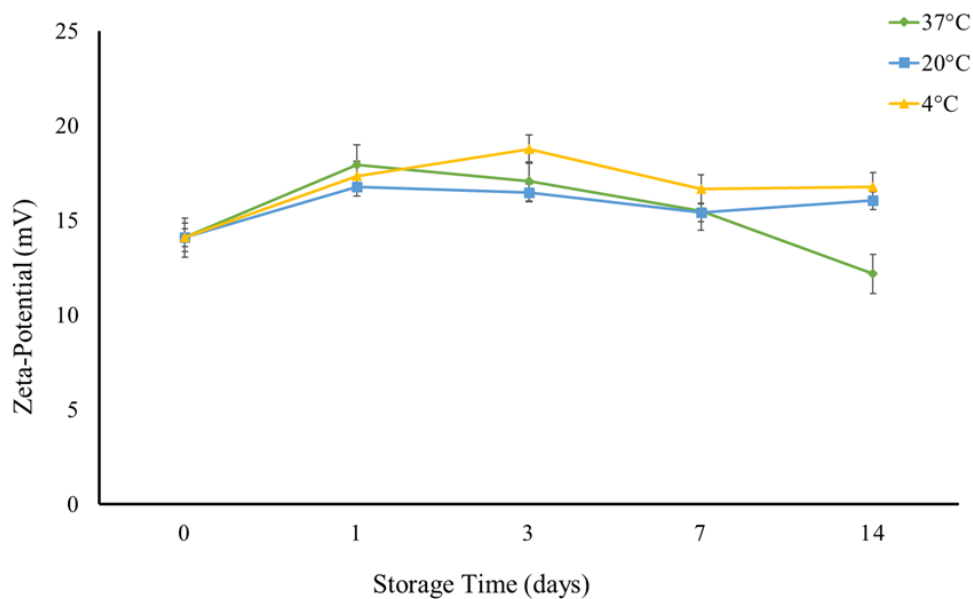
Fig. 2. 0.5% nanoemulsion shelf-life study on Zeta-Potential. Zeta-Potential is stable with mean particle diameter of selected nanoemulsions during 14 days of storage at three different temperature. Nanoemulsions were prepared using 5% oil (carvacrol + MCT of varying ratios), 5% surfactant (Tween80), and 90% aqueous phase (included deionized distilled water and 0.02%CTAB) at a stirring speed of 700 rpm at ambient temperature (25 °C)

2.5. Discussion

Initially, we examined the influence of organic and aqueous phase composition on the initial size of the oil droplets and Zeta-potential in positively charged nanoemulsions produced using spontaneous emulsification. Organic phase composition was varied by combining different mass ratios of carvacro) and MCT prior to emulsification. A present study (Flores et al., 2016) have applied 0.5% (v/v) carvacrol concentration with Tween 80 along with high-pressure homogenization that generated emulsions with smaller droplet size, lower

36

polydispersity index, and higher Zeta-potential. The presence of carvacrol and Tween 80 in the emulsions and the use of high-pressure homogenization decreased the emulsion contact angle because of the smaller droplet size and its greater surface interaction, thus improving its wettability properties.

Therefore, the lower concentrations (0.33, 0.5 and 1.0%) of carvacrol nanoemulsion were made in our study. The overall system composition reflected what has previously been reported 5.0% oil phase (carvacrol + MCT), 5.0% surfactant (Tween 80) and 90% aqueous phase (includes 0.02% CTAB). As the carvacrol concentration in the oil phase increased (from 0.33 to 0.5 and 1.0% v/v), the mean droplet diameter initially decreased (Table 6). To maintain a droplet size within the range generally desired for nanoemulsions, we adjusted the ratio between carvacrol and MCT (oil phase). For example, (Chang et al., 2013) presented the systems containing 2.5% carvacrol and 7.5% MCT (25% carvacrol and 75% MCT in oil phase) in the total nanoemulsion was created. In this work, the composition of three different concentrations of nanoemulsion were 0.33% (6.6% carvacrol and 93.4% MCT in oil phase), 0.5% (10% carvacrol and 90% MCT in oil phase) and 1.0% (20% carvacrol and 80% MCT in oil phase) formed mean droplet sizes of 220.8, 12.3 and 95.5 nm. This finding is in agreement with previous studies of nanoemulsion formation using spontaneous emulsification, as larger droplets can occur when too much MCT is added to the oil phase because the efficiency of spontaneous emulsification decreases. Consequently, an optimum MCT level is required (around 40%) to form stable nanoemulsions (Ryu et al., 2018).

In order to add delivery functionality, we applied 0.02% CTAB to give the nanoemulsions a positively charged nanoemulsion which exhibited stable and consistent Zeta-potential with three different concentrations (0.33, 0.5 and 1.0% v/v) of carvacrol nanoemulsions. The mean Zeta-potential generally did not fluctuate with different formulations, mean values from +11.3, +13.0, and 11.0 mV when the carvacrol concentration was increased from 0.33 to 0.5 and 1.0% (Table 6), respectively. Since droplet charge may have an important impact on nanoemulsion stability and antimicrobial efficacy, a recent study (Chang et al., 2015) showed that a 0.1% cationic surfactant was added in thyme nanoemulsion became positive charged (+18 mV), suggesting that at least some of the cationic surfactant molecules adsorbed to the oil droplet surfaces.

Overall, our shelf life study suggested that the nanoemulsions will likely stay stable in multiple application temperatures, ranging from refrigeration to body temperature (Fig. 1). As nanoemulsion droplet size stayed stable (from d >100, d < 300 nm) at three different temperatures (4°C, 20°C and 37°C) over 14 days.

At 4°C, the mean sizes ranged from 215.7-260.9 nm for days 1-14, then rapidly decreased by day 21 to 130.4 nm. This result is largely in agreement with (Chen et al., 2018) which presented their nanoemulsion with 10% carvacrol in oil phase was stable (d ≈ 150 nm) at 4°C in 28 days shelf life test.

 At 20°C, the mean sizes appeared very similar to 4ºC, with sizes ranging from 222.7-264.1 nm for days 1-14. Another carvacrol nanoemulsion systems in study (Chang et al., 2013), consisted of 10% carvacrol in oil phase (90% MCT in oil

phase) that were found stable (d ≈ 5 nm) at ambient temperature (25°C) for 30 days shelf life test.

When 0.5% carvacrol nanoemulsion storage at 37°C in this study, the mean diameter (Fig. 1) ranging from 130.4-294.9 nm over 14 days. Currently, there is no study presented carvacrol nanoemulsion (at desired concentration of 10% carvacrol in total oil phase) on 37°C for long term study. However, the study (Dey et al., 2018) demonstrated that a different type of nanoemulsion 1.5% (w/v) ω-3 PUFA rich fish oil plus 1% (wt /v) total surfactant (Tween 20 + Span 80) performed nanoemulsion oil droplet size (d ≈ 175 nm) stable at 37°C for 4 weeks.

To our knowledge, no prior studies have reported the behavior and shelf life of positively charged carvacrol nanoemulsions at different temperatures from different applications. Nevertheless, the mean Zeta-Potential (Fig. 2) fluctuated slightly but stayed stable (+12.1 to +18.7 mV) at three different temperatures (4°C, 20°C and 37°C) on 14 days shelf life test in this study. It is due to the fact in a more recent study (Kumar et al., 2018) exhibits the successful selection of Tween 80 surfactant from given CTAB by characterization on the basis of their surface active for stabilizing oil-in-water nanoemulsions

Overall, the 0.5% carvacrol nanoemulsion droplet size and Zeta-potential remained relatively stable at three distinct temperatures (4°C, 20°C and 37°C) for 14 days shelf life test. This is likely due to a higher amount (90% in total lipid phase) of MCT enhancing the long-term stability (Chang et al., 2013). In order to attempt to create smaller oil droplet size, we may increase the carvacrol

concentration (30 to 40%) in total lipid phase while lower the MCT concentration (70 to 60%) in total lipid phase. However, previous reports suggest that this may give the nanoemulsion unstable when more than 25% of carvacrol (in total oil phase) is used cause unstable nanoemulsions (Chang et al., 2013).

2.6. Conclusion

In sum, we optimized the formulation of and investigated the behavior of positively charged, spontaneously formed carvacrol nanoemulsions. Further, we evaluated the shelf life of these nanoemulsions at different potential application temperatures (refrigeration, room, and body temperature). In the wake of growing consumer demand for different "natural" products in a number of industries, our study broadly informs the development and study of functionalized carvacrol nanoemulsions that can be cheaply fabricated and are stable in a range of application temperatures.

CHAPTER 3

CARVACROL NANOEMULSION ANTIMICROBIC EFFICACY ON BACTERIOPHAGE MS2 AND *ESCHERICHIA COLI*

3.1. Abstract

The essential oils in plants contain complex mixtures with lipophilic and volatile secondary metabolites. The antimicrobial active ingredient of essential oils can be the dominant component, greater than 50% of the chemical composition in many cases. Additionally, the natural plant antimicrobials have a higher acceptance at public, therefore the natural essential oil active ingredients have been investigated for application on food to reduce microorganism transmission. Carvacrol has previously been demonstrated to have a moderate antiviral effect on noroviruses. Previous work has also demonstrated that restructuring essential oils in positively charged nanoemulsions can enhance their antimicrobial efficacy. The purpose of this work was to investigate if restructuring carvacrol in positively charged nanoemulsions could enhance the antinoroviral efficacy of carvacrol. Carvacrol nanoemulsions (0.5, 0.83% v/v) were dissolved in Dulbecco's phosphate-buffered saline (DPBS), and the median particle size was 122.7nm and 123nm— acceptable for nanoemulsions—and the median Zeta potential were -1.42 and -1.15mV at 0.5 and 0.83% carvacrol. Carvacrol nanoemulsions at 0.5 and 0.83% both displayed negligible viral reduction. Therefore, we investigated efficacy with different aqueous phase (nuclease-free distilled water) for dissolving carvacrol nanoemulsion. Due to carvacrol's low solubility in water, different concentrations of carvacrol nanoemulsion (0.5, 1.0% v/v) were produced by a low energy method (Nano-emulsification) in nuclease-free distilled water instead of DPBS. The median particle sizes were 112.3nm and 71.5nm, and Zeta-

potential were +12.9 and 9.6mV. When applied against MS2, carvacrol nanoemulsion (0.5%, 1.0% v/v carvacrol) with 60 min; 0.5% carvacrol nanoemulsion showed no reduction on MS2. However, 1.0% carvacrol nanoemulsion showed a 0.73 log reduction of MS2. In bacteria antiviral efficacy, carvacrol nanoemulsions (1.0% v/v) dissolved in nuclease-free distilled water leading to 0.35 log reductions of *Escherichia coli* after 15 min contact time. However, when contact time was increased to 30 and 60min, the treatment showed no log reduction of *Escherichia coli*.

3.2. Introduction

HuNoV is a major leading cause of foodborne illness, and now this pathogen is recognized as a leading cause of diarrhea for all ages of person. (Patel et al., 2009). NoVs are nonenveloped single-stranded RNA virus. The viral capsid typically is 27- 35 nm in diameter, and has a 7.5-7.7-kilobase in length of positive-sense genome that consists of three open reading frames (ORFs) (Glass et al., 2000; Jiang et al., 1993; Lambden et al., 1993). ORF1 codes for a non-structural polyprotein, ORF2 and ORF3 codes major (VP1) and minor (VP2) capsid proteins, respectively (Glasset et al., 2000; Prasad et al., 1999). The viruses have an icosahedral capsid that contain of 180 copies of the VP1 that self-assemble based on hydrophobic contacts, and the pI of VP1 makes it negatively charged in neutral pH (Smithet et al., 2019). One of the major challenges is their general resistance to many commonly used inactivation agents (Hirneisen et al., 2010) and the lack of natural disinfectants can efficiently inactive for norovirus capsid (Kamarasu et al., 2018). Therefore, we applied novel spontaneous Nano-emulsification method to form a carvacrol nanoemulsion against bacteriophage MS2 and *E. coli.*

The carvacrol with higher antibacterial properties contains a high percentage of phenolic compounds, causing irreversible damage to the bacterial membrane proteins and membrane

(Donsì et al., 2014). However, essential oils and their active components have only displayed moderate inactivation of noroviruses surrogate (<1-3 log10 reduction on MNV), therefore no food-grade, natural inactivation agents currently used in foods or on food contact surfaces (Gilling et al., 2014).

