INNOVATIVE STRATEGIES FOR THE CONTROL OF *CAMPYLOBACTER JEJUNI* IN AGRI-FOODS

by

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Abstract

The aims of this thesis project are to study the interactions between antimicrobials, identify the adaptive molecular mechanism of synergistic antimicrobial combinations, and develop an innovative antimicrobial packaging to control *Campylobacter jejuni* in raw chicken meat.

We identified additive and synergistic combinations consisting of cinnamon oil, encapsulated curcumin, and/or zinc oxide nanoparticles (ZnO NPs) using an innovative mathematical model to avoid some over- or under-estimation of synergism determined by the conventional methods.

The whole transcriptome sequence analysis (RNA-Seq) of single and combined treatments showed that each single antimicrobial played a different role in synergism, while combined treatments altered unique gene expressions that were not affected by any single treatment. Many of these genes are involved in signaling and chemotaxis, amino acid synthesis, protein translation, and/or bacterial cell wall synthesis. Unlike dual antimicrobial treatments, all single treatments resulted in under-expression of a major facilitator superfamily encoding gene involved in developing antimicrobial resistance. ZnO NPs alone altered the greatest number of genes and functions in *C. jejuni* compared to any other single, dual or triple antimicrobial treatments.

We included ZnO NPs into packaging material for the control of *Campylobacter* in raw chicken meat. Functionalized pad was placed underneath chicken meat to investigate the antimicrobial effect against *C. jejuni*. Immobilized ZnO NPs reduced *C. jejuni* from ~log 4 CFU/chicken sample to undetectable level during storage at 4°C. We validated no nanoparticle migration onto the chicken meat after treatment with immobilized ZnO NPs in the functionalized pad. Analysis by inductively coupled plasma-optical emission spectroscopy showed that Zn level increased from 0.02 to 0.17 mg/cm^2 in the treated chicken meat. Inactivation of *C. jejuni* was

associated with the increase of lactic acid produced by *Lactobacillus* in chicken meat in a pH dependent manner. Less than 5% of Zn^{2+} were released from ZnO NPs at neutral pH, while up to 88% were released when pH <3.5 within 2 d. This controllable conversion of immobilized ZnO NPs to free Zn^{2+} makes the approach safe and eco-friendly and can facilitate developing a novel intervention strategy for other high-risk foods including *Campylobacter*-contaminated chicken meat.

Lay Summary

No validated standard method for studying antimicrobial interactions is available. In this thesis, we compared the interactions of different antimicrobial combinations using various conventional methods and a novel mathematical model. We used *Campylobacter jejuni* as a foodborne pathogen model to study the antimicrobial synergism. We identified several effective synergistic antimicrobial combinations to reduce the risk of *Campylobacter*-associated food contamination. The antimicrobial mechanism of cinnamon oil, encapsulated curcumin and ZnO NPs and their antimicrobial interaction in binary or tertiary combinations for potential synergy were systematically investigated by using a genome wide sequence analysis including >1,600 genes in *C. jejuni*. Based on the knowledge gained from the aforementioned antimicrobial studies, we developed an innovative active packaging including immobilized ZnO NPs that could effectively inactivate *C. jejuni* in raw chicken meat.

Preface

With the supervision of Dr. Xiaonan Lu, my contribution to the three studies of this thesis were in idea development, experimental design, execution, data analysis, and co-writing the manuscripts. Chapter 2 is about the development of a novel mathematical model for studying antimicrobial interactions against *Campylobacter jejuni*. This study was published by Mohammed J. Hakeem, Khalid A. Asseri, Luyao Ma, Keng C. Chou, Michael E. Konkel and Xiaonan Lu in *Frontiers in Microbiology* (2019) 10, 1038. Mohammed J. Hakeem provided the idea of the study, conducted the experiments, and wrote the manuscript. Dr. Asseri contributed to the idea of the study and analyzed the data. Luyao Ma reviewed and edited the manuscript. Dr. Chou reviewed and helped the mathematical modeling. Dr. Konkel reviewed and edited the manuscript. Dr. Lu supervised, reviewed, and edited the manuscript. Chapter 3 is about the application of whole transcriptome sequencing analysis of antimicrobial synergism of plant-based antimicrobials and zinc oxide nanoparticles against *C. jejuni*. This study will be submitted for publication by Mohammed J. Hakeem, Jinsong Feng, Lina Ma and Xiaonan Lu. Chapter 4 is about the development of an innovative active packaging including immobilized zinc oxide nanoparticles to inactivate *C. jejuni* in raw chicken meat. This study will be submitted for publication by Mohammed J. Hakeem, Azadeh Nilghaz, Hwai Chuin Seah, Michael E. Konkel, and Xiaonan Lu.

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List of Abbreviations

ANOVA	analysis of variance
cDNA	complementary deoxyribonucleic Acid
CFU	colony forming unit
CO	cinnamon oil
CRISPR	clustered regularly interspaced short palindromic repeats
Ct	cycle threshold
d	day(s)
D-value	decimal reduction time
DMSO	dimethyl sulfoxide
EC	encapsulated curcumin
ED	Entner Doudoroff
FCR	feed conversion ratio
FDR	false discover rate
FICI	fractional inhibitory concentration index
g	gram or gravitational
GBS	Guillain–Barré syndrome
GIK	glucokinase
GO	gene ontology
GRAS	generally recognized as safe
h	hour(s)
HMS	hydrophobically modified starch
IC	inhibitory concentration

ICP-OES	Inductively coupled plasma - optical emission spectrometry
kV	Kilovolt
LogCPM	Logarithmic count per million
LogFC	Logarithmic fold change
M	molar
MAP	modified atmosphere packaging
MBC	minimum bactericidal concertation
Mbp	megabase pair
MH	Mueller-Hinton
MIC	minimum inhibitory concertation
min	minute(s)
MRS	DeMan, Rogosa and Sharpe agar
N	nitrogen or normal
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pfk	Phosphofructokinase
psi	pound per square inch
PVC	polyvinyl chloride
qPCR	quantitative PCR
RNA	ribonucleic acid
RNA-Seq	ribonucleic acid sequencing
ROS	reactive oxygen species

SD	standard deviation
SLD	spotty liver disease
spp.	species
t	tons
tRNA	transfer RNA
TSA	tryptic soy agar
v/v	volume per volume
VBNC	viable but nonculturable
w/v	weight per volume
ZnO NPs	zinc oxide nanoparticles

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A great gratitude goes to my parents (Jamal Hakeem and Nissar Musa). Great thanks for my siblings Layal, Suhayb, Fatmah, Lubnah, and Jafar. Last but certainly not least, I cannot thank my loving wife Basmah Bakheet and wonderful daughters Hala and Sama enough for accompanying me with love and support during my long graduate studies journey in Canada.

Dedication

I dedicate this thesis to my loving wife Basmah, and my beautiful daughters Hala and Sama. I could never reach this point without them. Hala and Sama, your father loves you more than you can imagen. I hope this dedication will inspire you to achieve your dreams one day. Hala I am so grateful to have you as my older daughter. I believe in you more than you ever know. Keep up the hard work, and I am sure you will be a wonderful and successful woman in the future. My baby girl Sama, I want you to know that your birth was the best event happened to us in many years. Your smiling face and happy personality helped me every day to write this thesis with pleasure.

Chapter 1: Introduction

1.1 Overview of *Campylobacter*

1.1.1 Morphology and diversity

Campylobacter species are Gram-negative, motile, and non-spore-forming bacteria. The name of "*Campylobacter*" originally came from the ancient Greek meaning "curved rod" although the unique shape of *Campylobacter* looks more like a spiral or helical one rather than a curved rod shape. *Campylobacter* can change its shape into filamentous or coccoid to adapt to the stressful conditions (Gaynor et al. 2005; Tresse, Alvarez-Ordóñez, and Connerton 2017). It was first isolated from a sheep abortion case and classified as a *Vibrio*-like bacterium (McFadyean and Stockman 1913; Martin B Skirrow 2006) and then renamed as *Campylobacter* after showing a clear different taxonomy profile from the *Vibrio* species (Véron and Chatelain 1973). The genus of *Campylobacter* consists of 26 species, 2 provisional species, and 9 subspecies (Bhunia 2018). *Campylobacter* bacteria are very diverse microorganisms not only on the species levels but also on the subspecies and strain levels (Vidal et al. 2016; Gaynor et al. 2005). Diversity includes differences in genetic and phenotypic characteristics as well as growth requirement, which may explain their presence in different hosts or ecological niches including different poultry and wild birds. Some *Campylobacter* species are flagellated with a single polar flagellum or bipolar flagella (*e.g.*, *C. jejuni*, *C. coli C. concisus* and *C. showae*), while fewer species (*e.g.*, *C. hominis* and *C. ureolyticus*) have no flagellum (Man 2011). The size of an individual *Campylobacter* cell is approximately 0.5-5 μm in length and 0.2-0.8 μm in width. The majority of *Campylobacter* species can grow under the microaerobic condition. Several species including *C. concisus*, *C. curvus*, *C. rectus*, *C. mucosalis*, *C. showae*, and *C. gracilis* require hydrogen during the microaerobic growth, while only a few species can grow under anaerobic conditions (Kaakoush et al. 2015; Lee et al. 2014).

1.1.2 Infections

Campylobacter is documented in 2019 to be the leading foodborne pathogen associated with the consumption of animal-source food products worldwide (M. Li et al. 2019). Numerous *Campylobacter* species can effectively colonize both humans and animals (Véron and Chatelain 1973). The classical symptoms of *Campylobacter* infections (called campylobacteriosis) include fever, severe watery or bloody diarrhea, cramps, and weight loss for 6 days on average in humans (Véron and Chatelain 1973; Kaakoush et al. 2015). Most infections are self-limiting and do not require medical therapy other than hydration and electrolyte balance (Acheson and Allos 2001). Antibiotic treatment is only applied either in severe cases or to the immunocompromised individuals. Although infection occurs in all ages, a population-based study from Denmark showed that toddlers (1 to 4) and young adults (15 to 24) are more susceptible to *Campylobacter* infections than others (Nielsen et al. 2013).

C. jejuni and *C. coli* are the major causes of campylobacteriosis in humans (Kaakoush et al. 2015). Several studies showed that infections of both *C. jejuni* and *C. coli* occur more frequently during the summer than other seasons (Nielsen et al. 2013; Bessède et al. 2014). *C. jejuni* infection is greater than *C. coli* in many countries, but *C. coli* is also an important species and reported to be the second most contributor to campylobacteriosis after *C. jejuni*. In fact, a comparison study of patients infected with either *C. jejuni* or *C. coli* showed that slightly older patients (34.6 compared to 27.5 years old) have a greater risk of being infected with *C. coli* than *C. jejuni* (Bessède et al. 2014).

Campylobacteriosis has also been linked to a range of gastrointestinal conditions, such as inflammatory bowel diseases (IBD), periodontitis, esophageal disease, functional gastrointestinal disorders, celiac disease, and colon cancer in humans (Véron and Chatelain 1973; Kaakoush et al. 2015). *C. jejuni* infections may lead to autoimmune disorders known as the Guillain-Barré syndrome (GBS) and Miller Fisher syndrome. According to an infection study of 111 volunteers, *C. jejuni* dosage correlates

with the colonization rate, but not with the development of illnesses (Black et al. 1988). The infectious dose to develop campylobacteriosis varied depending on the immunity and health status of the individuals. Only 800 cells were able to cause diarrhea to some volunteers, while other data showed that campylobacteriosis was developed with a dose as low as 360 cells (Hara-Kudo and Takatori 2011). In comparison, *C. hepaticus* is a chicken fatal bacterium that causes a spotty liver disease (SLD) and leads to significant losses in the poultry industry (Van et al. 2017)

1.1.3 Emerging species

Emerging *Campylobacter* bacteria include previously undetected species or species that were linked to increasing numbers of illnesses in the recent years and near future (Kaakoush et al. 2015). They include *C. concisus, C. curvus, C. fetus, C. gracilis, C. mucosalis, C. pinnipediorum, C. rectus, C. showae, C, sputorum, C. lari, C. ureolyticus, C. upsaliensis,* and *C. volucris.* The clinical importance and pathogenicity of emerging *Campylobacter* species have been reviewed (Man 2011; Costa and Iraola 2019). Available evidence showed that they could attach and invade human epithelial cells, alter intestinal barrier integrity, avoid host immune response, secrete toxins and invade macrophages. In contrast, the actual contribution of emerging *Campylobacter* species to campylobacteriosis is still not clear because available cultivation methods including hydrogen-enhanced microaerobic and anaerobic conditions failed to successfully grow these microbes under the laboratory condition (Kaakoush et al. 2015). This is due to several reasons including the slow growing nature of some fastidious species or individual strains, growth inhibition by antibiotics added in selective media, limited hydrogen source, presence of competitive microorganisms, and/or difficulties in identifying some *Campylobacter* species due to their morphological diversities. Nevertheless, hydrogen enhancement (generally 3-7%) in the microaerobic condition improved the detection of *C. concisus* from 0.03% to 1.92% (Casanova et al. 2015). Symptoms of *C.*

concisus infections and other *Campylobacter* bacterial infections are usually milder than that with *C. jejuni* and *C. coli*. However, emerging *Campylobacter* species are also important and require better isolation techniques for their detection and diagnosis. A previous report showed that infections of *C. concisus* and *C. fetus* were more common than infections of *C. jejuni* and *C. coli* in the elderly (68.4 years old) than young adults (28.6 years old) (Bessède et al. 2014). In conclusion, although *C. jejuni* and *C. coli* remain the leading cause of campylobacteriosis, more effective detection methods are required for a better understanding of how emerging *Campylobacter* bacteria evolve in the environment, transmit to agri-food systems, and contribute to campylobacteriosis.

1.1.4 Epidemiology

Available evidence suggests that campylobacteriosis incidence has been rising in both developed and developing countries in the recent years (Kaakoush et al. 2015). The size of *Campylobacter* outbreaks in different countries ranged from 10 to 100 cases between 2007 and 2013 (Kaakoush et al. 2015). Poultry and untreated water were the most reported sources of *Campylobacter* outbreaks. The number of *Campylobacter* cases in different countries within the same region can vary significantly. This is not only due to the unreported cases but also limited sensitivity of detection methods, population size and composition, variation in public health standards, intervention strategies, surveillance systems, food safety practices, and the prevalence of *Campylobacter* in natural reservoirs in different regions. The epidemiological data from Asia, Africa, and the Middle East shows that *Campylobacter* infection is prevalent in this region although the data is incomplete. The total number of *Campylobacter* infections in Canada was estimated to be about 145,350 cases per year (M. K. Thomas et al. 2013). British Columbia (BC) had an annual *Campylobacter* infection rate of 37.74 cases per 100,000 people (1,818 cases) in 2017 (BC Center for Disease Control 2017). In comparison, Japan had a rate of 1,512 cases per 100,000 people

(Kubota et al. 2011) and New Zealand had a rate of 161.5 per 100,000 people (Sears et al. 2011) within the last decade. In USA, the surveillance system, new regulations, and control strategies have contributed to the decline of several foodborne pathogens including *Salmonella*, *Listeria*, and *E. coli* O157:H7 from 2006 to 2014, but not *Campylobacter* and *Vibrio* (Crim et al. 2014). Altogether, both individual cases and outbreaks of campylobacteriosis are generally prevalent around the world.

Several risk factors that can lead to *Campylobacter* infections include traveling or person-to-person transmission, contact with animals, and consumption of contaminated food or water. Meta-analysis data suggest that international and domestic traveling was the most critical risk factor of *Campylobacter* infections, followed by the consumption of uncooked chicken meat, environmental exposure, and direct contact with the farm animals. A Canadian report showed that campylobacteriosis was responsible for the highest number of causes of travel-related diseases [123/446 cases (27.57%)] from 2005 to 2009 (Ravel et al. 2010). In addition, overlapping exists between risk factors. For example, travel-related diseases are frequently linked to the consumption of contaminated foods (Kaakoush et al. 2015). Although traveling abroad contributes to the overall *Campylobacter* transmission, the spread of antibiotic-resistant *Campylobacter* strains between countries and continents through international agri-food trade is also a considerable public health concern (Mughini-Gras et al. 2014).

1.1.5 Physiology

Campylobacter species are not only unique in their shape, but they also have a relatively small genome with unique cellular and molecular physiology compared to other foodborne pathogens. The first whole-genome sequencing analysis of *C. jejuni* (NCTC11168 strain) showed that the genome (1.6 Mbp) has uniquely a limited number of repeated sequences and no insertion or phage associated regions (Parkhill et al. 2000). Other reports showed that *C. jejuni* lacks the regulator *rpoS* (starvation/stationary phase sigma

factor) and their stationary-phase cultures are ununiformed dynamic populations unlike most of other bacteria (Kelly et al. 2001). This could be a survival strategy that *C. jejuni* uses to reduce its starvation stress during the stationary phase at least in some strains. Although the existence of stationary phase in *C. jejuni* is elusive, a transition from exponential to stationary phase was observed in *C. jejuni* populations with a number of changes in the transcriptomic and proteomic profiles between the two phases (Turonova et al. 2017). This data also suggests that the pleiotropic regulator *cosR* gene acts as a negative autoregulator and is alternative to *rpoS* gene in *C. jejuni* during the stationary phase of growth. In addition, *C. jejuni* is an asaccharolytic bacterium (*i.e.*, unable to break down carbohydrate for energy) due to the absence of some key glycolytic enzymes [*e.g.*, glucokinase (GIK) and phosphofructokinase (Pfk)] that involved in the functional Embden-Meyerhof-Parnas glycolysis pathway (Tresse, Alvarez-Ordóñez, and Connerton 2017). *Campylobacter* is also a chemo-organotrophic bacterium that oxidizes the chemical bonds in amino acids or intermediate molecules of tricarboxylic acid (Krebs) cycle as their energy and carbon source. Moreover, *C. jejuni* uses gluconeogenesis fueled by amino acids to generate glucose from non-carbohydrate sources. The Entner-Doudoroff (ED) pathway is used in bacteria for synthesizing pyruvate from extracellular glucose. A complete group of genes encoding ED pathway was identified in some rare *C. jejuni* and *C. coli* isolates (Vegge et al. 2016). Interestingly, this gene set increased the survival and biofilm formation in *Campylobacter*. Altogether, *C. jejuni* lacks many important stress response genes, but has developed different mechanisms to adapt to and survive in the new environmental and/or under stress conditions.

Campylobacter species have many unique growth requirements that can limit but not eliminate their prevalence outside warm-blooded hosts in foods and/or food environments. Most *Campylobacter* bacteria grow optimally at either 42°C (chicken body temperature) or 37°C (human body temperature), but none of them can grow below 30°C (**Table 1.1**) (Park 1996). The growth rate of most other bacteria

reduces gradually near their minimum growth temperature unlike *Campylobacter* that suddenly stops to grow below 30°C (Hazeleger et al. 1998). No growth adaptation of *C. jejuni* was observed below 30°C. This raises the question of how different the metabolic activity of *Campylobacter* is below and above the minimum growth temperature. This question will be answered below according to several reports about the survival of *Campylobacter* in food and food-related conditions. Moreover, *Campylobacter* is unable to survive under the ambient oxygen level due to several combined reasons (Mace et al. 2015). These include (i) limited tolerance against reactive oxygen species (ROS), (ii) incompetence of producing adequate antioxidant enzymes, (iii) low respiratory rate, and (iv) presence of oxygen-labile essential enzymes (Velayudhan et al. 2004). A few enzymes present in *Campylobacter* are believed to play a critical role in protecting the cells from oxygen tension. These include catalase, glutathione reductase, glutathione synthetase, peroxidase, and superoxide dismutase (Keener et al. 2004).

1.1.6 Survival in food and food-related conditions

Campylobacter is sensitive to food and food processing-related stresses. They are more sensitive to heat treatment compared to other foodborne pathogens. For example, the *D*-value of *E. coli* is 5 times higher than that of *C. jejuni* at 55°C (**Table 1.1**) (Rusin et al. 1997). Simply freezing at -15°C could reduce *C. jejuni* count by 3 log CFU/g in ground beef (Stern and Kotula 1982). Desiccation at room temperature inactivated *Campylobacter* within a few days (Michael P Doyle and Roman 1982). *Campylobacter* cannot survive for a long period of time on food contact surfaces, such as cutting boards, countertops, equipment or kitchen utensils. In contrast, *Campylobacter* can remain viable on fresh foods, such as ground beef (Stern and Kotula 1982), fresh produce (Kärenlampi and Hänninen 2004), fresh chicken (Blankenship and Craven 1982), and milk (Michael P Doyle and Roman 1982) during the entire shelf life up to 3 weeks. In addition, the combination of these wet and cold refrigeration conditions of fresh foods assists

Campylobacter in surviving on dry surfaces for a few weeks instead of a few days (MICHAEL P Doyle and Roman 1982; Stern and Kotula 1982).

Table 1.1. Growth limitations and heat sensitivity of *C. jejuni* and other common foodborne pathogens (Rusin et al. 1997)**.**

Bacteria	Temperature (°C)			Typical *D*-value at 55°C	Minimum water activity	Minimum pH	Oxygen requirement
	Min.	Opti.	Max.				
Listeria monocytogenes	0	37	45	4.5	0.92	4.4	Facultative
Staphylococcus aureus	7	37	48	3.0	0.83	4.0	Facultative
***Salmonella enterica* serotype Typhimurium**	5.2	37	46	4.7	0.93	3.8	Facultative
Escherichia coli	7	37	46	5.5	095	4.4	Facultative
Campylobacter jejuni	30	42	45	1.0	098	4.9	Microaerophilic (5 – 10% O_2)

Refrigeration is one of the most common food preservation methods either used alone or in combination with other antimicrobial strategies or food preservation methods such as the addition of preservatives, irradiation, or modification of atmosphere. The growth rates of the majority of microorganisms drop to the minimum or stop at refrigeration temperatures. However, fewer pathogenic and spoilage bacteria can grow from a few cells to a large number (*e.g.*, psychrotrophic bacteria) and cause serious food poisonings (Chan and Wiedmann 2008) or spoilage recall incidents that can be associated with food loss and negative impact on the economy (Pothakos et al. 2014). Food spoilage can have a *Pseudomonas* species (Q. Q. Zhang et al. 2012; Chouliara et al. 2007; Al-Nehlawi et al. 2013), lactic acid bacteria (Q. Q. Zhang et al. 2012; Chouliara et al. 2007; Doulgeraki et al. 2012), and *Brochothrix thermosphacta* (Q. Q. Zhang et al. 2012; Chouliara et al. 2007) are considered as the most problematic spoilage psychrotrophic bacteria in poultry meat. In contrast, *Campylobacter* and *Salmonella* are the most causes of human gastroenteritis due to poultry meat consumption (Rouger, Tresse, and Zagorec 2017). *Campylobacter* in poultry was ranked as the leading pathogen-food combination to cause health risks and negatively impact the economy (Batz, Hoffmann, and Morris Jr 2012). Kaakoush and others reported that poultry consumption was the most cause of campylobacteriosis outbreaks between 2007 and 2013 (Kaakoush et al., 2014). A more recent report showed that 28 campylobacteriosis outbreaks were linked to the consumption of chicken livers in USA between 2000 and 2016 (Lanier et al. 2018). Up to 90% of commercially available chicken meat in different regions has been identified to be contaminated by *Campylobacter* at ~log 4 CFU/carcass (Jorgensen et al. 2002; Willis and Murray 1997; Walker et al. 2019).

Cold stress response of *Campylobacter* is significantly different from other common foodborne pathogens. Although *Campylobacter* lacks cold shock proteins, this microbe can still be active during the shelf life of different refrigerated foods or during the winter season in the agro-ecosystem (Murphy,

Carroll, and Jordan 2006). Hazeleger and others compared the changes in the fatty acid composition of the membrane of coccoid-shaped *Campylobacter* cells with that of the spiral-shaped cells incubated at 4°C (Hazeleger et al. 1995). The change in the fatty acid composition in both groups was similar. In contrast, a significant change in the composition of fatty acids occurred when the cells were incubated either at 12°C or 25°C. This included a significant increase in the percentage of 16:0 and 18:0 fatty acids and a significant decline in the percentage of 14:0, 16:1 and 19:0 fatty acids. The same group reported in another study that the vital processes of *C. jejuni* including cellular respiration, catalase activity, energy generation, and protein synthesis were still be functional at 4°C, which was far below the minimum growth temperature at 30°C (Hazeleger et al. 1998). The total amount of ATP (*i.e.*, produced + consumed) as indicated by the respiration rate at 4°C was only 5% of that at 40°C, suggesting that *C. jejuni* has a relatively low metabolic activity at low temperatures. However, the concentration of the produced ATP at 4°C was almost 50% of that at 40°C. Physiological functions such as chemotaxis and aerotaxis were similarly observed at 4, 20, and 40°C, indicating that *C. jejuni* could normally move toward substrates even below 30°C. The effect of cold exposure (*i.e.*, 6°C for 24 h) on the thermal tolerance (*i.e.*, 56°C) of *C. jejuni* was compared with that of *E. coli* K-12 (Hughes et al. 2009). *C. jejuni* was more tolerant than *E. coli* K-12 to thermal treatment as the ratio of the unsaturated to saturated fatty acids did not change after cold exposure, which was different from that of *E. coli* K-12. In conclusion, *Campylobacter* not only remains viable at low temperatures but also maintains sufficient metabolic activity to survive and move to the favorable places even in the absence of cold shock proteins.

1.2 *Campylobacter* in poultry farms

1.2.1 Transmission into poultry farms

Poultry has been considered as the major source of food-related transmission of *Campylobacter* species to humans since the early years of poultry industry (M B Skirrow 1977). *C. jejuni* is a common commensal microorganism in chicken microbiome (Hendrixson and DiRita 2004; Awad et al. 2016; Ijaz et al. 2018). Poultry is also a reservoir of other *Campylobacter* species including *C. lari*, *C. upsaliensis*, and *C. concisus* (Kaakoush et al. 2014). This bacterium usually transmits horizontally from different environmental sources to flocks (Kaakoush et al. 2015; Sahin, Morishita, and Zhang 2002). It was reported that *Campylobacter* species are usually abundant in the surrounding environment of poultry farms, such as soil, water source, dust, surfaces and air (Ellis-Iversen et al. 2012). Animal feed and/or drinking water can transmit *Campylobacter* from the environment to poultry farms. Farmers and farm visitors who carry *Campylobacter* can also transmit this microbe to poultry farms. Several studies isolated *Campylobacter* from wild bird feces around poultry houses, suggesting that wild birds contributed to the transmission of this microbe into the poultry houses (Hiett et al. 2002; Craven et al. 2000). For example, a molecular subtype analysis showed that 12 *Campylobacter* strains isolated from the broiler flocks were closely related to a strain isolated from wild bird feces identified in the same farm environment (Hiett et al. 2002).

Other organisms including flies, insects, amoebae, yeasts and molds have been found to be also important routes of horizontal transmission of *Campylobacter* into poultry houses (Newell et al. 2011; Axelsson-Olsson et al. 2005). A lesser mealworm beetle and their larvae (*Alphitobius diaperunus*) were identified as important carriers of *C. jejuni* in the poultry facilities. They could transmit *C. jejuni* not only within batches but also cross-contaminate flocks in the successive rearing cycles (Hazeleger et al. 2008). In addition, microbial eukaryotes may act as a reservoir of *Campylobacter* in the environment. For example, numerous *C. jejuni* strains are able to invade, replicate, and remain viable inside an amoeba host

(*i.e., Acanthamoeba polyphaga*) (Axelsson-Olsson et al. 2005). Since eukaryotes are usually prevalent in both drinking water systems and microbial biofilms on farms (Snelling et al. 2006), it is highly possible that infected eukaryotes contribute to *C. jejuni* transmission to poultry infrastructure.

There has been a long controversy about whether *Campylobacter* can be transmitted vertically from one generation of poultry to the other (Cox et al. 2012). One study including 60,000 progeny parent breeders identified a lack of evidence for vertical transmission of *Campylobacter* to chickens (Callicott et al. 2006). All chickens used in the study were hatched from eggs of *Campylobacter*-colonized grandparent flocks. However, egg passage can lead to the transmission of fecal bacteria including *Campylobacter* and subsequently contaminate the shell, shell membrane, and albumen of newly laid and fertile eggs (Cox et al. 2012). This can lead to *Campylobacter* ingestion after the chicks emerge from their eggs, colonization and spread of *Campylobacter* in poultry houses. In contrast, vertical transmission is well-established in *Salmonella* as they contaminate the egg within the reproductive tract before the shell is formed or penetrate the eggshell and invade the yolk of the post-lay egg (Gast and Beard 1990; Miyamoto et al. 1997; H. Yang, Li, and Johnson 2001). In addition, *Salmonella* is the major cause of foodborne outbreaks linked to poultry eggs (Guard-Petter 2001), while *Campylobacter* egg-associated outbreaks are extremely rare (Finch and Blake 1985). A systematic review including a primary set of 4,316 references showed that *Campylobacter* was rarely isolated from the internal egg contents (Newell et al. 2011), which was also validated by several on-farm studies (J. Byrd et al. 2007; Callicott et al. 2006; Jacobs-Reitsma 1995; Jacobs-Reitsma et al. 1995; Kiess, Kenney, and Nayak 2007; Pearson et al. 1993; Petersen, Nielsen, and On 2001; Shanker, Lee, and Sorrell 1986; Smith et al. 2004; Van de Giessen et al. 1992). Therefore, improving biosecurity systems and applying effective intervention strategies are the key elements to limit the prevalence of *Campylobacter* in broiler farms.

1.2.2 Chicken colonization of *Campylobacter*

Colonization of *Campylobacter* in farm chickens occurs usually due to horizontal transmission from the environment, such as via drinking water or animal feed. Once *Campylobacter* enters the chicken flock, it spreads rapidly and colonizes the intestinal tracts (crap, small intestine, and ceca) of most chickens after one week (Newell et al. 2011; Beery, Hugdahl, and Doyle 1988; Shanker, Lee, and Sorrell 1990). The level of *C. jejuni* inside these niches could be as high as 10^9 cells/gram of intestinal tracts with no symptoms or noticeable harmful effects until slaughtering (Stern et al. 2001). One study reported that *C. jejuni* is not just a commensal bacterium in broiler chickens, but it can cause chronic inflammation, gut tissue damage, and diarrhea (Humphrey et al. 2014). In contrast, four combined and eight individual chicken genotypes showed no difference or negative effect on *C. jejuni* colonization and proliferation regardless of chicken growth rate or breed (Gormley et al. 2014).

Several factors affect chicken colonization by *Campylobacter*. These include chicken strain, *Campylobacter* strain, dosage of viable *Campylobacter* cells, and seasonality (Newell and Fearnley 2003). Colonization potential of chickens by some *Campylobacter* strains could be enhanced by 1,000-folds (Ringoir and Korolik 2003) or 10,000-folds (Cawthraw et al. 1996) under *in-vivo* experimental conditions, leading to the challenges to predict the ability of *Campylobacter* wild strains to colonize chicken flocks in the real commercial farms. There is generally a higher rate of colonization in summer than any other time of the year (Humphery, Henley, and Lanning 1993). The colonization level (Wallace et al. 1997) and type of strains (Hudson et al. 1999) are also seasonally dependent. Besides high temperature and humidity, poultry houses require more ventilation during summer, which exposes the birds to more *Campylobacter* from the outside environment than any other time of the year. Even individually caged birds showed a seasonal variation (increased to the peak in late April) in the fecal excretion of *C. jejuni*, suggesting that

the surrounding temperature affects bird colonization even under limited conditions of *C. jejuni* transmission (MICHAEL P Doyle 1984).

Moreover, geographical locations, flock size, and type of the production systems (*i.e.*, organic or conventional) can also influence the colonization of *Campylobacter* in chicken flocks (Newell and Fearnley 2003). According to a previous study, up to 100% of flock were *Campylobacter*-positive in the case of organic and free-range flocks (Heuer et al. 2001). This is probably due to the exposure to the outside environment and a longer time the birds require to grow to the slaughter size compared to the indoor reared flocks. In the cases where the colonization of *Campylobacter* identified at species level, *C. jejuni* was leading group by colonizing about 90% of *Campylobacter*-positive birds. The remaining ones were almost equally colonized by *C. coli* and *C. lari* (Uyttendaele et al. 1996). Several studies conducted in Europe suggested that the indoor-grown flocks were primarily colonized by one or two *C. jejuni* strains. Other studies conducted in North America and Australia showed that several *C. jejuni* strains usually colonized the indoor-grown flocks. This might be due to different levels of biosecurity standards in different counters as the incidences of *C. jejuni* colonization can be either due to the exposure to multiple sources consisting of different strains or a single source (*e.g.*, feed or water) consisting of multiple strains. Interestingly, Hald and co-authors reported that *C. jejuni* colonization was higher in a total of 88 randomly selected poultry flocks raised in Danish farms that fed external grains compared to farms that fed home-grown grains (Hald, Wedderkopp, and Madsen 2000).

Another important factor of chicken colonization is the adaptation capability and response of *Campylobacter* strains to the environmental conditions. For example, Gaynor and others identified a remarkable ability of *C. jejuni* to evolve rapidly during storage, culture, and condition passage (Gaynor et al. 2005). The colonization ability of *C. jejuni* 11168-O strain recognized as an excellent chicken colonizer was compared with *C. jejuni* 11168-GS clone recognized as a poor chicken colonizer after either aerobic

or anaerobic incubation. The anaerobic priming of 11168-GS increased its colonization while the aerobic passaging of 11168-O decreased its colonization compared to their original strains.

Some procedures, such as feed withdrawal and transportation, affect the presence of *Campylobacter* in live chickens before their arrival into the poultry processing plants. Feed withdrawal is a common commercial practice that the farmers remove the animal feeds from poultry houses 3-18 h before slaughtering (J. A. Byrd et al. 1998). The purpose of this practice is to clear the gastrointestinal tract and reduce the level of fecal materials in the body so as to minimize cross-contamination during poultry processing. Byrd and co-authors showed that feed withdrawal could increase the prevalence of *Campylobacter* in the crops of broiler chickens at the slaughter age (J. A. Byrd et al. 1998). *Campylobacter*-positive samples increased on average from 25% to 62.4% before and after feed withdrawal. The limitation of nutrients in the broiler crops might have resulted in a less diverse and competitive microbiota and subsequently enhance the growth of *Campylobacter*. Transportation from farms to processing plants has been identified as a critical harbor for the transmission and colonization of *Campylobacter* in live birds. This is due to the reuse of contaminated crates for shipping, animal hoarding, and induced-stress during the transportation of live birds from different flocks and/or farms to slaughterhouses (Slader et al. 2002; Newell and Fearnley 2003). Decontamination methods used for cleaning the reusable shipping crates for transportation was identified to be ineffective (Wedderkopp et al. 2001). Up to 70% *C. jejuni*-negative chickens became colonized after exposure to artificially contaminated shipping crates (Clark and Bueschkens 1988). Whyte and others demonstrated that poultry overcrowding and stress that induced during transportation could extensively increase the shedding of *Campylobacter* in fecal material of broilers and contributed to cross-contamination of their carcasses during processing (Whyte et al. 2001).

Cecal microbiota of broiler chickens changes corresponding to *C. jejuni* colonization regardless of the biological, environmental and technical variations (Awad et al. 2016; Ijaz et al. 2018; Connerton et al. 2018), indicating that *C. jejuni* acts as a commensal and a super colonizer in chicken cecal microbiota. Awad and co-authors identified that the microbial communities in the luminal and mucosa gut microbiome shifted in a timely manner during the growth of broiler chickens (Awad et al. 2016). A similar finding was observed when the chicken cecal microbiome was analyzed even without any artificial inoculation of *C. jejuni* (Ijaz et al. 2018). The critical periods for *C. jejuni* colonization ranged from 12 to 28 d of the broiler chicken age (Awad et al. 2016; Ijaz et al. 2018). Another report showed that *C. jejuni* appeared in 6-day old chicken birds (Connerton et al. 2018). The microbiota variation is usually influenced by the diet and microorganisms present in the surrounding environment, feed, and water (Connerton et al. 2018). For all of these reasons, it is quite challenging to inactivate *C. jejuni* once broiler chickens are colonized. Therefore, early prevention of *C. jejuni* colonization on poultry farms is very important to avoid further colonization. Although no symptom is associated with colonization of *C. jejuni* and *C. coli* in the broiler chickens, the high mortality rate might reflect the colonization prevalence (Powell et al. 2012). It was worth mentioning that no linear trend was observed between the mortality rate and *Campylobacter* colonization. This might be due to the high use of prophylactic antibiotics to reduce the mortality rate, but at the same time could promote the colonization and prevalence of antibiotic-resistant *Campylobacter* (Phillips et al. 2004). Taken together, both banning antibiotic use and developing alternative intervention strategies are required to have *Campylobacter*-free and healthy poultry on farms.

1.2.3 On-farm intervention strategies against *Campylobacter*

The early stage on-farm control of *Campylobacter* in broiler chickens has gained increasing attention during the last two decades because *Campylobacter* can effectively colonize chickens from the

early days of their lives and remain prevalent at a high level throughout the poultry processing line (**Table 1.2**). The potential of different intervention strategies by using vaccination, phage therapy, bacteriocins, probiotics, fatty acids, and essential oils has been investigated (**Table 1.3**). Each strategy has some advantages and disadvantages as summarized in **Table 1.3**. For example, numerous bacteriocins (antimicrobial peptides) produced by commensal bacteria from chicken gut microbiota, such as *Lactobacillus salivarius*, could effectively inactivate *Campylobacter* under both *in-vitro* and *in-vivo* experimental settings (Svetoch and Stern 2010). Specifically, the L-1077 bacteriocin was able to reduce >4 log CFU/g of *C. jejuni* in the cecal content. In a recent study, oral administration of three types of bacteriocins from *Lactobacillus salivarius* (OR-7) and *Enterococcus faecium* (E-760 and E50-52) were used in broiler chickens to investigate the development of resistance by *C. jejuni* (Mavri and Smole Možina 2013). CmeABC multidrug efflux pump in *C. jejuni* played an important role in intrinsic and acquired resistance against bacteriocins. Thus, combining bacteriocins with an efflux pump inhibitor might synergistically inactivate *C. jejuni* and prevent the development of antimicrobial resistance.

Table 1.2. Examples of the average prevalence and load of *Campylobacter* throughout the poultry processing chain.

