**THE EFFECTS OF MEAT JUICE ON BIOFILM FORMATION OF *CAMPYLOBACTER*
AND *SALMONELLA***

by

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Abstract

Campylobacter and *Salmonella* are leading causes of foodborne illnesses worldwide. Raw meat is the common food reservoir for *Campylobacter* and *Salmonella*. Both microbes are prevalent in the meat processing environment in the form of biofilms that contribute to cross-contamination and food poisoning. This thesis project applied raw meat juice (chicken juice and pork juice) as a minimally processed food model to study its effect on bacterial biofilm formation. Meat juice was collected during raw meat freeze-thaw process and sterilized by filtration. All biofilms were developed on solid-liquid interface. Biofilm formation level was determined by live cell staining, which is based upon the metabolic activity of bacterial cells. In polystyrene 96-well plates, over 25% (v/v) of meat juice supplemented in Mueller Hinton broth could significantly ($P < 0.05$) enhance the survival of *Campylobacter* in its biofilm. Luria-Bertani broth supplemented with 10% meat juice (v/v) could lead to a significant difference ($P < 0.05$) in *Salmonella* biofilm formation compared to that without meat juice supplementation. During the initial attachment stage of biofilm development, more bacterial cells were present on the substrate with meat juice residues compared to clean substrate alone. Meat juice particles on substrates supported biofilm formation of *Campylobacter* and *Salmonella* under both static condition and flow condition achieved in a microfluidic platform. Inverted fluorescence microscope was used to determine the thickness and 3-dimensional structure of biofilms in a microfluidic chamber. Further, the deficiency in biofilm formation by selective *Campylobacter* and *Salmonella* mutant strains could be recovered in the presence of meat juice particles. Meat juice residues on the substrate may act as the conditioning layer to support initial attachment and biofilm formation of *Campylobacter* and *Salmonella*. In conclusion, these results suggest a possible survival mechanism of *Campylobacter* and *Salmonella* in the meat processing environment. In application, thorough cleaning of meat residues during meat production and handling is considered to be critical in reducing the bacterial load of *Campylobacter* and *Salmonella*.

Preface

This dissertation is original, unpublished, independent work by the author, Jiaqi Li.

Table of Contents

Abstract.....	ii
Preface.....	iii
Table of Contents	iv
List of Tables	vii
List of Figures.....	viii
List of Abbreviations	xi
Acknowledgement.....	xii
Chapter 1 - Introduction	1
1.1 <i>Campylobacter</i> and <i>Salmonella</i>	1
1.1.1 Characteristics of <i>Campylobacter</i> and <i>Salmonella</i>	1
1.1.2 Pathogenesis and clinical aspects of <i>Campylobacter</i> and <i>Salmonella</i>	4
1.1.3 Food products associated with <i>Campylobacter</i> and <i>Salmonella</i>	7
1.1.3.1 Meat products.....	7
1.1.3.2 Egg and dairy products	8
1.1.3.3 Fruits and vegetables.....	9
1.2 Microbial biofilms	9
1.2.1 Characteristics of biofilms	9
1.2.2 Impact of biofilms on public health	11
1.2.3 Mechanism of biofilm formation	13
1.2.4 Factors affecting biofilm formation	14
1.2.5 Control strategy of biofilms	15
1.2.6 Laboratory assays to study biofilms	16
1.2.7 Biofilms formed by <i>Campylobacter</i> and <i>Salmonella</i>	18
1.3 Meat juice	21
1.3.1 Introduction to meat juice	21
1.3.2 Effects of meat juice on survival of <i>Campylobacter</i> and <i>Salmonella</i>	22
1.3.3 Effects of meat juice on <i>Campylobacter</i> and <i>Salmonella</i> biofilms.....	23

1.4 Objectives of the study.....	24
Chapter 2 - Effects of meat juice supplementation on biofilm formation of <i>Campylobacter</i> and <i>Salmonella</i>	26
2.1 Background	26
2.2 Materials and methods	27
2.2.1 Preparation of meat juice	27
2.2.2 Bacterial strains and culture preparation.....	28
2.2.3 Planktonic cell growth assay.....	28
2.2.4 Biofilm cultivation	29
2.2.5 TTC biofilm staining assay	29
2.2.6 Statistical analysis	30
2.3 Results and discussion	30
2.3.1 Growth of planktonic cells in meat juice	30
2.3.2 Biofilm formation in meat juice.....	32
2.4 Summary.....	38
2.5 Tables and figures	40
Chapter 3 - Effects of meat juice coating layer on initial attachment and biofilm formation of <i>Campylobacter</i> and <i>Salmonella</i>	51
3.1 Background	51
3.2 Materials and methods	53
3.2.1 Bacterial strains and cultures	53
See section 2.2.2.	53
3.2.3 Cell adhesion assay	53
3.2.4 Biofilm assay	53
3.2.5 Statistical analysis.....	54
3.3 Results and discussion	54
3.3.1 Cell adhesion to meat juice pre-coated surfaces	54
3.3.2 Biofilm formation on abiotic substrate pre-coated with meat juice.....	56
3.4 Summary.....	58
3.5 Tables and figures	59

Chapter 4 - Effects of meat juice coating on <i>Campylobacter</i> and <i>Salmonella</i> biofilm formation with respect to flow condition and cell motility	69
4.1 Background	69
4.2 Materials and methods	72
4.2.1 Bacteria strains and culture preparation.....	72
4.2.2 Biofilm assay for biofilm-defective mutant strains	72
4.2.3 Fabrication of microfluidic chip	73
4.2.4 Biofilm cultivation in microfluidic platform	73
4.2.5 Fluorescence microscopy	74
4.2.6 Statistical analysis	74
4.3 Results and discussion	75
4.3.1 <i>Campylobacter</i> and <i>Salmonella</i> biofilms under flow condition	75
4.3.2 Reduced biofilm formation compensated by pre-coated layer of meat juice	77
4.4 Summary.....	79
4.5 Tables and figures.....	80
Chapter 5 - Conclusions and significance of the study.....	87
References.....	90
Appendix - Supplementary tables and figures	117

List of Tables

Table 2.1. Bacterial strains with source and reference.	40
Table 2.2. Growth of <i>Campylobacter</i> planktonic cells in meat juice, shown as the mean of viable cell count $\pm$ standard deviation of the mean.	41
Table 2.3. Growth of <i>Salmonella</i> planktonic cells in meat juice, shown as the mean of viable cell count $\pm$ standard deviation.	42
Table 3.1. Attached <i>C. jejuni</i> F38011 cell number (shown as cell number /100 μm^2 of substrate surface) on pre-coating layer formed by meat juice.	59
Table 3.2. Attached <i>S. Typhimurium</i> SL1344 cell number (shown as cell number /100 μm^2 of substrate surface) on pre-coating layer formed by meat juice.	60
Table A.1. Characteristics of the meat juice after sterilization and calibration.	117

List of Figures

Figure 2.1. Formation level of <i>C. jejuni</i> F38011 biofilms on polystyrene 96-well plate with MH broth and gradient of meat juice.	43
Figure 2.2. Formation level of <i>C. jejuni</i> biofilms in polystyrene 96-well plate with MH broth, MH broth supplemented with meat juice (50%, v/v), and pure meat juice, respectively. a) chicken juice; b) pork juice.	44
Figure 2.3. Formation level of <i>C. jejuni</i> F38011 biofilms in glass slide chamber with MH broth and gradient of meat juice.	45
Figure 2.4. Formation level of <i>C. jejuni</i> strains biofilms formed in glass slide chamber with MH broth, MH broth supplemented with meat juice (50%, v/v), and pure meat juice, respectively. a) chicken juice; b) pork juice.	46
Figure 2.5. Formation level of <i>S. Typhimurium</i> SL1344 biofilms formed in polystyrene 96-well plate with LB broth and gradient of meat juice.	47
Figure 2.6. Formation level of <i>S. enterica</i> strains biofilms formed in polystyrene 96-well plate with LB broth, LB broth supplemented with meat juice (50%, v/v), and pure meat juice, respectively. a) chicken juice; b) pork juice.	48
Figure 2.7. Formation level of <i>S. Typhimurium</i> SL1344 biofilms in glass slide chamber with LB broth and gradient of meat juice.	49
Figure 2.8. Formation level of <i>S. enterica</i> biofilms in glass slide chamber with LB broth, LB broth supplemented with meat juice (50%, v/v), and pure meat juice, respectively. a) chicken juice; b) pork juice.	50
Figure 3.1. Formation levels of <i>C. jejuni</i> F38011 biofilms in polystyrene 96-well plate pre-coated with gradient of meat juice.	61
Figure 3.2. Biofilm formation levels of various <i>C. jejuni</i> strains in polystyrene 96-well plate pre-coated with meat juice.	62
Figure 3.3. Formation levels of <i>C. jejuni</i> F38011 biofilms in glass slide chamber pre-coated with gradient of meat juice.	63
Figure 3.4. Biofilm formation levels of various <i>C. jejuni</i> strains in glass slide chamber pre-coated with meat juice.	64
Figure 3.5. Formation levels of <i>S. Typhimurium</i> SL1344 biofilms in polystyrene 96-well plate pre-coated with gradient of meat juice.	65

Figure 3.6. Biofilm formation levels of various <i>S. Typhimurium</i> strains in polystyrene 96-well plate pre-coated with meat juice.	66
Figure 3.7. Formation levels of <i>S. Typhimurium</i> SL1344 biofilms in glass slide chamber pre-coated with gradient of meat juice.	67
Figure 3.8. Biofilm formation levels of various <i>S. Typhimurium</i> strains in glass slide chamber pre-coated with meat juice.	68
Figure 4.1. Design and configuration of a PDMS-based microfluidic chip with glass bottom for the cultivation of bacterial biofilms.	80
Figure 4.2. Representative fluorescence microscopic images of <i>C. jejuni</i> F38011-gfp biofilms grown in microfluidic chamber under static (A) and flow condition (B). The average fluorescence intensity of each biofilm sample is shown in panel (C) with background intensity subtracted.	81
Figure 4.3. Representative fluorescence microscopic images of <i>S. Typhimurium</i> SL1344-gfp biofilms grown in microfluidic chamber under static (A) and flow condition (B). The average fluorescence intensity of each biofilm sample is shown in panel (C) with background intensity subtracted.	82
Figure 4.4. Biofilm formation level of aflagellated <i>C. jejuni</i> F38011 mutants in polystyrene 96-well plate with and without meat juice pre-coated.	83
Figure 4.5. Biofilm formation level of aflagellated <i>C. jejuni</i> F38011 mutants on glass chamber with and without meat juice pre-coated.	84
Figure 4.6. Biofilm formation level of attachment-defective mutants of <i>S. Typhimurium</i> 14028 formed in polystyrene 96-well plate with and without meat juice pre-coated.	85
Figure 4.7. Biofilm formation level of attachment-defective mutants of <i>S. Typhimurium</i> 14028 formed on glass slide chamber with and without meat juice pre-coated.	86
Figure A.1. <i>C. jejuni</i> F38011 planktonic cell growth in MH broth and meat juice during a 30-day time course, respectively.....	118
Figure A.2. <i>S. Typhimurium</i> SL1344 planktonic cell growth in MH broth and meat juice during a 30-day time course, respectively.....	119
Figure A.3. Standard curve of <i>C. jejuni</i> F38011 cell count and OD value at 600nm.	120

Figure A.4. Standard curve of <i>Salmonella</i> cell count and OD value at 600nm. (a) <i>S. Enteritidis</i> ATCC33560; (b) <i>S. Typhimurium</i> SL1344	121
Figure A.5. Potential toxicity of TTC on <i>C. jejuni</i> F38011 and <i>S. Typhimurium</i> SL1344..	122
Figure A.6. Biofilm formation levels of <i>C. jejuni</i> F38011 and <i>S. Typhimurium</i> SL1344 in a 5-day time course.....	123
Figure A.7. Negative control of fluorescence images (no bacteria introduced).	124

List of Abbreviations

AI: autoinducer

BHI: Brain-Heart Infusion

CDC: U.S. Centre for Disease Control and Prevention

c-di-GMP: bis-(3'-5')-cyclic dimeric guanosine monophosphate

CFU: colony forming unit

CJ: chicken juice

C. jejuni: *Campylobacter jejuni*

CLSM: confocal laser scanning microscope

EPS: extracellular polymeric substances

FAO: Food and Agriculture Organization of the United Nations

FDA: U.S. Food and Drug Administration

LB: Luria-Bertani

MH: Müller Hinton

OD: optical density

P. aeruginosa: *Pseudomonas aeruginosa*

PDMS: poly-dimethylsiloxane

PJ: pork juice

S. aureus: *Staphylococcus aureus*

S. enterica: *Salmonella enterica*

S. Enteritidis: *Salmonella enterica* serovar Enteritidis

S. Typhimurium: *Salmonella enterica* serovar Typhimurium

TSB: tryptic soy broth

TPF: 1,3,5-triphenylformazan

TTC: 2,3,5-triphenyltetrazolium chloride

WHO: World Health Organization

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Chapter 1 - Introduction

1.1 *Campylobacter* and *Salmonella*

1.1.1 Characteristics of *Campylobacter* and *Salmonella*

Campylobacter was first isolated from aborted sheep by John McFadyean in 1906, described as a spiral microbe that resembles *Vibrio*. This first isolated strain was then defined as *C. fetus* in 1963, at which time the genus name *Campylobacter* was created (Nachamkin, Szymanski, & Blaser, 2008). *Campylobacter* was mainly known as a cause of veterinary disease for decades before it was recognized as a human pathogen in 1970s. In 1977, Skirrow and his team developed an efficient routine isolation method for *Campylobacter* bacteria (Skirrow & Benjamin, 1980). There are currently 17 species and 6 sub-species assigned to the genus *Campylobacter*, and genera *Campylobacter* and *Arcobacter* belong to the family *Campylobacteraceae* (Nachamkin et al., 2008).

The relatively late discovery of *Campylobacter* may be due to its fastidiousness to the growth environment and relatively low regeneration rate compared to other pathogenic bacteria (e.g., *Salmonella* and *Listeria*). *Campylobacter* is a typically spiral-shaped, Gram-negative, micro-aerophilic bacterium. The size of *Campylobacter* cells is 0.2 to 0.8- μm wide and 0.5 to 5- μm long. It is typically motile with a featured corkscrew-like motion motivated by a single polar unsheathed flagellum at one or both ends of the bacterial cell body. Replication occurs within a narrow temperature range of 32-47°C with the preference in a microaerobic condition (i.e., reduced oxygen level at 5%-10%). The normal level of oxygen in the environmental atmosphere (~21%) can be fatal to *Campylobacter*. *Campylobacter* grows best at pH range of 6.5-7.5 and it is sensitive to acidic condition (Alter & Scherer, 2006).

Substantial efforts were applied to reduce oxygen levels for optimum *Campylobacter* cultivation, such as supplementing blood as an oxygen scavenger into the culture media (Merino et al., 1986). US Food and Drug Administration (FDA) recommends a standard atmosphere consisting of 5% O₂, 10% CO₂ and 85% N₂ for the cultivation and enrichment of most *Campylobacter* species (Macé, Haddad, Zagorec, & Tresse, 2015).

The growth of *Campylobacter* is normally slower than other pathogenic bacteria. It takes over 18 h for *Campylobacter* culture to achieve stationary phase under their favourable condition (Fitzgerald, 2015). During growth, this microbe obtains energy from amino acids or tricarboxylic acid cycle intermediates instead of carbohydrates (Nachamkin et al., 2008). Hydrogen, formate, and succinate are commonly supplemented in the growth media as electron sources, although they are not necessary for all the species of *Campylobacter*. Gelatin, casein, starch, and tyrosine are not hydrolyzed by *Campylobacter* (Hofreuter, 2014). Culture media used in the laboratory for optimal growth of *Campylobacter* include Müller Hinton (MH) broth, Brucella broth, and Brain-Heart Infusion (BHI) broth (Tang, 2000).