Since the water solubility of carvacrol is as low as 0.11-0.83 g/l at room temperature (Chen et al., 2014), it is difficult to directly inactivate MS2 by only using carvacrol in a disinfectant solution. Although NoV do not have a lipid membrane, we hypothesize the positively charged nanoemulsions will still better deliver carvacrol to the negatively charged viral capsid that has hydrophobic contacts that hold the capsid together.

Nanoemulsions can enhance delivery of the active component against environmental stresses and increase the partition of the hydrophobic component to aqueous phase (Chang et al., 2013). The antimicrobial activity of carvacrol was found to be dependent on the composition of nanoemulsion (type of surfactant, concentration of oil phase, aqueous phase and ratio between carvacrol and MCT). The desired nanoemulsion droplet size and the solubility properties are defined to formulate a nanoemulsion. Additionally, the study (McClements et al., 2011) proved a small particle size of nanoemulsion (100nm-1000nm) that can improve physical stability and increased bioactivity of lipophilic active ingredients

Many essential oils have lower water solubility, which can lead to rapid nanoemulsion destabilization through a phenomenon known as Ostwald ripening, i.e., diffusion of the oil from small droplets to large droplets. Eventually, this leads to oil and aqueous phase separation, therefore the nanoemulsion may become unstable. Addition of highly lipophilic triglycerides (such as MCT) can prevents Ostwald ripening and stabilizes essential oil

nanoemulsions (Ziani et al., 2011).

Carvacrol oil-in-water nanoemulsions can stabilized by a nonionic surfactant (Tween 80), Tween 80 dissolved in sterile deionized distilled water will be prepared to determine the optimum interfacial composition to obtain the stable and smaller droplets with high antimicrobial efficacy. Addition of a cationic surfactant to nanoemulsions further enhances antimicrobial activity (Ziani et al., 2011). The mechanism of antibacterial action of essential oil is mainly based on the hydrophobicity of their constituent molecules. Indeed, the essential oil with higher antibacterial properties contain a high percentage of phenolic compounds, capable of interacting with the cytoplasmic membrane, causing its irreversible damage (Donsì et al., 2014).

Previous work for bacteria and fungi have demonstrated that restructuring essential oils into positively charged nanoemulsions can enhance antimicrobial efficacy by 1-2 log (Salvia-Trujillo et al. 2014). In this study, we hypothesize that incorporation of a strongly oxidizing essential oil (carvacrol) into a nanoemulsion with cationic surfactant (CTAB) will enhance delivery of a nanoemulsion to negatively charged norovirus particles. The 0.5 and 1.0% (v/v) concentration of carvacrol nanoemulsions were exam as a model microorganism of desired droplet size and charge.

The hypothesis of development of an efficacious carvacrol nanoemulsion (>4 log10 reduction of viral titer) that can be incorporated into foods or used on food contact surfaces is of significant interest to the food industry as well as for public health.

3.3. Materials and Methods

3.3.1. Bacterial Hosts and Cell Lines

Bacteriophage MS2 was kindly provided as a gift by L-A. Jaykus (North Carolina State University, Raleigh, NC) and its host *Escherichia coli* strain (ATCC 15597), were purchased from ATCC (Manassas, VA).

3.3.2. *Escherichia Coli* Preparation

Incubated *Escherichia coli* was kept at -80°C in a mixture of TSB containing 0.1% thiamine and 0.2% glucose and 50%v/v of glycerol as frozen stock. Before use, *Escherichia coli* was streaked in appropriate selective media (5ml TSB containing 0.1% thiamine and 0.2% glucose) with cultured tubes at 37°C in an atmosphere containing 5% CO_2 for 18 h.

3.3.3. Bacteriophage MS2 Plaque Assay

Incubated *Escherichia coli* from frozen stock in 5ml TSB containing 0.1% thiamine and 0.2% glucose at 37°C in an atmosphere containing 5% CO_2 for 18h. Adding 300ul of overnight *Escherichia coli* culture to inoculate at 29.7ml TSB containing 0.1% thiamine and 0.2% glucose in a 100ml conical flask. Incubate the *Escherichia coli* at 37 °C in an atmosphere containing 5% CO_2 using an incubator shaker (100 rpm) for 2h until the optical density at 600 nm of 0.60. As a guideline, an OD600 of 0.6 corresponds to approximately 2.6×10^8 CFU/ml for cultures of *Escherichia coli* strains. Warmed 1.0% TSA plates containing 0.1% thiamine and 0.2% glucose in 37°C for at least 1 hour prior to plaque assay beginning. Melted and tempered desired tubes of 9ml-0.5% TSA containing 0.1%thiamine and 0.2% glucose in a 50°C water bath. MS2 stock (3.18 $\times 10^{11}$ PFU/ml) was serially diluted in TSB containing 0.1% thiamine and 0.2% glucose, and 0.7 mL of diluted phage was mixed with 0.3 ml of 2-h *Escherichia coli* host. The 1-ml host-MS2 combination was then added to 9ml of 0.5% TSA containing 0.1%

thiamine, 0.1% calcium chloride and 0.2% glucose, mixed and poured on 1% TSA containing 0.1% thiamine and 0.2% glucose bottom agar plates, and incubated at 37°C overnight. To obtain accurate quantitative analyses of plaque numbers, petri plates should have relatively diluted MS2 samples (25 to 250 PFU/plate).

3.3.4. Propagation of Bacteriophage MS2

Selected plate with complete lysis and flooded with 3ml TSB 0.1% thiamine and 0.2% glucose. Gently scraped off the top layer of MS2 plaques formation surface into sterile 50ml tubes as over layer of complete lysis plaques suspensions. Bring volume to 40ml with TSB containing 0.1% thiamine and 0.2% glucose. Adding 0.2g of EDTA and 0.026g of lysozyme to each tube and vortex for 10s. Incubated each tube at 37°C in an atmosphere containing 5% CO_2 using an incubator shaker (100 rpm) for 2h. The over layer of complete lysis plaques suspensions were then centrifuged at 9280G for 10min. Adding supernatant in 0.22um sterilize filter and then aliquoted to 1ml storage cryogenic tubes. Storing at either 4°C for several weeks or -80°C for several years. Cryoprotectant (such as glycerol) is not necessary.

3.3.5. Antiviral Effects of Nanoemulsion on MS2

Incubated *Escherichia coli* from frozen stock in 5ml TSB containing 0.1% thiamine and 0.2% glucose at 37°C in an atmosphere containing 5% CO_2 for 18h. Adding 300ul of overnight *Escherichia coli* culture to inoculate at 29.7ml TSB containing 0.1% thiamine and 0.2% glucose in a 100ml conical flask. Incubate the Escherichia coli at 37°C in an atmosphere containing 5% CO_2 using an incubator shaker (100 rpm) for 2h until the optical density at 600 nm of 0.60. As a guideline, an OD600 of 0.6 corresponds to approximately 2.6×10^8 CFU/ml for cultures of *Escherichia coli* strains. Gently thawed MS2 stock (8.71×10^{10} PFU/ml) on ice, diluted in 1/100 (10ul MS2 in 990ul nuclease-free distilled water). Each concentration of sterile-dilute Nano-emulsion

(500ul) was mixed with an equal volume of MS2 (500ul) and incubated with gentle rotation for desired time (10-60 min) at room temperature. Sterile-dilute nano-emulsion was replaced with nuclease-free distilled water which also incubate with gentle rotation for desired time (10 to 60min) at room temperature used as the untreated controls. After incubation, treated MS2 and untreated control were neutralized in TSB containing 3% meat extract. MS2 plaque assays were performed using incubated 2-h *Escherichia coli*. MS2 treated with sterile-dilute nano-emulsion or water after neutralization with TSB containing 3% meat extract was serially diluted in TSB containing 0.1% thiamine and 0.2% glucose, and 0.7 ml of diluted phage was mixed with 0.3 ml of 2-h *Escherichia coli* host. The 1-ml host-MS2 combination was then added to 9ml of 0.5% TSA containing 0.1% thiamine, 0.1% calcium chloride and 0.2% glucose, mixed and poured on 1% TSA 0.1% thiamine and 0.2% glucose bottom agar plates, and incubated at 37°C overnight before counting.

3.3.6. Antibacterial Effects of Nanoemulsion on *Escherichia coli*

Incubated *Escherichia coli* from frozen stock in 5ml TSB containing 0.1% thiamine and 0.2% glucose at 37°C in an atmosphere containing 5% CO_2 for 18h. Adding 300ul of overnight *Escherichia coli* culture to inoculate at 29.7ml TSB containing 0.1% thiamine and 0.2% glucose in a 100ml conical flask. Incubate the *Escherichia coli* at 37 °C in an atmosphere containing 5% CO_2 using an incubator shaker (100 rpm) for 2h until the optical density at 600 nm of 0.60. As a guideline, an OD600 of 0.6 corresponds to approximately 2.6×10^8 CFU/ml for cultures of *Escherichia coli* strains. A 0.5ml-aliquot of incubated bacterial culture was mixed with 0.5ml of the carvacrol nanoemulsion and 9.0 ml of nuclease-free distilled water. To determine the inactivation kinetics, an aliquot was taken after 15, 30 and 60min of contact time. A control was performed with the same method, replacing the nanoemulsion by nuclease-free distilled

water. Minimum inhibitory concentration (MIC) were performed by using *Escherichia coli* treated with sterile-dilute nano-emulsion or water. Serially diluted treated and untreated *Escherichia coli* in TSB containing 0.1% thiamine and 0.2% glucose, and 0.1 ml of diluted cultures were poured on 1% TSA 0.1% thiamine and 0.2% glucose bottom agar plates. And incubated at 37°C overnight before counting.

3.3.7. Statistical analysis

Each experiment was performed in triplicate and all values are reported as the mean ± standard deviation (SD) by Microsoft Excel. Results from the plaque assay of treatments and non-treatment controls were statistically assayed in duplicate plates and two replicate analyses were made of each nano-emulsion sample.

3.4. Results

The degree to which restructuring carvacrol into positively charged nanoemulsions was investigated in this work. The levels of 0.5% and 1.0% carvacrol were chosen for a number of reasons. Based on work presented in Chapter 2, 0.5 to 1.0% carvacrol produced stable nanoemulsions with the desired droplet size (from 112.3to 95.5 nm) and Zeta-potential (from+13 to +11 mV). Additionally, previous work (Gilling et al., 2014) demonstrated these concentrations exhibited antiviral activity (nearing 4 log reduction after 24h) on MNV using 0.5% purified carvacrol. Therefore, 0.5 to 1.0% of carvacrol nanoemulsions were made in this study.