Stage	Source	Prevalence and/or load of *Campylobacter*	Reference
Farm	Broilers	87.5%, log 9 CFU/g of cecal content (*n* = 50)	(Stern et al. 2001)
	Feces	96.4%, 5.16 CFU/g of fecal content (*n* = 948)	(Stern and Robach 2003)
Transportation	Caecum	Log 6.5 CFU/g of cecal content	(Achen, Morishita, and Ley 1998)
	Feces	60 - 100% (*n* = 7 [10 flocks])	(Whyte et al. 2001)
Plant	Pre-scald	77%, >log 6 CFU/g of feather or skin (*n* = 40)	(Kotula and Pandya 1995)
	Defeathering	3.9 log 6 CFU/mL of carcasses rinse (*n* = 24)	(Berrang, Dickens, and Musgrove 2000)
	Evisceration	96-100%, log 2.7 CFU/carcass (*n* = 48)	(Northcutt et al. 2003)
	Pre-chill	98%, 4.75 CFU/mL of carcasses rinse (*n* = 450)	(Stern and Robach 2003)
	Post-chill	84.7%, 3.03 CFU/mL of carcasses rinse (*n* = 450)	(Stern and Robach 2003)
	Pre-wash	87%, log 4.78 CFU/mL of carcasses rinse (*n* = 30 [4 processing plants])	(Bashor et al. 2004)
	Post-wash	80%, log 4.30 CFU/mL of carcasses rinse (*n* = 30 [4 processing plants])	(Bashor et al. 2004)
Retail		90%, >log 4 CFU/carcass (*n* = 552)	(Walker et al. 2019)

Table 1.3. Advantages and disadvantages of different antimicrobial strategies against *Campylobacter* in poultry production.

Stage	Strategies	Advantage	Disadvantage
Farm	Vaccination	Preventive and promising (Neal-McKinney et al. 2014; Annamalai et al. 2013)	Expensive, highly specific, and difficult (Kaakoush et al. 2015; Saxena et al. 2013)
	Bacteriophages	Caused up to 5 log CFU/g reduction of *C. jejuni* in cecal content of commercial broiler flocks (Kittler et al. 2013)	Dilution in the gut over the time and development of resistance (Fischer et al. 2013; Labrie, Samson, and Moineau 2010)
	Bacteriocins	Caused >4 log CFU/g reduction of *C. jejuni* under *in-vitro* settings (Svetoch and Stern 2010)	Development of antimicrobial resistance by the multidrug efflux pump CmeABC (Mavri and Smole Možina 2013)
	Probiotics	Part of the chicken gut microbiota (Kaakoush et al. 2015)	Limited reduction of *C. jejuni* after 15 d of oral administration (Santini et al. 2010)
	Short chain fatty acids	Ability to invade the gut epithelium cells (Davidson, Sofos, and Branen 2005)	Limited reduction of *C. jejuni* under *in-vitro* settings (Davidson, Sofos, and Branen 2005)

1.3 *Campylobacter* in poultry processing plants

1.3.1 Overview

Poultry meat and eggs are important sources of dietary proteins, vitamins, and minerals. Poultry production is an intensively growing industry and chicken meat is one of the most produced meats around the world (**Figure 1.1**) (Ritchie and Roser 2017). The annual global amount of produced poultry meats has been rising by 10-folds within the last 50 years to approximately 102 million tons (**Figure 1.2**) (Ritchie and Roser 2017). Chicken is also one of the most sustainable major sources of dietary proteins as the feed conversion ratio (FCR; kg of feed/kg of edible weight) of chicken meat is only about 40% of the FCR of beef (Wilkinson 2011). As a large, diverse and vertically integrated system involving animal farming and food processing, poultry production can be a common source of foodborne outbreaks. Either live poultry or poultry meat are important sources of *Campylobacter* and other important foodborne pathogens (Kaakoush et al. 2015). Both on-farm and *in vivo Campylobacter* controls are challenging due to the complexity and diversity of both systems (**Table 1.3**). Alternatively, many studies have focused on controlling *Campylobacter* in the processing facilities.

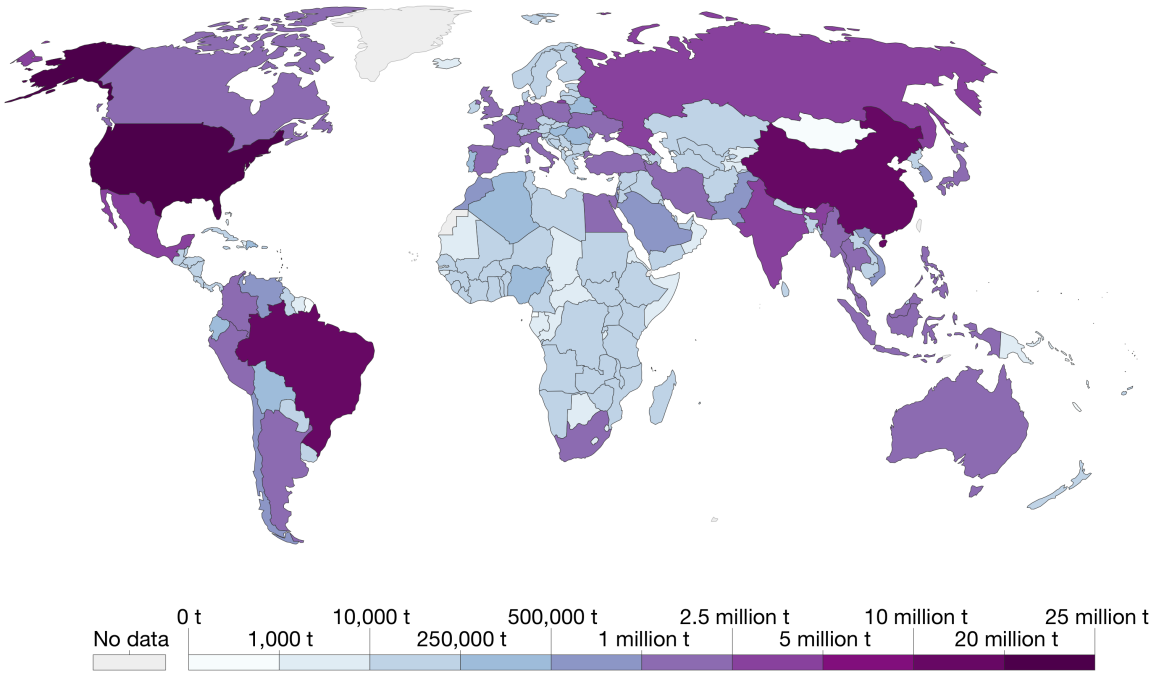


Figure 1.1. World map of poultry production (Ritchie and Roser 2017)**.**

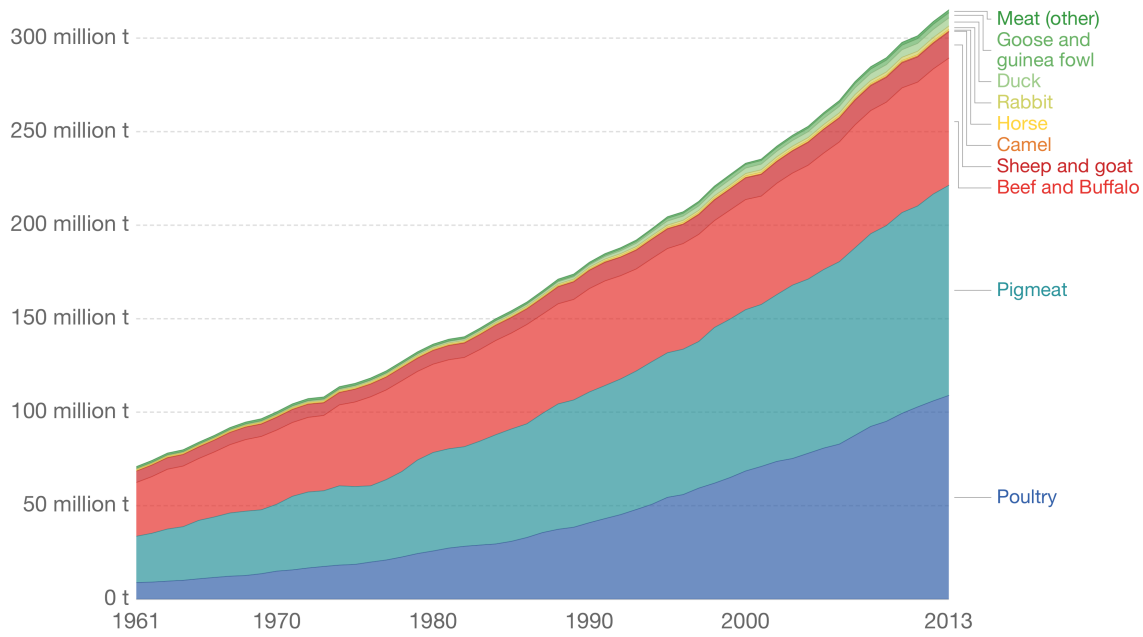


Figure 1.2.Global meat production of meats by different livestocks (Ritchie and Roser 2017)**.**

1.3.2 *Campylobacter* survival during poultry processing

Poultry processing is considered as an intensive procedure that requires highly trained personnel. One breach in sanitation or hygiene practices might end in several cases of foodborne illnesses. *Campylobacter* enters a processing plant through any potentially contaminated bird(s) at an initial count as many as 10^9 cells/g of cecal content (Beery, Hugdahl, and Doyle 1988; Stern et al. 2001). A single processing plant normally receives birds from multiple farms with variations in their ages, sizes, geographical locations, and production and biosecurity systems that increase the chance of *Campylobacter* contamination. Birds go through different processing steps starting from receiving and hanging until packaging. Processing consists of multiple critical points where *Campylobacter* starts to occur or increase in chicken carcasses (**Table 1.2**). Steps including scalding, defeathering, evisceration, nick removal, inside and outside (or inside-out) washing can all contribute to cross-contamination of *Campylobacter* in one way or another.

Scalding is a quick immersing of poultry carcasses into warm water (51-64°C) for a few seconds up to 2 min so as to loosen the skin follicles for defeathering. Berrang and others investigated the level of *Campylobacter*, total aerobic microbes, *E. coli*, and coliform throughout the poultry processing plant (Berrang and Dickens 2000). Total aerobic bacteria clearly decreased throughout the processing steps. In contrast, *Campylobacter* showed the highest recovery (increased from log 1.8 to 3.7 CFU/mL of chicken carcass rinse) compared to all the other bacteria after defeathering. Heating of poultry carcasses followed by chilling during the processing steps are essential in assisting practices for effective defeathering. However, this temperature fluctuation creates several challenges to control microorganisms including *Campylobacter*. For example, skin follicles remain open after scalding that allows bacteria to penetrate the skin and accumulate inside the follicles. Moreover, the follicles close again during chilling, making the poultry decontamination to be highly challenging.

In addition, a large shift appears in the native skin microbiome of chicken (C. J. Thomas and McMeekin 1980). The predominately Gram-positive skin microbiota (*e.g.*, *Micrococcus*) is usually detached and replaced by a population mixture consisting of a majority of Gram-negative bacteria. However, the alteration of chicken meat microbiome during processing varies based on multiple factors, including geographical location, season, and bird-to-bird. One common factor is that scalding liquidizes some fats on the skin that became part of chicken juice and other surrounding fluids. The liquid fat solidifies again during the chilling step and creates a lipid film on the surface of chicken meat. Both scanning electron microscopy and transmission electron microscopy showed that scalding and defeathering scraped off the epidermis cells of chicken skin that became smoother and less hydrophobic than normal after processing (C. J. Thomas and McMeekin 1980). The bacterial contaminants were identified within a protective fluid film formed

both on the surface and inside deep channels of chicken skin after chilling, which increases the presentence of microorganisms in chicken meat to be inactivated by antimicrobial agents during processing.

Moreover, evisceration is one of the most critical steps of cross-contamination. Colonized gastrointestinal tract of poultry birds carries a large number of *Campylobacter* bacteria that can spread in a wide range, especially in the case of gut leakage. Many in-plant studies confirmed that the number of *Campylobacter*-positive carcasses significantly increased after this process (Keener et al. 2004; Northcutt et al. 2003; Berrang, Ladely, and Buhr 2001). For example, *Campylobacter*-positive chicken thighs and breasts separately increased from 0 to 90% at a level between 2 and 3 log CFU/g after evisceration (Berrang, Ladely, and Buhr 2001). Another study identified that *Campylobacter* contamination level was higher on the breast meat than the thigh meat or drumstick (Kotula and Pandya 1995). Leaking of *Campylobacter* from the gut during evisceration can contaminate the lower half of the carcasses (breast and neck) more than the upper half (thighs and drumstick) as the birds are always hanged upside-down by the feet. The hanging necks of carcasses were also frequently determined to be *Campylobacter*-positive (Kotula and Pandya 1995).

Poultry carcasses require rapid cooling to prevent the growth of microorganisms after evisceration. Chilling and antimicrobial treatment are usually combined in many processing plants to save energy and rapidly inhibit bacterial growth by washing the carcasses with cold chlorinated water (Keener et al. 2004). Poultry carcasses are usually washed by dipping or spraying using chlorinated water to remove blood, tissue, fragments, and contamination after evisceration. Dipping can cross-contaminate carcasses under commercial processing conditions especially when processing a large number of birds at the time (Demirok et al. 2013; Bilgili et al. 2002; Bailey, Thomson, and Cox 1987). In contrast, spray washers tend to reduce the level of cross-

contamination on the chicken meat (Demirok et al. 2013; Keener et al. 2004). Several options of spraying systems for poultry carcasses are used in poultry industry that include brush, cabinet, and inside-out washing systems (Keener et al. 2004). The brush washing system is similar to a car washer where many rubber fingers are used with the aid of water to remove debris and wash the carcasses from the outside. The cabinet washing system contains multiple sprayers in an enclosed system to wash the outside of the carcasses. Inside-out system is a similar enclosed spraying system, but used for both external and internal washing at the same time. The machine rotates the carcasses and sprays them from the outside, while probes of single sprayers enter the intestinal cavities of carcasses to wash them from the inside. Many inside-out washing machines spray water at the pressure level between 40 to 180 psi to remove visible fecal contamination and fragments. Chlorine concentration ranged from 20 to 50 mg/L and water consumption ranged from 100 to 200 liter/min.

1.3.3 Antimicrobial treatments for poultry processing

Many laboratory-scale experiments showed that the approved antimicrobials such as acidified sodium, chlorite, cetylpyridinium, chlorine, chlorine dioxide, peroxyacetic acid, and trisodium phosphate could cause up to 5 log reduction of *Campylobacter* in chicken meat (**Table 1.3**). However, in-plant poultry washers have limited effect on inactivating *Campylobacter* in chicken meat regardless of the efficacy of antimicrobials, water temperature, or washing system (**Table 1.3**). This could be due to several factors including the presence of large molecules in chicken meat (*e.g.*, proteins and lipids) and *Campylobacter* in chicken skin due to changes induced by processing, sensitivity of chicken skin to heat, oxidation and discoloration, initial microbial load of carcasses, number of processed carcasses per min, interaction or masking of antimicrobials

(*e.g.*, chlorine) by organic materials in the processing water, water quality and survival of *Campylobacter* in recycled processing water, poor sanitation, accumulation of lipids, fecal materials, and/or organics at any point through the processing line. It is worth mentioning that there is no effective critical control (*i.e.*, killing) point in processing raw chicken similar to that of the pasteurization step for milk processing (Tresse, Alvarez-Ordóñez, and Connerton 2017).

1.3.4 Current situation of raw poultry product safety

The prevalence of *Campylobacter* in poultry products is clearly a major food safety challenge for many years. It is important to target chicken as a critical food vehicle of *C. jejuni* due to the high rate of contamination. More on-farm and in-plant control strategies became available in the recent years, but these strategies need improvement to enable effective inactivation of *Campylobacter* at an early stage or in chicken end-products. In 2015, the United State Department of Agriculture, the Food Safety and Inspection Service agency (USDA-FSIS) established a new *Campylobacter* and *Salmonella* performance standard for the contaminated poultry products, raw chicken parts (*e.g.*, breasts, thighs, wings), and not ready-to-eat (NRTE) poultry products (Crim et al. 2015). For example, 8 out of 51 *Campylobacter*-positive broiler carcasses is the maximum acceptable number of randomly tested samples. In 2018, new antimicrobial agents have been approved by the USDA-FSIS to be used in washing poultry carcasses during processing. These include peroxyacetic acid (a mixture of hydrogen peroxide and acetic acid), a mixture of calcium chloride, calcium hypochlorite, sodium chloride, calcium hydroxide, calcium carbonate, sodium triphosphate, and a combination of calcium chloride with sodium bisulfate (F. safety and inspection Service 2018). Although *Campylobacter* can be reduced to some extent by antimicrobials, they still might not be reduced to a safe level as only a few

hundred cells might cause human illnesses (Hara-Kudo and Takatori 2011; Black et al. 1988). In addition, in-plant antimicrobial treatment requires intensive amount of water to wash chicken carcasses. For example, a medium size poultry processing plant spends annually $0.5-1 million USD on average on water consumption for washing chicken carcasses and surfaces (W. C. Jackson 1999), but *Campylobacter* reduction is still insufficient.

1.4 Alternative strategies to control *C. jejuni* in agri-foods

1.4.1 Plant-based antimicrobials

Plant-derived compounds have been used for centuries in medicine, perfumery, cosmetics or being added to foods as oils, herbs or spices (Hyldgaard, Mygind, and Meyer 2012). For example, herbs and essential oils were initially used in medicine due to their antimicrobial, anti-inflammatory, or antioxidant effects, then their application expanded in agri-foods in the 19th century for their aroma and flavors. These antimicrobials are important secondary metabolites that play major roles in plant defense systems to protect them from microbial infections (Tajkarimi, Ibrahim, and Cliver 2010). It was estimated that ~3,000 essential oils have been identified and ~300 are commercially available for flavoring, fragments, or cosmetics (Van de Braak and Leijten 1999). In addition, essential oils can act as growth promoters in farm animals similar to antibiotics (Ahmadifar, Falahatkar, and Akrami 2011; Brenes and Roura 2010). A histology study showed that feeding different plant extracts to chicken broilers increased the thickness of the mucus layer in the glandular stomach and jejunum (Jamroz et al. 2006). These changes were associated with a large shift in gut microbiota that could hypothetically promote the growth of birds.

Cinnamon is one of the earliest spices used in human history and cinnamon oil is among the most studied essential oils due to its high antimicrobial potency (Ravindran, Nirmal-Babu, and

Shylaja 2003). The genus *Cinnamomum* consists of ~250 different species. *C. verum* and *C. cassia* are the most known and used herbal medicines or spices. These plants are the main natural sources of cinnamon. Cinnamon oil consists of several major antimicrobial compounds, including cinnamaldehyde (70-90%), 1-linalool, p-cymene, and eugenol (Davidson, Sofos, and Branen 2005). Aldehyde groups are reactive organic compounds that can crosslink covalently with proteins and nucleic acids through amine groups. Therefore, the mode of action of cinnamaldehyde is inconclusive. Several mechanisms can occur depending on the bioavailability and concentration of the system (Hyldgaard, Mygind, and Meyer 2012). For example, cytokinesis and less important functions can be inhibited due to the inhibition of different enzymes by cinnamaldehyde at a low concentration. ATPase inhibition occurs at the sub-lethal concentration, while the alteration of fatty acid composition of cell membrane, cell leakage and cell death occur at the lethal concentration.

In comparison, curcumin is the major active compound of the rhizome of turmeric (*Curcuma longa*). This golden spice is a phenolic pigment responsible for the yellow color of turmeric. Numerous studies have shown that curcumin can effectively inactivate both Gram-negative and Gram-positive bacteria (Tyagi et al. 2015; Kaur et al. 2010; Rudrappa and Bais 2008). However, limited studies have investigated the antimicrobial mechanism of curcumin. Blocking the assembly of the FtsZ protein essential for forming the FtsZ ring (*i.e*, Z ring) to initiate cell division in bacteria was identified to be the mode of action against *Bacillus subtilis* and *E. coli* (Kaur et al. 2010). In contrast, curcumin has been found to attenuate several virulence factors, including quorum sensing and biofilm formation in *P. aeruginosa* (Rudrappa and Bais 2008). A recent study examined the membrane permeability of *S. aureus, Enterococcus faecalis*, *E. coli* and *P. aeruginosa* after being treated with curcumin (Tyagi et al. 2015). A steady-state fluorescence

and flow cytometry analyses showed uptake in the extracellular propidium iodide (only enters intact bacterial cells by a permeabilizing agent) and leakage of calcein (only leak out of bacterial cells if there is membrane damage due to cell wall membrane damage) in both Gram-positive Gram-negative bacteria. Antimicrobial mechanism of curcumin is different depending on the bacteria studied and the assays used (Tyagi et al. 2015; Han et al. 2006; De et al. 2009; Kaur et al. 2010). More studies are still needed to confirm the antimicrobial mechanism(s) of the action of curcumin.

To the best of our knowledge, the specific antimicrobial mechanism of curcumin against *Campylobacter* has not been investigated. The effect of curcumin against *Helicobacter pylori*, a highly relevant bacterium to *Campylobacter*, has been repeatedly confirmed in several studies (Sarkar, De, and Mukhopadhyay 2016; Zaidi et al. 2009; Vetvicka, Vetvickova, and Fernandez-Botran 2016; Di Mario et al. 2007). One study that used a high-throughput screening of 5,000 chemical compounds discovered that the inhibition of *H. pylori* by curcumin was due to the inhibition of shikimate pathway (Han et al. 2006). This pathway is essential for the synthesis of aromatic amino acids (*e.g.*, phenylalanine, tryptophan, and tyrosine) in bacteria, fungi, and higher plants, but not in mammals. Targeting this particular pathway makes curcumin a very safe antimicrobial agent for human consumption. In fact, curcumin showed no toxicity on human health even used at a level as high as 8,000 mg per day (Hsieh et al., 2001; Lao et al. 2006). In addition, the antimicrobial activity of curcumin against *H. pylori in-vitro* (65 clinical isolates) and *in-vivo* during infections in mice were examined. The minimum inhibitory concentration (MIC) of curcumin ranged from 5-50 μg/mL regardless of genetic variation of the tested *Helicobacter* strains. Curcumin not only inactivated *H. pylori* during infection but also reduced the gastric damage induced by *H. pylori* infection to almost a normal state. Although limited studies have

identified the antimicrobial mechanism of curcumin, available evidence shows its great potential for preventing and treating bacterial contaminations and infections.

1.4.2 Metal oxide nanoparticles

Novel applications of nanotechnology and nanomaterials have gained great attention in the recent years. For example, the applications of metal oxide nanoparticles (*e.g.*, Al_2O_3, TiO_2, and ZnO NPs) could inactivate several foodborne pathogens in a variety of agri-food systems (Panea et al. 2014; Fernández et al. 2009; Akbar and Anal 2014). ZnO was identified to be more effective than other metal oxides (*e.g.*, CuO and Fe_2O_3) against both Gram-negative and Gram-positive bacteria (Azam et al. 2012). In addition, ZnO NPs was more effective against *C. jejuni* than other Gram-negative bacteria including *E. coli* O157:H7 and *S. enterica* (Xie et al. 2011). The direct contact of ZnO NPs (positively charged) with bacterial cell wall (negatively charged) by electrostatic force leads to destabilization and disruption of bacterial outer cell membrane. In addition, semi-conductive property of ZnO allows the generation of reactive oxygen species that can attack different cytoplasmic and extra-cytoplasmic targets after the binding (Sirelkhatim et al. 2015).

1.4.3 Synergism

1.4.3.1 Overview

Antimicrobial combinations have been used since the earliest days of the recorded history to treat illnesses and reduce sufferings (Chou 2006). Therapeutic use of the traditional Chinese herbs is a prime example. Indeed, antibiotic is one of the most important drug discoveries in the modern medicine. However, the emergence of antibiotic resistance to most available antibiotics

became a serious public health concern in the recent years and near future (de Kraker, Stewardson, and Harbarth 2016). A synergistic combination of antimicrobials can minimize some of the disadvantages associated with the use of antimicrobials, such as the development of bacterial resistance, high dosage, and limited effect (Chou 2006). Antimicrobial combination has been extensively studied to inactivate some highly challenging bacterial and viral infections including methicillin-resistant *Staphylococcus aureus* (MRSA) infection (An et al. 2011) and human immunodeficiency virus (HIV) infection (Gaibani et al. 2019).

Synergism is defined as an effect that is greater than the sum of multiple individual effects. Many approaches, hypotheses, methodologies, and models have been used to study the synergism in different fields, including microbiology, pharmacology and enzymology (Chou 2006). The definition of synergism is a very controversial topic due to the complexity of biological systems and some possible mathematical errors or pitfalls in the combinatorial studies. Some important concepts such as the difference between synergism and enhancement or potentiation are not fully clear. For example, if antimicrobial A has a quantifiable effect (*e.g.*, 10%), while antimicrobial B has no effect (*i.e.*, 0%), and their combination produces an effect greater than antimicrobial A (*e.g.*, 20%), then this is considered as an enhancement or potentiation, but not a synergistic interaction. In contrast, synergism is an effect greater than the sum of multiple quantifiable effects (*e.g.*, 10% + 10% = 30%). In addition, the additive effect has always to be less than 100%. For example, if antimicrobial A and B each affects 20%, the additive effect is not simply 40% because if each antimicrobial produces 70% effect the combined effect cannot be 140%. Chou and Talalay reported the fractional product equation to solve this issue [(1 - 0.7) (1 – 0.7) = 0.09] where the additive effect can never exceed 100% (Chou and Talalay 1984).

1.4.3.2 Methods for identifying antimicrobial synergism

Three methods are most used in antimicrobial combination studies. These include the disk diffusion method, time killing method and fractional inhibitory concentration index method (FICI) (Odds 2003; Zhou et al. 2016). Disk diffusion method is a simple visual test that relies on comparing bacterial inhibition zones of diffused (single and combined) antimicrobial agents in the agar plates. Time killing method shows how a bacterial population responds to the antimicrobial treatment at different time intervals in either broth or agar medium. It relies on monitoring the antimicrobial effect of single and combined antimicrobials by calculating the log reduction of lethal and sub-lethal concentrations over time. For example, if antimicrobial A caused 1 log reduction and antimicrobial B caused 1 log reduction, then the additive effect would be 2 log reduction. In this case, synergism would require effect greater than 2 log reduction (*e.g.*, 1 + 1 = 3). This method is labor-intensive and time-consuming. Thus, a few concentrations of antimicrobials are usually used and combined at a fixed ratio. In contrast, the FICI method (also called microdilution checkerboard) shows a clear visualization of positive/negative inhibitory interactions of multiple ratios of combined antimicrobials. It relies on constructing two antimicrobial combinations in a two-dimension array (*e.g.*, 96-well plate) and comparing the MICs of single and combined treatments. Synergy requires at least a four-fold reduction in the MIC of both antimicrobials combined (*i.e.*, FICI value of ≤ 0.5). The FICI method gained more popularity in the recent years as it is more restricted in identifying synergism, more comprehensive, and easier to construct and interpret than other methods.

1.4.3.3 Types and mechanisms of antimicrobial synergism

Different types of antimicrobial interactions can occur between antimicrobials depending on their origins and individual mechanisms. It is common to observe synergism between antimicrobials of different mechanisms and different targets (Oh, McMullen, and Jeon 2015; Jia et al. 2009). For example, combining efflux pump inhibitor(s) with an intracellular antimicrobial(s) can synergize to inactivate microorganisms that use efflux pumps to remove antimicrobials due to antimicrobial accumulation inside the cells (Oh, McMullen, and Jeon 2015). In addition, antimicrobials may synergize due to the complementary or facilitating collective actions (Jia et al. 2009). Although different antimicrobials may have different targets and mechanisms, they might have overlapping pathways at the molecular level. More importantly, synergism can be used to increase bacterial antibiotic susceptibility.

1.4.3.4 Applications

Bacteria develop resistance to antibiotics via different mechanisms (Mavri and Smole Možina 2013). These include the modification of a receptor or active site of the antibiotic target to prevent or reduce binding, production of enzymes that directly destruct or modify the antibiotics, and/or reducing the accumulation inside the cells by decreasing the outer cell membrane permeability or pumping out the antibiotics using efflux pumps. One of the best applications for antimicrobial synergism is to be against tolerant and/or resistant pathogens that require more than single or additive treatments. For example, Augmetin® is a common commercial antibiotic that consists of a combination of clavulanate acid and amoxicillin to inactivate different pathogens, including *β*-lactam resistant bacteria (12). The combination of clarithromycin and amoxicillin is part of the standard therapy for *H. pylori* stomach infections (11).

Plant-based antimicrobials are a great source of new alternative antimicrobials. Many recent studies showed that plant-based antimicrobials (*e.g.*, phenolic compounds) synergize with antibiotics (*e.g.*, amikacin, ceftriaxone, cephradine, methicillin, imipenem) (Oh and Jeon 2015) or metal oxide nanoparticles (Hemaiswarya and Doble 2010) against various microbes. Oh and Jeon reported synergistic interactions of several phenolic compounds (*e.g.*, gallic acid and taxifolin) in combination with ciprofloxacin or erythromycin against fluoroquinolones- and macrolides-resistant *C. jejuni* isolates (Oh and Jeon 2015). Phenolic compounds increased membrane permeability as determined by measuring the intracellular uptake of 1-*N*-phenylethylamine. As a result, accumulation of both antibiotics increased substantially inside the bacterial cells. Further testing showed that phenolic compounds increased 1-*N*-phenylethylamine accumulation in an isogenic (knockout) *cmeB* mutant more than that in a wild type *C. jejuni* strain. In addition, the expression level of CmeABC multidrug efflux pump was reduced by several phenolic compounds (*i.e.*, gallic acid and taxifolin). These findings indicated that phenolic compounds increased the influx rate and decreased the efflux rate of antibiotics.

1.4.4 Active packaging

1.4.4.1 Overview

Food packaging is one of the last steps in food processing to ensure that the food products are contained and delivered in the best condition. Packaging materials and/or the atmospheric condition inside the packaging are used to protect the foods from microbial growth, pathogen contamination, physical damage, chemical degradation, or other effects from the environment. Most of the commercially applied packaging technologies aim to preserve food quality and extend the shelf life of food products. Moreover, data regarding the use of active packaging to control

foodborne pathogens in potentially contaminated and high-risk foods is still limited compared to those for spoilage bacteria. For example, modified atmospheric packaging (MAP) was extensively studied for the control of spoilage microorganisms in a variety of food products, such as raw meats, fresh produce, and seafood products (McMillin 2008). In addition, recent technologies and intervention strategies that are used in food processing allow food packaging to be a suitable component of hurdle technology. Such approach might overcome the challenge of controlling the survival of frequently isolated pathogens from commercially available raw meats, such as *C. jejuni* in chicken, *V. parahaemolyticus* in seafood, and pathogenic *E. coli* in beef.

Fresh poultry, raw meats, and seafoods are considered as high-risk and highly perishable foods. Foods of animal origins including raw milk, raw cheese, and raw meats have a high content of moisture and nutrients. These factors form an ideal environment for rapid growth and/or long survival of many microorganisms, including both spoilage and pathogenic bacteria. Deferent bacterial have been commonly isolated form fresh chicken meats after processing. These include *Micrococcus*, Gram-positive rods*, Cytophaga-Flavobacterium*, *Pseudomonas,* and Enterobacteriaceae (C. J. Thomas and McMeekin 1980). Thomas and McMeekin identified that poultry carcasses originally carried *Micrococcus* as a part of the skin microbiome, but contamination with the psychrotrophic *Pseudomonas* appeared after processing (C. J. Thomas and McMeekin 1980). A whole-genome sequencing analysis showed that the Firmicutes (mainly Gram-positive) were the most abundant bacterial group based on the phyla level of raw chicken meat after processing (Kim et al. 2017).

Campylobacter and *Salmonella* are the most frequently isolated human pathogens from poultry products at the retail level (Rouger, Tresse, and Zagorec 2017). No correlation was established between the prevalence of these two poultry-associated pathogens in chicken carcasses

collected from 58 slaughterhouses during a 12-month period in France (Hue et al. 2011). Moreover, no correlation was established between the microbial load (*i.e*., total aerobic count, Enterobacteriaceae, and coliform) and the prevalence of *Campylobacter* in chicken and turkey fresh meat cuts (Fontanot et al. 2014). Thus, the prevalence of *Campylobacter* in poultry end products is random and unpredictable because transmission in farms and cross-contamination during processing can occur at any point, which is not always associated with any other microbial indicators. The large size of poultry industry and production scale makes the detection of *Campylobacter* more challenging in these products. Both *Campylobacter* and *Salmonella* can originally occur at a high level (up to 10^8 CFU/g) in the gastrointestinal tract of birds, but their prevalence in poultry meat varies depending on the cross-contamination incidents. A positive correlation was identified between *Campylobacter* level in chicken caeca and the end products (Hue et al. 2011). *C. jejuni* and *C. coli* were equally prevalent in chicken caeca, but *C. jejuni* was the most frequently isolated one from processed carcasses. These findings highlight the difficulty of preventing the presence of *C. jejuni* in poultry end products from *Campylobacter*-positive carcasses.

1.4.4.2 Poultry packaging options

Different packaging options are available for fresh poultry meats. Indeed, the packaging cost is one of the most important factors that can affect the decision of choosing a specific packaging type and material by the poultry producers. The most common and economic form of packaging in the retail marketplaces consists of a polystyrene poultry tray, an absorbing pad placed under the meat, and an overwrap clear film (Venkitanarayanan, Thakur, and Ricke 2019). In addition, these poultry trays can be packaged with a stretching barrier film and under MAP.

Aerobic and vacuum packaging of a whole bird with a stretch film are other options. MAP is commonly used as a preservation method to extend the shelf life of perishable foods, such as produce and fresh meat. MAP is defined as the replacement or removal of the atmospheric air surrounding the food before sealing in vapor-barrier materials (McMillin 2008). The ratio of N_2, CO_2, and O_2 are usually controlled in MAP based on food itself, quality characteristics, and aimed shelf life. One of the most important functions of this combination of gases is to form unfavorable atmosphere and inhibit the growth of many microorganisms so as to delay food spoilage. Regarding specific role of each gaseous component, N_2 is used as a filler when a large amount of oxygen is removed while CO_2 acts as an antimicrobial agent at high concentrations to inactivate microorganisms and oxygen is the most important gas influencing the microbial growth, quality characteristics and shelf life of foods (Venkitanarayanan, Thakur, and Ricke 2019). One way to evaluate the effectiveness of MAP is to measure the available CO_2 dissolved in foods. This result in the formation of carbonic acid that penetrates the bacterial cells and leads to a decrease in intracellular pH and metabolic activities. In addition, carbonic acid also inhibits the decarboxylating and non-decarboxylating enzymes and alters some of the membrane components affecting the membrane functionality. Although many spoilage bacteria can be inhibited by MAP, it supports and enhances the survival of *Campylobacter* due to the elevated level of CO_2 in the local microenvironment (Venkitanarayanan, Thakur, and Ricke 2019).

1.5 Research rationale and hypotheses

Campylobacter jejuni is one of the leading foodborne pathogens responsible for human gastroenteritis. No effective control method is available to prevent *Campylobacter* contamination either in poultry farms (Wagenaar, French, and Havelaar 2013) or poultry processing plants

(Oyarzabal 2005). Both of poultry farms and processing systems are complex and require intensive operations. In addition, the use of antibiotics has been banned as growth promoters so as to limit antibiotic resistance in different countries (Union 2003; Government of Canada 2018). The efficacy of many approved antimicrobials on the reduction of *Campylobacter* in poultry processing plants is also quite limited (Oyarzabal 2005; Capita et al. 2002). For all of these aforementioned factors, new generations of antimicrobial agents including novel synergistic antimicrobial combination is required for *Campylobacter* control and prevention in the agro-ecosystem. In Chapter 2, we hypothesized that combining plant-based antimicrobials with each other or with metal oxide nanoparticles could induce synergistic interactions against *C. jejuni*. Former studies demonstrated that the majority of the published studies between 1999-2015 on the synergetic effects of Chinese herbal medicine including antimicrobial combinations did not apply rigid mathematical methods (Zhou et al. 2016). Currently, there is no standard method to investigate antimicrobial interactions. Over 60% of dual antimicrobial studies used the FICI method and 36% applied the time-killing method during the past decade (Odds 2003). Each method has advantages and disadvantages, and generates incomparable outcomes (Rand et al. 1993; Odds 2003; Chou 2006; Noll et al. 2012; Zhou et al. 2016).

Knowledge of molecular mechanisms of synergistic combinations are important to define their best applications and provides strategies to discover other novel antimicrobial combinations. In Chapter 3, we hypothesized that each antimicrobial played a different role in the synergistic combinations. We will apply whole transcriptomic sequencing techniques to comprehensively investigate the synergistic antimicrobial mechanism against *C. jejuni* using the aforementioned antimicrobial agents.

Considering the challenges in controlling *C. jejuni* in poultry farms and processing plants, we aimed to develop an innovative antimicrobial packaging to reduce *C. jejuni* in raw chicken meat at the retail level. This novel strategy may offer protection to consumers away from *Campylobacter*-contaminated poultry meat at the final stage of food chain regardless of the initial contamination of poultry at farms and/or in the processing plants. In Chapter 4, we hypothesized that immobilization of ZnO NPs into the functionalized absorbing pad could inactivate *C. jejuni* without affecting antimicrobial efficacy and potency of ZnO NPs. We also hypothesized that immobilized ZnO NPs would not migrate from the functionalized absorbing pad to raw chicken meat and the release of Zn^{2+} from the immobilized ZnO NPs could contribute to the antimicrobial activity of the functionalized absorbing pad.

Chapter 2: A Novel Mathematical Model for Studying Antimicrobial Interactions against *Campylobacter jejuni*

2.1 Overview

The aim of this study is to investigate the antimicrobial synergistic effect against *Campylobacter jejuni*, a leading foodborne pathogen that causes human gastroenteritis, by cinnamon oil, encapsulated curcumin, and zinc oxide nanoparticles (ZnO NPs). We compared three approaches to study the antimicrobial interactions, including the time-killing method, the fractional inhibitory concentration index (FICI) method, and a mathematical concentration-effect model. Isobologram analysis was performed to evaluate the synergy in different combinations, and a median-effect equation was applied to identify the combinations of synergistic effects at median, bacteriostatic, and bactericidal reduction levels. The time-killing method overestimated the synergistic interaction between antimicrobials, while the FICI method failed to detect an existing synergistic phenomenon. This lack of accuracy and sensitivity was mainly due to combining antimicrobials without a deep understanding of their concentration-effect relationships. Our results showed that each antimicrobial had a unique concentration-effect curve. Specifically, encapsulated curcumin showed a sharp sigmoidal curve unlike cinnamon oil and zinc oxide nanoparticles. A mathematical model was applied to study the interaction between antimicrobials with a different shape of concentration-effect curve. We observed an additive effect of cinnamon oil/ZnO NPs and synergistic interactions of other binary combinations (cinnamon oil/encapsulated curcumin and ZnO NPs/encapsulated curcumin). The tertiary combination of cinnamon oil/ZnO NPs/encapsulated curcumin at IC_{25} (additive line <1-log CFU/mL) presented the greatest synergistic effect by reducing the bacterial population over 8-log CFU/mL. This mathematical

model provided an alternative strategy to develop a new antimicrobial strategy.