Salmonella is a pathogenic bacterium belonging to the family *Enterobacteriaceae*. It was first observed by Karl Eberth 1880 in the Peyer's patches and spleens of typhoid patients (Eckehart, 1967). In 1885, Daniel Salmon's group in the Veterinary Division of the United States Department of Agriculture discovered the microorganism later known as *Salmonella enterica* serovar Choleraesuis. The genus name “*Salmonella*” was officially proposed in the honour of Daniel Salmon in 1900. Although the nomenclature of *Salmonella* has been controversial, it is

now widely accepted that *Salmonella enterica* and *Salmonella bongori* are the only two species of *Salmonella* genus. *Salmonella enterica* is further divided into six sub-species that include over 2500 serovars, which are defined on the basis of the somatic O (lipopolysaccharide) and flagellar H antigens with credit to Kauffman-White classification (Brenner, Villar, Angulo, Tauxe, & Swaminathan, 2000).

Salmonella species are Gram-negative, rod-shaped, facultative anaerobic, predominantly motile bacteria with cell diameters between 0.7 and 1.5 μm and lengths from 2 to 5 μm , featured by multiple peritrichous flagella. Its optimal growth condition is 35-40°C with pH range of 6.5-7.5 (Bailey, Richardson, Cox, & Cosby, 2010). *Salmonella* is readily adapted to extreme environmental conditions (Humphrey, 2004). Most *Salmonella* serotypes can grow over the temperature range 7-48°C, and some serotypes can even grow at temperatures as low as 4°C (Chen, Wang, Su, & Chiu, 2013). This microbe is able to survive for extended periods in chilled and frozen foods (Neetoo & Chen, 2015). A degree of tolerance to extreme pH (3.7-9.5) and high concentration of salts (up to 5%) has been identified in *Salmonella* growth (Thayer, Muller, Buchanan, & Phillips, 1987).

Salmonella cells reproduce with a cell division interval of 40 minutes under optimal growth conditions (Lianou & Koutsoumanis, 2013). As a member of *Enterobacteriaceae*, *Salmonella* is characterized by its ability to metabolize citrate as the sole carbon source and lysine as a nitrogen source as well as the ability to produce hydrogen sulphide (Nataro, Bopp, Fields, Kaper, & Strockbine, 2015). *Salmonella* readily multiplies in several nutrient media that are widely used in the laboratory, such as tryptic soy broth (TSB) and Luria Bertani (LB) broth.

1.1.2 Pathogenesis and clinical aspects of *Campylobacter* and *Salmonella*

Campylobacter and *Salmonella* are two common pathogens that contribute to various foodborne illnesses worldwide. Pathogenesis derived from these two bacteria also leads to significant economic loss (Alter & Scherer, 2006; McEntire, Acheson, Siemens, Eilert, & Robach, 2014). In 2014, *Salmonella* maintained the highest incidence rate of 15.45 per 100,000 population in the United States among all foodborne bacterial pathogens. The second highest incidence rate was associated with *Campylobacter*, with an incidence rate of 13.45 per 100,000 people. Overall, *Campylobacter* and *Salmonella* together contributed to over 20% of all reported cases related to foodborne bacterial infections (CDC, 2015). Campylobacteriosis and salmonellosis have a comparable level of incidence in Canada with a rate of 23.11 and 22.19 per 100,000 people, respectively. According to the latest report, the incidence rate of campylobacteriosis in North America has increased in the recent five years while the number of incidences of salmonellosis has been maintained (Public Health Agency of Canada, 2015). Similar to that in North America, campylobacteriosis and salmonellosis have been massively identified and reported in Europe, Asia, and Australia (Kaakoush, Castaño-Rodríguez, Mitchell, & Man, 2015; Majowicz et al., 2010). Despite asymptomatic carriage in animals, *Campylobacter* and *Salmonella* are also important causes of veterinary diseases, such as livestock enteritis, septicaemia, and abortion (Nachamkin et al., 2008).

Most cases of *Campylobacter*-associated enteritis are sporadic, thus the sources of infection are difficult to identify. Numerous studies have reported that campylobacteriosis is mainly associated with undercooked or contaminated poultry meat (CDC, 2013). Large *Campylobacter*

outbreaks are rare (<1% reported cases) and these have been linked to raw milk, poultry liver pâté, salads, raw clams, garlic butter, fruits, and contaminated water. In comparison, *Salmonella* outbreaks occur more frequently (>5% reported cases). In the US, there were over 3,000 outbreaks associated with *Salmonella* from 2004-2011 (Ebel et al., 2016). A variety of food products were confirmed as *Salmonella* carriers in these outbreaks, including poultry and poultry meat, cucumbers, nuts and nut products, sprouts, cheese, pet foods, etc. (CDC, 2013).

Most *Campylobacter* species are pathogenic to human beings. By far, *C. jejuni* and *C. coli* play the most significant roles (WHO & FAO, 2009). *C. jejuni* contributes to over 80% of the reported infection cases over the past ten years (CDC, 2015). Other *Campylobacter* species, such as *C. fetus*, *C. lari*, and *C. upsaliensis*, are frequently isolated from wild or domestic animals, but human infections rarely occur (<5% of total campylobacteriosis), which may be due to the challenges in cultivation. Therefore, it is believed that the infections caused by species other than *C. jejuni* and *C. coli* are underestimated (Man, 2011).

Campylobacteriosis is an acute diarrheal disease caused by oral digestion of *Campylobacter* cells. The infection dose of *Campylobacter* can be less than 500 live cells. Digested *Campylobacter* cells are delivered to intestine and affect the colon and rectum. *Campylobacter* is generally considered to be invasive although the mechanism of uptake into epithelial cells remains unclear. There are no specialized type III secretion systems in *Campylobacter* that mediate invasion into the epithelial cells (Guerry, 2007). Instead, flagella filaments appears to function as a type III secretion organelle to secrete a number of proteins, some of which are reported to affect invasion (Konkel et al., 2004).

Clinical manifestation of campylobacteriosis involves bloody diarrhea, fever, abdominal pain, nausea, and vomiting, and the symptoms last for an average of 4.6 days. The disease is usually self-limiting. Children under five years old and young adults are most frequently affected by this microbe. Campylobacteriosis occasionally leads to complications, such as arthritis (*e.g.*, Reiter's syndrome). Reactive arthritis occurs in 1% of campylobacteriosis cases and 0.1% can suffer Guillain-Barré syndrome, a severe neurological disorder that leads to paralysis (Nachamkin et al., 2008).

Salmonella is a facultative intracellular pathogen and it is capable of living and reproducing either inside or outside of the infected cells. Serovars are clinically categorized as invasive (typhoidal) or non-invasive (nontyphoidal) based upon host preference and disease manifestations in humans. Nontyphoidal serovars are more common and usually cause self-limiting gastrointestinal diseases with similar symptoms as campylobacteriosis but no bloody diarrhea (Dekker & Frank, 2015). Live cells that successfully enter intestines can multiply in epitheliums and tissues and release endotoxins that lead to local immune responses (Chen et al., 2013). So far, ~2000 serotypes of nontyphoidal *Salmonella* have been identified and the leading ones associated with human infections are *Salmonella enterica* serovar Typhimurium, Enteritidis, and Newport (Bailey et al., 2010). My current study uses selective strains of nontyphoidal *S. Typhimurium* and *S. Enteritidis*.

Typhoidal serovars mainly include *Salmonella* serovar Typhi and Paratyphi, which can cause systemic infections in humans. After invading into intestinal mucosa, typhoidal strains

disseminate through a transient primary bacteremia. Some individuals can develop typhoid fever that involves high temperature ($>39^{\circ}\text{C}$), vomiting, headache, and sometimes complications (*e.g.*, neurologic involvement and intestinal perforation). The typhoidal salmonellosis occurs more frequently in developing countries (Dougan & Baker, 2014).

1.1.3 Food products associated with *Campylobacter* and *Salmonella*

1.1.3.1 Meat products

Campylobacter and *Salmonella* infections are highly related to poultry and poultry products. According to a recent report, the loads of *Campylobacter* and *Salmonella* in chicken meat in Canada were 49% and 32%, respectively (Public Health Agency of Canada, 2015). Consumption of undercooked chicken and inappropriate handling of chicken carcasses are regarded as the major causes of campylobacteriosis (CDC, 2015). Other meat products, such as beef and pork, are associated with reported infections as well but with a lower number. Less than 5% of ground beef and pork chops sampled from local grocery stores were tested to be positive for *Campylobacter* and *Salmonella* (Public Health Agency of Canada, 2015).

Campylobacter and *Salmonella* are intestinal habitants in many warm blooded animals (Boutrif & Schlundt, 2009). Over 80% of swine and beef manure and 10% of broiler chicken manure were tested to be positive for *Campylobacter*. The incidence of *Salmonella* in manure of broiler chicken, swine, and beef is 60%, 38%, and 9%, respectively (Public Health Agency of Canada, 2015). *Campylobacter* and *Salmonella* can spread and transmit to animal muscle during slaughtering and further processing (McEntire et al., 2014). Chicken skin was reported to maintain 7-fold higher *Campylobacter* numbers than the underlying muscle, indicating that

chicken was predominantly contaminated by bacteria in the processing environment, such as food contact surfaces (Hansson, Nyman, Lahti, Gustafsson, & Olsson Engvall, 2015). Although *Campylobacter* and *Salmonella* in raw meat can be eliminated by cooking over 75°C (Pouillot et al., 2012), inappropriate handling of meat carcasses during cooking can result in cross-contamination between raw meat and cooked meat (Guyard-Nicodème et al., 2013; Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003). Such cross-contamination is believed to pose a higher risk of food poisoning compared to the consumption of undercooked meat products (Luber, 2009).

1.1.3.2 Egg and dairy products

Besides poultry and meat products, egg and dairy products (*e.g.*, shell egg, raw milk, and unpasteurized cheese) are also regarded as common food vehicles for *Campylobacter* and *Salmonella*. As aforementioned in 1.1.3.1, *Campylobacter* and *Salmonella* are present in manure samples from chicken and cattle on the farm. The bacteria in the chicken's reproductive tract can directly transmit to eggs. Eggs and milk could also be contaminated by these bacteria in the breeding environment (Whiley & Ross, 2015). Over 90% of *Campylobacter* outbreaks in the recent ten years in the US were caused by unpasteurized milk (CDC, 2015). *Salmonella* was also associated with several raw milk and cheese outbreaks during 2004-2011 (Ebel et al., 2016). The largest multistate outbreak in US was associated with shell eggs contaminated by *S. Enteritidis*, resulting in an estimated 1,939 reported cases of illnesses (CDC, 2010).

1.1.3.3 Fruits and vegetables

The contamination rate of *Campylobacter* and *Salmonella* in fresh fruits and vegetables is not as high as that in animal-based products. The contamination of fresh produce with *Campylobacter* and *Salmonella* is usually heterogeneously spread and most likely present in low numbers, leading to challenges in routine sampling and diagnosis (Perez-Rodriguez, Begum, & Johannessen, 2014). There were a number of recent outbreaks caused by *Salmonella* contaminated fresh produce (CDC, 2016). Foodborne illness associated with fresh produce has become more common possibly due to the increased consumption (Andreoletti et al., 2013). The most frequently reported fresh produce contaminated by *Salmonella* are cucumbers, melons, and sprouts (CDC, 2016), estimated to be 20% of the outbreak cases that occurred in the US in the recent ten years.

1.2 Microbial biofilms

1.2.1 Characteristics of biofilms

A biofilm is a consortium of microorganisms in which cells associate with each other and adhere onto a surface (Ghannoum & O'Toole, 2004). The first time that biofilm was noticed could be traced back to the early 1970s, when Costerton and Cheng observed that some bacterial cells were embedded in a polysaccharide matrix and attached to the intestinal tissues of calves by using transmission electron microscopy (Cheng & Costerton, 1980). In the 1990s, numerous studies reported the discovery of bacterial biofilms on the implanted medical devices and in the oral environment. The treatment dose of antimicrobials that were effective in inactivating planktonic bacterial cells typically failed to eradicate bacterial biofilms (Costerton, 2004).

Biofilm is believed as the major form of microorganisms living outside laboratory to battle environmental stress (Branda & Kolter, 2004; Jefferson, 2004).

There are two major components of a biofilm, namely the sessile bacterial cells and a matrix of self-produced extracellular polymeric substances (EPS). EPS matrix comprises 50-90% dry mass of a biofilm varying among different types of biofilm-forming microorganisms. The production of EPS features the transition from planktonic cells to biofilms. The major compositions of EPS are microbial polysaccharides along with small amount of proteins, nucleic acids, and polymeric lipophilic compounds (Flemming & Wingender, 2010). EPS plays an important role in the survival of sessile cells. Specifically, EPS protect bacterial cells from environmental stress, mediate cell metabolism, and coordinate cell-to-cell communication (Stewart & Franklin, 2008).

Sessile cells within biofilms are physiologically and morphologically distinctive from their planktonic counterparts. Multi-cellularity of bacteria is highly behaved in biofilms, in which the cells establish and maintain long-term relationships with each other through chemical interactions (Branda & Kolter, 2004). A group of bacteria can produce and respond to small hormone-like molecules termed autoinducer (AI). This cell-to-cell communication process is named “quorum sensing” and it is the universal multicellular behavior in a bacterial community, allowing individual cells to behave on a population-wide scale in response to external variations (*e.g.*, environmental stress and antimicrobial treatment). Most quorum sensing processes can only be triggered in a large number of cells (Waters & Bassler, 2005). Quorum sensing is indispensable in EPS production and biofilm formation (Singh et al., 2000).

Production of EPS and multicellular cooperation lead to a successful life of bacteria in the environment. Sessile cells within a biofilm are much more resistant to various stresses and difficult to be inactivated compared to their planktonic counterparts. For instance, sodium hypochlorite, one of the most effective broad-spectrum antibacterial agents, requires a 600-fold increase in concentration to inactivate *Staphylococcus aureus* sessile cells within a biofilm than the planktonic cells (Luppens et al., 2002). Bacteria residing in a biofilm might be less susceptible to extreme conditions and antimicrobial treatments because these cells exhibit reduced metabolic activity and growth rate compared to the planktonic cells (Davies, 2003). In addition, bacterial cells in a biofilm can express specific protective factors, such as multidrug efflux pumps (Yamasaki, Wang, Hirata, Hayashi-Nishino, & Nishino, 2015) and stress-response regulons (Fuente-Núñez, Reffuveille, Fernández, & Hancock, 2013). EPS matrix might act as chemical adsorbent or physical barrier, thereby reducing the amount of antimicrobials to interact with bacterial cells in a biofilm (Stewart & Franklin, 2008). Further, biofilms are also resistant to other disinfecting treatments, such as heat treatment, radiation, and use of surfactants (Van Houdt & Michiels, 2010).

1.2.2 Impact of biofilms on public health

Biofilms can form on various surfaces, including biotic surface and abiotic surface. Their high prevalence and resistance to antimicrobial treatments lead to significant public health concerns. Biofilms formed by pathogenic microorganisms were identified in patient's organs and tissues, such as tooth surfaces (Zijnga et al., 2010), diabetic wounds (Kwiecinski, Kahlmeter, & Jin, 2015), lungs with cystic fibrosis (Ciofu, Tolker-Nielsen, Jensen, Wang, & Høiby, 2015), and gallstones (Marshall, Flechtner, La Perle, & Gunn, 2014). These biofilms and those on the

implanted devices result in significant morbidity and mortality cases (Shah, Tataara, D'Souza, Mikos, & Kasper, 2013).