3.4.1. Effect of 0.5 and 0.83% Carvacrol Nanoemulsions Dissolved in PBS on MS2

The 0.5 and 0.83% carvacrol nanoemulsions dissolved in PBS had no antiviral effect (Fig. 3). 2.5×10^7 and 2.7×10^7 viral titers of MS2 were observed across treatment groups (MS2 was treated with 0.5 and 0.83% carvacrol nanoemulsion) compared with untreated control (2.1×10^7) and neutralization control (2.1×10^5) by same treatment contact time (60min). Although treatment with PBS in different plaques population in

MS2, it was not significantly different from nuclease-free distilled water or PBS.
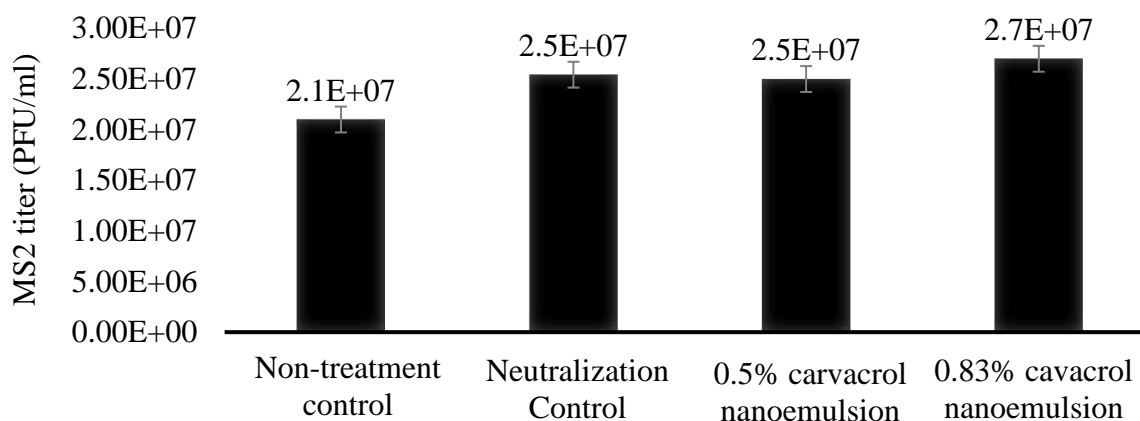

Fig. 3. Effect of 0.5 and 0.83% carvacrol nanoemulsions dissolved in PBS on MS2. At 60 min contact time, 0.5 and 0.83% carvacrol nanoemulsion dissolved in PBS shows no antiviral efficacy compared with non-treatment control.

3.4.2. Effect of 0.5 and 1.0% Carvacrol Nanoemulsions Dissolved in Nuclease-Free Distilled Water on MS2

The results of antiviral effect are shown in Fig. 4. The antiviral efficacy of MS2 was determined by comparison with the plaque reductions at the same treatment contact time (60min) at room temperature. 8.8×10^6 and 1.4×10^6 viral titers of MS2 were observed when MS2 treated with 0.5% and 1.0% carvacrol nanoemulsions 7.1×10^6 viral titers were observed at untreated control. The antiviral effect of 0.5% carvacrol nanoemulsion showed no antiviral activity than non-treatment control. However, carvacrol was examined at concentrations of 1.0%, 0.7 log reductions in comparison with the non-treatment controls at 60min contact time. In order to validate the experimental treatment protocol, 1.0% bleach showed complete inactivation, as

previously reported (Whitehead et al., 2010). Therefore, 0.5 and 1.0% carvacrol nanoemulsions dissolved in water showed lower efficacy on MS2 than carvacrol alone.
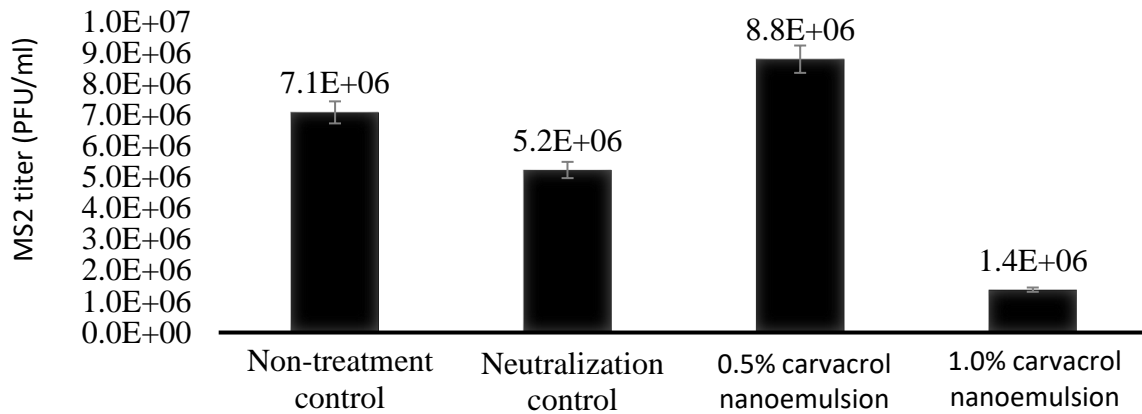

Fig. 4. Effect of 0.5 and 1.0% carvacrol nanoemulsions dissolved in nuclease-free distilled water on MS2. 0.5 and 1.0% carvacrol nanoemulsions dissolved in nuclease-free distilled water showed lower antiviral efficacy. The antiviral effect of 0.5% carvacrol nanoemulsion showed no antiviral activity than non-treatment control. However, carvacrol was examined at concentrations of 1.0%, a 0.7 log10 reductions in comparison with the non-treatment controls at 60 min contact time.

3.4.3. Effect of 1.0% Carvacrol Nanoemulsion Dissolved in Nuclease-Free Distilled Water on *Escherichia Coli* in Different Contact Time

In order to see if carvacrol nanoemulsions required a lipid membrane for efficacy, the antimicrobial effects of a 1.0% carvacrol nanoemulsion optimized against *Escherichia coli* showed in Fig. 5. The treatment on 1.0% carvacrol nanoemulsion dissolved nuclease-free distilled water shows low antibacterial efficacy. 2×10^7 CFU/ml bacterial titers were observed at non-treatment control and 9×10^6, 2×10^7 and 1.6×10^7 CFU/ml

were observed after contacted with 1% carvacrol nanoemulsion at 15, 30 and 60min. The results indicated that the 1.0% carvacrol nanoemulsions at 15, 30 and 60 min contact time achieved 0.35, -0.04 and 0.03 log10 bacteria colony reduction compare with non-treatment control. In order to present the experiment protocol, 1.0% bleach in 30 min contact time exams completely inactive antibacterial effect. In addition, no significant difference was observed between MS2 bacterial phage and *Escherichia coli* microorganism reduction on 1.0% nanoemulsion dissolved in nuclease-free distilled water,

Fig. 5. Effect of 1.0% carvacrol nanoemulsion dissolved in water on *Escherichia Co*li. In different contact time. 1.0% carvacrol nanoemulsion dissolved in nuclease-free distilled in water shows low antibacterial efficacy. At 15, 30 and 60 min contact time, 1.0% carvacrol nanoemulsion achieved 0.35, -0.04 and 0.03 log10 bacteria colony reduction compare with non-treatment control.

3.4.4. Effect of 1.0 and 10% Carvacrol Emulsion Dissolved in Water on *Escherichia Coli* in 60min Contact Time

The antimicrobial effects of 1.0% carvacrol optimized against *Escherichia coli* showed

51

in Fig. 6. The results indicated that the 1.0% carvacrol at 60 min contact time has no bacteria colony reduction. 1.9×10^7 CFU/ml was observed after contacted with 1% carvacrol compare with non-treatment control (1.7×10^7 CFU/ml). Additionally, the both results of 1% carvacrol nanoemulsion and 1% carvacrol showed the similar effect (no antibacterial reduction) on *Escherichia coli* at 60 min contact time. However, in 10% carvacrol treatment at same contact time, a 7.2 log10 reduction was achieved. Therefore, the higher concentration (10%) of carvacrol itself has higher antibacterial effect than lower concentration (1.0%).
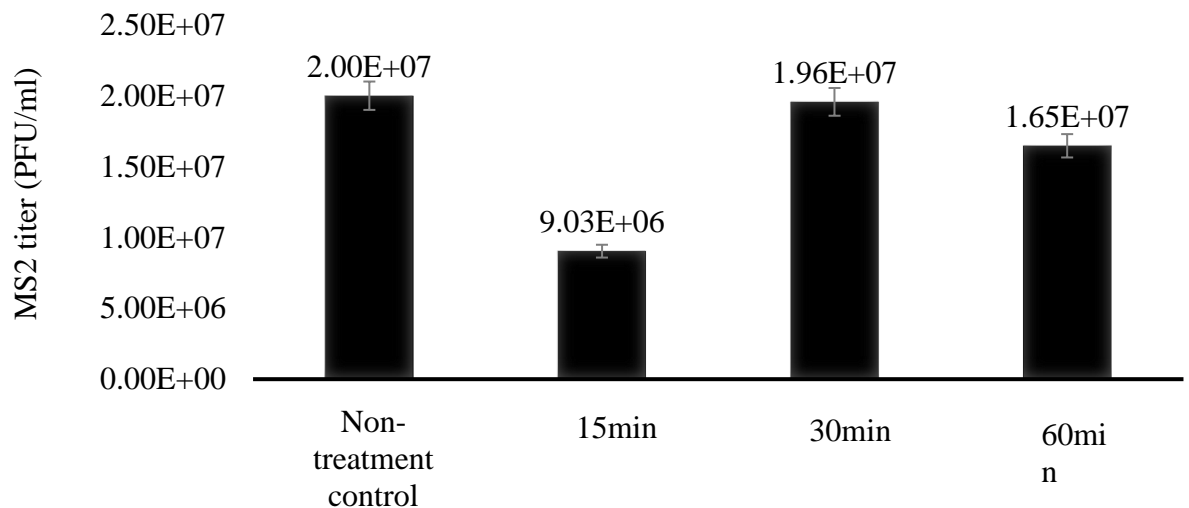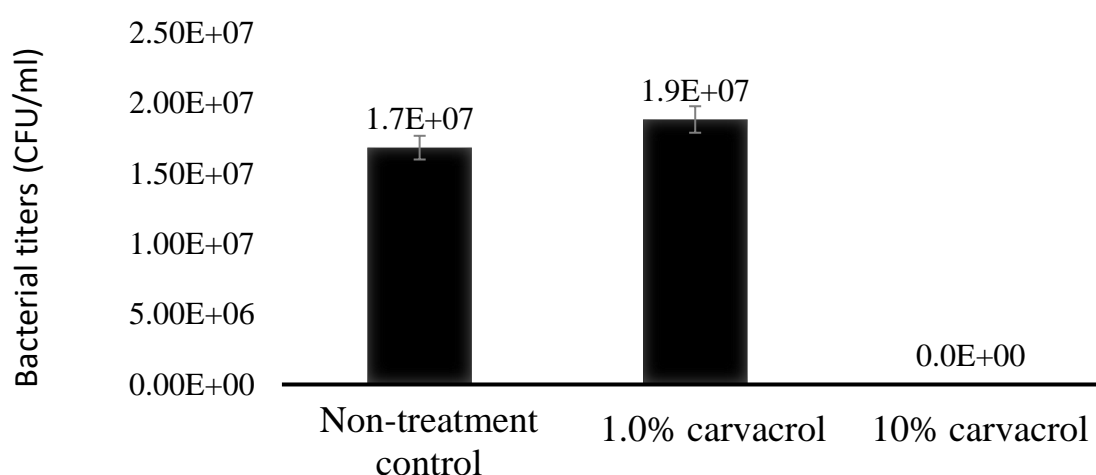

Fig. 6. Effect of 1.0 and 10% carvacrol emulsion dissolved in water on *Escherichia Coli*. In 60min contact time. 1.0% carvacrol essential oil dissolved in nuclease-free distilled water shows no antibacterial efficacy compared to non-treatment control However, 7.2 log10 reductions were observed at 10% carvacrol in comparison with the non-treatment controls at 60 min contact time.

3.5. Discussion

In this work, restructuring carvacrol in positively charged nanoemulsions did not enhance antiviral efficacy against bacteriophage MS2. The hypothesis that positively charged nanoemulsions would enhance delivery of carvacrol to the hydrophobic contacts that hold

the negatively charged viral protein capsid together was found not true. This suggests that nanoemulsions made using the low energy method may require the target microorganism to have a lipid membrane to enhance antimicrobial efficacy.

