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Growth and Biofilm Formation by Listeria Monocytogenes and Salmonella Spp. In Cantaloupe Extracts on Four Food-Contact Surfaces at 22°C and 10°C

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Growth and biofilm formation by *Listeria monocytogenes* and *Salmonella* spp. in cantaloupe extracts on four food-contact surfaces at 22°C and 10°C.

By

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Center for Disease Control and Food and Drug Administration reports indicate that cantaloupe is one of the five most likely fruits and vegetables to cause a foodborne disease outbreak. Cantaloupe is a potential hazardous food based on the FDA food code since it is capable of supporting pathogen growth due to its low acidity and high moisture content. The objectives of this study were to determine the effect of strain and temperature on growth and biofilm formation of *L. monocytogenes* and *Salmonella* spp. in cantaloupe flesh and peel extracts on different food-contact processing surfaces. Growth of *L. monocytogenes* and *Salmonella* strains was greater in high cantaloupe flesh and peel extract concentration at 22°C and 10°C. In 50 mg/ml of cantaloupe flesh or peel extract, the cell numbers of *L. monocytogenes* and *Salmonella* increased by 5.0-5.5 log CFU/ml in 40 h at 22°C and 1-3.5 log CFU/ml in 72 h at 10°C. In 2 mg/ml of cantaloupe flesh or peel extract, the cell numbers of *L. monocytogenes* and *Salmonella* increased by 4.0-4.5 log CFU/ml in 72 h at 22°C but no change in log CFU/ml in 72 h at 10°C. There were no differences ($P > 0.05$) among *L. monocytogenes* or *Salmonella* strains for biofilm formation in cantaloupe extracts, but biofilm formation was greater ($P < 0.05$) at high

temperature and high cantaloupe flesh or peel extract concentration. In 50 mg/ml cantaloupe flesh or peel extract, *L. monocytogenes* and *Salmonella* produced biofilms of 7 log CFU/coupon in 4 days at 22°C and 4-5 log CFU/coupon in 7 days at 10°C. In 2 mg/ml cantaloupe flesh or peel extract, *L. monocytogenes* and *Salmonella* produced biofilms of 5-6 log CFU/coupon in 4 days at 22°C and 3-4 log CFU/coupon in 7 days at 10°C. *L. monocytogenes* and *Salmonella* spp. formed less biofilms ($P < 0.05$) on buna-n rubber when compared to stainless steel, polyethylene and polyurethane surfaces. These findings indicate that a very low concentration of nutrients that are leaked from cantaloupe flesh or peel can induce growth and biofilm formation in *L. monocytogenes* and *Salmonella* spp. on different food-contact surfaces.

DEDICATION

This work is dedicated to my loving mother late Mrs. Cindrella Silva, my loving father Mr. Edmen Abeysundara and my husband Mr. Suranga Rajapaksha.

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CHAPTER I

INTRODUCTION

Consumption of fresh and minimally processed fruits is becoming more and more common due to an increased awareness of healthy eating. Production of fresh and minimally processed fruit products involve only mild food processing and preservation techniques. Therefore, these products may carry a comparatively large number of microorganisms, some of which may be potentially pathogenic to human (Danyluk et al., 2014). The Center for Disease Control (CDC) reported that the number of foodborne outbreaks linked to fresh produce in the United States of America (USA) has increased over the last few years (Walsh et al., 2014).

Cantaloupe associated outbreaks are mainly caused by *L. monocytogenes*, *Salmonella enterica*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, and by norovirus (Bowen et al., 2006; Walsh et al., 2014). From 1984 to 2002, there were 23 cantaloupe associate outbreaks which resulted in 1434 cases, 42 hospitalizations and 2 deaths occurred in the USA ((Bowen et al., 2006). In addition, cantaloupe was responsible for 56% of total melon related outbreaks from 1973 to 2011 in the USA ((Walsh et al., 2014).

L. monocytogenes is a Gram-positive, non-spore forming, facultative anaerobic, rod shape bacterium (Holch et al., 2013). Out of 13 known serotypes of *L. monocytogenes,* serotypes 1/2a, 1/2b and 4b account for approximately 95% of human

listeriosis cases (Kathariou, 2002). *L. monocytogenes* is found in a wide range of food products, which include fresh fruits, vegetables, fish, meat, milk and many types of raw and processed foods (Kramarenko et al., 2013). Fruits and vegetables are contaminated with *L. monocytogenes* at the farm via decaying vegetation, feces and effluents or by contaminated equipment during harvesting (Beuchat, 1996). Cantaloupe may also be contaminated with *L. monocytogenes* from contaminated washing water or equipment that was in the processing facility (Bowen et al., 2006). Among all foodborne pathogens, *L. monocytogenes* has the highest motility rate (Wang et al., 2015). The deadliest listeriosis outbreak in the USA occurred in 2011 and was associated with whole cantaloupe that was produced in Colorado. This outbreak resulted 147 total cases, 33 deaths and 1 miscarriage (Walsh et al., 2014). The USA has a zero tolerance policy for *L. monocytogenes* in ready-to-eat foods (RTE) such as minimally processed fresh fruits and vegetables. Therefore, the control of *L. monocytogenes* in the environment during cantaloupe processing is extremely important to avoid potential human health risks.

Salmonella enterica is a Gram-negative facultative anaerobe bacterium. Meat, poultry and eggs are the main source of *Salmonella* outbreaks. However, fruits, vegetables, milk, dairy products and fish products are also responsible for a significant number of *Salmonella* outbreaks in the USA every year (Jackson et al., 2013). The frequency of *Salmonella* spp. in comparison to other foodborne pathogens greater in cantaloupe products. From 1973 to 2011, *Salmonella* is responsible for 58% of total cantaloupe outbreaks that were reported to the CDC while *L. monocytogenes* was responsible only for 5% of outbreaks (Walsh et al., 2014). Out of over 2500 of known

Salmonella serovars, Poona, Newport and Javiana are highly associated with melon related outbreaks (Zarecki et al., 2013).

Biofilms are collections of microbial cell communities that were firmly attached to a surface and enclosed in a self-produced polysaccharide matrix. Biofilm is the predominant form of microorganisms as compared to planktonic cells (Chavez- Dozal and Nishiguchi, 2011). Studies have found that biofilm can exist as a single layer or multi-layers of microbial colonies with vertical and horizontal channels allowing flow of essential nutrients and water to inner layers of micro-colonies (Djordjevic et al., 2002). The persistence of biofilms on food-contact surfaces is a serious concern in the food industry since they can cause cross contamination of food products with pathogens that leads to food safety risks. Even though many studies on the growth and biofilm formation of *L. monocytogenes* and *Salmonella* spp. have been published, the majority of those studies were conducted in standard microbiology growth media that containing optimum level of nutrients. Therefore, the significance of data obtained from such studies to the predictable behavior of these foodborne pathogens is not very accurate in cantaloupe processing environments.

In cantaloupe processing environments, the presence of high or low concentrations of nutrients may exist due to leaking from cantaloupe flesh and peel. After approximately 5 weeks of fruit development, the flesh and peel of cantaloupe can contain up to 22 mg/g of free sugars and up to 4.5 mg/g of proteins based on fresh weight (Koubala et al., 2016). Therefore, juice that was leaked from cantaloupe flesh or peel can be highly favorable for growth and biofilm production by foodborne pathogens if a residue deposited on food-contact surfaces (Koubala et al., 2016). Stainless steel, glass,

plastic, rubber are frequently used in the food industry for food-contact processing surfaces (Di Bonaventura et al., 2008). The ability of *L. monocytogenes* and *Salmonella* Spp. to form biofilms on different food- contact surfaces in high and low concentration of cantaloupe extracts is important for the critical understanding of the behavior of these pathogens in cantaloupe processing environments. Therefore, the main objectives of this study were to the determine effects of strain and temperature on the growth and biofilm formation of *L. monocytogenes* and *Salmonella* spp. in cantaloupe flesh and peel extracts on different food-contact surfaces.

CHAPTER II

LITRATURE REVIEW

2.1 *Listeria monocytogenes*

L. monocytogenes is a Gram-positive, non-spore forming, facultative anaerobic, rod shape bacterium that is generally found in soil, decaying vegetation and animal feces (Farber and Peterkin, 1991). This microorganism is visible under a wide range of environmental conditions indicating temperatures from 0°C to 45°C, pH from 4.1 to 9.6 and NaCl concentrations from 14 to 20% (Jemmi and Stephan, 2006; McClure et al., 1989).

Thirteen serotypes of *L. monocytogenes* (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7) have been identified based on somatic and flagella antigen differences (Borucki and Call, 2003; Datta et al., 2013). These 13 serotypes can be further divided to 3 genetic lineage based on ribotyping and virulence gene polymorphism analysis. Lineage I include serotypes 1/2b, 3b, 4b, 4d, 4e, lineage II include serotypes 1/2a, 1/2c, 3a, and 3c and lineage III include serotypes 4a and 4c (Liu et al., 2006). Out of the 13 serotypes of *L. monocytogenes,* 1/2a, 1/2b and 4b account for approximately 95% of human listeriosis cases (Kathariou, 2002).