In food processing environments, most bacteria are able to develop biofilms on various inert abiotic surfaces, such as wood, stainless steel, and plastics. It is believed that biofilms are widely available in food industry. Commonly used disinfectants to clean food contact surfaces may not be fully effective to eradicate biofilms (Corcoran et al., 2014). Pathogen-containing biofilms formed on the direct and indirect food contact surfaces are considered to be one of the major risk factors of cross contamination during food processing (Giaouris et al., 2014; Simões, Simões, & Vieira, 2010; Sofos & Geornaras, 2010).

Foodborne pathogenic bacteria attached to the surfaces of fresh produce (*e.g.*, leafy greens) are associated with individual infections and outbreaks (Yaron & Römling, 2014). Fresh-cut produce can be contaminated during cutting and packaging steps. In a previous study, the use of chlorine in washing step failed to inactivate *Escherichia coli* cells on the leafy greens. These cells were shown as clusters encased in a self-produced matrix that were attached to the surface of the vegetables by using scanning electron microscopy (Lopez-Galvez, Gil, Truchado, Selma, & Allende, 2010). The biofilms of *Listeria*, *Pseudomonas*, *E. coli*, and *Bacillus* are also frequently identified in the dairy environment. Latorre and coauthors discovered *Listeria*-containing biofilms in the milking equipment, as a potential source of bulk tank milk contamination on a dairy farm where milk contamination was previously documented (Latorre et al., 2010). Additionally, biofilms are a common cause of biofouling in dairy filtration membranes, leading to reduced efficacy and shortened working life of the membranes (Tang, Flint, Bennett, &

Brooks, 2010). Similar membrane fouling problems were also identified in water treatment facilities (Flemming, 2002).

1.2.3 Mechanism of biofilm formation

A five-step process of *P. aeruginosa* biofilm development is widely accepted as a representative example of biofilm formation (Sauer et al., 2002). To form a biofilm, floating bacterial cells first loosely attach to a surface through van der Waals forces, electrostatic forces, and hydrophobic interactions at the cell-substrate interface (Palmer, Flint, & Brooks, 2007; Van Houdt & Michiels, 2010). Flagellar motility is required for *P. aeruginosa* to make contact with the substrate (Sauer et al., 2002). Bacterial adherence at this initial stage is reversible and mechanical forces can easily remove the bacterial cells (*e.g.*, rinsing) (Davis & Conner, 2007).

The second step is irreversible attachment of cells onto the surface, which is the most critical step in biofilm formation. Bacterial cells fix onto the surface by the production of exopolysaccharides and/or specific ligands (*e.g.*, pili or flagella) that may bind to the surface (Dunne, 2002). The quorum sensing system mediated by *P. aeruginosa* autoinducer-1 (PAI-1) is activated at this stage (Pearson et al., 1994; Sauer et al., 2002). Such cell-to-cell signaling is necessary for the production of EPS (Davies et al., 1998). Small cell clusters attached to the substrate can be observed. By the end of this stage, stronger physical or chemical treatments (*e.g.*, scraping, scrubbing, and chemical cleaners) are required to remove the bacteria from the surface (Davies, 2003). During the transition from reversible attachment to irreversible attachment, the dominant attaching factors alter from non-specific (*e.g.*, hydrophobic) interaction to specific responses mediated by bacterial cells (*e.g.*, secretion of exopolysaccharides).

Small cell clusters formed in the second step further proliferate and become layered at the third stage, namely “maturation-1” stage. Development of these micro-colonies is associated with PAI-2 mediated quorum sensing system (Pearson, Passador, Iglewski, & Greenberg, 1995; Sauer et al., 2002). A biofilm matrix is built up with accumulated bacterial cells and EPS matrix until the majority of the bacterial cells are segregated within the clusters. The dimension of cell clusters reaching its maximum marks the penultimate stage of biofilm development, namely “maturation-2” stage (Sauer et al., 2002). A mature biofilm can enter into the final stage, namely “dispersion”, with cells evacuating the cluster and becoming planktonic forms again (Sauer et al., 2002). The released cells can relocate and subsequently form a new biofilm (Giaouris et al., 2014). This dispersion action can be inherently mediated by quorum sensing (Petrova & Sauer, 2016) or accelerated by environmental stress, such as antibiotics, ultrasound, and enzymatic digestion (Dunne, 2002).

1.2.4 Factors affecting biofilm formation

Bacterial initial attachment can be influenced by a number of factors, including the surrounding environment and cell surface properties. Whether bacterial cells attach to a substrate or not depends upon the chemical and physical interaction between cell surface and substrate surface (Palmer et al., 2007). Charge and hydrophobicity of cell surface and physical properties of the attached surface (*e.g.*, roughness) can influence biofilm formation. Bacterial cells attach more rapidly to hydrophobic surfaces (*e.g.*, polystyrene) than hydrophilic materials (*e.g.*, glass and metals) (Donlan, 2002). A higher degree of roughness of a substrate basically provides a larger surface area that is available for attachment. Rugged surface can trap more bacterial cells during

the washing step (Teughels, Assche, Sliepen, & Quirynen, 2006). Additionally, the presence of bacterial surface appendages is believed to be critical to bacterial initial attachment to a substrate, such as flagella, fimbriae, pili, curli, and cellulose (Jonas et al., 2007; Sauer et al., 2002; Teplitski, Al-Agely, & Ahmer, 2006; Van Houdt & Michiels, 2010).

The subsequent growth of a biofilm can be affected by various environmental factors, such as atmospheric composition, temperature, nutrients, and hydrodynamic conditions (Barber, Werth, McRoberts, & Rybaka, 2014; Piras, Fois, Consolati, Mazza, & Mazzette, 2015; Speranza, Corbo, & Sinigaglia, 2011). These factors have different effects on the formation level of biofilms because various bacteria respond differently to these factors. For example, *Salmonella* produces more biofilms in a nutrient-poor medium, but *Listeria* develops more biofilms in a nutrient-rich medium (Stepanović, Ćirković, Ranin, & Švabić-Vlahović, 2004).

1.2.5 Control strategy of biofilms

The major strategy to prevent biofilm formation is to clean and disinfect the surfaces regularly using surfactants and alkali products (Midelet & Carpentier, 2004; Splendiani, Livingston, & Nicolella, 2006). Detectors of cleaning agents were developed to determine the frequency and efficiency of surface cleaning and estimate the possibility of biofilm development (Pereira, Mendes, & Melo, 2008; Philip-Chandy et al., 2000). An alternative strategy is the modification of substrate material. For example, selective antimicrobial agents (*e.g.*, nanoparticles and antimicrobial peptides) were used to coat the surface of the substrate material (Besinis, De Peralta, & Handy, 2014; Gao et al., 2011). Other studies reported that modification of the

physico-chemical features of substrate surface could reduce the chance of cell colonization and attachment (Rosmaninho et al., 2007; Whitehead, Colligon, & Verran, 2005).

A combination of chemical disinfectant and mechanical force (*e.g.*, rubbing) is recommended to inactivate bacterial biofilms. Most of the disinfection procedures are based upon the results of inactivating planktonic cells, which may not be able to fully eliminate bacteria in the form of biofilms (Simões et al., 2010). Recently, a few innovative strategies were developed to combat bacterial biofilms. For example, an ultrasonic treatment was able to remove twice as much biofilm on polypropylene as the swabbing method (Oulahal-Lagsir, Martial-Gros, Boistier, Blum, & Bonneau, 2000). In another study, the use of a multi-enzyme formulation could successfully remove biofilms formed by *S. aureus* and *P. aeruginosa*. This formulation contained enzymes that are capable of degrading microbial DNA, polysaccharides, proteins, and quorum-sensing molecules (Thallinger, Prasetyo, Nyanhongo, & Guebitz, 2013). In addition, bacteriophages can infect bacteria and could effectively control biofilm formation (Sutherland, Hughes, Skillman, & Tait, 2004).

1.2.6 Laboratory assays to study biofilms

1.2.6.1 Cultivation of biofilms

Biofilms can form at liquid-solid, air-liquid, and air-solid interfaces *in vitro*. A microtiter plate with multiple wells has been frequently used as a high-throughput method for the cultivation of bacterial biofilms (Müsken, Di Fiore, Römling, & Häussler, 2010). After bacterial culture is incubated in the plate with nutrient media, biofilms can be visualized as a layer attached to the bottom of the well. Compared to biofilms formed at air-liquid and air-solid interfaces, the

microtiter plate assay is more convenient to operate and subsequent quantification of biofilm formation is straightforward. A Calgary Biofilm Device is applied when the effect of gravity on cell attachment needs to be eliminated, or the formed biofilms need to be moved out of the plate. This device consists of a microtiter plate carrying multiple pegs on the lid that fit into each well. Biofilms can form on those pegs during incubation (Ceri et al., 1999; Harrison et al., 2006).

1.2.6.2 Quantitative analysis of biofilms

Crystal violet (CV) staining assay is widely used to determine biofilm formation level. Crystal violet binds to most biological substances with negative charges. The CV bond in a biofilm can be dissolved in organic solvents (*e.g.*, ethanol) and the concentration is measured at OD_{595nm}. CV staining is considered to be the most accurate assay to determine the quantity of biofilm biomass, including both sessile cells and polymer matrix (Müsken et al., 2010). Other biological dye, such as Congo Red, was also reported for the determination of biofilm formation level (Reuter, Mallett, Pearson, & van Vliet, 2010).

As the overall biomass of a biofilm is usually proportional to the density of sessile cells, methods that target the cell numbers within a biofilm are also applied to determine the biofilm formation level (Flemming & Wingender, 2010). The viable cell number within a biofilm can be determined by conventional plating method after the biofilm is detached (Arnold & Silvers, 2000). Biofilms can be detached by physical treatment (*e.g.*, scraping) or chemical treatment (*e.g.*, structure-breaking enzymes) (Chaignon et al., 2007; Wang, Ding, Wang, Xu, & Zhou, 2013). The live cells within biofilms could also be stained by metabolism dye, such as tetrazolium salts (Brown, van Vliet, Betts, & Reuter, 2013; Ferreira & Fernanda, 2016).

Confocal laser scanning microscope (CLSM) has been extensively used to study the morphological features of bacterial biofilms (Staudt, Horn, Hempel, & Neu, 2004). CLSM can generate high-resolution images because biofilms can be scanned spot-by-spot by the confocal laser. With good optical sectioning referred as depth or *z* resolution, CLSM allows three-dimensional reconstruction of a sample from images captured at different focal planes (Harrison et al., 2006). The application of CLSM is coupled with fluorescence staining, which can selectively bind to the specific components in a biofilm (e.g., live/dead cells and polysaccharides) (Boulos, Prévost, Barbeau, Coallier, & Desjardins, 1999; Neu, Swerhone, & Lawrence, 2001). Besides morphological characteristics, this technique is also able to quantify the thickness and formation level of biofilms by calculating fluorescence intensity (Feng et al., 2015; Staudt et al., 2004; Wagner, Ivleva, Haisch, Niessner, & Horn, 2009).

1.2.7 Biofilms formed by *Campylobacter* and *Salmonella*

Campylobacter jejuni is able to form monospecies biofilms on various abiotic materials, such as stainless steel, plastics, nitrocellulose membranes, and glass (Kalmokoff et al., 2006; Moe et al., 2010; Nereus IV & Chen, 2009; Reeser, Medler, Billington, Jost, & Joens, 2007; Reuter et al., 2010). Not all *C. jejuni* strains readily form biofilms and some strains can only form biofilms on the surface of specific substrate materials. The ability of *C. jejuni* to form biofilms is highly strain-specific and substrate-specific. *C. jejuni* exhibits much weaker biofilm forming capacity compared to other bacteria, such as *P. aeruginosa*, *E. coli*, *S. aureus*, and *S. enterica* (Teh, 2008). Some researchers failed to observe monospecies *C. jejuni* biofilms as highly structured as other bacteria commonly formed (Nereus IV & Chen, 2009; Teh, Lee, & Dykes, 2014). Joshua and

colleagues proposed that *C. jejuni* biofilms might occur in three different forms in liquid: (1) cells attached onto inert surfaces, (2) flocs (cell aggregates floating in the liquid), and (3) pellicles (cell aggregates at air-liquid interface). All three forms of *C. jejuni* cells were connected by polymer-like substances and this was observed by using scanning electron microscopy (Joshua, Guthrie-Irons, Karlyshev, & Wren, 2006). Therefore, *C. jejuni* biofilms in three different forms were all regarded as true biofilms, but not necessarily attach to a substrate surface.

Fragility of *Campylobacter* and its high prevalence in the environment seems to be contradictory. One possible survival mechanism of *Campylobacter* in nature is biofilm formation (Teh, Lee, & Dykes, 2014). Although *Campylobacter* is a weak biofilm former under their favorable condition (*i.e.*, microaerobic condition at 37-42°C), it is suggested that biofilm formation of *C. jejuni* could be stimulated in response to stresses. For example, biofilm formation by *C. jejuni* was increased under aerobic condition compared to that under microaerobic condition (Reuter et al., 2010). In another study, Dykes and coauthors identified that *C. jejuni* cells within its biofilm survived longer at lower temperatures (*i.e.*, 4°C and 10°C) than that at higher temperatures (*i.e.*, 25°C and 37°C) under stress conditions (Dykes, Sampathkumar, & Korber, 2003). However, other researchers reported that biofilm formation level of *C. jejuni* was reduced under oxygen and temperature stress (Reeser et al., 2007). The contradictory results among these studies may due to strain variation and inconsistent incubation condition.

To date, most studies of *C. jejuni* biofilms applied the favorable cultivation condition, which was far different from the actual environment (*e.g.*, poultry breeding house and food processing

plant) where *Campylobacter* persists. There is limited evidence suggesting the possible formation mode of *Campylobacter* biofilms in unfavorable environment. *C. jejuni* biofilms were cultivated under flow condition because *Campylobacter* bacteria are widely present in water piping and sinks (Buswell et al., 1998). In a previous study, *C. jejuni* was unable to form biofilms with a flow rate as low as 0.06 ml/min (Ica et al., 2012). Joshua and coauthors reported that *C. jejuni* readily formed biofilms in standing glass tubes, but no biofilms were observed when shaking cultivation was applied (Joshua et al., 2006). *Campylobacter* is likely to persist in multispecies biofilms and protected by existing biofilms formed by other bacteria (Ica et al., 2012; Teh, 2008).

Salmonella biofilm formation has been extensively investigated in various conditions, such as different types of abiotic surfaces, animal epithelial cells, and gallstones (Steenackers, Hermans, Vanderleyden, & De Keersmaecker, 2012). Almost all the tested *Salmonella* strains were able to form biofilms on plastics, metals, glass, cement, and rubber (De Oliveira et al., 2014; Hurrell, Kucerova, Loughlin, Caubilla-Barron, & Forsythe, 2009; Joseph, Otta, Karunasagar, & Karunasagar, 2001; Mangalappalli-Illathu, Lawrence, Swerhone, & Korber, 2008; Stepanović et al., 2004; H. Wang et al., 2013). The ability of *Salmonella* to form biofilms on these substrate surfaces might contribute to its persistence and transmission in food-related environment. Vestby and colleagues reported a positive correlation between the biofilm formation ability of 111 *Salmonella* strains isolated from feed and fish meal factories and their persistence in the factory environment (Vestby, Møretrø, Langsrud, Heir, & Nesse, 2009). Barker and Bloomfield studied the microbial environment in a family toilet, where the households experienced salmonellosis.

