UNDERSTANDING AND MANAGING THE RISK FROM *SALMONELLA* ON CUCUMBERS

by

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ABSTRACT OF DISSERTATION

Understanding and managing the risk from *Salmonella* on cucumbers

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Recent multistate outbreaks of salmonellosis directly associated with fresh cucumbers highlight the importance of understanding the factors that may influence or contribute to the risk of *Salmonella* contamination on cucumbers. The studies in this dissertation increase our limited knowledge of select factors on *Salmonella* behavior in cucumbers. The results in Chapter 2 show that significant bacterial transfer from cucumber skin to edible flesh portion and peeler occurred during peeling no matter what microorganisms exist on the surface of cucumber. There was no significant difference between transfer of *S*. Newport and *E. aerogenes*, indicating that *E. aerogenes* B199A could be used as surrogate for *S*. Newport in cross-contamination studies regarding cucumbers. The main finding from Chapter 3 was that curli and cellulose-producing *S*. Newport wild type showed better attachment to the surface of cucumber compared to non-producing mutant strains, but curli and cellulose did not have a significant influence on transfer of *S*. Newport to edible flesh portion of cucumber during peeling. These suggest that bacterial cell surface components including curli, cellulose, flagella, and

capsular polysaccharides are all involved in complex interaction of *S*. Newport with cucumber, but that other, more important mechanisms influence *Salmonella* attachment and transfer to cucumbers. Chapter 4 shows that *Salmonella* are able to survive on the surface of cucumbers stored at 7 and 21 °C for up to 7 days, whether the cucumber was waxed or un-waxed. Significant transfer of *Salmonella* from inoculated cucumbers to brushes and un-inoculated cucumbers was found during cucumber waxing. *Salmonella* remaining on the contaminated cucumbers after waxing survived up to 7 days, and *Salmonella* showed better survival on the cucumbers treated with a petroleum-based wax. These results suggest that survival and transfer of *Salmonella* vary depending on the type of wax coatings. The models developed in Chapter 5 predict the growth and survival of *Salmonella* on whole and cut cucumbers as a function of temperature and RH. These findings should be useful for future microbial risk assessments and predictions of *Salmonella* behavior in cucumbers to manage the risk of *Salmonella* with respect to cucumbers.

Dedication

LOMYFA

Thank you for being in my life.

Thank you for having me in your life.

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Table of Contents

List of Tables

List of Figures

Chapter 1. Literature Review

1.1. Cucumber production and safety

Cucumbers are one of the most economically important crops belong to *Cucurbitaceae* family commonly known as the cucurbits and gourds. Worldwide cucumber production has steadily increased and approximately 3% of the world's cucumber was produced in the United States in 2013. Imports of fresh cucumbers to the United States from other countries such as Mexico, Canada, and Honduras increase in winter months when the domestic production declines (1, 2). A total of 690 million pounds of fresh cucumbers was produced in the United States in 2014, and 180 million pounds were imported from other countries (3). Between 2004 and 2013, per capita use of fresh cucumber increased from 6.31 pounds to 7.42 pounds per person (4). With increased demand and imports of fresh cucumbers, the potential for multistate outbreaks of salmonellosis is cause for concern.

The Food Safety Modernization Act (FSMA) is designed to establish enforceable safety standards for produce farms and make importers accountable for verifying that imported food meets U.S. safety standards (5). The produce safety standards for growing, harvesting, packing, and holding produce include water quality standards, employee health and hygiene management, wild and domesticated animal control, biological soil amendment management, and proper use of equipment, tools, and buildings to minimize the risk of serious illness or death from consumption of contaminated produce.

As cucumbers may become contaminated at numerous points during growing, harvesting, processing, distributing, transporting, retailing and preparation in restaurants

or homes, it is important to understand and maintain good sanitation throughout production and handling of cucumbers to ensure safety for consumers.

Pre-harvest

Cucumbers are grown in warm weather conditions with ideal temperature between 65-75 °F and tolerate soil pH between 6.8-5.5 (6). Climatic production conditions and environment are important factors that affect the microbial safety of fresh produce (7). As fields on which livestock or wild animals have grazed or animal manure have been applied are more likely to be contaminated with enteric pathogens that can survive in soils for months or even years (8, 9), it is important to avoid establishing fields near animal operations or waste handling facilities and to ensure manure and biosolids are treated properly and should never have an opportunity to come into contact with fruits and vegetables. Another factor that can affect microbiological quality of produce is quality irrigation water and frost protection water. If water comes in contact with any edible portion of vegetables, it should be potable, i.e., of drinking water quality (10). Reuse of non-potable (grey or waste) water of uncertain quality has a high probability of containing pathogenic microorganisms and exacerbates produce contamination (11). The water should ideally be tested for the presence of coliform bacteria and it is recommended that fecal coliform level of wastewater should not exceed 1000 CFU/100 mL or MPN/100 mL (12). Other chemicals such as reconstituted pesticides applied to the field or crop can also be a source of contamination (11). Personal hygiene of farm workers is another important safety factor. Appropriate hand-washing facilities with portable toilets need to be provided and runoff from these facilities should be controlled properly to prevent contamination to the field. Infected workers who display symptoms

of illness should not be allowed to come in contact with fresh produce or any equipment that will contact the crops (10).

Harvest and Processing

Human and mechanical contact, washing, drying, cutting or slicing can impact the microbiological safety of fresh produce during harvesting and processing. Personal hygiene of farm workers, wild and domesticated animal exclusion, and good hygienic practices should be applied during harvest and at the packinghouse. Farm workers should not handle culled crops in the field, which may cause cross-contamination to healthy produce via workers' hands. The equipment and containers used in the field, in packinghouses, and for shipment should all be properly cleaned and sanitized. Animal and insect control in and around packing facilities, cleaning and sanitation of processing lines are all important in preventing cross-contamination to other products passing through the lines (7, 10). The water quality, types and concentration of disinfectants are also very important for the post-harvest washing of fresh produce to reduce the potential risk of cross-contamination in dump or hydro-cooling systems.

Distribution

The conditions of fresh produce storage and distribution can have profound effects on microbial safety. Maintaining appropriate temperatures in shipping or storage including loading and unloading docks is vital to control microbial contamination. It is also very important to transport fresh produce in clean, sanitary shipping containers or trailers (7, 10, 13).

Retailing and foodservice

Removal of fresh produce that has visible signs of decay or damage while setting up and rotating displays at retail can reduce the risk of pathogen contamination. Implementing sanitation procedures in the back room and display area is important to avoid cross-contamination between different foods or contamination by workers (10). Appropriate procedures for storing and displaying food, for excluding or restricting ill employees, for washing hands, date-marking, and for washing and sanitizing equipment should also be applied. Raw fruits and vegetables should be thoroughly washed with running tap water to remove surface dirt before being cut, combined with other food ingredients, cooked, served, or offered for human consumption in ready-to-eat form to avoid cross-contamination during food preparation at any foodservice operations or home (14).

1.2. *Salmonella*

Salmonella spp. are gram-negative, facultative anaerobic rod-shaped bacilli belonging to the family *Enterobacteriaceae*, with two species, *Salmonella enterica* and *bongori*, each of which contain multiple serovars (15, 16). *S. enterica* is divided into six subspecies designated by taxonomic names and referred to by roman numerals (17, 18). The species are differentiated biochemically and by genomic relatedness (19). The majority of currently classified human disease isolates belongs to *S. enterica* subspecies *enterica* designated by a name related to geographical place where the serovar was first isolated. The serotypes in other subspecies are designated by antigenic formulas (19, 20). The classification of specific serotype is based on the serological identification of somatic

(O) lipopolysaccharides (LPS) on the external surface of the bacterial outer membrane, and H (flagellar) antigens associated with the filamentous portion of the flagella and characterized host specificity or clinical syndromes (15, 21, 22). The four most common *S. enterica* serovars associated with human *Salmonella* infections are *S*. Enteritidis, *S*. Typhimurium, *S*. Newport, and *S*. Javiana (23).

1.3. Salmonellosis

Salmonella enterica is a leading cause of foodborne bacterial illnesses in humans, and is estimated to cause 1.2 million illnesses each year in the United States with more than 23,000 hospitalizations and 450 deaths (17, 24). Non-typhoidal *Salmonella* (NTS) serovars such as Typhimurium, Enteritidis, and Newport are serovars with broad host specificity, and are estimated to cause 11% of foodborne illnesses, 35% of foodborne hospitalizations, and 28% of foodborne death in the United States (24, 25). NTS infections may be transmitted by contaminated fruits, vegetables, or other plant products as well as contaminated animal-derived foods (22). Although animal-derived foods such as poultry, meat, eggs and dairy products are considered the most commonly implicated sources in *Salmonella* outbreaks (26), large multistate outbreaks of salmonellosis have been attributed to fresh produce and have increased over the last two decades (11, 27).

There have been at least four multistate outbreaks of salmonellosis linked to cucumbers recently in the United States. *Salmonella* had not been isolated from fresh cucumbers before these salmonellosis outbreaks occurred (28, 29), although *Listeria monocytogenes* and coliform bacteria such as *Enterobacter* have been found in

cucumbers (11, 30-32). The first reported *Salmonella* outbreak directly linked to contaminated cucumbers in the United States occurred in 2013, which was a multistate outbreak of *Salmonella* Saintpaul caused by imported cucumbers from Mexico (33). A *Salmonella* Newport outbreak implicating cucumbers occurred in 29 states and the District of Columbia in 2014, and was the largest multistate foodborne disease outbreak in the United States in that year (34). According to epidemiologic, microbiologic, and product traceback investigations performed by the Centers for Disease Control and Prevention (CDC), state and local health and agriculture departments and laboratories, and the U.S. Food and Drug Administration (FDA), it was the first multistate outbreak of *Salmonella* Newport linked to fresh produce items grown in the Delmarva region other than tomatoes (35). A multistate *Salmonella* Poona outbreak associated with imported Mexican cucumbers occurred in 2015, which resulted in 6 outbreak-related deaths and 204 hospitalizations among 907 cases in 40 states (36). Most recently, *Salmonella* Oslo infections attributed to Persian cucumbers sickened at least 14 people across 8 states. It was the fourth salmonellosis outbreak directly linked to fresh cucumbers since 2013 (37).

1.4. Bacterial transfer and cross-contamination

Cross-contamination is one of the major causes of foodborne disease outbreaks. Almost 25% of the foodborne outbreaks in Europe can be traced back to cross-contamination through food materials, unclean food surfaces or contaminated equipment, and food handlers (38). Cross-contamination also plays a significant role in transferring harmful pathogens to fresh produce at numerous points as fruits and vegetables are

frequently exposed to soil, insects, animals, or humans during growing, harvesting, packing processing, transporting, distributing, retailing and food preparation in the kitchen environment (11, 39).

Cross-contamination from the surface of fresh produce to edible flesh portion during cutting, slicing, or peeling can occur if the outer skin or rind of fresh produce is contaminated by pathogens (11, 26, 40-42). Moreover, during these preparation processes nutrients become more readily available and water is exuded from cut surfaces, which can support the growth of native microflora or pathogenic bacteria (11, 39, 43-45). Bacterial transfer from contaminated surface to the flesh portion of fresh produce during cutting or slicing can be affected by various factors such as concentration or type of organisms attached on the surface of food products, surface characteristics or type of food products, or processing procedures (40, 46-51). However, there is limited published information on cross-contamination during peeling of fresh produce. Quantifying all these factors that could cause cross-contamination is important to reduce and prevent infectious disease.

1.5. Bacterial attachment

The ability of bacteria to attach to the surface of fresh produce depends on intrinsic and extrinsic factors. These include cell structures and motility of the bacteria, surface physicochemical properties of both the bacterial cell and food materials, nutrient availability from the plant, pH, and temperature of the surrounding medium, and interaction with other organisms (11, 52-54).

Previous studies on *Salmonella* adhesion to fresh produce revealed that bacterial attachment to plant tissue is strongly strain dependent (55, 56). *S*. Typhimurium, Enteritidis and Senftenberg all showed stronger attachment to basil, lettuce or spinach leaves compared to other serovars including Agona and Heidelberg. In another study, *S*. Tennessee attached more strongly to iceberg and romaine lettuce leaves than other serovars including Newport, Negev, Thompson and Braenderup (52).

A study conducted by Barak et al. (57) found that specific genes necessary for animal virulence of *S*. *enterica* are also required for attachment and colonization of alfalfa sprouts, which includes *rpoS*, the general stress response regulator sigma factor, and *agfB*, the surface-exposed curli nucleator, and *agfD*, a transcription regulator from a thin aggregative fimbriae (Tafi or curli in *Escherichia coli*). These proteins have been reported to regulate the production of curli, cellulose and adhesins, synthesis of Tafi, and biofilm formation (57-59). Curli production has been attributed to both plant and mammalian adhesion through divergently expressed *agfBA(C)* and *agfDEFG* operon expressions, which are known as *csgBA(C)* and *csgDEFG* in *Escherichia coli* (59, 60). A study by Tan et al. (61) showed curli, cellulose, and flagella were all involved in *Salmonella* attachment to plant cell wall models. Lapidot and Yaron (62) also reported that *Salmonella* transfer and attachment to parsley are dependent on extracellular components of the biofilm such as cellulose and curli.

In addition to bacterial surface components including curli, flagella, and extracellular polysaccharides, the attachment of *Salmonella* to the surface of fresh produce may differ depending on specific properties of commodities. *Salmonella* serovars more strongly attach to lettuce leaves than to cabbage even though there was no

significant difference in *Salmonella* attachment on the condition (intact or cut) of the produce surface (52, 63). Kroupitski et al. (64) found distinct attachment properties of *S.* Typhimurium in older leaf parts and leaf regions near the petiole of romaine lettuce. Most attachment studies of *Salmonella* for fresh and fresh-cut produce have been performed with lettuce and tomato, and little is known about its attachment to other fresh produce, including cucumbers.

1.6. Bacterial growth and survival

The type and populations of microorganisms on fresh produce are affected by the type of produce, agronomic practices, geographical area of production, and weather conditions prior to harvest. Handling, storage conditions, processing, packaging, distribution, and retailing after harvesting also play important roles (7, 11, 44). Although various extrinsic and intrinsic factors such as pH, temperature, relative humidity, cohabitation with other microorganisms, nutrient availability, toxic compounds released by the plant, and presence of antimicrobials influence growth and survival of native microflora and pathogenic bacteria on fresh produce, very little is known about the microbial behaviors on cucumbers.

Cucumbers could be naturally contaminated (just as other fruits and vegetables may be contaminated) by soil, insects, animals, or humans during pre- and post-harvesting (39, 43, 45, 49). It has been reported that cucumbers have approximately 6-7 log CFU/piece (or g) of native microflora (31, 65). Abdul-Raouf et al. (66) demonstrated that the populations of native microflora on the sliced cucumber stored at 5, 12, and 21

°C increased significantly throughout a 7-day storage period, although the population of *E. coli* O157:H7 significantly declined during the storage of sliced cucumber at 5 °C and increased at 12 and 21 °C. In another study, the populations of *E. sakazakii* on the sliced cucumber stored at 4 °C gradually decreased as storage time progressed (67). However, significant increases in the populations of *E. sakazakii* occurred within 24 to 48 h at 12 °C and the sliced cucumbers supported the growth of *E. sakazakii* during storage at 25 °C, showing much shorter lag phase compared to those at 12 °C (67). Previous studies have reported that *S. enterica* serovars persist differently in tomato plant tissues (68-70), but related work has not been completed on cucumbers and very little is known about *Salmonella* behavior on cucumbers.

Chapter 2. Quantification of transfer of *Salmonella* Newport during cucumber peeling and evaluation of *Enterobacter aerogenes* B199A as its surrogate

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Key words: Native microflora, *Enterobacter aerogenes*, *Salmonella* Newport, cucumber, peeling, cross-contamination, risk assessment

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2.1. Abstract

Several multistate outbreaks of *Salmonella* linked to cucumbers recently occurred in the United States. Little is known about the degree to which bacteria can transfer from the surface of fresh produce items during peeling. This research quantified the transfer of *Salmonella* Newport and its potential surrogate *Enterobacter aerogenes* B199A from the surface of fresh cucumber to edible flesh or peeler during peeling. Fresh cucumbers were dip-inoculated with *S*. Newport or a food-grade strain of nalidixic acid resistant *E. aerogenes* (~10^9 CFU/mL) in a zip top plastic bag by massaging for 30 seconds. Half of each inoculated cucumber was hand peeled using a sterilized peeler. The unpeeled half, the peeled half (flesh), the removed peel and the peeler were all enumerated for the presence of *S*. Newport and *E. aerogenes*. This procedure was also repeated for un-inoculated fresh cucumber to determine the transfer rate of native microflora during peeling. Percent transfer was calculated by recipient surface/donor surface * 100. Transfer of native microflora, *E. aerogenes*, and *S*. Newport from cucumber skin to edible flesh and peeler occurred during peeling, although a majority of bacteria remained on the peel. The log percent transfer of *S*. Newport to edible flesh portion and peeler during peeling was lower than that of native microflora and *E. aerogenes* but there was no significant difference between the transfer of microorganisms. *E. aerogenes* B199A may be a useful surrogate for investigating the transfer of *S*. Newport in cross-contamination studies with respect to cucumbers.