Symptoms of listeriosis include fever, muscle ache, nausea, diarrhea, headache, stiff neck, loss of balance and convulsions. Listeriosis also causes fetal brainstem infections which can lead to meningitis and meningoencephalitis. The risk of contracting

listeriosis is greater in elderly people, pregnant women, and immune-compromised people when compared to general population (Schuchat et al., 1991). *L. monocytogenes* has a long incubation period in humans as compared to other foodborne pathogens which generally range from 1 to 67 days and depends on the type of microorganism (Goulet et al., 2013).

Conventional isolation and identification methods for *L. monocytogenes* generally involve selective enrichment and plating followed by characterization based on colony morphology, sugar fermentation and hemolytic properties. These methods are considered time consuming and therefore not suitable for the quality assurance of food with short shelf lives. Rapid testing methods such as enzyme-linked immunosorbent assay (Alexandre et al.), Real Time PCR (RT-PCR), multiplex-PCR, RNA RT-PCR and many methods are available to ensure the foods with shorter shelf lives (Gasanov et al., 2005; Välimaa et al., 2015).

2.1.1 *L. monocytogenes* occurrence in food

The majority of listeriosis outbreaks that occurred in the USA have been linked to the consumption of ready-to-eat frankfurters, deli meats, milk and dairy products. In addition, fresh produce such as, cantaloupe and sprouts were also associated with some of the major listeriosis outbreaks in recent years (McCollum et al., 2013; Tompkin, 2002).

Fruits and vegetables can get contaminated with *L. monocytogenes* at the farm by decaying vegetation, feces and effluents containing contaminants or by contaminated equipment during harvesting (Beuchat, 1996). Fresh fruits and vegetables can also become contaminated with *L. monocytogenes* through non-hygienic handling practices during food processing (Kovacevic et al., 2013). Meat or meat based products can get

contaminated with *L. monocytogenes* through feces contamination or by cross-contamination during processing (Hudson and Mead, 1989). Milk and dairy products are responsible for a significant number of listeriosis outbreaks in the USA (Sanaa et al., 1993). *L. monocytogenes* has been isolated from raw milk, pasteurized milk, cheese and ice cream. Poor quality of silage, poor cleanliness of cows, inadequate cleaning of milking and milk processing equipment and utensils have been identified as the main risk factors associated with listeria contaminations in milk and dairy products (Pak et al., 2002).

2.1.2 *L. monocytogenes* outbreaks in different countries

The CDC estimates that there are approximately 1600 illnesses and 260 deaths that occur annually due to listeriosis in the USA (Scallan et al., 2011). The Canadian Institute for Health Information and Hospital Morbidity Database reported that there were 124 hospitalizations and 29 deaths due to *L. monocytogenes* in 2006 in Canada (Thomas et al., 2015). Similarly, in Australia, *L. monocytogenes* has been responsible for 40% of the deaths due to foodborne illnesses from 1995 to 2000 (Gould et al., 2004). However, in China there have been zero deaths due to *L. monocytogenes* between 1994 to 2005 (Wang et al., 2007) while in Japan there have been only 83 cases of *L. monocytogenes* reported with no deaths from 1996 to 2002 (Miya et al., 2010).

2.1.3 Pathogenesis of *L. monocytogenes*

In order for *L. monocytogenes* to induce illness in humans, the contaminated food or water has to be ingested orally and bacterial cells have to penetrate the epithelial cells in the intestine. *L. monocytogenes* enters almost all adherent cells, but the efficiency of

uptake of microbial cells can vary depending on the type of the host cell. Normally macrophages and macrophage-like cell lines can internalize *L. monocytogenes* up to 20 bacteria per cell (Portnoy et al., 2002)

Internalization of *L. monocytogenes* cells to phagocytes is often mediated by one or more bacterial surface proteins. These proteins are collectively named internalins and includes Internalin A, B, C, D, E, F, G and H. Internalin A mediates *L. monocytogenes* adhesion and internalization into epithelial cells through interaction with host cell receptor that is termed E-cadherin (Schubert et al., 2002), while Internalin B is required for entry of *L. monocytogenes* into hepatocytes (Dramsi et al., 1995).

Once *L. monocytogenes* cells enter macrophages, they either get killed by cell immunity or escape into the cytosol with the help of the pore-forming protein, listeriolysin O (LLO). In addition to LLO, *L. monocytogenes* secretes two phospholipases C (PLCs) phosphatidylinositol-specific PLC (PI-PLC) and a broad-spectrum PLC (PC-PLC) which assists the escape of *L. monocytogenes*. When *L. monocytogenes* cells are in the cytosol, cell multiplication starts and polymerization of actin filaments that are present in the cytosol with the help of ActA to generate force to move intracellularly, thus causing damages to the host (Kocks et al., 1992; Portnoy et al., 2002).

2.1.4 Guidelines on *L. monocytogenes* in food

The USA has zero tolerance for *L. monocytogenes* in ready to eat foods (RTE foods) where if a single *L. monocytogenes* cell is present on such foods, alert of public and food recall is mandatory. In Australia and New Zealand, the tolerable level of *L. monocytogenes* in RTE foods in which growth of *L. monocytogenes* cannot occur is <100

CFU/g while in RTE foods in which growth of *L. monocytogenes* can occur, *L. monocytogenes* cannot be detected in 25g. In Canada, RTE foods are categorized into two main categories. In category I RTE foods (foods those support growth of *L. monocytogenes* throughout the shelf-life), the presence of *L. monocytogenes* is considered as a major health risk which requires the public alert and recall of the food product. Foods that belong to category II are subdivided into 2A (RTE food products in which limited growth of *L. monocytogenes* can occur) and 2B (food products in which the growth of *L. monocytogenes* cannot occur throughout the expected shelf-life). The allowed level of *L. monocytogenes* in RTE food belong to 2A is <100 CFU/g while for RTE foods in 2B, a low priority is given with regard to industry verification and control.

2.2 *Salmonella*

S. enterica is a Gram-negative, facultative anaerobic, rod shape bacterium. *S. enterica* can be found in the intestinal tract of domestic and wild animals (Carrasco et al., 2012). The genus *Salmonella* is divided into two species called *S. enterica* and *S. bongori*. *S. enterica* is further divided into six subspecies (*S. enterica* subsp. *enterica*; *S. enterica* subsp. *salamae*, *S. enterica* subsp. *arizonae*, *S. enterica* subsp. *diarizonae*, *S. enterica* subsp. *houtenae* and *S. enterica* subsp. *indica*) which has over 2500 serovars. These serovars are classified based on the presence of somatic (O) and flagellar (H) antigens (Cai et al., 2005).

The growth or survival of *S. enterica* is influenced by factors such as temperature, pH, water activity, nutrients, salt concentration etc. of the growth media. *S. enterica* can grow in media with water activity (a_w) ranging from 0.93 to >0.99, with a_w of 0.99 being the optimum. *S. enterica* has been shown to grow at temperature from 5.2-46.2°C.

However, the optimum growth temperate for this microorganisms is between 35-43°C. The heat resistance of *S. enterica* depends on other factors such as pH and water activity of growth media. Studies have found that *S. enterica* can grow in a pH range of 3.8-9.5 while 7-7.5 pH being the optimum condition. The other environmental factors such as temperature, water activity, salt concentration may affect the pH that *S. enterica* can grow (Buchanan and Phillips, 1990).

S. enterica infections can be transmitted by animal driven as well as plant driven food products. The CDC estimates that 1.2 million foodborne illnesses with 19,000 hospitalizations and 450 deaths happen in the USA due to non-typhoidal *S. enterica*. The hospitalization rate of *S. enterica* exceeds that of other foodborne pathogens such as *L. monocytogenes*, *Escherichia coli* and Norovirus (Scallan et al., 2011).

Studies have indicated that *S. enterica* serovars differ in their natural habitat and ability to cause human infections. Only a few serovars of *S. enterica* cause illnesses in human (Coburn et al., 2007; Yates, 2011). This is also evident in a study conducted by Jones et al. (2008) in which they found that only 687 *S. enterica* serovars caused 46639 human salmonellosis cases in the USA from 1996–2006. Of these 46639 cases, 22% required hospitalization, 5% caused invasive disease while only 0.5% caused death. *S. enterica* Choleraesuis and Dublin caused the highest hospitalization rate (60-67%).Serovar Dublin has the highest fatality rate (3%) while Newport has the lowest fatality rate (0.3%).

