**INVESTIGATING THE SYNERGISTIC ANTIMICROBIAL EFFECT OF
CARVACROL AND ZINC OXIDE NANOPARTICLES AGAINST CAMPYLOBACTER**

JEJUNI

by

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Abstract

Foodborne illness is a growing concern world-wide, and *Campylobacter* in particular has been reported to cause approximately 145,000 foodborne illness cases every year in Canada. A recent annual report from the Canadian Integrated Program for Antimicrobial Resistance Surveillance reveals an increasing trend of antibiotic-resistant *Campylobacter* isolated from poultry sources across Canada. The large number of foodborne illnesses and emergence of the resistant strains of *Campylobacter* pose a serious threat in the agri-food industry. Hence, there is increasing urgency to find alternatives to conventional antimicrobials to reduce the prevalence of *Campylobacter* in the food supply chain while reducing the likelihood of resistance.

Combining antimicrobials is a potential intervention strategy to reduce the growth of pathogens by expanding the spectrum of antimicrobial activity. In this study, carvacrol and zinc oxide nanoparticles (ZnO NPs) were investigated in regards to their synergistic antimicrobial effect against *C. jejuni*. The combination of these two agents for treatment is based upon current evidence of their individual antimicrobial activity. The objectives of this thesis project were to (1) determine the synergistic antimicrobial effect of carvacrol and ZnO NPs against *C. jejuni*, (2) investigate the macromolecular fingerprints and gene expression profile of *C. jejuni* after the combinational treatment, and (3) explain the potential mechanism of the synergistic antimicrobial effect.

In this work, a macrobroth dilution method was used to test the antimicrobial effect of the compounds against *C. jejuni*. The macromolecular fingerprints of *C. jejuni* cells treated with carvacrol and ZnO NPs were investigated using confocal micro-Raman spectroscopy, whereas Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) was employed to study the

variation in bacterial gene expression after the antimicrobial treatment. The synergistic antimicrobial effect of carvacrol and ZnO NPs against *C. jejuni* was clearly demonstrated using the time-kill curve. The macromolecular fingerprints and gene expression profile revealed the role of carvacrol in the synergistic antimicrobial effect against *C. jejuni*.

The results of this study provide fundamental knowledge about bacterial stress in response to the synergistic antimicrobial effects. This antimicrobial combination may be utilized as an intervention strategy to reduce the prevalence of *C. jejuni* in agri-foods.

Preface

A part of the work in Chapter 4, investigation of gene expression profile of *Campylobacter jejuni* after treated by carvacrol and zinc oxide nanoparticles, was conducted with the assistance and guidance from Dr. Lina Ma, Research Associate in the Lu Laboratory at the University of British Columbia.

The rest of the work in this thesis project was completed by the author, Gracia Windiasti, under the guidance of supervisor, Dr. Xiaonan Lu. The committee member, Dr. Pascal Delaquis and Dr. Kingsley Amoako, provided additional advice and support for this research.

The work in this thesis has not been previously published.

Table of Contents

Abstract.....	ii
Preface.....	iv
Table of Contents	v
List of Tables	ix
List of Figures.....	x
List of Abbreviations	xi
Acknowledgements	xiii
Dedication	xv
Chapter 1: Introduction, literature review, and research objectives.....	1
1.1 Introduction.....	1
1.2 Literature review	2
1.2.1 <i>Campylobacter</i>	2
1.2.1.1 History and taxonomy of <i>Campylobacter</i>	2
1.2.1.2 Reservoirs and clinical infections caused by <i>Campylobacter jejuni</i>	4
1.2.1.3 <i>Campylobacter jejuni</i> : characteristics and growth requirements.....	6
1.2.1.4 Pathogenicity and virulence of <i>Campylobacter jejuni</i>	7
1.2.1.5 Prevalence of <i>Campylobacter jejuni</i> in the agri-food system.....	8
1.2.2 Carvacrol.....	9
1.2.2.1 Chemical structure of carvacrol	9
1.2.2.2 Regulation, application, and toxicity level	10
1.2.2.3 Antimicrobial properties	11

1.2.3	Zinc oxide nanoparticles (ZnO NPs)	12
1.2.3.1	Structure and properties	12
1.2.3.2	Regulation, application, and toxicity	13
1.2.3.3	Antimicrobial properties	15
1.3	Research objectives	16
1.4	Hypotheses	16
Chapter 2: Investigation of antimicrobial effect of carvacrol and zinc oxide nanoparticles		
against <i>Campylobacter jejuni</i>		19
2.1	Introduction	19
2.2	Materials and methods	20
2.2.1	Preparation of <i>Campylobacter jejuni</i> overnight culture	20
2.2.2	Antimicrobial susceptibility of <i>Campylobacter jejuni</i> against carvacrol	21
2.2.3	Antimicrobial susceptibility of <i>Campylobacter jejuni</i> against zinc oxide nanoparticles	22
2.2.4	Synergistic antimicrobial effect of carvacrol and zinc oxide nanoparticles against <i>Campylobacter jejuni</i>	22
2.3	Results and discussion	23
2.3.1	Antimicrobial susceptibility of <i>Campylobacter jejuni</i> against carvacrol	23
2.3.2	Antimicrobial susceptibility of <i>Campylobacter jejuni</i> against zinc oxide nanoparticles	25
2.3.3	Synergistic antimicrobial effect of carvacrol and zinc oxide nanoparticles against <i>Campylobacter jejuni</i>	26
2.4	Conclusion	27

Chapter 3: Investigation of macromolecular fingerprints of <i>Campylobacter jejuni</i> after treatment with carvacrol and zinc oxide nanoparticles	33
3.1 Introduction.....	33
3.2 Materials and methods	35
3.2.1 Sample preparation for Raman spectral collection	35
3.2.2 Confocal micro-Raman spectroscopy	35
3.2.3 Raman spectral processing and chemometric analysis	36
3.3 Results and discussion	36
3.4 Conclusion	39
Chapter 4: Investigation of the gene expression profile of <i>Campylobacter jejuni</i> after treatment with carvacrol and zinc oxide nanoparticles	43
4.1 Introduction.....	43
4.2 Materials and methods	44
4.2.1 Ribonucleic acid sample preparation	44
4.2.2 Synthesis of complementary deoxyribonucleic acid (cDNA)	45
4.2.3 Real-time quantitative polymerase chain reaction (RT-qPCR)	46
4.2.4 Data analysis	46
4.3 Results and discussion	47
4.4 Conclusion	50
Chapter 5: The putative mechanism of the action of carvacrol and zinc oxide nanoparticles against <i>Campylobacter jejuni</i>, conclusion, and future research	53
5.1 The putative mechanism of the action of carvacrol and zinc oxide nanoparticles against <i>Campylobacter jejuni</i>	53

5.2	Conclusion	54
5.3	Future research.....	55
Bibliography		57
Appendix.....		80
Appendix A Chapter 2 supplementary information.....		80
A.1	The standard curve of <i>Campylobacter jejuni</i> strains used in the study	80
Appendix B Chapter 5 supplementary information.....		81
B.1	Scanning electron microscopy images of <i>Campylobacter jejuni</i>	81

List of Tables

Table 1-1 The antimicrobial effect of carvacrol and potential antimicrobial mechanism against foodborne pathogens	17
Table 1-2 The antimicrobial effect of ZnO NPs and possible antimicrobial mechanism against foodborne pathogens	18
Table 2-1 Change of viable cells of <i>C. jejuni</i> treated with different carvacrol concentrations over 24 hours of treatment at 37°C in a microaerobic condition	28
Table 2-2 Change of viable cells of <i>C. jejuni</i> cells treated with different concentrations of ZnO NPs over 24 hours of treatment at 37°C in a microaerobic condition	29
Table 2-3 Change of viable cells of <i>C. jejuni</i> treated with carvacrol, ZnO NPs, and mixtures of the compounds after 24 hours at 37°C in a microaerobic condition.....	30
Table 4-1 List of the target genes, functions, and primers sequences	51

List of Figures

Figure 1-1 The biosynthesis of carvacrol and thymol (Adapted from Nhu-Trang et al., 2006)...	10
Figure 1-2 The crystal structures of ZnO: (A) rocksalt, (B) zinc blende, and (C) wurtzite. (Adapted from Morkoç and Özgür, 2009)	13
Figure 2-1 Time-kill curve for a mixture of three strains of <i>C. jejuni</i> treated with (A) carvacrol or (B) ZnO NPs over 24 hours at 37°C with 10% CO ₂	31
Figure 2-2 Comparison of time-kill curves obtained with carvacrol, ZnO NPs, and mixtures of the compounds against <i>C. jejuni</i> over 24 hours at 37°C under 10% CO ₂ . In this figure, C denotes carvacrol and Z denotes ZnO NPs.	32
Figure 3-1 Raman spectral fingerprints of <i>C. jejuni</i> strain F38011 treated with (A) 12.5 µg/ml ZnO NPs, (B) 20.25 µg/ml carvacrol, (C) combination of carvacrol and ZnO NPs, and (D) untreated cells from 550 to 1650 cm ⁻¹	40
Figure 3-2 Three-dimensional principal component analysis (PCA) for the segregation of <i>C.</i> <i>jejuni</i> F38011 and treated with ZnO NPs, carvacrol, and combination of carvacrol and ZnO NPs using the wavenumber region of 550 to 1650 cm ⁻¹	41
Figure 3-3 The loading plot of (A) first-, (B) second-, and (C) third-principal component of the Raman spectra from 550 to 1650 cm ⁻¹	42
Figure 4-1 Relative gene expression of untreated <i>C. jejuni</i> F38011 (control) versus treated <i>C.</i> <i>jejuni</i> F38011 cells after 15 minutes of treatment.	52
Figure 5-1 The putative mechanism of stress response and inactivation of <i>C. jejuni</i> due to the synergistic antimicrobial effect of carvacrol and ZnO NPs.....	54

List of Abbreviations

APS – Average Particle Size

ATR – Adaptive Tolerance Response

ATP – Adenosine Triphosphate

cDNA – Complementary Deoxyribonucleic Acid

CFU – Colony Forming Unit

CIPARS – Canadian Integrated Program for Antimicrobial Resistance Surveillance

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

EAFUS – Everything Added to Food in the United States

EFSA – European Food Safety Authority

FCS – Food Contact Surfaces

FDA – Food and Drugs Administration

GBS – Guillain Barré Syndrome

GMP – Good Manufacturing Practices

GRAS – Generally Recognized as Safe

LDPE – Low-Density Polyethylene

LD₅₀ – Lethal Dose to kill 50% of population

MIC – Minimum Inhibitory Concentration

MBC – Minimum Bactericidal Concentration

NCBI – National Center for Biotechnology Information

NHP – Natural Health Product

NNHPD – Natural and Non-Prescription Health Product Directorate

NOEL – No Observed Effect Level

PBS – Phosphate Buffered Saline

PCA – Principal Component Analysis

PC – Principal Component

RNA – Ribonucleic acid

rRNA – Ribosomal Ribonucleic acid

ROS – Reactive Oxygen Species

RT-qPCR – Real Time Quantitative Polymerase Chain Reaction

SEM – Scanning Electron Microscopy

VBNC – Viable but Non-Culturable

v/v – Volume by Volume

w/w – Weight by Weight

ZnO NPs – Zinc Oxide Nanoparticles

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are the core motivation for me to dream big, always learn, explore, and sail away from the safe harbor.

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Dedication

I dedicate this thesis to my beloved parents for their unconditional love, heartfelt prayers, and endless support. They are my backbone and the greatest blessing in my life.