2.2. Introduction

Fresh cucumbers have recently been recognized as a vehicle in foodborne disease outbreaks since several multistate outbreaks of *Salmonella* directly linked to cucumbers have occurred in the United States (33, 34, 36, 37). *Salmonella* had not been isolated from fresh cucumbers before these salmonellosis outbreaks occurred (29, 71, 72), although *Listeria monocytogenes* and coliform bacteria such as *Enterobacter* have been found in cucumbers (11, 30-32). The first reported multistate *Salmonella* outbreak associated with contaminated fresh cucumbers occurred in 2013, where a total of 84 cases of *Salmonella* Saintpaul infections were caused by imported cucumbers from Mexico (33). The following next year, *Salmonella* Newport infections implicating fresh cucumbers were reported from 29 states and the District of Columbia, and was the largest multistate foodborne disease outbreak occurred in the United States in 2014 (34). Imported cucumbers from Mexico were also responsible for the multistate *Salmonella* Poona outbreak occurred in 2015, which resulted in 6 outbreak-related deaths and 204 hospitalizations in 40 states (36). Most recently, *Salmonella* Oslo infections attributed to Persian cucumbers sickened at least 14 people in 8 states.

Fresh produce including cucumbers could not only become contaminated from other contaminated foods and utensils due to unsafe food handling practices (49, 73, 74) but also cause cross-contamination from surface to edible flesh portion during food preparation in the kitchen environment if the surface of fresh produce is contaminated by pathogens (11, 41, 75). Cutting, slicing, shredding, or peeling can transfer pathogens from the surface of fresh produce to edible flesh portion through tools or hands (26, 39, 40, 42). Moreover, during these preparation processes more readily available nutrients

and water exuded from cut surfaces support the growth of native microflora and pathogenic bacteria, which can subsequently cause foodborne diseases (11, 39, 43, 44). However, little is known about the degree to which bacteria can transfer from the surface of fresh produce items during peeling.

Using nonpathogenic surrogate microorganisms provides one practical means for verifying process efficacy in food processing establishment without the use of the actual pathogens. The ideal surrogate would be a nonpathogenic that behavior similar to target microorganisms when exposed to similar environmental or processing conditions so that it could be used to predict microbial kinetics of target microorganism (76). Various nonpathogenic surrogates such as *Pediococcus* sp. NRRL B-2354, *Enterococcus faecium* ATCC 8459, *Saccharomyces cerevisiae*, and *Enterobacter aerogenes* B199A have been used in various intervention studies to reduce the risk of *Salmonella* contamination (77-79).

This study was undertaken to quantify the transfer of *Salmonella* Newport from the surface of cucumber to edible flesh portion or peeler during peeling and to evaluate *Enterobacter aerogenes* B199A as a nonpathogenic surrogate for *Salmonella* transfer on cucumbers. Bacterial transfer rates were determined between the inoculated surface of cucumber and edible flesh portion and between the inoculated surface of cucumber and peeler.

2.3. Materials and Methods

2.3.1. Cucumbers

Fresh cucumbers were purchased from a local supermarket (Somerset, NJ) and stored overnight at 5 °C until use. Prior to being inoculated, the cucumbers were spray washed with 70% ethanol to reduce nalidixic acid resistant microflora and then dried for 1 h with the biosafety cabinet fan running.

2.3.2. Bacterial strains and inoculation

Salmonella enterica Newport 96E01152C-TX (obtained from Dr. Barak, Department of Plant Pathology, University of Wisconsin-Madison) and nalidixic acid resistant *Enterobacter aerogenes* B199A were used in this study. Both organisms were grown in tryptic soy broth (TSB; Difco, BD, Sparks, MD) or TSB containing 50 μg/mL of nalidixic acid (Fisher Scientific, Pittsburgh, PA) if nalidixic acid resistant. After 24 h of incubation at 37 °C, cells were harvested by centrifugation (Allegra 21R centrifuge; Beckman Coulter, Brea, CA) at 5,000 × g for 10 min and then washed two times in 10 mL of phosphate buffer saline (PBS; 0.1M, pH 7.2) (Fisher Scientific). The cell pellets were suspended in PBS after final wash, yielding a solution of ~10^9 CFU/mL.

The cucumbers were dip-inoculated with 10 mL of *S.* Newport or *E. aerogenes* (~10^9 CFU/mL) in a zip top plastic bag by massaging for 30 s. The cucumbers inoculated with *S.* Newport were dried for 24 h in the biosafety cabinet with fan running. To determine the appropriate drying time for transfer of *E. aerogenes* on cucumbers as a surrogate microorganism, the cucumbers inoculated with *E. aerogenes* were sampled at 0, 2, 4, 8, 12, 16 or 24 h post inoculation.

2.3.3. Cross-contamination during peeling

Half the inoculated cucumber was hand peeled using a sterilized peeler to study the transfer of *S*. Newport and *E. aerogenes* from the cucumber skin to the edible flesh portion. After peeling, unpeeled half cucumber, peeled half (edible flesh portion), removed peel and contaminated peeler were placed into a Lab blender bag (Fisher Scientific) with 30 mL of PBS, and then massaged by hands for 30 s. This procedure was repeated for un-inoculated fresh cucumbers to determine the transfer rate of native microflora during peeling. The suspensions were serially diluted (1:10) in PBS, and 0.1 mL of each dilution was plated onto Hektoen Enteric agar (HE; Difco, BD) and MacConkey agar (Remel, Thermo Fisher Scientific, Waltham, MA) containing 50 μg of nalidixic acid per mL to enumerate *S*. Newport and *E. aerogenes*, respectively. Control samples were plated onto plate count agar (PCA; Difco, BD). The plates were incubated at 37 °C for 24 h and bacterial colonies were counted following the incubation. The population of *S*. Newport, *E. aerogenes* and native microflora was expressed in log CFU/piece for each portion of cucumber and peeler.

2.3.4. Data analysis

Individual transfer rate for each experiment (as well as mean, standard deviation, median, maximum transfer observed, minimum transfer observed, and range of transfer rates observed) was calculated using the following equation:

$$\text{Transfer rate } (\%) = \left(\frac{\text{CFU measured on peel, flesh portion, or peeler}}{\text{CFU measured on inoculated half cucumber}}\right) \times 100 \quad (1)$$

Transfer rates between samples were logarithmically transformed in order to create normally distributed data as per Schaffner (80). All results were analyzed using Tukey's

HSD test (SAS University Edition; SAS Institute Inc., Cary, NC, USA) to determine statistically significant differences in bacterial transfer during peeling.

2.4. Results

Bacterial transfer patterns (log percent transfer) from cucumber skin to edible flesh portion and peeler during peeling are shown in Figure 2.1 to 2.3. Transfer rates have also been characterized using six different statistical parameters (mean, standard deviation, median, maximum, minimum and range) as presented in Table 2.1 to 2.3 to provide a more detailed summary of the results.

The majority of *S.* Newport, native microflora, and *E. aerogenes* remained on the peel portion of cucumber, although significant bacterial transfer between the cucumber skin and the edible flesh portion and between the cucumber skin and the peeler occurred during peeling. Figure 2.1 shows the level of bacteria that remained on the peel portion of cucumber during peeling. There is no significant difference in the level of bacteria remaining on the peel portion of cucumber during peeling no matter what microorganisms were on the surface of cucumber. However, the *S.* Newport remaining on the peel portion of cucumber was lower than native microbiota (200.63%) and *E. aerogenes* (76.54-444.75%) on the peel portion except *E. aerogenes* at 8 h post inoculation (58.77%) as shown in Table 2.1 to 2.3. When the cucumber was dried for 8 h, the level of *E. aerogenes* remaining on the peel portion was similar to that of *S.* Newport on the peel portion of cucumber (65.30%).

The log percent transfer of *S*. Newport from cucumber skin to the edible flesh portion during peeling was the lowest among all microorganisms inoculated onto the surface of cucumber, while the highest transfer of *E. aerogenes* to the edible flesh portion was observed at 0 h drying. The lowest log percent transfer of *E. aerogenes* was shown at 16 h drying in which the transfer was highly variable but there was no significant difference between drying times from 0 to 24 h. As shown in Table 2.2, the percent transfer of *E. aerogenes* from the inoculated cucumber skin to the flesh portion decreased as drying time increased, with an average transfer rate range of 0.47 to 76.54%, but the overall percent transfer of *E. aerogenes* to edible flesh portion was noticeably higher than that of *S*. Newport to the edible flesh portion of cucumber during peeling (0.12%).

Similar to the bacterial transfer to the edible flesh portion, the log percent transfer of *E. aerogenes* to the peeler was the highest at 0 h post inoculation compared to the other drying times but there was no significant difference between drying times from 0 to 24 h. The average percent transfer of *Enterobacter* to the peeler ranged from 0.11 (2 h drying) to 4.00% (0 h drying). Unlike the *Enterobacter* transfer from the inoculated cucumber skin to the flesh portion, time dependent decrease in bacterial transfer to the peeler was not observed during peeling. The percent transfer of *S*. Newport from the cucumber skin to the peeler (0.20%; Table 2.1) showed a similar transfer rate when inoculated cucumber with *E. aerogenes* was dried for 24 h (0.26%; Table 2.3).

The log percent transfer of *S*. Newport to the edible flesh portion and peeler during the peeling of cucumber was lower than that of native microflora and *E. aerogenes,* and the overall transfer pattern of *E. aerogenes* was more similar to that of native microflora

rather than *S*. Newport. However, there was no significant difference in bacterial log percent transfer no matter what microorganisms present on the surface of cucumber.

2.5. Discussion

Previous studies have reported that bacterial transfer from contaminated surface to edible flesh portion of fresh produce during cutting or slicing can be affected by various factors such as concentration or type of organisms present on the surface of food products, surface characteristics or type of food products, or processing procedures (40, 46-51), while there is limited published information on the cross-contamination during peeling of fresh produce. Quantifying all these factors that could cause cross-contamination is very important to reduce and prevent foodborne disease.

Cucumbers could become contaminated by soil, insects, animals, or humans during pre- and post-harvesting (39, 43, 45, 49). There have been reported that cucumbers have approximately 6-7 log CFU/piece (or g) of native microflora (31, 65). In our study, the level of native microflora on the surface of cucumber ranged from 6.27 to 7.85 log CFU/piece but no bacterial transfer was observed when the concentration of native microflora on the surface of cucumber was below 6.30 log CFU/piece. Also, no bacterial transfer observed or detected when the final concentration of *S*. Newport or *Enterobacter* on the surface of cucumber after drying up to 24 h was below 5 log CFU/piece (data not shown). Selma et al. (74) demonstrated transference of *E. coli O157:H7* from cantaloupe surface to edible flesh portion during cutting. In their study, the edible flesh portion of cantaloupe inoculated with lower concentrations (3.3 log

CFU/rind) was negative for *E. coli* O157:H7 after cutting. Bacterial transfer from contaminated surface to edible flesh portion or utensils may not occur or may be below detection limits when the initial contamination levels are low, although the minimum amount of bacteria that cause cross-contamination may vary depending on the organisms.

Prior research has studied characteristics of organisms as an important factor that affects bacterial transfer. A study by Keskinen et al. (48) used six strains of *Listeria monocytogenes* to determine an effect of biofilm forming ability of *Listeria* on bacterial transfer rate from inoculated slicer blade to salami and roast turkey breast during slicing of meats. Their results indicated that a *Listeria* strain with strong biofilm forming ability transferred to a greater degree from blades to meat during slicing compared a weak biofilm forming strain. A study by Penteado et al. (50) examined cross-contamination from surface of mango to flesh portion during slicing with a knife inoculated with *Salmonella enterica* serovar enteritidis or *Listeria monocytogenes*. Their results indicated that bacterial transfer rates during slicing were dependent on the organisms inoculated on the knife blades.

Wang et al. (51) quantified the transfer of human norovirus and hepatitis A virus to peelers during peeling of contaminated carrots and celery. When the norovirus was inoculated on carrot and celery, the transfer rates to the peeler were 2.5 and 3.8 log PFU/utensil, respectively. The transfer rates of hepatitis A virus from the inoculated carrots and celery to the peeler were 2.9 and 3.8 log PFU/utensil, respectively. Their results showed that type of viruses or surface properties of fresh produce could affect the bacterial transfer between food materials and utensils during peeling.

A study conducted by Vadlamudi et al. (40) showed that food preparation procedures affect bacterial transfer from contaminated cantaloupe skin to edible portions. In their study, cutting cantaloupes after removing the contaminated rind effectively reduced the *Salmonella* transfer to edible portion compared to cutting cantaloupe prior to removing the rind. As fresh cucumbers are sliced or peeled before consumption, bacterial transfer rates from the surface of cucumbers to flesh portion could vary depending on preparation procedures like peeling or slicing.

Our data indicate that the percent transfer of *E. aerogenes* from the cucumber skin to the edible flesh portion during peeling decreased from 7.5 to 0.5% with extended drying time up to 24 h (Table 2.3). A similar result was obtained in a study by Keskinen et al. (48) where a significantly greater transfer of *Listeria* from inoculated slicer blades to salami and turkey was observed when the inoculated slicer blade was dried for 6 h before the slicing vs. 24 h drying. We speculate that extended drying times after inoculation with microorganisms not only has an impact on the changes in bacterial population on the surface of food products, but also may influence bacterial transfer rates during food preparation procedures such as peeling or slicing.

Previous studies conducted in our lab (79, 81, 82) and elsewhere (83) have successfully used nonpathogenic indicator microorganism, *E. aerogenes* B199A as a surrogate for *Salmonella* cross-contamination. In this study, the potential surrogate *E. aerogenes* B199A was evaluated for their transfer from cucumber skin to edible flesh portion or peeler during peeling of cucumber and the overall log percent transfer of *S.* Newport and *E. aerogenes* were not significantly different. These results suggest that *E.*

aerogenes B199A could be used as a conservative surrogate for *S*. Newport in cross-contamination studies on cucumbers.

The present study demonstrated that removing the peel portion of cucumber via peeling can reduce the contaminants on the surface of cucumber, but cross-contamination between the surface and the edible flesh portion and between the surface and the peeler occurred during peeling of cucumber. The log percent transfer of *E. aerogenes* B199A was not significantly different from that of *S*. Newport during the peeling of cucumber, which suggests that *E. aerogenes* B199A may be a useful surrogate for cross-contamination studies with respect to cucumber production and processing.

Table 2.1. Percent transfer of *S.* Newport from cucumber skin to peel, edible flesh portion, and peeler during peeling (n=20)

Transferred to	Mean	STDEV	Median	Maximum*	Minimum	Range
Peel	65.30	106.08	24.32	453.42	1.33	452.08
Flesh	0.12	0.16	0.04	0.60	0.00	0.60
Peeler	0.20	0.48	0.04	2.17	0.00	2.17

* Due to the variable nature of microbial counts in some cases apparent transfer exceeded 100%.

Table 2.2. Percent transfer of native microflora from cucumber skin to peel, edible flesh portion, and peeler during peeling (n=13)

Transferred to	Mean	STDEV	Median	Maximum*	Minimum	Range
Peel	200.63	302.18	103.57	1177.42	29.36	1148.06
Flesh	0.96	1.11	0.60	3.69	0.02	3.67
Peeler	1.39	1.36	0.56	3.69	0.04	3.65

* Due to the variable nature of microbial counts in some cases apparent transfer exceeded 100%.

Table 2.3. Percent transfer of *E. aerogenes* from cucumber skin to peel, edible flesh portion, and peeler during peeling according to different drying time after inoculation (n=4)

Drying time (h)	Transferred to	Mean	STDEV	Median	Maximum*	Minimum	Range
0	Peel	76.54	42.22	96.74	99.37	13.30	86.06
	Flesh	7.54	5.40	7.43	12.87	2.44	10.44
	Peeler	4.00	1.78	3.34	6.62	2.69	3.93
2	Peel	145.41	107.33	133.40	275.31	39.53	235.77
	Flesh	2.17	3.50	0.53	7.41	0.22	7.19
	Peeler	0.11	0.08	0.12	0.19	0.01	0.19
4	Peel	444.75	522.06	283.65	1167.54	44.18	1123.36
	Flesh	1.46	2.38	0.36	5.03	0.10	4.93
	Peeler	0.42	0.52	0.25	1.13	0.03	1.10
8	Peel	58.77	64.73	35.90	153.76	9.52	144.23
	Flesh	1.21	1.01	0.83	2.66	0.52	2.14
	Peeler	0.79	0.68	0.74	1.62	0.05	1.57
12	Peel	196.16	153.97	214.51	328.04	27.56	300.48
	Flesh	0.77	0.42	0.57	1.40	0.53	0.87
	Peeler	0.58	0.56	0.39	1.40	0.15	1.25
16	Peel	201.80	174.11	192.42	409.09	13.25	395.84
	Flesh	0.72	1.11	0.26	2.36	0.02	2.35
	Peeler	1.50	1.96	0.91	4.18	0.01	4.18
24	Peel	339.05	240.59	350.41	553.19	102.17	451.02
	Flesh	0.47	0.55	0.27	1.27	0.05	1.22
	Peeler	0.26	0.24	0.20	0.61	0.05	0.55

* Due to the variable nature of microbial counts in some cases apparent transfer exceeded 100%.