Salmonella cells were present in form of biofilm in the toilet bowl and could persist up to 4 weeks post infection (Barker & Bloomfield, 2000).

The quantity and morphology of monospecies *Salmonella* biofilms are strain-dependent and substrate-dependent (De Oliveira et al., 2014; Piras et al., 2015; Steenackers et al., 2012; Vestby et al., 2009). However, biofilm formation capacity of *Salmonella* is not greatly affected by environmental stress, such as temperature (Speranza et al., 2011). There was no significant difference among *Salmonella* biofilm formation levels at a temperature range of 16-35°C (De Oliveira et al., 2014). As a relatively strong biofilm former, *Salmonella* is highly adaptive to various conditions through cooperation of several complex regulation systems. For example, almost 30% of the functional genome in *S. Typhimurium* was regulated differently between agar and broth cultivation (Wang, Frye, McClelland, & Harshey, 2004). Major regulators involved in monospecies *Salmonella* biofilm formation include *csgD* (production of polymer components), c-di-GMP (cellulose synthesis), *RpoS* (general stress response), *BarA/SirA* (motility) and others (Gerstel & Römling, 2003; Kader, Simm, Gerstel, Morr, & Römling, 2006; Steenackers et al., 2012; Teplitski et al., 2006).

1.3 Meat juice

1.3.1 Introduction to meat juice

The exudate of frozen raw meat has been identified as an important source of bacterial contamination on the surfaces of food preparation benches in kitchen (Guyard-Nicodème *et al.*, 2013). Meat juice is the meat exudate collected from raw meat, containing similar chemical components to meat (Birk, Ingmer, Andersen, Jørgensen, & Brøndsted, 2004). An approximate

composition of sterile beef juice using the collection method described by Birk and others included 10% proteins, 0.2% lipids, 0.1% carbohydrates, 1.2% ashes, and 88% water (Birk et al., 2004; Graziella Midelet & Carpentier, 2002). With a non-thermal sterilization treatment (*e.g.*, filtration), sterile meat juice has been applied as a food-based model to mimic the nutrient environment during meat processing or in a meat matrix (Birk et al., 2004; Ferreira & Fernanda, 2016; Milillo & Ricke, 2010; Wang et al., 2013).

1.3.2 Effects of meat juice on survival of *Campylobacter* and *Salmonella*

Previous studies investigated the growth characteristics of *Salmonella* and *Campylobacter* cells in chicken juice. When cultivated in chicken juice, the growth rate of *C. jejuni* cells was significantly higher than in Brucella broth with other environmental factors defined (Brown *et al.*, 2014). Birk and others reported that the survival rate of *C. jejuni* was increased in chicken juice compared to Brain-Heart Infusion (BHI) broth once the microbes were under temperature stress (*i.e.*, 5°C and 48°C) (Birk et al., 2004).

C. jejuni has been identified to exhibit specific transcriptional responses to chicken juice. Compared to BHI broth used in the laboratory, chicken juice increased the expression of *luxS*, which is responsible for the production of quorum-sensing signaling molecules and bacterial adaptation to cold stress. A gene involved in O-linked flagellin glycosylation in *C. jejuni* was up-regulated in chicken juice, while the expression of haemin uptake and the peroxide stress response genes in *C. jejuni* were decreased (Ligowska, Cohn, Stabler, Wren, & Brøndsted, 2011). However, Gölz and coauthors identified that autoinducer-2 in *C. jejuni* 11168 lost its activity when the microbes were cultivated in chicken juice (Gözl, Adler, Huehn, & Alter, 2012).

Similar reduction was identified in other bacteria, such as *E. coli* O157:H7 and *S. Typhimurium*, in chicken juice compared to Brucella broth (Cloak, Solow, Briggs, Chen, & Fratamico, 2002).

Additionally, *S. Typhimurium* cells required a longer time to reach stationary phase in chicken juice than in BHI broth, although there was no significant difference in the viable cell number after 24-h incubation (Milillo & Ricke, 2010). The authors also investigated the pH change during *S. Typhimurium* cell growth in chicken juice and in BHI broth, and the former had a more stable pH during the overall growth period. Gene regulation and metabolism of *Salmonella* could vary in meat juice and laboratory media although no direct gene-related studies have been conducted so far.

1.3.3 Effects of meat juice on *Campylobacter* and *Salmonella* biofilms

There are limited studies on the interplay between meat juice and microbial biofilms. Brown and colleagues identified that chicken juice could enhance cell survival and biofilm formation level of selective *C. jejuni* strains using various substrates, such as polystyrene, stainless steel, and glass (Brown *et al.*, 2014). On the contrary, the growth of *Salmonella* within its biofilm was slower when it was formed in chicken juice compared to that in tryptic soy broth used in the laboratory (Wang, Ding, Wang, Xu, & Zhou, 2013). Except for bacterial inter-genus variation, differences in chicken samples and collecting methods may lead to different chemical compositions of chicken juice, therefore resulting in different experimental observation and conclusions.

Additionally, the mature biofilms of *Salmonella* grown in chicken juice showed “cloud-shaped” morphology on stainless steel, whereas the ones formed in TSB appeared “reticular-shape” (Wang et al., 2013). The authors also reported that the chemical composition of *Salmonella* biofilms was different in chicken juice and in TSB, suggesting that meat juice is a complex nutrient matrix that could play various roles in biofilm formation and bacterial cell survival. More comprehensive studies regarding the effects of meat juice on *Campylobacter* and *Salmonella* biofilm formation and their survival in the biofilms are therefore required.

1.4 Objectives of the study

Salmonella and *Campylobacter* are significant foodborne pathogens with high prevalence in food processing environment. The presence of meat residues in such an environment may support their biofilm formation and further increase the risk of cross contamination. Therefore, evaluating the effect of meat juice on *Salmonella* and *Campylobacter* biofilms under the condition that simulates the real meat-processing environment is critical to control and reduce biofilms, which is the central objective of this study.

Here, we propose three hypotheses:

- (1) Meat juice supports the growth of planktonic cells and facilitates the development of biofilms of *Campylobacter* and *Salmonella*.
- (2) Meat juice particles on substrate surface act as a conditioning layer that promotes initial attachment and biofilm formation of *Campylobacter* and *Salmonella*.
- (3) Deficiency in biofilm formation ability of *Campylobacter* and *Salmonella* under unfavourable conditions can be compensated by meat juice particles.

Accordingly, six objectives are proposed in this thesis project:

- (1) To investigate the growth of *Campylobacter* and *Salmonella* planktonic cells in meat juice.
- (2) To measure the biofilm formation level of *Campylobacter* and *Salmonella* in meat juice supplemented with culture media.
- (3) To investigate the attachment ability of *Campylobacter* and *Salmonella* cells on substrates with meat juice particles.
- (4) To measure the biofilm formation level of *Campylobacter* and *Salmonella* on substrates with meat juice particles.
- (5) To measure the biofilm formation level of *Campylobacter* and *Salmonella* with the presence of meat juice particles in a flow condition.
- (6) To investigate the effect of meat juice particles on biofilm formation ability of *Campylobacter* and *Salmonella* cells with biofilm-forming deficiency.

Chapter 2 - Effects of meat juice supplementation on biofilm formation of *Campylobacter* and *Salmonella*

2.1 Background

The formation of a biofilm is a complex process influenced by numerous environmental factors. Nutrients in the culture environment are one of the most fundamental aspects. For example, formation levels of *Campylobacter* biofilms varied significantly when they were cultivated with different nutrient broths (Reeser et al., 2007). Biofilms formed by *Salmonella* and *Campylobacter* have been mostly studied in well-defined nutrient media commonly used in laboratory and under experimental condition that are ideal for the growth of *Salmonella* and *Campylobacter*. However, these ideal conditions are not likely to be replicated in food processing environment. With the capability of adaptation to the environmental stresses, the growth and survival mode of bacteria could be distinct between laboratory and environmental situation. There is a lack of knowledge on the biofilms formed by *Campylobacter* and *Salmonella* in the meat processing environment, due to the difficulty in direct observation and investigation in such an environment. Meat juice is thus employed as a food-based model to create a condition that resembles the real situation in the current study.

According to the aforementioned literature review (section 1.4), meat juice may act as a complex nutrient matrix that can play a variety of roles in bacterial cell survival and biofilm formation. More comprehensive studies regarding the effects of meat juice on the development of *Campylobacter* and *Salmonella* biofilms and their survival within the biofilms are thus required. Here, chicken juice and pork juice was supplemented to the laboratory media, respectively, as chicken and pork are the most common meat contaminated by *Campylobacter* and *Salmonella*.

The objective of this chapter is to investigate how meat juices influence *Campylobacter* and *Salmonella* growth and biofilm formation, in comparison to the media that are commonly used in laboratory.

The biofilm formation level of *Campylobacter* and *Salmonella* in meat juice supplemented culture media was determined by a redox dye TTC (2,3,5 triphenyltetrazolium chloride). This TTC assay was selected instead of the conventional crystal violet (CV) assay because meat juice in media formed a precipitant layer onto the substrate and bound to CV molecules during biofilm cultivation, leading to inaccuracy in measuring the actual biofilm formation level (Brown et al., 2013; Ferreira & Fernanda, 2016; Hatzinger, Palmer, Smith, Peñarrieta, & Yoshinari, 2003). The supplemented TTC in culture media could be transformed to a red crystal (1,3,5-triphenylformazan, TPF) by live bacterial cells; therefore, the interference from the background (e.g., chicken juice particles) could be eliminated using this TTC assay. The concentration of TPF can be determined by its optical density value at wavelength of 500 nm, which indicates the number of live cells in the biofilms as well as biofilm formation level (Brown et al., 2013).

2.2 Materials and methods

2.2.1 Preparation of meat juice

Chicken juice and pork juice were collected by a frozen-thaw method described in a previous study (Birk *et al.*, 2004) with minor modification. Whole chicken and pork chops purchased from local grocery stores in Vancouver were frozen at -20°C and the thaw liquid was collected at 4°C. To eliminate large particles, collected meat exudates were centrifuged (12000 ×g, 10 min) and the supernatant was subsequently sterilized using a 0.22-µm filter (Millipore, Darmstadt,

Germany). Meat juice from several chickens and pork chops was mixed, respectively. Sterile meat juice was stored at -80°C and thawed overnight at 4°C before usage. The concentration of proteins in chicken juice and pork juice was determined by Bradford assay (Bradford reagent, Sigma-Aldrich, St. Louis, USA) and adjusted to 5 mg/ml using sterile water (Wang et al., 2013). The yield and pH of meat juice are listed in Table S1.

2.2.2 Bacterial strains and culture preparation

Bacteria strains used in the experiment were listed in Table 2.1. *C. jejuni* strains were routinely cultivated either on MH agar plate (Difco, New Jersey, USA) supplemented with 5% defibrinated sheep blood (Quad Five, Montana, USA) or in MH broth at 37°C in a microaerobic (10% CO₂) condition. *S. enterica* strains were grown either on LB agar plate or in LB broth (Difco, New Jersey, USA) at 37°C in an aerobic condition.

2.2.3 Planktonic cell growth assay

Overnight *C. jejuni* or *S. enterica* cultures (stationary phase) were adjusted to OD_{600nm}=0.3, referring to 10⁹ CFU/ml of *C. jejuni* and 10⁸ CFU/ml of *S. enterica* (Figure S3 and S4). This was followed by centrifugation at 8000 ×g for 2 min. The collected cell pellet was washed once by phosphate buffered saline (PBS, pH=7.4) and re-suspended in PBS. The cell suspensions were then added into MH/LB broth, chicken juice, and pork juice, respectively, followed by incubation at 37°C either in a microaerobic condition (*C. jejuni*) or an aerobic condition (*S. enterica*). The cell count was determined by plating 10-fold serial dilution of the bacterial culture on MH/LB agar plates at incubation periods of 0 h, 12 h, and 24 h.

2.2.4 Biofilm cultivation

A conventional static biofilm assay was applied with minor modification (Müsken et al., 2010). Overnight bacterial culture (stationary phase) was adjusted to $OD_{600nm}=0.3$, followed by centrifugation at $8000 \times g$ for 2 min. The collected cell pellet was washed once and re-suspended in PBS. The cell suspensions were added into MH/LB broth and chicken juice/pork juice supplemented MH/LB broth, respectively. The final concentration of bacterial cells was 10^8 CFU/ml for *C. jejuni* and 10^7 CFU/ml for *S. enterica*. The mixtures were introduced into polystyrene 96-well plate (Corning, Tewksbury, USA) or a slide chamber with glass bottom (Thermo Lab-Tek, Waltham, USA), followed by incubation at 37°C either in a microaerobic condition (*C. jejuni*) or aerobic condition (*S. enterica*) for 48 h in a static mode.

2.2.5 TTC biofilm staining assay

A live cell staining method was used to quantify the level of biofilm formation with minor modification (Brown et al., 2013; Knezevic & Petrovic, 2008). Cell suspension was removed and biofilms formed at the bottom were washed with PBS twice. MH/LB broth with 0.01% TTC (Sigma-Aldrich, St. Louis, USA) was then added, followed by incubation at 37°C either in a microaerobic condition for 24 h (*C. jejuni*) or in an aerobic condition for 2 h (*S. enterica*). The solution was then carefully removed, followed by drying each well in ambient air condition. The remaining red crystal was dissolved in 80% ethanol-20% acetone (v/v). Absorbance at 500 nm for each well in the plate was measured using a microtiter plate reader (SpectraMax, Sunnyvale, USA).

2.2.6 Statistical analysis

All the experiments were conducted at least in triplicates. Experimental results were reported as the averages of replicates $\pm$ the standard deviation. Student's t-test was used to compare pair of means and calculate *P* values.

2.3 Results and discussion

2.3.1 Growth of planktonic cells in meat juice

To investigate the possible influence of meat juice on the viability of *Campylobacter* and *Salmonella* cells, the growth of bacterial cells in meat juice was determined along with MH or LB broth as reference media. Both bacteria were cultured under their favorable condition as aforementioned (Section 2.2.4), and viable bacterial count was determined at 12 h and 24 h, respectively (Table 2.2 and Table 2.3). In order to observe further changes in cell viability after 2-day incubation period, a long-term incubation (up to 30 days) was conducted, with initial cell load of 10^4 CFU/ml. The cell density of *Campylobacter* and *Salmonella* within 30 days is shown in Figure S1 and Figure S2, respectively.

Overnight *C. jejuni* culture was added into the reference media (MH broth) or meat juice to achieve 10^7 CFU/ml as initial concentration. After 12 h incubation, all *C. jejuni* strains had approximately 0.5 log CFU/ml growth in MH broth, and subsequently achieved up to 10^{10} CFU/ml at 24 h after initial inoculation. However, *C. jejuni* strains 11168 and Human10 had one log reduction in chicken juice, and *C. jejuni* strain Human10 had one log reduction in pork juice post 12 h incubation. After 24 h incubation, all the *C. jejuni* strains cultivated in chicken juice and pork juice had significantly reduced cell loads compared with the ones cultivated in MH

broth, except for *C. jejuni* strain 81-116 in pork juice. Thus, meat juice was able to support cell growth of *C. jejuni* ATCC33560, 81-116 and F38011, although not as effective as MH broth. In the meantime, negative impact on the growth of *C. jejuni* strain 11168 and Human10 was identified, which was not consistent with the previous reports. Brown and coauthors reported that chicken juice could significantly increase the growth rate of *C. jejuni* strain 11168 within 24-h incubation compared to Brucella broth used in laboratory (Brown *et al.*, 2014). Although the similar method for the collection of chicken juice was described in that study, the concentration of nutrients in chicken juice was not reported and it might be different from that used in the current study. *C. jejuni* strains showed greater growth rate in MH broth than in Brucella broth (Tang, 2000); thus, the inconsistency may also be due to the difference in the growth rate of *C. jejuni* 11168 in both broths. Additionally, nutrient profiles, pH, and osmotic pressure of different media broths and chicken juice could be the potential factors that affect the growth of *Campylobacter*.