Chapter 1: Introduction, literature review, and research objectives

1.1 Introduction

According to the World Health Organization, there were 582 million cases of foodborne illness in 2010 caused by contaminated foods (Kirk et al., 2015). Amongst many foodborne pathogens, *Campylobacter* spp. was the 2nd leading foodborne pathogen and was responsible for 96 million cases of foodborne illness in 2010 (Kirk et al., 2015). This number only accounts for reported and diagnosed cases, but there may be many under-reported and un-diagnosed cases as well. In Canada, *Campylobacter* is responsible for over 145,000 cases of foodborne illness each year (Thomas and Murray, 2014). A recent report from FoodNet Canada 2014 indicated the prevalence of *Campylobacter* in chicken breast meat and suggested that the incidence of contamination was relatively constant from year to year (Public Health Agency of Canada, 2015). In 2014, almost half of the chicken breasts sampled in Canada tested positive for *Campylobacter*. In the United States, *Campylobacter* is included as a top five foodborne pathogen and it contributes to an estimated 12% of the total economic burden of foodborne illness and over 845,024 domestic incidences (Hoffman et al., 2015). The Centers for Disease Control and Prevention in the United States (2016) reported a 13% increase in domestic *Campylobacter* cases in 2013 compared to 2006 – 2008. Although *Campylobacter* may seem to be fragile in relation to its highly specific growth conditions, this microorganism has unique abilities to thrive in the food production environment, causing mild to life-threatening infections.

Aside from foodborne illness rates, the recent annual report of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) 2013 revealed an increasing trend of antibiotic-resistance in *Campylobacter* strains isolated from poultry across Canadian

provinces (Agunos et al., 2015). According to CIPARS, there was a 10% increase in ciprofloxacin-resistant *Campylobacter* isolated from poultry within the period of 2010 to 2013 (Agunos et al., 2015). The National Antimicrobial Resistance Monitoring System in the United States (2015) reported that 22% of *Campylobacter* strains isolated from chicken slaughter houses were resistant to ciprofloxacin, a common antibiotic used in poultry production. The emergence of resistant *Campylobacter* strains to common antibiotics is alarming, and poses a serious threat to the agri-food industry and public health.

Finding an alternative to conventional antibiotics is one of many possible intervention strategies to reduce the prevalence of resistant strains of *C. jejuni* in the agri-food system. The scientific community has been studying many alternatives to conventional antibiotics, such as bacteriophage, probiotics, and different compounds with antimicrobial properties (*e.g.* metals, nanoparticles, peptides, and essential oils). This study highlights the combination of two antimicrobials with different properties.

1.2 Literature review

1.2.1 *Campylobacter*

1.2.1.1 History and taxonomy of *Campylobacter*

Campylobacter was first discovered in 1886 when Theodore Escherich observed a bacterium in a colon of an infant who died from cholera infantum (Altekruse et al., 1999; Butzler, 2004). This discovery was not well received by the scientific community due to the unsuccessful attempt at isolating the bacterium. In 1913, *Campylobacter* was isolated from the

tissues of aborted sheep and misidentified as *Vibrio fetus* by MacFayden and Stockman (Altekruse et al., 1999). This was the first successful and inadvertent attempt to isolate *Campylobacter*, which established *Campylobacter* as an animal pathogen (Crushell et al., 2004). Jones and colleagues (1931) reported an infection in calves due to a *Campylobacter*-like bacterium and misidentified it as *Vibrio jejuni*. However, the isolation of the bacterium was again unsuccessful. Doyle (1944) described a similar type of *V. jejuni* isolated from an infection in swine and identified the bacterium as *V. coli*.

The first human infection and outbreak of *Campylobacter* was reported in 1938 when 357 prison inmates in the United States contracted campylobacteriosis by the consumption of contaminated milk (Butzler, 2004; Miller and Mandrell, 2005). *Campylobacter* was reported to be the causative agent of the outbreak, but again misidentified as *Vibrio* (Levy, 1946). The second reported human infection involved pregnant women and the agent was misidentified as *V. fetus* by Vincent and colleagues in 1947 (Butzler, 2004). King (1957) described the biochemical and serological features of *V. fetus* and its association with abortion in infected pregnant women and animals. *Campylobacter* was eventually isolated from blood and stool specimens from a patient suffering from hemorrhagic enteritis by Dekeyser and colleagues (1972), and the isolate was identified as *Campylobacter jejuni* (Butzler et al., 1973).

Prior to 1963, *Campylobacter* was misidentified as *Vibrio* spp. and grouped under the *Vibrio* genus due to similar cell morphology, immunological tests, and growth conditions to *V. cholera* (On, 2005; Smith and Taylor, 1919). Sébald and Véron (1963) determined that the result of the oxidative-fermentative test and G+C nucleotide ratio in *V. fetus* and *V. bubulus* were different from other *Vibrio*-like microorganisms. Unlike other *Vibrio* species, *V. fetus* and *V. bubulus* did not ferment glucose; hence Sébald and Véron (1963) proposed to re-classify these

Vibrio-like bacteria under the genus *Campylobacter* into *C. fetus* and *C. bubulus*. The classification of *Campylobacter* spp. was then widely accepted after the development of more comprehensive biochemical and immunological tests proposed by Véron and Chatelain (1973). Since then, many isolates of *Vibrio*-like microorganisms were re-classified as *Campylobacters*, such as *C. coli* and *C. jejuni*.

The advancement of selective media and genomic technology resulted in the reassignment of some *Campylobacter* spp. to different genera, such as *Arcobacter*, *Helicobacter*, *Dehalospirillum*, and *Sulfurospirillum* (Goodwin et al., 1989a,b; Vandamme et al., 1991; Vandamme and Goossens, 1992a, b). These genera are all from the family of Campylobacteraceae. As of 2015, the genus *Campylobacter* comprises 26 species and 10 subspecies (Public Health England, 2015).

1.2.1.2 Reservoirs and clinical infections caused by *Campylobacter jejuni*

Most of the *Campylobacter* species isolated from enterocolitic patients since 1977 have been identified as either *C. jejuni* or *C. coli*. The advancement of culturing methods for *Campylobacter* using selective media enabled scientists to conduct routine diagnostic testing on human and animal specimens, and enabled further investigation of common sources and transmission routes. In the mid-1980's, infection reports collected worldwide revealed that *C. jejuni* isolated from human infections were similar to those isolated from poultry, pigs, and sheep (Butzler, 2004). Since then, *C. jejuni* has been recognized as a zoonotic pathogen.

One species (*C. jejuni*) is responsible for the majority of infections and outbreaks (>90%). The remaining 5-10% of outbreaks are caused by other species, such as *C. coli* (Scharff, 2012). On a side note, Man (2011) emphasized the importance of studying emerging

Campylobacter species, such as *C. lari*, *C. fetus*, and *C. upsaliensis*, but their rate of infection and risk to human health remains unknown.

C. jejuni is transmitted through the fecal-oral route and its common reservoirs are domesticated animals, such as poultry, pigs, and cattle (Butzler, 2004; Epps et al., 2013). *C. jejuni* is a commensal microorganism in the gut of cattle (Cox et al., 2010) and also the predominant species found in the gastrointestinal tract of poultry and pigs (Cox et al., 2010) and other avian species (Hermans et al., 2011; Sahin et al., 2015).

C. jejuni is the most prominent species identified in human campylobacteriosis cases. *C. jejuni* infections in humans' results in acute diarrhea and inflammation of the digestive tract. The infectious dose of *C. jejuni* ranges from 500 to 10^4 cells, depending upon the nature of the host. The symptoms of campylobacteriosis in humans typically develop within 2 to 5 days (Butzler, 2004; Crushell et al., 2004). Immunocompromised populations are more prone to infection than healthy individuals. Some cases of *C. jejuni* infection can lead to serious illnesses, such as bacteraemia, Guillain-Baré Syndrome (GBS), and Miller-Fisher Syndrome (Butzler, 2004; Crushell et al., 2004; Epps et al., 2013). GBS and other neurodegenerative or paralytic conditions occur in roughly 0.1% of cases and can lead to a lifetime disabilities (Kaldor and Speed, 1984; Rhodes and Tattersfield, 1982). In these cases, both the strain of *C. jejuni* and host conditions can play major roles in the incidence of GBS (Crushell et al., 2004; Nachamkin et al., 1998). Taken together, these factors make *C. jejuni* infections and treatment options an important subject to investigate.

1.2.1.3 *Campylobacter jejuni*: characteristics and growth requirements

C. jejuni was first characterized in early 1970 when Dekeyser and colleagues were able to develop a filtration method to isolate the bacterium from fecal specimens of diarrhea patients (Butzler, 2004). *C. jejuni* is a non-spore forming, Gram-negative, S-shaped bacterial rod, ranging in size from 0.5- to 4.0- μm long and 0.2- to 0.9- μm wide (Cox et al., 2010). A single flagellum at either one or both ends of the cell aids in motility.

Based upon its growth characteristics, *C. jejuni* is considered a microaerophile (Kelly, 2008), requiring specific concentrations of CO_2 (3-10%), O_2 (3-5%), and N_2 (80-85%) in order to multiply and perform relevant metabolic activities (Kelly, 2008). Moreover, Doyle and Roman (1982) demonstrated that temperature and pH affected the growth and survival rate of *C. jejuni*. The optimal growth temperature for *C. jejuni* is 42°C; however, it can also grow over temperature range of 30 to 42°C (Kelly, 2008; Stintzi, 2003). Hazeleger and colleagues (1998) demonstrated that temperatures below 30°C resulted in suppression of growth and the cells were unable to propagate (Boucher et al., 1994; Hazeleger et al., 1998). Cellular respiration products and ATP production has been observed at temperatures as low as 4°C, leading to the conclusion that *C. jejuni* is “viable but non-culturable” (VBNC) at these temperatures (Hazeleger et al., 1998; Larazo et al., 1999; Rollins and Colwell, 1986).

In the laboratory setting, the optimum pH for *C. jejuni* growth ranges from 6.5 – 7.5 and pH of less than 4.9 or greater than 9.0 inhibits growth (Chaveerach et al., 2003; Doyle and Roman, 1982). Depending upon the strain and other environmental conditions, *C. jejuni* is able to survive below pH 4.9 (Axelsson-Olsson et al., 2010; Gill and Harris 1983; Shaheen et al., 2006) by developing an adaptive tolerance response (ATR) to harsh conditions (Murphy et al., 2003a,b; Reid et al., 2008; Ma et al., 2009).

Unlike other Gram-negative bacteria (*i.e. Escherichia coli* and *Salmonella*), *C. jejuni* utilizes organic acids (Vegge et al., 2009), amino acids (Guccione et al., 2008; Leach et al., 1997; Wright et al., 2009) and the intermediates of the citric acid cycle (Velayudhan and Kelly, 2002) for its metabolic activities. *C. jejuni* is a non-saccharolytic bacterium and several genotypic studies have confirmed the lack of genes responsible for the metabolism of carbohydrates (Gundogdu et al., 2007; Hofreuter et al., 2006; Parkhill et al., 2000). Likewise, the phenotypic study conducted by Line and colleagues (2010) confirmed the inability of *C. jejuni* to catabolize sugar molecules. However, a novel pathway for carbohydrate metabolism has been observed in *C. jejuni* (Muraoka and Zhang, 2011; Stahl et al., 2011).

1.2.1.4 Pathogenicity and virulence of *Campylobacter jejuni*

Although *C. jejuni* is a microaerophile and considered fragile in an oxygenated environment, the unique ability of this microbe to survive in the environment and infect humans is complex and yet to be fully understood. The pathogenicity and virulence factors of *C. jejuni* are mainly related to its motility, cell adherence, and production of bacterial toxins (Young et al., 2007). Several studies have highlighted the importance of flagella in attachment, invasion, and infection by *C. jejuni*, and movement towards the attachment site of the host cell (Young et al., 2007). Wassenar and Blaser (1999) showed the failure of aflagellated *C. jejuni* mutants to attach to host cells *in vitro*. Similarly, Yao and others (1994) conducted an *in vivo* study and validated the inability of aflagellated *C. jejuni* mutants to invade eukaryotic cells (*e.g.*, INT 407 cells).

1.2.1.5 Prevalence of *Campylobacter jejuni* in the agri-food system

As aforementioned, *C. jejuni* is a commensal bacterium in the gastrointestinal tract of poultry and pigs. As few as 35 cells of *C. jejuni* can initiate the colonization process in the chicken gut (Stern et al., 1988). *C. jejuni* cells are able to multiply and predominantly colonize the cecum of broilers at levels of up to 10^6 to 10^9 colony forming units per gram (CFU/g) within 24 hours. Recent evidence suggests that the presence of amoebas was a critical component for the survival mechanism of *C. jejuni* in the gastrointestinal tract of broilers (Axelsson-Olsson et al., 2010; Snelling et al., 2008). The survival mechanisms of *C. jejuni* in the gastrointestinal tract of broilers are continuously under investigation due to the complexity and multifactorial nature of the process (Newell, 2002).