Oral ingestion of *S. enterica* to cause human illnesses. There are four health conditions caused by *S. enterica*, Indicating enteric fever, diarrhea, bacteremia and chronic asymptomatic carriage. The gastroenteritis symptoms of salmonellosis may

include mild vomiting, fever, dehydration, headache or high fever, lethargy, abdomen and chest pain, meningitis and/or pneumonia. The infection period in human generally range from 8-72 h and symptoms may last for 2-7 days (Coburn et al., 2007). Carrasco et al. (2012) reported that *S. enterica* Enteritidis and Typhimurium are the most adapted serovars to human while Yates (2011) reported that Typhi and Paratyphi serovars cause enteric fever in human.

2.2.1 *Salmonella* occurrence in food and associated outbreaks

Meat, poultry and eggs are the main cause for *Salmonella* outbreaks. However, fruits, vegetables, milk, dairy products and fishery products also responsible for a significant number of *Salmonella* outbreaks in the USA. From 1998 to 2008, a total of 1491 confirmed *Salmonella* outbreaks have been reported to the CDC where 80% of cases are caused by a single serovar while 20% are caused by multiple serovars (Zarecki et al., 2013). In depth studies of these data indicate that there are notable relationships between *Salmonella* serovar and the type of food commodities that they are associated with. For example, *S. enterica* Enteritidis, Heidelberg, Hadar, and Infantis, cause outbreaks predominantly in animal-derived food commodities while *S. enterica* Newport, Braenderup and Typhimurium commonly cause outbreaks in fruit and vegetable products. Furthermore, *S. enterica* Poona, Newport and Javiana are highly associated with melon related outbreaks.

2.2.2 Pathogenesis of *Salmonella*

Salmonella cells have to be ingested orally to cause illnesses in human and animals. The nature of invasion of host cells varies depending on the type of serovar. *S.*

enterica Typhimurium infection in human and calves remains localized to intestinal and mesenteric lymph nodes while *S. enterica* Dublin can cause a more aggressive form of invasion in the host. The type III Secretion System (TTSS) is responsible for contact dependent secretion and delivery of *Salmonella* virulence proteins in to host cells. *S. enterica* uses two functionally distinct TTSS that are encoded on the pathogenicity islands SPI-1 and SPI-2 to transfer effector proteins into host cells. SPI-1 enables the bacterial invasion of epithelial cells while SPI-2 enables bacteria multiplying within membrane-bound vacuoles (Waterman and Holden, 2003). Dieye et al. (2009) have found that SPI-I helps *S. enterica* Typhimurium colonize in the cecum and spleen in the chicken. The effector protein SpiC, which is produce by SPI-II, is necessary for *S. enterica* to survive in macrophages during host cell invasion (Waterman and Holden, 2003).

2.3 Biofilms by *L. monocytogenes* and *Salmonella*

Biofilm is a collection of microbial cell communities that are firmly attached to a surface and embedded in an extra cellular matrix that is formed with polymeric substances. Biofilm cells are resistant to sanitizers, UV light and desiccation as compared to planktonic cells, predominantly due to the mechanical protection that is provided by the extra cellular polymeric matrix (Kumar and Anand, 1998). Bacteria form biofilms as their predominant form of living and exist as a single layer or multi-layers of microbial colonies with vertical and horizontal channels that distribute essential nutrients and water to inner layers (Djordjevic et al., 2002). Biofilm that are formed on food contact and non-food-contact surfaces in food industry can act as major sources of contaminations (Djordjevic et al., 2002).

Formation of biofilms is a very complex process and involves several steps. The first step is surface conditioning in which organic molecules from the environment are deposited on the hard surface. The second step is mass transport. In this step biologically active microbial cells are transported to the conditioned surface. In the third step, the microbial cells make initial attachments with the influence of Van Der Waals and electrostatic forces. In the final step, the attached cells initiate growth and form biofilm with the help of quorum sensing (Mizran et al 2015).

2.3.1 Factors affecting biofilm formation in *L. monocytogenes* and *Salmonella*

The process of biofilm formation is highly dependent on the nature of the growth media and microbial cell surface (Pan et al., 2010; Vatanyoopaisarn et al., 2000). The factors associated with growth media which affect the biofilm formation include nutrients, salt concentration, pH, temperature.

2.3.1.1 Temperature

L. monocytogenes cell attachment is greater at temperature around 30-37°C compared to low temperature conditions such as 4-10°C or greater temperature such as 42°C (Mai and Conner, 2007; Norwood and Gilmour, 2001). The temperature of growth media affects the hydrophobicity of the cell surface. At 30-37°C, the cell hydrophobicity is enhanced, which subsequently increases cell attachment to hard surfaces (Chavant et al., 2002; Di Bonaventura et al., 2008). Temperature also affects the flagella induced cell motility which can subsequently affect cell attachment at the beginning of biofilm production (Todhanakasem and Young, 2008; Vatanyoopaisarn et al., 2000). Di Bonaventura et al. (2008) reported that the motility of *L. monocytogenes* was drastically reduced at lower

temperature, where among 44 *L. monocytogenes* strains, 30 were motile at 22°C while only 4 strains were motile at 12°C.

2.3.1.2 Sodium chloride

NaCl residues can exist in the food processing environment and can enhance biofilm formation in foodborne pathogens. Jensen et al. (2007) reported that the presence of NaCl (0.5-5%) in TSB increases the adhesion of *L. monocytogenes* to plastic surfaces. In addition, Rode et al. (2007) stated that *Staphylococcus aureus* formed maximum biofilms in growth media contained a mixture of glucose (1%), NaCl (1%) and alcohol (<4%) as compared to growth media which contained each component separately. Xu et al. (2010) reported that hydrophobicity of *L. monocytogenes*, *S. aureus*, and *S.* enterica increased with increasing NaCl concentration from 0 to 8% while increasing cell auto-aggregation. Furthermore, the presence of NaCl in the growth media provides a heat protective effect that enhances the for growth and biofilm formation of foodborne pathogens such as *L. monocytogenes* and *S. aureus* (Juneja and Eblen, 1999; Rode et al., 2007).

2.3.1.3 pH

The pH of the growth medium plays an important role during biofilm formation in foodborne pathogens. *Pseudomonas fluorescens* produce more biofilms in neutral pH. This may be due to the high production of extra cellular polysaccharides in neutral pH conditions (Oliveira et al., 1994). Furthermore, the pH of the growth medium affects the hydrophobicity of the cell surface which subsequently affects the initial cell attachment and biofilm formation.

2.3.1.4 Surface

The properties of the solid surface are very important in bacterial biofilm production since they affect the initial cell attachment. Morgan and Wilson (2001) reported that surface roughness affects the early stages of biofilm formation of *Streptococcus oralis* on an acrylic surface. Moreover, cell attachment to hydrophilic surfaces such as stainless steel and glass is greater than hydrophobic surfaces such as buna-n rubber and plastic. The surfaces that are found in food industry can become rougher with continuous use and may form cracks which can trap food soils and bacteria cells. Jones et al. (1999) demonstrated that increase in surface roughness enhances the bacteria cell adhesion to polyethylene terephthalate bottles. Due to this reason food processing equipment are designed with highly polished and smooth surfaces.

2.3.1.5 Nutrient availability

The level of nutrients that are present in the growth media also affect the biofilm production of *L. monocytogenes* and *Salmonella*, while the effects may vary depending on the nature of the microorganism. For example, Stepanović et al. (2004) observed that *L. monocytogenes* produced a greater amount of biofilm in TSB when compared to 1/20 diluted TSB, while *Salmonella* spp. produce less biofilm in TSB when compared to 1/20 TSB. Moreover, Yang et al. (2014) reported that *S.* Enteritidis produced more cellulose in the extracellular polymeric substances matrix of biofilm when grown in 1/20 TSB as compared to an undiluted TSB.

2.3.1.6 Strain

L. monocytogenes serotype 1/2a strains forms biofilms with greater bacterial count than 1/2b and 4b (Borucki et al., 2003). Moreover, Di Bonaventura et al. (2008) reported that *L. monocytogenes* serotype 1/2c strains formed biofilms with more bacterial counts on stainless steel and glass when compared to strains belonging to serotype 1/2a, 1/2b or 4b at 37°C. However, it was recently reported that the biofilm formation ability of *L. monocytogenes* depends on the individual strains while the correlation between biofilm formation and *L. monocytogenes* serotype remained inconclusive (Doijad et al., 2015).

Vestby et al. (2009) reported that *Salmonella* spp. serovars differ for their ability to form biofilms. Studies indicate that, *S.* Agona and *S.* Montevideo formed more biofilms than *S.* Typhimurium. Similarly, Kroupitski et al. (2009) reported that *S.* Enteritidis, *S.* Virchow, *S.* Thompson, *S.* Typhimurium and *S.* Newport produced more biofilms compared to *S.* Hadar, *S.* Poona and *S.* Amager.