Salmonella exhibited different growth behaviour in meat juice compared with *Campylobacter*. The initial cell load of *Salmonella* was 10^2 CFU/ml and achieved $\sim 10^{10}$ CFU/ml after 12-h incubation. The bacterial cell loads remained stable from 12 h to 24 h. Remarkably, there was no significant difference ($P > 0.05$) in the cell counts of *Salmonella* grown in LB broth and chicken/pork juice for all the six strains tested, indicating that meat juice had no negative impact on the growth of *Salmonella* and could provide sufficient nutrients. Previous literature reported that viable cell count of *S. Typhimurium* was not significantly different after 24-h incubation in BHI broth and chicken juice under 37°C (Milillo & Ricke, 2010), which is in agreement with the current study.

Different growing behaviours of *Salmonella* and *Campylobacter* can be attributed to their variations in the inherent capability of adapting to the unfavourable conditions. *Campylobacter* is sensitive to different environmental stresses (Bolton, 2015) while *Salmonella* is more resistant to unfavourable conditions, such as high salt concentration and low pH (Humphrey, 2004). The pH of meat juice (~6.5) is lower than MH/LB media (~7.4). Both microbes can tolerate and multiply when pH is about 6.0, but in a sub-optimal condition. Another possible stress may come from the chemical compounds in meat juice, such as antibiotics used in livestock breeding (De Wasch et al., 1998). In addition, meat juice may not be as ideal as laboratory media in supporting the growth of bacteria in terms of nutritional availability. Compared to simple amino acids in media broth, macromolecules (*e.g.*, proteins) that serve as the nutrients in meat juice may not be easily digested by bacteria (Nachamkin et al., 2008).

2.3.2 Biofilm formation in meat juice

Formation levels of *Campylobacter* and *Salmonella* biofilms in meat juice supplemented media were determined. Different percentage of meat juice (*i.e.*, 10%, 25%, 50%, 75%, 100%, v/v) was mixed with the reference media (LB/MH broth), respectively, as the new culture media for cultivating biofilms. Glass and polystyrene were selected as substrate materials due to their extensive use in meat processing industry (Arnold & Silvers, 2000). After 2-day incubation under their favorable condition as aforementioned, the formed biofilms were washed and stained by TTC assay. The dose of TTC applied (0.01%) was tested to have no negative impact on cell viability (Figure S5). Since the biofilm level at post 2-day incubation started decreasing (Figure

S6), the 2-day old biofilms of *Campylobacter* and *Salmonella* can be regarded as the mature biofilms for characterization.

As shown in Figure 2.1, among all groups supplemented with various percentages of meat juice (*i.e.*, 10%, 25%, 50%, 75%, 100%, v/v), there was a significant increase ($P < 0.05$) in the formation level of *C. jejuni* F38011 biofilms as compared to the biofilms formed in MH broth without meat juice supplementation. Therefore, chicken juice and pork juice could significantly enhance biofilm formation and growth on polystyrene substrate.

Higher percentage of meat juice does not necessarily relate to a higher level of *C. jejuni* biofilm formation. Specifically, 50% (v/v) meat juice achieved the highest biofilm formation level. Although pure meat juice led to significantly ($P < 0.05$) higher level of biofilm formation than the control group (no meat juice added), the formation level of biofilm in pure meat juice was significantly ($P < 0.05$) lower than those cultivated with various concentrations of supplemented meat juice (Figure 2.1). Based upon the observation, meat juice in culture media could form a coating layer on the substrate during biofilm cultivation. Despite the nutritional effects of meat juice, the coating layer may act as an alternative substrate supporting biofilm formation. With a higher concentration of meat juice supplemented (*i.e.*, 10%-50%), meat sediments covered a larger area of the original substrate. Therefore, the biofilm formation levels were shown to increase along with a higher percentage of meat juice supplemented. However, the concentration of meat juice over 50% (*i.e.*, 75% and 100%) resulted in a decreased biofilm formation level. Biofilms may detach along with the meat particles during the washing step based upon the observation that a thicker coating layer of meat juice can be washed away more easily.

As indicated in section 2.3.1, viability of *C. jejuni* cells in meat juice was lower than that in MH broth, thus the decreased formation level of biofilm in pure meat juice could also be attributed to the lower cell count. This negative impact could be compensated by the coating effect because the formation level of biofilms cultivated in pure meat juice was significantly higher ($P > 0.05$) than that cultivated in media broth. Overall, 50% meat juice supplementation resulted in the highest formation level of *C. jejuni* biofilms on both substrates.

The formation levels of biofilms by other *C. jejuni* strains in polystyrene 96-well plate are shown in Figure 2.2. All *C. jejuni* strains showed similar trend of biofilm formation where pure meat juice resulted in less biofilm formation levels compared to 50% (v/v) meat juice supplementation, except for *C. jejuni* strain 81-116. *C. jejuni* 81-116 formed a similar level of biofilm in pure meat juice compared to that in 50% (v/v) meat juice supplemented. This result was consistent with cell viability in section 2.3.1, where *C. jejuni* 81-116 had the highest cell count among all the tested strains after the microbes were incubated in meat juice. For *C. jejuni* strains 11168 and Human10, the biofilm formation level in pure meat juice was significantly ($P < 0.05$) lower than the counterparts without supplementing meat juice. This was consistent to cell viability results, indicating that these two strains were more sensitive to meat juice than other *C. jejuni* strains.

C. jejuni biofilm formation by strain F38011 on borosilicate glass with the supplementation of meat juice was studied as well. Along with the increase in the concentration of meat juice supplemented, biofilm formation level of *C. jejuni* F38011 increased accordingly and achieved the highest amount with 50% meat juice supplemented (Figure 2.3). Although there was a

relative decrease in biofilm formation with 75% meat juice supplemented, the formation level of *C. jejuni* F38011 biofilm supplemented with various concentrations of chicken juice or pork juice was significantly higher ($P < 0.05$) than that without the addition of meat juice, except for that supplemented with 100% meat juice. *C. jejuni* cell viability could be affected by pure meat juice. In addition, the formed *C. jejuni* biofilms may be partially detached from the abiotic substrate during washing step because high content of meat juice particles can affect the stability of biofilm structure on the abiotic substrate. Different volumes of culture media were added in the polystyrene 96-well plate (200 μl /well) and glass chamber (500 μl /well), therefore the thickness of the meat juice coating layer could be different. This may explain the lower level of biofilm formed on glass substrate compared to that formed on polystyrene substrate (Figure 2.1) once 75% meat juice was supplemented. The difference in physical properties of substrate surface (*e.g.*, hydrophobicity and roughness) between polystyrene and glass may also contribute to the variations in biofilm formation level when the same type and concentration of meat juice were used (*e.g.*, 75% chicken juice).

Compared to *C. jejuni* F38011 biofilm formed on polystyrene supplemented with meat juice, the enhancement effect was more significant when the biofilm was formed on glass supplemented with meat juice. Specifically, the formation level of *C. jejuni* F38011 biofilm formed on glass supplemented with 25% and 50% chicken juice was 5-fold higher than without meat juice supplementation (Figure 2.3). In comparison, there was only 2-fold increase in the level of biofilm formed on polystyrene with the supplementation of the same type and concentration of meat juice compared to that cultivated in media broth alone (Figure 2.1).

The biofilm formation level of other *C. jejuni* strains on glass could be significantly ($P < 0.05$) enhanced by supplementing 50% meat juice into broth (Figure 2.4), although the extent of biofilm formation enhancement was strain-specific. Biofilm formation level of *C. jejuni* strains ATCC33560 and 81-116 on glass with pure meat juice was significantly higher ($P < 0.05$) than that without meat juice supplemented, but the other three *C. jejuni* strains (*i.e.*, 11168, Human10, and F38011) did not produce a higher level of biofilm than that without meat juice supplemented under the same cultivation condition.

The biofilm formation level of *S. Typhimurium* SL1344 in polystyrene 96-well plate was determined by TTC assay with a gradient of meat juice supplemented in LB broth (*i.e.*, 0%, 10%, 25%, 50%, 75%, and 100%, v/v). Both chicken juice and pork juice supplemented in broth ranging 10%-100% could significantly ($P < 0.05$) enhance the level of biofilms formed by *S. Typhimurium* SL1344 on polystyrene in broth alone (Figure 2.5). Generally, over 1-fold increase in biofilm formation level was observed. Similar to *C. jejuni* biofilms as aforementioned, the level of biofilm formed by *S. Typhimurium* SL1344 with pure chicken juice or pork juice was slightly lower than that supplemented with 75% meat juice. Because meat juice exhibited no negative impact on the viability of *Salmonella* with reference to LB broth (Table 2.3), the decrease in biofilm formation level in pure meat juice might be due to the detachment of the meat juice coating layer.

The levels of biofilms formed on polystyrene by other *S. enterica* strains were significantly ($P < 0.05$) enhanced by 50% and 100% meat juice supplemented in broth, with at least 1-fold increase than that without meat juice supplemented (Figure 2.6). The extent of enhancement of biofilm

formation caused by meat juice supplementation varied among different *S. enterica* strains. Similar to the observation as aforementioned in Figure 2.5, biofilms formed by some *S. enterica* strains (*i.e.*, *S. enterica* OEA2669, ME-14, and SL1344) in pure chicken juice exhibited decreased level than that with 50% chicken juice supplemented. No significant difference ($P > 0.05$) in the levels of biofilm formed between in 50% and 100% pork juice was observed among all six *S. enterica* strains.

The level of biofilms formed by *S. Typhimurium* SL1344 on glass substrate with meat juice supplemented is shown in Figure 2.7. The biofilm formation level was significantly ($P < 0.05$) enhanced with different concentrations of meat juice supplemented (*i.e.*, 10%-75%) than that in LB broth alone. The formation level of *S. Typhimurium* SL1344 biofilm in pure chicken juice was significantly lower ($P < 0.05$) than that supplemented with a lower concentration of chicken juice, and had no significant difference ($P < 0.05$) compared to that without chicken juice supplemented. In addition, the formation level of *S. Typhimurium* SL1344 biofilms in pure pork juice had no significant difference ($P > 0.05$) compared to that supplemented with 10% pork juice, but was significantly lower ($P < 0.05$) than that supplemented with 25%, 50% and 75% pork juice. Meat juice coating layer was more readily to detach from glass than polystyrene. The extent of enhancement of *S. Typhimurium* SL1344 biofilms caused by meat juice supplementation was lower on glass (up to 1.5-fold) compared to polystyrene (up to 3-fold).

The biofilm formation levels of other *S. enterica* strains on glass increased significantly ($P < 0.05$) with supplementing 50% meat juice compared to that without meat juice supplemented (Figure 2.8). The degree of biofilm formation enhancement was strain-specific. Biofilm

formation level of *S. enterica* strains OEA2669, 3512H, and PT-30 on glass supplemented with pure chicken juice was significantly higher ($P < 0.05$) than that without meat juice supplemented. However, the other two *S. enterica* strains (*i.e.*, ME-14 and ATCC43353) did not produce a significantly higher ($P > 0.05$) level of biofilm than that without meat juice supplemented under the same cultivation condition. Levels of biofilms formed by *S. enterica* strains OEA2669, ME-14, 3512H, and PT-30 in pure pork juice were significantly ($P < 0.05$) enhanced compared to that without pork juice supplemented, but this enhancement was not identified for *S. enterica* ATCC43353 biofilm.

In short, supplementation of meat juice up to 75% into MH broth could significantly enhance the biofilm formation levels of *C. jejuni*, but pure meat juice had no enhancement effect and even inhibited *C. jejuni* biofilm formation. The formation level of *S. enterica* biofilms was significantly enhanced with the supplementation of meat juice at any concentration between 10% and 100%. The enhanced bacterial biofilm formation was not due to cell viability in meat juice because *Salmonella* cells exhibited no growth difference between in media broth and meat juice and the growth rate of *Campylobacter* was even reduced in meat juice.

2.4 Summary

Viability of *Campylobacter* cells in meat juice was not as good as that in growth media. Some *C. jejuni* strains could not grow either in chicken or pork juice while other *C. jejuni* strains were able to multiply but not as fast as that in MH broth. When meat juice was supplemented in MH broth as the culture media (<100%, v/v), the formation level of *C. jejuni* biofilms was significantly ($P < 0.05$) higher than that in pure MH broth. The formation level of *C. jejuni*

biofilms in 100% meat juice was strain-specific and not necessarily higher than the ones in MH broth. Possible explanations include the detachment of meat juice coating layer and cell viability. Similar trend of enhancement in regard to *C. jejuni* biofilm formation was identified on both glass and polystyrene.

Viability of *Salmonella* cells showed no significant difference ($P > 0.05$) between the cultivation in LB broth and meat juice. Supplementing meat juice at different concentrations significantly ($P < 0.05$) enhanced *Salmonella* biofilm formation. There was a slight decrease in the formation level of biofilms with 100% chicken juice possibly due to the detachment of coating layer. Similar to *Campylobacter*, the extent of enhancement of *Salmonella* biofilm formation was strain-specific on both glass and polystyrene.

The enhanced biofilm formation of *Campylobacter* and *Salmonella* is not due to the improved cell viability in meat juice. Thus, the contribution from meat juice to bacterial biofilm formation may be derived from other factors. In the next chapter, the effect of meat juice coating layer on biofilm formation of *Campylobacter* and *Salmonella* will be further studied and discussed.

2.5 Tables and figures

Table 2.1. Bacterial strains used in this study.

Specie	Strain	Source	Reference
<i>C. jejuni</i>	F38011	Human clinical isolate	(Klena, Gray, & Konkel, 1998)
	ATCC33560	Bovine isolate	(Volokhov, Chizhikov, Chumakov, & Rasooly, 2003)
	11168	Human clinical isolate	(Parkhill et al., 2000)
	81-116	Human clinical isolate	(Pearson et al., 2007)
	Human10	Human clinical isolate	The current study
<i>S. enterica</i>	Enteritidis OEA2669	Human clinical isolate	The current study
	Enteritidis 3512H	Human clinical isolate	(Seo, Valentin-Bon, Brackett, & Holt, 2004)
	Enteritidis ATCC43353	Bovine isolate	(Draz & Lu, 2016)
	Enteritidis ME14	Human clinical isolate	(Guard-Petter, Keller, Rahman, Carlson, & Silvers, 1996)
	Enteritidis PT30	Human clinical isolate	(Isaacs et al., 2005)
	Typhimurium SL1344	Human clinical isolate	(Mills, Bajaj, & Lee, 1995)

Table 2.2. Growth of *Campylobacter* planktonic cells in meat juice, shown as the mean of viable cell count $\pm$ standard deviation of the mean.