Due to its ubiquitous nature, the transmission of *C. jejuni* to the chicken is likely occurs in the farm environment (Agunos et al., 2014; Newell et al., 2003; Sahin et al., 2002). Although feed and water are not the initial sources of *C. jejuni*, they can be contaminated by the farm environment thereby facilitating the wide spread of *C. jejuni* among chicken flocks (van de Giessen et al., 1998; Zimmer et al., 2003).

In the case of poultry products, the mishandling of poultry carcasses in the slaughterhouse can increase the probability of cross-contamination between raw and finished product through food contact surfaces (FCS). Although processing methods (*i.e.* freezing, chilling, and drying) may inactivate *C. jejuni*, appropriate product handling and effective sanitation methods of both FCS and non-FCS need to be enforced in the processing environment. Note that *C. jejuni* cells have been shown to remain viable and switch to different physiological processes under harsh environmental conditions. For instance, *C. jejuni* was reported to form a

dual-species biofilm along with *Pseudomonas aeruginosa* as one of its survival modes (Culotti and Packman, 2015).

With respect to foodborne illness outbreaks, human infections with *C. jejuni* are normally due to the consumption of raw or undercooked poultry products, contaminated fresh produce and water, and unpasteurized milk (Mandrell and Miller, 2006). Most foodborne outbreaks related to *C. jejuni* are sporadic because campylobacteriosis is usually a self-limiting disease. Hence, there may be numerous unreported cases (Mandrell and Miller, 2006).

1.2.2 Carvacrol

1.2.2.1 Chemical structure of carvacrol

Carvacrol ($C_{10}H_{14}O$), 5-isopropyl-2-methylphenol is a monoterpenoid phenolic compound that is the major constituent of oregano (*Origanum vulgare*) (De Falco et al., 2013; Friedman et al., 2002) and thyme (*Thymus vulgaris*) essential oil (Fachini-Queiroz et al., 2012). Carvacrol content may differ among essential oil products depending upon plant geographical location, harvest time, part of plants used for extraction (*i.e.* stem or leaves), and plant health (Schmidt, 2010). From the perspective of biosynthesis, *p*-cymene is the natural precursor of carvacrol and thymol in the plant. The hydroxylation of *p*-cymene results in the formation of carvacrol and thymol, depending upon which carbon on the benzene rings receives the hydroxyl group (Figure 1-1).

Figure 1-1 The biosynthesis of carvacrol and thymol (Adapted from Nhu-Trang et al., 2006)

1.2.2.2 Regulation, application, and toxicity level

The application of carvacrol in food products as a flavouring is approved under Everything Added to Food in the United States (EAFUS) status by the Food and Drugs Administration (FDA). The addition of carvacrol to food products must meet the criteria and serve the purpose of food additives. The European Food Safety Authority (EFSA) also approves the use of carvacrol as a food flavouring and feed additive with a maximum dose of 5 mg/kg of feed (EFSA, 2012). On the other hand, the application of carvacrol is approved and regulated under the Natural and Non-Prescription Health Products Directorate (NNHPD) in Canada as a flavour enhancer and for topical application (*i.e.* fragrance and skin ointment) under certain circumstances (Health Canada, 2016a). For instance, carvacrol can be used as a non-medicinal ingredient (*i.e.* flavour enhancer) as long as the daily usage level does not exceed 0.23 µg/kg body weight (Health Canada, 2016a). If the daily usage of carvacrol exceeds 0.23 µg/kg body

weight, product prescription may be required, as it is no longer considered as a Natural Health Product (NHP). The upper limit dosage of carvacrol for topical application is 0.5% (w/w) (Health Canada, 2016a).

According to Jenner and others (1964), the LD₅₀ value of carvacrol was 810 mg/kg body weight in rats, which was considered low toxicity level. Cytotoxicity effects were not observed in human cells at a maximum tested concentration of 90 µg/ml (Fachini-Queiroz et al., 2012). Currently, there is no standard and clear assessment of the recommended maximum dosage for human cells and/or the no-observed-effect-level (NOEL) of pure carvacrol.

1.2.2.3 Antimicrobial properties

Interestingly, carvacrol has been widely known to exhibit antimicrobial effects against planktonic and sessile cells of many foodborne pathogenic bacteria, such as *Salmonella* spp. (Knowles et al., 2005; Rattanachaikunsopon and Phumkhachorn, 2010), *C. jejuni* (van Alphen et al., 2012), vegetative cells of *Bacillus cereus* (Ultee et al., 2002), *Escherichia coli* O157:H7 (Burt et al., 2007), and *Listeria monocytogenes* (Guevara et al., 2015; Perez-Conesa et al., 2011). In addition, carvacrol has also been reported to act as antiviral, antioxidant (Jayakumar et al., 2012), antiparasitic (Lei et al., 2010), and antifungal agent (Ahmad et al., 2011; Dalleau et al., 2008).

The antimicrobial effect of carvacrol has been reported to inactivate both Gram-positive and Gram-negative bacteria (Ait-Ouazzou et al., 2012). Several studies were performed to determine the mechanism of action of carvacrol against pathogenic bacteria (Ultee et al., 2002; Veldhuizen et al., 2006). Ultee et al., (2002) implied that the hydrophobic nature of carvacrol allowed it to disrupt the permeability of cell membranes and negatively affect the cell's ion

gradient. Ultee and others (2002) observed the importance of the hydroxyl group in carvacrol structure by comparing the antimicrobial effect of carvacrol and cymene against vegetative cells of *Bacillus cereus*. The hydroxyl group of carvacrol acted as a delocalized electron system that disrupted the cell membrane potential, proton motive force system, and electron transport chain; hence it decreased the production of intracellular ATP (Ultee et al., 2002). Nonetheless, the benzene ring structure of carvacrol was also important to its antimicrobial activities (Veldhuizen et al., 2006). Table 1-1 summarizes the antimicrobial effect and potential mechanism of carvacrol action against both Gram-negative and Gram-positive bacteria.

1.2.3 Zinc oxide nanoparticles (ZnO NPs)

1.2.3.1 Structure and properties

ZnO is an inorganic compound, insoluble in water, and semiconductor of electricity that possesses electrochemical-coupling, pyroelectricity, and piezoelectricity properties (Wang, 2004). It has relatively high ionic binding activity with an isoelectric point of approximately 9.5; thus solubility at biological pH values is relatively low in comparison to other organic compounds (Wang, 2004). The strong ionic bond between Zn^{2+} and O^{2-} resulted in a high stability when it was subjected to high temperature and pressure (Sirelkhatim et al., 2015). ZnO has a symmetrical tetrahedral structure from a combination of Zn^{2+} surrounded by four O^{2-} ions (Wang, 2004). When forming a crystal matrix, ZnO tends to pack its tetrahedral structure in a cube, forming a wurtzite, rocksalt, and zinc blende crystal structure (Figure 1-2) (Morkoç and Özgür, 2009). Zincite wurtzite crystal is the mineral form of ZnO that is found naturally on earth, and it is the most thermodynamically stable crystal form of ZnO (Morkoç and Özgür, 2009). The

unique crystal structures of ZnO and its properties result in many industrial applications, such as pharmaceuticals, cosmetics, agriculture, and electronics (Kolodziejczak-Radzimska and Jesionowski, 2014). Regarding pharmaceutical and cosmetics uses, ZnO is applied and well-known for its use in the sunscreen, facial powders, and body lotion products.

Figure 1-2 The crystal structures of ZnO: (A) rocksalt, (B) zinc blende, and (C) wurtzite. (Adapted from Morkoç and Özgür, 2009)

The size of nano ZnO ranges from 1 to 100 nm and the crystal morphology varies depending upon synthesis method (Jalal et al., 2010; Ma et al., 2013; Ramani et al., 2012). The shape of ZnO NPs is related to the surface area, which affects the general properties of ZnO NPs, including its antimicrobial activity (Sirelkhatim et al., 2015). Tong and colleagues (2013) suggests that large surface area can increase the antimicrobial activity of ZnO NPs due to exposure of the polar surface plane of ZnO NPs to the negatively charged bacterial cell membrane.

1.2.3.2 Regulation, application, and toxicity

With respect to agricultural applications, ZnO is considered a generally recognized as safe (GRAS) food additive by FDA in the United States (21CFR182.8991). As a food additive,

good manufacturing practices (GMP) must be applied when adding ZnO in the foods destined for human consumption. The application of nanoparticles or nanomaterials of ZnO is evaluated on a case-by-case basis by the FDA (Zhong and Shah, 2012).

In Canada, ZnO is approved for use either as medicinal or a non-medicinal ingredient under the NNHPD. When ZnO is used as a mineral, it is considered as a Natural Health Product (Health Canada, 2016b). As a non-medicinal ingredient, ZnO is approved for use as a color additive and filler for topical applications. Currently, nanotechnology (*i.e.* nanoparticles and nanomaterials) in foods is regulated under the Novel Food Product regulation. Health Canada requires an evaluation of the application, rationale for usage, toxicological data, and a risk assessment of the novel food product(s).

In Europe, ZnO is listed as E6 food additive for animal feeds (EFSA, 2015). A recent report published by the EFSA also highlighted the approval of nano ZnO for use in FCS material (*i.e.* film or plastic of the food packaging). The EFSA also indicated that nano ZnO did not migrate out of its medium, but the soluble ionic form (Zn^{2+}) might. Emamifar and others (2011) demonstrated low levels of migration of Zn^{2+} ions from low-density polyethylene (LDPE) packaging into orange juice. Based upon this evaluation, the EFSA recommends 25 mg/person per day as the upper limit of ZnO NPs intakes (EFSA, 2016).

Current evidence evaluating the toxicology of ZnO NPs in mammalian cells is controversial. Several studies observed cytotoxic effects towards mammalian cells (Heng et al., 2010; Wang et al., 2006; Wang et al., 2008); however, most of these toxicological studies were conducted *in vitro* with direct addition of ZnO NPs to the cells. Additionally, these studies did not evaluate different exposure routes of ZnO NPs and their potential biotransformation before

being absorbed by the cells (Espitia et al., 2012). Controversially, cytotoxic effects were not observed in other *in vitro* studies (Colon et al., 2006; Noyhnek et al., 2010; Reddy et al., 2007).

Despite the controversy, the toxicity of ZnO NPs in mammalian cells was determined to be a function of size, shape, and concentration (Hyun-Joo et al., 2011). Further studies may need to consider and evaluate the properties of ZnO NPs, as well as its soluble ionic form while interpreting the toxicological data. Nonetheless, the ZnO NPs supplementation studies on domestic animals demonstrated beneficial effects on their immune systems (Sahoo et al., 2014) and for increasing milk production (Rajendran et al., 2013).

1.2.3.3 Antimicrobial properties

The antimicrobial properties of ZnO NPs are associated with their specific high-surface-area-to-volume ratio, size of particles, and shape. Several studies have reported the antimicrobial effects of ZnO NPs against many foodborne pathogens, such as *E. coli* O157:H7 (Liu et al., 2009), *C. jejuni* (Xie et al., 2011; Lu et al., 2012), *P. aeruginosa* (Feris et al., 2010), *Salmonella* spp. (Jin and Gurtler, 2010), and *Staphylococcus aureus* (Navale et al., 2015). Addition of ZnO NPs has been reported to generate reactive oxygen species (ROS), causing an oxidative stress to bacterial cells (Feris et al., 2010; Kumar et al., 2011). Others have put forth the claim that ZnO NPs may cause electrostatic disruption of the outer membrane and interrupt ion of normal cell membrane functionality, resulting in the leakage of intracellular materials (Liu et al., 2009). Furthermore, the solubility of Zn^{2+} may potentially affect metabolic activity and inhibit the growth of bacterial cells (Song et al., 2010). Table 1-2 summarizes the potential mechanisms responsible for the antimicrobial effect of ZnO NPs against both Gram-positive and Gram-negative bacteria.