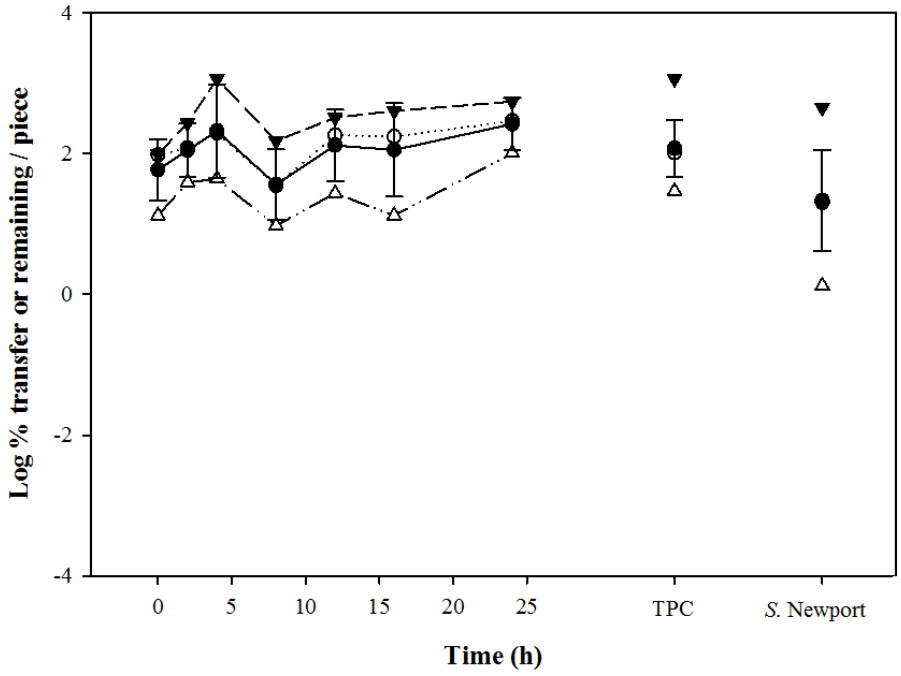

Figure 2.1. Log percent transfer or remaining of *S.* Newport, native microflora and *E. aerogenes* from cucumber skin to peel during peeling of cucumber according to different drying time after inoculation. Four different statistical parameters were used to characterize the log percent transfer rate or the level of microorganisms remained on the peel portion; mean (●), median (○), maximum (▼), and minimum (△).

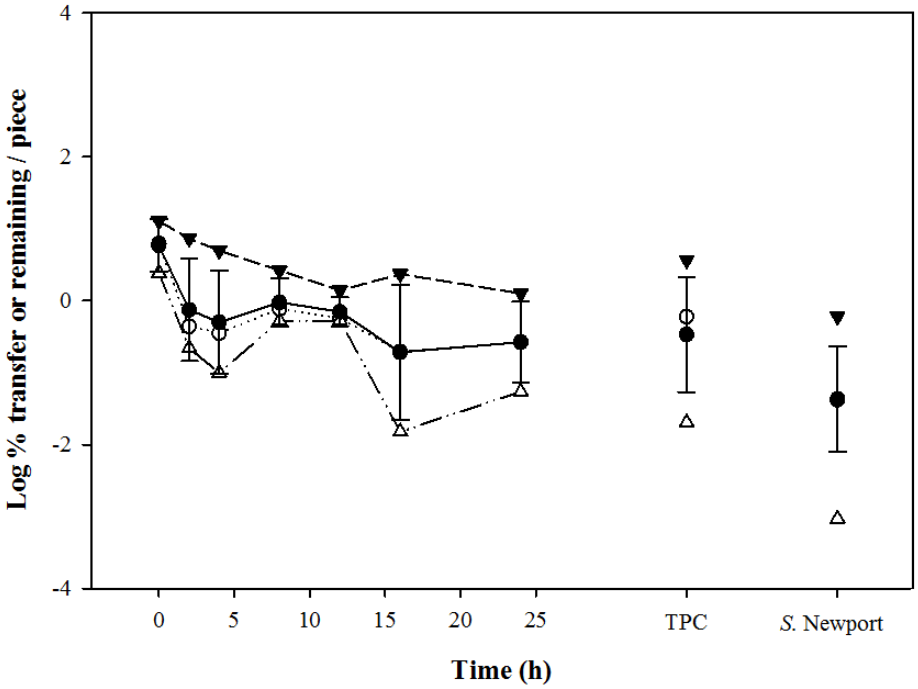

Figure 2.2. Log percent transfer or remaining of *S.* Newport, native microflora and *E. aerogenes* from cucumber skin to edible flesh portion during peeling of cucumber according to different drying time after inoculation. Four different statistical parameters were used to characterize the log percent transfer rate or the level of microorganisms remained on the peel portion; mean (●), median (○), maximum (▼), and minimum (△).

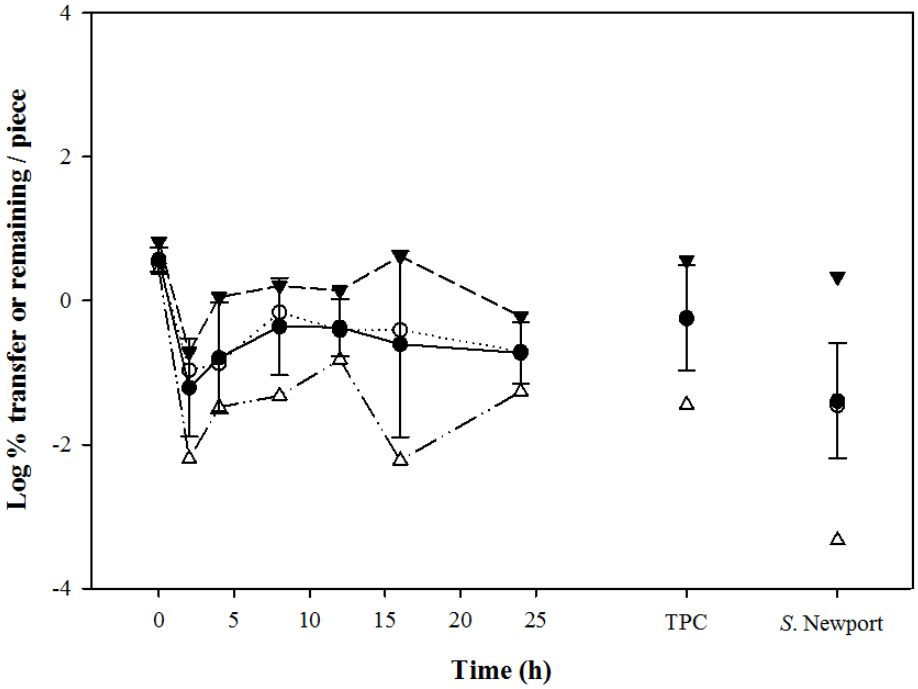

Figure 2.3. Log percent transfer or remaining of *S.* Newport, native microflora and *E. aerogenes* from cucumber skin to peeler during peeling of cucumber according to different drying time after inoculation. Four different statistical parameters were used to characterize the log percent transfer rate or the level of microorganisms remained on the peel portion; mean (●), median (○), maximum (▼), and minimum (△).

Chapter 3. The role of cell surface structure of *Salmonella* Newport on bacterial attachment and transfer in cucumbers

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3.1. Abstract

Fresh cucumbers have recently been recognized as a vehicle in foodborne disease outbreaks since several multistate outbreaks of salmonellosis directly linked to fresh cucumbers occurred in the United States. Little is known about microbial cell surface characteristics known to affect adhesion on subsequent bacterial cross-contamination and transfer. This study investigated the role of cell surface components of *S*. Newport on bacterial attachment and transfer in cucumbers. Wild type *S.* Newport and its transposon mutants, JDB 279 (*rpo*S::Tn*10*:lac:kan) and JDB 287 (Tn*10*:lac:kan insertion in the *agfD*/*agfB* intergenic region) were used to inoculate cucumbers. The attachment strength values (S_R) of *S.* Newport to the cucumber surface were calculated using standard methods. Bacterial transfer from cucumber skin to edible flesh portion and peeler during peeling was also quantified. The population of curli-positive *S.* Newport wild type attached to the cucumber surface was greater than curli-negative mutant strains (JDB 279 and JDB 287). The S_R value of *S.* Newport wild type on cucumber (0.998) was also higher than that of JDB 287 (0.996) and JDB 279 (0.995). During the peeling of cucumber, relatively higher log percent transfer of mutant strains to edible flesh portion and peeler was observed but the level of mutant strains remaining on the peel portion was also higher than that of wild type. This may be due to increased production of flagella-related genes and complex process of bacterial attachment and transfer involving many other factors. The results of this study suggest that curli and cellulose are required for enhancing *Salmonella* attachment to the surface of cucumbers but there may be more important mechanisms and factors for *Salmonella* transfer in cucumbers.

3.2. Introduction

Salmonella enterica is the most common cause of bacterial foodborne illnesses in humans and estimated to cause 1.2 million illnesses each year in the United States with more than 23,000 hospitalization and 450 deaths (17, 24). Although animal-derived foods such as poultry, meat, eggs and dairy products are considered the most commonly implicated sources in *Salmonella* outbreaks (26), large multistate outbreaks of salmonellosis have been attributed to fresh produce and have increased over the last two decades (11, 27). There have been at least four reported multistate outbreaks of salmonellosis directly linked to fresh cucumbers in the United States since 2013. Imported cucumbers from Mexico have been associated with *Salmonella* Saintpaul infections in 2013 and *Salmonella* Poona outbreak in 2015 (33, 36). *Salmonella* Newport infections implicating fresh cucumbers were the largest multistate foodborne disease outbreak in the United States in 2014 (35). According to epidemiological data, it was also the first multistate outbreak of *Salmonella* Newport linked to fresh produce items grown in Delmarva region other than tomato (35). Most recently, Persian cucumbers were implicated in *Salmonella* Oslo infections in 2016 (37).

Successful attachment of bacteria to plant surface is the first step in contamination of fresh produce. Bacterial cell surface components such as curli, cellulose, flagella, and extracellular polysaccharides are important for bacterial attachment and colonization on fresh produce (55, 57, 62, 84, 85). A study by Tan et al. (61) showed curli, cellulose, and flagella were all involved in *Salmonella* attachment to plant cell wall models. Lapidot and Yaron (62) also reported that *Salmonella* transfer and attachment to parsley are dependent on extracellular components of the biofilm such as cellulose and curli. Those components

are regulated by specific genes including *agfD*, *agfB,* and *rpoS* in *S. enterica* (57). *AgfD*, a transcriptional regulator of the LuxR superfamily, regulates thin aggregative fimbriae (Tafi or curli in Escherichia coli), cellulose and capsule production (59, 86). *RpoS*, the general stress response regulator sigma factor, plays an important role in the regulation of *agf* (Tapi) operon and other genes, as well as in biofilm formation (87-89). *AgfB*, the surface-exposed curli nucleator, was also reported to be important for *Salmonella* attachment to plant tissues (57).

Although *Salmonella* attachment and transfer to leafy vegetables has been investigated (52, 55, 63, 90), no studies reporting an interaction between cell surface components of *S*. Newport and cucumbers have been reported. This study was undertaken to investigate how bacterial cell surface structures play a role in attachment and transfer of *S*. Newport on cucumbers.

3.3. Materials and Methods

3.3.1. Bacterial strains and inoculum preparation

Salmonella enterica Newport 96E01152C-TX and its transposon mutants JDB 279 and JDB287 are described in Table 3.1. *S*. Newport wild type and its mutants were grown in tryptic soy broth (TSB; Difco, BD, Sparks, MD) and TSB containing 50 μg/mL of kanamycin (Sigma-Aldrich, St. Louis, MO), respectively. After 24 h of incubation at 37 °C, cells were harvested by centrifugation (Allegra 21R centrifuge; Beckman Coulter, Brea, CA) at 5,000 × g for 10 min and then washed two times in 10 mL of phosphate

buffer saline (PBS; 0.1M, pH 7.2) (Fisher Scientific, Pittsburgh, PA). The cell pellets were suspended in PBS after final wash, yielding a solution of ~10^9 CFU/mL.

3.3.2. Curli and cellulose expression

The Congo red binding assay was used to determine curli expression. *S*. Newport wild type and its mutants grown in Luria-Bertani (LB; Difco, BD) broth at 37 °C for 24 h was streaked onto Congo red indicator (CRI) plates that consisted of 10 g/L of casamino acid (Bacto, Becton Dickinson, Sparks, MD), 1 g/L of yeast extract (Bacto, Becton Dickins), 15 g/L of Bacto agar (Difco, BD), 20 mg/L of Congo red (MP Biomedicals, Solon, OH), and 10 mg/L of Coomassie brilliant blue (Bio-Rad Laboratories, Hercules, CA). The colonies were examined as an indicator of curli production following the incubation at 28 °C for 48 h. Curli-producing bacteria form red colonies on CRI plates (from binding of the dye with curli), while nonproducing cells form colorless colonies.

Cellulose production was further confirmed by streaking isolates onto LB plates containing 50 μg/mL of calcofluor white (fluorescent brightener 28, Sigma-Aldrich). Cellulose-producing bacteria form colonies that fluoresce under UV light.

3.3.3. Flagella staining

Flagella production was carried out with RYU flagella stain (Remel, Thermo Fisher Scientific, Waltham, MA) according to the recommendations of the manufacturer. Cells were then examined by phase-contrast microscopy.

3.3.4. Congo red negative staining of capsular polysaccharides

Capsular polysaccharides (CPS) production by *S*. Newport wild type and its mutants were detected by a negative staining based on Maneval's stain. Overnight culture

was mixed with one drop of 1% Congo red solution on a clean microscope slide and allowed to air dry for 5 min. The smears were counterstained with Maneval's stain solution (Carolina Biological Supply Company, Burlington, NC) for 1 min and gently washed with distilled water. Cells were examined by light microscope.

3.3.5. Attachment assay

Fresh cucumber was immersed in bacterial suspension (~10^9 CFU/mL) and gently agitated for 30 s to ensure even inoculation. The inoculated cucumber was dried in the biosafety cabinet for 24 h and washed with sterile distilled water for 1 min to remove loosely attached bacterial cells. In order to enumerate strongly attached bacteria, cucumber was placed into a Lab blender bag (Fisher Scientific) with 30 mL of PBS, and then massaged by hands for 30 s. The suspensions were serially diluted (1:10) in PBS, and 0.1 mL of each dilution was plated onto Hektoen Enteric agar (HE; Difco, BD) and HE with 50 μg/mL of kanamycin to enumerate *S*. Newport and its mutants, respectively. The plates were incubated at 37 °C for 24 h and bacterial colonies were counted following the incubation. The bacterial population was expressed in terms of attachment strength (S_R). The S_R values represent the percentage of the total population of bacteria strongly attached to the surface of cucumber and were calculated as described by Dickson and Koohmaraie (91) using the following equation:

$$S_R = \left(\frac{\text{Strongly attached bacteria}}{\text{Strongly attached bacteria + loosely attached bacteria}} \right) \quad (1)$$

3.3.6. Cross-contamination during peeling

To quantify the transfer of *S*. Newport wild type and its mutant strains from cucumber skin to edible flesh portion or peeler during peeling, fresh cucumber was dip-

inoculated with 10 mL of designated strain (~10^9 CFU/mL) in a zip top plastic bag by massaging for 30 s and dried for 24 h in the biosafety cabinet with fan running. Half of each inoculated cucumber was hand peeled using a sterilized peeler. After peeling, unpeeled half cucumber, peeled half (edible flesh portion), removed peel and contaminated peeler were enumerated as described above. The bacterial population was expressed in log CFU/piece for each portion of cucumber and peeler.

2.3.7. Data analysis

Individual transfer rate for each experiment was calculated using the following equation:

$$\text{Transfer rate (\%)} = \left(\frac{\text{CFU measured on peel, flesh portion, or peeler}}{\text{CFU measured on inoculated half cucumber}}\right) \times 100 \quad (2)$$

Transfer rates between samples were logarithmically transformed in order to create normally distributed data as per Schaffner (80). All results were analyzed using Tukey's HSD test (SAS University Edition; SAS Institute Inc., Cary, NC, USA) to determine statistically significant differences in bacterial transfer during peeling.

3.4. Results and Discussion

3.4.1. Production of cell surface components by *S.* Newport

Bacterial attachment, colonization, and biofilm formation on plant tissues are affected by bacterial cell surface components such as curli, cellulose, flagella, and extracellular polysaccharides (57, 61, 62, 85). The production of curli and cellulose are regulated by *agfB*, the surface-exposed curli nucleator, and *agfD*, a transcriptional

regulator belonging to the LuxR supermaily (57, 59). The general stress response regulator sigma factor, *rpoS* is required for transcription of *agf* oeprons in *Salmonella*, as well as biofilm formation and bacterial adhesions (57, 88, 92). *S*. Newport wild type and its transposon mutants, JDB 287 and JDB 279 with insertions in the intergenic region between *agfB* and *agfD*, and *rpoS* were used in this study to determine the role of cell surface components on bacterial transfer and attachment in cucumbers. *S*. Newport wild type showed strong curli and cellulose expression, whereas JDB 279 and JDB 287 produced neither curli nor cellulose on CRI plate and LB plates supplemented with calcofluor white, respectively (Figure 3.1). However, both *S*. Newport wild type and mutant strains showed production of flagella that has been known to be involved in bacterial adhesion to fresh produce (Figure 3.2) (55, 93). We also determined CPS production of *S*. Newport wild type and its mutants by Congo red negative staining. As shown in Figure 3.3, the production of CPS in both wild type and mutants was confirmed, showing clear zones surrounding the cells. CPS is tightly linked to the cell surface of bacteria and known to protect bacteria cells from adverse environmental stresses such as desiccation, to promote bacterial adhesion, and to facilitate the biofilm formation and colonization (94).