Therefore, in this study, we applied different concentrations of carvacrol nanoemulsions dissolved in different aqueous phase (nuclease-free distilled water) to exam antimicrobial efficacy on MS2 and *Escherichia coli*. In different concentration (0.5 and 1.0%) of carvacrol nanoemulsion dissolved in nuclease-free distilled water showed -0.09 and 0.7 $\log_{10}$ antiviral reduction; these two concentrations of nanoemulsion did not show significant reduction compared to the non-treatment control.

Various mechanisms, such as applied low concentration (0.5%) of carvacrol could decrease the antiviral efficacy. The results showed that 1% carvacrol nanoemulsion dissolved in water which has higher viral reduction (0.7 $\log_{10}$ reduction) than 0.5% nanoemulsion (-0.09 log reduction). Therefore, we could increase the higher ratio of carvacrol that may increase the antiviral efficacy. For example, we can raise carvacrol from 0.5 or 1.0% to 5% that may increase antiviral efficacy, and could be grounds for future work.

However, a previous study (Terjung et al., 2012) proved that essential oils prefer to stay at oil-in-water interfaces, therefore, the reduction of the specific interfacial area of emulsions by increasing the essential oil droplet size caused the increase of essential oil concentration in aqueous phase. When the concentration of carvacrol nanoemulsion is increased, the nanoemulsion droplet size may also increase—destabilizing the nanoemulsion. In addition, when raising carvacrol from 0.5 or 1.0% to 10%, we may reduce the concentration of surfactant (Tween 80) that may increase carvacrol nanoemulsion droplet.

To optimize carvacrol nanoemulsion droplet size, adding an appropriate ratio between oil and surfactant phase can helps formation of small droplets. In the field of nutraceutical delivery systems, nanoemulsion of appropriate droplet size is between 10 nm and 200 nm (Solans et al., 2005; Sagalowicz et al., 2010) because they can enhance the cell absorption by enabling passive mechanisms by pass through the cellular membrane due to their sub-cellular size (Donsì et al., 2012). Moreover, increasing the surfactant concentration help formulate the smaller droplets size because surfactant can lowers the interfacial tension at the oil-in-water interface (Chuesiang et al., 2019). Therefore, design criteria for the choice of the higher concentration in the formulation of the carvacrol nanoemulsion-based delivery system should be took into account on targeting bacteriophage MS2.

Since significant antimicrobial properties have been reported for carvacrol it has potential to be used as an antimicrobial agent in the food industry (Mazarei et al., 2019). In this study, antibacterial effects of 1.0% carvacrol nanoemulsion showed slight effect (0.35 log10 reduction) against *Escherichia coli* at 15 min contact time while, 1% carvacrol nanoemulsion showed no antibacterial effect at 30 and 60 min contact time on *Escherichia coli.*

As previous described in section 3, various mechanisms, such as applied low concentration of carvacrol, could decrease the antibacterial efficacy. The higher ratio of carvacrol essential oil may increase antibacterial efficacy. For example, raising carvacrol from 1.0% to 10% may increase antiviral efficacy. However, increasing the concentration of carvacrol nanoemulsion, can increase droplet size on nanoemulsion. The antimicrobial activity against *Escherichia coli* resulted to be significantly dependent on emulsion droplet size,

and not to be affected by emulsion formulation, in agreement with the behavior observed for the plant-base emulsions, in particular with nanoemulsions of sub-cellular mean droplet size (<200 nm) caused a higher inactivation (Donsì et al., 2012). Therefore, these results demonstrate that the 1% carvacrol nanoemulsion has low antibacterial efficacy in 15, 30 and 60 min contact time.

3.6. Conclusion

In conclusion, in this study, we applied different concentration of carvacrol nanoemulsion to evaluate whether the desire of droplet size and charged can enhance delivery to negatively charged viral capsid. The results showed low antimicrobial activities regardless of all concentration. However, 10% carvacrol showed strong antibacterial affect (7 $\log_{10}$ reduction), the results proved that carvacrol is an antimicrobial agent as reports from recent studies. In future work, we can raise carvacrol nanoemulsion concentration to 5%, that may increase antimicrobial compound for interacting with microorganism.

CHAPTER 4

CARVACROL EMULSION ANTIMICROBIC EFFICACY ON BACTERIOPHAGE MS2 AND *ESCHERICHIA ECOLI*

4.1. Abstract

Carvacrol is considered to improve food safety and the health of customers, and have been registered by U.S. Food and Drug Administration and recognized as safe component. Carvacrol is the antimicrobial monoterpene phenolic compounds which contains with lipophilic and volatile secondary metabolites that can thus interact with the cell physicochemical properties. In this study, the antimicrobial efficacy of carvacrol oil-in-water emulsions of concentrations of 0.1, 0.5, 1.0, and 10% were examined on bacteriophage MS2; 1.0 and 10% carvacrol were also examined on *Escherichia coli*. In addition, carvacrol oil-in-water emulsion stabilized by a nonionic surfactant (Tween 80) and cationic surfactant (CTAB) or in combination with anionic surfactant (sodium dodecyl sulfate) in inactivation of MS2 was also investigated. In antiviral efficacy, carvacrol at 0.1, 0.5, 1.0 and 10% have no reduction on bacteriophage MS2 at 60min contact time. In antibacterial efficacy, the 1.0% concentration showed no bacteria colony reduction on *E. coli* while 10% carvacrol showed 7.2 $\log_{10}$ reduction at 60min contact time. At ionic surfactant on antiviral efficacy test, 0.1% sodium dodecyl sulfate (SDS) showed no reduction while 0.2% CTAB and 0.25% SDS showed slight antiviral reduction (0.6 and 0.55 log reduction) on the phage. Moreover, a 2.5 $\log_{10}$ reduction was observed at 0.5% SDS on MS2. Due to there is no antiviral reduction when we applied carvacrol along on MS2, thus we added an appropriate amount of cationic or anionic surfactant to investigate whether additional ionic surfactant can improve antiviral efficacy. In combination 1% carvacrol with surfactant

study, incubated aliquot MS2 and cationic carvacrol emulsion contained (1% carvacrol plus 0.5% Tween 80 and 0.2% CTAB) at 60min, a 2 $\log_{10}$ reductions was observed on MS2. In anionic emulsion (contained 1% carvacrol plus 0.25 or 0.5% SDS), mixed separately 0.25 or 0.5% SDS with MS2 for 30 min and 60 min. At both 0.25 and 0.5% SDS, the ~4.6 log10 reduction were observed at two different contact time (30 and 60min). In this study, both cationic and anionic carvacrol emulsion can improve the antiviral efficacy, the results represent a step forward in improving food safety and reduce viral plaques colony by using carvacrol oil-in-water emulsion combining surfactant.

4.2. Introduction

Carvacrol, a monoterpene phenol, has emerged as a natural antimicrobial due to its wide spectrum activity against food spoilage and pathogenic fungi, yeast and bacteria (Nostro et al., 2012). Carvacrol is the primary component of oregano essential oil and has been identified as a natural economical food preservative (Lu et al., 2010; Obaidat et al., 2009). It has recently been reported that carvacrol could effectively reduce the infectivity of murine norovirus (MNV) (Gilling et al., 2014), a HuNoVs surrogate, and rotavirus (Pilau et al.,2011). The area of particular interest is their potential to inhibit some of the most serious foodborne pathogens, such as *Escherichia coli* (Božik et al. 2018) . However, the effectiveness of carvacrol oil-in-water emulsion plus ionic surfactant against bacteriophage MS2 and *Escherichia coli*, as well as its efficacy in food applications has yet to be explored.

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 inactive MS2 by only using carvacrol. Therefore, we applied surfactant that provide information about the solute-solvent interactions. The surface-active properties describe the interaction of surfactant molecules between two phases, a desired amount of surfactant can adsorb with their molecular arrangement and reduce surface tension (Danov et

al., 2012), that improve the carvacrol solubility in solution.

In oil-in-water emulsion system, the solubility of essential oil can be altered by using non-ionic surfactants (Tween 80). The polysorbate family of surfactants (included Tween 80), can be used by mixing water, the surfactant, and essential oil using a vortex mixer (Chen et al., 2014). Additionally, a study (Kumar et al., 2018) suggested that non-ionic surfactant also help forms a stable oil-in-water emulsion. Therefore, adding an appropriate amount of Tween80 for suitability in carvacrol emulsion system could be took in account in this study.

Addition of a cationic surfactant to emulsions has been shown to further enhance antimicrobial activity (Ziani et al., 2011). The mechanism of antibacterial effect of essential oil is mainly based on the hydrophobicity of their constituent molecules. In the case of virus, the positively charged compounds can adsorb on viral capsid by also electrostatic interaction which inhibit viral adsorption on host cells. However, the microbial activities of cationic compounds have mainly been focused on pathogenic bacterial but they were less studied on viruses (Pan et al., 2006).

SDS, as a surfactant compound (Singer et al.,1993), makes the liquid spread more easily and can lower the interfacial tension between two liquid (Li et al., 2013), which facilitates the organic compound to penetrate the cell membrane and accumulate. Previous research suggested SDS is able to cause significant damages to viral structures of both enveloped and nonenveloped viruses; the result on combinations of SDS with chlorinated water improved inactivation of HuNoV surrogates on fresh vegetables and Fruits (Predmore et al., 2011). Another study (Zhou et al., 2017) also presents that the treatment of washing with levulinic acid plus SDS was able to reduce MNV-1 and MS2 on strawberry (Aydin et al., 2013).

In this study, we hypothesize that incorporation of nonionic (Tween 80) plus cationic (CTAB), or anionic (SDS) surfactant will enhance the efficacy of a strongly oxidizing essential oil active compound (carvacrol) on bacteriophage MS2 and *Escherichia coli*. The development of an efficacious natural disinfectant (>4 log10 reduction of viral titer) that can be incorporated into foods or used on food contact surfaces is of significant interest to the food industry as well as for public health.

4.3. Materials and Methods

4.3.1. Bacterial Hosts, and Cell Lines

Bacteriophage MS2 was kindly provided as a gift by L-A. Jaykus (North Carolina State University, Raleigh, NC) and its host *Escherichia coli* strain (ATCC 15597), were purchased from ATCC (Manassas, VA).

4.3.2. *Escherichia Coli* Preparation

Incubated *Escherichia coli* was kept at -80°C in a mixture of TSB containing 0.1% thiamine and 0.2% glucose and 50%v/v of glycerol as frozen stock. Before use, *Escherichia coli* was streaked in appropriate selective media (5ml TSB containing 0.1% thiamine and 0.2% glucose) with cultured tubes at 37°C in an atmosphere containing 5% CO_2 for 18 h.

4.3.3. Bacteriophage MS2 Plaque Assay

Incubated *Escherichia coli* from frozen stock in 5mL TSB containing 0.1% thiamine and 0.2% glucose at 37°C in an atmosphere containing 5% CO_2 for 18h. Adding 300ul of overnight *Escherichia coli* culture to inoculate at 29.7ml TSB containing 0.1% thiamine and 0.2% glucose in a 100ml conical flask. Incubate the *Escherichia coli* at 37 °C in an atmosphere containing 5% CO_2 using an incubator shaker (100 rpm) for 2h until the optical density at 600 nm of 0.60. As a guideline, an OD600 of 0.6 corresponds to approximately 2.6×10^8 CFU/ml for cultures of *Escherichia coli* strains. Warmed 1.0% TSA plates containing 0.1% thiamine

and 0.2% glucose in 37 °C for at least 1 hour prior to plaque assay beginning. Melted and tempered desired tubes of 9ml-0.5% TSA containing 0.1%thiamine and 0.2% glucose in a 50°C water bath. MS2 stock (3.18×10^{11} PFU/ml) was serially diluted in TSB containing 0.1% thiamine and 0.2% glucose, and 0.7 mL of diluted phage was mixed with 0.3 mL of 2-h *Escherichia coli* host. The 1-ml host-MS2 combination was then added to 9mL of 0.5% TSA containing 0.1% thiamine, 0.1% calcium chloride and 0.2% glucose, mixed and poured on 1% TSA containing 0.1% thiamine and 0.2% glucose bottom agar plates, and incubated at 37°C overnight. To obtain accurate quantitative analyses of plaque numbers, petri plates should have relatively diluted MS2 samples (25 to 250 PFU/plate).