Growth media	Time	ATCC33560	11168	Human10	81-116	F38011
	0 h	7.08 $\pm$ 0.08	7.22 $\pm$ 0.10	7.03 $\pm$ 0.05	7.31 $\pm$ 0.06	7.12 $\pm$ 0.11
MH	12 h	7.46 $\pm$ 0.12	7.95 $\pm$ 0.21	7.36 $\pm$ 0.14	7.95 $\pm$ 0.28	7.84 $\pm$ 0.81
broth	24 h	9.42 $\pm$ 0.37	9.96 $\pm$ 0.51	9.10 $\pm$ 0.66	9.88 $\pm$ 0.47	9.94 $\pm$ 0.26
Chicken	12 h	6.89 $\pm$ 0.72	6.32 $\pm$ 0.38*	6.39 $\pm$ 0.29*	7.74 $\pm$ 0.36	7.24 $\pm$ 0.88
juice	24 h	6.37 $\pm$ 0.24*	5.87 $\pm$ 0.62*	6.42 $\pm$ 0.65*	8.71 $\pm$ 0.81*	8.88 $\pm$ 0.30*
Pork	12 h	7.38 $\pm$ 0.33	7.48 $\pm$ 0.23	6.25 $\pm$ 0.45*	7.97 $\pm$ 0.84	7.31 $\pm$ 0.93
juice	24 h	8.21 $\pm$ 0.62*	8.66 $\pm$ 0.81*	5.81 $\pm$ 0.54*	9.22 $\pm$ 0.66	8.54 $\pm$ 0.90*

*Pair comparison with the counterpart in MH broth group (Student's t-test, $P < 0.05$)

Table 2.3. Growth of *Salmonella* planktonic cells in meat juice, shown as the mean of viable cell count $\pm$ standard deviation.

Growth media	Time	OEA2669	ME-14	3512H	PT-30	ATCC43353	SL1344
	0 h	2.21 $\pm$ 0.03	2.36 $\pm$ 0.13	2.29 $\pm$ 0.12	2.01 $\pm$ 0.09	1.95 $\pm$ 0.04	2.31 $\pm$ 0.07
LB	12 h	9.29 $\pm$ 0.53	10.45 $\pm$ 0.91	10.12 $\pm$ 0.88	9.88 $\pm$ 0.91	9.84 $\pm$ 1.07	10.84 $\pm$ 1.07
broth	24 h	9.42 $\pm$ 1.23	9.23 $\pm$ 0.92	10.33 $\pm$ 0.96	10.28 $\pm$ 0.97	10.54 $\pm$ 1.66	9.74 $\pm$ 1.23
Chicken juice	12 h	9.79 $\pm$ 0.53	9.35 $\pm$ 0.65	10.01 $\pm$ 1.02	10.82 $\pm$ 0.76	9.93 $\pm$ 0.78	9.73 $\pm$ 1.39
	24 h	9.82 $\pm$ 1.11	8.87 $\pm$ 0.83	9.93 $\pm$ 0.79	9.96 $\pm$ 0.49	9.49 $\pm$ 1.22	9.02 $\pm$ 0.96
Pork juice	12 h	9.09 $\pm$ 0.89	9.45 $\pm$ 0.87	9.12 $\pm$ 1.29	9.78 $\pm$ 0.45	9.24 $\pm$ 1.01	9.44 $\pm$ 0.86
	24 h	8.78 $\pm$ 1.13	9.43 $\pm$ 0.59	9.79 $\pm$ 0.69	9.99 $\pm$ 0.56	9.45 $\pm$ 0.38	9.15 $\pm$ 0.95

* denotes significant difference (Student's t-test, $P < 0.05$).

Figure 2.1. Formation level of *C. jejuni* F38011 biofilms in polystyrene 96-well plate with MH broth and gradient of meat juice.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 2.2. Formation level of *C. jejuni* biofilms in polystyrene 96-well plate with MH broth, MH broth supplemented with meat juice (50%, v/v), and pure meat juice, respectively. a) chicken juice; b) pork juice.

* denotes significant difference (Student's t-test, $P < 0.05$).

Figure 2.3. Formation level of *C. jejuni* F38011 biofilms in glass slide chamber with MH broth and gradient of meat juice.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 2.4. Formation level of *C. jejuni* strains biofilms formed in glass slide chamber with MH broth, MH broth supplemented with meat juice (50%, v/v), and pure meat juice, respectively. a) chicken juice; b) pork juice.

* denotes significant difference (Student's t-test, $P < 0.05$).

Figure 2.5. Formation level of *S. Typhimurium* SL1344 biofilms formed in polystyrene 96-well plate with LB broth and gradient of meat juice.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 2.6. Formation level of *S. enterica* strains biofilms formed in polystyrene 96-well plate with LB broth, LB broth supplemented with meat juice (50%, v/v), and pure meat juice, respectively. a) chicken juice; b) pork juice.

* denotes significant difference (Student's t-test, $P < 0.05$).

Figure 2.7. Formation level of *S. Typhimurium* SL1344 biofilms in glass slide chamber with LB broth and gradient of meat juice.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 2.8. Formation level of *S. enterica* biofilms in glass slide chamber with LB broth, LB broth supplemented with meat juice (50%, v/v), and pure meat juice, respectively. a) chicken juice; b) pork juice.

Chapter 3 - Effects of meat juice coating layer on initial attachment and biofilm formation of *Campylobacter* and *Salmonella*

3.1 Background

To form a biofilm, bacteria first irreversibly attach to a surface and continuously aggregate and produce EPS. A mature biofilm may disperse and release bacterial cells to continuously contaminate other contact surfaces, leading to further biofilm formation (Giaouris *et al.*, 2014). Initial attachment of bacteria is regarded as the most critical step for biofilm formation. It can be influenced by different factors (section 1.2.4), such as substrate surface properties (Nguyen, Fegan, Turner, & Dykes, 2012; Yang, Kumar, Zheng, & Yuk, 2015).

In Chapter 2, we identified that supplemented meat juices resulted in enhanced biofilm formation of *Campylobacter* and *Salmonella*. Interestingly, *Salmonella* cell viability showed no difference between meat juice and reference media. Meat juice even posed negative impact on *Campylobacter* cell viability. Therefore, there should be a working mechanism to facilitate *Campylobacter* and *Salmonella* biofilm formation that is independent from cell growth. During biofilm development, the sediments of meat juice could form a visible layer binding upon the surface of abiotic substrates. Here, we hypothesize that the pre-coated layer of meat juice serves as a conditioning surface to facilitate cell attachment and subsequent biofilm formation.

Previous studies reported that bacterial biofilms preferentially form on biotic surface (*e.g.*, vegetable leaves) rather than abiotic surface (*e.g.*, glass) (Heindl *et al.*, 2014). Foods or food residues facilitate bacteria to attach and form biofilm on food contact surfaces (Bernbom *et al.*, 2009). Horse serum protein on stainless steel, polypropylene, and wooden surfaces could

significantly increase the attachment of *S. Typhimurium* (Moore *et al.*, 2007). Coating with whey proteins increased the attachment of dairy microbes to stainless steel, rubber, and glass surfaces (Speers and Gilmour, 1985). Survival of *S. Enteritidis* and *C. jejuni* on stainless steel was increased with the presence of milk and chicken fillet residues (Kusumaningrum *et al.*, 2003). Whitehead and others deposited various food residues (*i.e.*, meat and fish extracts, oil, carbohydrates, and milk caseins) onto stainless steel, forming heterogeneously covered surfaces. They identified that *E. coli* cell attachment was different among surface types and increased along with the percentage of area covered by food particles. The highest *E. coli* attachment rate was observed on the surfaces covered with fish extracts (Whitehead *et al.*, 2010). Using scanning electron microscope, Brown and coauthors identified that *C. jejuni* cells preferentially attached to chicken meat particles compared to glass (Brown *et al.*, 2014). The increased bacterial affinity to substrate surface was attributed to changed surface physiochemical properties and enlarged surface area caused by food residues (Bower *et al.*, 1996). Bacterial cells also had the tendency to locate and aggregate onto the areas with rich nutrients (Dat, Hamanaka, Tanaka, & Uchino, 2010).

Here, polystyrene and glass surfaces were pre-coated with meat juice particles before bacterial inoculation instead of simultaneous addition of meat juice and bacterial culture. The pre-coating process is to simulate the food contact surfaces lack of thorough cleaning. The objective of this chapter was to evaluate how pre-coating of meat juice could influence initial attachment and biofilm formation level of *Campylobacter* and *Salmonella*, thereby providing a possible explanation to the formation enhancement in these biofilms identified in Chapter 2.

3.2 Materials and methods

3.2.1 Bacterial strains and cultures

See section 2.2.2.

3.2.3 Cell adhesion assay

Glass chamber or polystyrene 96-well plate was first coated with meat juice precipitates. MH/LB broth, MH/LB broth mixed with meat juice (10% and 50%, v/v) or pure meat juice was individually added to the substrate. After overnight incubation at 37°C, the solution was removed and coating layer formed on the bottom was washed once with PBS. Then, diluted overnight cultures of *Campylobacter* and *Salmonella* (10^7 CFU/ml) were individually introduced onto the coating layer. The bacterial cultures were stayed for 2 h at 37°C to allow initial attachment (Moe *et al.*, 2010). Unattached cells were then removed and the conditioned substrate was washed twice with PBS. The remaining adhered cells were detached with 0.1% Trypsin-EDTA (Sigma-Aldrich, St. Louis, USA) and incubated at 22°C for 15 min (Lu *et al.*, 2012). The trypsin solution was subsequently 10-fold diluted and plated on MH/LB agar, followed by determining the attached cell number.

3.2.4 Biofilm assay

Glass chamber or polystyrene 96-well plate was first pre-coated with meat juice, as described in section 3.2.3. Overnight bacterial culture was adjusted to $OD_{600nm}=0.3$, followed by ten-fold dilution with MH/LB broth to achieve 10^8 CFU/ml for *C. jejuni* or 10^7 CFU/ml for *S. enterica*. Cell suspension was added into each well of polystyrene 96-well plate (200 μ l) and glass slide chamber (500 μ l), followed by incubation at 37°C under a microaerobic condition for *C. jejuni* or

aerobic condition for *S. enterica* for 48 h in a static mode. The bacterial biofilms were subsequently stained and determined using TTC assay as described in section 2.2.5.

3.2.5 Statistical analysis

All the experiments were conducted at least in triplicates. Results were reported as the averages of replicates $\pm$ the standard deviation. Student's t-test and one-way ANOVA were used to calculate *P* values among treatment groups.

3.3 Results and discussion

3.3.1 Cell adhesion to meat juice pre-coated surfaces

We firstly evaluated the effect of meat juice coating layer on the initial attachment of *C. jejuni* and *S. enterica* cells. The meat juice particles could form a thin layer closely bond upon polystyrene and glass surfaces, which was still present after basic washing step. After the bacterial cell suspension made contact with the coated substrate surface, un-attached cells were removed and attached cell numbers were determined. The attachment time was as short as possible (2 h) to exclude the possible influence of nutrients in coating layer on bacterial growth. The attached cell number is summarized in Table 3.1 and Table 3.2, indicating the effect of meat juice coating on bacterial cell attachment.

Approximately 32 *C. jejuni* cells attached to polystyrene and 71 *C. jejuni* cells were attached to glass per 100 μm^2 when no meat juice was added into the coating solution. Ten percent chicken juice or pork juice in the coating solution resulted in significantly ($P < 0.05$) more cell attachment onto the glass surface, with cell number of 91.5 and 102.3 per 100 μm^2 , respectively.

The attached number of *C. jejuni* cells on polystyrene increased significantly ($P < 0.05$) once 50% or 100% meat juice was supplemented. However, polystyrene supplemented with 10% meat juice failed to give a significantly ($P > 0.05$) higher attached number of *C. jejuni* cells on polystyrene compared to that without meat juice supplementation. Thus, the enhancement effect of pre-coating on bacterial initial attachment was not as strong on polystyrene as that on glass.

Ten percent meat juice in the coating solution led to a significant difference ($P < 0.05$) in attached *Salmonella* cell numbers on both substrates compared to that without pre-coating. A higher concentration of meat juice (*i.e.*, 50% and 100%) contributed to a continuous increase in cell attachment to both substrates. Similar to *C. jejuni*, the degree of increase in attached *Salmonella* cell numbers was more extensive on glass than that on polystyrene. Specifically, there was comparable number of attached *Salmonella* cells on both polystyrene and glass (~ 40 cells/100 μm^2). This number increased to ~ 200 cells per 100 μm^2 on polystyrene coated with 50% meat, but over 300 cells per 100 μm^2 on glass coated with the same concentration of meat juice.

In sum, glass and polystyrene substrates coated with meat juice particles became more readily for attached *Campylobacter* and *Salmonella* cells. Higher percentage of meat juice in the coating solution resulted in increase of bacterial attachment because more area of the original abiotic substrate was covered by meat juice particles. There was no significant difference ($P > 0.05$) in attached cell numbers between the supplementation with 50% meat juice and 100% meat juice, indicating that the surface of the abiotic substrate might be fully covered by meat juice particles once the concentration reached to 50%. Attached numbers of *Campylobacter* and *Salmonella*

cells per 100 μm^2 on glass was always higher than that on polystyrene and this could be attributed to different volume of bacterial culture introduced onto the substrates (200 μl on polystyrene vs. 500 μl on glass).

3.3.2 Biofilm formation on abiotic substrate pre-coated with meat juice

Over 10% meat juice in the coating solution led to significant increase ($P < 0.05$) in biofilm formation of *C. jejuni* F38011 compared to that without meat juice supplemented (Figure 3.1). Noticeably, there was no significant decrease ($P > 0.05$) in biofilm formation supplemented with pure meat juice compared to that supplemented with 50% or 75% meat juice, which was not consistent to the results in the previous chapter that biofilm formation decreased once the concentration of meat juice reached to 100%. The pre-coated meat juice layer was washed before the introduction of bacterial culture for biofilm cultivation, which may greatly reduce the chance to shed meat particles together with the bonded biofilms when the formed biofilms were washed.

The levels of biofilms formed by selective *C. jejuni* strains on polystyrene pre-coated with pure meat juice were determined (Figure 3.2). All of the five tested strains produced significantly ($P < 0.05$) higher level of biofilms on polystyrene pre-coated with meat juice compared to that on polystyrene alone. The enhancement of biofilm formation was different among various strains with F38011 achieving the highest increase (*i.e.*, 2.5-fold). Similar to that on polystyrene, the formation level of *C. jejuni* F38011 biofilms on glass substrate pre-coated with a gradient of meat juice (*i.e.*, 10%-100%, v/v) was significantly higher ($P < 0.05$) than that without meat juice supplementation (Figure 3.3). A higher concentration of meat juice in the coating solution led to a higher level of *C. jejuni* biofilm formation. The enhancement effect on biofilm formation with

pre-coated meat juice was more pronounced on glass than that on polystyrene, with about 5-fold and 2-fold increase of biofilms cultivated in pure meat juice, respectively. In addition, pure meat juice coating could significantly ($P < 0.05$) enhance the formation level of biofilms by various *C. jejuni* strains compared to that without meat juice supplemented (Figure 3.4).

S. Typhimurium SL1344 formed significantly ($P < 0.05$) higher levels of biofilms on polystyrene pre-coated with a gradient of meat juice (*i.e.*, 10%-100%, v/v). Biofilm formation levels increased along with the increase in meat juice concentration in coating solution (Figure 3.5). A similar enhancement trend of *S. Typhimurium* SL1344 biofilms formed on glass pre-coated with meat juice was observed as well (Figure 3.7). Pure meat juice coating also effectively enhanced the biofilm formation of other *S. enterica* strains on both polystyrene (Figure 3.6) and glass (Figure 3.8). Overall, the enhancement ranged between 1-fold and 5-fold among different *S. enterica* strains.