2.2 Introduction

Campylobacter is one of the leading bacterial causes of human infectious diseases worldwide. In Canada, this microorganism causes ~145,350 cases of foodborne illness per year (M. K. Thomas et al. 2013). *C. jejuni* is the most common species that accounts for ~80% of campylobacteriosis with a relatively low infectious dose (~500-800 cells) (Nachamkin 2008). *C. jejuni* infections usually lead to nonfatal and self-limiting gastroenteritis, including watery diarrhea, nausea, and vomiting; however, severe autoimmune neurological disorders such as Guillain-Barré syndrome may occur in immunocompromised individuals (Kaakoush et al. 2015). Transmission of *C. jejuni* is commonly through the consumption of meat product (*e.g.*, poultry and beef), raw milk, and/or contaminated drinking water. For all of these reasons, there is an urgent need to develop a new strategy of antimicrobial usage to reduce the prevalence of *Campylobacter* in the environment and agri-food products.

A combination of antimicrobials may target different bacterial sites and lead to synergistic interaction. This may form a novel and effective strategy of antimicrobial usage to reduce the prevalence of *Campylobacter* infections. Synergy is defined as an effect produced by two or more agents greater than the sum of their individual effects combined (*i.e.*, additive effect) (Chou 2006). Synergy requires a lower concentration of each agent to either increase or maintain the antimicrobial effect. Three methods have been used to study the synergism between antimicrobials, including disk diffusion, time-killing, and fractional inhibitory concentration index (FICI) methods (Odds 2003; Zhou et al. 2016). Currently, there is no standard method for studying antimicrobial interactions. Over 60% of the dual antimicrobial studies used the FICI method and

36% applied the time-killing method during the past decade (Odds 2003). Each method has advantages and disadvantages, and generates different outcomes that may not be comparable with each other (Rand et al. 1993; Odds 2003; Chou 2006; Noll et al. 2012; Zhou et al. 2016). For instance, the time-killing method investigates bactericidal effect over time, while the FICI method studies bacteriostatic effect after one time point (*e.g.*, 24 h). Both methods are established on a linear concentration-effect curve of antimicrobials, which can result in either over or under-estimation of interaction(s). According to a recent report, only 40 out of 86 studies published between 1999-2015 used rigid mathematical methods to accurately study the synergetic effects of Chinese herbal medicine (Zhou et al. 2016). Although advanced pharmacological methods are widely used to study drug combination effect in the pharmaceutical and biomedical sciences, few of these studies are related to antimicrobials research (Odds 2003; Mora-Navarro et al. 2015; Zhou et al. 2016).

The isobologram is commonly conducted in drug combination studies to identify and evaluate drug interactions. The combined drugs are assumed to be equally effective, but the dose-response of each combined drug is not always similar (Tallarida 2001). In-depth studies have indicated that even if two drugs have the same effect at the reference concentration (*e.g.*, minimum inhibitory concentration (MIC)), this equivalence may not occur at their sub-concentrations (Tallarida 2001; Chou 2006). Quantitative assessment is therefore essential to identify dose-response of individual drugs and distinguish these situations when the shapes of dose-response curves are not similar. Methods used for the study of drug interactions can be valid only if both drugs have hyperbolic dose-response curves. Drawing an accurate additive line based on every pair of concentrations can overcome this limitation regardless the assay used, shape of dose-response curve, and the level of interaction. Even if an interaction exists, not every pair of

concentration result in the same level of interaction. Additional analysis involving the use of a median-effect equation can provide a more comprehensive understanding of interactions between antimicrobials.

The objective of the current study was to compare the time-killing method and FICI method with a mathematical model and provide an easy and accurate approach to study the effect of dual antimicrobials. We used a nonlinear mathematical concentration-effect model to evaluate the synergistic interactions of three representative antimicrobials (*i.e.,* cinnamon oil, encapsulated curcumin and ZnO NPs) against *C. jejuni* as a foodborne pathogen model. The advantage of using the mathematical model is not only to identify or evaluate the synergy but also to avoid some of the possible mathematical errors and increase the sensitivity to detect an existing synergistic interaction. To the best of our knowledge, this was the first study to apply a mathematical model that could accurately evaluate the antimicrobial interactions at median (50% reduction), bacteriostatic, and bactericidal levels. This approach can be generalized to quantitatively evaluate any type of dual antimicrobial treatment against microorganisms for different applications.

2.3 Material and methods

2.3.1 Chemicals and reagents

Hydrophobically modified starch (HMS) HI-CAP^{TM} 100 was donated from Ingredion Canada Inc. (Mississauga, ON). Cinnamon oil and curcumin purified from turmeric powder were purchased from Sigma-Aldrich (Oakville, ON). A powdered form of ZnO NPs (size: 40-100 nm, surface area: 12-24 m^2/g) was obtained from Alfa Aesar (Haverhill, MA). Acetic acid, acetonitrile, chloroform and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Oakville, ON).

2.3.2 Preparation, extraction and quantification of encapsulated curcumin

To increase the water solubility, curcumin was encapsulated by HMS according to the protocol described in a previous study (Yu and Huang 2010). Briefly, 1.8 g of curcumin was added into 1% (w/v) HMS solution, homogenized at 8,000 rpm for 10 min by using Omni Mixer Homogenizer (Omni, Kennesaw, GA), followed by stirring for 24 h at room temperature. The suspension was centrifuged at 11,617 ×*g* for 5 min. Supernatant was collected and filtered through a 0.45-μm nylon syringe membrane (Milliporesigma, Mississauga, ON). Filtered encapsulated curcumin was placed at -80°C for 4 h and then freeze-dried at -53°C using a 12-L Console Labconco freeze-dryer (Kansas city, MO) for 24 h. The freeze-dried product was packaged and stored at -20°C for further use. The negative control was that of HMS processed without the addition of curcumin.

Encapsulated curcumin was extracted and quantified as follows. First, 1.5 mL of double-deionized water was added to 0.5 g of freeze-dried encapsulated curcumin. The curcumin aqueous solution was then mixed with an equal volume of chloroform in a 10-mL glass tube. Two-phase liquid-liquid emulsions were obtained and then stirred at 200 rpm for 10 min to remove any free curcumin. Next, the emulsion was mixed with chloroform at the same volume, followed by vortex for 10 min and stirring at 200 rpm for overnight. Chloroform paste was collected then filtered through a 0.45-μm membrane before quantification. The extracted curcumin was analyzed using an Agilent 1260 HPLC system coupled with a diode array detector (HPLC-DAD; Agilent Technology, Santa Clara, CA). An aliquot (20 μL) of sample was injected into a SUPELCOSIL™ C_{18} column (300 × 4.6 mm, 3 μm; Sigma-Aldrich, Oakville, ON) and eluted at a flow rate of 1.0 mL/min and ambient temperature. The extracted curcumin was analyzed using an Agilent 1260 HPLC system coupled with a diode array detector (HPLC-DAD; Agilent Technology, Santa Clara,

CA). An aliquot (20 μL) of sample was injected into a SUPELCOSIL™ C_{18} column (300 × 4.6 mm, 3 μm; Sigma-Aldrich, Oakville, ON) according to the protocol described in a previous study (Jayaprakasha, Rao, and Sakariah 2002). The mobile phase consisted of (A) 2% acetic acid (v/v) in water and (B) acetonitrile. A linear gradient was conducted from 45% B to 65% B within 8 min, then to 90% B before 10 min, and returned to 45% B before 15 min. Chromatograms were obtained at 425 nm. The retention time of curcumin was 7.5 min.

2.3.3 Bacterial strains

The *C. jejuni* F38011 (clinical isolate), human10 (clinical isolate) (J. Li et al. 2017) and ATCC 33560 (bovine feces isolate) strains were routinely cultivated either on *Campylobacter* agar (OXOID, Nepean, ON) plates supplemented with 5% defibrinated sheep blood (Alere, Stittsville, ON) for 48 h or in Mueller-Hinton (MH) broth with constant shaking for 18 h at 37°C in a microaerobic environment (*i.e.*, 10% CO_2). Overnight *C. jejuni* cultures were individually prepared to 1×10^9 CFU/mL by adjusting OD_{600} value. Then, a cocktail culture was prepared by combining equal volumes of each of the three cultures for further antimicrobial testing.

2.3.4 Investigation of synergistic antimicrobial effect using time-killing method

The minimum bactericidal concentrations (MBC) of cinnamon oil, ZnO NPs, and encapsulated curcumin against *C. jejuni* were separately determined using time-killing method (Wiegand, Hilpert, and Hancock 2008). A series of two-fold dilutions from 1-100 ppm of antimicrobials were individually mixed with *C. jejuni* cocktail, with the initial cell count of 10^5 CFU/mL (**Table 2.S1**), and the survival cells were determined after different time intervals using the conventional platting assay. The MBC value was used as the reference concentration for

synergistic antimicrobial testing. Different concentrations (2×, 1×, 0.5× and 0.25× MBC) of cinnamon oil were individually combined with the MBC of ZnO NPs and *C. jejuni* cocktail culture (**Table 2.S1**). To identify the interaction between antimicrobials, the results of individual antimicrobial treatments were compared to that of the combined antimicrobial treatment. Log reduction was calculated by subtracting the bacterial count in treated group from the control group at the same time point. The experiment was conducted in triplicates.

2.3.5 Investigation of synergistic antimicrobial effect using fractional inhibitory concentration index method

The interaction of antimicrobial effects between cinnamon oil, encapsulated curcumin, and ZnO NPs was also evaluated using the FICI method. The minimum bacteriostatic concentration (MIC) of single and dual antimicrobials was determined then the FICI of each combination was identified as follows: FICI = (MIC of A in combination / MIC of A alone) + (MIC of B in combination / MIC of B alone), where A and B represent a different antimicrobial agent. Tests were performed at FICI values of 0.3, 0.4, 0.5, and 1 in order to identify the type of interactions between antimicrobials. The minimum FICI value that inhibited bacterial growth was defined as synergistic (≤ 0.5), additive (0.5-4), or antagonistic (>4) (Odds 2003). Antimicrobials were prepared in fresh MH medium containing *C. jejuni* cocktail with an initial cell density of 10^5 CFU/mL. The procedure was similarly applied in all standardized susceptibility methods (Wiegand, Hilpert, and Hancock 2008). The inhibitory effect was reported as either positive or negative based upon the clarity (+) or turbidity (-) of the tested bacterial cultures. The experiment was conducted in triplicates.

2.3.6 Investigation of synergistic antimicrobial effect using mathematical modelling

2.3.6.1 Concentration-effect curves of single antimicrobials

A broad range of concentrations of cinnamon oil, ZnO NPs, and encapsulated curcumin were tested against *C. jejuni* strain F38011 to identify the concentration-effect curve after a 3 h of treatment. Each antimicrobial was prepared at a series of concentrations (**Table 2.S2**) and then individually mixed with *C. jejuni* at an initial concentration of 10^8 CFU/mL, followed by incubation at 37°C for 3 h in a microaerobic environment. Antimicrobial testing was performed using the conventional plating assay. The experiment was conducted in triplicates. Antimicrobial effect was reported as a percent and log reduction of the bacterial cells. Concentration-effect curves were generated using Prism 5 software (GraphPad, San Diego, CA).

2.3.6.2 Preparation of antimicrobial combinations

Concentration-effect data were used to prepare different pairs of concentrations based on their potencies (*e.g.* IC_{20} + IC_{20}). The inhibitory concentrations (*e.g.* IC_{20}) used for preparing pairs of concentrations were determined by:

$$I \qquad (1)$$

where I represents the inhibitory concentration (*e.g.* IC_{20}), F denotes the cell percentage reduction (*e.g.*, 20%), H stands for the Hill slope, and IC_{50} is the inhibitory concentration that gives 50% reduction of the cells. The theoretical additive effect (*e.g.* the sum effect of IC_{20} + IC_{20}) was calculated by the fractional product method (Chou and Talalay 1984):

$$(1 - f_1)\,(1 - f_2) = V_{1\&2}$$

$$\text{Theoretical additive effect (\%)} = () \qquad (2)$$

where f represents the fraction of cell reduction (CFU/mL) by single antimicrobials (*i.e.* f_1 and f_2) and $V_{1\&2}$ represents the theoretical fractional concentration of viable cells after treatment with two antimicrobials. Combined effect can be synergistic, additive or antagonistic. Synergistic effect takes place when the combined effect is greater than the theoretical additive effect, while additive or antagonistic effects take place when the combined effect is equal to or lower than the theoretical additive effect, respectively. The maximum theoretical additive effect used in this study was 82% (<1 log) CFU/mL to ensure that the antimicrobial combinations do not completely inactivate the entire bacterial population. In this case, the 18% remaining population accounts for (>7 log) CFU/mL.

2.3.6.3 Isobologram

Isobologram analysis was used to investigate the interaction effect (synergistic, antagonistic, or additive interaction) between different binary combinations. Sets of equally effective concentrations were selected to generate isobolograms according to the method described in a previous study (Chou and Talalay 1984). The IC_{50} of each single antimicrobial was used to draw an additive line between the combined antimicrobials. Reduction of antimicrobials was calculated according to:

(3)

where R represents the reduction of antimicrobial concentration. The isobolograms were generated using Prism 5.

2.3.6.4 Median-effect plot

A systematic analysis of the concentration-effect data of single and combined antimicrobials was conducted to generate a median effect plot. The data were normalized by Chou's median effect equation (Chou 2006) as follows:

(4)

where *C* represents the concentration of antimicrobial(s). The median effect plot was generated using Prism 5.

2.3.7 Statistical analysis

Prism software (version 5.01: GraphPad Software Inc., San Diego, CA) was used for statistical analysis and the graphs generation. The time-killing data were analyzed by one-way ANOVA, followed by post hoc Tukey's test for multiple comparisons. A *P* value was adjusted at 0.05 or less to define statistically significant differences between and within groups.

2.4 Results and discussion

2.4.1 Conventional methods used to study the antimicrobial interactions

2.4.1.1 Time-killing method

The time-killing method was used first to study single and combined antimicrobial treatments. Cinnamon oil, ZnO NPs, and encapsulated curcumin showed bactericidal activity at 1× MBC and 2× MBC after 3, 6, 12, and 24 h ($P < 0.0001$) (**Figure 2.1A**). The 0.5× MBCs of all single antimicrobials had a mild effect with ≤1 log reduction and no significant effect compared to the control groups at all time points ($P > 0.05$) (**Figure 2.1**). The combination of cinnamon oil and ZnO NPs at low concentrations (≤ 0.5× MBC) significantly enhanced the antimicrobial effect.

For example, ZnO NPs at 0.5× MBC and cinnamon oil at 0.25× MBC resulted in a 6.24 log reduction of *C. jejuni* after 12 h of treatment ($P < 0.0001$) (**Figure 2.2**), while the same concentrations of both single antimicrobials showed no significant difference ($P > 0.05$) compared to the control group even after 24 h (**Figure 2.1A&B**).

DMSO (1%) did not significantly affect cell viability ($P > 0.05$) (**Figure 2.1A**), indicating that the antimicrobial effect of cinnamon oil solution was not due to the solvent. Similarly, up to 100 ppm of free curcumin (without encapsulation or HMS) and 1% HMS had no effect on the viability of *C. jejuni* ($P > 0.05$) (**Figure 2.1C**). The effects of 1% DMSO or 1% HMS with and without antimicrobial agents (4× MIC) were significantly different after 3, 6, 12, and 24 h ($P < 0.0007$) indicating no interaction between these compounds and antimicrobials against cell viability (**Figure 2.1A&C**).

Synergism was identified when two antimicrobials resulted in a greater log reduction than the sum of their individual effects. In the current study, the single treatment of cinnamon oil (0.5× MBC) or ZnO NPs (0.5× MBC) induced a ≤1 log reduction after 24 h ($P > 0.05$) (**Figure 2.1A&B**). An additive effect was considered if the combined treatment caused ~2 log reduction after 24 h. Interestingly, the combination of cinnamon oil (0.5× MBC)/ZnO NPs (1× MIC) resulted in 5 log reduction within 6 h of treatment ($P < 0.0001$) (**Figure 2.2**), inducing additional log-reduction (CFU/ mL) compared to the additive effect. Therefore, cinnamon oil and ZnO NPs were considered to have a synergistic effect against *C. jejuni*. Although previous studies used this approach to study antimicrobial synergic effect (Ghosh et al. 2013; Ha and Kang 2015; Huq et al. 2015), it measures the effect on the basis of logarithmic scale, leading to potential overestimation of the interaction between the two antimicrobials. For example, the 0.5× MBC of all single antimicrobials showed ≤1 log reduction (≤ 90% of bacterial population) (**Figure 2.1** and **Figure**

2.2). If 90% of bacterial cells can be inactivated by one antimicrobial at the 0.5× MBC, it is likely that the other antimicrobial can easily inactivate the remaining population (~10% of the viable cells) at its 0.5× MBC when both antimicrobials are applied at the same time. It is not reasonable to draw an additive line for two antimicrobials based on their log reductions because the sum of the two combined concentrations may fully inactivate the entire bacterial population. Although the time-killing method has many advantages including that the antimicrobial effects are monitored over time, our current results showed that this method overestimated the synergism between cinnamon oil and ZnO NPs. Thus, other methods were further applied to investigate the synergism between the selected antimicrobials.

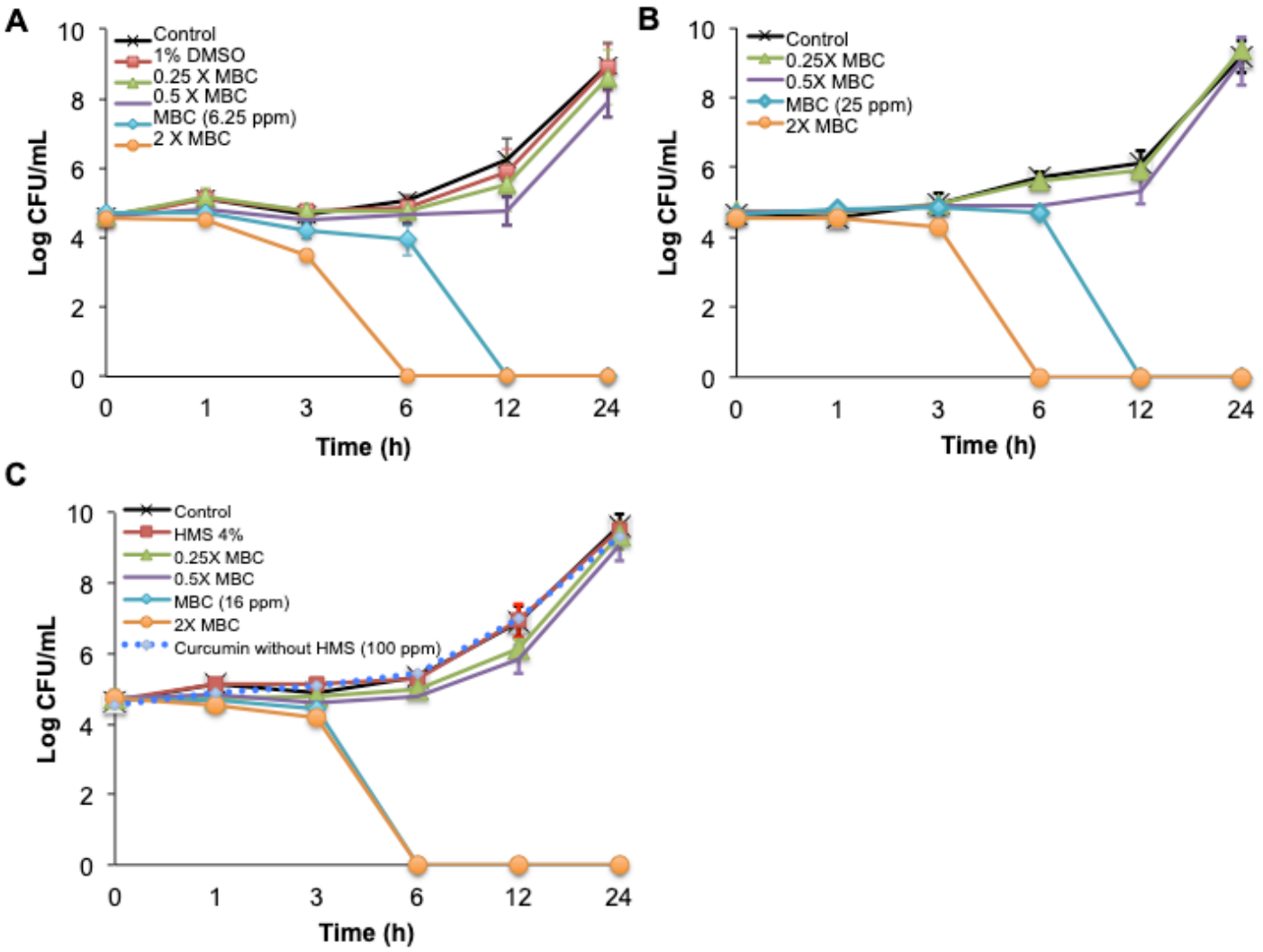


Figure 2.1. Time-killing curves of *C. jejuni* cocktail with single antimicrobial treatment at 37°C in a microaerobic condition.

The antimicrobial effect of (A) cinnamon oil, (B) zinc oxide nanoparticle (ZnO NP), and (C) encapsulated curcumin against *C. jejuni* is summarized. Control represents the untreated cells, MBC is the minimum bactericidal concentration, and HMS reflects hydrophobically modified starch. Error bars represent the standard deviation (n = 3, duplicates).

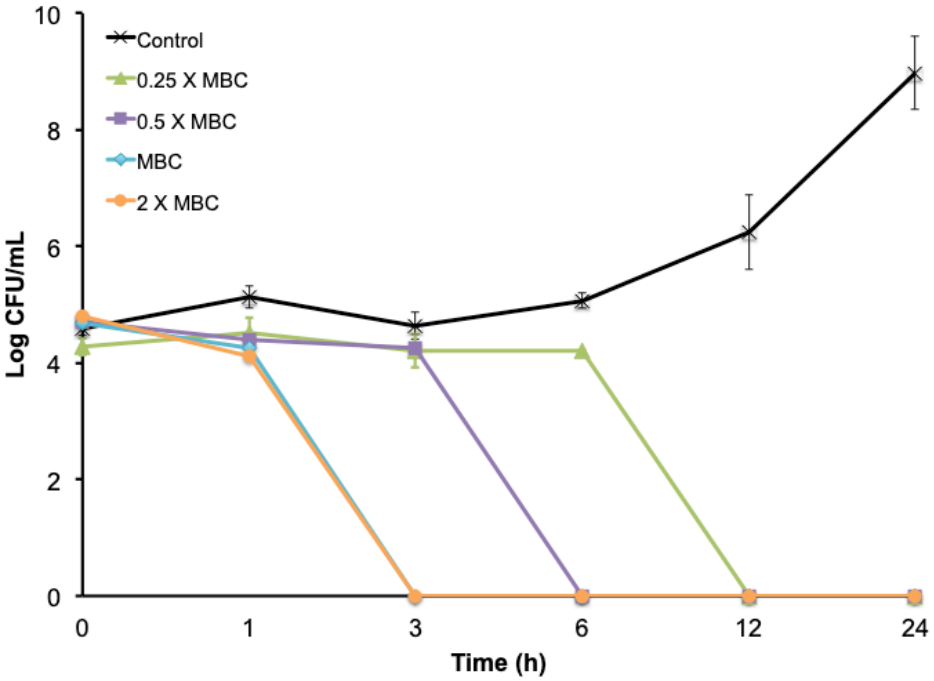


Figure 2.2. Time-killing curves of *C. jejuni* cocktail treated with different concentrations of cinnamon oil (0.25 – 2 X MBC) combined with 0.5 X MBC of ZnO NP (MBC = 25 ppm) at 37°C in a microaerobic condition. Control represents the untreated cells. MBC is the minimum bactericidal concentration. Error bars represent the standard deviation (n = 3, duplicates).

2.4.1.2 FICI method

The FICI method has been frequently used to study antimicrobial interactions because it is simple and can test many concentrations simultaneously (Odds 2003). The FICI method relies on combining multiple sub-MICs. The minimum inhibitory concentration at specific FICI value indicates the type of interaction. The additive effect takes place when the FICI is >0.5-1 (Odds 2003). A lower FICI value indicates a higher synergism while a higher FICI value indicates antagonism. We used the FICI method to study the interactions between the binary combinations of cinnamon oil/ZnO NPs, cinnamon oil/encapsulated curcumin, and ZnO NPs/encapsulated curcumin. *C. jejuni* cocktails (5 log CFU/mL) were treated with antimicrobials for 24 h. All of the combinations did not show any inhibitory effect at a FICI value of 0.3, 0.4, or 0.5 (**Table 2.1**), indicating that no synergistic interaction occurred. In comparison, all combinations inhibited the growth of *C. jejuni* at a FICI value of 1, demonstrating an additive effect. Unlike the time-killing method, the FICI method did not show any synergistic interactions between tested antimicrobials (**Figure 2.2** and **Table 2.1**).

Both the time-killing method and the FICI method rely on the assumption that antimicrobials are equally effective at MICs/sub-MICs or MBC/sub-MBC. However, even when two antimicrobials at MICs cause similar effects, the antimicrobial effect at their sub-MICs might be totally different from each other depending on the shape of their concentration-effect curve (Tallarida 2006). It is difficult to predict the additive effect by combining two antimicrobials with different concentration-effect shapes. The extreme difference between two antimicrobials in their concentration-effect relationship highlights the challenge to predict the additive line of antimicrobial combinations in the conventional methods. Moreover, the potency of each antimicrobial can be unequal when they are mixed at 0.50× or 0.25× MICs (**Figure 2.3**). Friedman

and colleagues tested the antimicrobial effects of 96 essential oils and 23 purified oil compounds against *C. jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica* and obtained different shapes of concentration-effect curve (Friedman, Henika, and Mandrell 2002). Although this is a common problem, none of the conventional methods take this into consideration. Our results (**Table 2.1)** agreed with a previous study demonstrating the inability of FICI method to identify synergism between synergistic antimicrobials (Lambert and Lambert 2003). The limitation is simply due to different dose-response of the combined antimicrobials. Therefore, it is important to use alternative methods to discover new synergistic combinations that are not detected by the conventional methods. Taken together, FICI may not be able to identify an existing synergism between antimicrobials if they have different concentration-effect curves.

Table 2.1. Antimicrobial effect of binary combinations of cinnamon oil, zinc oxide nanoparticle and encapsulated curcumin on *C. jejuni* cocktail.

FICI*	Fraction of MIC		A/B	Cinnamon oil/ ZnO NPs	Cinnamon oil / Encapsulated curcumin	ZnO NPs/ Encapsulated curcumin
	A	B				
0	0	0		+	+	+
1	0.5	0.5		-	-	-
1	0.25	0.75		-	-	-
1	0.75	0.25		-	-	-
0.5	0.25	0.25		+	+	+
0.4	0.2	0.2		+	+	+
0.3	0.15	0.15		+	+	+

The turbidity of the bacterial culture was observed after 24 h of incubation at 37°C in a microaerobic condition. Initial bacterial count was 10^5 CFU/mL. The "+" donates turbid and the "–" indicates clear (n = 3, duplicates). The average bacterial concentration in the turbid samples was 9.69 ± 0.27 log CFU/mL. FICI: fractional inhibitory concentration index.*

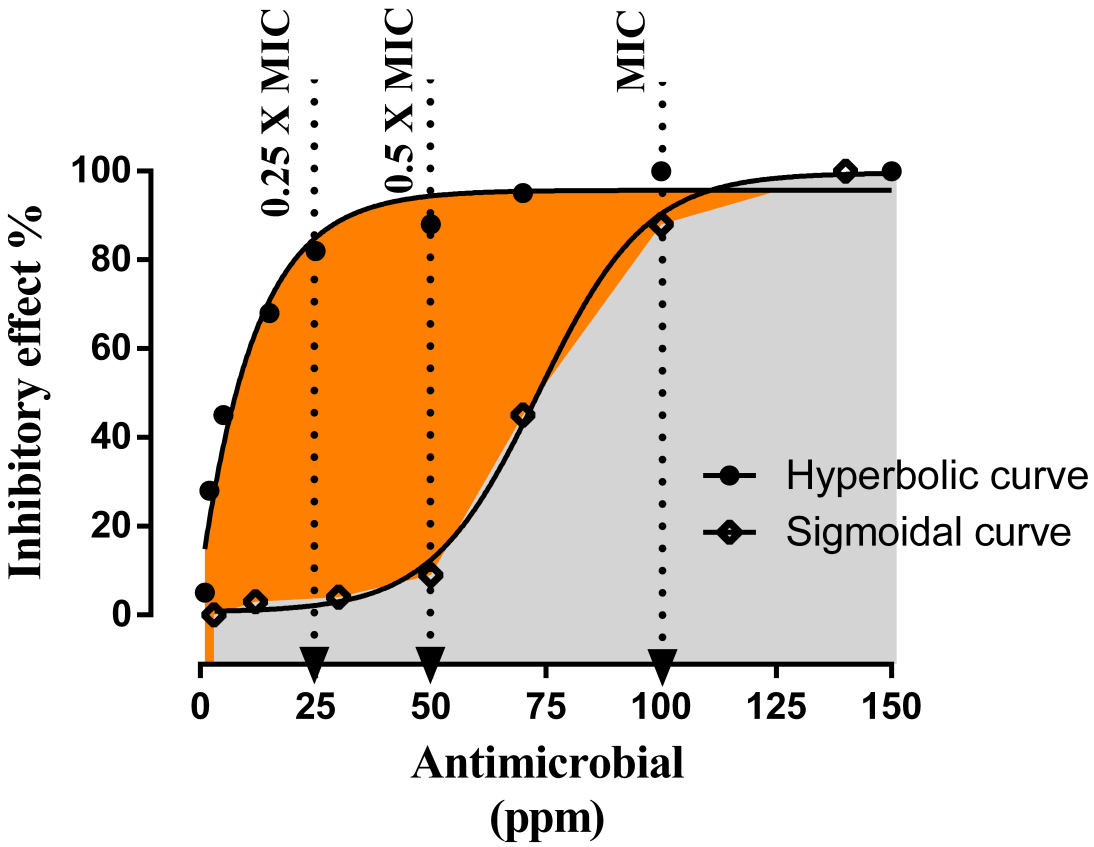


Figure 2.3. Predicted example of combining two antimicrobials based on their sub-MICs. Inhibitory effect represents the percentage of inactivated cells. Increasing the concentration in the hyperbolic curve from 50 to 100 ppm raises the inhibitory effect by 5% (from 85 to 90%). Increasing the concentration in the sigmoidal curve from 50 to 100 ppm raises the inhibitory effect by more than 3-fold (from 18 to 88%).

2.4.2 Mathematical modelling used to study the antimicrobial interactions

A nonlinear mathematical concentration-effect model was used to combine antimicrobials in a more detailed manner using a selected strain and a selected time point. The isobologram and median-effect equation were used to analyze the data (Chou and Talalay 1984; Tallarida 2001; Chou 2006; Tallarida 2006). The idea was to combine antimicrobials based on their quantitative potencies (*i.e.*, IC_{40} + IC_{40}) instead of sub-MICs or sub-MBCs. Potency is the antimicrobial effect of a specific concentration (*e.g.*, IC_{50}) of an antimicrobial agent, while efficacy it the maximum effect induced by an antimicrobial. *C. jejuni* strain F38011 was selected because it was isolated from an individual with bloody diarrhea (O'Loughlin et al. 2015), effectively colonizes the chicken gastrointestinal tract (Klena, Gray, and Konkel 1998), and causes illness in mice and pigs (O'Loughlin et al. 2015). Only a single time point (*i.e.,* 3 h) was selected to study the concentration-effect curve for each antimicrobial because 3 h was identified to be the minimum time period to cause *Campylobacter* reduction by single or dual antimicrobials (**Figure 2.1** and **Figure 2.2**). Similarly, another study tested the antimicrobial effect of ZnO NPs and identified that 3 h of treatment was the minimum time to reduce the count of *C. jejuni* (Xie et al. 2011). Taken together, different steps and considerations were used in a nonlinear concentration-effect mathematical model to study the antimicrobial interactions.

2.4.2.1 Concentration-effect curves

All antimicrobials demonstrated a nonlinear regression between the concentrations and antimicrobial effects (**Figure 2.4**). Cinnamon oil and ZnO NPs showed a similar shape of concentration-effect curve with a slight difference, while encapsulated curcumin presented a sharp sigmoidal curve. IC_{50} is a representative concentration used to evaluate the potency of drugs or

antimicrobials. The lower the IC_{50} the more potent is the drug. Up to thirteen concentration-effect points were used to generate high quality concentration-effect curves. Some points showed no effect; some were on the slope; and some were on the plateau of their concentration-effect curves. Other methods, such as the FICI, rely on one reference concentration (MIC) for studying antimicrobial interactions. We used multiple data points and identify the IC_{50} as a representative concentration (located on the middle of the concentration-effect slope) to determine nine other reference concentrations. The IC_{50} of cinnamon oil, ZnO NPs, and encapsulated curcumin were identified to be 0.90, 1.20 and 1.48 log ppm, respectively (**Figure 2.4**). All three individual antimicrobials had the same efficacy because they were all able to reduce bacterial population over 99% at 1.39 log ppm for cinnamon oil, 1.54 log ppm for ZnO NPs, and 1.60 log ppm for encapsulated curcumin (**Figure 2.4**).

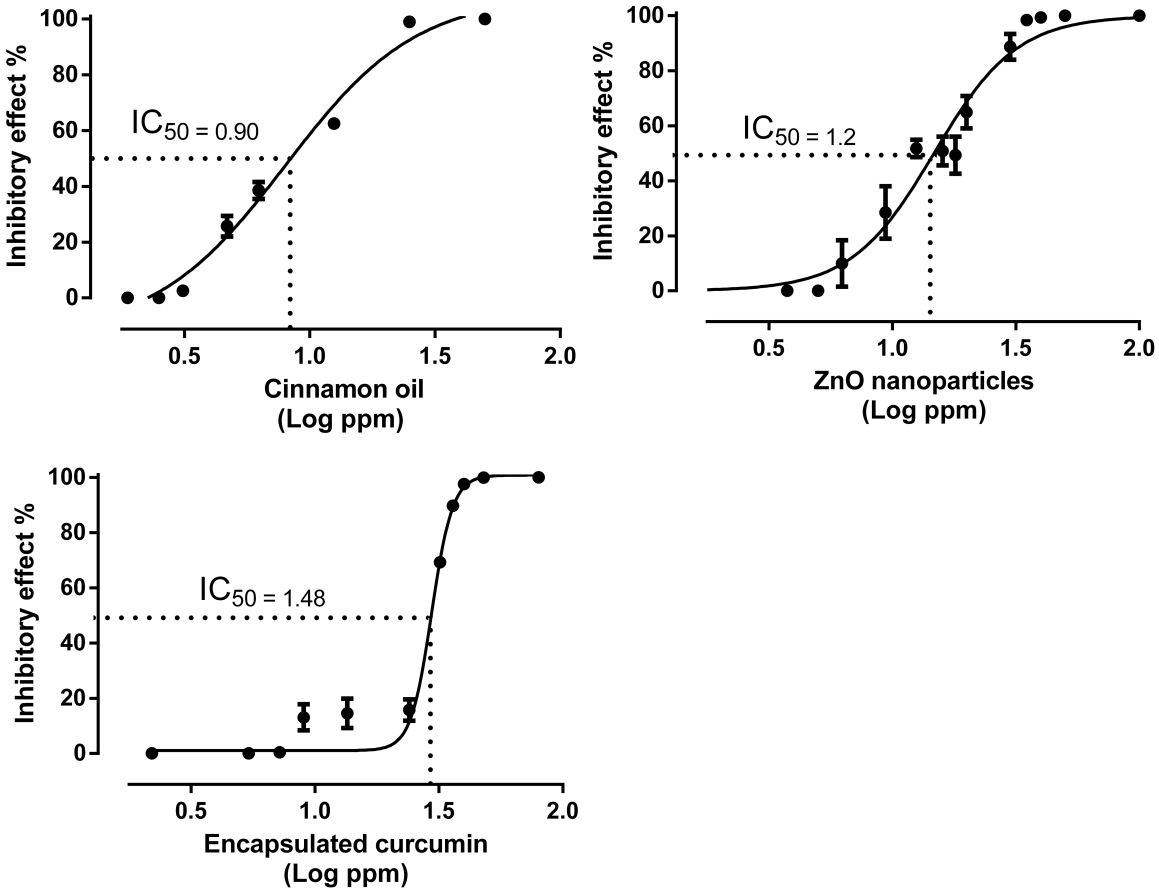


Figure 2.4. Concentration-effect curves for cinnamon oil (A), ZnO NPs (B), and encapsulated curcumin (C) against *C. jejuni* strain F38011 after 3 h treatment at 37°C in microaerobic condition. Inhibitory effect represents the percentage of inactivated cells. Error bars represent the standard deviation (n = 3, duplicates).

2.4.2.2 Isobologram

The isobologram is a diagram that is commonly used to identify the type of interaction of a combination by comparing the concentrations of two single agents (x and y-axes) and their combination (axial point) (**Figure 2.5**). The IC_{50} of single antimicrobial cinnamon oil (7.92 ppm), ZnO NPs (14.75 ppm) and encapsulated curcumin (29.63 ppm) were used to draw additive lines. The additive effect is indicated if the combined (IC_{50}) data point is on the additive line and synergism is indicated if the combined IC_{50} data point is below the additive line. The combination of cinnamon oil/ZnO NPs (4.14+10.37 ppm) indicated an additive interaction (**Figure 2.5A**). In contrast, cinnamon oil/encapsulated curcumin (0.523+11.70 ppm) (**Figure 2.5b**) and encapsulated curcumin/ZnO NPs (11.70+4.14 ppm) (**Figure 2.5C**) indicated synergistic interaction. Synergism between cinnamon oil and encapsulated curcumin resulted in the reduction of antimicrobial concentrations by 93.40% and 60.51%, respectively (**Figure 2.5B**). In comparison, ZnO NPs and encapsulated curcumin were reduced by 81.76% and 60.51%, respectively (**Figure 2.5C**). Taken together, up to 93.40% of the antimicrobial concentrations were reduced due to the synergistic interactions.