4.3.4. Propagation of Bacteriophage MS2

Selected plate with complete lysis and flooded with 3mL TSB 0.1% thiamine and 0.2% glucose. Gently scraped off the top layer of MS2 plaques formation surface into sterile 50ml tubes as over layer of complete lysis plaques suspensions. Bring volume to 40ml with TSB containing 0.1% thiamine and 0.2% glucose. Adding 0.2g of EDTA and 0.026g of lysozyme to each tube and vortex for 10s. Incubated each tube at 37°C in an atmosphere containing 5% CO_2 using an incubator shaker (100 rpm) for 2h. The over layer of complete lysis plaques suspensions were then centrifuged at 9280G for 10 min. Adding supernatant in 0.22um sterilize filter and then aliquoted to 1mL storage cryogenic tubes. Storing at either 4°C for several weeks or -80°C for several years. Cryoprotectant (such as glycerol) is not necessary.

4.3.5. Carvacrol Emulsion Preparation.

Purified carvacrol (>98%) was purchased from Sigma-Aldrich (St. Louis, MO), Polyoxyethylene-80 (Tween 80) and Hexadecyltrimethylammonium bromide (CTAB) was purchased from (Markham, ON), were added with nuclease-free distilled water to form emulsion's aqueous phase. Sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich (St. Louis, MO), was added with nuclease-free distilled water to form emulsion's aqueous

phase. Initially, few different amounts of purified carvacrol were mix with nuclease-free distilled water to form different concentration (0.1, 0.5, 1.0 and 10% v/v) of carvacrol emulsion. Nuclease-free distilled water (Thermo Fisher, Waltham, MA) was added with 0.2% of cationic surfactant (CTAB) and 0.5% Tween 80 for 1 min by using a vortex at room temperature to form an aqueous phase. A 1.0% carvacrol was then added at aqueous phase then all components were mixed for 1 min at 25 °C to make a cationic antimicrobial emulsion. In combining different surfactant with purified carvacrol to form emulsion, a 0.1, 0.25 and 0.5% (v/v) concentration of SDS were separately added with nuclease-free distilled water for 1 min by using a vortex at room temperature to form an aqueous phase. A 1.0% (v/v) carvacrol was then separately added to aqueous phase (with different concentration of SDS) then all components were mixed for 1 min at 25 °C to make an antimicrobial emulsion.

4.3.6. Antiviral Effects of Carvacrol Emulsion on MS2.

Incubated *Escherichia coli* from frozen stock in 5ml TSB containing 0.1% thiamine and 0.2% glucose at 37°C in an atmosphere containing 5% CO_2 for 18 h. Adding 300ul of overnight *Escherichia coli* culture to inoculate at 29.7ml TSB containing 0.1% thiamine and 0.2% glucose in a 100ml conical flask. Incubate the *Escherichia coli* at 37 °C in an atmosphere containing 5% CO_2 using an incubator shaker (100 rpm) for 2h until the optical density at 600 nm of 0.60. As a guideline, an OD600 of 0.6 corresponds to approximately 2.6×10^8 CFU/mL for cultures of *Escherichia coli* strains. Gently thawed MS2 stock (8.71×10^{10} PFU/ml) on ice, diluted in 1/100 (10ul MS2 in 990ul sterile nuclease-free distilled water). First, 500ul of 0.1, 0.5, 1.0 and 10% concentration of carvacrol emulsion were separately mixed with an equal volume of MS2 (500ul) and incubated with gentle rotation for desired time (60 min) at room temperature. In cationic antimicrobial, 500ul emulsion (contained 1.0% carvacrol, 0.2% CTAB and 0.5% Tween 80) was mixed with an equal volume of MS2 (500ul) and incubated with gentle rotation for desired time (60 min) at room temperature. Another emulsion was also tested

on suspension assay, 500ul of 1.0% carvacrol emulsion contained with a different concentration (0.1, 0.25 and 2.5% of SDS) was mixed with an equal volume of MS2 (500ul) and incubated with gentle rotation for two different desired time (30 and 60min) at room temperature. Antimicrobial carvacrol emulsion were replaced with sterile nuclease-free distilled water which also incubate with gentle rotation for desired time (30 and 60 min) at room temperature used as the untreated controls. After incubation, treated MS2 and untreated control were neutralized in TSB containing 3% meat extract. MS2 plaque assays were performed using incubated 2-h *Escherichia coli*. MS2 treated with 1.0% carvacrol emulsion or water after neutralization with TSB containing 3% meat extract was serially diluted in TSB containing 0.1%thiamine and 0.2% glucose, and 0.7 ml of diluted phage was mixed with 0.3 ml of 2-h *Escherichia coli* host. The 1-ml host-MS2 combination was then added to 9ml of 0.5% TSA containing 0.1% thiamine, 0.1% calcium chloride and 0.2% glucose, mixed and poured on 1% TSA 0.1% thiamine and 0.2% glucose bottom agar plates, and incubated at 37°C overnight before counting.

4.3.7. Antibacterial Effects of Carvacrol Emulsion on *Escherichia Coli*.

Incubated *Escherichia coli* from frozen stock in 5ml TSB containing 0.1% thiamine and 0.2% glucose at 37°C in an atmosphere containing 5% CO_2 for 18 h. Adding 300ul of overnight *Escherichia coli* culture to inoculate at 29.7ml TSB containing 0.1% thiamine and 0.2% glucose in a 100ml conical flask. Incubate the *Escherichia coli* at 37 °C in an atmosphere containing 5% CO_2 using an incubator shaker (100 rpm) for 2h until the optical density at 600 nm of 0.60. As a guideline, an OD600 of 0.6 corresponds to approximately 2.6×10^8CFU/mL for cultures of *Escherichia coli* strains. A 500ul-aliquot of incubated bacterial culture was mixed with 500ul of different concentration (1.0 and 10%) of carvacrol emulsion and 9.0 mL of sterile nuclease-free distilled water. To determine the inactivation kinetics, an aliquot was taken after 15, 30 and 60 min of contact time. A control was performed with the same method,

replacing the carvacrol emulsion by sterile nuclease-free distilled water. Minimum inhibitory concentration (MIC) were performed by using *Escherichia coli* treated with carvacrol emulsion or water. Serially diluted treated and untreated *Escherichia coli* in TSB containing 0.1% thiamine and 0.2% glucose, and 0.1 ml of diluted cultures were poured on 1% TSA 0.1% thiamine and 0.2% glucose bottom agar plates. And incubated at 37°C overnight before counting.

4.3.8. Statistical Analysis.

Each experiment was performed in triplicate and all values are reported as the mean ± standard deviation (SD) by Microsoft Excel. Results from the plaque assay of treatments and non-treatment controls were statistically assayed in duplicate plates and two replicate analyses were made of each nano-emulsion sample.

4.4. Results

4.4.1. Carvacrol and Its Surfactants Antiviral Effect on MS2

Low concentration (0.1, 0.5 and 1.0%) of carvacrol emulsion show low inactivation effect (<0.05 log reduction) on MS2 (Fig. 7.); although treatment with increasing the concentration on carvacrol to 10%, the results still showed no antiviral effect on the phage. At 0.2% CTAB treatment, a slight reduction (0.6 log) on bacteriophage MS2 showed as viral titers in Although, in 0.1% SDS showed no inactivation effect on bacteriophage MS2, a 0.55 and 2.5 $\log_{10}$ reduction were observed when treated with 0.25 and 0.5% SDS at same treatment contact time showed as viral titers in Fig. 8.
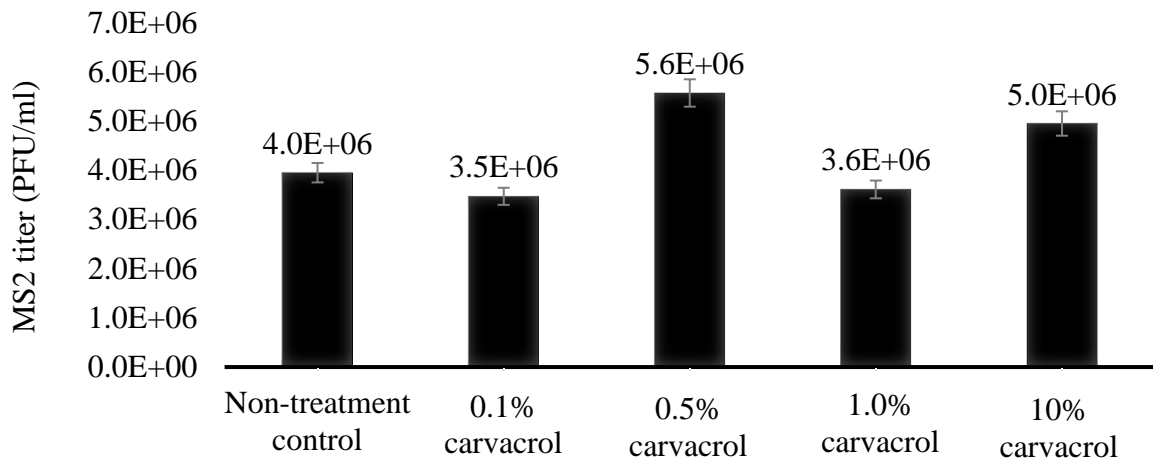
Fig. 7. Carvacrol antiviral effect on bacteriophage MS2. Effect of 0.1, 0.5,1.0 and 10% carvacrol against bacteriophage MS2 at 60 min treatment contact time.
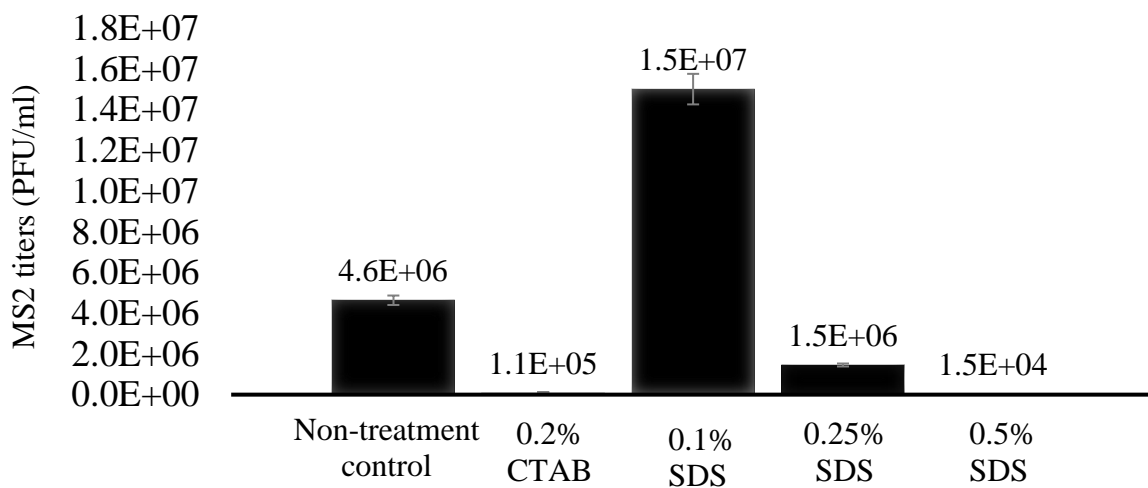

Fig. 8. Cationic and anionic surfactant antiviral effect on bacteriophage MS2.