3.4.2. Effect of cell surface structures on *Salmonella* transfer and attachment to cucumbers

The population of *S*. Newport wild type and its mutant strains, JDB 279 and JDB 287 attached to the surface of cucumber ranged from 5.08 to 5.54 log CFU/cucumber. The highest S_R value was that of *S*. Newport wild type (0.998), followed by JDB 279

(0.996) and JDB 287 (0.995). Higher S_R value means stronger adhesion of bacteria to the surface of cucumber as indicated by relative inability of washing treatment to remove the bacterial cells from cucumber. *S*. Newport wild type showed stronger attachment and higher S_R value on the surface of cucumber compared to the mutant strains but no significant differences was observed. However, these mutant strains, JDB 279 and JDB 287 were reduced in attachment to alfalfa sprouts compared to the wild type in a study conducted by Barak et al. (57). Differences in their findings and our results presented here may be due to the use of different produce type or methodology to evaluate bacterial attachment to fresh produce. Patel et al. (52) reported that *Salmonella* attachment to lettuce and cabbage are different and that stronger attachment of *Salmonella* serovars was found on lettuce than on cabbage, regardless of the condition (cut or intact) of the produce surface.

During the peeling of cucumber, the majority of *S*. Newport and its mutants, JDB 279 and JDB 287 remained on the peel portion of cucumber, although bacterial transfer between the cucumber skin and edible flesh portion and between the cucumber skin and peeler occurred (Figure 3.4). *S*. Newport wild type remaining on the peel portion of cucumber was lower than mutant strains, JDB 287 and JDB 279. The highest log percent transfer to the edible flesh portion and peeler was observed when cucumber was inoculated with JDB 287, followed by JDB 279 and wild type. These results indicate that deficiencies in curli and cellulose may not affect the transfer of *S*. Newport during the peeling of cucumber. However, other study revealed that extracellular components of biofilm such as curli and cellulose are important in bacterial attachment, transfer and survival, and transfer of S. Typhimurium deficient in curli and cellulose to parsley was

lower than that of wild type (62). The discrepancies in the roles of curli and cellulose in bacterial transfer may be due to the differences in bacterial species or serotypes, produce type, or methodology to evaluate bacterial transfer to fresh produce.

In addition to the importance of curli and cellulose for strengthening the bacterial attachment and transfer to fresh produce (57, 62, 85, 95, 96), the importance of flagella has been supported by results of previous studies in which flagella-deficient *S. enterica* exhibited reduced attachment and internalization on basil and lettuce leaves (55, 93). Moreover, a study conducted by Tan et al. (61) demonstrated that flagella played a more important role than cellulose and curli in attachment of *S.* Typhimurium to plant cell wall models, although both curli and cellulose also aided in attachment. However, the main function of flagella is to confer motility and chemotaxis and to stimulate the host immune response, not to mediate adhesion (97). According to Ogasawara et al. (98), *fliE* and *fliEFGH* operons for flagella formation are directly repressed by *csgD* that is the key regulator for curli production in *E. coli*. In their study, *csgD* mutant strains deficient in curli and cellulose increased *fliE* promoter activity compared to wild type. Although the role of flagella on bacterial transfer to fresh produce needs to be further elucidated, we carefully speculate that *S.* Newport strains used in our study, JDB 279 and JDB 287 deficient in curli and cellulose may have increased production of flagella-related genes that could help mutant strains to transfer better to the edible flesh portion and peeler during peeling of cucumber, showing higher log percent transfer compared to the wild type (Figure 3.4).

CPS in the outermost layer of cell plays an important role in bacterial colonization and biofilm formation by facilitating cell adhesion to host cells (94, 99, 100). However,

the attachment of *S*. Typhimurium DT104 was not mediated by CPS and CPS alone was not sufficient for bacterial attachment, although it was a biofilm matrix component along with curli and cellulose (101). Since very little is known about the involvement of CPS in bacterial attachment and transfer on fresh produce, additional studies involving foodborne pathogens that lack CPS would further clarify the role of CPS on bacterial behavior on fresh produce.

The results of our study demonstrate that curli and cellulose-producing *S*. Newport wild type showed higher attachment to the surface of cucumber compared to non-producing mutant strains. However, curli and cellulose are not believed to be important cell surface components in the transfer of *S*. Newport to cucumbers. In addition to curli and cellulose, bacterial cell surface components including flagella and CPS are all involved in complex interaction of *S*. Newport with fresh produce and there may be more important mechanisms for *Salmonella* attachment and transfer to cucumbers.

Table 3.1. *S. enterica* Newport strains used in this study

Identification	Description	Reference
96E01152C-TX	Isolated from alfalfa sprouts	(102)
JDB 279	*rpoS*::Tn*10*:lac:kan	(57)
JDB 287	Tn*10*:lac:kan insertion in the *agfD/agfB* intergenic region	(57)

Table 3.2. Adhesion of *S*. Newport to cucumber (n=5)

Bacterium	Log CFU/cucumber	S_R*
S. Newport 96E01152C-TX	5.54 ± 0.63	0.998 ± 0.001
S. Newport JDB 279	5.23 ± 0.70	0.996 ± 0.003
S. Newport JDB 287	5.08 ± 0.40	0.995 ±0.004

* The S_R value represents the strength of attachment.

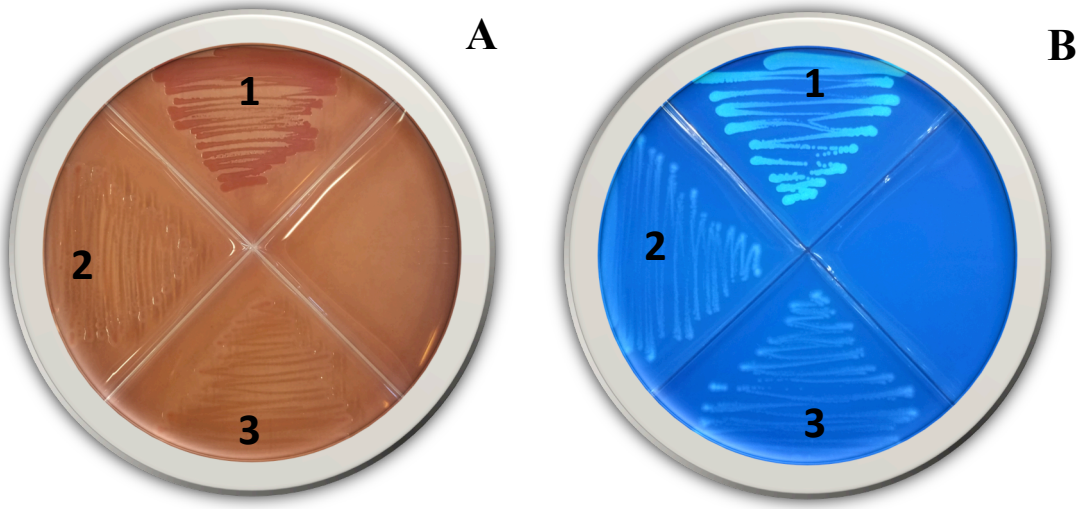

Figure 3.1. Phenotypic characterization of *S.* Newport wild type (section 1), JDB 287 (section 2) and JDB 279 (section 3) grown on Congo red indicator plates (A) and LB containing calcofluor (B) at 28 °C for 48 h.

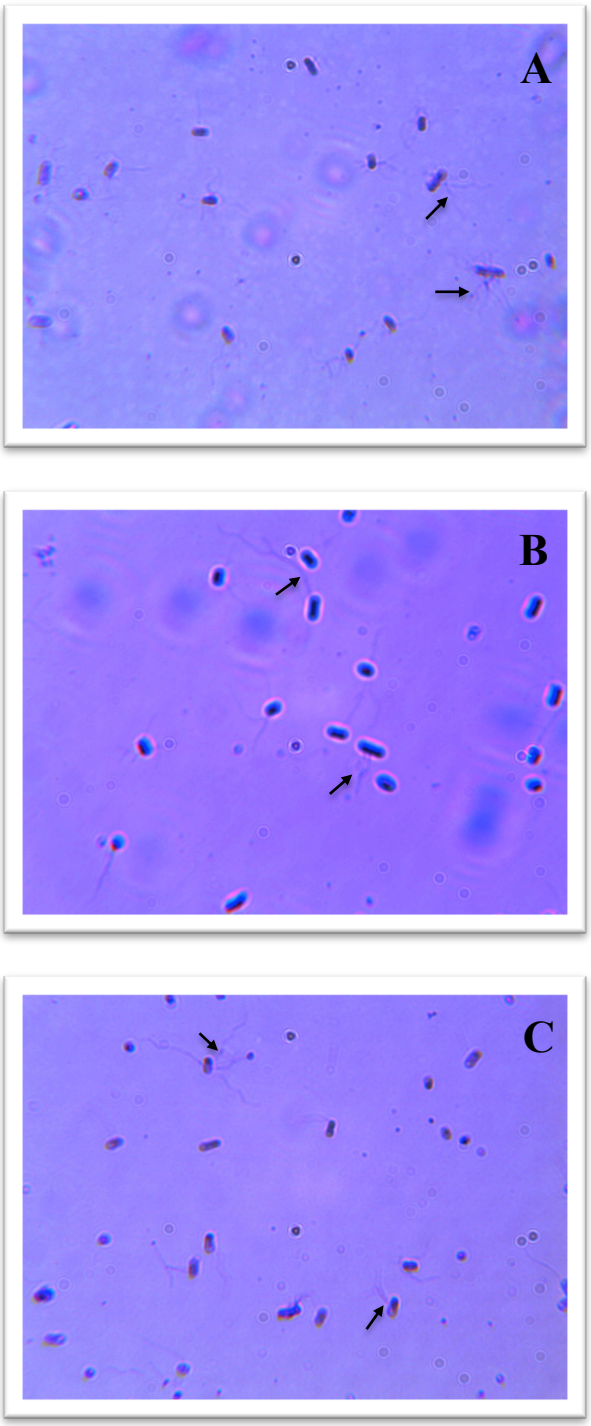

Figure 3.2. Flagella of *S.* Newport wild type (A), JDB 287 (B) and JDB 279 (C) stained with RYU flagella stain. Flagella are denoted by arrows.

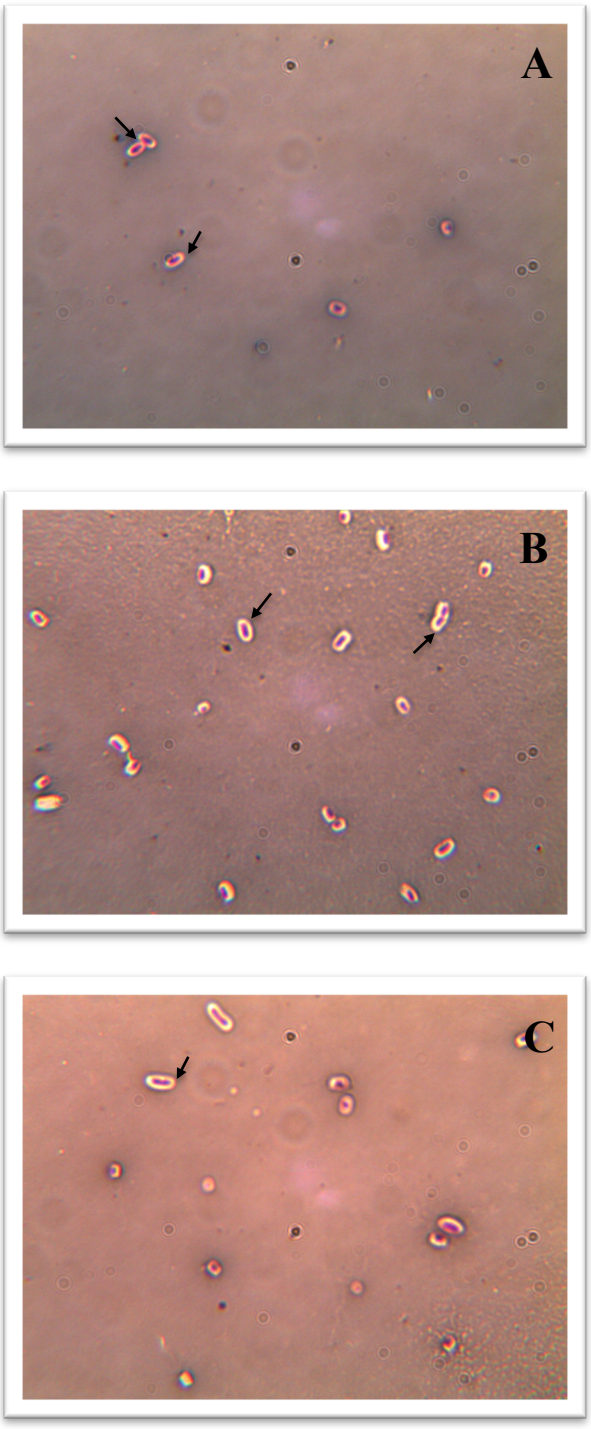

Figure 3.3. Negative staining of *S.* Newport wild type (A), JDB 287 (B) and JDB 279 (C). Clear halos suggestive of the presence of capsular polysaccharides are denoted by arrows.

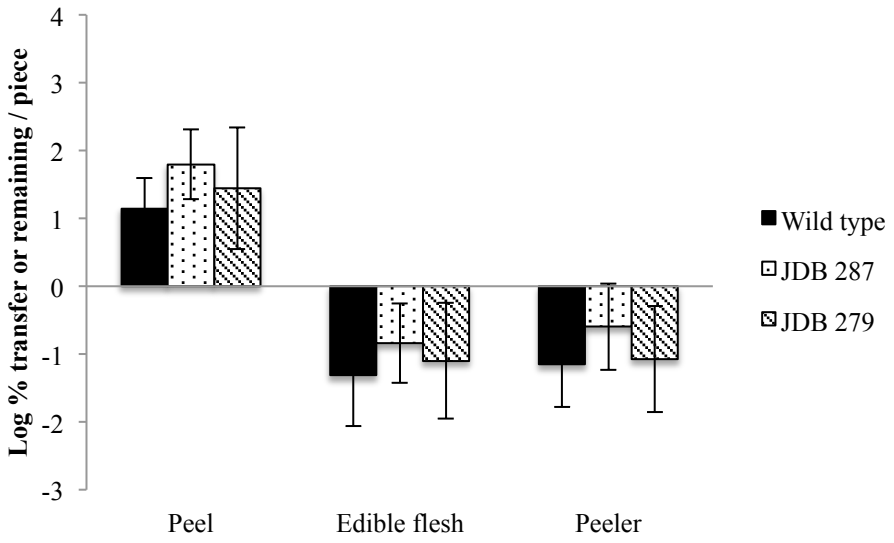

Figure 3.4. Log percent transfer or remaining of *S*. Newport wild type, JDB 279 and JDB 287 from cucumber skin to peel, edible flesh portion, and peeler during peeling (n=20).

Chapter 4. Survival and transfer of *Salmonella* on fresh cucumbers during waxing

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4.1. Abstract

Cucumbers can become contaminated at numerous points during pre- and post-harvesting and have been implicated in several multistate outbreaks of salmonellosis. Many cucumbers found in retail markets have been waxed to make them visually appealing and retard moisture loss. This waxing may affect bacterial survival. This study assessed the survival of *Salmonella* on waxed and un-waxed cucumbers and the potential for *Salmonella* cross-contamination during the waxing process. Fresh un-waxed cucumbers were spot-inoculated with a four-strain cocktail of *Salmonella enterica* before or after wax treatment. Three different wax coatings (mineral oil, vegetable oil, or petroleum based wax) were applied manually, using polyethylene brushes. Samples were enumerated following storage for 1, 2, 3, and 7 days. *Salmonella* concentration was expressed in CFU/cucumber and *Salmonella* transfer from inoculated cucumber to brush or to un-inoculated cucumber was quantified. Higher *Salmonella* concentrations were observed on waxed cucumber during the first 72 h but the final concentration on un-waxed cucumber (2.16 log CFU/cucumber) was higher than on waxed cucumber (1.48 log CFU/cucumber) after 168 h. *Salmonella* cells did transfer from contaminated un-waxed cucumbers to brushes used for waxing and then to un-inoculated cucumbers during waxing. Significantly higher log percent transfer to brushes was observed when cucumbers were waxed with vegetable oil (0.71 log percent, $p=0.00441$) vs. mineral oil (0.06 log percent) or petroleum (0.05 log percent). Transfer to un-inoculated cucumbers via brushes was also quantified (0.18 to 0.35 log percent transfer). *Salmonella* remaining on the contaminated cucumbers after waxing survived up to 7 days, and *Salmonella* survived better on the cucumbers with a petroleum-based wax. This study shows

significant bacterial transfer during waxing. Survival and transfer of *Salmonella* were affected by the type of wax coatings. These findings should be useful in managing *Salmonella* contamination in cucumbers during post-harvest handling.