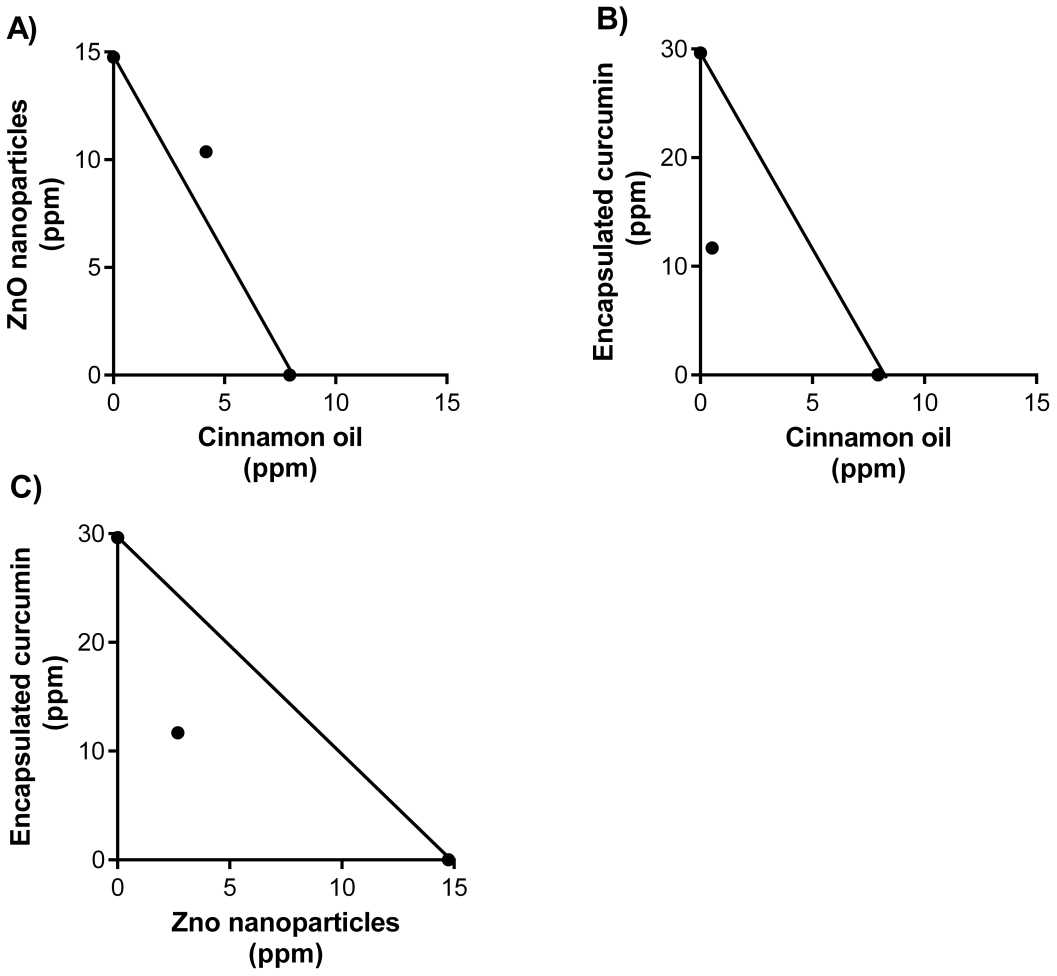


Figure 2.5. Isobologram analysis of binary combinations of cinnamon oil/ZnO NPs (A), cinnamon oil/encapsulated curcumin (B), ZnO NP/encapsulated curcumin (C) against *C. jejuni* strain F380I1 after 3 h treatment at 37°C in a microaerobic condition. The additive lines connect the IC_{50} of each single antimicrobial. Axial points show the IC_{50} of antimicrobial combinations. An axial point on the additive line indicates additive interaction. An axial point under the additive line indicates synergistic interaction (n = 3, duplicates).

2.4.2.3 Median-effect curves

The median-effect principle has been employed to analyze the dose-response data in enzymatic, cellular, and animal studies. A few studies have used this approach to study the antimicrobial effects, such as the ones of using the median-effect equation to investigate the antiviral synergistic effect against HIV and influenza virus A (Asin-Milan et al. 2014; Kulkarni et al. 2014; Belardo et al. 2015). Most studies used the ComboSyn software to generate the median-effect plot (Chou 2010). However, this software does not consider the shape of dose-response. Hence, it cannot overcome some of the possible mathematical errors in combinational studies. In the current study, we used nonlinear concentrations-effect data to generate the median-effect plot and identified the overall effect of antimicrobials and their combinations in a wide range of concentrations. By using the median-effect principle, more meaningful data were provided because the IC_{50} shown in the isobologram was not sufficient for treatments (**Figure 2.5**).

Figure 2.6 shows the median-effect curves of single antimicrobials and their combinations at a broad range of concentration-effect relationship between 0 to >8 log reduction of bacterial cells. Cinnamon oil was identified to be the most potent antimicrobial because its median-effect curve reached to the maximum effect (>8 log) first followed by ZnO NPs, and then the encapsulated curcumin (**Figure 2.6**). Although we did not study the mode of action of single or dual antimicrobials, the median-effect curves of cinnamon oil and ZnO NPs had the same slope, suggesting that they might work with a similar mechanism (**Figure 2.6A**). The primary antibacterial functions of cinnamon oil and ZnO NPs are both related to the disruption of bacterial cell wall and cell membrane (Davidson, Sofos, and Branen 2005; Xie et al. 2011). In contrast, curcumin acts as a cytokinesis inhibitor by direct interaction with FtsZ (Rai et al. 2008), an essential bacterial cell-division protein. This might explain why the encapsulated curcumin

showed a sharp sigmoidal concentration-effect curve (**Figure 2.4**) and a very steep median-effect curve (**Figure 2.6B,C&D**).

Several pairs of the fixed ratio concentrations (from IC_{10} to IC_{40}) were used to generate the median-effect plot. The median-effect line of cinnamon oil/ZnO NPs was almost horizontal and unable to cross the MIC (*i.e.*, additive) line (**Figure 2.6A** and **Table 2.2A**). In comparison, all other combinations showed synergistic interactions and were able to cause antimicrobial reduction about three times greater (3 log-reduction) than the additive line (>1 log reduction) (**Figure 2.6B&C** and **Table 2.2B&C**). The tertiary combination of cinnamon oil/ZnO NPs/encapsulated curcumin had the greatest synergistic interaction among the tested combinations because mixing three concentrations of IC_{25} (additive line 57%) resulted in over 8 log reduction of bacterial viable cells (**Figure 2.6D** and **Table 2.2D**). The median-effect curves of all-synergistic combinations were parallel with the curve of the encapsulated curcumin (**Figure 2.6B&C**), suggesting that encapsulated curcumin played an important role in the synergistic interaction.

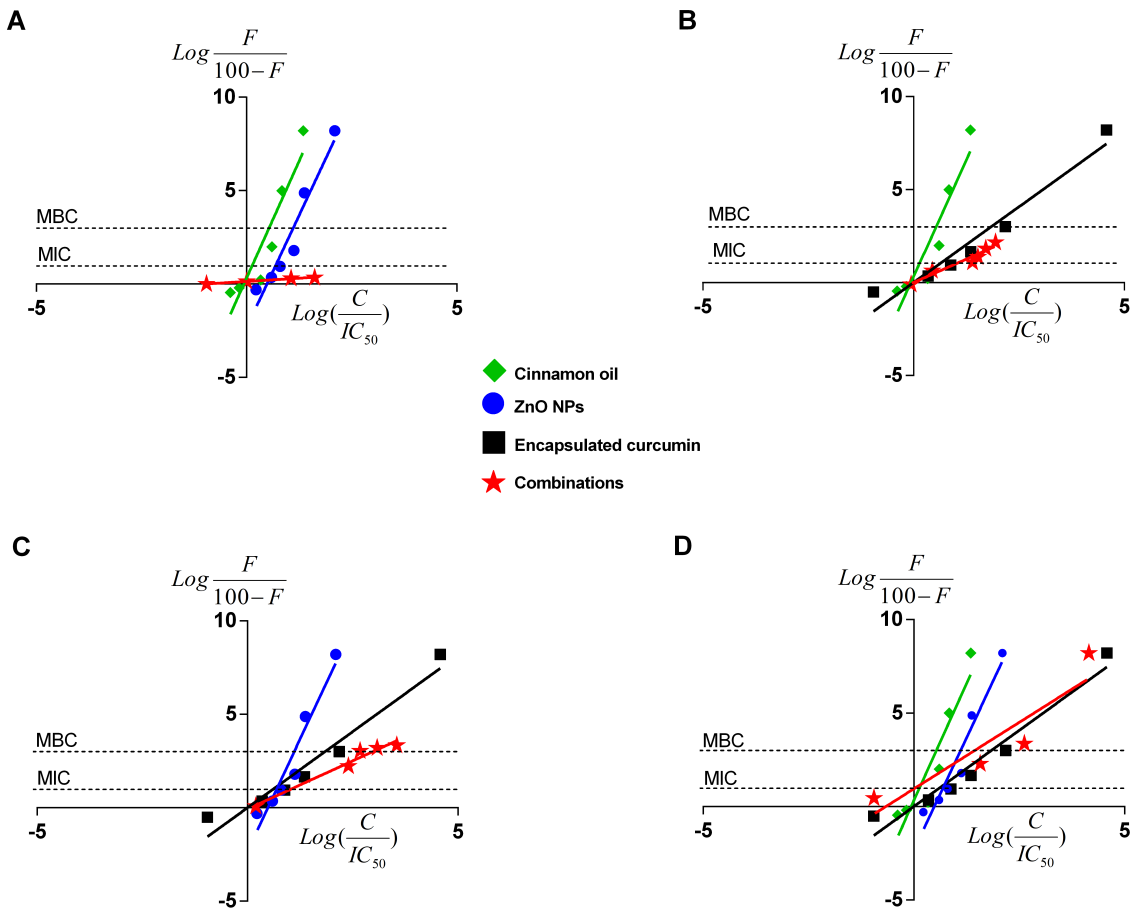


Figure 2.6. Median-effect plots of cinnamon oil, ZnO NP, and encapsulated curcumin against *C. jejuni* F38011 strain after 3-h treatment at 37°C in a microaerobic condition. Data were plotted for single or dual treatments as binary (A - C) or tertiary combinations (D). *F* represents the percentage reduction of bacterial cells. *C* means the antimicrobial concentration and *H* is the slope hill of concentration-effect curves. Each line represents the effect of single or combinational antimicrobial treatment. The "0" on the x-axis represents median-effect and the "0" on the y-axis represents a 50% reduction of viable cells. The two unconnected lines show the antimicrobial effects at MIC (90% reduction) and MBC (99.9% reduction) of viable cells. The line slope represents the potency of the antimicrobial treatment (*n* =3, duplicates).

Table 2.2. Binary (A - C) and tertiary (D) combinations of cinnamon oil, encapsulated curcumin, and zinc oxide nanoparticle against *C. jejuni* F38011 after 3 h treatment at 37°C in microaerobic condition (*n* = 3, duplicates).

A	Cinnamon oil potency (ppm)		ZnO NPs potency (ppm)		Additive line %	Effect %	Interaction	Log reduction	SD.
	IC_{10}	(2.16)	IC_{10}	(6.78)	19	0.00	Additive	0.00	0.124
	IC_{15}	(2.84)	IC_{15}	(8.11)	27	26.99	Additive	0.14	0.027
	IC_{25}	(4.14)	IC_{25}	(10.37)	43	50.00	Additive	0.30	0.205
	IC_{40}	(6.24)	IC_{40}	(13.55)	64	55.42	Additive	0.35	0.020
	IC_{30}	(4.81)	IC_{60}	(18.53)	72	48.57	Additive	0.29	0.076
	IC_{60}	(10.09)	IC_{30}	(11.42)	72	45.09	Additive	0.26	0.255
	IC_{10}	(2.16)	IC_{80}	(27.05)	82	56.44	Additive	0.36	0.128
	IC_{80}	(18.04)	IC_{10}	(6.78)	82	72.80	Additive	0.57	0.068

B	Cinnamon oil potency (ppm)		Enc. curcumin potency (ppm)		Additive line %	Effect %	Interaction	Log reduction	SD.
	IC_{1}	(0.52)	IC_{1}	(11.70)	2	44.58	Synergistic	0.26	0.187
	IC_{5}	(1.38)	IC_{5}	(14.38)	9	81.64	Synergistic	0.74	0.573
	IC_{10}	(2.16)	IC_{10}	(22.97)	19	92.56	Synergistic	1.13	0.012
	IC_{15}	(2.84)	IC_{15}	(24.02)	27	95.98	Synergistic	1.40	0.052
	IC_{25}	(4.14)	IC_{25}	(25.53)	43	98.53	Synergistic	1.83	0.523
	IC_{40}	(6.24)	IC_{40}	(27.29)	64	99.34	Synergistic	2.18	0.462
	IC_{30}	(4.81)	IC_{60}	(29.51)	72	99.42	Synergistic	2.24	0.264
	IC_{60}	(10.09)	IC_{30}	(26.16)	72	99.63	Synergistic	2.43	0.012
	IC_{10}	(2.16)	IC_{80}	(32.43)	82	99.99	Synergistic	4.45	0.168
	IC_{80}	(18.04)	IC_{10}	(22.97)	82	99.95	Synergistic	3.29	0.048

C	Enc. curcumin potency (ppm)		ZnO NPs potency (ppm)		Additive line %	Effect %	Interaction	Log reduction	SD.
	IC_{1}	(11.70)	IC_{1}	(2.69)	2	49.08	Synergistic	0.29	0.071
	IC_{5}	(14.38)	IC_{5}	(5.80)	9	54.91	Synergistic	0.35	0.552
	IC_{10}	(22.97)	IC_{10}	(6.78)	19	99.42	Synergistic	2.24	0.050
	IC_{15}	(24.02)	IC_{15}	(8.11)	27	99.91	Synergistic	3.05	0.419
	IC_{25}	(25.53)	IC_{25}	(10.37)	43	99.94	Synergistic	3.21	0.059
	IC_{40}	(27.29)	IC_{40}	(13.55)	64	99.96	Synergistic	3.35	0.019
	IC_{30}	(26.16)	IC_{60}	(18.53)	72	99.99	Synergistic	8.21	0
	IC_{60}	(29.51)	IC_{30}	(11.42)	72	99.99	Synergistic	8.21	0
	IC_{10}	(22.97)	IC_{80}	(27.05)	82	99.99	Synergistic	5.12	0
	IC_{80}	(32.43)	IC_{10}	(6.78)	82	99.99	Synergistic	8.21	0

D	Cinnamon oil potency (ppm)		ZnO NPs potency (ppm)		Enc. curcumin potency (ppm)		Additive line %	Effect %	Interaction	Log reduction	SD.
	IC_{1}	(0.52)	IC_{1}	(2.69)	IC_{1}	(11.70)	3	50.00	Synergistic	0.21	0.031
	IC_{5}	(1.38)	IC_{5}	(5.80)	IC_{5}	(14.38)	14	55.06	Synergistic	0.35	0.026
	IC_{10}	(2.16)	IC_{10}	(6.78)	IC_{10}	(22.97)	27	99.49	Synergistic	2.29	0.002
	IC_{25}	(4.14)	IC_{25}	(10.37)	IC_{10}	(22.97)	50	99.66	Synergistic	2.46	0.093
	IC_{15}	(2.84)	IC_{15}	(8.11)	IC_{15}	(24.02)	43	99.96	Synergistic	3.36	0.150
	IC_{25}	(4.14)	IC_{10}	(6.79)	IC_{25}	(25.53)	50	99.99	Synergistic	8.21	0
	IC_{10}	(2.16)	IC_{25}	(10.37)	IC_{25}	(25.53)	50	99.99	Synergistic	8.21	0
	IC_{25}	(4.14)	IC_{25}	(10.37)	IC_{25}	(25.53)	57	99.99	Synergistic	8.21	0

2.5 Conclusion

In conclusion, conventional methods either overestimated or failed to detect the exiting synergic antimicrobial interactions due to the undefined concentration-effect curves. We were able to identify reference concentrations and evaluate the synergism even between antimicrobials with different concentration-effect curves. Up to 93.40% of the antimicrobial concentrations were reduced while maintaining the same effect due to synergistic interactions. Cinnamon oil and ZnO NPs acted differently from the encapsulated curcumin. We propose that this mathematical modelling can aid in developing new synergistic combinations to potentially reduce the prevalence and survival of foodborne pathogens as well as open the door to discover new mechanisms of dual antimicrobials.

Chapter 3: Whole Transcriptome Sequencing Analysis of Antimicrobial Synergism of Plant-based Antimicrobials and Zinc Oxide Nanoparticles against *Campylobacter jejuni*

3.1 Overview

The increasing emergence of antibiotic resistance and continuous banning of the use of antibiotics in animal farms poses the requirement to identify new antimicrobial treatment for agri-food production. Synergistic combination of antimicrobials can provide many advantages such as the decrease in dosage, treatment time, and antimicrobial resistance. We investigated the antimicrobial mechanism of cinnamon oil, encapsulated curcumin, ZnO NPs, and their dual and triple combinations against *C. jejuni* using whole transcriptome sequence analysis (RNA-Seq). Single treatments altered the expression of genes involved in similar functions, such as motility, cell permeability, and bacterial cell wall synthesis and maintenance. Each antimicrobial played a different role in synergism. Cinnamon oil increased cell permeability to allow other antimicrobials to accumulate inside the cells. Encapsulated curcumin and ZnO NPs disrupted bacterial cell wall and cell membrane against the same membrane protein targets. The combined treatment of cinnamon oil and encapsulated curcumin altered the expression of some unique genes, such as flagellar sigma factor regulating a group of genes involved in motility as well as genes involved in signaling and chemotaxis. All of the single treatments under-expressed a major facilitator superfamily encoding gene involved in developing antimicrobial resistance, which was different from that by the dual antimicrobial treatment. Triple antimicrobial treatment had systematically overexpressed genes involved in amino acid synthesis, protein translation, and membrane protein synthesis. The identified key genes involved in synergism are potential targets to develop other

effective antimicrobial combinations against *C. jejuni*, which can minimize the disadvantage of developing antimicrobial resistance of this microbe due to single antimicrobial usage.

3.2 Introduction

New sustainable, effective, and safe antimicrobial strategies are consistently required due to global emergence of antibiotic resistance. Alternative antimicrobials including antimicrobial peptides, bacteriophages, essential oils, and bacteriocins seem promising, but they are not immune to antimicrobial resistance. For example, the advanced machinery CRISPR-Cas systems that bacteria use to develop resistance against bacteriophage attack is now well interpreted (Labrie, Samson, and Moineau 2010). The use of antibiotics has been banned as growth promoters to limit antibiotic resistance in several countries including the European Union and Canada (Union 2003; Government of Canada 2018). A new generation of antimicrobial agents including novel synergistic antimicrobial combination is needed because these can be used not only for medical treatment but also for pathogen control and prevention in the agro-ecosystem.

Synergism is defined as a combined effect greater than the sum of multiple individual effects (Chou 2006). Theoretically, synergism occurs when two or more antimicrobials work based on different mechanisms. For example, several recent reports identified novel synergistic antimicrobial combinations of plant-based antimicrobials and metal oxide nanoparticles with different synergistic mechanisms (Hensley, Tierney, and Crowder 2011; Ghosh et al. 2013; Hakeem et al. 2019; Krychowiak et al. 2014; Sharifi-Rad et al. 2014; Xue et al. 2018). In contrast, overlapping mechanisms result in either additive or antagonistic interaction that occurs when two similar antimicrobials, such as two essential oils or two metal oxide nanoparticles, are combined for use together. Knowledge of molecular mechanism of synergistic antimicrobial combinations

can facilitate their agri-food and medical applications. Development of alternative multi-component and multi-targeted antimicrobials has shown a great success in treating some of the most chronic bacterial infections. For example, the standard triple therapy for *Helicobacter pylori* infections includes a proton pump inhibitor and two antibiotics, namely clarithromycin to inhibit proteins involved in bacterial growth and amoxicillin to inhibit bacterial cell wall synthesis (Megraud 1997). Combining multi-target antimicrobial agents might not only increase the sum of their individual effects (*i.e.*, synergism) but also minimize their usage and the potential of antimicrobial resistance. In addition, antimicrobial combinations are commonly used to treat antibiotic resistant bacteria. For example, clavulanate acid and amoxicillin are combined in the commercial drug Augmetin® (Zimmermann, Lehar, and Keith 2007). Clavulanate acid inactivates the β-lactamase resistance so as to maintain the efficacy of amoxicillin against bacterial cell wall transpeptidase.

Plant-based antimicrobials have been gaining more interests due to their low cost, broad effects against different microorganisms including antibiotic-resistant bacteria, and broad mechanisms involving extracellular and/or intracellular targets. Plant-based antimicrobials can directly disrupt bacterial cell wall and cell membrane, elongate the entire structure of bacterial cell wall, increase membrane permeability, induce coagulation of cytoplasm, and/or inhibit the replication and transcription of proteins and/or nucleic acids (Nazzaro et al. 2013). The antimicrobial mechanisms of the actions of plant-based antimicrobials have been classified into two different categories, namely membrane and non-membrane mechanisms of action (Rempe et al. 2017). However, there is no clear line between these two categories due to a high diversity of antimicrobial compounds in a single plant oil or extract. Even a single compound of plant-based antimicrobials can inactivate bacteria by different mechanisms. For example, cinnamaldehyde is

the major component of cinnamon oil, inhibits different cytokinesis enzymes at low concentration, acts as an ATPase inhibitor at sub-lethal concentrations, and disrupts bacterial cell wall at lethal concentrations (Hyldgaard, Mygind, and Meyer 2012). As another example, curcumin is the major phenolic compound in turmeric and it can cause cell filamentation by inhibiting the FtsZ, a protein that plays critical roles in bacterial cytokinesis assembly [reviewed in (Margolin 2005; Erickson, Anderson, and Osawa 2010)]) and increase the GTPase activity of the FtsZ (Rai et al. 2008; Kaur et al. 2010). This disorder drives the bacterial cells to elongate without division and eventually leads to cell death.

The US Food and Drug Administration has approved zinc oxide (ZnO) as generally recognized as safe (GRAS) since 2013 (Xie et al. 2011; Liu et al. 2009; Hernández-Sierra et al. 2008). ZnO is part of many commercial products of cosmetics, sunscreens, and sunglasses due to its small particle size and excellent absorbance property (Stark et al. 2015). Materials can change their physical and chemical properties when they are prepared in very small size, such as the nanoscale. Ionic metal oxide nanoparticles have unique crystal morphologies and a large number of edges, corners, and reactive surface sites. ZnO NPs have shown great antimicrobial effect against a broad range of pathogenic bacteria including *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Salmonella enterica* serotype Enteritis, and *Listeria monocytogenes* (Xie et al. 2011). ZnO NP is a potent antimicrobial that mainly acts as disrupting agent towards cell membrane due to the electrostatic interaction between the positively charged surface of nanoparticles and negatively charged surface of bacterial cells (Arakha et al. 2015). In addition, ZnO NPs can accumulate at bacterial cell membrane, generate reactive oxygen spices (ROS), and release antimicrobial Zn ions (Zn^{2+}), altogether contributing to its antimicrobial effect. Combining plant-

based antimicrobials with metal oxide nanoparticles can theoretically affect multiple targets and lead to synergistic interactions against various bacteria.

Whole transcriptome sequencing analysis (RNA-seq) can study molecular mechanisms of stress responses of microorganisms. RNA-Seq has replaced DNA microarray to investigate bacterial transcriptional profiles (Croucher and Thomson 2010). Many recent studies have applied RNA-Seq to investigate the whole transcriptome of different pathogenic bacteria after antimicrobial treatments and/or under environmental stresses. These included but not limited to *C. jejuni* treated with metal oxide nanoparticles and ajoene (Xue et al. 2018), *E. coli* O157:H7 treated with sub-lethal concentrations of thymol, carvacrol, and trans-cinnamaldehyde (Yuan et al. 2018), *S. enterica* after desiccation and starvation in peanut oil (Deng, Li, and Zhang 2012), and *L. monocytogenes* under cold stress on vacuum-packaged smoked salmon (Tang et al. 2015).

Understanding the molecular mechanism of synergistic antimicrobial combinations can provide clues to explore novel and unique antimicrobial treatment. Multiple targets of combined antimicrobials can reside in the same or different pathways. Some multi-target antibiotic combinations, such as penicillin and clavulanate, are used to treat bacterial infections (Jia et al. 2009). In addition, plants are rich sources for new antimicrobials to be used individually or in combination against antibiotic-resistant bacteria. For example, combining membrane-disrupting plant-based compounds (*e.g.*, phenolics) with antibiotics (*e.g.*, amikacin, ceftriaxone, cephradine, methicillin, and imipenem) resulted in synergism due to the fast and easy access for antibiotics to the damaged bacterial cell wall (Hemaiswarya and Doble 2010; Amin et al. 2015; Oh and Jeon 2015). As another example, combining efflux pump inhibitor(s) with an intracellular antimicrobial synergistically inactivated microorganisms that use efflux pumps to remove antimicrobials (Oh and Jeon 2015). Taken together, combining antimicrobials that work on the basis of different

mechanisms not only minimizes the development of resistance by single antimicrobial agents but also facilitates discovering new mechanisms used to design novel antimicrobial combinations. The aim of this study was to investigate the phenotypic and transcriptomic changes in *C. jejuni* by single and combined antimicrobials treatments.

3.3 Material and methods

3.3.1 Chemical reagents and culture methods

Cinnamon oil and curcumin powder were obtained from Sigma-Aldrich (Oakville, ON). ZnO NPs powder with the particle size of 40-100 nm and surface area of 12-24 m^2/g was purchased from Alfa Aesar™ (Haverhill, MA). *C. jejuni* was routinely grown on Mueller-Hinton (MH) agar (OXOID™, Nepean, ON) plates with 5% defibrinated sheep blood supplementation (Alere™, Stittsville, ON) for 48 h. For the preparation of bacterial culture, individual colonies were suspended in MH broth and then incubated at 37°C in a microaerobic condition (85% N_2, 10% CO_2, 5% O_2) for 18 h with constant shaking at 175 rpm. Liquid cultures were routinely adjusted at an initial concentration of 1×10^9 CFU/mL.

3.3.2 RNA-Seq

Synergistic interactions between plant-based antimicrobials and metal oxide nanoparticles against *C. jejuni* were identified in Chapter 2 (Hakeem et al. 2019). These included cinnamon oil/encapsulated curcumin, ZnO NPs/encapsulated curcumin, and cinnamon oil/ZnO NPs/encapsulated curcumin. In the current study, we investigated the molecular mechanisms of those synergistic combinations against *C. jejuni*. *C. jejuni* F38011 culture (10^8 CFU/mL) was either treated or untreated (negative control) for 15 min with single or combined antimicrobials at

37°C in a microaerobic condition for 15 min with constant shaking at 175 rpm. Adjusted concentrations of overnight cultures were diluted by 10-fold and incubated for 2 h in new fresh MH medium. The antimicrobials treatments were interduce at the same incubation conditions. Single treatments were conducted at bactericidal concentrations of 8 ppm of tetracycline (positive control), 50 ppm of cinnamon oil, 100 ppm of ZnO NPs, and 40 ppm of encapsulated curcumin. Cinnamon oil/encapsulated curcumin combined at 12.5 ppm/20 ppm and encapsulated curcumin/ZnO NPs combined at 20 ppm/25 ppm were developed as dual antimicrobial treatments. Cinnamon oil/encapsulated curcumin/ZnO NPs combined at 12.5 ppm/20 ppm/25 ppm was developed as triple antimicrobial treatment.

Treated and untreated bacterial cultures were placed on ice immediately after incubation. Bacterial cells were then collected by centrifugation at 8,000 ×*g* for 3 min at 4°C. Total RNA was extracted from either treated or untreated *C. jejuni* cells using the GENEzol™ TriRNA Pure Kit (Froggabio, North York, ON). Extraction was performed following the manufacturer's protocol with some modifications. Briefly, bacterial cells were exposed to lysozyme and GENEzol™ reagent, followed by DNase treatment for total RNA extraction, homogenization, and purification, respectively. Each sample was loaded into a spin column for RNA binding and washing. Purified RNA sample was individually examined using Nanodrop1000 spectrophotometer (ThermoFisher, Massachusetts, USA), gel electrophoresis, and Agilent bioanalyzer RNA nano chip (Agilent Technologies, Santa Clara, CA) for assessing both quantity and quality.

The Hiseq 2500 system (Illumina, CA) was used for conducting a high-throughput transcriptome analysis of negative control (untreated), positive control (tetracycline), single and combined antimicrobial treatments of cinnamon oil, encapsulated curcumin, and ZnO NPs against *C. jejuni*. RNA-Seq data analysis involving alignment was performed according to a previous

report (Trapnell et al. 2012) and the whole genome of *C. jejuni* NCTC 11168 was used as the reference (Parkhill et al. 2000). Mapping transcripts to the reference genome, calculation of transcription levels, and visualization of the differentially expressed genes were performed using the CLC genomics workbench software (CLCBio, Cambridge, MA, United States). A cut-off of ≤0.05 and ≥2 log fold change was applied for data visualization. The molecular functions and processes of deferentially expressed genes were identified using the Gene Ontology (GO) term tools (http://geneontology.org) (Ashburner et al. 2000).

3.3.3 Quantitative polymerase chain reaction (qPCR)

Several genes were used to validate RNA-Seq results by conducting qPCR (**Table 3.1**). RNA samples were standardized to ~500 ng. The synthesis of cDNA was performed using the Superscript VILO Synthesis kit (Invitrogen, CA) and the synthesis program followed several steps: 25°C for 10 min, 42°C for 60 min, 85°C for 5 min, and then hold at 4°C. The cDNA samples were mixed with the Power SYBR Green PCR Master Mix (Thermofisher, MA) and qPCR was run on an ABI Prism 7000 Fast Instrument (Life technologies, CA). Fold change of differentially expressed genes was calculated using the Ct values (Schmittgen and Livak 2008). Housekeeping *rpoA* gene was used as an internal control (Ritz et al. 2009).

Table 3.1. A list of selected genes, functions and primer sequence used for RNA-Seq data validation by qPCR.

Gene tag	Function	Primer sequence (5' – 3')	
Cj0061c	RNA polymerase sigma factor for flagella operon	F	TGCTAAAAGAGCCGCCTAAA
		R	GCTGGCATGTAAGAAAGCACA
Cj0371c	flagella surface structure protein and motility	F	GTGCAAGTGGCGGGTCTATTT
		R	AAACCAATAATAGCGGGCAACA
Cj0372c	glutathionylspermidine synthase activity	F	CTGCAGCGCAAGAAGTGATTA
		R	AGGCTTGCCATCAAGTCCAC
Cj0481	protein folding and lysine synthesis	F	GCAAGTGGCGGGTCTATTT
		R	TCAAACCAATAATAGCGGGCAA
Cj0759	protein folding	F	TTTAGCGCAAACGAGGGAGA
		R	AGCTCTTGCTTGATGGCTTCT
Cj0888c	valyl-tRNA aminoacylation	F	TTTAGCGCAAACGAGGGAGA
		R	AGCTCTTGCTTGATGGCTTCT
tRNA-Leu	Leucine ligase, and protein assembly	F	CGAGTGGTGAAACTGGTAGAC
		R	GGACTCGAACCGACACAAG
Cj1221	protein folding	F	TATGGGCGCTTCACTTGTAA
		R	CCTCGATAGGATTTGCACCT
Cj1595	RNA polymerase A, transcription initiation	F	CGAGCTTGCTTTGATGAGTG
		R	AGTTCCCACAGGAAAACCTA

3.3.4 Scanning Electron Microscopy (SEM)

SEM was used to determine the interaction between single, dual, and triple antimicrobial treatments of cinnamon oil, ZnO NPs, and encapsulated curcumin against *C. jejuni*. An adjusted bacterial culture of ~10^8 CFU/mL was either treated or untreated (negative control) with single or combined antimicrobials at 37°C in a microaerobic condition for 3 h with constant shaking at 175 rpm. Single treatments were conducted at bactericidal concentrations of 18 ppm of cinnamon oil, 27 ppm of ZnO NPs, and 32 ppm of encapsulated curcumin. Cinnamon oil/encapsulated curcumin were combined at 6.25 ppm/16 ppm and ZnO NPs/encapsulated curcumin were combined at 13.5 ppm/16 ppm. The antimicrobial concentration used for SEM was lower than that for RNA-Seq (15 min) because the former treatment time (3 h) was longer than the latter one (15 min).

3.4 Results

C. jejuni clinical strain F38011 was used as the bacterial model. The cDNA library was constructed and RNA-Seq was run on the HiSeq 2500 system. Sequencing length was 75 bp and each library yielded in an average of 29.4 million reads. The reads were mapped to the reference genome of *C. jejuni* NCTC 11168 of >1,600 genes. The sequence coverage at the whole genome and gene levels indicated good quality of the RNA-Seq data (**Figure 3.S1**). Differentially expressed genes after various single and combined antimicrobial treatments were identified. Each single and combined treatment had a unique transcriptional pattern of differentially expressed genes. A heatmap combined with hierarchical clustering analysis was generated using the differentially expressed genes of the whole bacterial genome (**Figure 3.1A**). Dual and triple treatments were similar but not identical, while every single treatment showed a distinct pattern of differentially expressed genes. Transcriptional profile of tetracycline (positive control) showed the

highest level of either over- or under-expression compared to other single and combined antimicrobial treatments. Some gene overlapping was identified between different single treatments at a cut-off of ≤0.05 and ≥2 log fold change (**Figure 3.1B**). Gene ontology analysis was performed to compare the adaptive mechanisms of single treatments (**Table 3.2-3.4**) with the combined treatments against *C. jejuni* (**Table 3.5**). Details will be provided in each of the following sections.

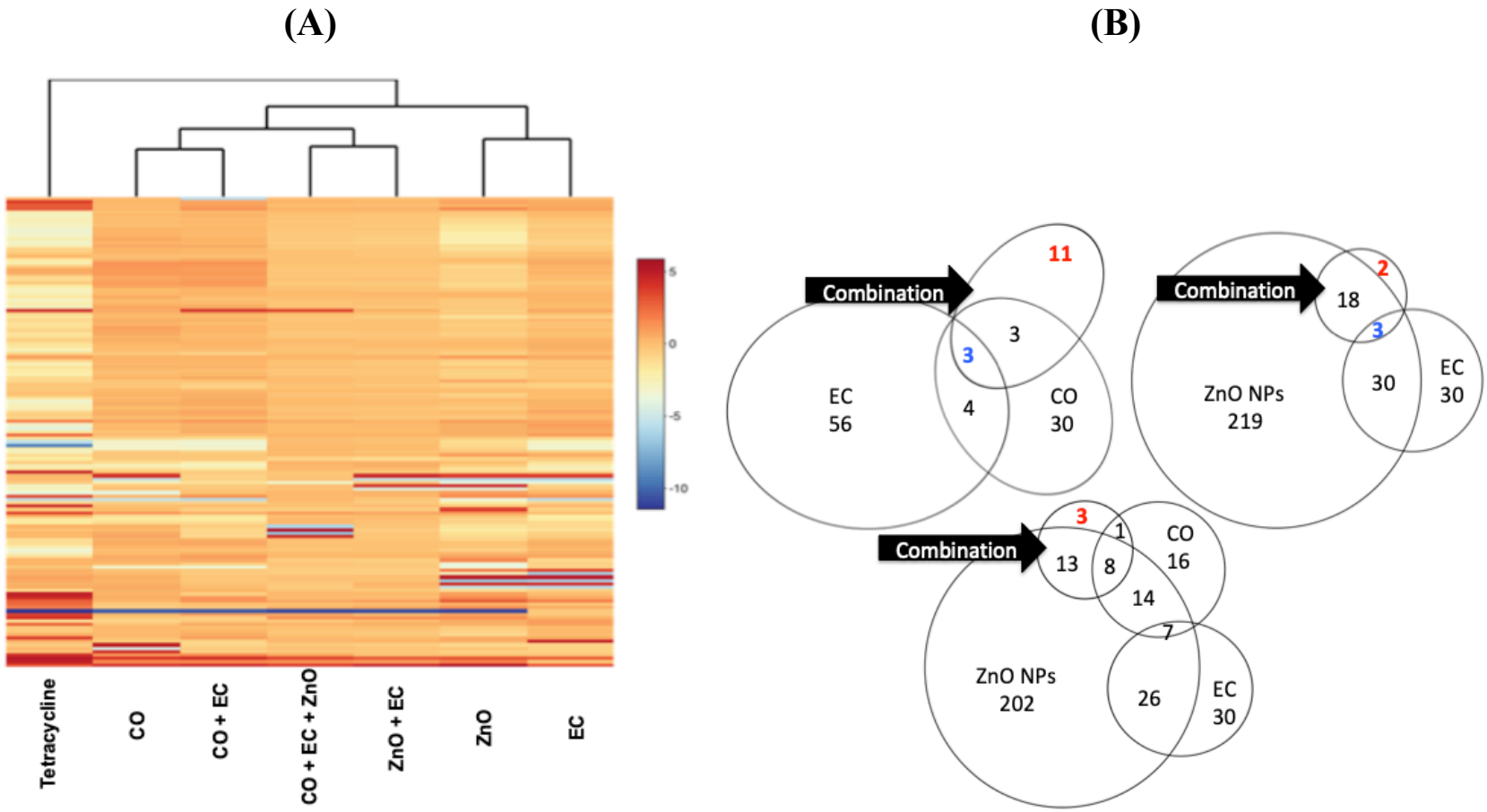


Figure 3.1. Heatmap (A) and Venn diagram (B) of differentially expressed genes in *C. jejuni* F38011 after 15 min of single and combined antimicrobial treatments at 37°C in a microaerobic condition. CO denotes cinnamon oil, ZnO NPs denote zinc oxide nanoparticles, and EC denotes encapsulated curcumin. The color key in (A) shows the color score of differently expressed genes from -10 to 5 fold change. Red color in (B) shows the number of unique differentially expressed genes by combined treatments only. Blue color in (B) shows the number of overlaying differentially expressed genes by individual and combined treatments.