1.3 Research objectives

The prevalence of *Campylobacter* particularly in poultry production, the numbers of foodborne illness caused by *Campylobacter*, and the emergence of antibiotic-resistant *Campylobacter* pose a serious threat to the health and wellbeing of our agri-food system. Therefore, there is a need to find an alternative to conventional antibiotics that can reduce the prevalence of *Campylobacter* in the food supply chain while eliminating the risk of antibiotic resistance.

The primary objective of this thesis project was to investigate the synergistic antimicrobial effect of carvacrol and ZnO NPs against *C. jejuni*. The macromolecular fingerprints and gene expression profile of *C. jejuni* treated with the single and combined antimicrobial agents were investigated and discussed in this thesis project. Based upon data collected, a possible mechanism of action for carvacrol and ZnO NPs against *C. jejuni* was established from this study.

1.4 Hypotheses

Several hypotheses were tested in this thesis project:

1. The combined treatment of carvacrol and ZnO NPs show a synergistic antimicrobial effect against *C. jejuni* at 37°C with 10% CO₂ over 24 hours of incubation.
2. The macromolecular fingerprints of *C. jejuni* cells exposed to carvacrol and ZnO NPs are significantly different than those observed with either agent alone.
3. The gene expression profile of *C. jejuni* cells exposed to carvacrol and ZnO NPs are significantly different than those observed with either agent alone.

Table 1-1 The antimicrobial effect of carvacrol and potential antimicrobial mechanism against foodborne pathogens

Target microorganisms	Potential mechanism from the findings	References
<i>C. jejuni</i>	Disruption of flagellar function motility and invasion of epithelial cells	van Alphen et al., 2012
<i>E. coli</i> O157:H7	Disruption of flagellar synthesis, motility, and induction of heat-shock proteins	Burt et al., 2007
<i>Bacillus cereus</i> (vegetative cells)	Decreased membrane potential, disruption of proton motive force and electron transport chain, and depletion of the ATP pool	Ultee et al., 1999; Ultee et al., 2002
<i>S. Typhimurium</i>	Disintegration of cell membrane by increasing membrane permeability	Lambert et al., 2001
<i>L. monocytogenes</i>	Damage to outer cell and cytoplasmic membranes by targeting phospholipid structure at pH = 7 and protein structure at pH = 4	Ait-Ouazzou et al., 2012

Table 1-2 The antimicrobial effect of ZnO NPs and possible antimicrobial mechanism against foodborne pathogens

Target microorganisms	Potential mechanism from the findings	References
<i>C. jejuni</i>	Induced up-regulation of oxidative stress genes (<i>katA</i> and <i>ahpC</i>) and stress response genes (<i>dnaK</i>), causing morphological change to coccoid form, and damaging membrane integrity	Xie et al., 2011
<i>E. coli</i> O157:H7	Electrostatic interaction, membrane damage, and leakage of intracellular materials	Liu et al., 2009
<i>P. aeruginosa</i>	Electrostatic interaction and oxidative stress response and disruption of membrane integrity	Feris et al., 2010
<i>S. aureus</i> and <i>S. Typhimurium</i>	Oxidative stress by the generation of ROS	Navale et al., 2015

Chapter 2: Investigation of antimicrobial effect of carvacrol and zinc oxide nanoparticles against *Campylobacter jejuni*

2.1 Introduction

Prior to investigating the synergistic antimicrobial effect of carvacrol and ZnO NPs against *C. jejuni*, the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each compound need to be investigated. MIC is defined as the minimum concentration of an antimicrobial agent that can inhibit the growth of a particular microorganism under defined conditions over 24 hours (Koneman and Allen, 1997). MBC refers to the minimum concentration that is required to achieve a bactericidal effect of $\geq 99.9\%$ cell death (Doern, 2014; Koneman and Allen, 1997).

To investigate the antimicrobial effect of two or more antimicrobials in combination, several methods have been proposed, including the checkerboard assay, the fractional inhibitory concentration index, and time-kill curve (Koneman and Allen, 1997). In this project, the time-kill curve was employed to investigate the synergistic antimicrobial effect of carvacrol and ZnO NPs against *C. jejuni*. Although the time-kill curve method is more laborious and time-consuming compared to other methods, this method provides a dynamic observation of the bactericidal effect of the antimicrobial agents (Doern 2014; Koneman and Allen, 1997).

When combining two or more antimicrobials, there are three possible responses: additive (or indifferent), antagonistic, and synergistic. The additive antimicrobial effect refers to an equal response of the antimicrobial combination to that achieved with single antimicrobials added together (Koneman and Allen, 1997; Pillai et al., 2005). In other words, the response of

antimicrobial combination is autonomous. The antagonistic antimicrobial effect is observed when the response achieved with antimicrobial combination is less than the response of either single antimicrobial (Koneman and Allen, 1997; Pillai et al., 2005). The definition of synergistic antimicrobial effect is slightly different depending upon the selected method. Using the time-kill curve method, the synergistic antimicrobial effect is observed when the treatment of two or more compounds result in a minimum of a 2-log (100-fold) decrease in growth of a particular microorganism under specified conditions compared to the treatment of the most active single antimicrobial agent (Koneman and Allen, 1997; Pillai et al., 2005). In other words, the combined treatment of two or more compounds should result in at least 3-log growth inhibition of the targeted microorganism if the most active single antimicrobial agent inhibit the growth of the microorganism by 1-log unit.

2.2 Materials and methods

2.2.1 Preparation of *Campylobacter jejuni* overnight culture

A mixture of three *C. jejuni* strains was used to test antimicrobial susceptibility, including strain ATCC 33560 and two clinical isolates (*i.e.*, F38011 and human clinical 10). Each strain was cultured individually on Mueller-Hinton agar (Difco, New Jersey, USA) supplemented with 5% defibrinated sheep blood (Quad Five, Montana, USA) and incubated at 37°C for 48 hours under 10% CO₂. *C. jejuni* colonies of each strain were inoculated into 7 ml Mueller-Hinton broth (pH = 7) (Difco, New Jersey, USA) and incubated for 18 hours at 37°C under 10% CO₂ with shaking. After an overnight incubation period, each culture was adjusted to OD_{600nm} = ~0.3 (1×10^9

CFU/ml) and mixed in an equal volumes. Refer to Appendix A.1 for the adjustment of cell numbers. The bacterial mixture was then diluted to $\sim 1 \times 10^8$ CFU/ml.

2.2.2 Antimicrobial susceptibility of *Campylobacter jejuni* against carvacrol

The macrodilution broth method adopted from the Clinical Laboratory & Standard Institute (2006) and Nature Protocols (Wiegand et al., 2008) was conducted with minor modifications. Carvacrol (purity, $\geq 98\%$; Food Chemical Codex, Food Grade) was purchased from Sigma Aldrich (Density = 0.976 g/ml; molecular weight = 150.22 g/mol). One hundred microliters of carvacrol was dissolved in 900 μ l of dimethyl sulfoxide (DMSO) (Fisher Bioreagents, purity $>99\%$) in two 1:10 (v/v) serial dilutions. From the last dilution series, carvacrol was diluted to 1 mg/ml in Mueller-Hinton broth. Six concentrations of carvacrol were prepared from the 1 mg/ml working solution to make up a final concentration of 13.5 μ g/ml, 18 μ g/ml, 20.25 μ g/ml, 27 μ g/ml, 40.5 μ g/ml, and 54 μ g/ml respectively, accounting for the addition of the *C. jejuni* cocktail. Two control groups were prepared without the addition of carvacrol, which were Mueller-Hinton broth alone and with 0.54% (v/v) DMSO. The total volume of the samples was 2 ml. Treatment and control samples were incubated at 37°C under 10% CO₂ with shaking for 0, 4, 8, 12, and 24 hours. At each time point, each sample was mixed thoroughly, serially diluted in sterile 1× phosphate buffered saline (PBS) (pH = 7.4), and plated on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. Enumeration of the viable cells was performed after 48 hours of incubation at 37°C under 10% CO₂. The experiment was conducted in duplicate and repeated three times.

2.2.3 Antimicrobial susceptibility of *Campylobacter jejuni* against zinc oxide nanoparticles

ZnO NanoArc ZN-0605 was purchased from Alfa Aesar, Thermo Fisher Scientific Chemicals, Inc. (surface area of 12 – 24 m²/g, average particle size (APS) of 40 – 100 nm). A stock solution of 1 mg/ml ZnO NPs was prepared in Mueller-Hinton broth. A 1:10 (v/v) dilution was performed to develop a working solution of 0.1 mg/ml from which three concentrations of ZnO NPs were prepared, again accounting for the addition of the *C. jejuni* cell cocktail to make up a final concentration of 12.5 µg/ml, 25 µg/ml, and 50 µg/ml. Mueller-Hinton broth without ZnO NPs was prepared as a control. The total volume of the samples was 2 ml. The samples were incubated at 37°C under 10% CO₂ with shaking for 0, 4, 8, 12 and 24 hours. At each time point, each sample was mixed thoroughly, serially diluted in sterile 1× PBS (pH = 7.4), and plated on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. Enumeration of the viable cells was performed after 48 hours of incubation at 37°C under 10% CO₂. The experiment was conducted in duplicate and repeated three times.

2.2.4 Synergistic antimicrobial effect of carvacrol and zinc oxide nanoparticles against *Campylobacter jejuni*

The time-kill curve method adapted from Koneman and Allen (1997) was used to test the synergistic antimicrobial effect of ZnO NPs and carvacrol against *C. jejuni*. Working solutions of ZnO NPs (0.1 mg/ml) and carvacrol (1 mg/ml) were prepared separately in Mueller-Hinton broth. Two concentration combinations of carvacrol:ZnO NPs were prepared with the addition of a $\sim 1 \times 10^8$ CFU/ml (OD₆₀₀ = ~0.03) *C. jejuni* cocktail to make up a final concentrations of 18:12.5 µg/ml and 20.25:12.5 µg/ml. Positive control groups were prepared with the addition of

either carvacrol or ZnO NPs at the same concentration used in the combination. A negative control was prepared without the addition of carvacrol and (or) ZnO NPs to the *C. jejuni* cocktail. The total volume of the samples was 2 ml. The treatment and control groups were incubated at 37°C under 10% CO₂ with shaking condition for 0, 8, 12, and 24 hours. At each time point, each sample was mixed thoroughly, serially diluted in sterile PBS, and plated on Mueller-Hinton agar plate supplemented with 5% defibrinated sheep blood. Enumeration of the viable cells was performed after 48 hours of incubation at 37°C under 10% CO₂. The experiment was conducted in duplicate and repeated three times.

2.3 Results and discussion

2.3.1 Antimicrobial susceptibility of *Campylobacter jejuni* against carvacrol

In this study, six concentrations of carvacrol ranging from 13.5 to 54 µg/ml were investigated for their antimicrobial effects against a mixture of three strains of *C. jejuni*. The positive control is shown as 0 µg/ml, which refers to the *C. jejuni* culture in Mueller-Hinton broth without the addition of carvacrol and/or DMSO (Figure 2.1). The negative control contained 0.54% (v/v) DMSO because this was the upper limit of DMSO in the carvacrol solution. The growth of *C. jejuni* in the control increased from $\sim 10^8$ to $\sim 10^9$ CFU/ml over 24 hours at 37°C under 10% CO₂. There was no significant difference ($P > 0.05$) in the cell population between positive and negative control, indicating that 0.54% (v/v) DMSO did not have antimicrobial effects against *C. jejuni*. In other words, $\leq 0.54\%$ (v/v) DMSO was not a confounding factor and it did not interfere with the effect of carvacrol in the assay. Several authors have reported that $\leq 1\%$ (v/v) DMSO does not affect the visible growth of both Gram-

positive and Gram-negative bacteria (Basch and Gadebusch 1968; Nostro et al., 2000; Wadhwani et al, 2008).

The interaction between concentration of carvacrol and treatment time contributed to variable growth inhibition of *C. jejuni* over 24 hours. A concentration of 13.5 µg/ml carvacrol alone did not significantly inhibit the growth of *C. jejuni* over 24 hours ($P > 0.05$). Increasing the carvacrol concentration to 18 µg/ml resulted in inhibition of *C. jejuni* (Figure 2-1A).