2.3.1.7 Other factors

The conditioning of the hard surface is an essential step in biofilm formation. Stainless steel and Teflon interact chemically with milk proteins more than other surfaces which can increase or decrease the subsequent bacterial cell attachment (McGuire and SWARTZEL, 1989). For example, Barnes et al. (1999) reported that milk proteins such as a-casein, b-casein, k-casein, and a-lactalbumin reduce the adhesion of *S. aureus* and *L. monocytogenes* cells to stainless steel surfaces.

2.3.2 Genetic regulation of biofilm production

Biofilm production by foodborne pathogens is regulated by numerous gene regulators (Hall-Stoodley and Stoodley, 2002). Todhanakasem and Young (2008) reportedthat flagella and motility mutants of *L. monocytogenes* (*flgL* and *motA)* had less ability to attach to 96-well polyvinyl chloride microtiter plates as compared to wild type cells. This suggests that flagella based motility is important for biofilm formation. However, these researchers observed no significant difference in biofilm production among these mutants and wild type cells in the later stages of biofilm development. This suggests that flagella and cell motility are not important to *L. monocytogenes* during biofilm maturation. Similarly, Vatanyoopaisarn et al. (2000) reported that flagella act as an adhesive structure during early stages of cell attachment in *L. monocytogenes* and mutation in *flaA* gene reduces initial cell attachment by 10-fold as compared to wild-type cells at 22°C. Moreover, flagella help to keep microbial cells together within the biofilms. Most bacterial cells naturally possess a net negative charge which tends to keep microbial cells apart from each other. However, the fimbria, flagella and lipopolysaccharides that are present in cell surface create a hydrophobic nature around the cells that keeps the cells together overcoming negative repulsive forces.

The agr (accessory gene regulator) has an important role in biofilm formation of *L. monocytogenes* (Riedel et al., 2009; Rieu et al., 2007). Among the 4 main genes that belonging to the agr regulator (*agrA*, *agrB*, *agrC*, and *agrD*), mutation in *agrA* and *agrD* significantly reduces biofilm formation in *L. monocytogenes* as compared to wildtype cells.

Lemon et al. (2010) reported that *L. monocytogenes* lacking a *PrfA* regulator showed significantly less adhesion to 96-well polyvinyl chloride microtiter plates as compared to parent cells. The known function of *PrfA* regulator is switching the extra cellular *L. monocytogenes* to intracellular pathogen by expressing multiple virulence genes in *L. monocytogenes*. Vander Veen and Abee (2010) reported that sigB, the major stress response regulator in *L. monocytogenes* is required to form biofilm in *L. monocytogenes* where mutant of sigB forms weak biofilms when compared to wild type *L. monocytogenes* cells.

LuxS is another gene that has an important role in biofilm formation of *L. monocytogenes*. However, Sela et al. (2006) reported that unlike *agrA*, *agrD*, *PrfA* and *SigB* mutants, *luxS* mutants produce 19-fold denser biofilms on glass surface as compared to the parent strain of *L. monocytogenes,* EGD serovar 1/2a. Moreover, Alonso et al. (2014) reported that mutation in genetic loci, *dltABCD* and *phoPR* significantly reduces biofilm production in *L. monocytogenes* on 96-well polyvinyl chloride microtiter plates as compared to wild type cells.

2.4 Foodborne disease outbreaks associated with cantaloupes

Cantaloupe (*Cucumis melo* L.) belongs to the family of cucurbitaceae along with squash, pumpkin, cucumber and watermelon. In the USA, cantaloupe is mainly grown in California, Arizona, Colorado, Georgia, Maryland, Pennsylvania, South Carolina and Texas. To meet the year-round demand, cantaloupe is imported from other countries such as Mexico, Guatemala, Honduras and Costa Rica. According to the USDA National Agricultural Statistic Service (NASS) and Economics, Statistics and Marketing Information System (ESMIS), the leading state in cantaloupe production in the USA is

California, which produced 1491.2 million pounds in 2014. The farm value for cantaloupe has gradually increased from $13.8/100lb in 2004 to $17.2/100lb in 2012. It is important to note that even though there was an increasing trend in farm value from 2004 to 2012, the per capita consumption of cantaloupe has rapidly declined from 11.1 lb. in 2002 to 7.6 lb. in 2012. One possible reason for this could be high frequency of food outbreaks associated with cantaloupe products during that time period.

There are several cantaloupe varieties grown in the USA. The majority of these varieties have similar taste but can differ in flesh color, peel texture, size and shape. The reticulatus cantaloupe variety is round in shape, has an orange color mesocarp and light brown color netted-like rind. The average pH of cantaloupe ranges from pH 6.4-6.7. The cantaloupe flesh is rich in water (86-91%) and dietary fiber while low in proteins and lipids. Therefore, cantaloupe is not considered as an energy dense fruit. However, proximate analysis of cantaloupe reveals that it is rich in vitamin A and C and therefore considered high in anti-oxidant capacity. Moreover, cantaloupe is rich in minerals such as Mg, Ca, Cu, Mn, Na while poor in K (Laur and Tian, 2011). The titratable acidity of cantaloupe is approximately 0.5-0.8 mg/ml (citric acid). Cantaloupe has a total phenolic content of 0.3-0.5 µg/g (catechine), total antioxidant capacity of 90-570 nmol/ g (ascorbic acid) and a total flavonoids of 31-63 µg/g (ctechine eq). (Maietti et al., 2012).

Due to low acidity and high water content, cut cantaloupe is considered as a potentially hazardous food according to the USFDA Food code. Since cantaloupe is grown close to the ground, there is a moderate to high risk of pathogenic contaminations that is associated with soil and animals than some other fruits. Specifically, the netted rind makes cantaloupe more prone to microbial contaminations. Cantaloupe is one of the

leading cause of outbreaks. There have been 34 outbreaks associated with consumption of different melons (cantaloupe, watermelon, honeydew melon) reported to the CDC from 1973-2011. These outbreaks have resulted in a total of 3602 illnesses, 322 hospitalizations, 46 deaths and 3 fetal losses. More importantly, 56% of these outbreaks have been caused by contaminated cantaloupe. *Salmonella*, Norovirus, *Campylobacter jejuni*, *Escherichia coli*, *L. monocytogenes* are reported to be the most common foodborne pathogens in cantaloupe products. Among these foodborne pathogens, *Salmonella* have caused 58% while *L. monocytogenes* caused 5% of cantaloupe associated outbreaks (Walsh et al., 2014). Bowen et al. (2006) reported that 23 outbreaks associated with cantaloupe have been reported to the CDC from 1984-2002 resulting 1434 illnesses, 42 hospitalizations and 2 deaths. According to their analysis, all these outbreaks have been caused by five serotypes of *Salmonella*, *C. jejuni*, *E. coli* O157:H7 and Norovirus. Among these pathogens, *Salmonella* has caused 39% of outbreaks, 59% of illnesses and 91% of hospitalizations. It is noteworthy that there have been no listeria outbreaks reported in cantaloupe from 1984-2003.

The most-deadliest listeriosis outbreak in the USA history since 1920 which occurred in 2011 was associated with whole cantaloupe produced by Jenesen farm, Colorado. Through August to October 2011, this outbreak resulted 147 total cases, 33 deaths and 1 miscarriage (McCollum et al., 2013). The investigations conducted by FDA stated that 12 out of 39 environmental samples that were taken in the cantaloupe processing facility were positive for *L. monocytogenes* strains that caused illnesses in people. All these positive samples have been collected from equipment used for cantaloupe processing. All the environmental samples collected from cantaloupe

production field were negative for *L. monocytogenes* suggesting that *L. monocytogenes* contamination has occurred inside the processing plant from contaminated equipment. The investigators have noted several possibilities for *L. monocytogenes* contamination which include inadequate facility and equipment design that can preclude effective cleaning and sanitation.

One year after the 2011 listeriosis outbreak, a *Salmonella* outbreak hit the cantaloupe industry with cantaloupe produced by Chamberlain Farms Produce of Owensville, Indiana. This outbreak spread in 24 states and resulted 261 illnesses, 94 hospitalizations and 3 deaths. The investigations conducted by FDA revealed that cantaloupe contaminations have primarily occurred at the field from contaminated soil and then inside the processing plant from contaminated equipment.

Collectively, these investigations show that maintaining a clean environment in cantaloupe processing facilities is extremely important to avoid cantaloupe outbreaks since even a small number of pathogenic bacteria can deposit on processing surfaces and promote growth and biofilm formation when appropriate conditions are met creating very dangerous consequences.