4.2. Introduction

Fresh cucumbers have been associated with several multistate outbreaks of salmonellosis in the United States in recent years (33, 34, 36, 37). The recent U.S. Food and Drug Administration microbiological surveillance sampling program reported an incidence of 1.8% positive for *Salmonella* in 1,558 samples of cucumbers, where contaminated cucumbers were either produced domestically (39.3%) in the United States or imported (60.7%) from other countries (103). Cucumbers are typically grown on the ground and can become contaminated by soil, insects, animals, equipment or humans during pre- and post-harvesting, which has been implicated in microbial cross-contamination on fresh produce (8, 39, 43, 45, 49, 104). Research have pointed to importance of cleaning and sanitation of processing lines as critical to prevent cross-contamination to other products passing through the lines (7, 10). Mechanical brushes commonly used in produce processing for washing, sanitizing, or waxing could also play an important role in cross-contamination in packing and processing facilities (105, 106), but very little is known about bacterial contamination or transfer that might occur during produce waxing.

Most unwrapped cucumbers in retail markets have likely been waxed to retard moisture loss, maintain textural quality and external appearance, and to improve shelf life and marketability of cucumbers during handling and marketing (107, 108). Edible films and coatings used as a preservation for cucumber and other fresh produce are composed of lipids, polysaccharides, proteins or resins, and may be incorporated with antimicrobial agents, minerals, vitamins, colors or flavors to enhance their functions (107, 109-111). Wax coating materials are generally selected based on their gas permeability, water

solubility and sensory properties to enhance or replace natural wax layer for a specific product (109, 112, 113). Selection of proper coating materials and their beneficial aspects or adverse effects on fresh produce have been studied over the last two decades (107, 108, 110-112, 114). The impact of wax coating materials and waxing process in bacterial behavior in fresh produce including cucumbers is still unclear. This study was undertaken to determine the effect of wax coatings on *Salmonella* survival on the surface of cucumber during storage. *Salmonella* cross-contamination during cucumber waxing was also quantified. Transfer rates were determined between inoculated cucumber and brush and between contaminated brush and un-inoculated cucumber.

4.3 Materials and Methods

4.3.1. Bacterial strains and inoculum preparation

Four strains of *Salmonella enterica* provided by Dr. Joshua Gurtler (Eastern Region Research Center, USDA, Wyndmoor, PA) were used for all experiments: *Salmonella* Newport H1275 (sprout outbreak), Stanley H0558 (sprout outbreak), Montevideo G4639 (tomato outbreak), and Saintpaul 02-517-1 (cantaloupe outbreak). *Salmonella* strains were made resistant to 100 μg/mL of nalidixic acid (Fisher Scientific, Pittsburgh, PA) by serial exposure to increasing (1:2) concentrations of nalidixic acid in tryptic soy broth (TSB; Difco Becton Dickinson & Co., Sparks, MD) incubated at 37 °C for 24 h. A frozen culture of each strain was streaked onto tryptic soy agar (TSA; Difco, BD) supplemented with 50 μg/mL of nalidixic acid, and incubated at 37 °C for 24 h prior to each experiment. An isolated colony was transferred to 10 mL of TSB supplemented

with 50 μg/mL of nalidixic acid, and incubated at 37 °C for 24 h. The culture was centrifuged at 5,000 × g for 10 min (Allegra 21R centrifuge; Beckman Coulter, Brea, CA) and then washed twice with 0.1M, pH 7.2 phosphate buffer saline (Fisher Scientific). The washed cell pellets were suspended in phosphate buffer saline (PBS) after final wash and then combined to produce a cocktail of four strains for inoculating cucumber samples, yielding approximately 8.5 log CFU/mL of inoculum.

4.3.2. Inoculation of cucumber samples for survival study

Fresh un-waxed and waxed cucumbers were purchased from a local farm market (Somerset, NJ) and stored at 5 °C until use. The inoculum (50 μL) was spot-inoculated onto the surface of cucumber and allowed to dry for 2 h in the biosafety cabinet with fan running. Cucumbers were analyzed for a population of *Salmonella* after storage for 0, 1, 3 and 7 days at 7 and 21 °C.

4.3.3. Waxes

Samples of commercial wax coatings were obtained from Decco (Monrovia, CA) and Pace International (Wapato, WA). The detailed formulations of commercial wax coatings are proprietary but available information is shown in Table 4.1. All wax coatings were used at full strength as recommended by manufacturers.

4.3.4. Cross-contamination during waxing

Fresh un-waxed cucumber was washed with tap water and then dried for 2 h with the biosafety cabinet fan running. Prior to waxing, the cucumber was spot-inoculated with 50 μL of *Salmonella* suspension (~8.5 log CFU/mL) and dried for 2 h. Twenty microliters of wax coating were applied manually onto the surface of inoculated

cucumber and distributed evenly over using polyethylene brush. Cucumbers were sampled for *Salmonella* after storage for 0, 1, 3 and 7 days at 21 °C. Brushes were also collected to quantify *Salmonella* transfer from inoculated cucumber to brush or reused to wax un-inoculated cucumber to quantify *Salmonella* transfer between contaminated brush and uninoculated cucumber.

4.3.5. *Salmonella* recovery

Twenty and two milliliters of PBS were added to a Lab blender bag (Fisher Scientific) containing cucumber and brush, respectively prior to hand massaging for 30 s. Samples were serially diluted in PBS, and 0.1 mL of each dilution was plated onto Hektoen Enteric agar (HE; Difco, BD) with 50 μg/mL of nalidixic acid. The plates were incubated at 37 °C for 24 h, bacterial colonies were counted and log CFU/cucumber or brush were calculated.

4.3.6. Data analysis

Individual transfer rate for each experiment was calculated using the following equation:

$$\text{Transfer rate (\%)} = \left(\frac{\text{CFU measured on brush or cucumber}}{\text{CFU measured on inoculated cucumber or contaminated brush}}\right) \times 100 \quad (1)$$

Transfer rates between samples were logarithmically transformed in order to create normally distributed data as per Schaffner (80). All results were analyzed using Tukey's honest significant difference (HSD) test (SAS University Edition; SAS Institute Inc., Cary, NC, USA) to determine statistically significant differences in bacterial transfer during cucumber waxing.

4.4. Results and Discussion

4.4.1. *Salmonella* survival on waxed and un-waxed cucumbers

Salmonella population recovered from waxed and un-waxed cucumbers during storage at 21 °C is shown in Figure 4.1. Higher concentration of *Salmonella* was observed on the surface of waxed cucumbers during the first 3 days compared to that on un-waxed cucumbers. The same trend was found in *Salmonella* population on the waxed and un-waxed cucumbers stored at 7 °C (Figure 4.2) and a significant decrease in *Salmonella* population was observed at both 7 and 21 °C over the storage period. Although waxing treatment on the surface of cucumber did not significantly influence *Salmonella* population recovered from waxed or un-waxed cucumbers, the final concentration of *Salmonella* on un-waxed cucumber was higher than on waxed cucumber at the end of storage.

A study conducted by Pao (115) demonstrated that applying wax coating reduced microbial contaminants on the surface of orange fruit. Wax coatings on the surface of fresh produce extend shelf life and minimize quality changes by reducing moisture loss, respiration rate, wilting, and deterioration (107, 108, 116). El Ghaouth et al. (108) demonstrated that chitosan coating reduced decay in cucumber and bell pepper caused by *Botrytis cinerea*, species of *Erwinia* and *Alternaria*. Presence of post-harvest pathogens such as *Botrytis cinerea* or *Penicillium* spp. could alter the pH of plant tissues and influence growth and survival of human pathogens (117). Populations of *E. coli* O157:H7 and *L. monocytogenes* increased on un-waxed apple surface and sliced apple decayed by

Glomerella cingulata (118, 119). On the other hand, a study revealed that growth and survival of *E. coli* O157:H7 and *S*. Muenchen on waxed and unwaxed apples were influenced by storage temperature (120). Significant reduction in population of *E. coli* O157:H7 and *S*. Muenchen on waxed apples stored at 2 °C was found compared to un-waxed apples, but bacterial population was higher on waxed apples during 6 weeks storage at 21 °C. Along with storage temperature, relative humidity, type of wax coatings, concentration of wax are all important for physical and microbial quality of fresh produce (107, 108, 113, 114, 116, 121).

4.4.2. *Salmonella* survival on cucumber after waxing treatment

Salmonella remaining on inoculated cucumbers after applying wax coatings using polyethylene brushes survived up to 7 days (Figure 4.3). At the initial stage of storage, the highest *Salmonella* population was observed on the surface of cucumber waxed with sample C (3.75 log CFU/cucumber), followed by sample B (3.20 log CFU/cucumber) and sample A (3.19 log CFU/cucumber). *Salmonella* survived better on the cucumber with petroleum-based wax (sample C) during the first 3 days compared to the other wax coatings, although all *Salmonella* population decreased over the storage period no matter what wax coating was applied.

Selecting a proper wax coating is crucial as each produce has different skin resistance, gas diffusion, respiration and transpiration rate (110, 112). In a study that determined the effect of edible coatings on green bell peppers, mineral oil-based wax coating was identified as most desirable to reduce moisture loss and to maintain fruit firmness and freshness rather than cellulose-based and protein-based wax coatings (114).

Dou and Ismail (116) have also pointed to the importance of wax components and concentrations. Commercial shellac wax coatings induced higher pitting incidence and increased decay on citrus fruits than diluted shellac and resin solutions and non-treated fruits. It has been suggested that shellac-based wax coating forms diffusion barriers that reduce respiration rate resulting in low internal oxygen concentration and high carbon dioxide and ethanol concentration, which accelerates deterioration in fresh produce (121).

4.4.3. Cross-contamination during cucumber waxing

Salmonella transfer from inoculated cucumbers to brushes occurred during cucumber waxing using no matter what wax coating was applied onto the surface of cucumbers as shown in Figure 4.4. Significantly higher log percent transfer to brush was observed from cucumber waxed with sample B (0.71 log percent transfer, $p=0.004$) compared to sample A (0.63 log percent transfer) and sample C (0.05 log percent transfer). Subsequent *Salmonella* transfer from contaminated brushes to un-inoculated cucumbers was also observed during cucumber waxing (Figure 4.5). Although not significantly different, *Salmonella* transfer to un-inoculated cucumber was highest with sample C (0.35 log percent transfer), followed by sample B (0.20 log percent transfer) and sample A (0.18 log percent transfer). Salmonella transfer to the surface of un-inoculated cucumber was greatest when cucumber was treated with petroleum-based wax coating (Figure 4.3, sample C) using polyethylene brushes, just as *Salmonella* survival on the surface of cucumber was greatest after this waxing treatment. These data suggest that petroleum-based wax coating may enhance the survival and transfer of *Salmonella* on the surface of cucumber compared to mineral oil-based and vegetable oil-based wax coatings.

Bacterial cross-contaminations between fresh produce and brush rollers during packinghouse process have been reported (105, 106, 122). Wang et al. (122) found that brush rollers in commercial packinghouse were heavily contaminated with mesophilic aerobic bacteria, yeast, and mold before and after tomato waxing. Pao et al (105) demonstrated cross-contamination with *Salmonella* from polyethylene roller brushes to tomato surface, and brushing tomatoes on inoculated roller brushes introduced *Salmonella* onto the fruit surface at the level of 5.7 log CFU/cm^2. Brush bristles used in produce waxing could harbor pathogenic bacteria, leading further cross-contamination as well as entrapping bacterial cells in protected sites on the epidermis of fresh produce by rubbing or brushing during waxing process (120, 123). Kenney et al. (123) showed that *E. coli* O157:H7 cells become enmeshed in natural wax platelets and lenticels on the surface of apples by rubbing. Bacterial cells attached in protected sites on the surface of fresh produce are difficult to inactivate or remove during washing or sanitizing (75, 123).

The results of this study demonstrate that *Salmonella* are able to survive on the surface of cucumbers stored at 7 or 21 °C for up to 7 days no matter whether the cucumber was waxed or un-waxed. *Salmonella* could transfer from inoculated cucumbers to brushes during cucumber waxing, and subsequent bacterial transfer to un-inoculated cucumber from contaminated brush was observed. Our study also shows that survival and transfer of *Salmonella* vary depending on the type of wax coatings. These findings should be useful in managing *Salmonella* contamination in cucumbers during post-harvest handling and further research regarding the commercial scale of cucumber processing should provide deep insight into good agricultural and best management practices to enhance microbial safety of fresh cucumbers.

Table 4.1. Commercial wax coatings used in this study

Wax code	Formulation base	Produce	Feature
A	Mineral oil, petrolatum and paraffin	Tomatoes, cucumbers, peppers, zucchini, and other smooth-skin commodities	Enhances appearance and reduces weight loss due to evaporation
B	Food grade vegetable and/or shellac	Nectarines, plums, peaches, and other round fruits and vegetables	Vegetable oil-based protective coating for stone fruit and vegetables
C	Petroleum	Stone fruit and other soft fruits and vegetables	Protective coating for fruits and vegetables

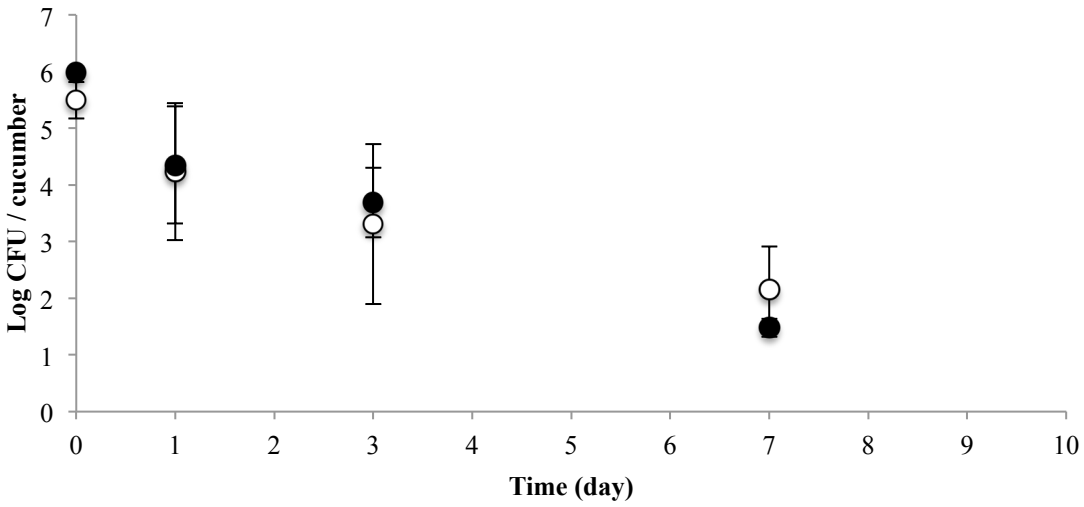

Figure 4.1. Survival of *Salmonella* on waxed (●) and un-waxed (○) cucumbers during storage at 21 °C. Data represent means ± standard errors (n=3).

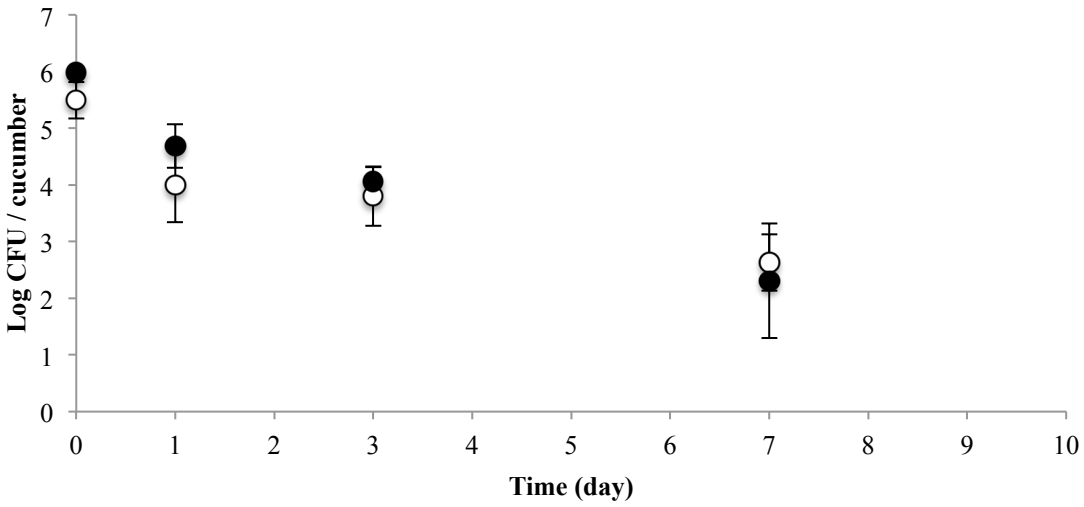

Figure 4.2. Survival of *Salmonella* on waxed (●) and un-waxed (○) cucumbers during storage at 7 °C. Data represent means ± standard errors (n=3).