Growth of *C. jejuni* was inhibited for 12 hours and increased by 1-log unit after treatment with 18 µg/ml carvacrol ($P < 0.05$). Although there was a slight decrease in *C. jejuni* cells treated with 20.25 µg/ml carvacrol over the first 8 and 12 hours, this treatment did not significantly ($P > 0.05$) lower the cell population over the full course of the experiment (Table 2-1). After 12 hours of incubation, carvacrol may be depleted in the liquid medium, resulting in a slight increase in *C. jejuni* cells. Also, it is possible that cells recovered from sub-lethal injuries and resumed growth. Depletion of carvacrol may also have occurred due to its volatility and the thermal-labile nature of carvacrol's underlying terpenoid structure. Terpenoids undergo thermal degradation (McGraw et al., 1999; Turek and Stintzing, 2013) and structural re-arrangement with prolonged high-temperature incubation (Turek and Stintzing, 2013).

Based upon the growth and statistical analysis in Table 2-1 and Figure 2-1A, the MIC of carvacrol ranged from 18 to 27 µg/ml and the MBC of carvacrol was 27 µg/ml. As mentioned earlier, the growth of *C. jejuni* cells treated with 18 µg/ml carvacrol was inhibited, and 18 µg/ml was included in the MIC range of carvacrol. Higher concentration of carvacrol, (27 µg/ml) led to bactericidal effect and prevented growth of *C. jejuni* for 24 hours of incubation ($P < 0.05$). Exposure to ≥ 27 µg/ml carvacrol resulted in an 8-log CFU/ml killing effect against *C. jejuni* cells over 24 hours of incubation.

Several authors have reported that carvacrol was effective to cause bacteriostatic and bactericidal effect to *C. jejuni in vitro* (Johnny et al., 2010; van Alphen et al., 2012) and *in vivo* (Arsi et al., 2014). The antimicrobial effects of carvacrol observed from this study were in agreement with these *in vitro* studies (Johnny et al., 2010; Lambert et al., 2001; van Alphen et al., 2012), even though the measured MIC and MBC values were slightly different. This difference could be due to initial amount of culture, type of media, and treatment condition applied in the study.

2.3.2 Antimicrobial susceptibility of *Campylobacter jejuni* against zinc oxide nanoparticles

Three concentrations of ZnO NPs (12.5, 25, and 50 µg/ml) were investigated for their antimicrobial effects against the *C. jejuni* cocktail. The positive control was shown as 0 µg/ml in Figure 2-1B, referring to no addition of ZnO NPs. The untreated cells of *C. jejuni* were able to grow to approximately 8×10^9 CFU/ml. In this experiment, the growth of *C. jejuni* was affected by the interaction of treatment time and different concentrations of ZnO NPs.

Growth of *C. jejuni* treated was unaffected by 12.5 µg/ml ZnO NPs. When the concentration of ZnO NPs was increased two-fold (25 µg/ml), the maximum population achieved was ~1 log CFU/ml lower than in control (Figure 2-1B). Cell population was significantly lowered by 3 log CFU/ml after 8 hours of exposure to 50 µg/ml ZnO NPs. As shown in Figure 2-1B, the cell population was lowered by ~7 log CFU/ml after 24 hours ($P < 0.05$). Based upon this observation, the MIC of ZnO NPs ranged from 25 to 50 µg/ml and the MBC of ZnO NPs was 50 µg/ml.

ZnO NPs have been shown to inhibit the growth of Gram-negative pathogenic bacteria, such as *E. coli* O157:H7 (Liu et al., 2009), *P. aeruginosa* (Azam et al., 2012), *S. Typhimurium* (Navale et al., 20015), and *C. jejuni* (Xie et al., 2011; Lu et al., 2012). The results of the current study demonstrated similar antimicrobial activity of ZnO NPs against *C. jejuni*. MIC values were in the same range as those reported by Xie and others (2011).

2.3.3 Synergistic antimicrobial effect of carvacrol and zinc oxide nanoparticles against *Campylobacter jejuni*

The concentrations selected for each antimicrobial agent was based on prior susceptibility testing. The negative controls contained no carvacrol or ZnO NPs (Figure 2-2). The interaction between the factors treatment and time was significant ($P < 0.05$), implying that both factors contributed to the inhibition of *C. jejuni* (Table 2-3).

Both concentrations of carvacrol and ZnO NPs exerted synergistic antimicrobial effect against *C. jejuni* over 24 hours of incubation. The first combination consisted of 20.25 µg/ml carvacrol : 12.5 µg/ml ZnO NPs and the second 18 µg/ml carvacrol : 12.5 µg/ml ZnO NPs. Both combinations exerted bactericidal effect and lowered *C. jejuni* cell population by ~8 log CFU/ml over 24 hours ($P < 0.05$).

The first combination inhibited the growth of *C. jejuni* by an approximately 2.25-log unit within 8 hours of treatment, which was more than 100-fold cells growth inhibition than the single treatment. Similarly, the second combination treatment lowered the maximum cell population approximately 1.44 log CFU/ml within 8 hours of treatment, which was over a 2-log growth inhibition compared to the single antimicrobial treatment. When the exposure time was increased to 24 hours, both combinations resulted in the killing-effect (Figure 2-2). In contrast, the single

treatment exerted bacteriostatic effect and prevented the growth of cells. These results were thus indicative of a synergistic antimicrobial effect along with exposure time.

Synergy between essential oils or component of essential oils and metal oxide nanoparticles against bacteria cells was observed previously (Behzad and Sani 2016; Biasi-Garbin et al., 2015; Ghosh et al., 2013; Krychowiak et al., 2014; Scandorieiro et al., 2016). Most of these studies utilized the time-kill curve method as well to observe the synergistic antimicrobial effect and all emphasized that treatment and exposure time contribute to the synergistic antimicrobial effect. Similar to this work, Scandorieiro and colleagues (2016) observed bactericidal effect against both Gram-positive and Gram-negative multi-drug resistant bacterial over a 7-hour-incubation period when treated with sub-inhibitory concentrations of oregano essential oil and silver nanoparticles (Ag NPs).

2.4 Conclusion

Treatment with carvacrol or ZnO NPs alone was observed to inhibit the growth of *C. jejuni* cells depending upon the concentration and treatment time. Treatment with 18 µg/ml and 20.25 µg/ml carvacrol showed bacteriostatic effect, whereas treatment with 12.5 µg/ml ZnO NPs did not show inhibition effect against *C. jejuni*. The combination of carvacrol (18 or 20.25 µg/ml) and ZnO NPs (12.5 µg/ml) showed synergistic antimicrobial effects against *C. jejuni* within 8 hours of treatment. These combinations showed bactericidal effect against *C. jejuni* and lowered the maximum cell population by at least more than 100-fold compared to treatment with either carvacrol or ZnO NPs. To the best of our knowledge, this study was the first one to observe the synergistic antimicrobial effect of carvacrol and ZnO NPs against *C. jejuni* cells *in vitro*.

Table 2-1 Change of viable cells of *C. jejuni* treated with different carvacrol concentrations over 24 hours of treatment at 37°C in a microaerobic condition

Carvacrol concentration (µg/ml)	Change of viable cells (log ₁₀ CFU/ml)			
	4 hours	8 hours	12 hours	24 hours
0	-0.81 ± 0.05 ^{JKL}	-1.40 ± 0.14 ^{LMN}	-1.73 ± 0.17 ^N	-1.42 ± 0.09 ^{MN}
0 [0.54% (v/v) DMSO]	-0.64 ± 0.20 ^{IJK}	-1.64 ± 0.14 ^{MN}	-1.75 ± 0.08 ^N	-1.80 ± 0.09 ^N
13.5	-0.28 ± 0.10 ^{GHIJ}	-0.52 ± 0.26 ^{HIJK}	-1.08 ± 0.39 ^{KLM}	-1.39 ± 0.23 ^{LMN}
18	-0.04 ± 0.13 ^{GH}	-0.14 ± 0.49 ^{GHI}	-0.20 ± 0.69 ^{GHI}	-1.09 ± 0.17 ^{KLM}
20.25	-0.01 ± 0.13 ^{GH}	0.92 ± 0.28 ^F	1.09 ± 0.15 ^F	0.17 ± 0.30 ^G
27	0.85 ± 0.27 ^F	3.07 ± 0.31 ^D	4.47 ± 0.16 ^B	8.05 ± 0.16 ^A
40.5	1.35 ± 0.38 ^F	3.82 ± 0.21 ^C	4.74 ± 0.08 ^B	8.04 ± 0.08 ^A
54	2.10 ± 0.14 ^E	4.21 ± 0.21 ^{BC}	8.03 ± 0.08 ^A	8.03 ± 0.08 ^A

Change of viable cells count was calculated as difference between viable cells (log₁₀ CFU/ml) at 0 hour and at 4, 8, 12, or 24 hours per treatment and; results are shown as mean ± standard deviation (n=3); treatment, incubation time, and interaction were significant ($P < 0.05$); superscript letters (A-N) show the significant differences between treatments and incubation time ($P < 0.05$).

Table 2-2 Change of viable cells of *C. jejuni* cells treated with different concentrations of ZnO NPs over 24 hours of treatment at 37°C in a microaerobic condition

ZnO NPs concentration (µg/ml)	Change of viable cells (log ₁₀ CFU/ml)			
	4 hours	8 hours	12 hours	24 hours
0	-0.81 ± 0.05 ^{EFG}	-1.36 ± 0.11 ^{FG}	-1.73 ± 0.17 ^G	-1.47 ± 0.15 ^{FG}
12.5	-0.64 ± 0.18 ^{EF}	-0.76 ± 0.36 ^{EFG}	-1.46 ± 0.05 ^{FG}	-1.27 ± 0.32 ^{FG}
25	0.07 ± 0.05 ^{DE}	0.53 ± 0.44 ^D	0.47 ± 0.60 ^D	0.53 ± 0.69 ^D
50	0.40 ± 0.28 ^D	2.78 ± 1.44 ^C	3.99 ± 0.34 ^B	7.67 ± 0.16 ^A

Change of viable cells count was calculated as difference between viable cells (log₁₀ CFU/ml) at 0 hour and at 4, 8, 12, or 24 hours per treatment and; results are shown as mean ± standard deviation (n=3); treatment, incubation time, and interaction were significant ($P < 0.05$); superscript letters (A-G) show the significant differences between treatments and incubation time ($P < 0.05$).

Table 2-3 Change of viable cells of *C. jejuni* treated with carvacrol, ZnO NPs, and mixtures of the compounds after 24 hours at 37°C in a microaerobic condition

Concentration (µg/ml)		Change of viable cells (log ₁₀ CFU/ml)		
Carvacrol	ZnO NPs	8 hours	12 hours	24 hours
0	0	-1.20 ± 0.09 ^H	-1.49 ± 0.14 ^H	-1.49 ± 0.37 ^H
20.25	0	0.24 ± 0.51 ^G	-0.02 ± 0.37 ^G	1.13 ± 0.20 ^{EF}
18	0	-1.26 ± 0.91 ^C	-0.95 ± 0.18 ^H	0.45 ± 0.44 ^{FG}
0	12.5	-1.25 ± 0.12 ^H	-1.54 ± 0.21 ^H	-1.31 ± 0.20 ^H
20.25	12.5	2.25 ± 0.35 ^C	3.19 ± 0.33 ^B	8.01 ± 0.08 ^A
18	12.5	1.44 ± 0.68 ^{DE}	1.73 ± 0.82 ^{BC}	7.95 ± 0.06 ^A

Change of viable cells count was calculated as difference between viable cells (log₁₀ CFU/ml) at 0 hour and at 8, 12, or 24 hours per treatment and; results are shown as mean ± standard deviation (n=3); treatment, incubation time, and interaction were significant ($P < 0.05$); superscript letters (A-H) show the significant differences between treatments and incubation time ($P < 0.05$).

Figure 2-1 Time-kill curve for a mixture of three strains of *C. jejuni* treated with (A) carvacrol or (B) ZnO NPs over 24 hours at 37°C with 10% CO₂

Figure 2-2 Comparison of time-kill curves obtained with carvacrol, ZnO NPs, and mixtures of the compounds against *C. jejuni* over 24 hours at 37°C under 10% CO₂. In this figure, C denotes carvacrol and Z denotes ZnO NPs.