CHAPTER III

GROWTH AND BIOFILM FORMATION BY *LISTERIA MONOCYTOGENES* IN CANTALOUPE FLESH AND PEEL EXTRACTS ON FOUR FOOD-CONTACT SURFACES AT 22°C AND 10°C

3.1 Abstract

The nationwide listeriosis outbreak that occurred in the USA during 2011 demonstrated the importance of preventing cantaloupe contamination with *Listeria monocytogenes* (*Lm*) within farm and processing environments. The objectives of this study was to determine the effects of strain and temperature on growth and biofilm formation of *Lm* in cantaloupe flesh and peel extracts on different food-contact surfaces. Growth of *Lm* strains was greater at high concentration of cantaloupe extracts and temperature in comparison to low concentration and temperature. For 50 mg/ml of cantaloupe extract inoculated with 3 log CFU/ml, the growth of *Lm* was 8.5 log CFU/ml in 32 h at 22°C and 6-7 log CFU/ml in 72 h at 10°C. For 2 mg/ml of cantaloupe extract that was inoculated with 3 log CFU/ml, the growth of *Lm* was 7-7.5 log CFU/ml in 72 h at 22°C and 3.5 log CFU/ml in 72 h at 10°C. There were no differences ($P < 0.05$) among *Lm* strains for biofilm formation in cantaloupe extracts, but biofilm formation was greater at high temperature and high concentration. For 50 mg/ml cantaloupe extracts inoculated with 3 log CFU/ml, the biofilm formation of *Lm* on stainless steel surface was approximately 7 log CFU/coupon at 22°C in 4-7 days and 5-6 log CFU/coupon at 10°C in

7 days. For 2 mg/ml cantaloupe extracts inoculated with 3 log CFU/ml, the biofilm formation of *Lm* on the stainless steel surface was approximately 5-6 log CFU/coupon at 22°C and 4-4.5 log CFU/coupon at 10°C in 7 days. The biofilm formation by cantaloupe outbreak strain *Lm* 2011L-2625 in cantaloupe extracts was least on buna-n rubber when compared to stainless steel, polyethylene and polyurethane surfaces. These findings show that a very low concentration of nutrients from cantaloupe flesh or peel can induce *Lm* growth and subsequent biofilm formation on different food-contact processing surfaces.

3.2 Introduction

Listeria monocytogenes is a Gram-positive rod shape, non-spore forming, facultative anaerobic, bacterium which is responsible for listeriosis in humans and animals (Gandhi and Chikindas, 2007). Among all the foodborne pathogens, *L. monocytogenes* is the most dangerous due to its high mortality rate of 20-25% (Todd and Notermans, 2011). Listeriosis outbreaks are associated with fresh or frozen fruits and vegetables, raw milk, processed dairy products, and ready-to-eat meat products (Kramarenko et al., 2013; Wang et al., 2013). The Center for Disease Control estimates that 1600 illnesses and 260 deaths occur annually due to listeriosis in the United States (Scallan et al., 2011). The largest multi-state listeriosis outbreak in the USA in 2011 was associated with whole cantaloupes which caused 33 deaths and one miscarriage (McCollum et al., 2013). Among the 13 known serotypes of *L. monocytogenes*, strains that belong to serotypes 1/2a, 1/2b and 4b are mostly associated with foodborne listeriosis outbreaks (Borucki et al., 2003; Nelson et al., 2004) .

L. monocytogenes is commonly found in soil, sewage and fecal matter (Locatelli et al., 2013). Cantaloupe can often be contaminated with pathogens that are associated

with soil and animals since it is a fruit that is grown close to the ground. The outer rind of cantaloupe fruit provides an excellent surface for pathogenic microorganisms to attach and hide (Webb et al., 2015a, b). *L. monocytogenes* cells that are attached to the rind can contaminate cantaloupe-processing environments and promote growth and subsequent biofilm formation on food-contact and non-food contact surfaces when appropriate environmental conditions exist. The investigations conducted by U.S. Food and Drug Administration during the multi-state cantaloupe outbreak in 2011 have found the presence of *L. monocytogenes* on the food-contact and non-food contact surfaces of the cantaloupe processing facility of Jensen farm, Colorado (McCollum et al., 2013).

A biofilm is a collection of microbial cell communities that are attached to a surface and embedded in a self-produced matrix that consists of polysaccharides (Vestby et al., 2009; Whitehead and Verran, 2015). Biofilms are the most predominant form of bacteria in food processing environments since microbial cells have extra protection from desiccation, UV light, antimicrobials and sanitizing agents (Bernbom et al., 2011; Vestby et al., 2009). Biofilms formed on the food-contact surfaces can cause cross-contamination when improperly cleaned, thus creating a serious food safety issue.

L. monocytogenes is capable of adhering to various types of food-contact surfaces that are found in the food processing environments (Di Bonaventura et al., 2008). Attachment and biofilm formation of *L. monocytogenes* is influenced by the physiochemical properties of environment (nutrients, temperature, pH, salt concentration) as well as the cell surface (hydrophobicity, flagellation and motility) (Di Bonaventura et al., 2008; Kalmokoff et al., 2001). Survival, growth and biofilm formation in standard microbiology growth media such as tryptic soy broth and brain heart infusion may not

reflect the actual behavior of *L. monocytogenes* in food processing environments. This is because the media contain optimum levels of all of the nutrients that are necessary for microbial growth. Beuchat et al. (1986) studied the growth of *L. monocytogenes* in cabbage juice at 5°C and observed that *L. monocytogenes* was capable of growing in sterile cabbage juice containing <5% NaCl. In contrast, *L. monocytogenes* growth was observed when ≤10% NaCl was present in tryptic phosphate broth. Kim et al. (2006) studied the biofilm formation of *Enterobacter sakazakii* in different growth media at 25°C and found that *E. sakazakii* did not form biofilms on the stainless steel containing lettuce juice broth but produced biofilms on the same surfaces in infant formula broth. This finding suggests that the growth and biofilm formation of foodborne bacterial pathogens can drastically differ depending on the growth media.

In cantaloupe processing environments, *L. monocytogenes* can be exposed to nutrients that are leaked from cantaloupe fruits. Both cantaloupe flesh and peel contain nutrients such as sugar, proteins, carotenoids, vitamin C and various minerals that are essential for microbial growth (Koubala et al., 2016). There are no published data on the growth and biofilm formation of *L. monocytogenes* in cantaloupe residue that persists on the food processing surfaces. Penteado and Leitao (2004a) studied the growth of *L. monocytogenes* in low-acid fruit pulps (melon, watermelon and papaya) at 10°C, 20°C and 30°C. According to their study, the growth of *L. monocytogenes* varied depending on the fruit type.

Even though food-soils are removed from the cantaloupe processing environments routinely, residues may persist on the food-contact surfaces when they are improperly cleaned (Fryer and Asteriadou, 2009). There is a need for critical

understanding of the survival, growth and biofilm formation that reflects the actual behavior of *L. monocytogenes* in cantaloupe residue when it is present on food processing surfaces. Therefore, the objectives of the present study were to determine the effects of strain, temperature, nutrient level, and food-contact surface on the growth and biofilm formation of *L. monocytogenes* in cantaloupe flesh and peel extracts.

3.3 Materials and methods

3.3.1 Bacterial strains and culture conditions

L. monocytogenes strains that were used in this study are described in Table 3.1. The working stock cultures of these strains were prepared by inoculating a colony grown on PALCAM agar onto tryptic soy agar that contained 0.6% yeast extract (TSAYE) slants and incubated at 37°C for 24 h. These working stocks were stored at 4°C for 4-5 weeks. The overnight culture of each strain was prepared by inoculation of 10 ml of tryptic soy broth that contained 0.6% yeast extract (TSBYE) from the working stocks and incubated at 37°C for 18 to 20 h with shaking at 150 rpm (Imperial III incubator, Lab-Line Instrument Inc., IL, USA).

3.3.2 Food-contact surfaces preparation

Four types of food-contact surfaces that are commonly used in the cantaloupe processing industry were obtained (Table 3. 2) and were cut into 1 cm×1.5 cm size coupons. These coupons were soaked in an alkali detergent for 1 h and washed thoroughly with deionized water. Washed stainless steel coupons were autoclaved at 121°C for 15 min. Buna-n rubber, ultra-high molecular weight polyethylene and thermoplastic polyurethane coupons were surface sterilized by submerging in 100%

alcohol for 15 min prior to washing with sterile water to eliminate alcohol residue. Sterile coupons were surface dried inside a biosafety cabinet prior to use.

3.3.3 Preparation of cantaloupe extracts

Cantaloupe (*Cucumis melo* L. var. reticulatus) fruits were obtained from a local supermarket and washed under running tap water for 1 min. The peel was then removed and the flesh was deseeded. In order to prepare cantaloupe flesh and peel extracts, 100 g of cut cantaloupe flesh or peel was blended with 400 ml of deionized water for 30 seconds using a juice extractor (Hamilton beach, Model: 53514). The blended flesh and peel extracts were then filtered using a filter bag (BA6141/STR) and centrifuged (2500 × g) (Beckman, Model TJ-6 centrifuge) for 5 min to eliminate coarse particles. Cantaloupe extracts were autoclaved at 121°C for 15 min and stored at room temperature until needed. These cantaloupe extracts (200 mg/ml) were further diluted in physiological saline to obtain 50 mg/ml and 2 mg/ml of concentrations, respectively, based on the fresh weight of the cantaloupe.