4.4.2. Cationic and Anionic Carvacrol Emulsion Antiviral Effect on MS2

The results of antiviral effect are shown in Fig. 9. The antiviral efficacy of bacteriophage MS2 was determined by comparison with the viral reductions at 60 min treatment contact time at room temperature. 2.7×10^5 viral titers were observed at cationic carvacrol nanoemulsion (contained 1% carvacrol 0.5% Tween 80 plus 0.2% CTAB) compared with non-treatment control (1.5×10^7 viral titers). 2.3×10^1 and 1.9×10^1 viral titers of MS2 were observed when MS2 treated with anionic carvacrol emulsion (contained 1% carvacrol plus 0.25 or 0.5% SDS). The antiviral effect of cationic carvacrol emulsion (contained 1% carvacrol, 0.2% CTAB and 0.5% Tween 80) achieved 2 $\log_{10}$ reduction on MS2 compared to non-treatment control. When applied anionic carvacrol emulsion (contained 1% carvacrol and either 0.25 or 0.5% SDS), both were achieved 5.3 $\log_{10}$ reductions on MS2.
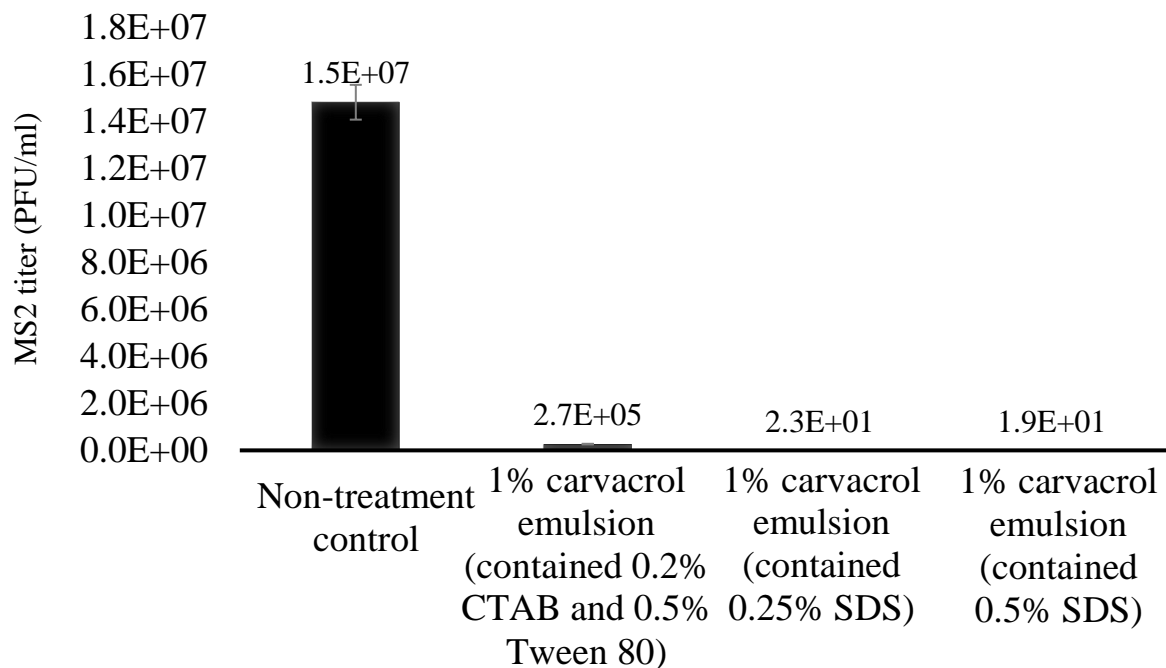

Fig. 9. Cationic and anionic carvacrol emulsion antiviral effect on MS2 at 60 min contact time. Cationic carvacrol emulsion (contained 1% carvacrol, 0.2% CTAB and 0.5% Tween

80) and anionic carvacrol emulsion (contained 1% carvacrol and 0.25% or 0.5% SDS) antiviral effect on MS2.

4.4.3. Anionic Carvacrol Emulsion Antiviral Effect on MS2 at 30 min Contact Time

The results of antiviral effect are shown in Fig. 10.6×10^1 and 1×10^2 viral titers of MS2 were observed when MS2 treated with anionic carvacrol emulsion (contained 1% carvacrol plus 0.25 and 0.5% SDS) and 4.6×10^6 viral titers were observed at untreated control. The antiviral efficacy of MS2 was determined by comparison with the log_{10} reductions at the 30 min treatment contact time at room temperature. In 30 min of treatment contact time, the antiviral effect of anionic carvacrol emulsion contained different concentration (0.25 and 0.5%) of SDS. A 4.8 log reduction was overserved in MS2 with 1% carvacrol emulsion (contained 0.25% SDS) and a 4.6 log reduction was overserved in MS2 with 1% carvacrol emulsion (contained 0.5% SDS). Therefore, anionic carvacrol emulsion contains with different concentration of SDS, all have strong antiviral efficacy on the phage at 30 min contact time showed in Fig. 10.
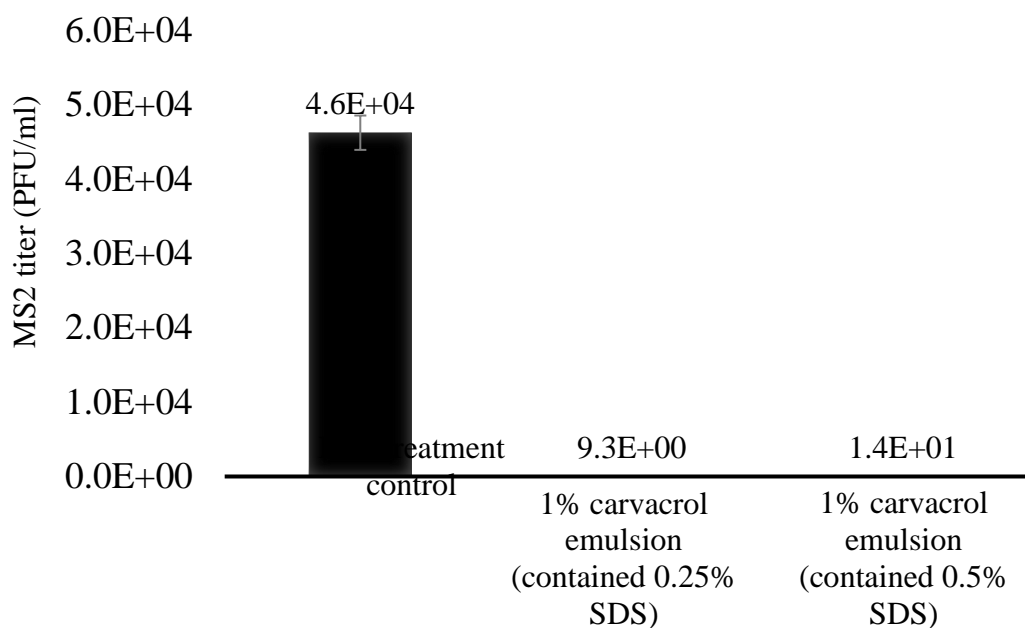
Fig. 10. Anionic carvacrol emulsion antiviral effect on MS2 at 30 min contact time. Anionic carvacrol emulsion (contained 1% carvacrol, 0.25 or 0.5% SDS) antiviral effect on MS2at 30 min contact time.

4.4.4. Effect Of 1.0 And 10% Carvacrol Emulsion Dissolving in Water on *Escherichia Coli* in 60min Contact Time.

The antimicrobial effects of 1.0% carvacrol optimized against *Escherichia coli* showed in Fig.6. The results indicated that the 1.0% carvacrol at 60 min contact time has no bacteria colony reduction (1.9 $\times 10^7$ CFU/ml bacterial titers compare with non-treatment control 1.7$\times$107 CFU/ml). However, in 10% carvacrol treatment at same contact time, a 7.2 log10 reduction was achieved. Therefore, the higher concentration (10%) of carvacrol essential has higher antibacterial effect than lower concentration (1%).

4.5. Discussion

We hypothesized carvacrol oil-in-water emulsion could improve antiviral efficacy, however 0.1, 0.5, 1.0 and 10% carvacrol emulsion dissolved in water shows no antiviral efficacy. There is no viral reduction compare occurred following 0.1, 1.0 and 10% treated carvacrol than non-treatment control at 60 min treatment contact time. In addition, in antimicrobial efficacy, the results indicated that 1.0% carvacrol emulsion at 60 min contact time has no bacteria colony reduction. However, in 10% carvacrol emulsion treatment at same contact time (60 min), a 7.2 log reduction was achieved. It has been reported that hydrophobicity of carvacrol could be an advantage for inducing antibacterial properties. It is well known that lipophilic compounds possess a high affinity for cell membranes and their insertions induce changes in membrane physicochemical properties. The interactions of antimicrobial compounds and cell membranes are considered to affect both the lipid ordering and the bilayer stability, resulting in a membrane integrity decrease and potential depolarization (Arfa et al., 2006). In addition, a study by (Ultee et al., 1998) presented the carvacrol concentration increases, more of the compound is expected to dissolve in the membrane and more damage of the membranes appears.

Since the water solubility of carvacrol is low, we investigated the ability of three representative surfactants (Tween 80) to enhance solubility to enhance carvacrol antimicrobial efficacy. The solubility of essential oil can be altered by using a neutral surfactant Tween 80 (Chen et al. 2014) and addition of a cationic surfactant to emulsions can further enhances antimicrobial activity (Ziani et al. 2011) due to the positive charge of CTAB. A 0.6 log reduction was achieved when only apply 0.2% CTAB dissolved in nuclease-free distilled water, the results showed the cationic surfactant has slight antiviral effect on bacteriophage MS2.

In 1% carvacrol emulsion (contained 0.5% Tween plus 0.2% CTAB), Tween 80 helps oil-in-water emulsion form smaller droplet size, the surfactant is favored by inequality in the hydrocarbon chain length (Wang et al. 2009). The unequal surfactant chain length is expected to lead to a more disarranged surfactant/oil interface, hence, producing a region of enhanced oil mixing (Eastoe et al. 2003). In order to find an antiviral effect on bacteriophage MS2, the positively charged compounds, including CTAB may enhance emulsions antiviral activity (Ly-Chatain et al., 2013). Additionally, the positively charged compounds can adsorb on viral capsid by also electrostatic interaction which inhibit viral adsorption on host cells (Pan et al., 2006). At pH acid, phages have a neutral or positive charge leading to a reduction the absorption of cationic compounds on phage. A lower cell viability have been observed when the cell surface charge change from negative to positive (Ly-Chatain et al., 2013). In this study, when we applied 0.2% CTAB surfactant along against bacteriophage MS2, a 0.6 log10 reduction was achieved. Therefore, we made a carvacrol emulsion (1.0% carvacrol plus 0.5% Tween 80 and 0.2% CTAB), a 2 log10 reduction on MS2 was observed at 60 min contact time. Although results showed a moderate antiviral efficacy, carvacrol emulsion (1.0% carvacrol plus 0.5% Tween 80 and 0.2% CTAB) proved that the additional of CTAB cause further

antiviral effect (compared with 1% carvacrol along) on MS2.

In addition, we also applied 1.0% carvacrol emulsion plus a different concentration (0.25 and 0.5%) of SDS to exam the antiviral efficacy on bacteriophage MS2. Anionic surfactants (SDS), which can reduce the surface tension of water by adsorbing at the liquid-gas or liquid-liquid interface and thus can potentially enhance the removal of viruses from fresh produce. Another alternative is that the surfactants are able to directly denature the virus, resulting in inactivation during sanitization (Predmore et al., 2011).