Chapter 3: Investigation of macromolecular fingerprints of *Campylobacter jejuni* after treatment with carvacrol and zinc oxide nanoparticles

3.1 Introduction

Raman spectroscopy was selected to investigate the effects of single or combined antimicrobial treatment on the macromolecular fingerprints of *C. jejuni*. The macromolecular fingerprints of *C. jejuni* cells can provide details in regards to changes in the chemical composition of bacterial cell membranes and cell walls after antimicrobial treatment. Raman spectroscopy is considered as a vibrational spectroscopic technique because it measures the Raman scattering effect of molecules. The principle of Raman scattering effect was first discovered by Sir Chandrasekhara Venkata Raman and Kariamanickam Srinivasa Krishnan in 1928 (Ferraro et al., 2003). This discovery led to the development of the Raman spectroscopic instrumentation.

The Raman scattering effect describes difference in energy between scattered photons and the incident light beam (Banwell and McCash, 1994). When incident light collides with a molecule, photons are scattered in several different ways, depending upon its interaction with the electrons of the molecule. The collision of incident light and molecule causes the electrons to enter into an unstable excited state, which gives off a photon upon returning to its ground state, resulting in elastic scattering of photons. In this case, the energy of the scattered photons is equal to the energy of the incident light. This scattering is also referred to as Rayleigh scattering (Banwell and McCash, 1994). However, there are molecules that do not return to their ground state, but remain in a slightly higher energy state, resulting in the inelastic scattering of photons.

When inelastic scattering occurs, the energy of the scattered photons is no longer equal to the energy of the incident light. The energy of the scattered photons is either higher or lower than the energy of incident light. This concept is known as the Raman scattering effect (Banwell and McCash, 1994).

During the collision process, some molecules do not return to their ground state because of the changes in their polarizability as a result of an induced-dipole moment (Ferraro et al., 2003). This phenomenon offers an advantage when studying the molecular properties of a compound. By using Raman spectroscopy, the information about the manners and types of vibrational modes of the molecules can be collected by measuring the difference between the energy of the scattered photons and the incident light (Ferraro et al., 2003).

Numerous studies have employed Raman spectroscopy to study bacterial stress and sub-lethal injuries as a result of treatment by conventional and natural antimicrobials. Assmann and colleagues (2015) utilized Raman spectroscopy to study the antimicrobial effect of vancomycin against *Enterococcus faecalis* and identified the differences between the untreated and treated cells using a cluster analysis model. In another study, Raman spectroscopy was employed to investigate the sub-inhibitory effects of amikacin and it was able to detect the different biochemical changes between treated cells with different concentrations of amikacin versus the untreated cells (López-Díez et al., 2005). Feng and colleagues (2014) utilized Raman spectroscopy to study the mode of action of garlic-derived compounds against *Cronobacter sakazakii*, an opportunistic pathogen found in dry infant formula. These studies have successfully applied Raman spectroscopy to evaluate biochemical variations between the treated and untreated bacterial cells.

3.2 Materials and methods

3.2.1 Sample preparation for Raman spectral collection

C. jejuni strain F38011 was treated with carvacrol and ZnO NPs, either alone or in combination. Treatments preparation and incubation conditions were the same as in the antimicrobial susceptibility experiment. Following 6 hours of incubation, 1 ml of each treatment was transferred to a 1.5-ml microcentrifuge tube. Samples were centrifuged at $13,000 \times g$ for 2 minutes for the collection of bacterial pellets. The supernatant was removed and cell pellets were washed twice with 500 μ l sterile distilled deionized water to ensure minimal treatment residues. Cell pellets were then re-suspended in 10 μ l of water to reach at least $OD_{600nm} = \sim 0.3$. Bacterial samples (1 μ l) were transferred onto a gold-coated microarray chip (Thermo Scientific Inc., Waltham, MA). Samples on the chip were air-dried for 10 minutes before collection of Raman spectra. Each experiment was repeated in triplicate.

3.2.2 Confocal micro-Raman spectroscopy

To determine the macromolecular fingerprints of *C. jejuni* under the stressed and sub-lethal condition, a confocal micro-Raman spectroscopic system was used. The system includes a Leica microscope (Leica Biosystems Inc., Germany) and a Raman spectroscopic platform (Renishaw, United Kingdom). A near-infrared laser ($\lambda = 785$ nm) was used for spectral collection. Each bacterial sample prepared on the gold-coated microarray chip was placed under a standard stage of the microscope and observed using a 50 $\times$ objective lens. Raman spectra of bacterial samples were collected using a 300 mW incident laser with 10-s exposure time at wavenumbers 400-3000 cm^{-1} . Raman spectral features were collected from 5 random locations per sample cell; samples

were tested in triplicate. In total, 15 spectra were collected per sample. Data was collected using WiRE v.3.0 software (Renishaw, Gloucestershire, United Kingdom)

3.2.3 Raman spectral processing and chemometric analysis

The Raman spectra were evaluated in the region of 550-1650 cm^{-1} . The baseline of each spectrum collected was corrected with a fifth-order polynomial fitting, followed by a five-point boxcar smoothing using Vancouver Raman Algorithm software (The University of British Columbia). Processed spectra were normalized to the intensity at 1450 cm^{-1} using OMNIC v.8.2 Software (Thermo Scientific Inc., Waltham, MA). Multivariate statistical analysis was then employed to analyze the spectral data. Specifically, a principal component analysis (PCA) model was constructed using MATLAB R2016a software (MathWorks, United States) with in-house programs and codes.

3.3 Results and discussion

Raman spectroscopy was utilized to determine chemical variations in bacterial cells after 6 hours of antimicrobial treatment. Sampling time points were selected based on the time-kill curve data shown in Figure 2-2, when the growth of *C. jejuni* cells were inhibited by 1 log CFU/ml with the combined treatment of carvacrol and ZnO NPs and the synergistic antimicrobial effect was observed. The amount of bacterial cells sampled at 6 hours was estimated to be at least 10^7 CFU/ml, which might have contained a mixture of healthy, dead, and injured bacterial cells.

Raman spectra for each sample were normalized based upon the prominent band at 1450 cm^{-1} , assigned to the deformation of C-H functional groups in lipids and carbohydrates

(Movasaghi et al., 2007). The raw Raman spectra of the four bacterial samples appeared similar based upon visual judgement. However, several minor variations were observed in raw Raman spectra at 725, 785, 1080, and 1575 cm^{-1} . The bands at 725, 785, and 1575 cm^{-1} are associated with the ring-breathing modes of DNA/RNA bases, such as adenine, cytosine, guanine, thymine, and uracil (Movasaghi et al., 2007). These bands in the spectra of *C. jejuni* cells treated with carvacrol or the combined treatment had a lower intensity than that in the spectra of *C. jejuni* cells treated with ZnO NPs only, and the untreated cells. This suggests that genetic materials in the cells are potentially being damaged or disrupted due to carvacrol alone.

Furthermore, the intensity of the band at 1080 cm^{-1} from the spectra of *C. jejuni* cells treated with carvacrol or the combination treatment also appeared to be lower than that of the spectra of *C. jejuni* cells treated with ZnO NPs and untreated cells. This band is related to phospholipids or phosphate vibrations of the phosphodiester groups in nucleic acids (Movasaghi et al., 2007). The lipid bilayer structure of the bacterial cell membrane is comprised of approximately 40% phospholipids and the cell membrane is the first line of defence of the bacterial cells against antimicrobials. The lower intensity of the Raman spectral feature suggests that there may be changes in the phospholipid structure of the outer cell membrane which again can be primarily attributed to carvacrol. A similar observation was identified in a study conducted by Ait-Ouazzou and colleagues (2013), in which the assigned peak of 1080 cm^{-1} was different for the carvacrol-treated cells versus untreated cells. Ait-Ouazzou and colleagues (2013) implied that the phospholipids structure of the outer bacterial cell membrane was most likely affected by carvacrol treatment at neutral pH.

Other prominent bands appeared on each of the Raman spectrum of *C. jejuni* cells were assigned to 825, 850, 1004, and 1034 cm^{-1} . The bands at 825 and 850 cm^{-1} are attributed to

phosphodiester and single bond stretching of amino acids and polysaccharides, respectively (Movasaghi et al., 2007). Bands at 1004 and 1034 cm^{-1} are normally shown in the spectra of most bacterial cells, which are associated with phenylalanine in the cell membrane (Movasaghi et al., 2007; Schuster et al., 2000).

A three-dimensional PCA model was then constructed and showed that three tight clusters were formed using Raman spectra derived from the four *C. jejuni* samples. The first principal component (PC1) accounted for 55.4% of the total explained variance in the separation, while the PC2 (15.7%) and PC3 (8.5%) still attribute to some variations. The PCA model accounts for the variance of both wavenumbers and intensity of the Raman spectra, which are attributed to different functional groups and their concentration, respectively. Untreated cells and cells treated with ZnO NPs overlapped significantly with each other and they were clearly separated from the clusters of *C. jejuni* cells treated with carvacrol or the combinational treatment (Figure 3-2). This clustering analysis implied that carvacrol had more of a role in the synergistic antimicrobial effect against *C. jejuni* than ZnO NPs.

The absolute coefficients in the loading plot showed the wavenumbers that contributed to the variances in the Raman spectroscopic-based PCA model (Figure 3-3). According to PC1, PC2, and PC3 on the loading plot, the wavenumbers 725, 785, 1004, 1080-1090, 1215-1242, 1478, and 1572-1578 cm^{-1} contributed most to the variances that segregate the *C. jejuni* samples into three clusters. These wavenumbers are associated with the changes in the nucleic acids (725, 785, 1572-1578 cm^{-1}), carbohydrates (1082 cm^{-1}), lipids (1080 and 1083-1090 cm^{-1}), and proteins (1004, 1215-1242, and 1478 cm^{-1}) of the bacterial cells (Movasaghi et al., 2007; Talari et al., 2015). The vibrational modes in the lipid structures may be associated with phospholipids (1,080 cm^{-1}), C-C or PO_2^- stretch (1087 – 1090 cm^{-1}), and C-C vibration in the acyl backbone in

the lipid structure (1081 and 1087 cm^{-1}) on the outer membrane of *C. jejuni* (Schuster et al., 2000; Movasaghi et al., 2007; Talari et al., 2015). The protein bands are associated with the phenylalanine (1004 cm^{-1}), amide III, C-N, and C=N=C stretching (1215 - 1242 and 1478 cm^{-1}) (Movasaghi et al., 2007) that are potentially contributing to the outer membrane as glycosylated extracellular proteins and capsular lipopolysaccharide of *C. jejuni* (Moran et al., 1991).

3.4 Conclusion

The macromolecular fingerprints of *C. jejuni* cells treated with the combined treatment of $20.25\text{ }\mu\text{g/ml}$ of carvacrol and $12.5\text{ }\mu\text{g/ml}$ of ZnO NPs were different from the cells treated with the $12.5\text{ }\mu\text{g/ml}$ ZnO NPs alone and the untreated cells. The cells treated with the combined treatment could not be segregated from the cells treated with $20.25\text{ }\mu\text{g/ml}$ carvacrol. Based upon the macromolecular fingerprints, the majority of the synergistic antimicrobial effect against *C. jejuni* can be attributed to carvacrol. Based on altered band patterns, carvacrol most likely disrupts genetic material and causes changes to the phospholipids of the outer cell membrane. Therefore, the mechanism of the synergistic antimicrobial effect of carvacrol and ZnO NPs potentially includes both internal and external cellular damage.