3.3.4 Determination of physical/chemical properties of cantaloupe extracts

The pH of sterile cantaloupe flesh and peel extracts was determined using a calibrated pH meter (Accumet Basic, AB15 pH meter, Fisher Scientific). Brix values were determined using a refractometer (RHB-18ATC, GxPro). In order to determine the dry weight of cantaloupe flesh or and peel extract, 25 ml of each extract was transferred to a pre-weighed 50 ml centrifuge tube. These extracts were freeze dried until a constant weight was obtained. The dry weight was calculated using the following equation.

$$\text{Dry weight } \left(\frac{\text{mg}}{\text{ml}}\right) = \frac{(\text{Post dry weight of the tube} - \text{Weight of the empty tube})\text{mg}}{\text{Volume of extract (ml)}} \quad (3.1)$$

3.3.5 Evaluation of growth of *L. monocytogenes* in cantaloupe extracts at different temperatures

Growth of *L. monocytogenes* EGD (Bug600), 2011-2624 and G1091 in 50 and 2 mg/ml of cantaloupe flesh and peel extracts at 22°C and 10°C was studied. The overnight cultures of these strains were prepared as described previously. One ml volumes of overnight cultures were centrifuged for 5 min (9000 ×g, MARATHON 21000R, Fisher Scientific). The resulting cell pellets were resuspended in 1 ml of physiological saline to obtain 9 log CFU/ml of stationary phase cells.

Cantaloupe flesh or peel extracts (50 and 2 mg/ml) were inoculated with the *L. monocytogenes* stationary phase cells to yield an initial cell concentration of 3 log CFU/ml. The inoculated cantaloupe extracts were incubated at 10°C (VWR, Scientific Products, Sheldon manufacturing, INC., OR, 97113) and at room temperature (22°C). Cell numbers were enumerated for 72 h at 8 h intervals by serially diluting an aliquot from the cell suspensions in physiological saline and plating on tryptic soy agar that was enriched with 0.6% yeast extract, Escullin and Ferric ammonium citrate (TSA-YEEF). These plates were incubated at 37°C (Imperial III Incubator, Lab line instrument Inc., IL, USA) for 48 h to obtain colony forming unit (CFU) counts.

3.3.6 Calculation of generation time

The generation time (g) was calculated from the slope of the line of the semi logarithmic plot of exponential growth using the mean of 4 replications. The equation of

g = 0.301/slope of the semi logarithmic plot of exponential growth was used to calculate the generation time as described previously (Penteado and Leitao, 2004a, b).

3.3.7 Evaluation of biofilm formation by different strains of *L. monocytogenes* in cantaloupe extracts at different temperatures

One ml from each overnight culture (Table 3.1) was centrifuged, and the resulting cell pellets were resuspended in 1 ml of physiological saline. Cantaloupe flesh or peel extracts (50 and 2 mg/ml) were inoculated with stationary phase cells to obtain a 3 log CFU/ml initial cell concentration. Sterile stainless steel coupons were placed in polystyrene 24-well plates with one coupon per well. Each well was filled with 2 ml of cell suspension that was prepared in either cantaloupe flesh or peel extract. These coupons were incubated at 22°C or 10°C for 1, 4 or 7 days.

3.3.8 Enumeration of biofilms

In order to enumerate biofilm formation at the end of 1, 4 or 7 days, used growth media was removed from the wells and coupons were washed twice by adding 2.75 ml of physiological saline to remove loosely bound cells. Washed coupons were transferred into plastic tubes (15 ml-Tronado^{TM}) each containing 5 ml of 0.1% peptone water with 0.02% Tween 80 and five sterile glass beads. Tubes were vortexed for 1 min (Vortex mixer, Labnet International, Inc, Edison, NJ, USA) and aliquots of detached cell suspensions were serially diluted in physiological saline. Each dilution was plated on TSA-YEEF and incubated at 37°C for 48 h.

3.3.9 Effect of the nature of the food-contact surface on biofilm formation by *L. monocytogenes* 2011-2625 in cantaloupe extracts at different temperatures

Stainless steel, buna-n rubber, ultra-high molecular weight polyethylene and thermoplastic polyurethane coupons were placed in 24-well plates with one coupon per well. *L. monocytogenes* 2011L-2625 cell suspension of 2 ml at 3 log CFU/ml was prepared in cantaloupe flesh or peel extract (50 and 2 mg/ml) and added to each well. These coupons were incubated at 22°C and 10°C for 1, 4 or 7 days. At the end of each incubation period, biofilm formation on the four different surfaces was enumerated as described previously.

3.3.10 Statistical analysis

A completely randomized design with a 2-way factorial structure and 4 replications was used. Mean values (log CFU/ ml or log CFU/coupon) were calculated using four values obtained and analysis of variance with Tukey's honestly significant difference test ($P < 0.05$) was performed to separate means where significant differences occur. For the comparison study of biofilm formation among 6 strains, the two factors included strain and temperature. For the comparison of average biofilm formation of *L. monocytogenes* in 1, 4 and 7 days, the days were compared with combination of concentration and temperature combination. For the comparison of biofilm formation of *L. monocytogenes* 2011-2625 on different food contact surfaces, the two factors included surfaces and temperature ($P < 0.05$).

3.4 Results

3.4.1 Growth curves of *L. monocytogenes* in cantaloupe extracts

The growth rate of *L. monocytogenes* in cantaloupe flesh and peel extracts was greater at higher cantaloupe extract concentration and higher temperature. The growth curves of *L. monocytogenes* in cantaloupe flesh at 50 and 2 mg/ml at 22°C and 10°C are shown in Figure 3.1. In 50 mg/ml cantaloupe flesh, the cell numbers of *L. monocytogenes* Bug600, 2011L-2625 and G1091 increased by 5.5 log CFU/ml in 32-40 h at 22°C and remained stable at that level afterwards, while *L. monocytogenes* increased by 3.5 log CFU/ml in 72 h at 10°C (Fig. 3. 1ACE). In 2 mg/ml cantaloupe flesh extract, the cell numbers of *L. monocytogenes* strains Bug600, 2011L-2625 and G1091 were increased by 4.5 log CFU/ml in 56 h at 22°C and 0.5 log CFU/ml in 72 h at 10°C (Fig. 3. 1BDF). In 50 and 2 mg/ml cantaloupe flesh extract, the cell number of *L. monocytogenes* from 8 – 72 h was greater ($P < 0.05$) at 22°C when compared to 10°C.

A similar pattern was also observed for the growth of *L. monocytogenes* in cantaloupe peel extract at 50 and 2 mg/ml concentrations at 22°C and 10°C (Fig. 3.2). In 50 mg/ml cantaloupe peel extract, the cell numbers of *L. monocytogenes* Bug600, 2011L-2625 and G1091 were increased by 5.5 log CFU/ml at 22°C in 24-40 h and remained stable at that level afterwards, while *L. monocytogenes* strains were increased by 3-4 log CFU/ml at 10°C in 72 h (Fig. 3. 2ACE). In 2 mg/ml cantaloupe peel extract, the cell numbers of *L. monocytogenes* strains Bug600, 2011L-2625 and G1091 were increased by 4-4.5 log CFU/ml in 48-56 h at 22°C and 0.5 log CFU/ml at 10°C in 72 h (Fig. 3. 2BDF). For 50 and 2 mg/ml cantaloupe peel extract, the cell number of *L. monocytogenes* from 8 – 72 h was greater ($P < 0.05$) at 22°C when compared to 10°C.

The lag phase of *L. monocytogenes* strains was less than 8 h in 50 and 2 mg/ml cantaloupe flesh or peel extracts at 22°C while it was extended to 16-18 h in 50 mg/ml or 72 h in 2 mg/ml cantaloupe extracts at 10°C.

In 50 mg/ml cantaloupe extracts, the generation time was markedly increased by 2-4 h by shift of temperature from 22°C to 10°C for all *L. monocytogenes* strains tested. In 2 mg/ml cantaloupe extract, the generation time of *L. monocytogenes* extended by about 2-4 h beyond the corresponding generation time in 50 mg/ml cantaloupe extract at 22°C. Since no exponential growth was observed during the duration of 72 h, the generation time was not calculated for *L. monocytogenes* in 2 mg/ml at 10°C.