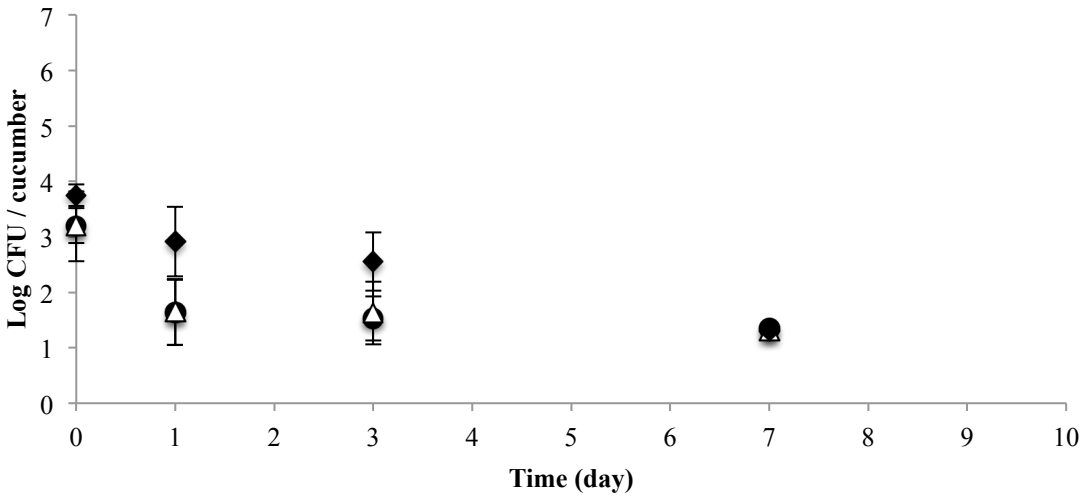

Figure 4.3. Survival of *Salmonella* remaining on the contaminated cucumbers after waxing with sample A (mineral oil-based, ●), sample B (vegetable oil-based, △), and sample C (petroleum-based, ◆). Data represent means ± standard errors (n=3).

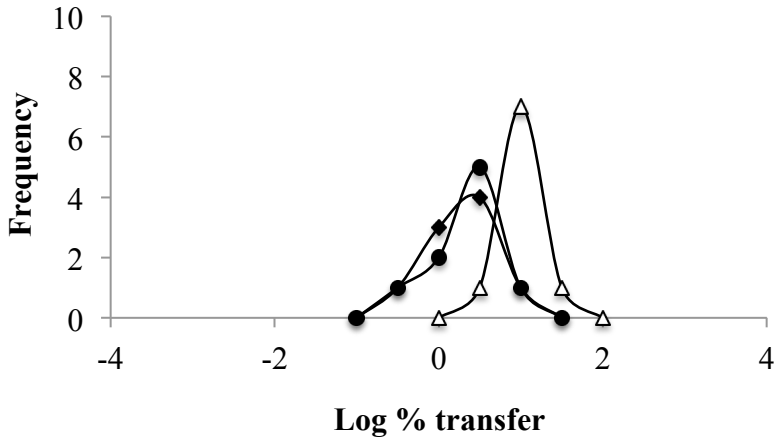

Figure 4.4. Frequency distributions of log percent transfer of *Salmonella* from contaminated cucumber surface to brush during cucumber waxing with sample A (mineral oil-based, ●), sample B (vegetable oil-based, △), and sample C (petroleum-based, ◆). Frequency is the number of times a particular log percent transfer occurred within a target data set (n=9).

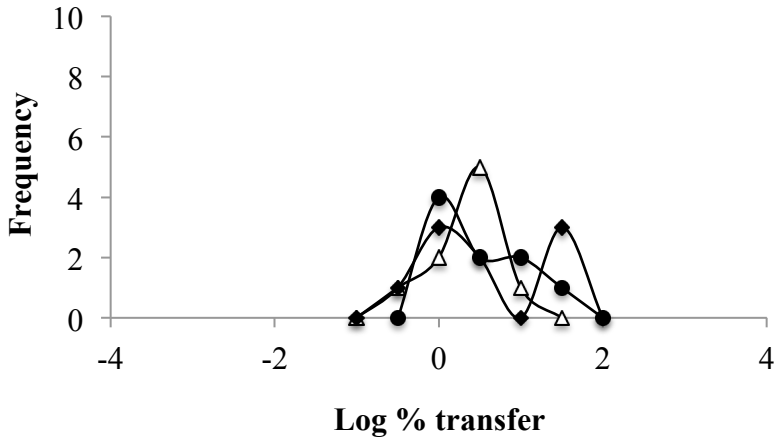

Figure 4.5. Frequency distributions of log percent transfer of *Salmonella* from contaminated brush to un-inoculated cucumber during cucumber waxing with sample A (mineral oil-based, ●), sample B (vegetable oil-based, △), and sample C (petroleum-based, ◆). Frequency is the number of times a particular log percent transfer occurred within a target data set (n=9).

Chapter 5. Modeling the growth and survival of *Salmonella* in whole and cut cucumbers as a function of temperature and relative humidity

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Key words: *Salmonella*, cucumber, temperature, relative humidity, modeling, risk assessment and management

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5.1. Abstract

Recent multistate outbreaks of salmonellosis associated with fresh cucumbers underscore the importance of understanding *Salmonella* behavior in cucumbers at different storage conditions. No validated models which describe the impact of environmental factors on growth and survival of *Salmonella* in cucumbers currently exist. This study developed mathematical models to predict the growth and survival of *Salmonella* on whole and cut cucumbers at different temperature and relative humidity (RH) storage conditions. Fresh cucumbers were purchased from a local supermarket and used in whole or cut cucumber experiments. Whole or cut cucumbers were spot-inoculated with a four-strain cocktail of *Salmonella enterica*. All strains were originally linked to fresh produce outbreaks and were made resistant to nalidixic acid. Inoculated cucumbers were placed in desiccators containing saturated salt (lithium chloride, potassium carbonate, and potassium sulfate) used to create controlled RH environments (~15, 50, 100% RH) at 7, 14, and 21 °C. RH in the desiccators increased due to the presence of cucumber samples to approximately 40-50, 70-80, and 100% RH. Samples were enumerated at appropriate time intervals ranging from 0 to 240 h. Predictive models were developed using Baranyi and Roberts equation as a primary model and estimated kinetic parameters were fitted into a polynomial equation or square root (or Ratkowsky) equation for secondary models. *Salmonella* on whole and cut cucumbers showed better survival and slower growth at lower temperatures. RH had no impact on *Salmonella* growth on cut cucumber. RH did affect *Salmonella* survival on whole fresh cucumber, with the greatest decline in *Salmonella* populations observed at 15% RH. When a polynomial equation was used to describe the maximum death rate and the degree of

decline of *Salmonella* on whole fresh cucumber as a function of temperature and RH, a linear trend with high R^2 values (>0.98) was observed. The maximum growth rates for *Salmonella* on cut cucumber depended on temperature but not RH and ranged from 0 to 0.18 log CFU/h. The square root model for *Salmonella* was $SQRT(\mu) = 0.0297 * (T - 6.52)$, with a high R^2 value (0.98). The models in this study will be useful for future microbial risk assessments and predictions of *Salmonella* behavior in the cucumbers to manage the risk of *Salmonella* with respect to cucumbers.

5.2. Introduction

Fresh and fresh-cut produce market has rapidly grown with the increase in consumption of fruits and vegetables over the past two decades (45, 124). With increased demand for fresh and fresh-cut produce, the number of foodborne disease outbreaks associated with fruits and vegetables has been increased since surveillance began in 1973 in the United States (40, 117, 125). One of most common pathogens associated with fruits and vegetables is *Salmonella*. This organism is a leading cause of foodborne bacterial illnesses and is estimated to cause 1.2 million illnesses each year in the United States with more than 23,000 hospitalizations and 450 deaths (17, 24, 126). There have been at least four reported multistate outbreaks of salmonellosis directly linked to fresh cucumbers in the United States since 2013 (33, 34, 36, 37). Cucumbers were not considered a typical vehicle in produce-related human infections before those outbreaks occurred. This series of multistate outbreaks have elevated cucumbers to one of the primary fresh produce commodities linked to salmonellosis.

Native microflora and pathogenic microorganisms on fresh and fresh-cut produce are affected by various extrinsic and intrinsic factors (11, 52-54). Storage conditions including temperature, and relative humidity (RH) are two of the most important environmental factors needed to maintain the quality and shelf life of fruits and vegetables (127-131). *Salmonella* and other pathogenic microorganisms can survive on the surface of fresh produce at room temperature and their growth can be accelerated on the fresh-cut produce with readily available nutrients and water released from edible flesh portion (42, 67, 129, 132, 133). Microbial survival and growth are favored by high RH (>80%) conditions (127), and such high RH condition have been reported to be favorable

for growth and survival of *Salmonella* on tomatoes and apples (129, 133). Very little is known about the effect of temperature and RH on bacterial behavior in cucumbers, although the recommended storage conditions needed to maintain the quality of cucumber without chilling injury, wilting, shrinkage, or loss of flavor and nutrients has been reported (128).

Many predictive models describing single or combined effect of temperature, RH, pH and/or storage time on bacterial growth and survival kinetic parameters on fresh and fresh-cut produce have been developed (130, 134-137). No validated models currently exist however, which describe the impact of environmental factors on growth and survival of *Salmonella* in cucumbers. The purpose of this study was to develop mathematical models capable of predicting growth and survival of *Salmonella* on whole and cut cucumbers as a function of temperature and RH.

5.3. Materials and Methods

5.3.1. Bacterial strains and inoculum preparation

Four strains of *Salmonella enterica* provided by Dr. Joshua Gurtler (Eastern Region Research Center, USDA, Wyndmoor, PA) were used for all experiments: *Salmonella* Newport H1275 (sprout outbreak), Stanley H0558 (sprout outbreak), Montevideo G4639 (tomato outbreak), and Saintpaul 02-517-1 (cantaloupe outbreak). *Salmonella* strains were made resistant to 100 μg/mL of nalidixic acid (Fisher Scientific, Pittsburgh, PA) by serial exposure to increasing (1:2) concentrations of nalidixic acid in tryptic soy broth (TSB; Difco Becton Dickinson & Co., Sparks, MD) incubated at 37 °C

for 24 h. A frozen culture of each strain was streaked onto tryptic soy agar (TSA; Difco, BD) supplemented with 50 μg/mL of nalidixic acid, and incubated at 37 °C for 24 h prior to each experiment. An isolated colony was transferred to 10 mL of TSB supplemented with 50 μg/mL of nalidixic acid, and incubated at 37 °C for 24 h. The culture was centrifuged at 5,000 × g for 10 min (Allegra 21R centrifuge; Beckman Coulter, Brea, CA) and then washed twice with 0.1M, pH 7.2 phosphate buffer saline (Fisher Scientific). The washed cell pellets were suspended in phosphate buffer saline (PBS) after final wash and then combined to produce a cocktail of four strains, yielding approximately 8.5 log CFU/mL of inoculum.

5.3.2. Inoculation of cucumber samples

Fresh cucumbers were purchased from a local supermarket (Somerset, NJ) and used in whole or cut cucumber experiments. Whole cucumber was spot-inoculated with 50 μL of the cocktail, resulting in ca. 10^6 CFU/cucumber prior to drying in the biosafety cabinet for 2h to conduct *Salmonella* survival study. Fifty microliters of inoculum (10^3 CFU/20 g sample) were spot-inoculated onto the cut cucumber and allowed to dry for 1 h to characterize *Salmonella* growth on the cut cucumber at different temperature and RH storage condition.

5.3.3. Controlled environmental condition

After inoculation and drying, the inoculated whole or cut cucumber samples were transferred into RH controlled desiccators. Saturated solutions of lithium chloride, potassium carbonate and potassium sulfate (Fisher Scientific) were prepared to equilibrate the atmosphere of desiccators to approximately 15, 50, and 100% RH,

respectively. RH in the desiccators increased due to the cucumber samples to approximately 40-50, 70-80, and 100% RH as recorded by temperature and RH logger (EL-USB-2-LCD, Lascar electronics, Erie, PA). The desiccators were stored at 7, 14, or 21 °C and cucumber samples were enumerated at appropriate time intervals ranging from 0 to 240 h.

5.3.4. *Salmonella* recovery

Whole cucumber was placed into a Lab blender bag (Fisher Scientific), containing 20 mL of PBS, and rubbed by hands for 30 s. Ten milliliters of PBS were added to a bag containing cut cucumber prior to homogenization using stomacher (Dynatech Laboratories, Alexandria, VA) for 1 min. Samples were serially diluted in PBS, and 0.1 mL of each dilution was plated onto Hektoen Enteric agar (HE; Difco, BD) with 50 μg/mL of nalidixic acid. The plates were incubated at 37 °C for 24 h, bacterial colonies were counted and *Salmonella* populations were expressed as log CFU/cucumber or CFU/g cut cucumber.

5.3.5. Development of growth and survival models

Experimental data collected from survival and growth of *Salmonella* on whole and cut cucumbers were separately fitted to Baranyi and Roberts model (138) using DMFit web edition (Institute of Food Research, Norwich, UK).

To evaluate the effect of temperature and RH on *Salmonella* survival on whole cucumber, estimated survival kinetic parameters, maximum death rate (DR) and degree of decline (where log reduction equals initial bacterial counts minus final bacterial counts)

obtained from the Baranyi and Roberts model were fitted into quadratic polynomial equation:

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_1^2 + a_4X_2^2 + a_5X_1X_2 \quad (1)$$

where Y is any of the survival kinetic parameters, X_1 is temperature, X_2 is RH, a_i (where "i" represents any number from 0 to 10) are coefficients.

The square root (or Ratkowsky) model:

$$\sqrt{\text{Growth Rate}} = b^*(T - T_0) \quad (2)$$

was used to describe the maximum growth rate (GR) of *Salmonella* on cut cucumber as a function of temperature since GR obtained from Baranyi and Roberts model depended only on temperature, not on RH. In this model, b is regression coefficient, T is temperature and T_0 is a theoretical minimum temperature for *Salmonella* growth, where T was given in °C.

5.4. Results and Discussion

5.4.1. Modeling survival of *Salmonella* on whole cucumber

Salmonella survival on whole fresh cucumber during storage at different temperature (7, 14, and 21 °C) and RH (15, 50, and 100%) is shown in Figure 5.1, 5.2 and 5.3. The greatest survival of *Salmonella* on whole cucumber was observed at 100% RH, followed by at 50 and 15% RH. Effect of temperature on *Salmonella* survival on whole cucumber during the storage period up to 240 h was not as marked compared to that of RH. Combined effect of temperature and RH on bacterial behavior has been studied (129-131, 133, 137), although most of the studies evaluating environmental

factors controlled only temperature, not RH. Prior studies have reported that bacterial survival and growth were influenced by combined temperature and RH, but significance or degree of their relative impacts on bacterial behavior varied depending on microorganism and produce items tested.

Experimental data obtained in this study was fitted to Baranyi and Roberts model to develop models predicting *Salmonella* survival on whole fresh cucumber under different storage condition. *Salmonella* survival kinetic parameters are shown in Table 5.1. The R^2 values for the primary models ranged from 0.616 to 0.998, indicating relatively good fit. At the highest RH condition, *Salmonella* on whole fresh cucumber presented highest DR, while the highest log reduction was observed at 15% RH. There was no noticeable impact of temperature on DR found but the log reduction of *Salmonella* on whole fresh cucumber decreased with rise in temperature. Tian et al. (129) revealed the importance of temperature and RH affecting reduction of *S.* Typhimurium on the surface of apples. In their study, significantly higher reduction of *S.* Typhimurium was achieved when unblemished or bruised apples were stored at low temperature (4 vs 15 °C) and RH condition (68 vs 85-100%). Conversely, Stine et al. (139) found no trend in the effect of RH on bacterial survival on the surface of cantaloupe and longer survival of most of microorganisms on surfaces of lettuce and bell peppers in dry condition (22.7-24.8% RH) vs humid condition (45.1-90.3% RH). These differences may be due to the different storage temperature, RH, or types of produce tested.

The effect of temperature and RH on *Salmonella* survival on whole fresh cucumber is more clearly shown in contour plots (Figure 5.4). Although temperature is one of the major environmental factors that influence bacterial behavior on foods (140,

141), DR in our study was affected only by RH, not by temperature (Figure 5.4A). However, the effect of temperature on log reduction of *Salmonella* on whole fresh cucumber was magnified with increase in RH, showing the lowest log reduction at the highest temperature at 100% RH (Figure 5.4B). High temperature and RH favor microbial growth and survival, and even small fluctuation in temperature at high RH can cause condensation of water, which greatly increases proliferation of microorganisms (127, 128).

A quadratic polynomial equation was used to describe DR and log reduction of *Salmonella* on whole fresh cucumber as a function of temperature and RH (Table 5.2 and 5.3). Insignificant variables and their interactions in full models were removed, which decreased the R^2 by 0.02 and 0.04 in the reduced models of DR and log reduction, respectively. With higher *F* values, the variables remaining in the reduced model were highly significant ($P<0.0001$). The significant coefficients ($p<0.0001$) and R^2 of the equations expressing the dependence of *Salmonella* survival kinetic parameters on temperature and RH are as follows:

$$DR = -0.16090 + 0.00396(RH) - 0.00003(RH)(RH) \quad (3)$$

$$\text{Log reduction} = 4.48385 - 0.00199(T)(RH) \quad (4)$$

where T is temperature in °C and RH is relative humidity (%). The DR and log reduction models had high R^2 (>0.95) and were significant at the 0.0001 level (Table 5.2 and 5.3). The coefficient values for each of the variables in the models and their *P* values are also shown in Table 5.2 and 5.3.