Figure 3-1 Raman spectral fingerprints of *C. jejuni* strain F38011 treated with (A) 12.5 $\mu\text{g/ml}$ ZnO NPs, (B) 20.25 $\mu\text{g/ml}$ carvacrol, (C) combination of carvacrol and ZnO NPs, and (D) untreated cells from 550 to 1650 cm^{-1}

Figure 3-2 Three-dimensional principal component analysis (PCA) for the segregation of *C. jejuni* F38011 and treated with ZnO NPs, carvacrol, and combination of carvacrol and ZnO NPs using the wavenumber region of 550 to 1650 cm^{-1}

Figure 3-3 The loading plot of (A) first-, (B) second-, and (C) third-principal component of the Raman spectra from 550 to 1650 cm^{-1}

Chapter 4: Investigation of the gene expression profile of *Campylobacter jejuni* after treatment with carvacrol and zinc oxide nanoparticles

4.1 Introduction

Real-Time Quantitative PCR (RT-qPCR) allows us to see a specific region of nucleic acids in a particular sample being amplified in real time. In conventional PCR, the first process is the denaturation of double stranded DNA where a high temperature is used to dissociate double stranded DNA into single stranded DNA. The second step is called “annealing”, where a specific primer binds to the specific region of a template DNA. Finally, the polymerase continues the process by adding the subsequent sequences of DNA template in the direction of 3’ to 5’, resulting in amplicons as the PCR products. In the RT-qPCR, hydrolysis probe (*e.g.* Taqman polymerase) and double-stranded DNA-intercalating dye (*e.g.* SYBR Green I dye), are the two common assays used to detect and quantify the amount of amplicons (Arya et al., 2005; Navarro et al., 2015).

The hydrolysis probe assay utilizes a single stranded DNA probe that is attached to the fluorescence molecule at a terminal base and quencher molecule at the other terminal base to generate fluorescence signals. When both molecules are attached onto the probe, the quencher absorbs the fluorescence as long as they are in close proximity to each other. In the annealing step, both primer and probe attach onto their complementary sites on the DNA template strand. As aforementioned, the polymerase will add complementary nucleotides; however, it also has a 3’-5’ exonuclease activity engineered into it. This additional feature digests the double stranded DNA sites, replaces it with the new nucleotides, and breaks down the oligonucleotide probe. This causes a release of the fluorophore and quencher into solution. As a result, the fluorophore

is no longer quenched, allowing fluorescence to occur when excited with the appropriate incident light. For each amplification process of a single stranded DNA, a fluorescence molecule is released from its neighbouring quencher. Therefore, the fluorescence intensity is equivalent to the number of amplicons produced.

Conversely, the SYBR Green I dye does not require a sequence-specific probe design. During the denaturation process in PCR, the SYBR Green I dye cannot bind tightly to the single strand of DNA, resulting in a very low and negligible fluorescence signal. The annealing and polymerization steps of PCR results in generation of double-stranded DNA to which the SYBR Green I dye tightly intercalates along DNA's minor groove. When the SYBR Green I dye binds to the double stranded DNA, it produces a strong fluorescent signal that is detected in the qPCR instrument. This fluorescence signal is equivalent to the number of amplicons produced during the PCR reaction. Note that SYBR Green dye I can bind to any double stranded DNA, including non-specific amplicons (*i.e.* primer dimers). Post amplification process, a melting curve (fluorescence reported versus temperature) is generated to ensure the specificity of the assay. In this study, the SYBR Green I dye RT-qPCR assay was utilized to determine the relative quantity of target gene expression in *C. jejuni* after treatment with carvacrol and ZnO NPs, either alone or in combination.

4.2 Materials and methods

4.2.1 Ribonucleic acid sample preparation

C. jejuni strain F38011 was treated with carvacrol, ZnO NPs, and a combination of carvacrol and ZnO NPs tested previously. *C. jejuni* strain F38011 overnight culture was adjusted to $OD_{600nm} =$

~0.3 (~10⁹ CFU/ml) and diluted to ~1 × 10⁷ CFU/ml in Mueller-Hinton broth. This culture was then incubated at 37°C with 10% CO₂ for 4 hours to obtain 1 × 10⁸ CFU/ml. At this point, the antimicrobial treatments were then introduced to the *C. jejuni* culture and incubated for 15 minutes. The treatment preparation and incubation conditions were the same as the antimicrobial susceptibility experiments. A positive control of *C. jejuni* cells treated with 6.25 µg/ml of tetracycline was prepared using Mueller-Hinton broth as the solvent. After 15 minutes of incubation, 1 ml of each treatment was transferred to a 1.5-ml microcentrifuge tube. The bacterial samples were centrifuged at 12,000 ×g for 2 minutes to collect bacterial cell pellets. The supernatant was discarded and the bacterial cell pellets were washed twice using 500 µl of sterile distilled deionized water to ensure minimal residues of treatment. The total RNA was extracted from each sample using a GENEzol TriRNA Pure Kit GZX050 from Geneaid Biotech Ltd., New Taipei City, Taiwan. The procedure of RNA extraction followed the protocol, which was provided by the kit with some modification. Nanodrop1000 spectrophotometer (Thermofisher, Massachusetts, USA) was utilized to quantify the amount and quality of the RNA after extraction. RNA samples were run on a 1.5% agarose gel to confirm the RNA quality as well.

4.2.2 Synthesis of complementary deoxyribonucleic acid (cDNA)

Synthesis of cDNA was carried out using a Superscript VILO cDNA Synthesis kit (Invitrogen, California, USA). The amount of RNA was standardized to ~500 ng copies for generating the first-strand of cDNA. Each 20-µl reaction mixture contained 4 µl of 5× VILO Reaction Mix, 2 µl 10× SuperScript Enzyme Mix, ~2 µl of RNA sample, and ~12 µl of DNA/RNase free water. Each sample tube was mixed gently and kept in an ice bath. The cDNA synthesis program was

25°C for 10 minutes, 42°C for 60 minutes, and then terminated at 85°C for 5 minutes. The cDNA samples were kept at 4°C for further usage.

4.2.3 Real-time quantitative polymerase chain reaction (RT-qPCR)

The cDNA samples were diluted to a ratio of 1:500 (v/v) in DNA/RNase free water. RT-qPCR reagent used was from Applied Biosystems Power SYBR Green PCR Master Mix (ThermoFisher, Massachusetts, USA) and the instrument used was a ABI Prism 7000 Fast Instrument (Life Technologies, California, USA). Primer3-BLAST software (NCBI, Maryland, USA) was utilized to design the forward and reverse primers. Table 3-1 shows a complete list of the sequence of primers. Each 25-μl of reaction contained 12.5 μl of 2× SYBR Green PCR Master Mix, 0.5 μM of each primer, 2 μl of DNA template, and 10.4 μl of nuclease-free water. The amplification program was 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The melting curve stage was performed continuously at 95°C for 30 seconds and 60°C for 15 seconds. The experiment was prepared in triplicate and repeated at least for three times.

4.2.4 Data analysis

The *rpoA* gene served as a reference gene for data normalization. The relative quantity of gene expression data was analyzed using a comparative threshold cycle ($2^{-\Delta\Delta C_T}$) method, where C_T is the critical threshold cycle value for the amplified gene. The following expression was applied to analyze the data: $\Delta\Delta C_T = \Delta C_T(\text{treated sample}) - \Delta C_T(\text{untreated sample})$ and $\Delta C_T = C_T(\text{target gene}) - C_T(\text{rpoA})$. The result was analyzed using a two-tailed t-test to compare the pair means

between the treatment with α value of 0.05 with at least equal to or greater than 2-fold change in the relative gene expression.

4.3 Results and discussion

The investigation of the gene expression profile of *C. jejuni* F38011 cells involved a quantitative analysis of relative gene expression pattern between the pairs of treatment groups. Twelve target genes were selected and grouped into four categories based upon the functional class of each gene. These four categories include genes responsible for housekeeping, oxidative stress defense, general stress response, and pathogenicity (*e.g.*, adherence and virulence). The relative expression of these 12 target genes was compared to the expression of the reference gene (*i.e.*, *rpoA*). Ritz and others (2009) validated that the transcription level of *rpoA* in *C. jejuni* was relatively constant either under normal physiological conditions or stressed conditions (Ritz et al., 2009). This reference gene is responsible for synthesizing the alpha subunit of RNA polymerase which is required for initiating transcription in the cells. Therefore, *rpoA* gene is a suitable reference gene to study the stress response profile of *C. jejuni*.

The results in Figure 4-1 showed that the gene expression of untreated *C. jejuni* cells was essentially the same as the *C. jejuni* cells treated with 0.54% (v/v) DMSO ($P < 0.05$). Therefore, 0.54% (v/v) DMSO was not considered as a confounding factor when examining the subsequent data. In contrast, the expression of *thiC*, *katA*, *sodB*, *dnaK*, *mreB*, *cmeC*, and *clpA* of *C. jejuni* cells treated with 6.25 $\mu\text{g/ml}$ of tetracycline was significantly down-regulated by 2-fold ($P < 0.05$) compared to the untreated *C. jejuni* cells and other treatment groups. The susceptible break-point of tetracycline was $\leq 4 \mu\text{g/ml}$ (FDA 2010; CIPARS 2013) and therefore a slightly higher concentration of the susceptible break-point was selected as the positive control treatment.

This gene expression profile suggests that *C. jejuni* F38011 might have a different stress response when treated with 6.25 µg/ml tetracycline. Tetracycline may have a different mechanism of action compared to carvacrol. Therefore, most of the stress response genes (*dnaK*, *mreB*, *cmeC*, *clpA*) were affected in a different direction than the cells treated with carvacrol alone or the combinational treatment.

In the current study, the general stress response genes (*dnaK*, *grpE*, and *groEL*) were 4.7 to 5.4-fold up-regulated in *C. jejuni* cells ($P < 0.05$) treated with 20.25 µg/ml carvacrol and the combination of 20.25-µg/ml carvacrol with 12.5 µg/ml ZnO NPs. However, the level of *dnaK* and *grpE* expression between the cells treated with carvacrol and the combinational treatment were not significantly different ($P > 0.05$). Although the level of *groEL* expression was statistically different ($P < 0.05$), there was only 1-fold change in *groEL* expression level between these treatments, and thus the difference was not relevant in this situation.

These targeted genes (*dnaK*, *grpE*, and *groEL*) are mostly associated with the global stress response and the synthesis of heat-shock proteins (DnaK, GrpE, and GroEL), which are critical to assisting the cells in preventing degradation, or aggregation of proteins, as well as maintaining cytosolic activity in the Gram-negative bacteria (Arsène et al., 2000; Hayes and Dice, 1996; Mayhew et al., 1996). The up-regulation of *dnaK*, *grpE* and *groEL* indicates an increasing production of heat-shock proteins, indicating the *C. jejuni* cells were experiencing stressful conditions (Garbe, 1992).

Reid and colleagues (2008) demonstrated that *dnaK*, *grpE*, and *groEL* were highly up-regulated in response to an acidic environment. These general stress response genes were also induced in *C. jejuni* cells under starving conditions (Klančnik et al., 2006). A recent study of the synergistic antimicrobial effect of carvacrol and cinnamaldehyde against *Acinetobacter*

baumannii observed similar up-regulation of *dnaK* and *groEL* in the cells treated with the combined treatment and carvacrol alone (Montagu et al., 2016). Similarly, Burt and colleagues (2007) observed a high level of GroEL protein production in *E. coli* cells treated with carvacrol. Both *A. baumannii* and *E. coli* are opportunistic Gram-negative pathogenic bacteria that share similar genetic profiles of 16S rRNA to *C. jejuni*. That aside, the up-regulation of *dnaK*, *grpE* and *groEL* may indicate that carvacrol treatment induces stress conditions to *C. jejuni* cells. The misfolding and/or disaggregation of protein on the outer membrane of the cells might possibly occur in the *C. jejuni* cells treated with the combinational treatment and carvacrol alone.

Conversely, none of the oxidative stress response genes were significantly changed for *C. jejuni* cells treated with 12.5 µg/ml ZnO NPs, 20.25 µg/ml carvacrol and the combination of antimicrobials ($P > 0.05$). This conflicts with the results of another study which showed high expression levels of *katA* and *ahpC* in *C. jejuni* cells treated with ZnO NPs (Xie et al., 2011). The conflicted result may be due to the difference in the applied concentration and the shapes of the ZnO NPs used to treat *C. jejuni* cells, the selected strain of *C. jejuni*, different primers sequence for *katA* and *ahpC*, and the use of a different RT-qPCR assay (SYBR Green versus TaqMan). Xie and others (2011) treated *C. jejuni* cells with 0.1 mg/ml of ZnO NPs for the gene expression analysis, which was approximately 10 times higher than the concentration used in our current study. The treatment of 12.5 µg/ml ZnO NPs did not result in a killing-effect against *C. jejuni* cells, whereas 1 mg/ml ZnO NPs resulted in a lethal-effect to *C. jejuni* cells. The shape and surface area of ZnO NPs also contribute to the antimicrobial activity against pathogenic bacteria (Ma et al., 2013; Stanković et al., 2013; Talebian et al., 2013). Xie and colleagues (2011) used larger surface area (35 m²/g) of ZnO NPs and smaller size of nanoparticles (~30 nm) than the surface area (12 – 24 m²/g) and size (40 – 100nm) of this study. As aforementioned, the

shape of ZnO NPs depends on the synthesis process of nanoparticles (Jalal et al., 2010; Ramani et al., 2012). This study purchased a commercial ZnO NPs powder, thus the shape and synthesis process of ZnO NPs was proprietary.