3.4.2 Influence of strain and temperature on biofilm formation by *L. monocytogenes* on stainless steel surface containing cantaloupe extracts

Biofilm formation by *L. monocytogenes* strains was greater in 50 mg/ml cantaloupe extract at 22°C ($P < 0.05$) as compared to 2 mg/ml at 10°C. In 50 mg/ml cantaloupe flesh extract, biofilm formation of *L. monocytogenes* strains was 5-6 log CFU/coupon in 1 d and 7 log CFU/coupon in 4 or 7 d at 22°C. At 10°C biofilm formation was 1.5 to 3.5 logs less ($P < 0.05$) than that observed at 22°C (Fig. 3. 3ACE). In 2 mg/ml cantaloupe flesh extract, biofilm formation by *L. monocytogenes* was 4.5 log CFU/coupon in 1 d or 5-6 log CFU/coupon in 4 or 7 d at 22°C. At 10°C biofilm formation was 1 to 2.5 logs less ($P < 0.05$) than that at 22°C (Fig. 3. 3BDF). With one exception, there were no differences ($P < 0.05$) in biofilm formation among the six strains of *L. monocytogenes* in cantaloupe flesh extract under all environmental condition tested. For 50 mg/ml cantaloupe flesh extract, *L. monocytogenes* Bug600 had 1 log less biofilm formation in 1 day as compared to other strains at 22°C.

A similar pattern was also observed for the biofilm formation by six strains of *L. monocytogenes* in cantaloupe peel extract at 50 and 2 mg/ml concentrations at 22°C and 10°C (Fig. 3. 4). In 50 mg/ml cantaloupe peel extract, biofilm formation of *L. monocytogenes* strains was approximately 6 log CFU/coupon in 1 d and 7 log CFU/ coupon in 4 or 7 d at 22°C. At 10°C biofilm formation was 1.5 to 4 logs less ($P < 0.05$) than that at 22°C (Fig. 3. 4ACE). In 2 mg/ml cantaloupe peel extract, biofilm formation by *L. monocytogenes* strains were approximately 4-5 log CFU/coupon in 1 d or 5-6 log CFU/coupon in 4 or 7 d at 22°C while their corresponding biofilm formation at 10°C was 1.5 to 3 logs less ($P < 0.05$) than that observed at 22°C (Fig. 3. 4BDF). No significant differences in biofilm formation were observed among the six strains of *L. monocytogenes* in cantaloupe peel extract under all environmental condition tested with the exception of 50 mg/ml cantaloupe flesh extract in 1 day at 10°C, where *L. monocytogenes* G1091 had 1 log greater biofilm formation as compared to other strains.

In 50 mg/ml cantaloupe flesh or peel extract, there was no increase ($P < 0.05$) in average biofilm formation by *L. monocytogenes* observed from 4 to 7 d at 22°C, but there was 1.5 - 2.0 log increase observed at 10°C (Table 3. 5). In 2 mg/ml cantaloupe flesh or peel extract, there was no increase ($P < 0.05$) in average biofilm formation by *L. monocytogenes* strains observed from 4- 7 d at both 22°C and 10°C.

3.4.3 Biofilm formation by *L. monocytogenes* 2011L-2625 on different food-contact surfaces containing cantaloupe extracts

In 50 mg/ml cantaloupe flesh extract inoculated with 3 log CFU/ml, *L. monocytogenes* 2011L-2625 biofilm formation was approximately 6.5 log CFU/coupon in 1 d or 7 log CFU/coupon in 4 or 7 d on stainless steel, polyethylene and polyurethane

surfaces 22°C. The corresponding biofilm formation on buna-n rubber was 2.0-3.5 logs less ($P < 0.05$) than that observed on the other three surfaces at 22°C (Fig. 3. 5ACE). In 50 mg/ml cantaloupe flesh extract, *L. monocytogenes* 2011L-2625 formed 1-2 log CFU/coupon biofilm on all the four surfaces in 1 d at 10°C. However, *L. monocytogenes* 2011L-2625 formed 5-6 log CFU/coupon biofilms on stainless steel, polyethylene and polyurethane surfaces in 4 or 7 d at 10°C while its corresponding biofilm formation on buna- n rubber was 2 logs less ($P < 0.05$) than that observed on other surfaces at 10°C.

In 2 mg/ml cantaloupe flesh extract, *L. monocytogenes* 2011L-2625 biofilm formation was approximately 4 log CFU/coupon in 1 d or 6 log CFU/coupon in 4 or 7 d on the stainless steel, polyethylene and polyurethane surfaces. The corresponding biofilm formation on buna-n rubber was approximately 1.5 logs less ($P < 0.05$) than that observed on the other three surfaces at 22°C (Fig. 3. 5BDF). In 2 mg/ml cantaloupe flesh extract, biofilm formation by *L. monocytogenes* 2011L-2625 was approximately 2 log CFU/coupon on all the four surfaces in 1 d at 10°C. However, *L. monocytogenes* 2011L-2625 formed 5.5-6 log CFU/coupon of biofilm on the stainless steel, polyethylene and polyurethane surfaces in 4 or 7 d while its corresponding biofilm formation on buna-n rubber was 1-1.5 logs less ($P < 0.05$) than that observed on the other surfaces at 10°C.

In 50 mg/ml cantaloupe peel extract, *L. monocytogenes* 2011L-2625 biofilm formation was approximately 6.5-7.0 log CFU/coupon in 1, 4 or 7 d on stainless steel, polyethylene and polyurethane surfaces while its corresponding biofilm formation on buna-n rubber was 2.5-3.5 logs less ($P < 0.05$) than that observed on the other three surfaces at 22°C (Fig. 3. 6ACE). In 50 mg/ml cantaloupe peel extract, *L. monocytogenes* 2011L-2625 formed 1-2 log CFU/coupon biofilm on all four surfaces in 1 d at 10°C.

However, *L. monocytogenes* 2011L-2625 formed 4.5-6.0 log CFU/coupon biofilms on stainless steel, polyethylene and polyurethane surfaces in 4 or 7 d while its corresponding biofilm formation on buna-n rubber was 1-2 logs less ($P < 0.05$) than that observed on other surfaces at 10°C.

In 2 mg/ml cantaloupe peel extract, *L. monocytogenes* 2011L-2625 biofilm formation was 4-5 log CFU/coupon in 1 d or 6 log CFU/coupon in 4 or 7 d on stainless steel, polyethylene and polyurethane surfaces. The corresponding biofilm formation on buna-n rubber was approximately 1-2 logs less ($P < 0.05$) than that observed on the other surfaces at 22°C (Fig. 3. 6BDF). In 2 mg/ml cantaloupe flesh extract, biofilm formation by *L. monocytogenes* 2011L-2625 was approximately 2 log CFU/coupon on all the four surfaces in 1 d at 10°C. However, *L. monocytogenes* 2011L-2625 formed 6 log CFU/coupon biofilms on stainless steel, polyethylene and polyurethane surfaces in 4 or 7 d while the corresponding biofilm formation on buna-n rubber was 1-2 logs less ($P < 0.05$) than that observed on other surfaces at 10°C.

Overall, biofilm formation of *L. monocytogenes* 2011L-2625 on buna-n rubber was significantly lower than the biofilm formation on stainless steel, polyethylene and polyurethane surfaces in 50 and 2 mg/ml cantaloupe flesh and peel extract concentrations at 22°C and 10°C.

3.5 Discussion

In the cantaloupe processing environments, nutrients from cantaloupe fruits can leak and accumulate on food contact surfaces when improperly cleaned. Such residues may promote the growth and subsequent biofilm formation by *L. monocytogenes* on the food processing surfaces. This study focused on the critical understanding of the survival,

growth and biofilm formation that reflected the actual behavior of *L. monocytogenes* in cantaloupe residue if present at two concentrations on varying processing surfaces. The pH of both cantaloupe flesh and peel extracts were around 6.4-6.5 and was optimum for *L. monocytogenes* growth. The brix value indicated that cantaloupe flesh extract contained more soluble solids than the peel extract which was also reflected in the dry weight that was calculated for each extract. This was probably due to the high sugar content in cantaloupe flesh when compared to peel.

Results indicate that concentration as low as 2 mg/ml of cantaloupe flesh and peel extract is an adequate substrate for *L. monocytogenes* to induce growth. The influence of temperature on the growth rate of *L. monocytogenes* was evident in 50 mg/ml cantaloupe flesh extract where all *L. monocytogenes* strains yielded a doubling time of approximately 2 h at 22°C and 4-6 h at 10°C. Similarly, Penteado and Leitao (2004a) and de Modelos (2007) observed that the generation time of *L. monocytogenes* ScottA in cantaloupe pulp increased from 0.84 h to 1.74 h or from 1.74 h to 7.12 h, respectively, when the growth temperature was decreased from 30°C to 22°C or from 22°C to 10°C.

The concentration of cantaloupe extract in the growth media also affected the generation time of *L. monocytogenes.* For example*,* for 50 mg/ml cantaloupe extracts, *L. monocytogenes* strains yielded a generation time of approximately 2 h as compared to 4.5-5.5 h for 2 mg/ml cantaloupe extract at 22°C. Overall, the lesser temperature and decreasing nutrient concentration in the growth media, there was an increase in the doubling time that inversely decreased the growth rate of *L. monocytogenes*.