In the present study, we tested the different concentration (0.1, 0.25 and 0.5%) of SDS to exam the anionic surfactant antiviral efficacy. Although 0.1% SDS showed no antiviral reduction, 0.5 and 2.5 $\log_{10}$ reduction were observed when applied 0.25 and 0.5% SDS on bacteriophage MS2. Therefore, we tested 1% carvacrol emulsion in combination with 0.25 and 0.5% concentration of SDS surfactants at two different contact times (30 and 60 min). In all cases, two different concentration of SDS show significant antiviral efficacy at two different contact time (30 and 60min). These results presented at 0.25 an 0.5% SDS concentration in treatment with 30 and 60 min contact time, treated phage were all observed a >4.6 log viral reduction on MS2. This is due to the fact that the viricidal activity of surfactants for sexually transmitted mechanism in viral capsid (Howett et al., 1999); the capsid protein of norovirus surrogate became aggregated after incubation with SDS and that the structure of viral capsid was severely altered (Predmore et al., 2011).

4.6. Conclusion

Taken together, in anionic carvacrol nanoemulsions antiviral activities, the results suggest that SDS as well as other surfactants can be useful in the inactivation of both enveloped and

nonenveloped. Viruses. In future study, we can apply lower concentration of carvacrol to combine with SDS. Since 1% carvacrol combine with 0.5% Tween 80 plus 0.2% CTAB or 1% carvacrol combine with 0.2 or 0.5% SDS can show the strong antiviral efficacy. To lower down the 1% carvacrol emulsion to 0.5% or 0.1% may still show the reduction on the phage.

REFERENCES

A. Ultee, L.G.M. Gorris and E.J. Smid. 1998. Bactericidal activity of carvacrol towards the food-borne pathogen Bacillus cereus Bactericidal activity of carvacrol towards the food-borne pathogen Bacillus cereus. (SEPTEMBER 1998). https://doi.org/10.1046/j.1365-2672.1998.00467.x.

Anton, N., & Vandamme, T. F. (2009). The universality of low-energy nano-emulsification. International Journal of Pharmaceutics, 377(1–2), 142–147. https://doi.org/10.1016/j.ijpharm.2009.05.014.

Arfa, A. Ben, Combes, S., Gontard, N., & Chalier, P. (2006). Antimicrobial activity of carvacrol related to its chemical structure. 43, 149–154. https://doi.org/10.1111/j.1472-765X.2006.01938.

Avato, P., & Argentieri, M. (2018). Plant biodiversity: phytochemicals and health. Phytochemistry Reviews, 17(4), 645–656. https://doi.org/10.1007/s11101-018-9549-1.

Aviles, B., Klotz, C., Smith, T., Williams, R., & Ponder, M. (2013). Survival of Salmonella enterica Serotype Tennessee during Simulated Gastric Passage Is Improved by Low Water Activity and High Fat Content. Journal of Food Protection, 76(2), 333–337. https://doi.org/10.4315/0362-028X.

B. J¨onsson, B. Lindman, K. Holmberg, B. Kronberg, Surfactants and Poly- mers in Aqueous Solution, John Wiley & Sons, Chichester, 1998.

Božik, M., Hovorková, P., & Klouček, P. (2018). Antibacterial Effect of Carvacrol and Coconut Oil on Selected Pathogenic Bacteria. Scientia Agriculturae Bohemica, 49(1), 46–52. https://doi.org/10.2478/sab-2018-0008.

Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in foods: a review. Int. J. Food Microbiol. 94: 223-253.

Chang, Y., McLandsborough, L., & McClements, D. J. (2013). Physicochemical properties and antimicrobial efficacy of carvacrol nanoemulsions formed by spontaneous emulsification. Journal of Agricultural and Food Chemistry, 61(37), 8906–8913. https://doi.org/10.1021/jf402147p.

Chang, Y., McLandsborough, L., & McClements, D. J. (2015). Fabrication, stability and efficacy of dual-component antimicrobial nanoemulsions: Essential oil (thyme oil) and cationic surfactant (lauric arginate). Food Chemistry, 172, 298–304. https://doi.org/10.1016/j.foodchem.2014.09.081.

Che Man, Y. B., & Marina, A. M. (2006). Medium chain triacylglycerol. In F. Shahidi (Ed.),Nutraceutical and specialty lipids and their coproducts(pp. 27e56). Boca Raton: Taylor & Francis Group.

Chen, E., Cao, L., McClements, D. J., Liu, S., Li, B., & Li, Y. (2018). Enhancement of physicochemical properties of whey protein-stabilized nanoemulsions by interfacial cross-linking using cinnamaldehyde. Food Hydrocolloids, 77, 976–985. https://doi.org/10.1016/j.foodhyd.2017.11.047.

Cromeans, T., Park, G. W., Costantini, V., Lee, D., Wang, Q., Farkas, T., … Vinjé, J. (2014). Comprehensive comparison of cultivable norovirus surrogates in response to different inactivation and disinfection treatments. Applied and Environmental Microbiology, 80(18), 5743–5751. https://doi.org/10.1128/AEM.01532-14.

Dey, T. K., Banerjee, P., Chatterjee, R., & Dhar, P. (2018). Designing of ω-3 PUFA enriched biocompatible nanoemulsion with sesame protein isolate as a natural surfactant: Focus on enhanced shelf-life stability and biocompatibility. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 538(December 2017), 36–44. https://doi.org/10.1016/j.colsurfa.2017.10.066.

Donsì, F., Annunziata, M., Vincensi, M., & Ferrari, G. (2012). Design of nanoemulsion-based delivery systems of natural antimicrobials: Effect of the emulsifier. Journal of Biotechnology, 159(4), 342–350. https://doi.org/10.1016/j.jbiotec.2011.07.001.

Donsì, F., Cuomo, A., Marchese, E., & Ferrari, G. (2014). Infusion of essential oils for food stabilization: Unraveling the role of nanoemulsion-based delivery systems on mass transfer and antimicrobial activity. Innovative Food Science and Emerging Technologies, 22, 212–220. https://doi.org/10.1016/j.ifset.2014.01.008.

Dunkin, N., Weng, S., Coulter, C. G., Jacangelo, J. G., & Schwab, K. J. (2017). Reduction of Human Norovirus GI, GII, and Surrogates by Peracetic Acid and Monochloramine in Municipal Secondary Wastewater Effluent. Environmental Science and Technology, 51(20), 11918–11927. https://doi.org/10.1021/acs.est.7b02954.

Ettayebi,K., Crawford,S.E., Murakami, K., Broughman, J.R.,Karandikar, U.,Tenge,V.R., Neill, F.H., Blutt, S.E., Zeng, X.L., Qu, L., Kou, B., Opekun, A.R., Burrin, D., Graham, D.Y., Ramani, S., Atmar, R.L., Estes, M.K., 2016. Replication of human noroviruses in stem cell-derived human enteroids. Science 353, 1387–1393.

Flores, Z., San Martín, D., Villalobos-Carvajal, R., Tabilo-Munizaga, G., Osorio, F., & Leiva-Vega, J. (2016). Physicochemical characterization of chitosan-based coating-forming emulsions: Effect of homogenization method and carvacrol content. Food Hydrocolloids, 61, 851–857. https://doi.org/10.1016/j.foodhyd.2016.07.007.

Geun Woo Park and Mark D. Sobsey. (2011). Simultaneous Comparison of Murine Norovirus, Feline Calicivirus, Coliphage MS2, and GII.4 Norovirus to Evaluate the Efficacy of Sodium Hypochlorite Against Human Norovirus on a Fecally Soiled Stainless Steel Surface.

Gilling, D. H., Kitajima, M., Torrey, J. R., & Bright, K. R. (2014). Antiviral efficacy and mechanisms of action of oregano essential oil and its primary component carvacrol against murine norovirus. Journal of Applied Microbiology, 116(5), 1149–1163. https://doi.org/10.1111/jam.12453.

Girard, M., Ngazoa, S., Mattison, K., & Jean, J. (2010). Attachment of Noroviruses to Stainless Steel and Their Inactivation, Using Household Disinfectants. Journal of Food Protection, 73(2), 400–404. https://doi.org/10.4315/0362-028X-73.2.400.

Glass PJ, White LJ, Ball JM, Leparc-Goffart I, Hardy ME, Estes MK. 2000. Norwalk virus open reading frame 3 encodes a minor structural protein. J. Virol. 74(14):6581–91.

Govaris, A., Solomakos, N., Pexara, A. and Chatzopoulou, P. S. (2010). The antimicrobial effect of oregano essential oil, nisin and their combination against Salmonella enteritidis in minced sheep meat during refrigerated stor- age. Int. J. Food Microbiol. 137:175–180.
.
Graham, C., Boyle, D., & Connor, N. (2017). North Korea fires second missile over Japan as US tells China and Russia to take "direct action." The Telegraph, 71(2), 912–920. https://doi.org/10.1128/AEM.71.2.912.

Gupta, A., Eral, H. B., Hatton, T. A., & Doyle, P. S. (2016). Nanoemulsions: Formation, properties and applications. Soft Matter, 12(11), 2826–2841. https://doi.org/10.1039/c5sm02958a.

Ha, J. H., Choi, C., Lee, H. J., Ju, I. S., Lee, J. S., & Ha, S. Do. (2016). Efficacy of chemical disinfectant compounds against human norovirus. Food Control, 59, 524–529. https://doi.org/10.1016/j.foodcont.2015.04.040.

Ha, Ji-Hyoung, Sung H Kim, and Hee-Min Lee. "Efficacy of Combination Treatment with Sodium Metasilicate and Sodium Hypochlorite for Inactivation of

Norovirus on Fresh Vegetables." Foodborne Pathogens and Disease 15.2 (2018):73-80.

Hirneisen, K. A., Black, E. P., Cascarino, J. L., Fino, V. R., Hoover, D. G., & Kniel, K. E. (n.d.). Viral Inactivation in Foods : A Review of Traditional and Technologies.

Hotta M, Nakata R, Katsukawa M, Hori K, Takahashi S, Inoue H (2010) Carvacrol, a component of thyme oil, activates PPARa and suppresses COX-2 expression. J Lipid Res 51:132–139.

Howett, M. K., et al. 1999. A broad-spectrum microbicide with virucidal activity against sexually transmitted viruses. Antimicrob. Agents Chemother. 43:314–321.

Illarruel-lopez, A. V. (2012). Virolysis of Feline Calicivirus and Human GII.4 Norovirus following Chlorine Exposure under Standardized Light Soil Disinfection Conditions. 75(1), 79–84. https://doi.org/10.4315/0362-028X.JFP-11.

J. Eastoe, 3, Microemulsions, Surfactant Chem (2003) 59–95, https://doi.org/10. J. Food Prot. 73 (12), 2270–2275.

Jiang X, Wang M, Wang K, Estes MK. 1993. Sequence and genomic organization ofNorwalk virus. Virology 195:51–61.

Jones, M.K., Watanabe, M., Zhu, S., Graves, C.L., Keyes, L.R., Grau, K.R., Gonzalez- Hernandez, M.B., Iovine, N.M., Wobus, C.E., Vinje, J., Tibbetts, S.A., Wallet, S.M., Karst, S.M., 2014. Enteric bacteria promote human and mouse norovirus infection of B cells. Science 346, 755–759.

K.D. Danov, P.A. Kralchevsky, The standard free energy of surfactant adsorption at air/water and oil/water interfaces: theoretical vs. empirical approaches, Colloid J.74 (2012) 172–185.

Kamarasu, P., Hsu, H.-Y., & Moore, M. D. (2018). Research Progress in Viral Inactivation Utilizing Human Norovirus Surrogates. Frontiers in Sustainable Food Systems, 2(December). https://doi.org/10.3389/fsufs.2018.00089.

Kingsley, D. H., Vincent, E. M., Meade, G. K., Watson, C. L., & Fan, X. (2014). Inactivation of human norovirus using chemical sanitizers. International Journal of Food Microbiology, 171, 94–99. https://doi.org/10.1016/j.ijfoodmicro.2013.11.018.