3.4.1 Transcriptomic and phenotypic changes in *C. jejuni* by single antimicrobial treatment

Single treatment of cinnamon oil altered the expression of 39 genes in *C. jejuni*. These include genes encoding for proteins dedicated to different functions, including membrane synthesis, cell permeability, motility, and chemotaxes (**Table 3.2**). Most affected genes were involved in the synthesis and assembly of several extracellular components. For example, the Cj0144 gene encoding for chemotaxis signal transduction protein in *C. jejuni* (Cj0144) was upregulated by 2.26-fold change. Overexpression level of the Cj0481 gene involved in protein folding (19.46-fold change) indicated that membrane proteins were unfolded due to the treatment by cinnamon oil. Many genes that participate in the synthesis of amino acids including lysine, arginine, and aspartate were over-expressed by over 2 folds. The Cj0147c gene that is involved in cell maintenance, redox homeostasis, and glycerol ether metabolism was over-expressed by 2.16-fold change. Moreover, cinnamon oil altered gene expression of the Cj1621, which is involved in DNA replication and repair system, by 31.63-fold change. In contrast, the gene expression level of flagellum encoding protein (Cj0371) was under-expressed by -9.09. Other genes that participate in the synthesis of membrane and plasma proteins such as Cj0865 and Cj01621 were also under-expressed. The Cj0482 gene that is involved in the synthesis of transmembrane transport protein and cell permeability was under-expressed by -36.11 folds. *C. jejuni* treated cells had a rod shape as examined by SEM (**Figure 3.2B**), which was different from the untreated cells (**Figure 3.2A**). However, no obvious cell membrane damage was observed. Altogether, cinnamon oil altered the expression levels of *C. jejuni* genes involved in the synthesis, assembly, and permeability of cell membrane as well as motility and chemotaxis.

Table 3.2. Differentially expressed genes in *C. jejuni* treated by cinnamon oil (50 ppm).

	Gene tag	GO Term and/or Function	Fold change
Over-expression	Cj0144	GO:0007165; chemotaxis signal transduction protein	2.26
	Cj0481	GO:0051082; unfolded protein binding and lysine biosynthesis	19.46
	Cj0145	Twin-arginine translocation protein	2.33
	Cj0150c	GO:0004069; aspartate biosynthesis and aminotransferase protein	2.05
	Cj0762c	GO:0004069; aspartate biosynthesis	2.06
	Cj0147c	GO:0045454; cell redox homeostasis, GO:0006662; glycerol ether metabolic process	2.16
	Cj1620	GO:0003677; DNA replication and repair	31.64
Under-expression	Cj0371	Lipoprotein involved in flagellar motility	-9.09
	Cj0426	GO:0016887; ATPase activity and ABC transporter	-6.49
	Cj0484	GO:0055085; major facilitator superfamily	-5.16
	Cj0482	Transmembrane transport protein	-36.11
	Cj0865	GO:0005886; plasma membrane, GO:0016021; integral component of membrane	-2.23
	Cj1621	Periplasmic protein	-23.41

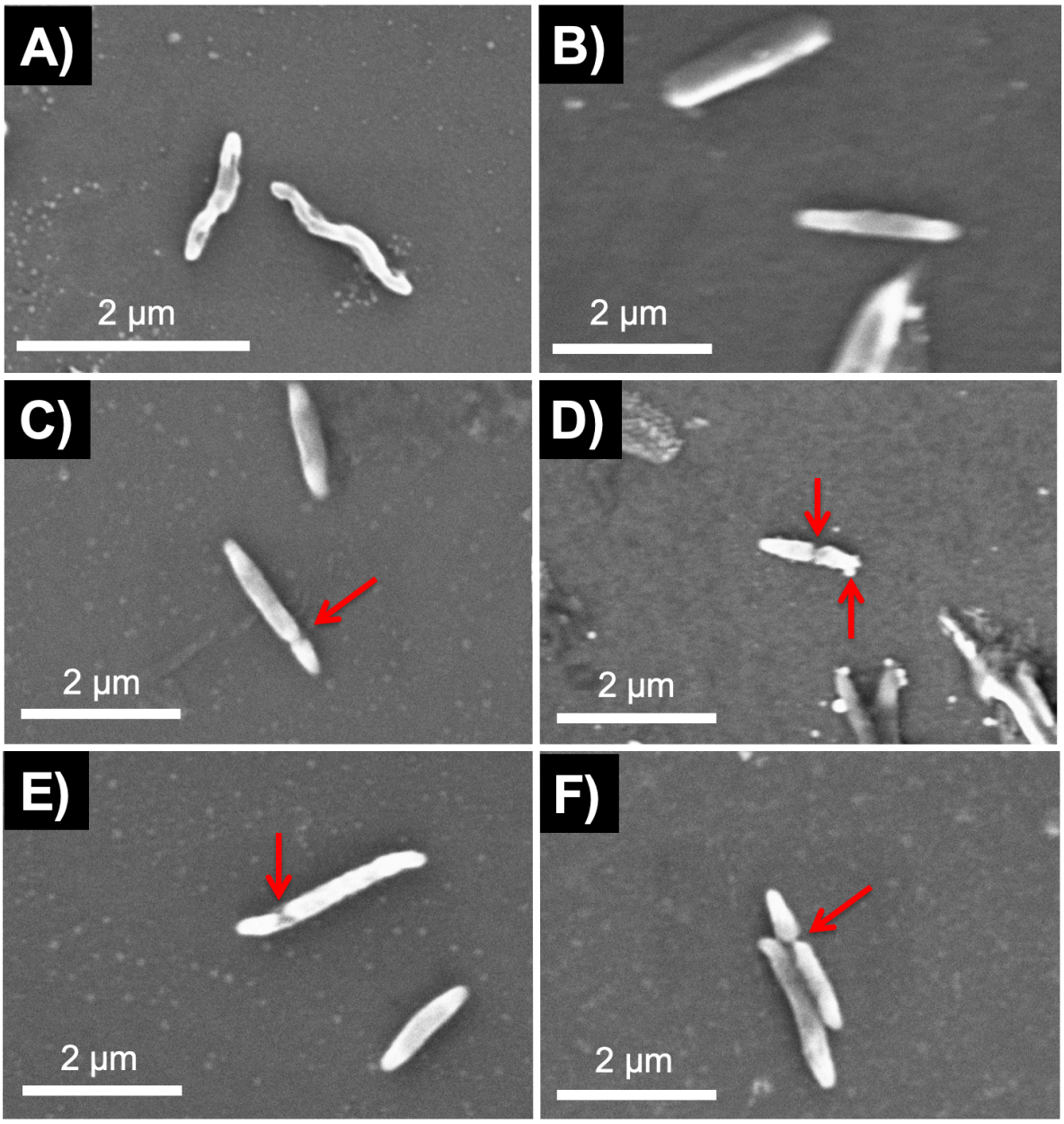


Figure 3.2. Representative scanning electron microscopic (SEM) images of untreated (A) and treated *C. jejuni* with cinnamon oil (B), encapsulated curcumin (C), zinc oxide nanoparticles (D), cinnamon oil along with encapsulated curcumin (E), and encapsulated curcumin along with zinc oxide nanoparticles (F).

Encapsulated curcumin altered the expression of 63 different genes in *C. jejuni*. Similar to cinnamon oil, encapsulated curcumin had several extracellular and intracellular targets. Several genes related to the membrane and flagella synthesis were over-expressed (**Table 3.3**). These included the Cj0481 and Cj942c genes involved in protein folding and membrane permeability, respectively. In addition, the Cj1497c flagellum encoding protein gene was over-expressed by 27.54 folds. Many genes related to intracellular components that are involved in respiration and energy production were under-expressed. For example, the Cj426 gene related to ATPase activity was under-expressed by over -2 folds. Other genes involved in the synthesis of DNA and plasma membrane proteins were under-expressed (*e.g.*, Cj0864, Cj0865 and Cj0888c). SEM images showed that encapsulated curcumin changed the cell from spiral to rod shape and caused some damages on bacterial outer cell wall (**Figure 3.2C**). Altogether, encapsulated curcumin was able to alter the expression levels of genes involved in the metabolism of extracellular and intracellular components of *C. jejuni*.

Table 3.3. Differentially expressed genes in *C. jejuni* treated by encapsulated curcumin (40 ppm).

	Gene tag	GO Term and/or Function	Fold change
Over-expression	Cj1497c	GO:0071973; flagellum-motility	27.54
	Cj0481	GO:0051082; unfolded protein binding and lysine biosynthesis	14.01
	Cj1626	periplasmic protein	6.95
	Cj0942c	GO:0065002; intracellular transmembrane transport	11.90
	Cj0304c	GO:0009102; biotin synthesis protein	2.05
	Cj1202	GO:0009086; methionine synthetic process	3.57
Under-expression	Cj0484	GO:0055085; major facilitator superfamily	-14.87
	Cj0426	GO:0016887; ATPase activity and ABC transporter	-4.80
	Cj0862c	GO:0016829; lyase activity, GO:0016874; ligase activity	-2.11
	Cj0864	GO:0005886; plasma membrane, GO:0016021; integral component of membrane	-3.57
	Cj0865	GO:0005886; plasma membrane, GO:0016021; integral component of membrane	-2.11
	Cj0888c	GO:0006438; valyl-tRNA aminoacylation	-2.17

ZnO NPs disrupted bacterial cell wall and altered the expression of 270 genes in *C. jejuni*. These included a broad range of gene categories related to cell envelop, flagella, oxidative stress, broad regulatory function proteins, heat shock proteins, ions transport, as well as the biosynthesis of amino acids and fatty acids (**Table 3.4**). Up to 33.36 folds of increase in the expression level of genes was responsible for the regulation of both outer- and plasma membrane proteins (Cj0944c, Cj1626c, Cj0942c, Cj0946). For example, the Cj0946 gene that is involved in membrane lipoprotein and channel protein synthesis was over-expressed by 20.97 folds. In addition, RNA polymerase sigma factor for flagella operon Cj0061c was over-expressed by 3.22 folds after the treatment by ZnO NPs. Other genes involved in broad regulatory functions (*i.e*., Cj0422 and Cj1387c) were over-expressed, indicating a shift in the transcriptome profile of *C. jejuni* after treatment with ZnO NPs. Many genes involved in the synthesis of amino acids (Cj0130 and Cj0134) and lipid biosynthesis (Cj0132 and Cj1409) were under-expressed by ZnO NPs treatment. These observations indicate that ZnO NPs had a broad effect against multiple membrane targets that were different from the treatment by cinnamon oil and encapsulated curcumin. SEM images showed that ZnO NPs were able to disrupt *C. jejuni* cells (**Figure 3.2D**). ZnO NPs were accumulated on the surface of *C. jejuni* cells and cell size shrunk to almost half of the untreated cells.

Table 3.4. Differentially expressed genes in *C. jejuni* treated by ZnO NPs (100 ppm).

	Gene tag	GO Term and/or Function	Fold change
Over-expression	Cj0061c	GO:0006352; RNA polymerase sigma factor for flagellar operon	3.22
	Cj1339c	GO:0071973; flagellum-motility	2.60
	Cj0481	GO:0051082; unfolded protein binding and lysine biosynthesis	15.21
	Cj0944c	Preplasmic protein	33.36
	Cj1626c	Preplasmic protein	8.85
	Cj0942c	GO:0065002; intracellular protein transmembrane transport	3.75
	Cj0946	Membrane lipoproteins and porins	20.97
	Cj0422c	Broad regulatory functions	2.21
	Cj1387c	Broad regulatory functions	2.78
	Cj1202	GO0009086; methionine biosynthesis	7.33
Under-expression	Cj0484	GO:0055085; major facilitator superfamily	-14.78
	Cj0128c	GO:0046854; phosphatidylinositol phosphorylation	-2.27
	Cj0129c	GO:0043165; outer membrane assembly	-3.22
	Cj0130	GO:0006571; tyrosine biosynthesis	-2.85
	Cj0134	GO:0009088; threonine biosynthesis	-4.73
	Cj0132	GO:0009245; lipid biosynthesis	-3.88
	Cj1409	GO:0006633; fatty acids biosynthesis	-2.04
	Cj0195	GO:0044780; flagellum assembly	-3.10

3.4.2 Transcriptomic and phenotypic changes in *C. jejuni* by combined antimicrobial treatments

Some gene overlapping was observed between single and combined treatments, indicating that they shared some of the same gene targets (**Figure 3.3**). More interestingly, some unique genes were only differentially expressed by some but not all the combined antimicrobial treatments (**Table 3.5**). Combination of cinnamon oil/encapsulated curcumin altered 11 genes that were not affected by any of the two singe treatments. Gene Ontology analysis indicated that combined antimicrobials targeted unique biological pathways. Both single treatments of cinnamon oil and encapsulated curcumin as well as their combination over-expressed or under-expressed genes that are involved in flagellum-motility (**Figure 3.4**). These included the Cj0371 gene involved in flagella lipoprotein synthesis and the Cj1497c gene involved in flagellum motility. Moreover, the Cj0061c gene that encodes for RNA polymerase sigma factor for flagellar operon was only over-expressed in the combined treatment of cinnamon oil and encapsulated curcumin. Similarly, an outer membrane protein-encoding gene (Cj0190c) was over-expressed in dual or triple treatments consisting of cinnamon oil and encapsulated curcumin, which was different from that by the single treatments. Damage on *C. jejuni* cell wall was observed using SEM, which was similar to single antimicrobial treatments (**Figure 3.2E**). The gene Cj0484 that encodes for proteins involved in the major facilitator superfamily synthesis was under-expressed by -5.16 and -14.87 folds after single treatment with cinnamon oil and encapsulated curcumin, respectively (**Table 3.2** and **3.3**). In contrast, the combined treatment of these two antimicrobials altered the expression level of the Cj0484 gene by -2.79 folds only (**Table 3.5**).

Combining encapsulated curcumin with ZnO NPs altered the expression levels of genes involved in the synthesis and maintenance of the bacterial cell wall (**Table 3.5**). For example, the

expression level of the Cj0481 (protein folding gene) increased by this combination from 14.01 to 23.57-fold change compared to single treatments. Another gene involved in the synthesis of membrane lipoprotein and porins (Cj0423) was only under expressed in the combined treatment by -2.29-fold change (**Table 3.5**), but not in any of the single treatments of encapsulated curcumin or ZnO NPs. Damage on bacterial cell wall was observed using SEM, which was also similar to that by single antimicrobial treatments (**Figure 3.2C, D, and F**).

Tertiary combination of cinnamon oil/encapsulated curcumin/ZnO NPs had uniquely altered the expression level of genes involved in the synthesis of an outer membrane protein (Cj0190c), bacterial cell wall permeability (Cj0888c; ABC transporter and ATP binding protein), and protein translation (tRNA-Leu; leucine ligase) (**Table 3.5**). Affected genes by the tertiary combination had an impact on the regulation of many other genes involved in amino acid biosynthesis, molecule transportation, and membrane permeability (**Figure 3.9**). The effect of combined treatments affected different biological processes and was more selective compared to all single treatments (**Figure 3.3-3.5**). In contrast, many single treatments altered the expression of a broad range of genes involved in different biological processes, such as signal transduction, metal ion transport, cell redox hemostasis, protein folding, flagellum motility and regulation of pH. In addition, we validated that RNA-Seq results of several differentially expressed genes after treatments with cinnamon oil, encapsulated curcumin and their combinations were consistent to the corresponding qPCR results (**Figure 3.6**).

Table 3.5. Differentially expressed genes in *C. jejuni* treated by tertiary combination of cinnamon oil (CO), encapsulated curcumin (EC) and ZnO NPs (ZnO).

Gene tag	GO Term and function	Fold change		
		CO/EC	EC/ZnO	CO/EC/ZnO
Cj0061c	GO:0006352; RNA polymerase sigma factor for flagellar operon	2.46	1.32	1.28
Cj0372	GO:0008885; glutathionylspermidine synthase, glutathione metabolism, antioxidant activity	-9.06	1.04	1.09
Cj0423	GO:0016021; membrane lipoproteins and porins	-1.28	-2.29	-1.96
Cj0484	GO:0055085; major facilitator superfamily	-2.79	1.17	-9.21
Cj0371	GO:0005886; plasma protein	-10.98	-1.01	1.02
Cj0190c	Outer membrane protein	18.62	1.33	13.39
tRNA-Leu	Leucine ligase, and protein assembly	-2.08	-1.49	41.00
Cj0888c	GO:0006438; valyl-tRNA aminoacylation	-2.12	-1.17	20.74
Cj0481	GO:0051082; unfolded protein binding and lysine biosynthesis	-1.66	23.57	-1.14

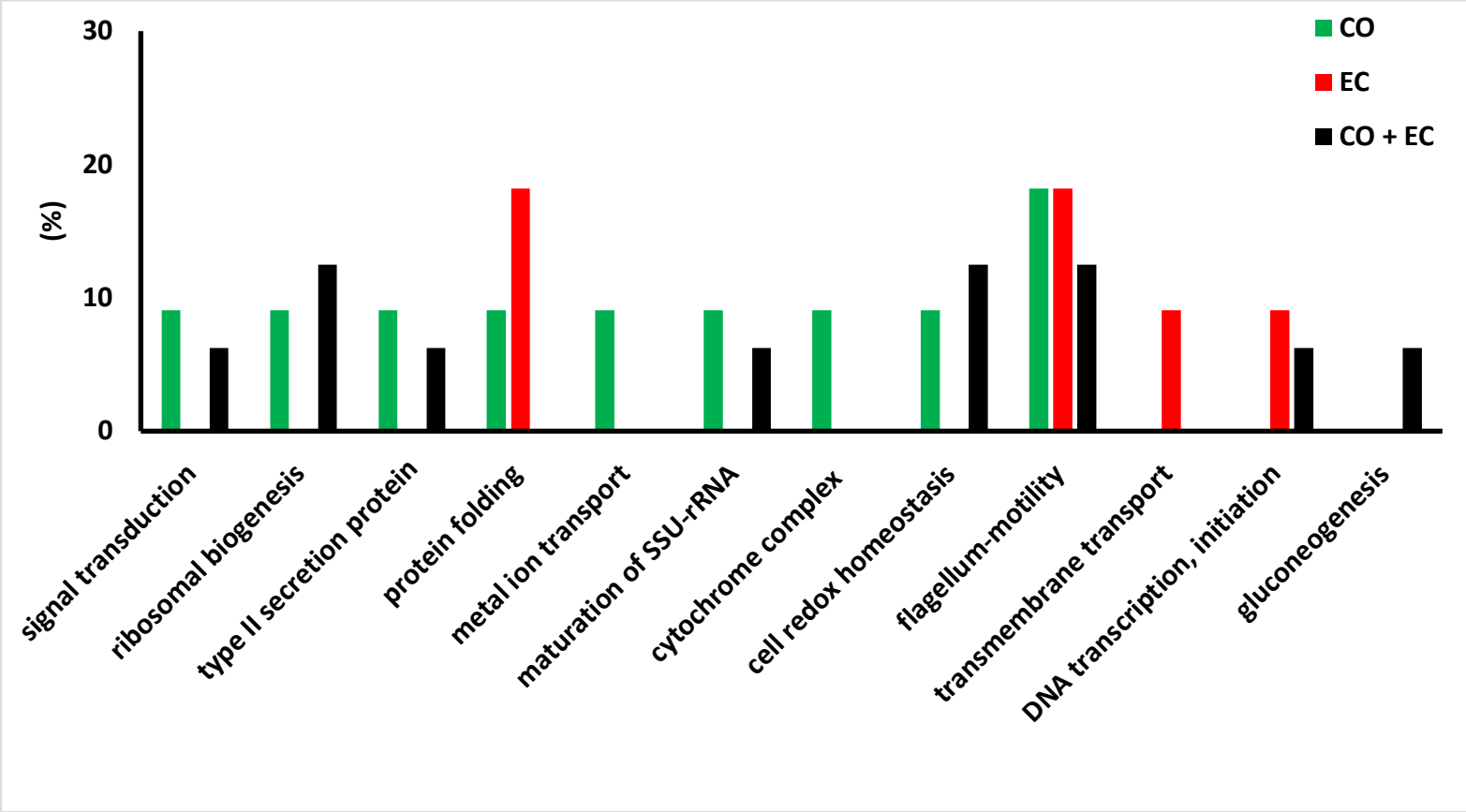


Figure 3.3. Biological processes of Gene Ontology of overexpressed genes in *C. jejuni* F38011 after 15 min of single and dual antimicrobial treatments at 37°C in a microaerobic condition. CO denotes cinnamon oil and EC denotes encapsulated curcumin.

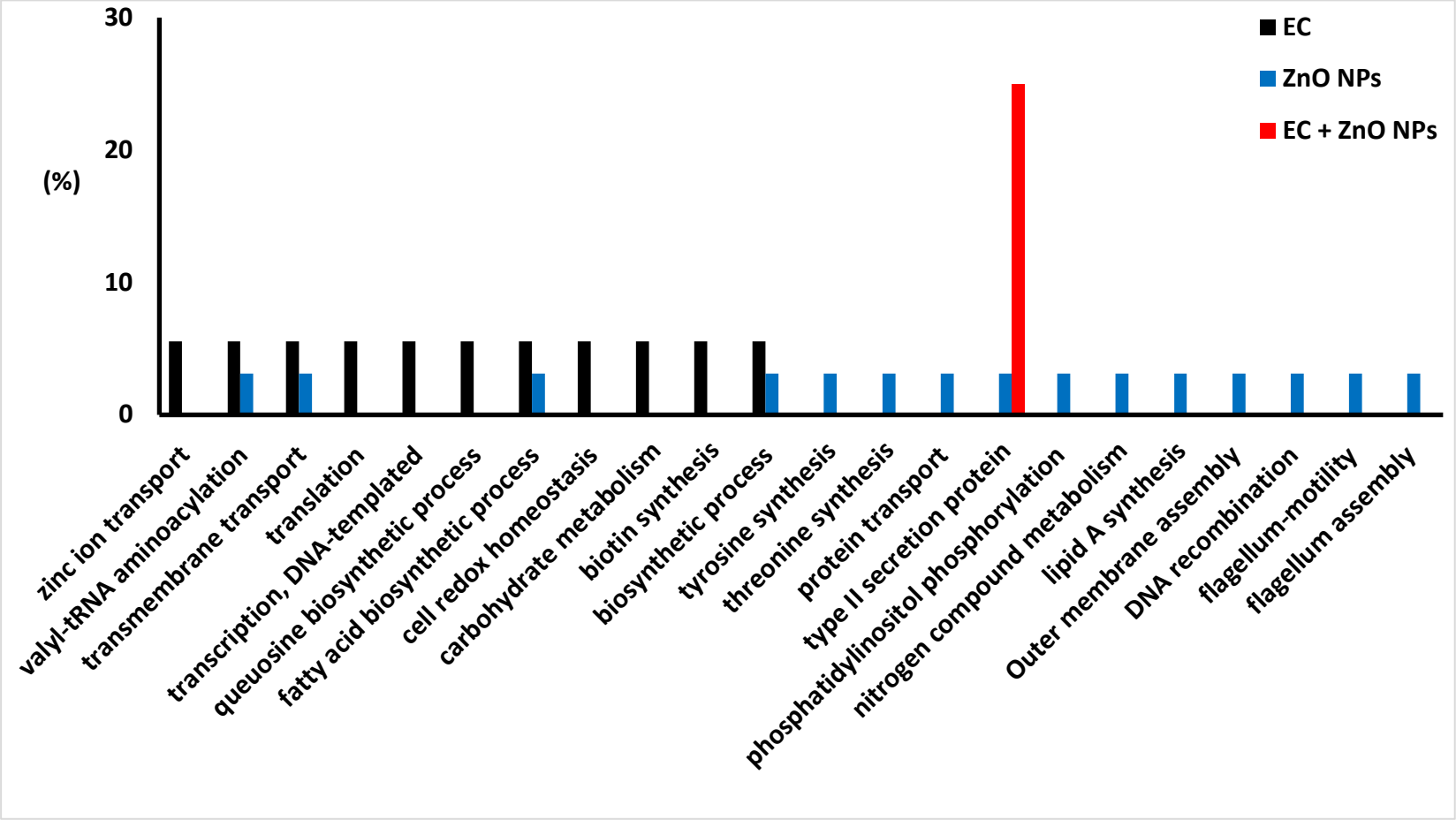


Figure 3.4. Biological processes of Gene Ontology of under-expressed genes in *C. jejuni* F38011 after 15 min of single and dual antimicrobial treatments at 37°C in a microaerobic condition. EC denotes encapsulated curcumin.

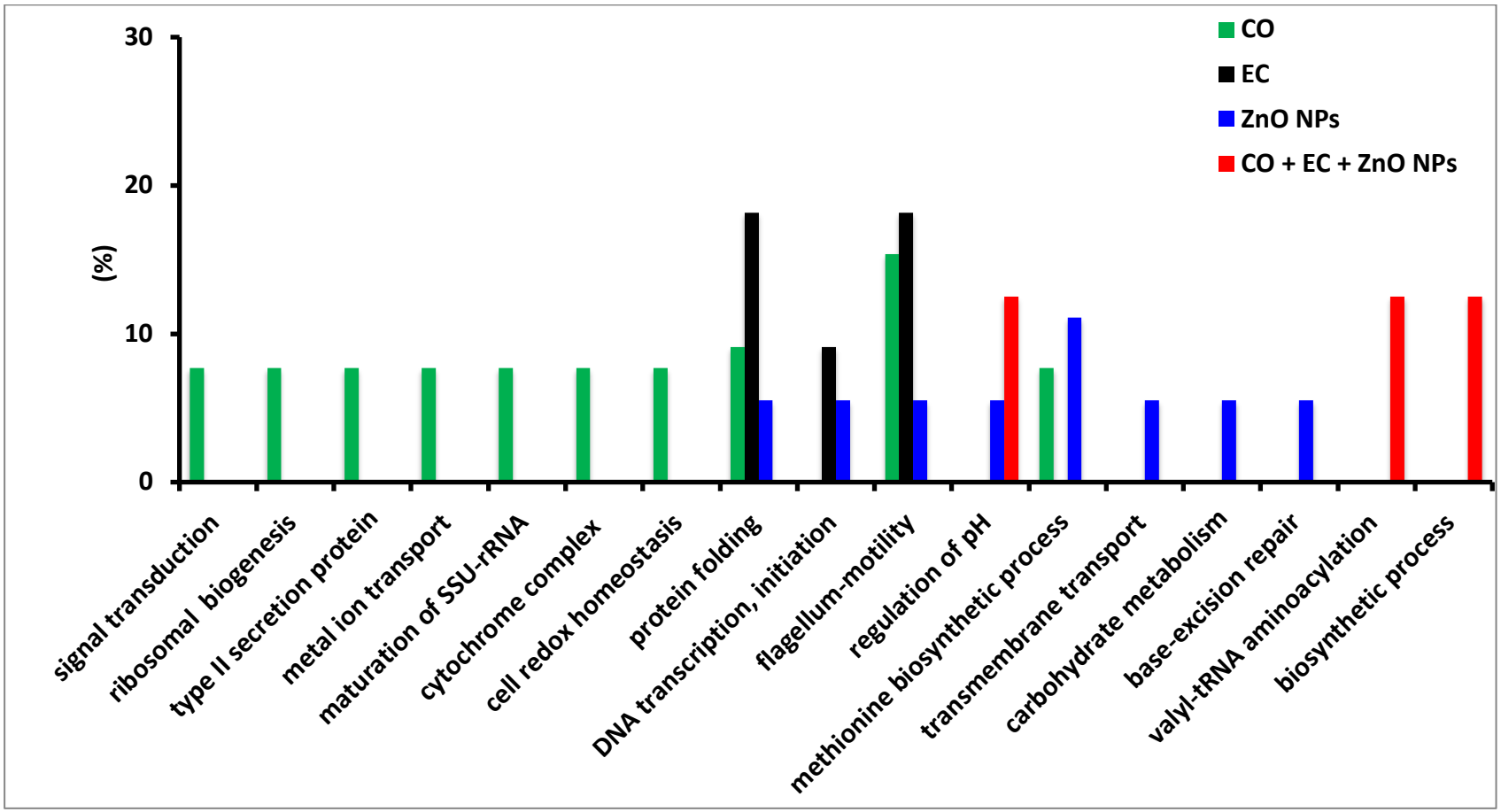


Figure 3.5. Biological processes of Gene Ontology of overexpressed genes in *C. jejuni* F38011 after 15 min of single and triple antimicrobial treatments at 37°C in a microaerobic condition. CO denotes cinnamon oil and EC denotes encapsulated curcumin.

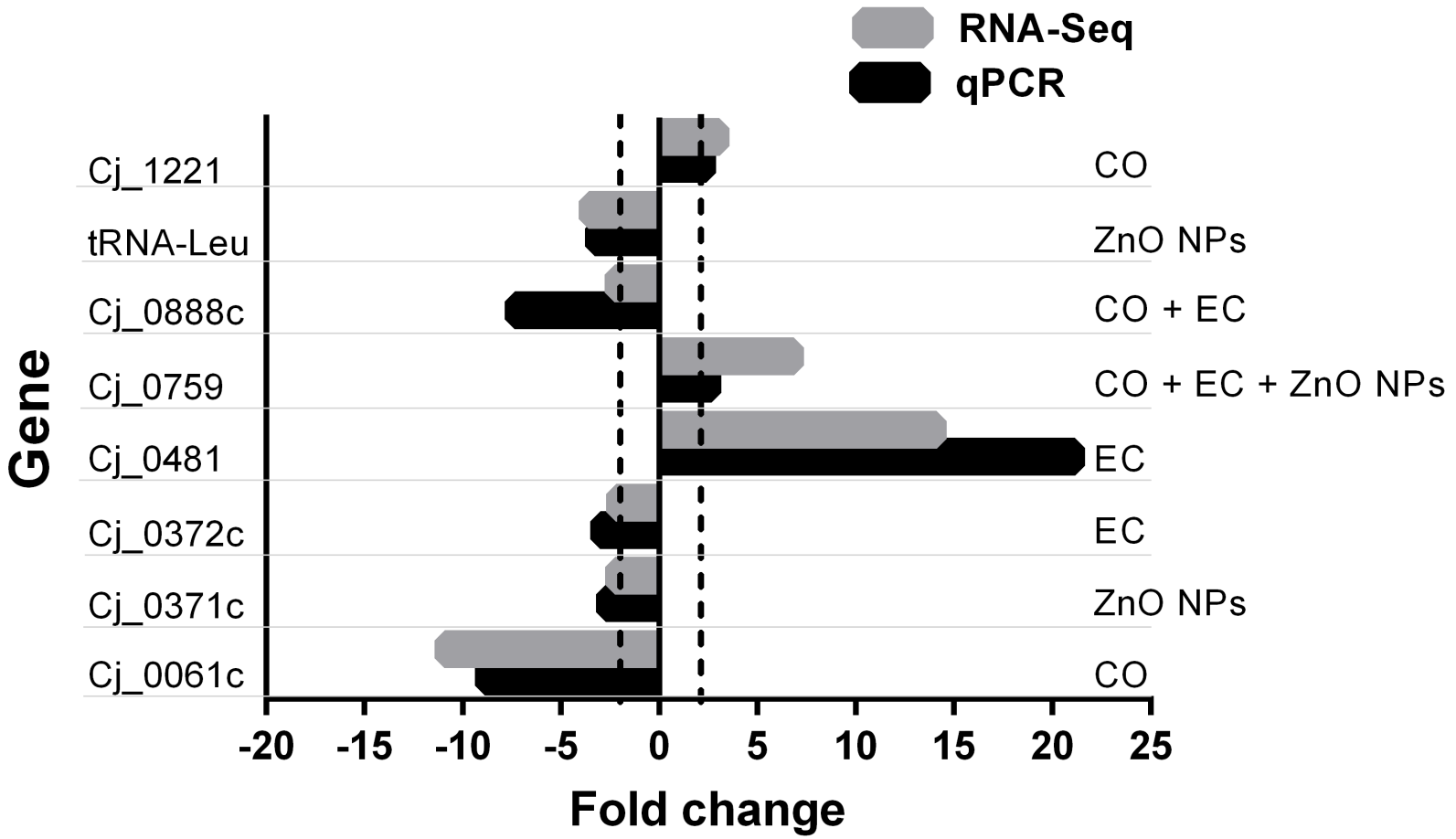


Figure 3.6. Validation of RNA-Seq data by using qPCR. Expression levels of nine genes were selected from the treatment of cinnamon oil, encapsulated curcumin or their combinations.

3.5 Discussion

The annual numbers of illnesses linked to several foodborne pathogens including *Salmonella*, *Listeria*, and *E. coli* O157:H7 have been declined in the past decade due to the improved surveillance systems, new regulations, and more effective intervention strategies (Crim et al. 2014). However, the number of illnesses linked to *Campylobacter* remains high. Developing novel synergistic antimicrobial combinations is important to control *Campylobacter* in the agro-ecosystem. Understanding the molecular adaptive mechanisms of synergistic antimicrobial combinations can effectively inactivate pathogens, reduce the dosage of individual antimicrobials, and potentially minimize the development of antimicrobial resistance. RNA-Seq can provide an unbiased and high-throughput screening of differentially expressed genes to identify the adaptive mechanisms of bacterial cells treated with synergistic antimicrobial combinations. We identified dual and triple synergistic combinations against *C. jejuni* in our previous study (Hakeem et al. 2019). These included cinnamon oil/encapsulated curcumin, ZnO NPs/encapsulated curcumin, and cinnamon oil/ZnO NPs/encapsulated curcumin. In this study, we investigated the adaptive mechanisms of these synergistic combinations against *C. jejuni*.

The antimicrobial mechanisms of actions of cinnamon oil (Zhou et al. 2016; Di Pasqua et al. 2007), curcumin (Kaur et al. 2010; Rai et al. 2008) and ZnO NPs (Xie et al. 2011; Arakha et al. 2015) have been reported in the previous studies. Phenols, terpenes, and aldehydes are the major active compounds among the plant-based antimicrobials. Disruption of bacterial cell wall by plant-based antimicrobials is well known. In addition, hydroxyl group is responsible to alter fatty acids and phospholipids on the outer membrane and can inhibit some important intracellular enzymes. For example, the compositions of membrane fatty acids and/or surface proteins can be affected due to the inhibition of some catalyzing enzymes that participate in their biosynthesis process.

Pasqua and others showed that cinnamaldehyde had the greatest effect on the alteration of fatty acids in both Gram-positive (*B. thermosphacta* and *S. aureus*) and Gram-negative bacteria (*E. coli* O157:H7, *S.* Typhimurium, and *P. fluorescens*) compared to thymol, limonene, eugenol, and carvacrol (Di Pasqua et al. 2007). The percentage of unsaturated fatty acids declined by 41%-67%, while the percentage of one saturated fatty acid (*i.e.*, heptadecanoic acid [C17:0]) increased by >70% after treatment with cinnamaldehyde. In addition, Gill and co-authors identified that cinnamaldehyde could cause cell leakage of small ions due to the alteration of proton motive force without leakage of larger cell components, such as ATP (Gill and Holley 2004). In addition, curcumin as the major phenolic compound in turmeric can cause cell elongation without division due to the inhibition of the FtsZ protein assembly. ZnO NPs mainly act as a membrane-disrupting antimicrobial due to electrostatic interaction between positively charged surface of nanoparticles and negatively charged surface of bacterial cells (Arakha et al. 2015). Taken together, we hypothesize that combining plant-based antimicrobials with each other or with ZnO NPs can synergistically affect multiple molecular targets in *C. jejuni*.

Our results showed that cinnamon oil altered the expression of several genes involved in bacterial cell wall synthesis and cell permeability, including the Cj0144, Cj0371, Cj0481, Cj0482, Cj0484, Cj0865, and Cj1621 genes (**Table 3.2**). SEM validated the morphological variation of *C. jejuni* cells from spiral (**Figure 3.2A**) to rod shape after the treatment with cinnamon oil (**Figure 3.2B**). These findings were in agreement with the results reported by Pasqua and others who observed structural alteration in bacterial cell wall envelope after treatment with cinnamaldehyde (Di Pasqua et al. 2007). Another study showed that cinnamon oil could damage the outer membrane and caused the loss of membrane selective permeability and subsequently cell death (Bouhdid et al. 2010). Using RNA-seq, another study validated that cinnamaldehyde could induce

heat-tolerance adaptive response in *E. coli* O157:H7 (Yuan et al. 2018). Thus, the use of cinnamon oil may induce the risk of developing heat-tolerant bacteria during food processing. Combining cinnamon oil with other antimicrobials could overcome some potential risks associated with its single use in food applications.

Encapsulated curcumin altered the expression levels of genes involved in membrane synthesis and permeability as well as respiration and energy production. These included the Cj0426, Cj0888c, Cj0481, Cj0864, Cj0865, Cj0942, and Cj1626 genes (**Table 3.3**). Previous studies showed that curcumin could block the assembly of the FtsZ protein that is responsible for the formation of the Z ring to allow bacterial cells to divide (Kaur et al. 2010; Rai et al. 2008). Other studies demonstrated that curcumin acted as a non-competitive inhibitor of dehydrogenase involved in the shikimate pathway in *H. pylori* (De et al. 2009; Han et al. 2006). In contrast, encoding FtsZ gene (Cj0696) and shikimate dehydrogenase gene (Cj0405) were not differentially expressed after the treatment with curcumin in our current study. De and co-authors showed that inhibition of different *H. pylori* strains by curcumin did not always depend on the shikimate pathway due to the variation in uptake and efflux ability of different bacterial strains (De et al. 2009). In addition, we observed under-expression (-14.87-fold change) of the Cj0484 gene in *C. jejuni*. Similarly, the same gene was also under-expressed after treatment with either cinnamon oil (-5.16-fold change) or ZnO NPs (-14.78-fold change). This gene is involved in the synthesis of major facilitator superfamily protein that acts as a membrane transporter for small molecules, ions, and drugs. Thus, *C. jejuni* might under-expressed this gene to limit the uptake of individual antimicrobials. A recent study of using steady-state fluorescent spectroscopy and flow cytometry showed that curcumin could damage bacterial cell wall and caused leakage of cytoplasmic content in both Gram-positive (*S. aureus* and *E. faecalis*) and Gram-negative (*E. coli* and *P. aeruginosa*)

bacteria (Tyagi et al. 2015), which was consistent to our current findings as curcumin was identified to alter the expression level of genes involved in protein folding and synthesis of outer and plasma membranes.

Although single treatment of ZnO NPs altered the expression of a broad range of genes coding for extracellular and intracellular proteins, the treatment had some specific and unique targets. For example, the RNA polymerase sigma factor for flagella operon Cj0061c (*FliA*) was over-expressed by 3.22 folds after the treatment with ZnO NPs. This gene is a sigma factor that is necessary for the formation of flagella as it regulates many other genes responsible for flagella synthesis. Sigma factors are proteins utilized in bacteria to inhibit the transcription of specific proteins for adaptation and stability in new environment and niches (Kelly et al. 2001). No other sigma factor encoding gene was affected by any other single antimicrobial treatment.