One pitfall of the RT-qPCR technique is that it does not provide a whole genomic profile that includes all possible genes of *C. jejuni*. It might be possible that the target genes responsible for the stress response of *C. jejuni* cells against ZnO NPs were not covered in this study. For instance, the putative zinc transporter genes of *C. jejuni* (ZnuABC and ZnuT) may have a role in the stress response profile of *C. jejuni* treated with ZnO NPs; however, these putative genes were not included due to unclear characterization of zinc metabolism in *C. jejuni* (Stahl et al., 2012). Further analysis using whole transcriptome shotgun sequencing (RNA-seq) may provide a comprehensive understanding of the whole transcriptional level of *C. jejuni* cells after treatment with the combinational antimicrobial treatment versus the single antimicrobial treatment.

4.4 Conclusion

The expression levels of the general stress response genes, *dnaK*, *grpE*, and *groEL* were highly up-regulated in the gene expression profile of *C. jejuni* cells after treatment with carvacrol alone and the combination of 20.25 µg/ml carvacrol and 12.5 µg/ml ZnO NPs. None of the targeted genes had altered expression levels in *C. jejuni* cells treated with ZnO NPs alone. Based upon this gene expression profile, carvacrol may play a major role in the putative mechanism of the action of the synergistic antimicrobial effect of carvacrol and ZnO NPs against *C. jejuni* by inducing the expression level of heat-shock related-genes.

Table 4-1 List of the target genes, functions, and primers sequences

Function	Gene	Primer	Primer Sequence (5' - 3')	Reference
Housekeeping				
RNA polymerase A, initiation of transcription	<i>rpoA</i>	F	CGAGCTTGCTTTGATGAGTG	Ritz et al., 2009
		R	AGTTCCCACAGGAAAACCTA	
Involved in thiamin biosynthesis pathway	<i>thiC</i>	F	TTATCTTTGGGCGATGCTTT	Ritz et al., 2009
		R	CATCCCAAGCCCTTTGAGTA	
Oxidative stress				
Catalase, defence against hydrogen peroxide	<i>katA</i>	F	CAAACAGCTATGATAATAGCC	Haddad et al., 2012
		R	GGAGCATATCTTTGTGCTACG	
Superoxide dismutase, defence against superoxide	<i>sodB</i>	F	TGGCGGTTCATGTCAAAGTA	Xie et al., 2011
		R	ACCAAAACCATCCTGAACCA	
Alkyl hydroxyperoxide reductase, defence against hydroperoxides	<i>ahpC</i>	F	CGTTCTTGCTTGATGCTGAT	This study
		R	CAGGACAAACTTCACCATGC	
General stress response				
Chaperone, chaperonin, heat shock protein	<i>dnaK</i>	F	CGGTATGCCACAAATCGAAG	Xie et al., 2011
		R	GCTAAGTCCGCTTGAACCTG	
Rod-shape determinant	<i>mreB</i>	F	GAGCCTTCTGTTGTGGCAGTT	Xie et al., 2011
		R	AGCGGATCATTTTTTCAGTCAT	
Multidrug efflux pump	<i>cmeC</i>	F	CAAGCTGCTGCTCAATTAGG	This study
		R	TCGATTGCTTGGAGCATTTA	
ATP-dependent ClpAP protease, abnormal protein degradation	<i>clpA</i>	F	GGCTCATCCTGATTTAAGCAA	This study
		R	ACCAAGCTCATTGCTTTCTTT	
Chaperone, chaperonin, heat shock protein	<i>grpE</i>	F	GTGCAAACGCTGAATTTGA	This study
		R	TCATCTTGGCATTTCGACATT	
Chaperonin, heat-shock protein	<i>groEL</i>	F	TATGGGCGCTTCACTTGTA	This study
		R	CCTCGATAGGATTTGCACCT	
Virulence				
Adhesion fibronectin binding protein	<i>cadF</i>	F	TTCTATGGTTTAGCAGGTGGAG	Koolman et al., 2016
		R	TTACACCCGCGCCATAAT	
Formation and expression of flagellin	<i>flaA</i>	F	GTTGCTCATCCATCGCTTTA	This study
		R	TGGCGATAGCAGATTCTTTG

Figure 4-1 Relative gene expression of untreated *C. jejuni* F38011 (control) versus treated *C. jejuni* F38011 cells after 15 minutes of treatment.

Data were analyzed using the comparative critical threshold ($\Delta\Delta C_T$) method. A ratio greater than one (>1) indicates up-regulation of gene expression and a ratio below one (<1) indicates down-regulation. *Targeted gene of the treatment was significantly different than the control ($P < 0.05$)

Chapter 5: The putative mechanism of the action of carvacrol and zinc oxide nanoparticles against *Campylobacter jejuni*, conclusion, and future research

5.1 The putative mechanism of the action of carvacrol and zinc oxide nanoparticles against *Campylobacter jejuni*

Figure 5-1 shows the putative mechanism of action of carvacrol and ZnO NPs against *C. jejuni* over 24 hours. This putative mechanism is derived from the summary of macromolecular fingerprints and gene expression profile of *C. jejuni* cells after the combined treatment, as well as scanning electron microscopy (SEM) observations (Figure B.1). Within 15 minutes, the expression level of the general response genes (*dnaK*, *grpE*, and *groEL*) significantly increased. Over 6 hours of treatment carvacrol may contribute to chemical changes in *C. jejuni* cells, which was indicated by segregation on the PCA model and Raman spectral features. Carvacrol may affect phospholipids structure on the outer cell membrane and disrupt the DNA/RNA-base materials in *C. jejuni* cells. Additionally, the SEM images (Figure B.1) reveal that leakage of intracellular material may occur in cells after 6 hours of treatment. ZnO NPs were observed to attach onto the surface of *C. jejuni* cells, which potentially induced a morphological change (Figure B.1). In this case, ZnO NPs were hypothesized to amplify the killing-effect of carvacrol against *C. jejuni* between 6 to 24 hours of incubation by damaging the outer cell membrane, resulting in bacterial inactivation. The synergistic antimicrobial effect of carvacrol and ZnO NPs against *C. jejuni* was observed within 8 hours of treatment based upon the time-kill curve and it became more evident with a prolonged incubation time of 24 hours.

In the current study, the role of ZnO NPs was not well observed in the macromolecular fingerprints and gene expression profile of *C. jejuni* possibly due to potentially low or non-

inhibitory concentration of ZnO NPs tested and limited sampling of the time-points. The ZnO NPs concentrations used for treatment in the current study were very low, which may make it difficult to resolve their effects on the macromolecular fingerprints and gene expression profiles. Several studies had shown that ZnO NPs induce morphological changes on the cell surface, increased membrane permeability, and elevated oxidative stress (Xie et al., 2011; Liu et al., 2009).

Figure 5-1 The putative mechanism of stress response and inactivation of *C. jejuni* due to the synergistic antimicrobial effect of carvacrol and ZnO NPs

5.2 Conclusion

In this thesis project, the synergistic antimicrobial effect of carvacrol and ZnO NPs against *C. jejuni* was demonstrated for the first time using the time-kill curve. The putative mode of action of carvacrol and ZnO NPs was developed for the first time based upon the macromolecular fingerprints and gene expression profiles that were observed. The macromolecular fingerprints of *C. jejuni* cells treated with the combined treatment could be

segregated from the cells treated with ZnO NPs and the untreated cells, but clustered together with the cells treated with carvacrol alone. A similar pattern was also observed in the gene expression profile, in which gene expression of *C. jejuni* cells treated with carvacrol had similar up-regulation of *dnaK*, *groEL*, and *grpE* as cells subjected to the combined treatment. Based upon the macromolecular fingerprints and gene expression profiles, carvacrol was likely to be the major contributor to antimicrobial activity and the synergistic effect. Further studies on physiological and morphological aspects may be conducted to evaluate the role of ZnO NPs in the inactivation of *C. jejuni*.

The results from this study provide fundamental knowledge to understand bacterial inactivation and stress response consequent to synergistic antimicrobial treatment with an essential oil and metal oxide nanoparticles. This antimicrobial combination may potentially be utilized as an intervention strategy to reduce the prevalence of *C. jejuni* in the environment and the agri-food system as a whole.

5.3 Future research

The current study adopted the time-kill curve to evaluate the synergistic antimicrobial effect of two compounds. Future studies may need to adopt different methods (*e.g.*, checkboard assay) and compare the results to provide a more comprehensive evaluation of the synergism. Future work should also investigate the morphological and physiological aspects of *C. jejuni* cells under the stress of an antimicrobial agent, as well as its phenotypic characteristics, to generate a comprehensive overview of the possible mechanism of the action of these two antimicrobial agents. Understanding the multilayer mechanism of the presentation of bacterial

stress due to a synergistic effect of two antimicrobials is important to develop the most effective and beneficial intervention strategies to inactivate *C. jejuni*.

Note that this study documented the synergistic antimicrobial effect of carvacrol and ZnO NPs against *C. jejuni in vitro*. In a real situation, the most effective delivery system for these two antimicrobials with different properties and natures would need to be considered before conducting an *in vivo* study. Perhaps a nano-emulsion of carvacrol coated with ZnO NPs may be worth investigation as a delivery system for the *in vivo study* in the hope of using these antimicrobials for application in food packaging materials and food contact surfaces.

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Appendix

Appendix A Chapter 2 supplementary information

A.1 The standard curve of *Campylobacter jejuni* strains used in the study

Figure A.1 Standard curve of the growth of *Campylobacter jejuni* strain (A) human clinical isolate 10, (B) ATCC 33560, and (C) F38011 in Mueller-Hinton broth incubated at 37°C in a microaerobic condition

Appendix B Chapter 5 supplementary information

B.1 Scanning electron microscopy images of *Campylobacter jejuni*

The preparation of the *C. jejuni* strain F38011 culture and antimicrobial treatment followed the procedure in section 2.2.1 and 2.2.4, respectively. *C. jejuni* strain F38011 was treated with the combinational concentration of carvacrol (20.25 µg/ml) and ZnO NPs (12.5 µg/ml), carvacrol alone, and ZnO NPs alone. After 6 hours of antimicrobial treatment, the cell pellets were collected by centrifugation. The cell pellets were rinsed for three times with phosphate buffer and fixed with 2.5% glutaraldehyde in 0.1 mol⁻¹ phosphate buffer (pH = 7.35) overnight at room temperature. The fixed bacterial cells were then rinsed with sterile water and dehydrated in a series of 25%, 50%, 70%, 60%, 90% ethanol (10 minutes each). Next, the cells were further dehydrated twice with 100% ethanol (15 minutes each) and freeze-dried immediately in a Christ Alpha 1-4 lyophilizer (Christ, Osterode, Germany). Then, the samples were mounted onto the SEM stubs and sputter coated with a thin layer of gold. The coated samples were observed under a FEI Nova200 NanoLab scanning electron microscope (FEI, Hillsboro, USA) using an accelerating voltage of 5 kV.

Figure B.1 Scanning electron microscopy (SEM) images of *C. jejuni* strain F38011 revealed cellular leakage and damage when (B) treated with the combinational of carvacrol (20.25 μg/ml) and ZnO NPs (12.5 μg/ml) compared to the (A) untreated cells. The morphological change of *C. jejuni* strain F38011 cells was observed when the cells was treated with (C) carvacrol (20.25 μg/ml), and (D) treated with ZnO NPs (12.5 μg/ml).