Kitajima, M., Tohya, Y., Matsubara, K., Haramoto, E., Utagawa, E., & Katayama, H.

(2010). Chlorine inactivation of human norovirus, murine norovirus and poliovirus in drinking water. Letters in Applied Microbiology, 51(1), 119–121. https://doi.org/10.1111/j.1472-765X.2010.02869.x.

Koromyslova, A. D., White, P. A., & Hansman, G. S. (2015). Treatment of norovirus particles with citrate. Virology, 485, 199–204. https://doi.org/10.1016/j.virol.2015.07.009.

Kumar, N., & Mandal, A. (2018). Thermodynamic and physicochemical properties evaluation for formation and characterization of oil-in-water nanoemulsion. Journal of Molecular Liquids, 266, 147–159. https://doi.org/10.1016/j.molliq.2018.06.069.

Lambden PR, Caul EO, Ashley CR, Clarke IN. 1993. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science 259(5094):516–19.

Li, X., & Chen, H. (2015). Evaluation of the pgmfor high-pressure-inactivation studies using murine norovirus and tulane virus. Applied and Environmental Microbiology, 81(2), 515–521. https://doi.org/10.1128/AEM.02971-14.

Li, X., Huang, R., & Chen, H. (2017). Evaluation of Assays to Quantify Infectious Human Norovirus for Heat and High-Pressure Inactivation Studies Using Tulane Virus. Food and Environmental Virology, 9(3), 314–325. https://doi.org/10.1007/s12560-017-9288-2.

Li, Y., & Wu, C. (2013). Enhanced inactivation of Salmonella Typhimurium from blueberries by combinations of sodium dodecyl sulfate with organic acids or hydrogen peroxide. Food Research International, 54(2), 1553–1559. https://doi.org/10.1016/j.foodres.2013.09.012.

Lima Mda S, Quintans-Junior LJ, de Santana WA, Martins Kaneto CM, Pereira Soares MB, Villareal CF (2013) Anti- inflammatory effects of carvacrol: evidence for a key role of interleukin-10. Eur J Pharmacol 699:112–117..

Liu, P., Kim, M., Schlesinger, D., Kranz, C., Ha, S., Ha, J., … Moe, C. (2015). Immunomagnetic separation combined with RT-qPCR for determining the efficacy of disinfectants against human noroviruses. Journal of Infection and Public Health, 8(2), 145–154. https://doi.org/10.1016/j.jiph.2014.08.007.

Liu, P., Yuen, Y., Hsiao, H.M., Jaykus, L.A. and Moe, C. (2010) Effectiveness of liquid soap and hand sanitizer against Norwalk virus on contaminated hands. Appl Environ Microbiol 76, 394–399.

Lou, F., Huang, P., Neetoo, H., Gurtler, J. B., Niemira, B. A., Chen, H., … Li, J. (2012). High-pressure inactivation of human norovirus virus-like particles provides evidence that the capsid of human norovirus is highly pressure resistant. Applied and Environmental Microbiology, 78(15), 5320–5327. https://doi.org/10.1128/AEM.00532-12.

Ly-Chatain, M. H., Moussaoui, S., Vera, A., Rigobello, V., & Demarigny, Y. (2013). Antiviral effect of cationic compounds on bacteriophages. Frontiers in Microbiology, 4(MAR), 1–6. https://doi.org/10.3389/fmicb.2013.00046.

M.S. Jovic, D. Manojlovic, D.M. Stankovic, A. Millc, M. Sentic, G. Roglic, Volta metricbehavior of mesotrione using silver/amalgam electrode, Int. J. Environ. Res. 7(2013) 165–172.

Manuel, C. S., Moore, M. D., & Jaykus, L. A. (2015). Destruction of the capsid and genome of GII.4 human norovirus occurs during exposure to metal alloys containing copper. Applied and Environmental Microbiology, 81(15), 4940–4946. https://doi.org/10.1128/AEM.00388-15.

Manuel, C. S., Moore, M. D., & Jaykus, L. A. (2017). Efficacy of a disinfectant containing silver dihydrogen citrate against GI.6 and GII.4 human norovirus. Journal of Applied Microbiology, 122(1), 78–86. https://doi.org/10.1111/jam.13331.

Mason, T. G., Wilking, J. N., Meleson, K., Chang, C. B., & Graves, S. M. (2006). Nanoemulsions: Formation, structure, and physical properties. Journal of Physics Condensed Matter, 18(41), 635–666. https://doi.org/10.1088/0953-8984/18/41/R01.

Mazarei, Z., & Rafati, H. (2019). Nanoemulsification of Satureja khuzestanica essential oil and pure carvacrol; comparison of physicochemical properties and antimicrobial activity against food pathogens. Lwt, 100(October 2018), 328–334. https://doi.org/10.1016/j.lwt.2018.10.094.

McClements, D. J., & Rao, J. (2011). Food-Grade nanoemulsions: Formulation, fabrication, properties, performance, Biological fate, and Potential Toxicity. Critical Reviews in Food Science and Nutrition, 51(4), 285–330. https://doi.org/10.1080/10408398.2011.559558.

Montazeri, N., Manuel, C., Moorman, E., Khatiwada, J. R., Williams, L. L., & Jaykus, L. A. (2017). Virucidal Activity of Fogged chlorine dioxide- and hydrogen peroxide-based disinfectants against Human norovirus and its surrogate, feline calicivirus, on hard-to-reach surfaces. Frontiers in Microbiology, 8(JUN), 1–9. https://doi.org/10.3389/fmicb.2017.01031.

Norovirus, H., Moorman, E., Montazeri, N., & Jaykus, L. (2017). crossm Efficacy of Neutral Electrolyzed Water for. 83(16), 1–11.

Oyi AR, Onaolapo JA, Obi RC (2010): Formulation and an- timicrobial studies of coconut (Cocos nucifera Linne) oil. Research Journal of Applied Sciences, Engineering and Technology, 2, 133–137.

Pan, Y., Lee, A., Wan, J., Coventry, M. J., Michalski, W. P., Shiell, B., et al. (2006). Antiviral properties of milkproteins and peptides. Int. Dairy J.16, 1252–1261.

Park, G. W., Collins, N., Barclay, L., Hu, L., Prasad, B. V. V., Lopman, B. A., & Vinjé, J. (2016). Strain-specific virolysis patterns of human noroviruses in response to alcohols. PLoS ONE, 11(6), 1–12. https://doi.org/10.1371/journal.pone.0157787.

Patel, M.M., Hall, A.J., Vinje, J., Parashar, U.D., 2009. Noroviruses: a comprehensive review. J. Clin. Virol. Off. Publ. Pan Am. Soc. Clin. Virol.

Piętka-Ottlik, M., Lewińska, A., Jaromin, A., Krasowska, A., & Wilk, K. A. (2016). Antifungal organoselenium compound loaded nanoemulsions stabilized by bifunctional cationic surfactants. Colloids and Surfaces A: Physicochemical and EngineeringAspects, 510, 53–62. https://doi.org/10.1016/j.colsurfa.2016.07.062.

Predmore and Li, 2011 A. Predmore, J. Li Enhanced removal of a human norovirus surrogate from fresh vegetables and fruits by a combination of surfactants and sanitizers Applied and Environmental Microbiology, 77 (2011), pp. 4829-4838.

Richards, G. P. 2012. Critical review of norovirus surrogates in food safety research: rationale for considering volunteer studies. Food Environ. Virol. 4:6–13.

Ryu, V., McClements, D. J., Corradini, M. G., & McLandsborough, L. (2018). Effect of ripening inhibitor type on formation, stability, and antimicrobial activity of thyme oil nanoemulsion. Food Chemistry, 245(October 2017), 104–111. https://doi.org/10.1016/j.foodchem.2017.10.084.

Salvia-Trujillo, L., Rojas-Graü, M. A., Soliva-Fortuny, R., & Martín-Belloso, O. (2014). Impact of microfluidization or ultrasound processing on the antimicrobial activity against Escherichia coli of lemongrass oil-loaded nanoemulsions. Food Control, 37(1), 292–297. https://doi.org/10.1016/j.foodcont.2013.09.015.

Sánchez, G., Aznar, R., Martínez, A., & Rodrigo, D. (2011). Inactivation of Human and Murine Norovirus by High-Pressure Processing. Foodborne Pathogens and Disease, Vol. 8, pp. 249–253. https://doi.org/10.1089/fpd.2010.0667.

Sato, J., Miki, M., Kubota, H., Hitomi, J., Tokuda, H., Todaka-Takai, R., &

Katayama, K. (2016). Effects of disinfectants against norovirus virus-like particles predict norovirus inactivation. Microbiology and Immunology, 60(9), 609–616. https://doi.org/10.1111/1348-0421.12435

Shan, B., Cai, Y. Z., Sun, M. and Corke, H. (2005). Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. J. Agric. Food Chem. 53:7749–7759.

Sido, R. F., Huang, R., Liu, C., & Chen, H. (2017). High hydrostatic pressure inactivation of murine norovirus and human noroviruses on green onions and in salsa. International Journal of Food Microbiology, 242, 1–6. https://doi.org/10.1016/j.ijfoodmicro.2016.11.003

Singer M.M., Tjeerdema R.S. (1993) Fate and Effects of the Surfactant Sodium Dodecyl Sulfate. In: Ware G.W. (eds) Reviews of Environmental Contamination and Toxicology. Reviews of Environmental Contamination and Toxicology, vol 133. Springer, New York, NY

Smith, H. Q., & Smith, T. J. (2019). The dynamic capsid structures of the noroviruses. Viruses, 11(3), 1–18. https://doi.org/10.3390/v11030235.

Smith-Palmer, A., J. Stewart, and L. Fyfe. 1998. Antimicrobial properties of plant essential oils and essences against five important food-borne pathogens. Lett. Appi. Microbiol. 26:118-122.

Solans, C., Izquierdo, P., Nolla, J., Azemar, N., Garcia-Celma, M.J., 2005. Nanoemulsions. Current Opinion in Colloid and Interface Science 10, 102–110. sulfate. In: Reviews of Environmental Contamination and Toxicology. Springer, pp. 95–149.

T. Tadros, P. Izquierdo, J. Esquena, C. Solans, Formation and stability of nanoemulsions, Adv. Colloid Interf. Sci. 108–109 (2004) 303–318.

Tuladhar, E., Hazeleger, W. C., Koopmans, M., Zwietering, M. H., Duizer, E., & Beumer, R. R. (2015). Reducing viral contamination from finger pads: Handwashing is more effective than alcohol-based hand disinfectants. Journal of Hospital Infection, 90(3), 226–234. https://doi.org/10.1016/j.jhin.2015.02.019.

Wang, L., Dong, J., Chen, J., Eastoe, J., & Li, X. (2009). Design and optimization of a new self-nanoemulsifying drug delivery system. Journal of Colloid and Interface Science, 330(2), 443–448. https://doi.org/10.1016/j.jcis.2008.10.077.

Wang, Q., Zhang, Z., & Saif, L. J. (2012). Stability of and attachment to lettuce by a culturable porcine sapovirus surrogate for human caliciviruses. Applied and

Environmental Microbiology, 78(11), 3932–3940. https://doi.org/10.1128/AEM.06600-11
washing with thyme oil, thymol, and carvacrol as compared with chlorine treatment.

Whitehead, K., & McCue, K. A. (2010). Virucidal efficacy of disinfectant actives against feline calicivirus, a surrogate for norovirus, in a short contact time. American Journal of Infection Control, 38(1), 26–30. https://doi.org/10.1016/j.ajic.2009.03.015.

Ziani, K., Chang, Y., McLandsborough, L., & McClements, D. J. (2011). Influence of surfactant charge on antimicrobial efficacy of surfactant-stabilized thyme oil nanoemulsions. Journal of Agricultural and Food Chemistry, 59(11), 6247–6255. https://doi.org/10.1021/jf200450m.