Plant-based antimicrobials share some common antimicrobial targets, but their working mechanisms are not identical (Hyldgaard, Mygind, and Meyer 2012). Although SEM images showed that both single antimicrobial treatments (*i.e.*, cinnamon oil and encapsulated curcumin) caused some membrane damages (**Figure 3.2B** and **D**), RNA-Seq data indicated that they targeted at different genes coding for different membrane proteins (**Table 3.2** and **Table 3.3**). More interestingly, combining cinnamon oil with encapsulated curcumin against *C. jejuni* resulted in the alteration of expression levels of 11 unique genes that were not affected by any of their single treatment (**Figure 3.1**). These included the RNA polymerase sigma factor for flagellar operon that regulates many other genes involved in flagella synthesis and motility (Cj0061c). In addition, the outer membrane encoding gene (Cj0190c) was over-expressed only in the combined treatment of cinnamon oil and encapsulated curcumin, which could be due to the collective synergistic effect of these two antimicrobials on the same target. In addition, the Cj0484 involved in the synthesis

of major facilitator superfamily proteins was only under-expressed at -2.79-fold change compared to -5.16-fold change and -14.87-fold change by single treatment of cinnamon oil and encapsulated curcumin, respectively. This major facilitator superfamily includes membrane proteins that allow the movement of small molecules from the surrounding environment into bacterial cells. Less transcriptional response of this gene induced by the combined treatment than single treatment indicated that this combined treatment may reduce the potential of *C. jejuni* to develop antimicrobial resistance. Such a synergistic treatment by two plant-based antimicrobial agents can be a good candidate as an alternative to antibiotics used in animal feeds so as to prevent the colonization of *C. jejuni* in live chickens.

Combining encapsulated curcumin with ZnO NPs resulted in unique transcriptional response of *C. jejuni* cells. For example, the Cj0484 coding for a major facilitator superfamily protein was not differentially expressed in the combination of encapsulated curcumin and ZnO NPs (**Table 3.5**), but was under-expressed by -14.78-fold change by the single treatments. This was due to the use of a low concentration of each antimicrobial for the synergistic combination compared to single antimicrobial treatment. Thus, this combination can potentially reduce the development of antimicrobial resistance against single antimicrobial treatments. In addition, this combination uniquely altered the expression level of two genes that were not affected by any single treatment. This included the under-expression (-2.29-fold change) of the Cj0423 gene that is involved in the synthesis of membrane lipoproteins and porins (**Table 3.5**). Many other membrane targets encoding genes were also affected by this combination, which were similar to the effect by their single antimicrobial treatments. For example, the expression level of the Cj0481 (protein folding gene) increased by 23.57 folds by the combination treatment, which was greater than the expression level induced by any of their single treatments. This gene was the target of all the tested

single antimicrobial treatment (**Table 3.3-3.5**) and has interactions with other genes responsible for amino acid synthesis, cell permeability, and cell uptake (**Figure 3.7-3.9**).

As aforementioned, every single antimicrobial agent resulted in a unique and broad antimicrobial transcriptomic response with some overlapping. The effect of three antimicrobials combined covers a broad range of bacterial cell wall components, synthesis of amino acids and inhibition of protein synthesis and translation (**Table 3.3-3.5**). We combined these antimicrobials at a lower concentration than that used for single treatment due to their synergistic interaction, explaining why either dual or triple combination altered the expression of fewer genes compared to that by the single treatment. Although only 25 genes were altered by the tertiary combination, some of these genes are critical to be involved in antimicrobial synergism of this combination. For example, some genes are involved in the synthesis of an outer membrane protein (Cj0190c), bacterial cell wall permeability (Cj0888c; ABC transporter and ATP binding protein), and protein translation (tRNA-Leu; leucine ligase). Increase in cell permeability can enhanced the intracellular concentration of each antimicrobial inside the cells. In addition, affected genes also have interactions with other genes that are responsible for other amino acids synthesis and energy production (**Figure 3.10**).

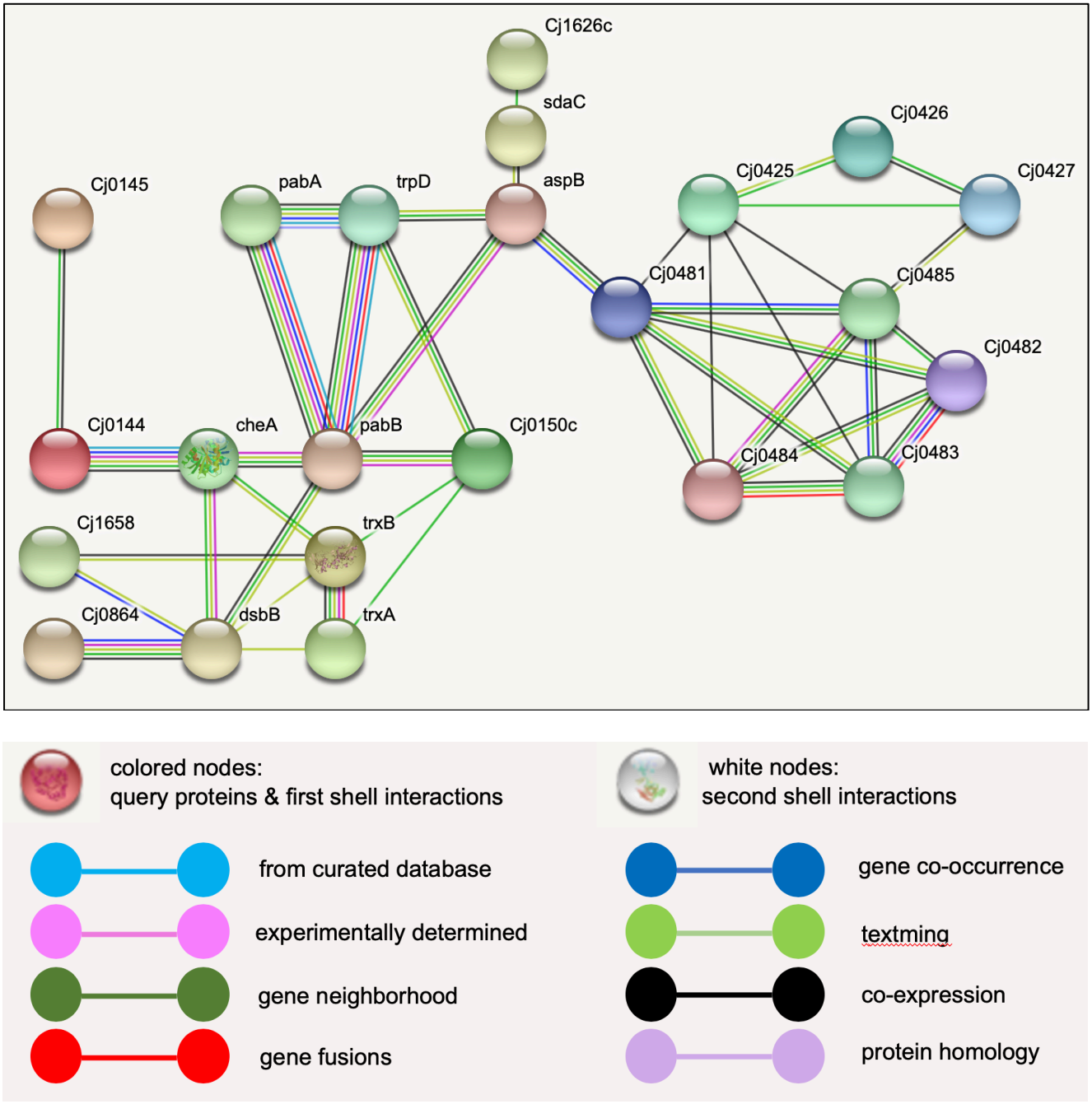


Figure 3.7. *C. jejuni* altered genes by cinnamon oil and their interactions with other relevant genes.

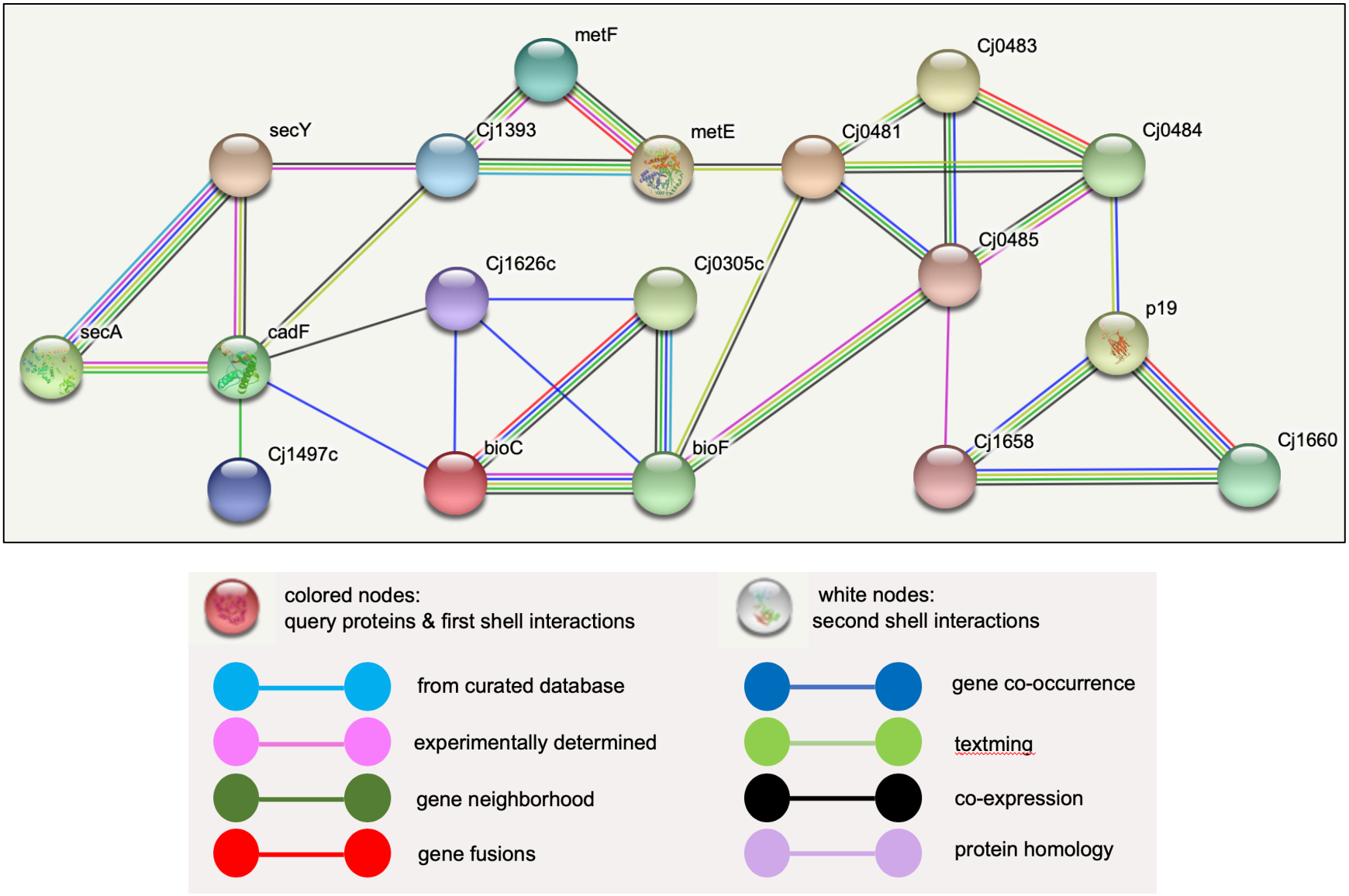


Figure 3.8. *C. jejuni* altered genes by encapsulated curcumin and their interactions with other relevant genes.

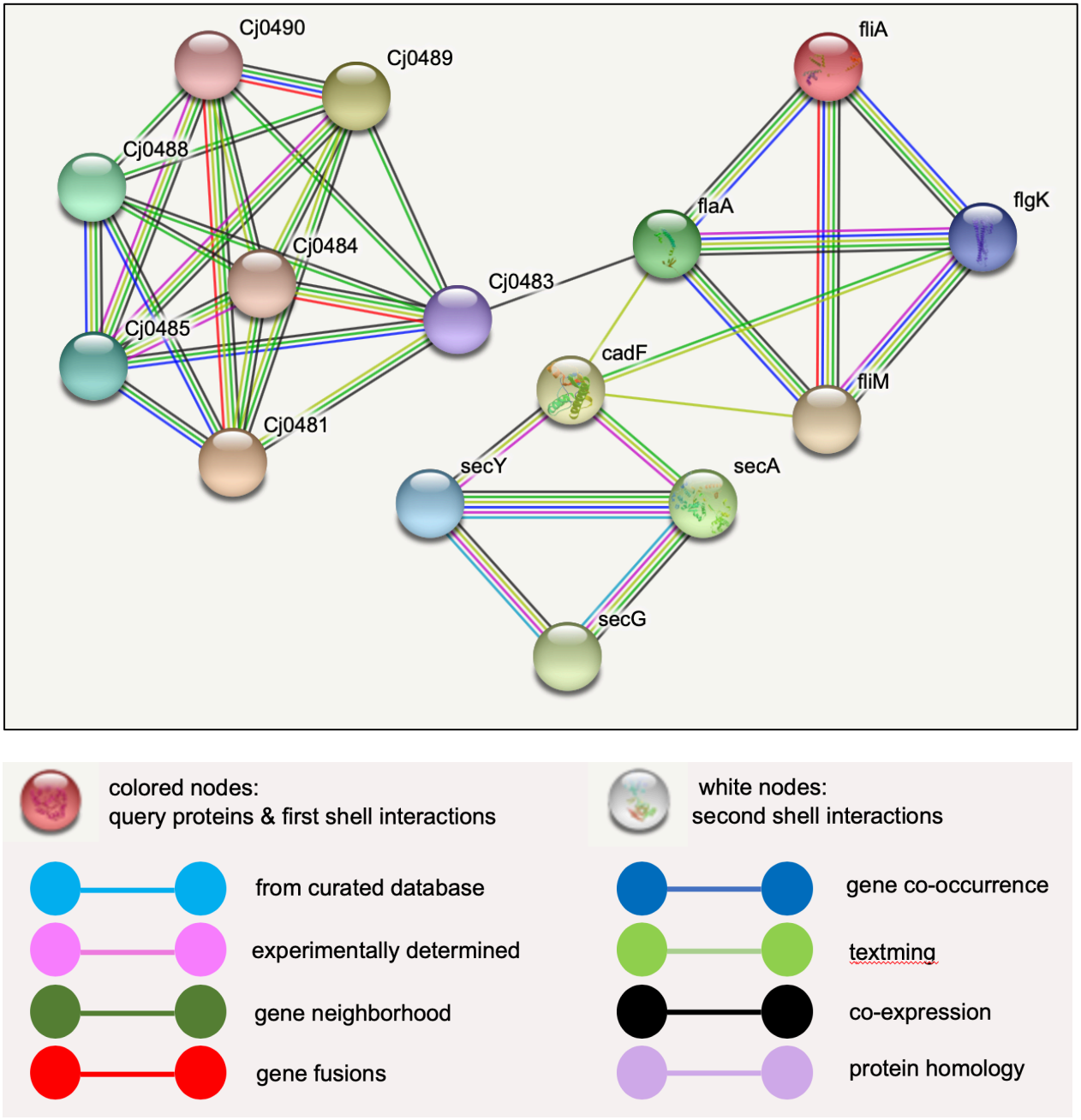


Figure 3.9. *C. jejuni* altered genes by ZnO NPs and their interactions with other relevant genes.

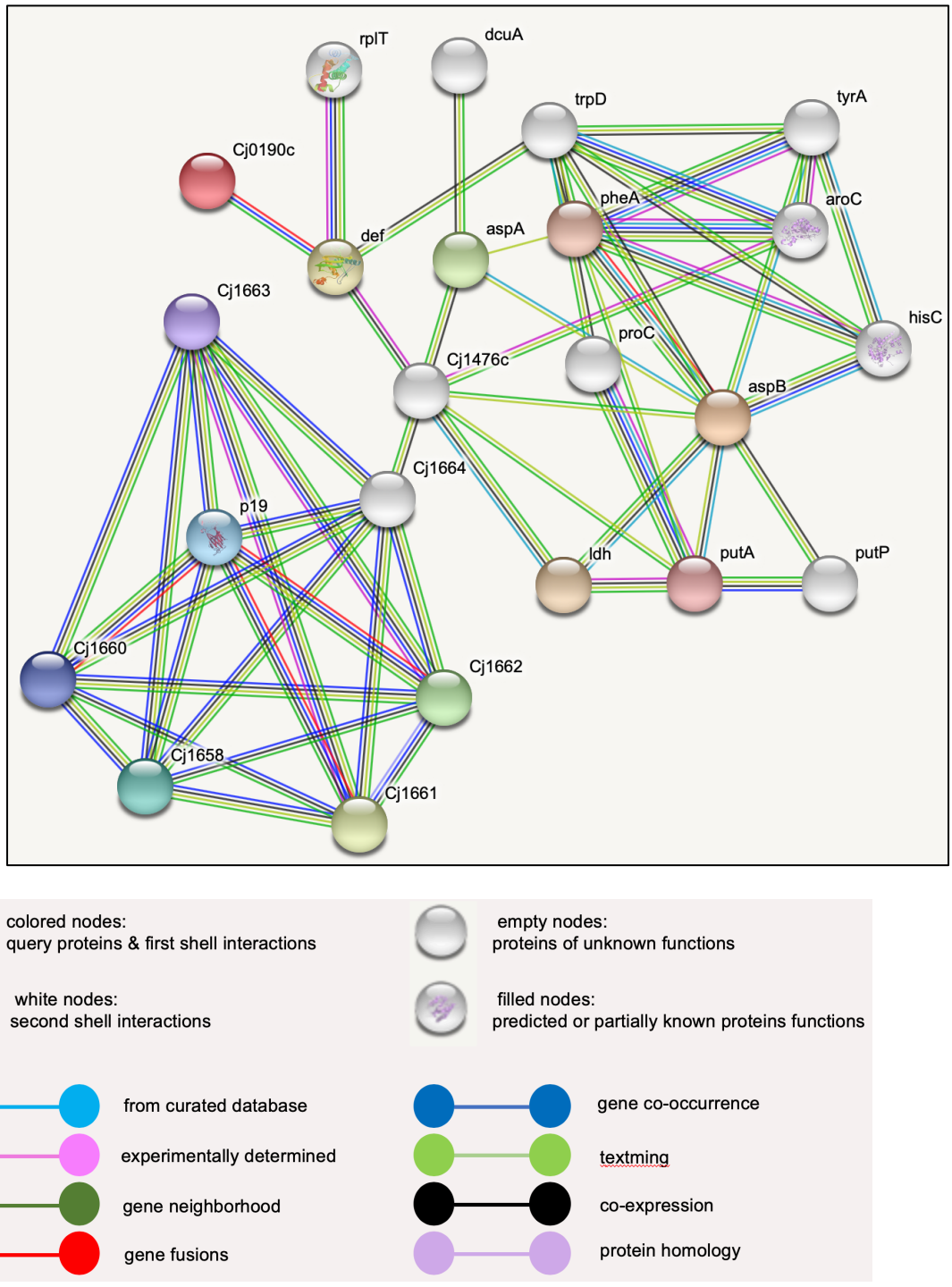


Figure 3.10. *C. jejuni* altered genes by tertiary combination of cinnamon oil, encapsulated curcumin and ZnO NPs and encapsulated curcumin and their interactions with other relevant genes.

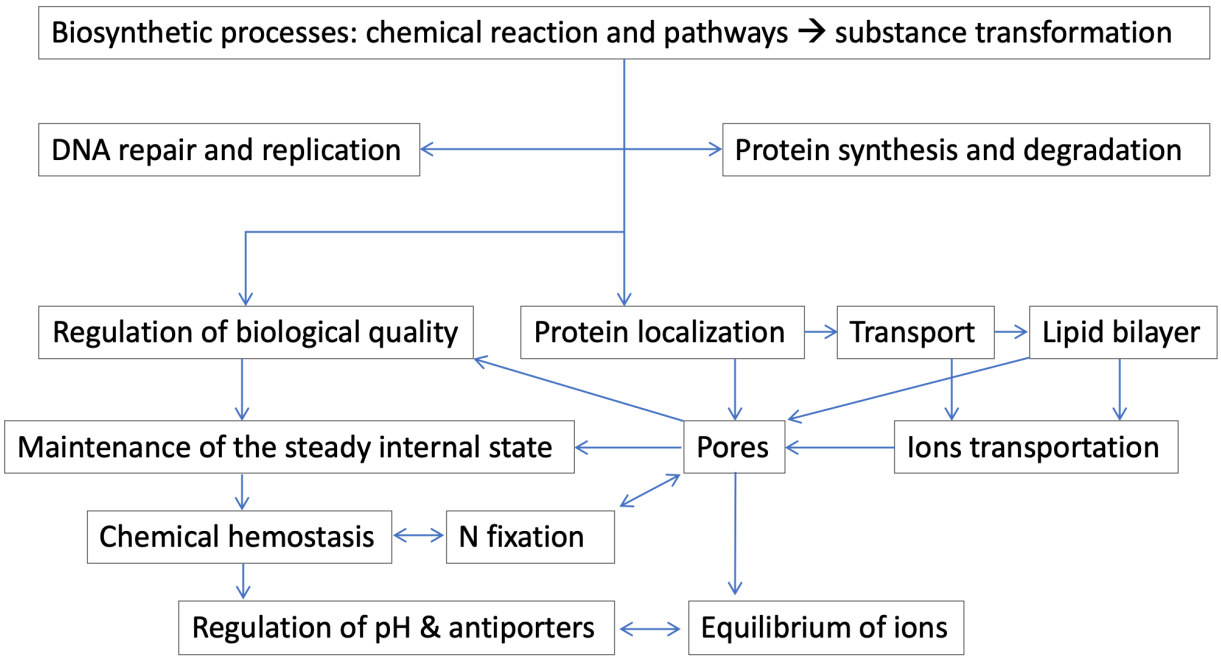


Figure 3.11. Overexpression map of the biosynthetic processes of tertiary combination of cinnamon oil, encapsulated curcumin and ZnO NPs and encapsulated curcumin in *C. jejuni*.

3.6 Conclusion

We investigated the antimicrobial mechanism of cinnamon oil, encapsulated curcumin, ZnO NPs, and their dual and triple combinations against *C. jejuni* using RNA-Seq. Single treatments altered the expression of genes involved in similar functions, such as motility, cell permeability, and bacterial cell wall synthesis and maintenance. Each antimicrobial played a different role in synergism. Cinnamon oil increased cell permeability to allow other antimicrobials to accumulate inside the cells. Encapsulated curcumin and ZnO NPs disrupted bacterial cell wall and cell membrane towards the same membrane protein targets. Combined treatment of cinnamon oil and encapsulated curcumin resulted in a unique transcriptional response including over-

expression of flagellar sigma factor regulating genes involved in motility, signaling and chemotaxis. All single treatments under-expressed a major facilitator superfamily encoding gene involved in developing antimicrobial resistance, which was different from that by the dual antimicrobial treatment. Triple antimicrobial treatment overexpressed genes involved in amino acid synthesis, protein translation, and membrane protein synthesis. Further investigation of these antimicrobials for agri-food applications is needed so as to replace antibiotics in animal farms or use as alternative antimicrobials during food processing.

Chapter 4: Development of Active Packaging Including Immobilized Zinc Oxide Nanoparticles to Inactivate *Campylobacter jejuni* in Raw Chicken Meat

4.1 Overview

Zinc oxide nanoparticles (ZnO NPs) are regarded as a safe and stable antimicrobial that can inactivate different bacteria by several potential working mechanisms. We aimed to include ZnO NPs into the packaging material for the control of *Campylobacter* in raw chicken meat. ZnO NPs were first incorporated into 3D paper tubes to identify the lethal concentration against *C. jejuni* that was selected to develop the 2D functionalized absorbing pads by ultrasound-assisted dipping technique. Functionalized pad was placed underneath chicken meat to investigate the antimicrobial effect against *C. jejuni* and the predominant chicken microflora (*i.e.*, *Lactobacillus* and psychrotrophic bacteria) at 4°C within 8 d of storage. Immobilized ZnO NPs at 0.856 mg/cm^2 reduced *C. jejuni* from ~log 4 CFU/chicken sample to undetectable level after 3 d of storage at 4°C, but had no effect on chicken microflora. Scanning electron microscopy validated that no nanoparticle migration onto the chicken meat after treatment with immobilized ZnO NPs in the functionalized pads. Analysis by inductively coupled plasma-optical emission spectroscopy showed that Zn level increased from 0.02 to 0.17 mg/cm^2 in the treated chicken meat. Inactivation of *C. jejuni* was associated with the increase of lactic acid produced by *Lactobacillus* in chicken meat in a pH dependent manner. Less than 5% of Zn^{2+} were released from ZnO NPs at neutral pH, while up to 88% were released when pH < 3.5 within 2 d. Taken together, functionalized absorbing pads inactivated *C. jejuni* in fresh chicken meat by the immobilized ZnO NPs along with the controllable released Zn^{2+}.

4.2 Introduction

As a microaerobic bacterium, *C. jejuni* is adapted to grow rapidly from the early days of chicken life (Beery, Hugdahl, and Doyle 1988; Shanker, Lee, and Sorrell 1990) and hence it can be routinely detected in the chicken carcasses (Newell and Fearnley 2003). *Campylobacter* is documented in 2018 to be the leading cause of foodborne zoonosis worldwide (Carron et al. 2018). Although chicken is not usually consumed raw, *C. jejuni* is frequently transmitted through the undercooked chicken and cross-contaminate other foods and/or food contact surfaces. Numerous reports in Europe and North America demonstrated that about 70-90% of all commercial raw chicken products have been tested to be *Campylobacter* positive (Jorgensen et al. 2002; Willis and Murray 1997). Other food vehicles such as unpasteurized dairy products, fresh produce, untreated water, and shellfish have been on the race (Geissler et al. 2017). Recent epidemiology studies indicated that poultry is the main reservoir and route of transmission of campylobacteriosis to humans (Kaakoush et al. 2015; Acheson and Allos 2001). The health burden and negative impact on the economy and individuals caused by *Campylobacter*-contaminated poultry products have been ranked as the number one pathogen-food combination among 14 foodborne pathogens in 12 different food categories (Batz, Hoffmann, and Morris Jr 2012).

Campylobacter contamination is a challenge to poultry industry. No prevention method (*e.g.*, vaccination) is available and classical intervention strategies on farms (Wagenaar, French, and Havelaar 2013) or in the slaughterhouses (Oyarzabal 2005) have limited impact on the reduction of *Campylobacter* loads due to the complexity of both systems. Effective and sustainable *Campylobacter* control is highly challenging due to the large number of birds, high microbial load and diversity, and rapid horizontal transmission of this microbe within and between batches (Marotta et al. 2015). For example, evisceration is one of the most critical points of poultry

carcasses contamination due to a large amount of *Campylobacter* cells in the small intestine and fecal materials (Bryan and Doyle 1995). Nevertheless, Berrang and co-workers isolated *Campylobacter* from the skin of chicken carcasses even before evisceration or any contact with the internal organs (Berrang, Dickens, and Musgrove 2000), indicating that other parts/fluids (*e.g.*, feathers, blood, and/or water) can also be involved in the cross-contamination by this bacterium. Many other factors including the farm and plant design, waste management, water quality and washing systems can have a significant influence on the prevalence of *Campylobacter* in the final poultry and food products as well.

The survival of *Campylobacter* as an obligate microaerobic bacterium in raw poultry products from the slaughterhouses to the retail outlets remains a puzzle to many researchers (Tresse, Alvarez-Ordóñez, and Connerton 2017). The overall strict requirement of this fastidious microbe to be either isolated or cultivated in the laboratory settings make itself to appear as a weak survivor and/or an easy target to be inactivated in the agri-food system. However, *Campylobacter* can be frequently detected in raw chicken meat and also occur at a level as high as 4-6 log CFU/chicken carcass (Bashor et al. 2004; Stern and Robach 2003; Kim et al. 2017). The attachment of *C. jejuni* to the contact surfaces can be associated with the development of bacterial biofilms that extend its survival outside hosts, but this could not fully explain the long term survival of culturable cells in the processing plants (I. H. R. J. M. Slavik 2008). In response to the processing relevant conditions, such as extreme pH, temperature fluctuation and starvation, *C. jejuni* can enter the viable but non-culturable (VBNC) state, which cannot be detected by the conventional plating assay (D. N. Jackson et al. 2009). A recent study identified several emerging hyper-aerotolerant *C. jejuni* clones isolated from raw chicken meat with a potential impact on human *Campylobacter* infections (Oh, McMullen, and Jeon 2015). It is generally agreed that the

intervention strategies used in the poultry industry are not sufficient to overcome the safety concern associated with this microorganism (Oyarzabal 2005; Bashor et al. 2004; M. F. Slavik et al. 1994).

Antimicrobial treatments for chicken meat showed significant reduction on *Campylobacter* (up to 5 log CFU/mL) in the laboratory settings (L. Zhang et al. 2018), but had very limited effect on its reduction (0.5-1.5 log CFU/mL) in the large processing plants (Bashor et al. 2004; M. F. Slavik et al. 1994). The U.S. Department of Agriculture (USDA)'s Food Safety and Inspection Service (FSIS) has proposed several antimicrobial agents for the control of *Campylobacter* in poultry slaughterhouses (F. S. and I. Service 2018). The efficacy of many approved antimicrobials (*e.g.*, acidified sodium, chlorite, cetylpyridinium, chlorine, chlorine dioxide, peroxyacetic acid, and trisodium phosphate) on the reduction of *Campylobacter* in poultry processing plants has been reported in several review articles (Oyarzabal 2005; Capita et al. 2002). For example, single or combined antimicrobial agents with heat and/or cold shock(s) could reduce *Campylobacter* load by 1-2 log CFU/mL of carcasses rinse. Although the use of commercial antimicrobials may not be effective to reduce *Campylobacter*, they are still widely used to try to prevent cross-contamination (Food Safety and Inspection Service, 2018). To date, there is no effective control strategy, recommendation, or guideline that can be applied to eliminate or at least significantly reduce *Campylobacter* in raw poultry products (Tresse, Alvarez-Ordóñez, and Connerton 2017). The use of commercial antimicrobial agents in the poultry industry is limited due to their low inactivation effect on pathogens, chemical residues as potential risks to humans, equipment corrosion, large water consumption, and high cost (Kim et al. 2017). Existing control methods also have other disadvantages. For examples, dipping can contribute to cross-contamination between batches and advanced spraying equipment are not always affordable.

Active antimicrobial packaging is an effective control method to enhance the safety of some high-risk food products, such as raw meat and fresh produce. It is usually applied for quality preservation, shelf life extension and food safety, with a major focus on the control of food freshness and inhibition of spoilage microflora (Panea et al. 2014). The study of integrating antimicrobials into the packaging material to inactivate pathogenic bacteria in foods is still in its infancy. One example was the use of immobilized bacteriophages to control *Escherichia coli* O104:H4 in alfalfa sprouts and *Listeria monocytogenes* in cantaloupes in ready-to-eat meat (Lone et al. 2016). The stability of antimicrobials in the packaging material is a major concern due to the dehydration nature of paper (Anany et al. 2011). Absorbing pads starts to be used under fresh meat to absorb moisture and fluids so as to maintain the quality and freshness of red meat, poultry, and fish. Chicken juice is not only a rich source of nutrients for the survival and growth of microorganisms, but also identified to enhance the surface attachment and biofilm formation of foodborne pathogens including *C. jejuni* (Brown et al. 2014). The area under the packed meat is difficult to reach by the conventional active packaging approach, such as modified atmosphere packaging (MAP) and aerosolized antimicrobial treatments. Alternative antimicrobials are thus required to develop novel active packaging technique used in agri-food industry.

Nanotechnology improves the functionality, physical and chemical properties of materials at nanoscale and generates sustainable industrial applications. Metal oxide nanoparticles are potential candidates for food packaging applications due to their antimicrobial effect and stability under the harsh conditions. Several studies demonstrated some successful applications of incorporating metal oxide nanoparticles to control spoilage and pathogenic bacteria in different food products (Panea et al. 2014; Fernández et al. 2009; Akbar and Anal 2014), as well as other biomedical and drug delivery applications (Lam et al. 2012). ZnO is a stable antimicrobial agent

that inactivates bacterial cells by several mechanisms involving different chemical species, such as Zn^{2+} and reactive oxygen species (ROS) (Pasquet et al. 2014). It is generally recognized as safe by the U.S. Food and Drug Administration (21CFR182.8991) (FDA 2016). In a previous study, ZnO NPs were reported to be more effective on inactivating both Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*E. coli*, and *Pseudomonas aeruginosa*) bacteria compared to CuO and Fe_2O_3 NPs (Azam et al. 2012). A few studies indicated that *C. jejuni* was more susceptible to ZnO NPs than other major foodborne pathogens (Azam et al. 2012; Xie et al. 2011; Liu et al. 2009; Hernández-Sierra et al. 2008). For instance, 0.005% (w/v) ZnO NPs could eliminate >8 log CFU/mL of *C. jejuni* within 3 h. In contrast, at least a 8- to 16-fold higher concentration was required to inactivate *E. coli* O157:H7 and *S. enterica* (Xie et al. 2011). Direct contact of ZnO NPs (positively charged) with bacterial cell wall (negatively charged) by electrostatic force leads to destabilization and disruption of bacterial outer membrane. In addition, semi-conductive property of ZnO allows the generation of ROS that can attack different cytoplasmic and extra-cytoplasmic targets. A previous study validated that treating *C. jejuni* with ZnO NPs could lead to significant over-expression of several oxidative stress response genes, including *katA* and *ahpC*, as well as the disruption of bacterial cell membrane (Xie et al. 2011).

No single control method is yet known to fully address *Campylobacter* contamination in the poultry industry. Controlling *Campylobacter* in the end production of raw chicken is a promising strategy that might replace or at least enhance ineffective mitigation strategies being used. The objective of this study was to investigate the antimicrobial efficacy of an innovative functionalized absorbing pad by immobilized ZnO NPs to control *C. jejuni* in the raw poultry products.

4.3 Material and methods

4.3.1 Chemical reagents and bacterial strains

ZnO NPs (size: 40-100 nm, surface area: 12-24 m^2/g) were obtained from Alfa Aesar™ (Haverhill, MA). Spruce-Pine-Fir softwood kraft paper sheet was donated from the Paper and Pulp Centre at The University of British Columbia (Vancouver, BC). Two clinical *C. jejuni* isolates (F38011 and human10), one bovine fecal isolate (ATCC 33560), and one chicken isolate (1173) were routinely cultivated on Mueller-Hinton (MH) agar (OXOID™, Nepean, ON) plates supplemented with 5% defibrinated sheep blood (Alere™, Stittsville, ON) for 48 h. Fresh bacterial cultures were prepared by suspending *C. jejuni* colonies in MH broth with constant shaking for 18 h at 37°C in a microaerobic condition (85% N_2, 10% CO_2, 5% O_2). Equal volumes of adjusted *C. jejuni* cultures were combined as a cocktail at an initial concentration of 1×10^9 CFU/mL.

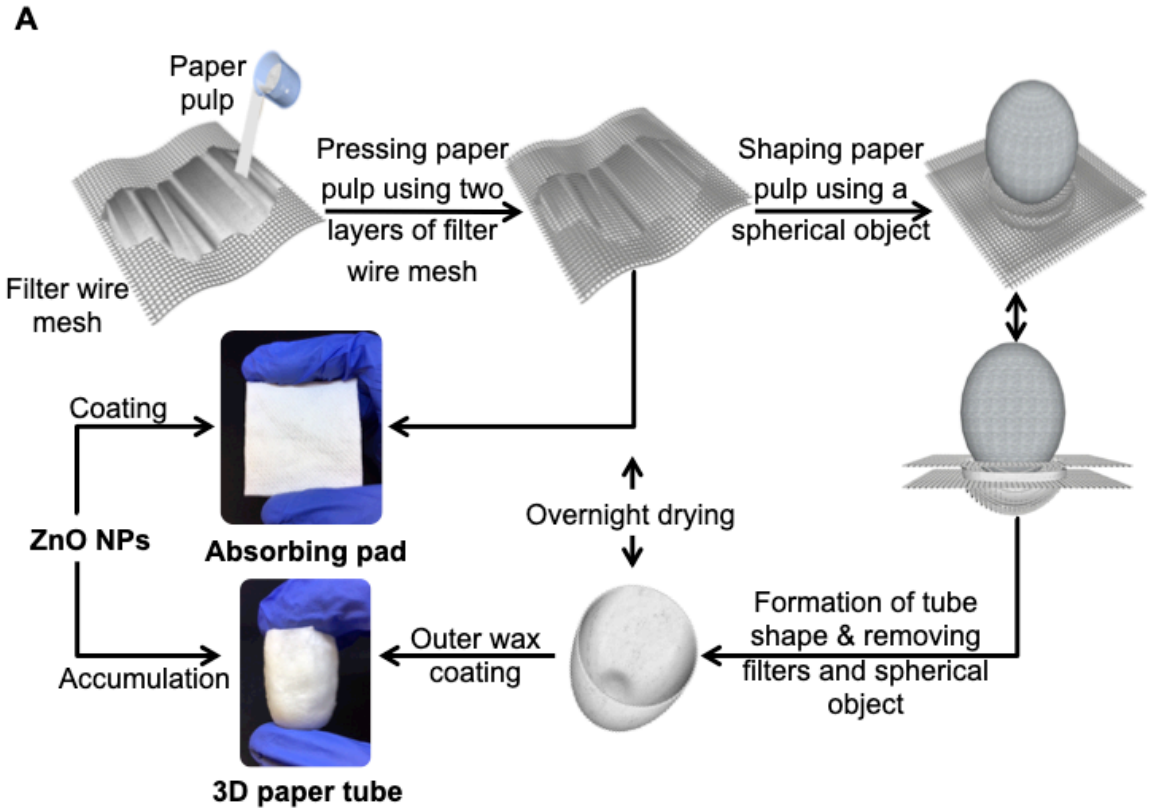
4.3.2 Development and antimicrobial functionalization of 3D paper tubes and 2D absorbing pads

Three-dimensional (3D) paper tubes and two-dimensional (2D) absorbing pads were designed and developed as shown in **Figure 3.1A**. Briefly, Spruce-Pine-Fir softwood kraft paper sheet was cut into a few smaller sheets before soaking into distilled water for 10 min to allow it to absorb water. The paper sheets were then mixed with water and blended for 10 min using a blender to obtain a homogeneous paper pulp. Then, water was removed from the homogenized pulp by squeezing and drying at 22°C for overnight. The dry paper pulp (4 g) was immersed into distilled water again to form a homogenous mixture, which was subsequently applied and pressed between two layers of filter wire meshes to remove the excess water and obtain paper sheets with the surface area of 25 cm^2 (5×5 cm). This paper sheet was then removed from the wire meshes and dried at 22°C for overnight. To make 3D

paper tubes, the applied paper pulp between the wire meshes was shaped using a spherical object before removing the meshes and allows it to dry. Finally, all paper tubes were coated with wax from the outside to make them to be water-resistant that can enable holding the bacterial liquid culture.