Predicted DR and log reduction of *Salmonella* on whole fresh cucumber can be determined with reduced model equations or the contour plots. Figure 5.5 shows the

effect of temperature and/or RH on the predicted DR and log reduction of *Salmonella* on whole fresh cucumbers. Although DR of *Salmonella* was predicted to increase with the rise in RH (Figure 5.5A), the greatest inactivation of *Salmonella* on whole fresh cucumber is predicted to achieve at low RH at low temperature condition (Figure 5.5B). While low RH and temperature conditions may benefit food safety, wilting and shrinkage due to moisture loss can be caused by storage at low RH and chilling injury symptoms such as pitting and yellowing could be developed in cucumber stored at 7 °C or below (107, 128). Recommended storage temperature (10 °C) and RH (65-90%) needed to maintain the quality of cucumber reported previously (128) may reduce *Salmonella* contamination on whole fresh cucumber, where ca. 3 log CFU/cucumber reduction is predicted.

5.4.2. Modeling growth of *Salmonella* on cut cucumber

Salmonella on cut cucumbers showed faster growth at a higher temperatures, while RH had no impact on *Salmonella* growth on cut cucumber during the storage period up to 120 h, as shown in Figure 5.6 to 5.8. *Salmonella* population increased by approximately 4 log CFU/g on cut cucumber stored at 21 °C during the first 24 h. A similar but slower growth trend was observed at 14 °C and the maximum population of *Salmonella* ranged between 10^6 and 10^7 after storage for 72 h. *Salmonella* did not grow on cut cucumber storage at 7 °C but did survive up to 120 h. Tian et al. (129) reported that effect of RH on population of *S.* Typhimurium on cut apple varied depending on storage temperature. In their study, no difference in *Salmonella* population for storage at three RH conditions (68, 85, and 100%) at 15 °C was found, while significantly highest

reduction was observed on cut apple stored at lowest RH at 4 °C. *S*. Montevideo on the surface of intact tomato was also influenced by temperature and RH (133). Marked increase in *Salmonella* population on tomatoes was shown at 97% RH during 10 days storage at 30 °C compared storage at 60, 75, and 85% RH. *Salmonella* population growth on tomatoes at 22 °C was lower than at 30 °C and no growth was observed at 60% RH. The combined effect of temperature and RH on *Listeria monocytogenes* on the surface of cucumbers has also been studied (131). During storage at 10, 20, and 30 °C and at 53 and 90% RH, *Listeria* grew only on cucumber and not lettuce and parsley which were also tested. Faster growth of *Listeria* found at a higher temperatures and the effect of RH was magnified with a decrease in temperature, showing a marked difference in *Listeria* population at 53 and 90% RH. Although temperature and RH are both important factors that may affect bacterial behavior on fresh and fresh-cut produce, their impact clearly varies depending on microorganisms, produce type, state (intact or cut), storage temperature and RH.

Growth kinetic parameters of *Salmonella* on cut cucumber at 7, 14, and 21 °C and at 15, 50, and 100% RH obtained by fitting the data to Baranyi and Roberts model are shown in Table 5.4. *Salmonella* on cut cucumber had the highest GR at 21 °C, followed by 14 and 7 °C. Since *Salmonella* did not grow during storage period at 7 °C, GR was 0 and final (maximum) value could not be obtained. There was no marked difference in GR at 15, 50, and 100% RH at each temperature condition. The combined effect of temperature and RH on GR on cut cucumber is more clearly shown in contour plot (Figure 5.9), which indicates that the GR for *Salmonella* on cut cucumber depends only on temperature, and not RH. A clear lag phase was not evident on the growth curves at 7

and 21 °C and DMFit did not estimate lag time at those temperatures, so lag time of *Salmonella* on cut cucumber was not modeled. The square root model gave a good fit as shown in Figure 5.10 (R^2=0.98).

$$\sqrt{\text{Growth Rate}} = 0.0297(T - 6.5185) \quad (5)$$

where T is temperature in °C. This model was significant at the 0.0001 level with high *F* value (342.02, data not shown).

Storage temperature of fresh-cut produce is crucial not only to maintain the quality attributes but also to reduce microbial contamination. The U.S. Food and Drug Administration recommends that fresh-cut produce should be stored at less than or equal to 5 °C for safety control (142), which has been shown to prevent growth of *E. sakazakii* and *E. coli* O157:H7 on cut cucumbers (66, 67). *S.* Choleraesuis also did not grow on cut cucumber stored at 4 °C but survived well up to 48 h without a decrease in population (132), and the same study demonstrated that *S.* Choleraesuis populations on cut cucumber significantly increased to 7.1 log CFU/g at 25 °C over 48 h. The same trend for *Salmonella* growth on cut cucumber stored at 7, 14, 21 °C and at 15, 50, and 100% RH was observed in our study. The results and model developed in our study should be useful in predicting *Salmonella* behavior on cut cucumber under various storage temperatures during processing, marketing and storage in consumers homes.

The models developed in this study are the first to describe the effect of temperature and RH on *Salmonella* behavior on whole and cut cucumbers. Our results showed a significant impact of combined temperature and RH on *Salmonella* inactivation on whole fresh cucumber and importance of storage temperature to ensure microbial safety on cut cucumber, which can be applied to validate current and future intervention

strategies for control *Salmonella* contamination in cucumbers. These models will be also useful for future microbial risk assessments and predictions of *Salmonella* behavior in cucumbers to manage the risk of *Salmonella* with respect to cucumbers.

Table 5.1. Survival kinetic parameters of *Salmonella* on whole cucumber at different temperature and RH conditions [a]

Temp (°C)	RH (%)	R^2	SE of Fit	Initial Value (Log CFU/cucumber)	Final Value (Log CFU/cucumber)	Reduction (Log CFU)	Maximum Death Rate
21	100	0.616	0.127	5.437 ± 0.117	5.007 ± 0.074	0.4297	-0.012 ± 0.008
	50	0.957	0.238	5.207 ± 0.157	2.492 ± 0.275	2.7150	-0.020 ± 0.003
	15	0.973	0.251	5.375 ± 0.231	1.723 ± 0.145	3.6520	-0.116 ± 0.016
14	100	0.703	0.318	5.048 ± 0.216	3.828 ± 0.320	1.2197	-0.010 ± 0.005
	50	0.880	0.509	4.816 ± 0.333	1.382 ± 0.629	3.4340	-0.024 ± 0.006
	15	0.998	0.193	5.466 ± 0.177	1.469 ± 0.112	3.9970	-0.089 ± 0.012
7	100	0.899	0.366	5.622 ± 0.270	2.825 ± 0.405	2.7970	-0.015 ± 0.003
	50	0.969	0.307	5.636 ± 0.244	1.619 ± 0.230	4.0170	-0.030 ± 0.004
	15	0.998	0.085	5.640 ± 0.085	1.365 ± 0.043	4.2750	-0.116 ± 0.005

[a] SE of fit, standard error of fit; Reduction (Initial value minus final value).

Table 5.2. Statistics for the effects of temperature and RH on maximum death rate of *Salmonella* on whole cucumber

	Full model		Reduced model [a]	
R^2	0.981655		0.967195	
F value	32.106421		88.449409	
No. of variables	9		9	
Variable [b]	Coefficients	*P* value	Coefficients	*P* value
Intercept	-0.198505	0.007166	-0.160897	<0.0001
T	0.006122	0.240605	-	-
RH	0.003945	0.005326	0.003960	0.000137
(T)(RH)	0.000001	0.953697	-	-
$(T)^2$	-0.000210	0.241941	-	-
$(RH)^2$	-0.000025	0.008945	-0.000025	0.000671

[a] All variables significant at the 0.001 level.

[b] T, temperature (°C); RH, relative humidity (%).

Table 5.3. Statistics for the effects of temperature and RH on log reduction of *Salmonella* on whole cucumber

	Full model		Reduced model [a]	
R^2	0.993729		0.954001	
F value	95.083716		145.177077	
No. of variables	9		9	
Variable [b]	Coefficients	*P* value	Coefficients	*P* value
Intercept	4.660850	0.002898	4.483849	<0.0001
T	-0.077015	0.362896	-	-
RH	0.020187	0.117834	-	-
(T)(RH)	-0.001470	0.014481	-0.001994	<0.0001
$(T)^2$	0.001987	0.481683	-	-
$(RH)^2$	-0.000252	0.036675	-	-

[a] All variables significant at the 0.001 level.

[b] T, temperature (°C); RH, relative humidity (%).

Table 5.4. Growth kinetic parameters of *Salmonella* on cut cucumber at different temperature and RH conditions [a]

Temp (°C)	RH (%)	R^2	SE of Fit	Initial Value (Log CFU/cucumber)	Final Value (Log CFU/cucumber)	Maximum Growth Rate
21	100	1.000	0.002	2.305 ± 0.002	7.090 ± 0.002	0.179 ± 0.000
	50	0.998	0.086	2.273 ± 0.080	6.957 ± 0.086	0.170 ± 0.005
	15	0.998	0.085	2.275 ± 0.080	6.733 ± 0.085	0.170 ± 0.006
14	100	0.978	0.285	2.191 ± 0.260	6.356 ± 0.209	0.076 ± 0.016
	50	0.871	0.570	2.292 ± 0.521	5.699 ± 0.409	0.066 ± 0.034
	15	0.957	0.361	2.095 ± 0.263	5.994 ± 0.365	0.049 ± 0.009
7	100	0.874	0.055	2.401 ± 0.039	-	0.000
	50	0.988	0.030	2.383 ± 0.022	-	0.000
	15	0.990	0.029	2.405 ± 0.018	-	0.000

[a] SE of fit, standard error of fit

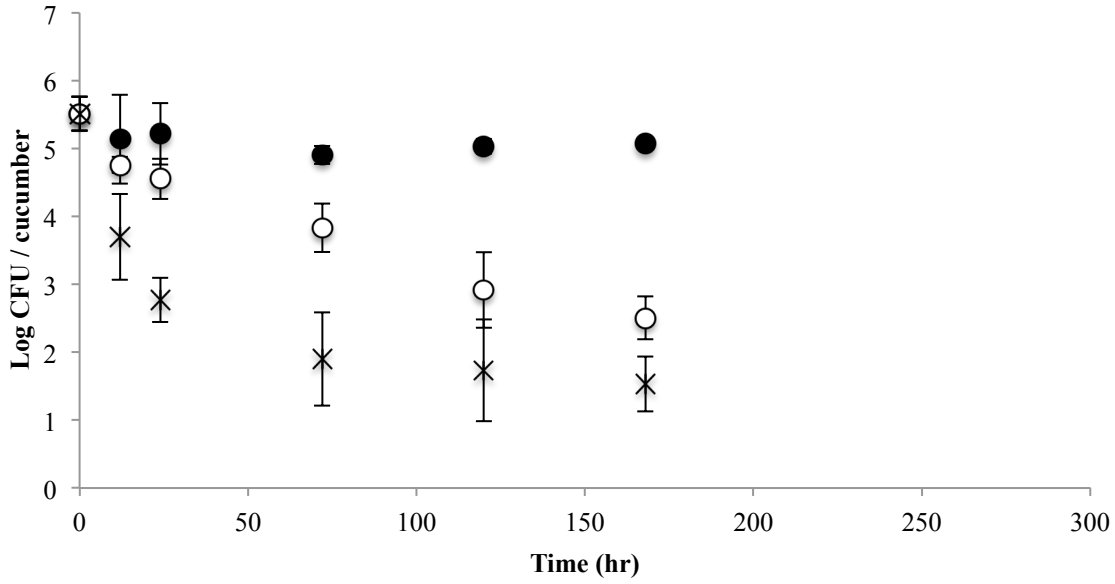

Figure 5.1. *Salmonella* survival on whole cucumbers during storage at 21 °C and at 15 (∗), 50 (○), and 100 (●)% RH.

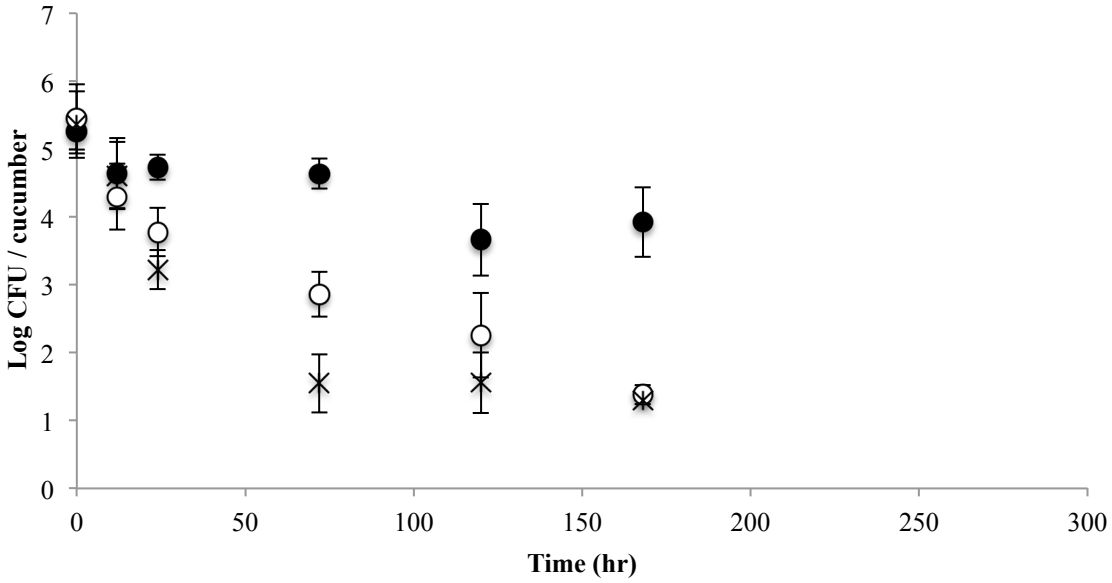

Figure 5.2. *Salmonella* survival on whole cucumbers during storage at 14 °C and at 15 (∗), 50 (○), and 100 (●)% RH.

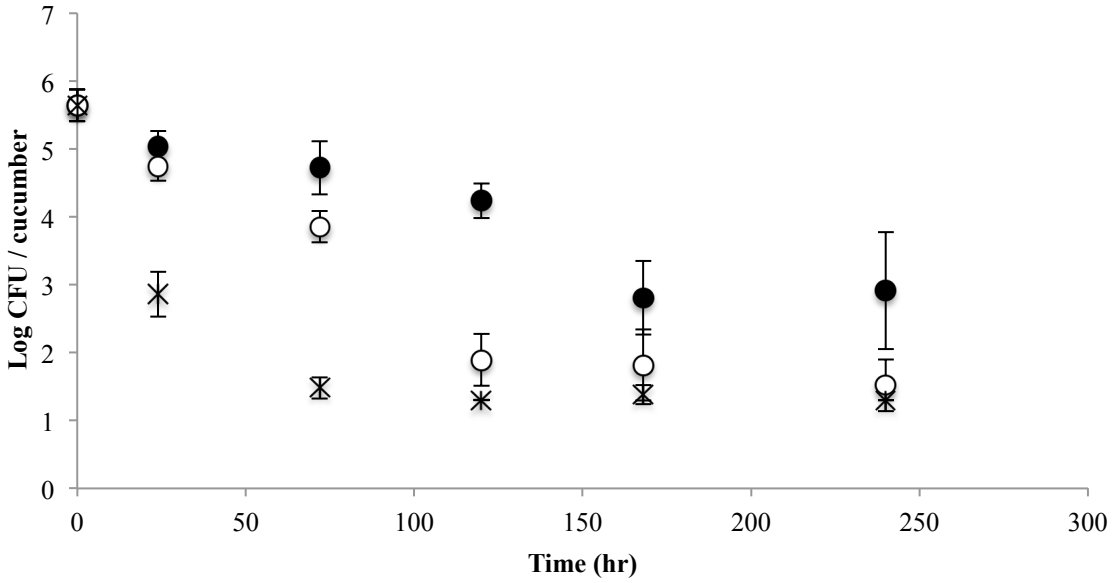

Figure 5.3. *Salmonella* survival on whole cucumbers during storage at 7 °C and at 15 (∗), 50 (○), and 100 (●)% RH.