Interestingly, different *S. enterica* strains formed various levels of biofilms on both substrates with or without meat juice pre-coating. For example, *S. Enteritidis* PT-30 produced the lowest level of biofilm on polystyrene without meat juice coating among all strains (Figure 3.6). On the contrary, this strain developed the highest level of biofilm on glass substrate with or without meat juice coating among all strains (Figure 3.8). In contrast, *S. Enteritidis* 3512H formed a higher level of biofilm on polystyrene than that on glass, which was opposite to *S. Enteritidis* PT-30. Therefore, there may be considerable variations in cell-substrate surface interaction among *S. enterica* strains.

Finally, chicken juice and pork juice contributed to a comparable degree in biofilm enhancement. Therefore, both meat juices could lead to a similar coating effect.

3.4 Summary

In short, meat juice pre-coating layer extensively facilitated the initial attachment and further biofilm formation of *Campylobacter* and *Salmonella* on polystyrene and glass surfaces. Pre-coating treatment of meat juice on polystyrene and glass led to significantly ($P < 0.05$) more attached cell number of *Campylobacter* and *Salmonella* within 2-h incubation. Over 10% (v/v) chicken juice or pork juice in the coating solution resulted in significantly increased ($P < 0.05$) biofilm formation level of *C. jejuni* F38011 and *S. Typhimurium* SL1344 on both glass and polystyrene substrates. The degree of increase in biofilm formation level varied among different *C. jejuni* and *S. enterica* strains.

The substrates with meat juice pre-coating treatment simulated the food contact surfaces lack of thorough cleaning in the real situation. It is suggested that, if no frequent and sufficient cleaning action is conducted, meat exudate in meat processing area is able to form such a bonded layer that can support bacterial biofilm formation. The importance of thorough cleaning on surfaces involved in food processing is highlighted.

Further, the results in this chapter illustrated the underlying mechanism of the results in Chapter 2, which is, the supplemented meat juice in media enhanced biofilm formation of *Campylobacter* and *Salmonella* by means of forming a conditioning layer to facilitate bacterial cell attachment.

3.5 Tables and figures

Table 3.1. Attached *C. jejuni* F38011 cell number (shown as cell number /100 μm^2 of substrate surface) on pre-coating layer formed by meat juice.

Substrate	% Meat juice in coating solution (v/v)			
	0%	10%	50%	100%
Polystyrene + Chicken juice	32.7 $\pm$ 5.6 ^{a*}	34.5 $\pm$ 2.5 ^a	43.2 $\pm$ 7.3 ^b	50.4 $\pm$ 4.4 ^b
Glass + Chicken juice	71.0 $\pm$ 8.1 ^a	91.5 $\pm$ 7.4 ^b	150.0 $\pm$ 12.2 ^c	148.5 $\pm$ 8.4 ^c
Polystyrene + Pork juice	32.7 $\pm$ 5.6 ^a	31.9 $\pm$ 4.7 ^a	44.6 $\pm$ 5.1 ^b	49.3 $\pm$ 7.9 ^b
Glass + Pork juice	71.0 $\pm$ 8.1 ^a	102.3 $\pm$ 8.3 ^b	153.0 $\pm$ 8.8 ^c	151.0 $\pm$ 9.5 ^c

*Significant difference determined by one-way ANOVA ($P < 0.05$).

Table 3.2. Attached *S. Typhimurium* SL1344 cell number (shown as cell number /100 μm^2 of substrate surface) on pre-coating layer formed by meat juice.

Substrate	% Meat juice in coating solution (v/v)			
	0%	10%	50%	100%
Polystyrene + Chicken juice	41.0 $\pm$ 0.9 ^a	124.1 $\pm$ 40.1 ^b	203.0 $\pm$ 28.9 ^c	204.2 $\pm$ 59.1 ^c
Glass + Chicken juice	37.0 $\pm$ 0.3 ^a	140.9 $\pm$ 17.8 ^b	347.3 $\pm$ 53.3 ^c	373.8 $\pm$ 23.0 ^c
Polystyrene + Pork juice	41.0 $\pm$ 0.9 ^a	97.0 $\pm$ 30.1 ^b	184.1 $\pm$ 44.0 ^c	235.0 $\pm$ 54.9 ^c
Glass + Pork juice	37.0 $\pm$ 0.3 ^a	161.7 $\pm$ 25.0 ^b	405.3 $\pm$ 23.2 ^c	352.7 $\pm$ 74.4 ^c

*Significant difference determined by one-way ANOVA ($P < 0.05$).

* denotes significant difference (Student's t-test, $P < 0.05$).

Figure 3.1. Formation levels of *C. jejuni* F38011 biofilms in polystyrene 96-well plate pre-coated with gradient of meat juice.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 3.2. Biofilm formation levels of various *C. jejuni* strains in polystyrene 96-well plate pre-coated with meat juice.

* denotes significant difference (Student's t-test, $P < 0.05$).

Figure 3.3. Formation levels of *C. jejuni* F38011 biofilms in glass slide chamber pre-coated with a gradient of meat juice.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 3.4. Biofilm formation levels of various *C. jejuni* strains in glass slide chamber pre-coated with meat juice.

* denotes significant difference (Student's t-test, $P < 0.05$).

Figure 3.5. Formation levels of *S. Typhimurium* SL1344 biofilms in polystyrene 96-well plate pre-coated with a gradient of meat juice.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 3.6. Biofilm formation levels of various *S. Typhimurium* strains in polystyrene 96-well plate pre-coated with meat juice.

* denotes significant difference (Student's t-test, $P < 0.05$).

Figure 3.7. Formation levels of *S. Typhimurium* SL1344 biofilms in glass slide chamber pre-coated with a gradient of meat juice.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 3.8. Biofilm formation levels of various *S. Typhimurium* strains in glass slide chamber pre-coated with meat juice.

Chapter 4 - Effects of meat juice coating on *Campylobacter* and *Salmonella* biofilm formation with respect to flow condition and cell motility

4.1 Background

Fluid hydrodynamic conditions influence biofilm architecture as well as metabolism of sessile cells in the biofilm. Shear force is regarded as a negative factor that can inhibit biofilm growth. Kim and colleagues identified that high flow rates with high Reynolds number suppressed the growth of bacterial biofilms (Kim *et al.*, 2013). Water streaming with high flow velocity is an effective treatment to detach bacterial biofilms (Zacheus *et al.*, 2000). In addition, flow media leads to a higher utilization and exchange rate of nutrients than static cultivation, which may be beneficial to biofilm growth (Reuben, Banas, Banas, & Swarup, 2014). In a natural state, the flow media confers an ecological advantage on the formation of cooperative multi-species biofilms, because biofilms developed in a flow condition have a higher chance to get contact with newly arriving microorganisms (Ghannoum & O'Toole, 2004). The structural morphology (Mangalappalli-Illathu, Lawrence, & Korber, 2009), chemical composition (Mangalappalli-Illathu *et al.*, 2008), and physical strength (Shen *et al.*, 2015) of bacterial biofilms formed in flow condition could be significantly different from the counterparts formed in static condition, needless to mention the potentially extensive variations in gene regulation and responses of sessile cells within the biofilms (Persat *et al.*, 2015).

Biofilms developed in food processing environments always encounter flow condition, especially those formed in sinks and sewage vessels (Buswell *et al.*, 1998). The detached bacterial cells from biofilms in sewage water become sources of further cross contamination (Zimmer, Barnhart, Idris, & Leebe, 2003). Biofilms formed on food contact surfaces (*e.g.*, a

cutting board) should survive from washing step before they can transfer to food products. In Chapter 2 and 3, a conventionally used static biofilm assay was selected in the current study to investigate the influence of meat juice on the formation of *Campylobacter* and *Salmonella* biofilms under a well-controlled manner. However, static cultivation condition could not represent the actual food processing environment that bacteria live. There is a lack of literature that report the effect of meat juice on *Campylobacter* or *Salmonella* biofilms formed under flow condition.

Here we introduced a cultivation mode with continuous flow of culture media/nutrients in a microfluidic platform to study biofilm formation. Microfluidic “lab-on-a-chip” platform has been developed and applied for the characterization of bacterial biofilms (Bruchmann, Sachsenheimer, Rapp, & Schwartz, 2015; Feng et al., 2015; Karimi, Karig, Kumarc, & Ardekani, 2015; Kim et al., 2013). Compared to the conventional macro-scale flow cell for cultivating bacterial biofilms, microfluidic system requires smaller volumes of media. Further, it allows accurate spatial and temporal control for biofilm formation (Atencia, Morrow, & Locascio, 2009; Choi et al., 2007; Lin et al., 2004; Mosier & Cady, 2011; Skolimowski et al., 2010). Thus, it could closely mimic the environmental condition for the study of bacterial biofilms.

Besides hydrodynamic conditions, cell surface structures (*i.e.*, flagella and fimbriae) are believed to influence bacterial initial attachment and subsequently biofilm formation. Flagellar motility is critical to initial cell-to-surface contact and further biofilm formation for various bacteria, such as *E. coli* (Pratt & Kolter, 1998), *L. monocytogenes* (Vatanyoopaisarn et al. 2000; Lemon et al. 2007; Todhanakasem and Young 2008) and *C. jejuni* (J. S. Kim, Park, & Kim, 2015; Moe et al.,

2010; Svensson, Pryjma, & Gaynor, 2014). *C. jejuni* aflagellated or flagella paralyzed mutants are all defective in biofilm formation. Flagella-mediated motility allows for necessary cellular movement along substrate surfaces, which may confer benefits on biofilm initial development and further spread. Flagella themselves may serve as direct linkage on the cell-substrate interface (Van Houdt & Michiels, 2010).

However, the role of flagella can be widely divergent in various types of bacteria. Distinctive from *Campylobacter*, the production of flagella in *Salmonella* hindered its biofilm formation (Jonas *et al.*, 2007). Flagellar gene knockout mutants of *Salmonella* exhibited enhanced biofilm formation ability (Teplitski, Al-Agely, & Ahmer, 2006). Instead, type 1 fimbriae are the most common adhesins in *Salmonella* and other Enterobacteriaceae (Klemm & Schembri, 2000), and play a critical role in irreversible attachment for *E. coli* (Ren, Bedzyk, Thomas, Ye, & Wood, 2004) and *S. Enteritidis* (Austin, Sanders, Y, & Collinson, 1998) to stainless steel. Expression of *fim* gene cluster was required for attachment and biofilm formation of *S. Typhimurium* on biotic surfaces, including cholesterol gallstones (Gonzalez-Escobedo & Gunn, 2013) and epithelial cells (Boddicker, Ledebor, Jagnow, Jones, & Clegg, 2002). *S. Typhimurium* mutants lacking the production of type 1 fimbriae failed to form comparable level of biofilms to the wild-type *S. Typhimurium* strain on polystyrene (Teplitski *et al.*, 2006).

In the natural environment where bacteria live in an unfavourable condition, the production of those surface structures might be inactivated temporarily or permanently (Sandhya Marathe *et al.*, 2016; Wan, Leptos, & Goldstein, 2014). In other words, bacterial cells living in food processing environment are not as active and motile as those under ideal laboratory conditions. Therefore,

several *Campylobacter* and *Salmonella* mutants that are deficient in biofilm formation were selected in the current study. Their biofilm formation capabilities were determined with and without the support from meat juice particles.

4.2 Materials and methods

4.2.1 Bacteria strains and culture preparation

Green fluorescence protein (GFP) gene tagged mutants of *C. jejuni* F38011 (Mixer, Klena, Flom, Siegesmund, & Konkel, 2003) was routinely cultured on MH agar plate or MH broth supplemented with 50 µg/ml kanamycin. *C. jejuni* F38011 aflagellated mutant strains (*AflaAB* and *AflgL*) were routinely cultured on MH agar plate or MH broth supplemented with 2 µg/ml tetracycline (Neal-McKinney & Konkel, 2012). *S. Typhimurium* SL1344 with GFP gene tagged strain (Valdivia, Cormack, & Falkow, 1999), *S. Typhimurium* 14028 wild-type and CA513 (14028*ΔsirA*) (Altier, Suyemoto, Ruiz, Burnham, & Maurer, 2000) were cultured in LB broth. *S. Typhimurium* BA1557 (14028 *fimI*1557::MudJ) and BA746 (14028 *sirA*3::cam) were cultured in LB broth supplemented with kanamycin (50 µg/ml) and chloramphenicol (15 µg/ml), respectively (Teplitski *et al.*, 2006).

4.2.2 Biofilm assay for biofilm-defective mutant strains

Overnight cultures of *C. jejuni* F38011 and *S. Typhimurium* 14028 mutants were prepared in MH/LB broth supplemented with appropriate antibiotics. After adjusted to OD_{600nm}=0.3, each bacterial culture was centrifuged (8000 ×g, 2 min) and cell pellet was washed once with PBS to remove antibiotic residues. Bacterial pellet was then re-suspended and diluted with MH/LB broth to a final concentration of 10⁸ CFU/ml for *C. jejuni* or 10⁷ CFU/ml for *S. enterica*. The cell

suspensions were added into polystyrene 96-well plate or glass chamber pre-coated with meat juice, followed by incubation under a microaerobic condition for *C. jejuni* or aerobic condition for *S. enterica* at 37°C for 48 h in a static mode. The developed biofilms were stained and quantified using TTC assay as described in section 2.2.5.

4.2.3 Fabrication of microfluidic chip

A microfluidic platform was constructed and used for flow cultivation of biofilms (Figure 4.1). The platform consists of a glass substrate on the bottom and a poly-dimethylsiloxane (PDMS) (CiDRA Precision, Wallingford, USA) layer on the top, which was fabricated using soft lithography technique (Qin, Xia, & Whitesides, 2010). In each microfluidic chip, the PDMS layer consists of two in/outlet of microchannels with two individual cultivation chambers. The size for each microchannel was 400 μm (width) $\times$ 80 μm (height). The microchamber for biofilm cultivation was designed as a circle shape with dimensions of 300 μm (radius) $\times$ 80 μm (height). PDMS layer was bonded with glass slide by oxygen plasma treatment (Xiong, Chen, & Zhou, 2014). This microfluidic chip was connected with syringes on a syringe pump (CorSolutions, Ithaca, USA) by biocompatible tubing (Nordson, Loveland, USA), allowing stable and precise control of flow rate in the microfluidic chip.

4.2.4 Biofilm cultivation in microfluidic platform

The developed microfluidic platform was first sterilized by washing with 70% ethanol, followed by rinsing with sterile water. MH/LB broth with or without supplementation of 50% meat juice was introduced into the microchamber, followed by overnight incubation without flow at 37°C in aerobic condition. Overnight cultures of GFP strains (10^9 CFU/ml for *C. jejuni* and 10^8 CFU/ml

for *S. enterica*) were injected into the microchamber (flow rate: 0.01 ml/min). The bacterial culture fulfilling the microchamber was statically maintained at 37°C for 2 h for *S. enterica* in a aerobic condition and 8 h for *C. jejuni* in a microaerobic condition, allowing cell attachment onto the substrate. The attached bacterial cells were then exposed to flowing media (flow rate=0.001 ml/min for *S. enterica* and 0.0002 ml/min for *C. jejuni*) at 37°C for up to 72 h either in a microaerobic condition (*C. jejuni*) or an aerobic condition (*S. enterica*). For static cultivation, the flow rate was set as zero after introducing culture media. The formed bacterial biofilms were washed with flow media (flow rate: 0.01 ml/min) to remove planktonic or loosely bond bacterial cells, followed by direct determination using fluorescence microscopy.

4.2.5 Fluorescence microscopy

Inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany) was employed to determine the formation level of bacterial biofilms in the microfluidic chip. Fluorescence images were obtained using green fluorescence laser channel at 488 nm with 100× oil objective. At each random location of the biofilm sample, fluorescence images were collected at 50 slices within the z-stack range of 50 µm. Biofilm 3-dimensional reconstruction and fluorescence intensity calculation were conducted using the Zeiss software (Carl Zeiss, Oberkochen, Germany).