ZnO NPs suspension was prepared at the concentration of 10,000 ppm and then placed in a fixed power sonicator (Fisher Scientific™ model FS110, Ottawa, ON) for 10 min. The pH was adjusted to 8 by using 0.1 M NH_4OH to make it consistent to the alkalinity of the paper (Ghule et al. 2006). Two-fold serial dilutions from 100 to 1 ppm of ZnO NPs aqueous suspensions were individually added to different paper tubes. The negative control was the paper tube without the addition of ZnO NPs. The tubes were kept at 22°C for overnight to dry and allow the absorption by electrostatic attraction and hydrogen bonding between ZnO NPs and cellulose fibers (Zhao and Doyle 2006). Next, 3D tubes were washed by using distilled water for three times to remove any free ZnO NPs and then dried at 80°C for 45 min.

In addition, ZnO NPs were also immobilized on the 2D absorbing pads according to the protocol described in a previous study with some modifications (Ghule et al. 2006). Briefly, two different ZnO NPs suspensions at the concentration of 1,000 ppm and 10,000 ppm were separately prepared under sonication and pH adjustment conditions as aforementioned. Spruce-Pine-Fir softwood kraft paper sheet was used to make flat and thick (2 mm) paper sheets as the substrates to generate functionalized absorbing pads. Specifically, paper substrates were attached face down to ZnO NPs suspension (**Figure 4.1B**). Paper substrates were dipped under sonication for 5 min, followed by being immersed into distilled water for washing three times to remove the free nanoparticles. The coated papers were detached from the dipping setup and dried at 80°C for 45 min. By using ZnO NPs suspensions at 1,000 ppm and 10,000 ppm, two different concentrations of ZnO NPs in the functionalized absorbing pads were generated accordingly.


A
Paper pulp
Filter wire mesh
Pressing paper pulp using two layers of filter wire mesh
Shaping paper pulp using a spherical object
Coating
ZnO NPs
Absorbing pad
Overnight drying
Formation of tube shape & removing filters and spherical object
Outer wax coating
Accumulation
3D paper tube

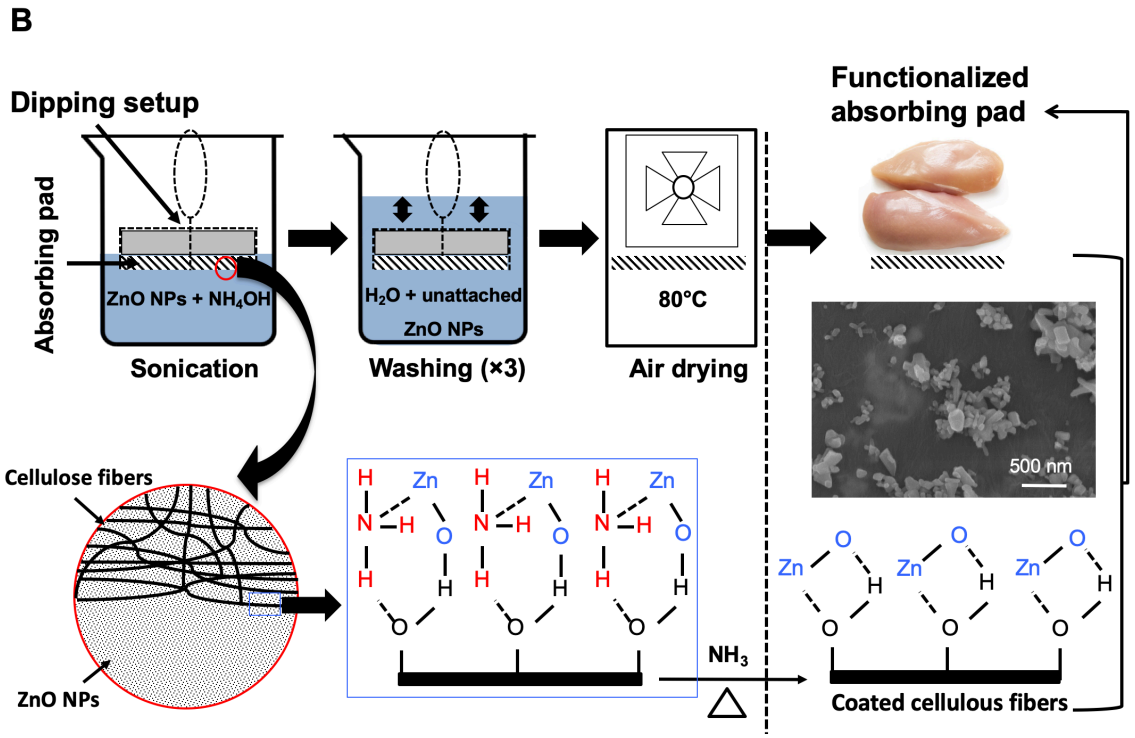

B
Dipping setup
Absorbing pad
ZnO NPs + NH4OH
Sonication
H2O + unattached ZnO NPs
Washing (×3)
80°C
Air drying
Functionalized absorbing pad
500 nm
Cellulose fibers
ZnO NPs
NH3
Coated cellulous fibers

Figure 4.1. Schematic diagrams of the development of 2D absorbing pads and 3D paper tubes (A), and functionalization of the absorbing pads by ZnO nanoparticles (B). The 3D paper tubes were functionalized by filling them with diluted ZnO NPs suspensions and allowed them to dry at 22°C for 24 h. Attached ZnO NPs onto the cellulose fibers were observed and confirmed by scanning electron microscopy (SEM).

4.3.3 Testing the antimicrobial effect of functionalized 3D paper tubes and 2D absorbing pads on *C. jejuni* F38011

Both functionalized paper products (*i.e.*, functionalized 3D tubes and functionalized 2D absorbing pads) were sterilized by autoclave for 15 min at 121°C before the antimicrobial testing. The functionalized 3D tubes were filled with 1.5 mL of *C. jejuni* F38011 culture in early stationary phase (1×10^8 CFU/mL) and incubated with constant shaking (175 rpm) at 37°C in a microaerobic condition. Microbial counts were determined after 3 h of incubation by plating assay and the minimum bactericidal concentration (MBC) of immobilized ZnO NPs was consequently identified. In addition, the growth of *C. jejuni* F38011 was examined with either uncoated or coated absorbing pads with ZnO NPs using the diffusion assay (Gittard et al. 2009). Three different concentrations (1×10^2, 1×10^5, and 1×10^8 CFU/mL) of *C. jejuni* F38011 (1 mL) in early stationary phase were separately added onto MH agar plates supplemented with 5% defibrinated sheep blood and dried for 30 min at 22°C. Both coated and uncoated absorbing pads (~1 cm^2) were then applied onto the surface of *C. jejuni*-inoculated plates and incubated at 37°C for 48 h in a microaerobic condition until *C. jejuni* lawns were developed. Visual comparison of the size of inhibition zones was applied to determine the antimicrobial effect of the coated absorbing pads.

4.3.4 Investigating the antimicrobial effect of functionalized absorbing pads to control *C. jejuni* cocktail in raw chicken meat

Packages of boneless chicken breasts were obtained from local grocery stores in Vancouver (BC, Canada) and used immediately after the arrival to the laboratory. The chicken breasts were cut into pieces of 25 g, aseptically mixed in a container, and aged at 4°C for overnight to obtain an evenly distributed microflora on the chicken surfaces. Each piece of chicken breast was placed into a sterile Petri dish and inoculated with a cocktail of four strains of *C. jejuni* (*i.e*., F38011, Human 10, ATCC 33560, 1173). Initial inoculum of *C. jejuni* was adjusted to 4 log CFU/sample, which is the commonly observed level of this microbe in raw chicken products (Stern and Robach 2003; Kim et al. 2017; Luber and Bartelt 2007). Inoculated chicken samples were separately treated by absorbing pads with two different concentrations of ZnO NPs and a negative control (*i.e.*, absorbing pad with no ZnO NPs). One uninoculated and untreated control group were also included to track the native *Campylobacter* load in the chicken samples. After treatment, absorbing pads were aseptically removed and chicken samples were placed in 225 mL of phosphate buffered saline (PBS, pH = 7.4), followed by manual massaging for 10 min. Chicken rinses were serially diluted and plated onto Campy-Cefex agar (Oyarzabal et al. 2005), DeMan, Rogosa and Sharpe agar (MRS) (Fisher Scientific™, Ottawa, ON), and Tryptic Soy Agar (TSA) (VWR, Mississauga, ON) plates. Plates were separately incubated at 42°C, 30°C and 7°C. Bacterial counts were enumerated for *C. jejuni* and *Lactobacillus* after 72 h of incubation and psychrotrophic bacteria after 3-7 d of incubation. When necessary, up to 10 mL of chicken rinse was directly plated on ten Campy-Cefex agar plates (1 mL each) to reduce the limit of detection of the plating assay. Each sample was tested at least in triplicate.

4.3.5 Testing the antimicrobial effect of functionalized absorbing pads against individual *C. jejuni* strains in raw chicken meat

Chicken breasts were decontaminated to remove the native microflora before testing the effect of functionalized absorbing pads on each individual *Campylobacter* strain. Briefly, chicken breasts were soaked in 3% (v/v) hydrogen peroxide for 2 min under constant shaking condition (100 rpm). Samples were air-dried for 2 min and soaked in 98% (v/v) ethanol for another 1 min following the same procedure. A sterilized knife was then used to remove the outer layer of chicken and cut the chicken meat into pieces of 5 g or 5 cm^2 (2.236 cm × 2.236 cm). Random portions of decontaminated chicken meat (5 g) were separately placed in the stomacher bags with 45 mL of PBS and then manually massaged for 10 min. Chicken rinses were plated on TSA and incubated at 37°C for at least 48 h to confirm the absence of native microflora. Four strains of *C. jejuni* (*i.e.*, F38011, Human 10, ATCC 33560, 1173) were inoculated individually onto the surface of chicken samples and then treated with either coated or uncoated absorbing pads for 3 d at 4°C. Samples were collected after 0 d and 3 d. Absorbing pads were removed and chicken samples were placed in 45 mL of PBS and then manually massaged for 10 min. Chicken rinses were plated on MH agar plates with 5% defibrinated sheep blood. Each sample was tested at least in triplicate.

4.3.6 Determination of Zn levels in both functionalized absorbing pads and treated chicken meat

Quantification of Zn levels in both functionalized absorbing pads and treated chicken meat products was conducted according to the protocol described in a previous study (Song et al. 2014) with modifications. Briefly, ZnO NPs were spiked on 4 cm^2 (equivalent to 0.2 g) of absorbing pads at the concentrations of 0, 50, 250, 625, 1250, and 2500 ppm to generate a calibration curve for

quantification. Similarly, ZnO NPs were spiked on 5 cm^2 (equivalent to 5 g) of chicken meats at the concentrations of 0, 0.1, 0.2, 1, 2, 10, 20, 50, 400 ppm to quantify the migration level of Zn to chicken meat. Samples of both groups (*i.e.*, functionalized absorbing pads and treated chicken meats) were separately transferred to ceramic crucibles and incinerated at 550°C for 2.5 h. Obtained samples were placed in 10 mL of 6 N HCl and diluted by adding 30 mL of double distilled water. The remained ashes were removed by filtration through Whatman No. 42 paper (Millipore sigma®, Oakville, ON). The supernatant was collected and analyzed using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Varian® model 725ES, Agilent Technologies, Inc., USA) to quantify Zn levels. Each sample was tested at least in triplicate.

Electron microscopy was conducted to determine if ZnO NPs were coated on the surface of functionalized absorbing pads or migrated to the treated chicken meat using a Hitachi®-S4700 Field Emission scanning electron microscope (SEM) (Tokyo, Japan). Chicken samples were cut into ~0.5 cm thin slices, stored at -80°C for overnight, and then lyophilized at -53°C by using a 12-L Console Labconco freeze-dryer (Kansas City, MO) until complete dryness. Both samples were cut into ~1 cm^2 pieces and sputter coated with platinum to get an electron conductive surface. The negative controls of chicken samples for SEM study were processed without the treatment of functionalized absorbing pads or addition of ZnO NPs, and the positive control was chicken meat spiked with ZnO NPs at 0.22 mg/cm^2. Images were collected at the accelerating voltage of 2.0 kV and a magnification range of 0.5-1,000 μm.

4.3.7 Determination of the release of Zn^{2+} at different pH levels

Different pH values (*i.e.*, 3, 3.5, 4, 5, 6, and 7) were prepared in 200 mL of deionized water and adjusted by 10% (v/v) lactic acid. Five milliliters at each pH value were used to prepare ZnO

NPs suspension by sonication for 10 min and then placed in a floating dialysis device with a molecular weight cut-off of 8-10 kD (Spectra-Por® Float-A-Lyzer® G2; Millipore sigma®, Oakville, ON). Each dialysis device was placed against 195 mL of deionized water with lactic acid at different pH values. ZnO NPs suspensions were dialyzed for 48 h in dark under stirring at 160 rpm and at 4°C. The dialysates were collected after 24 h and 48 h, and then analyzed using ICP-OES to quantify the level of the released of Zn^{2+}. Each sample was tested at least in triplicate.

4.3.8 Statistical analysis

Prism6® (version 6.01, GraphPad Software Inc., San Diego, CA) was used for graph generation and statistical analysis. Data were analyzed using one-way ANOVA, followed by the post hoc Tukey's test for multiple comparisons. *P* value was adjusted at 0.05 or lower. All of the experiments were conducted at least in triplicate.

4.4 Results

4.4.1 Determination of MBC and inhibition zones of immobilized ZnO NPs against *C. jejuni*

The 3D paper tubes were designed to determine bactericidal concentrations of the immobilized ZnO NPs against *C. jejuni* in broth. Immobilized ZnO NPs into paper tubes had a concentration-dependent bactericidal effect against *C. jejuni* F38011 after 3 h of incubation at 37°C in a microaerobic condition (**Figure 4.S1**). For example, immobilized ZnO NPs at 25 ppm could reduce ~90% of bacterial population and 50 ppm could inactivate >99% of bacterial population (*i.e.,* 2.61 log reduction). Immobilized ZnO NPs at 100 ppm reduced the bacterial population to undetectable level (>8 log reduction). Thus, 100 ppm of ZnO NPs was identified as the MBC according to the definition of the lowest concentration that leads to no observed bacterial

growth in the broth medium (Andrews 2001). In contrast, immobilized ZnO NPs at 3.12, 6.25, and 12.5 ppm did not significantly ($P > 0.05$) affect *C. jejuni* viability compared to the negative control. These 3D paper tubes were developed to determine the MBC of immobilized ZnO NPs against *C. jejuni*, which was the prerequisite to further generate the effective functionalized absorbing pads. Diffusion assay was then used to test the effect of functionalized absorbing pads on inactivating *C. jejuni* F38011 grown on MH agar medium according to a previously established method (Gittard et al. 2009). The functionalized absorbing pads showed clear inhibition zones against all of the tested bacterial concentrations (*i.e.*, 10^2, 10^5, and 10^8 CFU/plate) as shown in **Figure 4.S2B**. Taken together, the functionalized paper products with ZnO NPs were able to effectively inactivate *C. jejuni* in the pure culture.

4.4.2 Quantification and imaging of ZnO NPs in the functionalized absorbing pads

ICP-OES was used to quantify the immobilized ZnO NPs into the absorbing pads. Only 7.51% and 8.56% of the initial ZnO NPs suspensions at 1000 ppm and 10,000 ppm, respectively, were determined to immobilize into the absorbing pads, respectively. The final Zn concentration in the two absorbing pads was separately determined to be 0.856 ± 0.082 mg/cm^2 and 0.075 ± 0.012 mg/cm^2. SEM images confirmed that ZnO NPs were successfully coated onto the surface of individual cellulose fibers and formed a thin layer around individual cellulose fibers after 5 min of sonication-assisted dipping (**Figure 4.2B**), including small individual nanoparticles with different shapes (mainly irregular and spherical shapes). In addition, unattached free ZnO NPs were not observed in the cavities between the coated cellulose fibers. In contrast, a smooth and uncoated surface of cellulose fibers was observed for the uncoated group (**Figure 4.2A**). Taken together, the ICP-OES analysis and SEM imaging validated that a certain percentage of ZnO NPs were

successfully coated onto the surface of individual cellulose fibers and form the functionalized absorbing pads.

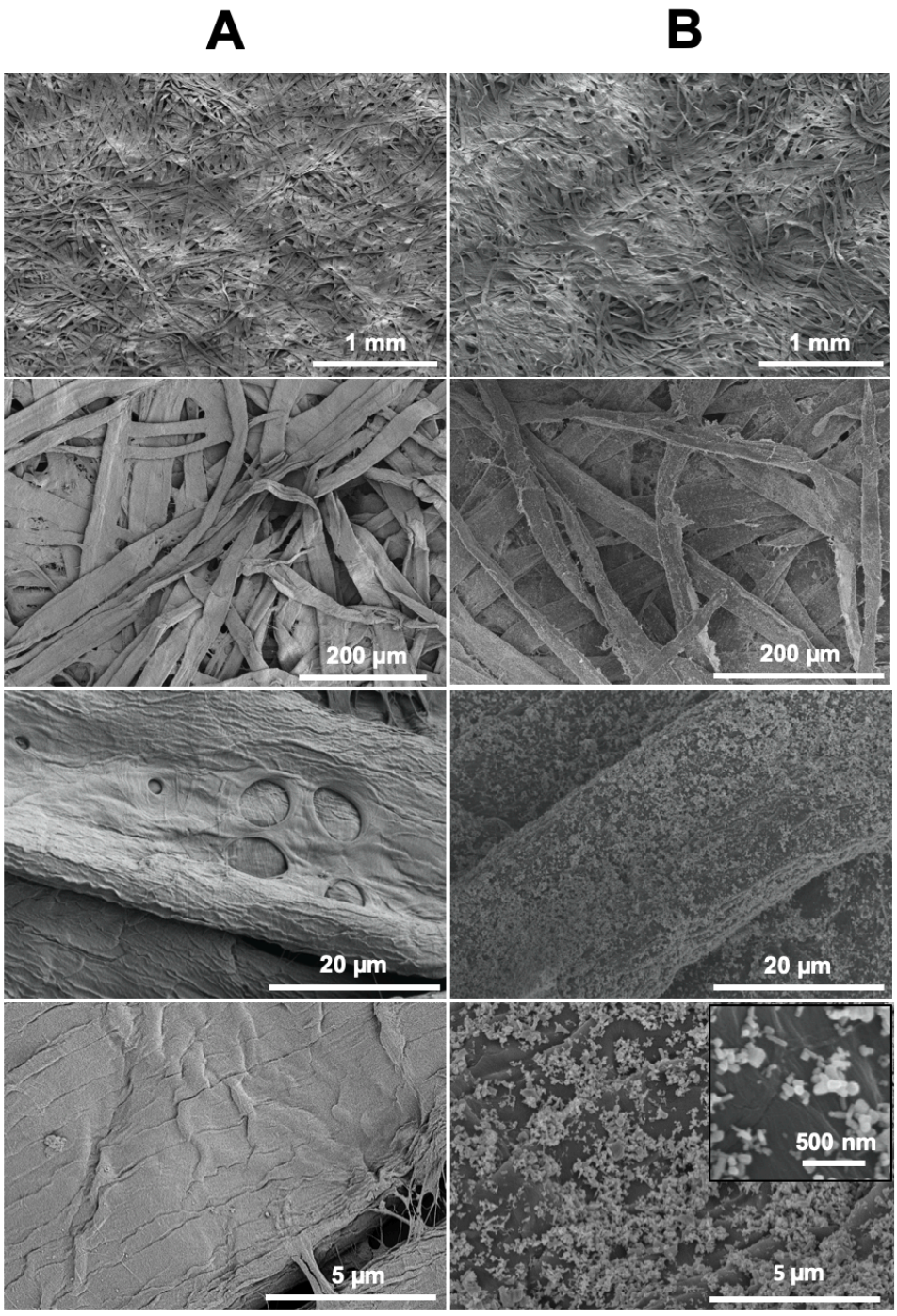


Figure 4.2. Representative scanning electron microscopic (SEM) images of uncoated (A) and coated (B) absorbing pads with immobilized ZnO nanoparticles at 0.856 mg/cm^2. Coating was conducted using a sonication/ultrasound-assisted dipping technique for 5 min, followed by immersing in distilled water for washing three times and drying at 80°C for 45 min. SEM accelerating voltage was 2.0 kV and the magnification ranged from 0.5-1,000 μm (n = 7).

4.4.3 Inactivation of *C. jejuni* cocktail on raw chicken meat by the functionalized absorbing pads

Inactivation effects of the functionalized absorbing pads on *C. jejuni*-contaminated chicken meat were investigated at both refrigeration and abuse temperatures (*i.e.*, 4°C and 7°C). The initial inoculum of *C. jejuni* cocktail (*i.e.*, log 4 CFU/sample) did not increase in chicken meat during 8 days at 4°C in any of the treated or untreated groups (**Figure 4.3**). *C. jejuni* on the untreated chicken meat was able to remain viable during the storage at 4°C for 8 d (**Figure 4.3A**) regardless the cold stress and the presence of a relatively high level of competitive bacteria in the chicken microflora, such as *Lactobacillus* and psychrotrophs (**Figure 4.3B and C**). Two different concentrations of immobilized ZnO NPs in the absorbing pads (0.075 and 0.856 mg/cm^2) were tested against *C. jejuni* cocktail inoculated on the raw chicken meat at 4°C. As shown in **Figure 4.3A**, the functionalized absorbing pads with ZnO NPs at 0.075 mg/cm^2 did not have a significant antimicrobial effect ($P > 0.05$) against *C. jejuni* after 3 and 5 days of storage but had ~0.5 log CFU/sample reduction at day 8 ($P \leq 0.001$). In comparison, the functionalized absorbing pads with ZnO NPs at 0.856 mg/cm^2 caused the reduction of 1.45 log CFU/sample after 3 days, followed by a further decrease to undetectable level (≤ 500 cells) after 5 and 8 days of storage. Based on the detection limit of the plating assay, no native *Campylobacter* spp. was detected in chicken samples at all tested temperatures and time points (data not shown). The total count of other predominant microflora including the undefined *Lactobacillus* and psychrotrophic bacteria were identified to

be 4-5 log CFU/g after 3 d, and 6-9 CFU/g after 8 d (**Figure 4.3B and C**). No interaction between the predominant microflora and the functionalized absorbing pads was observed at all time points at 4°C (**Figure 4.3B and C**). In addition, no reduction of *C. jejuni* in chicken meat products was observed after 24 h of storage at 7°C (**Figure 4.S3A**). Predominant microflora in the raw chicken meat grew rapidly at 7°C within 24 h and were not affected by the functionalized absorbing pads (**Figure 4.S3B** and **C**). No further time points were investigated due to the high level of psychrotrophs (~6 log CFU/g) after only 24 h of incubation at this abuse temperature. Altogether, significant reduction of *C. jejuni* cocktail by the functionalized absorbing pads was observed after 5 d at 4°C.

The antimicrobial effect of the functionalized absorbing pads with immobilized ZnO NPs at 0.856 mg/cm^2 on individual C. *jejuni* strain was determined to be strain dependent (**Figure 4.S4**). In particular, the clinical strains (F38011 and Human 10) were reduced to undetectable level after 3 d at 4°C and the other two strains isolated from agri-foods were more tolerant to the treatment by the functionalized absorbing pads. The bovine isolated strain ATCC 33560 was more susceptible to the immobilized ZnO NPs in the functionalized pads than strain 1173 isolated from chicken, with variation of ~1.5 log CFU/sample. Taken together, immobilized ZnO NPs (0.856 mg/cm^2) in the functionalized pads were able to eliminate two *C. jejuni* strains after 3 d (**Figure 4.S4**) and inactivate all four tested strains after 5 d on raw chicken meat (**Figure 4.3A**)

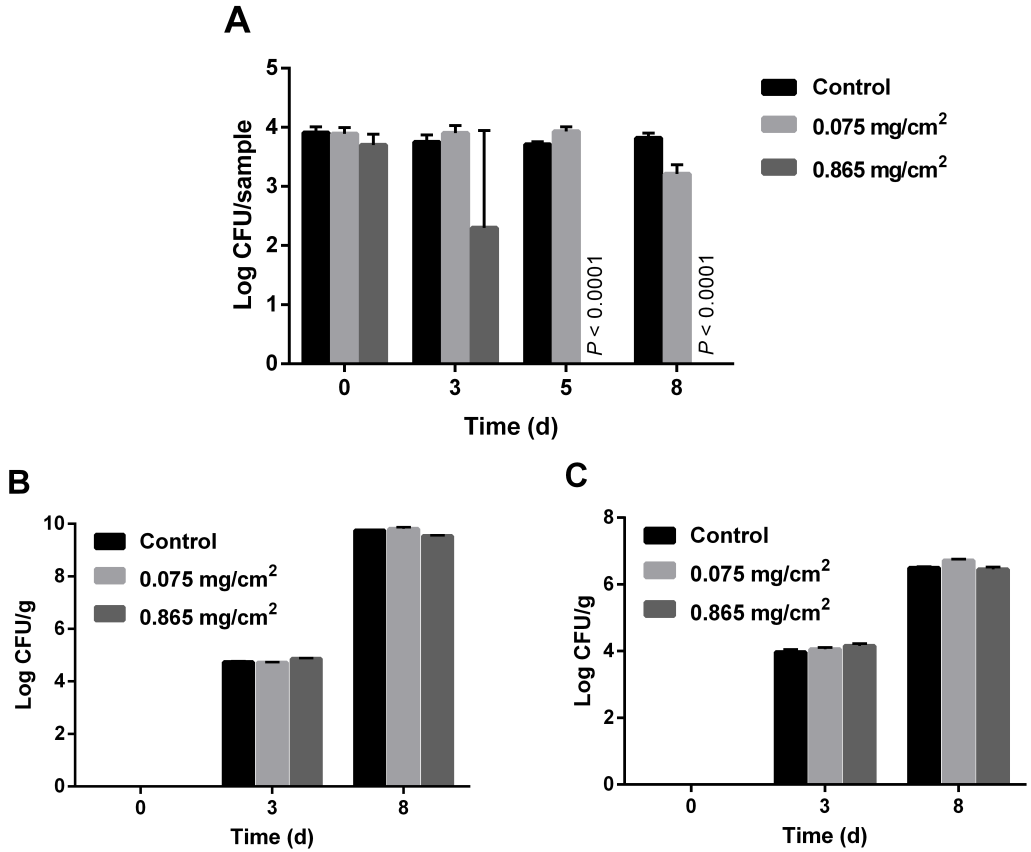


Figure 4.3. The count of *Campylobacter jejuni* cocktail (F38011, Human10, 1173, and ATCC 33560) (A), *Lactobacillus* (B), and psychrotrophs (C) on raw chicken breasts stored at 4°C with or without functionalized absorbing pads including immobilized ZnO nanoparticles at 0.075 and 0.856 mg/cm2 for 8 d. Campy-Cefex was used as the plating assay for enumerating *C. jejuni*, and plates were incubated at 42°C in a microaerobic condition. DeMan, Rogosa and Sharpe agar (MRS) and Tryptic Soy Agar (TSA) were used as the plating assay to separately enumerate *Lactobacillus* and psychrotrophs and the plates were incubated aerobically at 30°C (48 h) and 7°C (72 h), respectively. Limit of detection was determined to be 500 CFU/sample (*i.e.*, 25 g of chicken breast). Data were analyzed by using one-way ANOVA, followed by the post hoc Tukey's test for multiple comparisons.

4.4.4 Migration of Zn from the functionalized absorbing pads to chicken meats

In parallel, the migration of Zn from the functionalized absorbing pads to chicken meats was investigated. Zn levels in the treated chicken meats continuously increased from 0.02 mg/cm^2 to 0.41 mg/cm^2 in 8 d at 4°C, as determined by using ICP-OES (**Figure 4.4)**. SEM images of the treated chicken meat surface were collected to investigate the presence of ZnO NPs after 5 d treatment with the functionalized absorbing pads including ZnO NPs at 0.856 mg/cm^2. During this time period, Zn level increased from 0.02 to 0.22 mg/cm^2 (**Figure 4.4**) and all *C. jejuni* strains reduced to undetectable level (**Figure 4.3A**), but no ZnO NP was observed on the surface of chicken meat with the treatment of functionalized absorbing pads at 5 d (**Figure 3.6C**) or the untreated chicken meat as the negative control (**Figure 4.5A**). In comparison, clear accumulation of ZnO NPs on the surface of chicken meat was observed for the positive control (**Figure 4.5B**). In conclusion, no ZnO NPs was observed in chicken meat by using the functionalized absorbing pads while Zn ion level increased along with the reduction of *C. jejuni* counts to undetectable level.

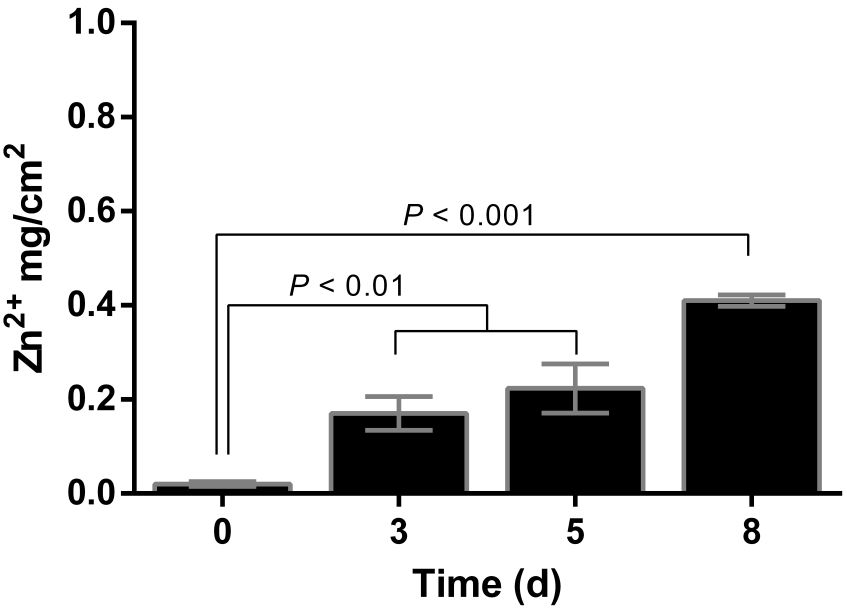


Figure 4.4. Quantification of Zn ion in treated chicken meats with immobilized ZnO NPs at 4°C for up to 8 days. Treated chicken pieces were spiked with ZnO NPs to generate calibration curves for the quantification. Standards and samples were ashed at 550°C for 2.5 h to remove the organic contents. Samples were then analyzed using inductively coupled plasma-optical emission spectroscopy (ICP-OES). Each data point is the mean of three replicates and error bars represent the standard deviations of the means. Data were analyzed by using one-way ANOVA, followed by the post hoc Tukey's test for multiple comparisons (n = 3).

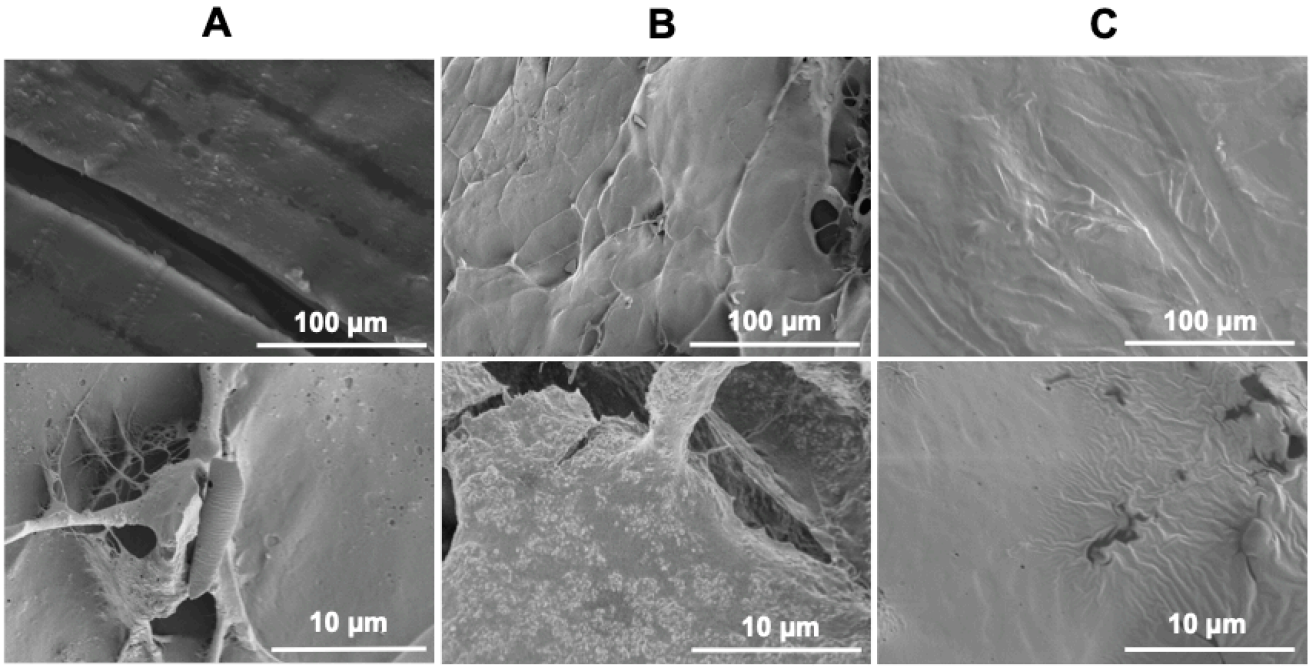


Figure 4.5. Representative scanning electron microscopic (SEM) images of chicken meat either untreated or treated with the functionalized absorbing pads including immobilized ZnO nanoparticles, or directly spiked with ZnO nanoparticles. Chicken samples include untreated sample (A), spiked sample with ZnO nanoparticles at 0.22 mg/cm^2 (B), and treated sample with the functionalized absorbing pads including immobilized ZnO nanoparticles at 0.856 mg/cm^2 for 5 d (C). All samples were lyophilized for 24 h before imaging. SEM accelerating voltage was 2.0 kV and the magnification ranged from 10-200 µm (n = 7).

4.4.5 Release of Zn ions at different pH levels

We then investigated the release of Zn^{2+} from ZnO NPs mimic to the storage condition of refrigerated raw chicken meat (neutral to acidic pH at 4°C). There was a minor release (2.8% – 6.0%) of Zn^{2+} at pH values of 4, 5, 6, and 7 after 24 h and 48 h of storage at 4°C (**Figure 4.7**). The pH 3.5 was identified as the cut-off value to release Zn^{2+} by using lactic acid. The largest release of Zn^{2+} (*i.e.*, 47% and 88% after 24 h and 48 h, respectively) was observed at pH of 3. The release

of Zn^{2+} could be controlled at different pH values in the presence of lactic acid and at refrigeration condition.

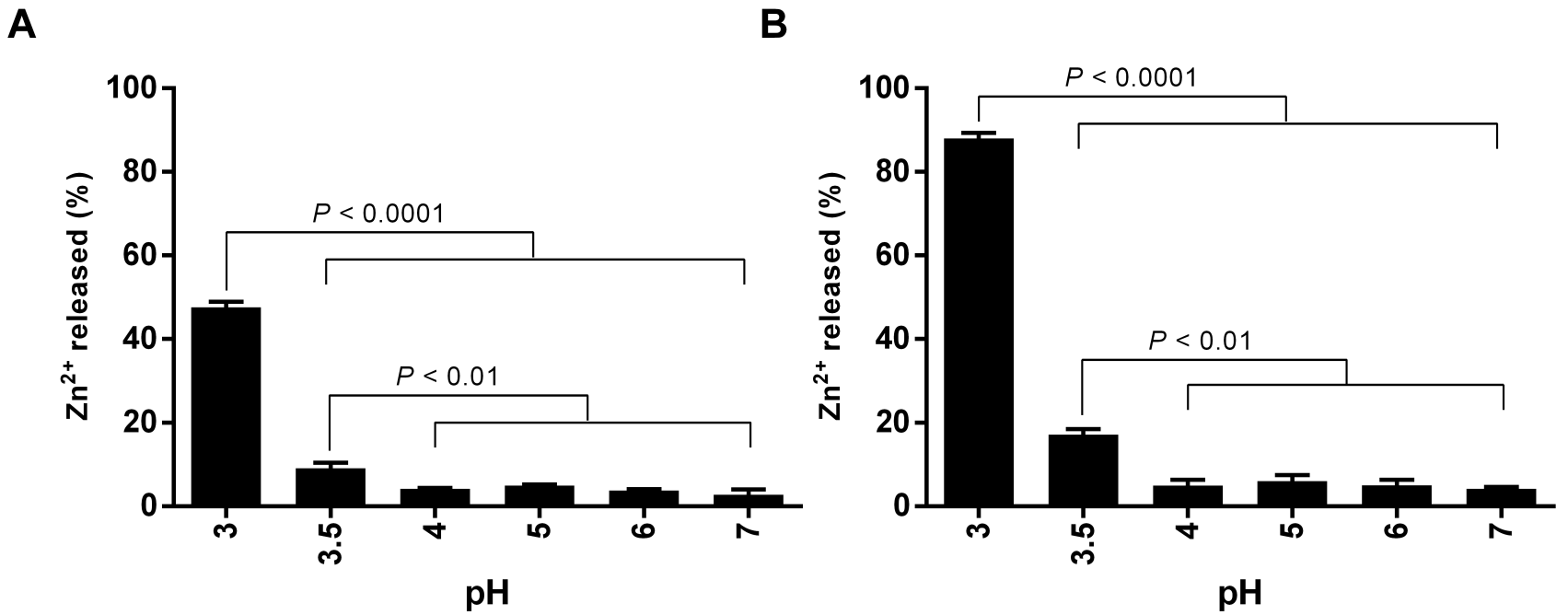


Figure 4.6. Quantification of Zn ions released by ZnO nanoparticles in the dialysis devices at pH 3-7 at 4°C. The pH levels were adjusted using 10% (v/v) lactic acid. Dialysate samples of Zn ions were collected and then analyzed by using inductively coupled plasma-optical emission spectroscopy (ICP-OES) after 24 h (A) and 48 h (B). Serial dilutions of ZnO nanoparticles were used to generate a calibration curve for the quantification of Zn ions. Each data point is the mean of three replicates and error bars correspond to the standard deviations of the means. Data were analyzed by using one-way ANOVA, followed by the post hoc Tukey's test for multiple comparisons.