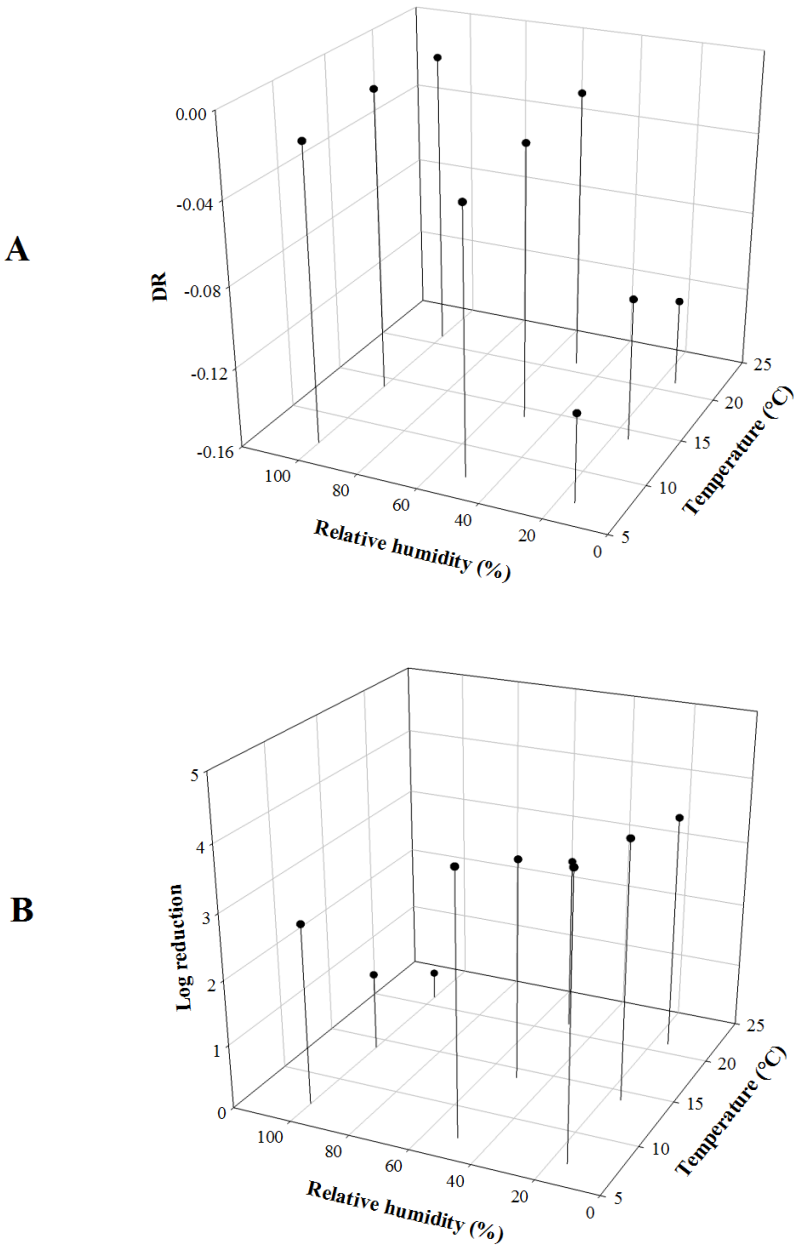

Figure 5.4. Comparison of maximum death rate (A) and log reduction (B) for *Salmonella* survival on whole cucumber at different temperature and RH. DMFit web edition was used to model the survival of *Salmonella* from all experimental observations, fitting data to Baranyi and Roberts model.

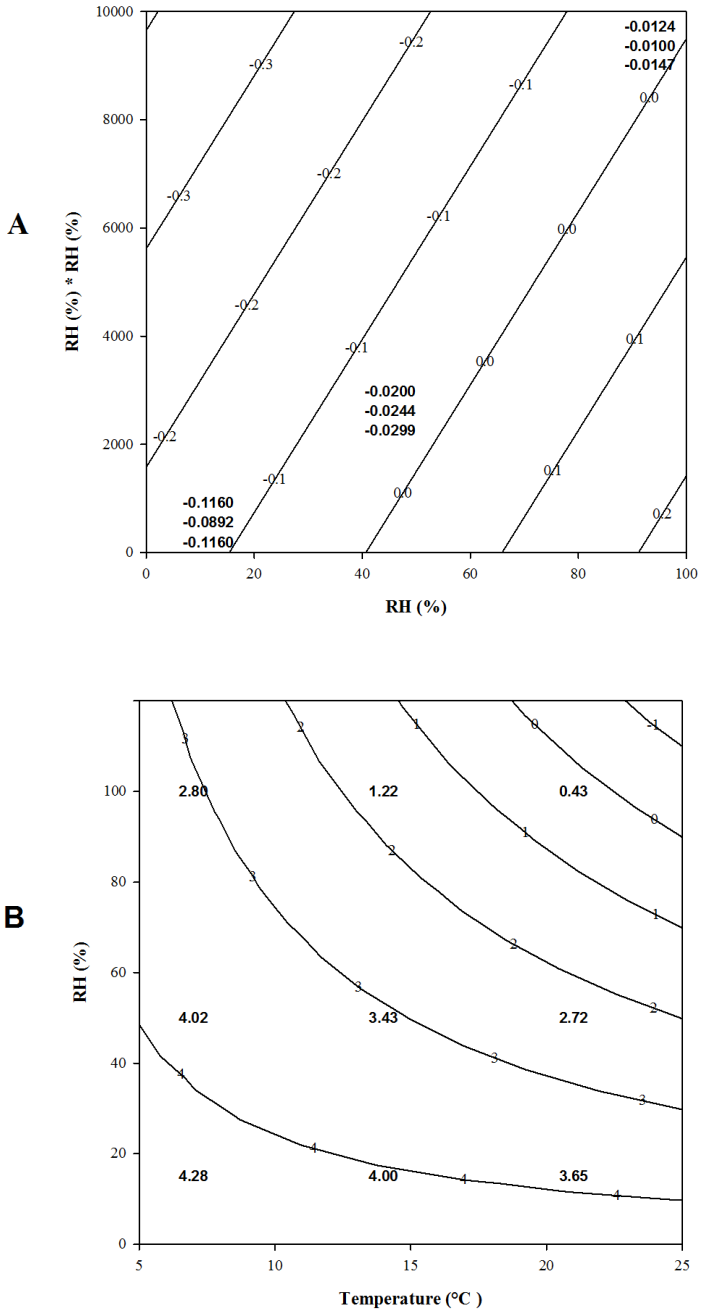

Figure 5.5. Effect of temperature and/or relative humidity on predicted maximum death rate and log reduction (contour line in A and B) of *Salmonella* on whole fresh cucumber. Boldfaced numbers are experimental data used to generate the model predictions.

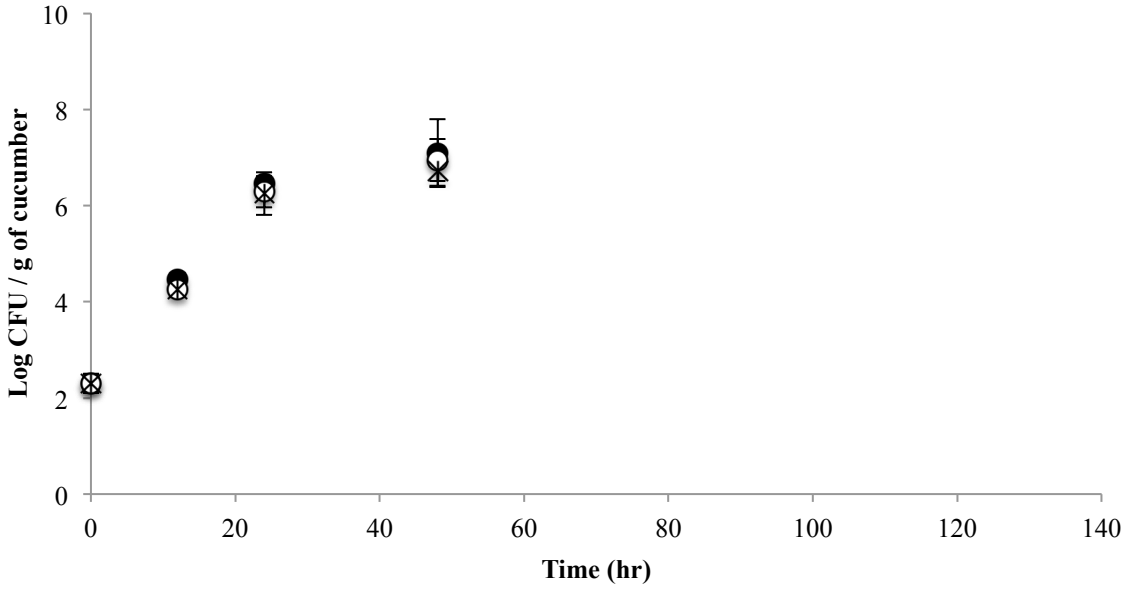

Figure 5.6. *Salmonella* growth on cut cucumbers during storage at 21 °C and at 15 (∗), 50 (○), and 100 (●)% RH.

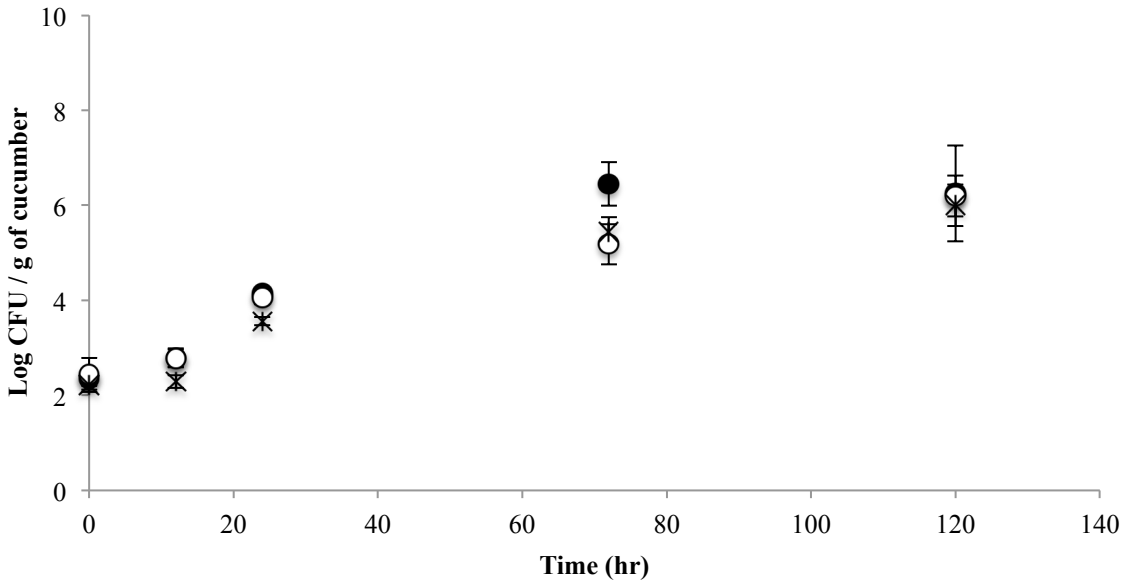

Figure 5.7. *Salmonella* growth on cut cucumbers during storage at 14 °C and at 15 (∗), 50 (○), and 100 (●)% RH.

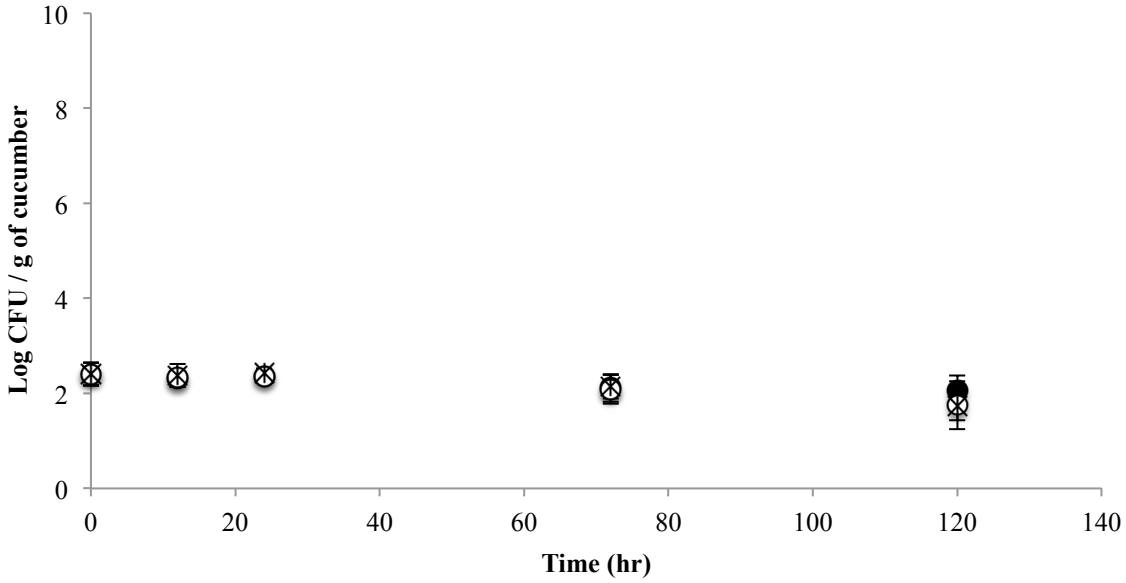

Figure 5.8. *Salmonella* growth on cut cucumbers during storage at 7 °C and at 15 (∗), 50 (○), and 100 (●)% RH.

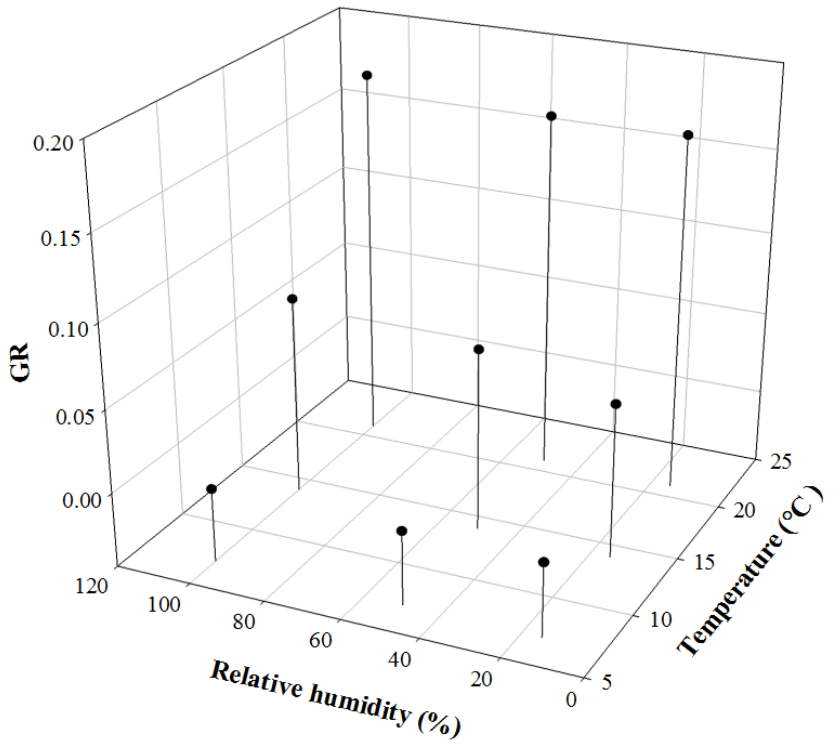

Figure 5.9. Comparison of maximum growth rates for *Salmonella* growth on cut cucumber at different temperature and RH. DMFit web edition was used to model the growth of *Salmonella* from all experimental observations, fitting data to Baranyi and Roberts model.

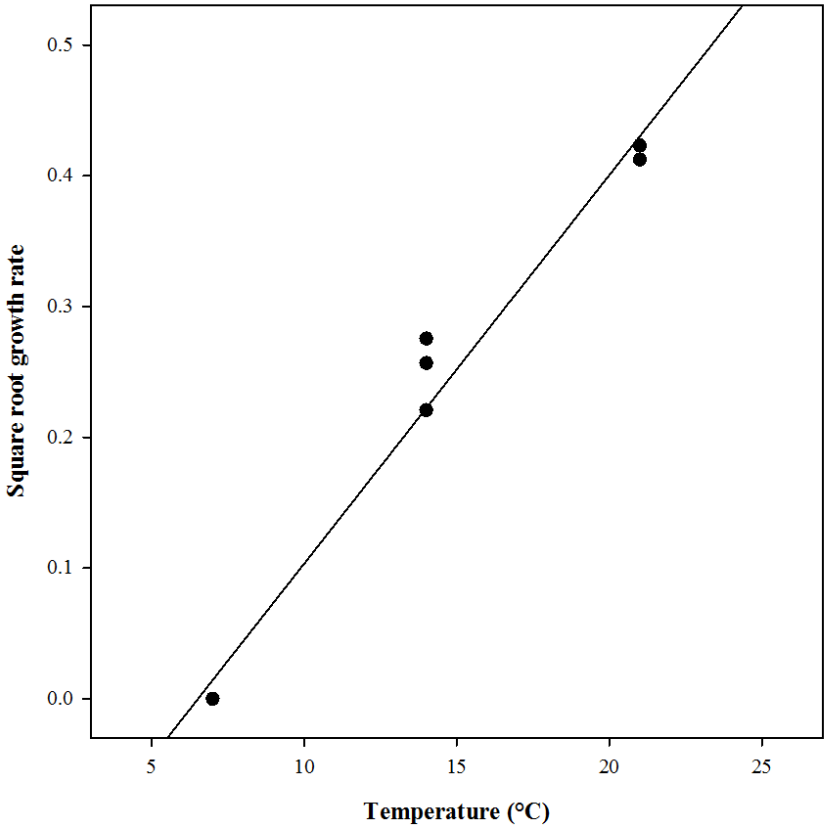

Figure 5.10. Effect of temperature on predicted square root growth rate (solid line) of *Salmonella* on cut cucumber. Solid circles (A) are experimental data used to generate the model predictions.

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