4.2.6 Statistical analysis

All the experiments were conducted at least in triplicates. Results were reported as the averages of replicates $\pm$ the standard deviation. Student's t-test was used to compare pair of means and calculate *P* values.

4.3 Results and discussion

4.3.1 *Campylobacter* and *Salmonella* biofilms under flow condition

Campylobacter and *Salmonella* biofilms formed under flow condition were characterized in terms of fluorescence images and formation level. The biofilms were formed by *Campylobacter* and *Salmonella* strains that are constitutively tagged with GFP. The fluorescence intensity of images represented bacterial counts and densities within a biofilm. A microfluidic platform was applied to cultivate biofilm in an accurate control mode of flow rate and shear force, aiming to investigate if pre-coated layer of meat juice can assist in biofilm development under shear force stress. A control experiment was conducted in the same microfluidic platform under static cultivation, as the reference to flow condition. For another negative control experiment, culture broth without the addition of bacteria was introduced with the identical operation (Figure S7).

As shown in 3-D reconstruction fluorescence images [Figure 4.2 (A)], *C. jejuni* was observed to form “blanket-like” biofilms in the microchamber under static mode. The biofilms were flat and compact with smooth surface and uniform structure. There was no obvious difference in surface morphology between biofilms formed with and without pre-coated layer of meat juice. However, fluorescence intensity of *C. jejuni* biofilm with pre-coated layer of meat juice was significantly higher ($P < 0.05$) than that without pre-coated layer.

In contrast, biofilm development under flow condition exhibited distinctive architectural features [Figure 4.2 (B)]. *C. jejuni* cells formed a heterogeneous biofilm with microcolonies sparsely distributed on the substrate without pre-coated layer. With pre-coating chicken juice or pork juice, a thicker biofilm was formed, exhibiting rugged topographic features with unevenly

distributed fluorescence intensity. The average fluorescence intensity of *C. jejuni* biofilms was significantly different ($P < 0.01$) with and without pre-coated layer, indicating that pre-coating of meat juice could massively enhanced *C. jejuni* biofilm formation level.

Topographic variations in biofilms under static and flow cultivation can be possibly attributed to different mechanisms of biofilm formation under these conditions. In static mode, *C. jejuni* cells were propelled to the substrate mainly by means of gravity and chemotaxis towards a lower oxygen content. In the meantime, *C. jejuni* cells experienced transverse force of fluid under flow condition, thus the capability of *C. jejuni* cells to attach to the substrate (e.g., production of flagella) become the most critical factor. The attaching capability depends upon both the inherent adhering activity of cells and substrate surface properties (Van Houdt & Michiels, 2010). The inherent adhering activities differ among individual cells at different growth stages (Chavant, Martinie, Meylheuc, Bellon-Fontaine, 2002). As reported in Chapter 3, *C. jejuni* cells exhibited higher affinity to attach to meat particles than abiotic surfaces. Because meat juice particles were unevenly distributed on the substrate, this contributed to the heterogeneity of cell attaching compactness over surface.

The higher level of *C. jejuni* biofilm formation under flow condition than that under static cultivation condition could be explained by the following reasons. Firstly, the supporting effect of pre-coated meat juice becomes prominent when shear velocity is present. Secondly, *C. jejuni* cells might have developed biofilms more resistant to shear force under flow condition, while some cells were loosely attached and fragile to the washing step under static condition. A recent

study reported that biofilms formed under high velocity exhibited stronger cohesion to water channels than that formed under low velocity (Wang et al., 2014).

S. Typhimurium developed biofilms with similar morphology with and without pre-coated layer of meat juice under static conditions [Figure 4.3 (A)]. The structure of *S. Typhimurium* biofilm was homogeneous, with cells loosely connected and dispersed over biofilm matrices. In contrast, *S. Typhimurium* biofilms had entirely different architectures under flow condition [Figure 4.3 (B)]. *S. Typhimurium* cells were compactly associated in the biofilm matrix without pre-coated meat juice, with some microcolonies evenly distributed. For biofilms formed with pre-coating layer of chicken juice or pork juice, “mushroom-like” structures were observed with a higher thickness compared to that without coating meat juice. Overall, biofilm formation level of *S. Typhimurium* increased when the pre-coated layer of meat juice was available under both static and flow condition [Figure 4.3 (C)]. The degree of enhancement of *S. Typhimurium* biofilm levels under flow condition was higher than the ones under static condition.

4.3.2 Reduced biofilm formation compensated by pre-coated layer of meat juice

Bacterial cells may lose surface motile structures in food processing environment, leading to the lack of ability to form biofilms. Selective mutants of *C. jejuni* and *S. Typhimurium* with genetically modified deficiency of attachment to abiotic surfaces and biofilm formation were used in the current study. These mutants were cultivated to form biofilms on glass and polystyrene surfaces, as well as on these substrates pre-coated with meat juice particles. We aimed to identify the role of pre-coated layer of meat juice in biofilm formation by these mutants, compared to the wild-type strain with normal capability of forming biofilms.

Both aflagellated mutants of *C. jejuni* (*i.e.*, $\Delta flaAB$ and $\Delta flgL$) showed significant deficiency ($P < 0.05$) in biofilm formation on polystyrene and borosilicate glass compared to the wild-type strain, with TTC values less than 0.1 (Figure 4.4). FlaA and FlaB are the proteins associated with the production of flagellar filament components, while FlgL is responsible for the formation of flagellar hook components (Nachamkin, Szymanski & Blaser, 2008). Our results validated the importance of flagellar structure in *C. jejuni* biofilm formation. Noticeably, with support from both chicken juice and pork juice pre-coating treatment on polystyrene and glass, both *C. jejuni* flagella mutants were able to form significantly more ($P < 0.001$) biofilms than that without meat juice pre-coating, with TTC values higher than 0.4. These biofilm formation levels were comparable to the biofilm on corresponding un-coated surfaces formed by the wild-type strain.

Gene *fimI* in *S. Typhimurium* is responsible for encoding the major component (*i.e.*, fimbrin) of type 1 fimbriae, which is mediated by *sirA/barA* regulatory system (Teplitski et al., 2006). *S. Typhimurium* mutants with type 1 fimbriae functional disorder formed significantly lower ($P < 0.05$) biofilms on both glass and polystyrene compared to the wild-type strain (Figure 4.5). In the presence of pre-coated layer of meat juice, the biofilm formation of these mutants was recovered to a comparable or higher level than the biofilms formed by wild type strain on un-coated abiotic surfaces.

Taken together, deficiency in biofilm formation by aflagellated *Campylobacter* and *Salmonella* mutants could be effectively compensated by the pre-coated layer of meat juice on abiotic substrate (*i.e.*, glass and polystyrene). Thus, meat juice particles not only enhanced biofilm

formation ability of normal cells, but also massively facilitated the recovery of biofilm formation ability of the cells in lack of surface structures (*i.e.*, flagella and type 1 fimbriae). In addition, meat juice pre-coating treatment could enhance biofilm formation levels regardless the bacterial strains were capable or defective in forming biofilms. Therefore, the enhancing effect of meat juice on *Campylobacter* and *Salmonella* biofilms is associated with the changes in surface properties of the inert substrate, instead of nutritional chemotaxis of the bacteria.

4.4 Summary

Pre-coating of chicken juice and pork juice on borosilicate glass and polystyrene could significantly enhance biofilm formation of *Campylobacter* and *Salmonella* under flow condition. The degree of increase caused by meat juice particles was higher than the counterpart biofilms formed under static cultivation. The presence of meat juice particles did not lead to obvious difference in biofilm morphology of *Campylobacter* and *Salmonella*.

Campylobacter and *Salmonella* biofilms exhibited distinctive structural morphology and surface topographic characteristics between static and flow cultivation. *Campylobacter* and *Salmonella* biofilms formed under static condition were homogeneously distributed with smooth surface, while the biofilms formed under shear stress were bumpy and rough with high heterogeneity.

Campylobacter and *Salmonella* mutants lacking cell surface structures (*i.e.*, flagella and type 1 fimbriae) necessary for initial attachment and biofilm formation were used for further investigation. Their deficiency in biofilm formation could recover in the presence of pre-coated meat juice particles, with reference to the biofilms formed by the corresponding wild-type strains

on bare abiotic surfaces. The mechanism of biofilm enhancement was validated to be associated with the properties of substrate surface altered by the pre-coating treatment.

4.5 Tables and figures

Figure 4.1. Design and configuration of a PDMS-based microfluidic chip with glass bottom for the cultivation of bacterial biofilms.

(A)

(B)

(C)

Statistical significance was determined using Student's t-test (* denotes $P < 0.05$, ** denotes $P < 0.01$). In this figure, MH: Müller Hinton broth, CJ: chicken juice, and PJ: pork juice.

Figure 4.2. Representative fluorescence microscopic images of *C. jejuni* F38011-gfp biofilms grown in microfluidic chamber under static (A) and flow condition (B). The average fluorescence intensity of each biofilm sample is shown in panel (C) with background intensity subtracted.

(A)

(B)

(C)

Statistical significance was determined using Student's t-test (* denotes $P < 0.05$, ** denotes $P < 0.01$). In this figure, LB: Luria-Bertani broth, CJ: chicken juice, and PJ: pork juice.

Figure 4.3. Representative fluorescence microscopic images of *S. Typhimurium* SL1344-gfp biofilms grown in microfluidic chamber under static (A) and flow condition (B). The average fluorescence intensity of each biofilm sample is shown in panel (C) with background intensity subtracted.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 4.4. Biofilm formation level of aflagellated *C. jejuni* F38011 mutants in polystyrene 96-well plate with and without meat juice pre-coated.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 4.5. Biofilm formation level of aflagellated *C. jejuni* F38011 mutants in glass chamber with and without meat juice pre-coated.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 4.6. Biofilm formation level of attachment-defective mutants of *S. Typhimurium* 14028 formed in polystyrene 96-well plate with and without meat juice pre-coated.

*Significant difference in pair comparison with the control group (Student's t-test, $P < 0.05$).

Figure 4.7. Biofilm formation level of attachment-defective mutants of *S. Typhimurium* 14028 formed in glass slide chamber with and without meat juice pre-coated.

Chapter 5 - Conclusions and significance of the study

Chicken juice and pork juice collected from raw meat carcasses were validated to be an effective promoting factor on biofilm formation of *Campylobacter* and *Salmonella*. The enhancement effect of meat juice on bacterial biofilms was attributed to the formation of a conditioning layer on the inert substrates (*i.e.*, polystyrene and borosilicate glass) by meat juice particles. Biofilm formation level of *Campylobacter* and *Salmonella* could be enhanced by meat juice pre-coating treatment under both static and flow cultivation. Biofilm-defective mutants of *Campylobacter* and *Salmonella* could form considerable levels of biofilms with the pre-coating layer of meat juice.

Campylobacter and *Salmonella* prefer to attach onto biologically conditioned surfaces rather than inert abiotic surfaces as the initial stage of biofilm formation, indicating that the existence of food residues in processing and handling areas can play a positive role in the formation of *Campylobacter* and *Salmonella* biofilms. Both chicken juice and pork juice performed similar enhancing capability on *Campylobacter* and *Salmonella* biofilm formation. Studies using other types of food ingredients, such as fish, milk, oil, and carbohydrates, could also lead to the enhancement of biofilm formation, suggesting that the facilitation by food residues might be universally effective on biofilms formed by various bacteria (Dat et al., 2010; Kuda, Shibata, Takahashi, & Kimura, 2015; Graziella Midelet & Carpentier, 2002; Sofos & Geornaras, 2010; Whitehead, Smith, & Verran, 2010). The development of bacterial biofilms on direct or indirect contact surfaces is a critical risk factor that contributes to the transmission of foodborne pathogens. Findings in this study emphasize the importance of thorough cleaning of food contact surfaces.

This thesis project applied biofilm cultivation conditions that mimic the real situation in meat processing industry in the following three aspects:

(1) Meat juice was applied as a minimally processed food-based model. We validated the feasibility of using meat juice to investigate its impact on foodborne pathogens and their corresponding biofilms. *Campylobacter* and *Salmonella* cells could multiply in chicken juice and pork juice, indicating that meat juice is the possible nutrient source for bacteria in the real environmental and food processing conditions.

(2) The biofilm formation of *Campylobacter* and *Salmonella* was investigated under both static and flow cultivation conditions. Both conditions can be encountered in the meat processing environment, such as a cutting board or sewage piping, where *Campylobacter* and *Salmonella* are believed to persist. This was achieved in a microfluidic platform that allows biofilm cultivation in a well-controlled manner. My current study validated the potential use of microfluidic “lab-on-a-chip” platform in bacterial biofilm studies.

(3) We selected several mutants of *Campylobacter* and *Salmonella* in lack of attachment capacity because bacteria could lose this function when they are exposed to the environmental stresses. Meat juice particles could lead to an increase in biofilm formation, regardless of attachment ability and motility, indicating that the enhancement was more likely due to alteration in surface physiochemical properties by meat particles instead of nutritional attraction.

By characterizing *Campylobacter* and *Salmonella* biofilms under a simulated meat processing environment, we obtained a more comprehensive understanding of bacterial survival outside the laboratory. The contribution of meat juice to bacterial biofilm formation provided an explanation

to the high prevalence of *Campylobacter* and *Salmonella* under unfavorable conditions, such as aerobic and antimicrobial stresses. Meat juice can therefore be employed as a vehicle to investigate bacterial responses to environmental stresses.

The knowledge received from this study can contribute to the development of mitigation strategies against *Campylobacter* and *Salmonella* in such an environment in the future. However, more knowledge is needed in terms of composition of meat juice, especially the meat juice residue on the substrate surface. Further study could focus on the interplay between specific meat juice components (*e.g.*, types of proteins) and bacterial biofilm formation. We can then develop the strategy targeting specific components to achieve the maximum efficacy of biofilm reduction.

Lastly, only monospecies biofilms were investigated in the current study. In the natural state, different species of microorganisms are more likely to live in a cooperative and interactive community of a biofilm. Multispecies biofilms therefore should be studied using meat juice as a food-based model.

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Appendix - Supplementary tables and figures

Table A.1. Characteristics of meat juice after sterilization and calibration.

	source	yield	pH	protein content
chicken juice	whole chicken	~180 ml/chicken	6.52±0.02	5 mg/ml
pork juice	pork chop	~115 ml/4 chops	6.38±0.04	5 mg/ml

Figure A.1. *C. jejuni* F38011 planktonic cell growth in MH broth and meat juice during a 30-day time period.

Figure A.2. *S. Typhimurium* SL1344 planktonic cell growth in MH broth and meat juice during a 30-day time period.

Figure A.3. Standard curve of *C. jejuni* cell count and OD value at 600 nm.

(a)

(b)

Figure A.4. Standard curve of *Salmonella* cell count and OD value at 600 nm. (a) *S. Enteritidis* ATCC33560; (b) *S. Typhimurium* SL1344.

Figure A.5. Potential toxicity of TTC on *C. jejuni* F38011 and *S. Typhimurium* SL1344, shown as the log reduction of *C. jejuni* F38011 and *S. Typhimurium* SL1344 cells after cultured in TTC supplemented MH/LB broth for 24 hours.

Figure A.6. Biofilm formation levels of *C. jejuni* F38011 and *S. Typhimurium* SL1344 in MH/LB media during a 5-day time course.

Figure A.7. Negative control of fluorescence images (no bacteria introduced). a) no meat juice pre-coated; b) pure chicken juice pre-coated; c) pure pork juice pre-coated.