4.5 Discussion

Controlling *Campylobacter* in poultry is highly challenging in both farms (Wagenaar, French, and Havelaar 2013) and slaughterhouses (Oyarzabal 2005). So far, no commercial vaccine or antimicrobial treatment is available to reduce the number of *Campylobacter* in the cecum of poultry once this bacterium successfully colonizes flocks (Wagenaar, French, and Havelaar 2013). Advanced biosecurity systems might be the only option to prevent the presence of *Campylobacter* in poultry farms. However, this approach is more costly and challenging than targeting *Campylobacter* at the endpoint of farm-to-fork pathway (*i.e.,* poultry meat) as bacterial transmission can occur via multiple routes, such as environment, food chain, water, and direct contact (Wagenaar, French, and Havelaar 2013). In addition, poultry carcasses collected from different farms might have distinct *Campylobacter*-contamination levels, but they end up in the same poultry processing plant. Thus, *Campylobacter*-positive birds can pollute the food contact surfaces, equipment and water system of processing plants, leading to cross-contamination of clean batches of poultry carcasses. The current available antimicrobial agents can only achieve a 1-2 log CFU/mL reduction of *Campylobacter* in poultry plants (Oyarzabal 2005; Capita et al. 2002). Due to the inefficient *Campylobacter* control measures, the incidence of human campylobacteriosis via the consumption of contaminated poultry products has remained stable during the past decade (Kaakoush et al. 2015; M B Skirrow 1977; CDC 2016). It is generally believed that *Campylobacter*-free chicken meat at the retail level is unreachable even with the use of available intervention strategies and fast/sensitive detection methods (Havelaar et al. 2007). However, the mitigation of *Campylobacter* in post-chill poultry products can remarkably reduce the campylobacteriosis risk. For example, a risk assessment study demonstrated that reducing *Campylobacter* by 3 log CFU/g of chicken intestinal contents in slaughterhouses, or by 1 log

CFU/carcass in the raw chicken end-products can reduce *Campylobacter*-infection risks by 50-90% (European Food Safety Authority, 2011). Taken together, a new generation of antimicrobials and mitigation strategies need to be investigated to enhance the safety of raw poultry meats.

Nanotechnology offers a variety of novel approaches to improve the quality and safety of foods. Several organic and inorganic food-grade nanomaterials can be used as antimicrobial agents. For example, ZnO NPs can be utilized to control different foodborne pathogens in high-risk foods, including raw and processed meat products. Several studies have shown that ZnO NPs could effectively inactivate a broad range of pathogens, such as *Staphylococcus aureus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella enterica* and *C. jejuni* (Azam et al. 2012; Xie et al. 2011; Liu et al. 2009; Hernández-Sierra et al. 2008). However, the application of metal oxide nanoparticles in foods is still at its infancy due to the general health concerns of nanomaterials in agri-food products from different stakeholders, such as consumers, regulators, and the food industry (McClements and Xiao 2017). ZnO NPs should not be added directly to food products, but instead they can be incorporated into food packaging materials for the inactivation of foodborne pathogens. In the current study, we developed an innovative antimicrobial packaging as an alternative strategy that can reduce *C. jejuni* in raw chicken meat at the retail level. This novel strategy offers consumers with the protection from *Campylobacter*-contaminated poultry meat at the final stage of food chain regardless the contamination levels of poultry in farms or processing plants. Considering the potential migration of ZnO NPs from food packaging materials onto chicken meat, we immobilized ZnO NPs at minimum lethal concentrations against *C. jejuni* and also monitored the presence of ZnO NPs on the surface of the treated chicken meats.

Two types of paper-based active packaging (*i.e.*, 3D tubes and 2D pads) were developed for the inactivation of *C. jejuni*. First, broth dilution method was performed to identify the MBC

value of immobilized ZnO NPs in 3D paper tubes. The identified MBC (*i.e.,* 100 ppm) of the functionalized 3D tubes was selected to develop the 2D functionalized absorbing pads, achieving a final surface area concentration of ZnO NPs at 0.856 mg/cm^2. The attachment efficiency of ZnO NPs onto 2D functionalized absorbing pads was only 10% by using the ultrasound-assisted dipping technique. This relatively low attachment efficiency was mainly due to the repeated washing steps (*i.e.,* 3× washing) for the removal of any unattached ZnO NPs (**Figure 4.1B**). This washing step was of great importance because the absence of unattached nanoparticles could ensure no migration of free ZnO NPs from the functionalized absorbing pads to poultry meat products. In brief, the functionalized absorbing pads were dipped into 1,000 ppm of ZnO NPs suspension for 5 min under sonication and then washed three times to obtain a minimum lethal concentration of immobilized ZnO NPs (*i.e.,* 0.1 mg/cm^2). *C. jejuni* F38011 was selected as the representative strain for antimicrobial testing of both 3D and 2D functionalized paper products (**Figure 4.S1 and 4.S2**). This strain has been reported to infect humans, mice, and pigs (O'Loughlin et al. 2015) and could effectively colonize the gastrointestinal tract of live chickens (Klena, Gray, and Konkel 1998). To investigate the impact of immobilization on the antimicrobial activity of ZnO NPs, we compared the antimicrobial efficacy of immobilized ZnO NPs in 3D paper tubes with free ZnO NPs in solution (Xie et al. 2011; Hakeem et al. 2019). Previous studies reported that 100 ppm of free ZnO NPs could cause ≥8 log CFU/ml reduction of *C. jejuni* after only 3-h treatment in microaerobic condition with constant shaking at 37°C (Hakeem et al. 2019) or static condition at 42°C (Xie et al. 2011). Immobilized ZnO NPs into 3D paper tubes showed consistent antimicrobial efficacy to that of the free ZnO NPs after the same incubation condition (**Figure 4.S1**). Given that immobilization did not influence the antimicrobial effect of ZnO NPs, we immobilized ZnO NPs

into 2D functionalized absorbing pads at MBC level (0.1 mg/cm^2) for further food packaging application.

Functionalized absorbing pads with immobilized ZnO NPs were used for the inactivation of *C. jejuni* in raw chicken meat. Quantification of immobilized ZnO NPs into the functionalized absorbing pads dipped in 1,000 ppm of ZnO NPs suspension by ICP-OES showed that the final concentration of immobilized ZnO NPs was 0.075 mg/cm^2. This absorbing pad showed a limited inhibition zone when it was applied to inactivate *C. jejuni* by using the diffusion assay (**Figure 4.S2A**). It also showed limited antimicrobial effect against a cocktail of four *C. jejuni* strains on the surface of chicken meat products. Specifically, <1 CFU/sample log reduction was obtained after 8 d of treatment at 4°C (**Figure 4.3A**). A few factors could contribute to the reduced antimicrobial effect of this absorbing pad against *C. jejuni* in chicken meat. Firstly, other tested *C. jejuni* strains (*i.e.*, Human 10, ATCC 33560, 1173) could be more tolerant to ZnO NPs than *C. jejuni* F38011 (**Figure 4.4S**). Secondly, complex chicken matrices (*e.g.,* lipids and proteins in blood and chicken juice) and/or native chicken microflora might form protection armors around *C. jejuni* that can reduce the antimicrobial effect of ZnO NPs. Thirdly, *C. jejuni* could become more tolerant to antimicrobial treatment at 4°C than that at 37°C due to a reduced metabolic activity. In fact, *C. jejuni* does not grow below 30°C, but remains viable, perform respiration and responds normally to chemotaxis and aerotaxis even at 4°C (Hazeleger et al. 1998). This was consistent with our data as *C. jejuni* level did not decline in chicken meat for up to 8 d if not treated with the immobilized ZnO NPs (**Figure 4.3**). This highlights the need for an effective intervention strategy to control *C. jejuni* in the poultry packages during cold-chain transportation and storage.

To achieve sufficient inactivation of *C. jejuni* in raw chicken meat, functionalized absorbing pads with a higher concentration of immobilized ZnO NPs dipped in 10,000 ppm of

ZnO NPs suspension were developed. The final concentration of the immobilized ZnO NPs was determined to be 0.856 mg/cm^2. This level of immobilized ZnO NPs showed clear inhibition zones on *C. jejuni* lawns regardless the initial concentrations of this microbe (**Figure 4.S2B**).

Both functionalized absorbing pads were used to test the antimicrobial effect of immobilized ZnO NPs against a cocktail of *C. jejuni* on the surface of raw chicken meat. The immobilized ZnO NPs at 0.856 mg/cm^2 was able to reduce 4-log *C. jejuni* strains in chicken after 3 d treatment at 4°C (**Figure 4.3A & 4.S4**), which was equivalent to 5.60-log reduction of this microbe per 1 kg of chicken meat. Such a high contamination level has been the maximum level of *Campylobacter* reported in raw chicken products so far (Stern and Robach 2003; Kim et al. 2017; Luber and Bartelt 2007). It is noteworthy that the interaction between the immobilized ZnO NPs and *C. jejuni* was relatively slow and followed a time- and strain-dependent manner. A significant reduction ($P < 0.0001$) was observed after 3 d for two clinical strains (F38011 and Human 10) while the other two food-isolated strains (ATCC 33560 and 1173) were more resistant to the immobilized ZnO NPs (**Figure 4.S4**). This could be due to the ecology and adaptation and/or the different membrane surface charge of different strains. A significant reduction ($P < 0.05$) of all the tested *C. jejuni* strains was observed after 5-d storage at 4°C (**Figure 4.3A**).

Although the antimicrobial effect of ZnO NPs is well known, the antimicrobial working mechanism has not been fully investigated yet. Several previous studies reported that Zn^{2+} had a minor contribution to the antimicrobial effect of ZnO NPs due to its low dissociation (Pasquet et al. 2014; Jiang et al. 2016). However, the antimicrobial role of Zn^{2+} depends on several factors, including environmental conditions (*e.g.,* pH), exposure time, UV irradiation, presence of other substances or microorganisms, and physiochemical properties of nanoparticles (*e.g.,* size, shape, porosity, and concentration). For example, a recent study investigated the antimicrobial interaction

between *E. coli* and ZnO NPs along with their released compounds, such as Zn^{2+} and ROS (Jiang et al. 2016). Polyvinyl chloride (PVC) film coated with ZnO NPs was able to induce a potent antimicrobial affect even without a direct contact with bacterial cells. Unlike free ZnO NPs, ZnO NPs coated on the PVC films did not induce any outer membrane damage (Xie et al. 2011), but disrupted the intracellular integrity of carboxyfluoreses in-filled liposome and caused the leakage of K^+. The release of Zn^{2+} did not reduce bacterial populations while ROS played a role in the lethality of the immobilized ZnO NPs at neutral pH within 3 h of treatment. However, the authors failed to consider the antimicrobial effect of ZnO NPs at different pH levels. In another study, several microorganisms including *E. coli, P. aeruginosa*, *S. aureus* and *C. albicans* were identified to be sensitive to ZnO NPs and the contribution of Zn^{2+} to these bactericidal effects varied from 15%-100% (Pasquet et al. 2014). This study also identified that the release rate of Zn^{2+} was more consistent in buffered solution than distilled water within 24 h. The dissolution profiles of ZnO NPs varied on the basis of particle characteristics and morphologies (*e.g.*, size, surface area and type). Smaller nanoparticles released a higher amount of Zn^{2+}. Altogether, the contribution of Zn^{2+} to the overall antimicrobial effect of ZnO NPs is multi-factorial.

The correlation between time/pH and the release of Zn^{2+} from ZnO NPs was previously studied in simulated uterine solution (Z. Yang and Xie 2006). The contribution of Zn^{2+} to the overall antimicrobial effect of ZnO NPs was negligible at neutral pH, whereas a greater contribution of Zn^{2+} was obtained at a lower pH (Z. Yang and Xie 2006). The release of Zn^{2+} at a low pH was due to the reaction between ZnO and proton ions ($ZnO + 2H^+ \rightarrow Zn^{2+} + H_2O$) (Z. Yang and Xie 2006). This chemical dissociation may control antimicrobial delivery, such as the release of Zn^{2+}, in food packaging applications. Zn^{2+} was identified to have potent antimicrobial activity (McDevitt et al. 2011) and could treat several bacterial infectious diseases, such as pneumonia,

diarrhea, and prostatitis (McDevitt et al. 2011; Cho et al. 2002). Bacterial cells maintain the intracellular concentration of Zn^{2+} at a low level (10^{-4} M) even when they are grown in Zn^{2+}-rich environment, such as soil, oysters, and red meat (Eide 2006). The antimicrobial effect of Zn^{2+} is mainly a result of unspecific binding to the intracellular proteins and enzymes that are involved in protein synthesis and important metabolic pathways (Cerasi, Ammendola, and Battistoni 2013). These include proteins that are involved in DNA transcription and translation (Chivers 2007), sigma factor proteins (Campbell et al. 2007), tRNA synthesis proteins (Miller, Hill, and Schimmel 1991), RNA polymerases (Scrutton, Wu, and Goldthwait 1971) and ribosomal proteins (Hensley, Tierney, and Crowder 2011). As far as we know, Zn^{2+} release from ZnO NPs immobilized into food packaging materials has not been investigated yet.

In the current study, we observed a slow antimicrobial effect of immobilized ZnO NPs against *C. jejuni* on the surface of raw chicken meat. The level of Zn increased in chicken meat over time as determined by using ICP-OES (**Figure 4.4**), but no nanoparticle was observed by using SEM (**Figure 4.5**). Thus, we hypothesized that the increase of lactic acid generated by *Lactobacillus* in the packaging system including chicken meat, chicken juice, and functionalized absorbing pads allowed the slow release of Zn^{2+}, which eventually led to the inactivation of *C. jejuni* after 3-5 d at 4°C (**Figure 4.3**). Additional experiment showed a significant and controllable release of Zn^{2+} from ZnO NPs in the presence of lactic acid at different pH levels (**Figure 4.6**). Such a controlled release of Zn^{2+} not only improves the inactivation speed against *Campylobacter* in chicken meat but also expands the applications of ZnO NPs-functionalized absorbing pads to control other more tolerant pathogenic and spoilage microorganisms in agri-food commodities. It is noteworthy that the immobilized ZnO NPs might also partially disrupt bacterial cell wall and cell membrane via electrostatic interactions and thus allow the accumulation of extracellular Zn^{2+}

into the cells. However, further mechanism study is required to fully disclose the entire antimicrobial working mechanism of this active packaging application. The conversion of ZnO NPs to Zn^{2+} makes this packaging system an eco-friendly approach as the remaining functionalized absorbing pads only consist biodegradable materials (*e.g.,* cellulose) and Zn^{2+}.

4.6 Conclusion and future directions

Controlling *Campylobacter* in raw chicken meat is still a significant challenge to date. We developed a novel ionic antimicrobial release method to control this foodborne pathogen in the end-product of raw chicken meat. This functionalized packaging was able to reduce *C. jejuni* from ~log 4 CFU/sample to undetectable level after 3 d at 4°C. No migration of ZnO NPs from the functionalized absorbing pads to chicken meat was identified, while Zn level increased during chicken storage. We also identified that the presence of lactic acid was associated with a significant increase in the release of Zn ions from the functionalized absorbing pads to chicken meat. This novel approach can extend the applications of nanotechnology and replace the ineffective water consumption and costly conventional control methods used in poultry industry. Furthermore, this study opened the door for several future studies, such as the development of multilayer (consisting of a layer with nano-encapsulated organic acids) functionalized absorbing pads with ion-controlled release system to inactivate pathogens in a commercial scale of food production.

Chapter 5: Conclusion and Future Directions

The first hypothesis of this thesis project was that combining plant-based antimicrobials with each other or with metal oxide nanoparticles in binary or tertiary combinations induces synergistic interactions against *C. jejuni*. We selected three potent and unique antimicrobials of different origins to test individually and in combination. These include one plant oil (cinnamon oil), one spice (encapsulated curcumin), and ZnO NPs. Conventional methods including the time killing method and the FICI were identified to over- or under-estimate the synergism. Using time-killing method might lead to a potential overestimation of synergism as the combined effect is normally compared to the single effects on the basis of logarithmic scale with unrepayable additive line. FICI method relies on combining multiple sub-MICs. The minimum inhibitory concentration at specific FICI value indicates the type of interaction. It is difficult to predict the additive effect by combining two antimicrobials with different concentration-effect shapes. The extreme difference between two antimicrobials in their concentration-effect relationship highlights the challenge to predict the additive line of antimicrobial combinations by the conventional methods. The limitation is simply due to different dose-response of the combined antimicrobials. Therefore, it was important to use alternative methods to discover new synergistic combinations that are not detected by the conventional methods.

Some additive combinations and some synergistic combinations were identified using a nonlinear mathematical concentration-effect model. Up to 93.40% antimicrobial concentrations were reduced while maintaining the same effect at IC_{50} due to synergism between antimicrobials. The median-effect data showed that cinnamon oil was the most potent antimicrobial, followed by ZnO NPs and encapsulated curcumin. All of the median-effect curves of combined antimicrobials were parallel with the curve of the encapsulated curcumin. In addition, reducing the inhibitory

concentration of encapsulated curcumin resulted in less synergism between antimicrobials compared to either cinnamon oil or ZnO NPs. Thus, encapsulated curcumin played an important role in all synergistic interactions. This mathematical modelling can aid in developing new synergistic combinations to potentially reduce the prevalence and survival of foodborne pathogens as well as discover new mechanisms of synergistic antimicrobials.

After investigating the interactions between antimicrobials, we identified their synergistic adaptive molecular mechanisms in *C. jejuni*. We hypothesized that each antimicrobial plays a different role in synergistic combinations. Cinnamon oil increases the bacterial cell wall permeability and allows the accumulation of other antimicrobials in a more effective manner (synergistic) than single treatments. Furthermore, encapsulated curcumin elongates *C. jejuni* cells and increases their susceptibility to other antimicrobials that can disrupt bacterial cell wall. Finally, ZnO NPs damages *C. jejuni* cell wall, allowing other antimicrobials to accumulate inside the cells.

RNA-Seq showed that each single antimicrobial resulted in different transcriptional patterns. Cinnamon oil altered the expression of several genes involved in bacterial cell wall synthesis and cell permeability. Curcumin was identified to alter the expression level of genes involved in protein folding and synthesis of outer and plasma membranes. ZnO NPs altered the greatest number of genes and functions in *C. jejuni* compared to any other single, dual or triple antimicrobial treatments. Specifically, RNA polymerase sigma factor for flagella operon Cj0061c (*FliA*) was over-expressed by 3.22 folds by ZnO NPs treatment. No other sigma factor encoding gene was affected after any other single treatments.

Every single and combined antimicrobial treatment resulted in a unique transcriptomic response with some overlapping. The effect of three antimicrobials combined covered a broad range of bacterial cell wall components, synthesis of amino acids and inhibition of protein

synthesis and translation. We combined these antimicrobials in tertiary combination at lower concentrations than that in the single treatments due to their synergistic interactions. Dual and triple combined treatments altered some unique genes that were not affected by any of their single treatment counterparts. Many of these genes are involved in signaling and chemotaxis, amino acid synthesis, protein translation, and/or bacterial cell wall synthesis. Unlike dual antimicrobial treatments, all single treatments resulted in under-expression of a major facilitator superfamily encoding gene involved in developing antimicrobial resistance. Such synergistic combinations can be a good candidate as an alternative to antibiotics used in animal feeds and to prevent the colonization of *C. jejuni* in live chickens at an early stage.

Prevalence of *Campylobacter* in raw poultry remains one of the major food safety challenges. Novel mitigation strategies are required to ensure the safety and quality of poultry products. We developed a functionalized absorbing pad to control *C. jejuni* in raw chicken meat using immobilized ZnO NPs due to its high antimicrobial activity, great stability, and small size. Using an ultrasound-assisted technique, we immobilized ZnO NPs inside the absorbing pads at the MBC against *C. jejuni*. We investigated the antimicrobial effect against *C. jejuni* during storage of raw chicken meat at 4°C. The functionalized absorbing pad reduced *C. jejuni* from ~log 4 CFU/chicken sample to an undetectable level. No nanoparticle was migrated onto chicken meat after treatment with the functionalized absorbing pads.

Inactivation of *C. jejuni* by immobilized ZnO NPs in the functionalized packaging was due to direct contact with nanoparticles and release of antimicrobials. Analysis by inductively coupled plasma-optical emission spectroscopy showed that Zn level increased from 0.02 to 0.17 mg/cm^2 in the treated chicken meat. Inactivation of *C. jejuni* was also associated with the increase of *Lactobacillus* in raw chicken meat in a pH-dependent manner. We observed that less than 5% of

Zn^{2+} were released from ZnO NPs at neutral pH, while up to 88% were released when pH <3.5 within 2 d at 4°C. This controllable conversion of immobilized ZnO NPs to free Zn^{2+} makes the approach safe and eco-friendly and paves the ways to develop a novel intervention strategy for other high-risk foods including *Campylobacter*-contaminated chicken meat. This approach extends the applications of nanotechnology and reduces the ineffective water consumption and costly conventional control methods used in the poultry industry, such as machinery dipping and spraying with the conventional antimicrobials. Future studies on the development of multilayer functionalized absorbing pads with a controlled release system of ions to inactivate pathogens in a commercial scale of food production might expand the application of this strategy.

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Appendices

Appendix A: Chapter 2 supplementary tables

Antimicrobials	Concentrations of antimicrobial agents (ppm)
Cinnamon oil	0, 1.56, 3.12, 6.25, 12.5
ZnO NPs	0, 6.25, 12.5, 25, 50
Encapsulated curcumin	0, 4, 8, 16, 32
Cinnamon oil + 12.5 ppm ZnO NPs	0, 1.56, 3.12, 6.25, 12.5

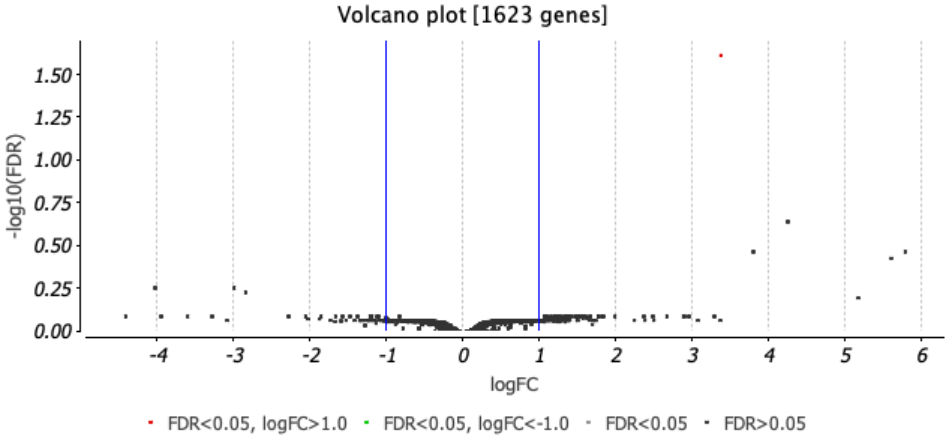
Table S1. Working concentrations of antimicrobial agents in time-killing method.

Table S2. Working concentrations of antimicrobial agents to generate concentration-effect curves.

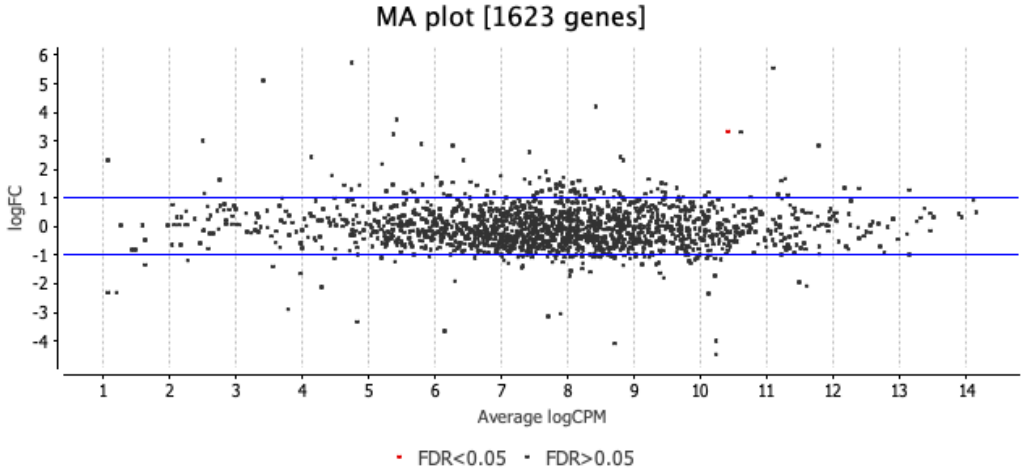
Antimicrobials	Concentrations of antimicrobial agents (ppm)
Cinnamon oil	0, 1.87, 2.5, 3.12, 4.68, 6.25, 12.5, 25, 50
ZnO NPs	0, 1.25, 3.75, 6.25, 9.37, 16, 18, 20, 25, 30, 35, 40, 50, 100
Encapsulated curcumin	0, 2.20, 5.40, 7.2, 9, 13.50, 24, 32, 36, 40, 48, 80

Appendix B: Chapter 3 supplementary figures

A



B



C

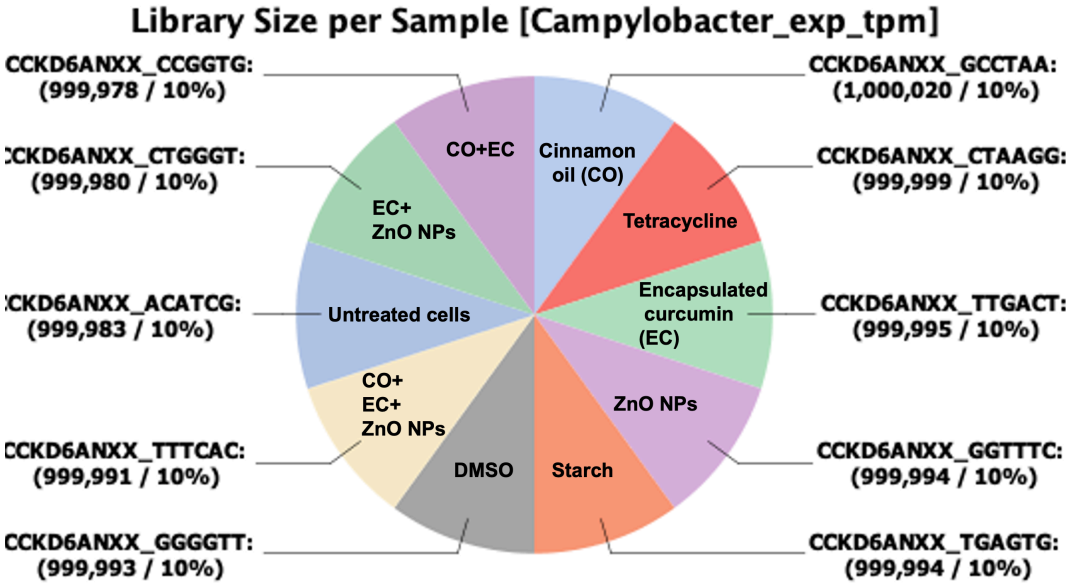


Figure 3.S1 Volcano plot (A), MA plot (B), and library size per sample (C) of *C. jejuni* F38011 after 15 min of single and combined antimicrobial treatments at 37°C in a microaerobic condition.

Appendix C: Chapter 4 supplementary figures

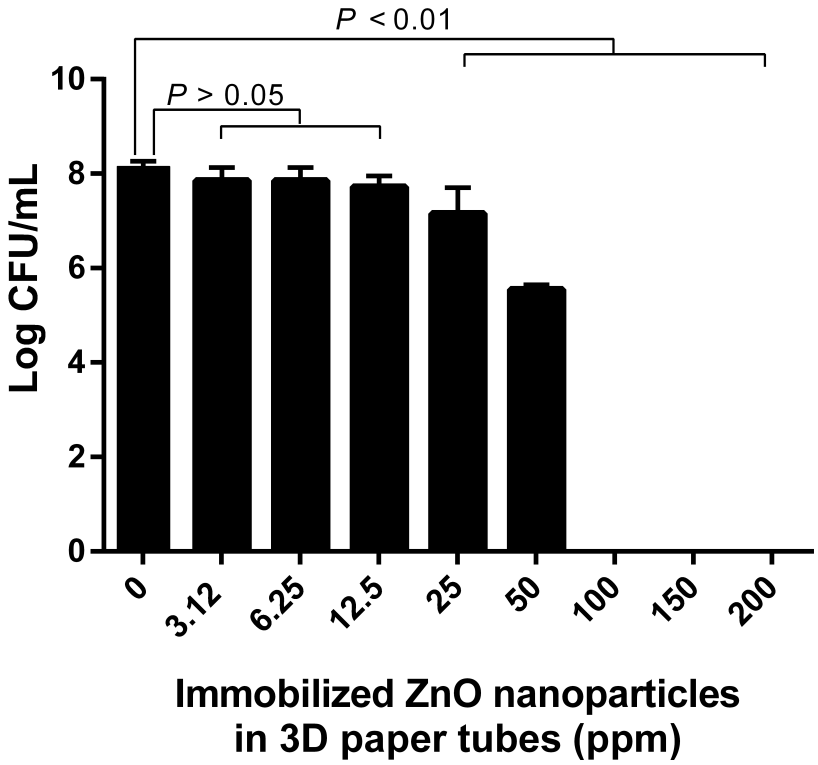


Figure 4.S1. Broth-dilution susceptibility test of the functionalized 3D paper tubes with ZnO nanoparticles. *C. jejuni* F38011 culture was placed into the functionalized 3D paper tubes with different concentrations of immobilized ZnO nanoparticles for 3 h at 37°C in a microaerobic condition with constant shaking at 175 rpm. The survived number of *C. jejuni* after this treatment was determined using the plating assay, followed by the identification of minimum bactericidal concentration (MBC) of immobilized ZnO NPs. Error bars represent the standard deviation. Data were analyzed by one-way ANOVA, followed by the post hoc Tukey's test for multiple comparisons (n = 3).

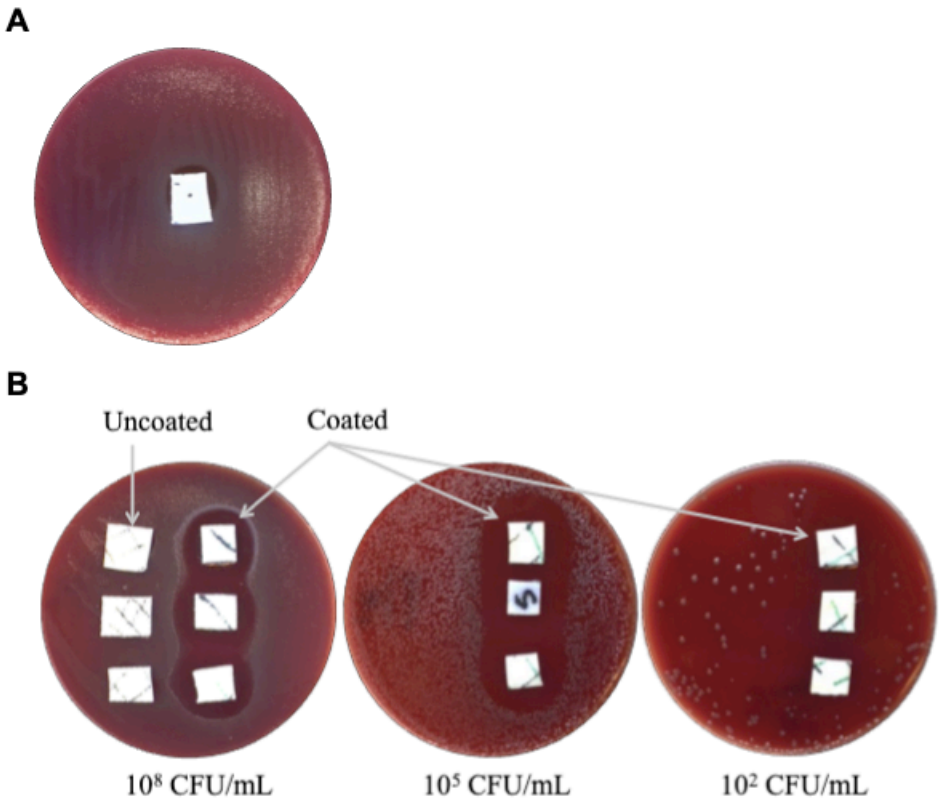


Figure 4.S2. Inhibition zones of the functionalized absorbing pads with immobilized ZnO nanoparticles against *C. jejuni* F38011 at 0.075 (A) and 0.856 mg/cm2 (B). Each piece of paper in the vertical order represents one batch of the functionalized absorbing pads. Bacterial lawns were grown on Mueller-Hinton agar supplemented with 5% defibrinated sheep blood and imaged after 48 h of incubation at 37°C in a microaerobic condition.

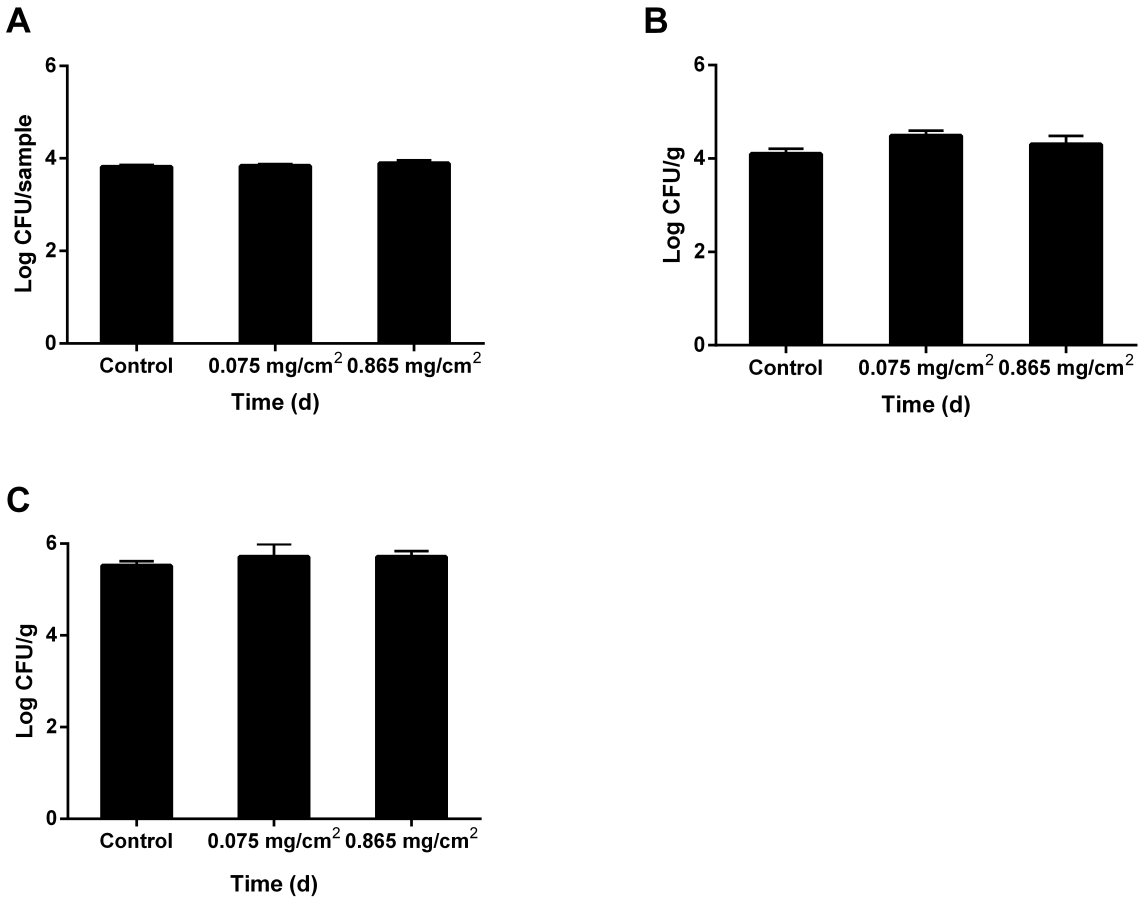


Figure 4.S3. The counts of *Campylobacter jejuni* cocktail (F38011, Human10, 1173, and ATCC 33560) (A), *Lactobacillus* (B) and psychrotrophs (C) on raw chicken breasts stored at 7°C with or without the functionalized absorbing pads including immobilized ZnO nanoparticles for 24 h. Limit of detection was determined to be 500 CFU/sample (*i.e.*, 25 g of chicken breast). Campy-Cefex, DeMan, Rogosa and Sharpe agar (MRS) and Tryptic Soy Agar (TSA) were used as the plating assay of each corresponding bacterium and plates were incubated at 42°C (in a microaerobic condition), 30°C and 7°C (in an aerobic condition), respectively. Data were analyzed by using one-way ANOVA, followed by the post hoc Tukey's test for multiple comparisons (n = 3).

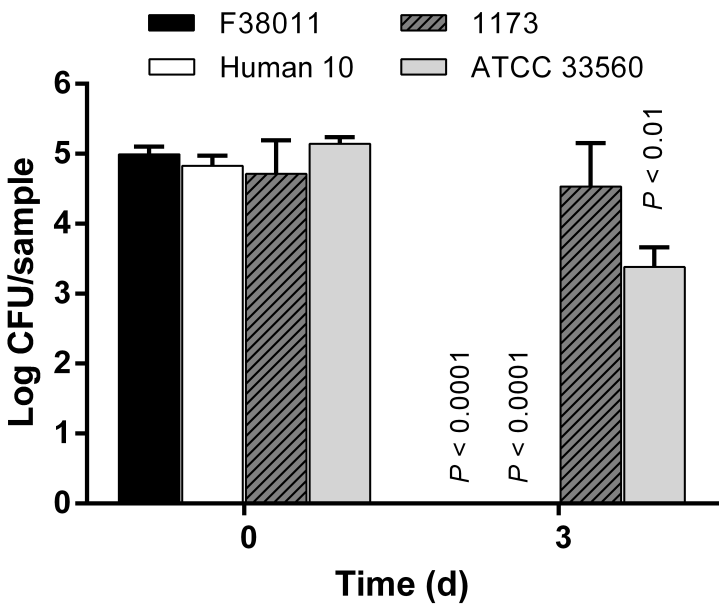


Figure 4.S4. The count of individual *Campylobacter jejuni* strain on the raw chicken breast stored for 3 days at 4°C with the functionalized absorbing pads including immobilized ZnO nanoparticles at 0.856 mg/cm^2. *C. jejuni* F38011 and human10 are clinical isolates; ATCC 33560 is a bovine fecal isolate; 1173 is a chicken isolate. Limit of detection was determined to be 500 CFU/sample (*i.e.*, 25 g of chicken breast). Mueller-Hinton agar supplemented with 5% defibrinated sheep blood was used as the plating assay and the plates were incubated at 42°C in a microaerobic condition. Data were analyzed by using one-way ANOVA, followed by the post hoc Tukey's test for multiple comparisons (n = 3).