Results from the current study indicate that low temperature (10°C) is not a barrier for *L. monocytogenes* growth in cantaloupe processing environments. For

example, all *L. monocytogenes* strains showed rapid growth at 10°C in 50 mg/ml cantaloupe extracts. However, there was no significant growth until 72 h in 2 mg/ml cantaloupe extracts suggesting that the growth of *L. monocytogenes* was inhibited at 10°C when the available nutrients were limited.

A stainless steel surface was used for the comparison study of biofilm formation by *L. monocytogenes* strains belonging to serotypes 1/2a, 1/2b and 4b since it is the most commonly found surface in food processing environments (Hilbert et al., 2003). With few exceptions, there were no differences ($P < 0.05$) in biofilm formation among the six *L. monocytogenes* strains that were tested in cantaloupe flesh or peel extract under different environmental conditions. Previously, it was reported that *L. monocytogenes* strains belonging to serotype 1/2a formed biofilms with greater bacterial counts than 1/2b and 4b (Borucki et al., 2003). Di Bonaventura et al. (2008) also reported that *L. monocytogenes* strains belonging to serotype 1/2c formed biofilms with greater bacterial counts on stainless steel and glass when compared to strains belonging to serotype 1/2a, 1/2b or 4b at 37°C. However, it was recently reported that the ability of *L. monocytogenes* to form biofilms depends on the individual strains while the correlation between biofilm formation and *L. monocytogenes* serotype remained inconclusive (Doijad et al., 2015).

L. monocytogenes biofilm formation was greater ($P < 0.05$) at 22°C when compared to 10°C in 1, 4 or 7 days in both 50 and 2 mg/ml cantaloupe flesh concentrations. Similarly, Di Bonaventura et al. (2008) reported that *L. monocytogenes* biofilm formation was greater at 37°C or 22°C when compared to 12°C or 4°C. This may be due to the increase in cell hydrophobicity with increasing temperature, which

enhances the subsequent cell attachment to hard surfaces (Chavant et al., 2002; Di Bonaventura et al., 2008). Flagella induced cell motility is required for cell attachment and the initiation of biofilm formation (Todhanakasem and Young, 2008; Vatanyoopaisarn et al., 2000). Di Bonaventura et al. (2008) found that the motility of *L. monocytogenes* was drastically reduced at lower temperature, where among 44 *L. monocytogenes* strains, 30 were motile at 22°C while only 4 strains were motile at 12°C. This suggests that at 10°C, the decreased motility of *L. monocytogenes* could result in lower cell attachment and subsequently less biofilm formation on stainless steel as compared to 22°C in cantaloupe extract. Ochiai et al. (2014) reported that the persistent *L. monocytogenes* strains that were obtained from a chicken processing plant had enhanced ability to alter biofilm formation in response to changing the temperature from 30°C to 37°C when compared to non-persistent *L. monocytogenes* strains. These findings suggest that regulation of biofilm formation through temperature control in *L. monocytogenes* may be significant for its survival and growth in food processing environments.

Biofilm formation of foodborne pathogens under very low concentrations of nutrients has been studied by many researchers (Solomon et al., 2005; Stepanović et al., 2004; Yang et al., 2014). Foodborne pathogens may have access to different levels of nutrients depending on where they persist in food processing facilities. In the present study, it was observed that the decreasing cantaloupe extract concentration from 50 to 2 mg/ml decreased *L. monocytogenes* biofilm formation at 22°C and 10°C in 7 days. Stanley and Lazazzera (2004) reported that starvation could induce more biofilm formation in *Bacillus subtilis*. The starvation condition of *Bacillus subtilis* promoted

higher activation of transcription factors such as RpoS and Spo0A that are needed for biofilm formation. Yang et al. (2014) reported that *S. Enteridis* produced more cellulose in the extracellular polymeric substances matrix of biofilm when grown in 1/20 TSB as compared to an un diluted TSB. However, the effects of nutrient density of the growth medium on biofilm formation may depend on the nature of the microorganism. Stepanović et al. (2004) observed that *L. monocytogenes* produced a greater amount of biofilm in TSB when compared to 1/20 TSB, while *Salmonella* Spp. showed the opposite behavior. Similarly, Solomon et al. (2005) reported that *Salmonella* Spp. from clinical samples promoted greater biofilm formation in 1/20 TSB when compared to TSB while the meat isolate *Salmonella* Spp. from meat samples did not differ in biofilm formation as the nutrient density in the growth media varied.

Various materials such as stainless steel, glass, polyurethane, buna-n rubber and polyethylene are used in the food processing and retail environments as food-contact surfaces. *L. monocytogenes* cells adhere to a wide variety of surfaces found in the food processing and retail environments (Beresford et al., 2001). It was observed that *L. monocytogenes* formed biofilms on stainless steel, buna-n rubber, polyethylene, polyurethane surfaces in cantaloupe extracts at 50 and 2 mg/ml concentrations at both 22°C or 10°C. However, regardless of the growth temperature or the concentration of cantaloupe extract, *L. monocytogenes* 2011L-2526 formed 1-3.5 logs less biofilm on buna-n rubber as compared to stainless steel, polyethylene or polyurethane. Previously, Ronner and Wong (1993) also reported that *L. monocytogenes* and *Salmonella* formed less biofilm on buna-n rubber than stainless steel surface. Surfaces such as glass and stainless steel are hydrophilic while rubber and plastic surfaces are more hydrophobic

(Stepanović et al., 2004). Bonsaglia et al. (2014) reported that *L. monocytogenes* formed more biofilm on hydrophilic materials as compared to hydrophobic materials regardless of the growth temperature while Chavant et al. (2002) reported that *L. monocytogenes* biofilm formation was more rapid on hydrophilic surfaces as compared to hydrophobic materials. Even though polyethylene and polyurethane are hydrophobic, biofilm formation on those surfaces was as great as stainless steel in this study.

In summary, results indicate that *L. monocytogenes* can induce significant growth and biofilm formation on different food-contact surfaces in very low concentrations of cantaloupe flesh and peel extracts. The growth and biofilm formation of *L. monocytogenes* was greater in 50 mg/ml cantaloupe flesh or peel extract concentration at 22°C as compared to 2 mg/ml cantaloupe flesh or peel extract at 10°C. No major differences were found among the *L. monocytogenes* strains tested for biofilm formation in cantaloupe extracts. *L. monocytogenes* formed less biofilm on buna-n rubber than stainless steel, polyurethane and polyethylene containing cantaloupe extract. These findings illustrate the critical importance of appropriate cleaning and sanitizing of cantaloupe processing surfaces because even the presence of small sediments of cantaloupe residue on the food-contact surfaces can promote growth and subsequent biofilm formation in the processing environments, which creates serious food safety risks.

Table 3.1 *L. monocytogenes* strains tested in this study.

L. monocytogenes	Serotype	Isolation source	Obtained from
2011L-2625	1/2a	Cantaloupe outbreak 2011	UGA
EGD (Bug600)	1/2a	Clinical	Institute Pasteur, France
2011-2624	1/2b	Cantaloupe outbreak 2011	UGA
F8385	1/2b	Carrot	UGA
ScottA	4b	Clinical	FDA
G1091	4b	Coleslaw	UGA

UGA, University of Georgia
FDA, Food and Drug Administration, USA

Table 3.2 Description of food-contact surfaces tested in this study.

Material	Thickness (inch)	Manufacturer	Used for
Stainless steel (304 #4)	0.018	Thyssen Krup	Table tops, Utensil & equipment surface
Nitrile buna rubber	0.031	Warco Nitrite	Conveyer belt
Thermoplastic polyurethane	0.01	Habasit	Conveyer belt
Ultrahigh molecular weight polyethylene	0.125	Sibe Automation	Cutting boards, Conveyer belt, Table tops

Table 3.3 Physical and chemical properties of cantaloupe extracts.

Physical/chemical property	Cantaloupe flesh extract	Cantaloupe peel extract
pH	6.4	6.5
Brix (°B)	1.6	0.6
Fresh weight (mg/ml)	200	200
Dry weight (mg/ml) *	1.7 ± 0.3	6.9± 0.3

*Results expressed as a mean of four repetitions and standard deviation.

Table 3.4 Generation time of *L. monocytogenes* in cantaloupe extracts at 10°C and 22°C

L. monocytogenes strain	Cantaloupe extract	Generation time of *L. monocytogenes* (h)			
		50 mg/ml		2 mg/ml	
		10°C	22°C	10°C	22°C
EGD (Bug600)	Flesh	5.9 (0.93)	2.0 (0.95)	NA	5.4 (0.83)
2011L-2624	Flesh	4.4 (0.95)	2.0 (0.91)	NA	4.6 (0.86)
G1091	Flesh	4.1 (0.89)	2.2 (0.99)	NA	5.2 (0.83)
EGD (Bug600)	Peel	5.6 (0.98)	1.5 (0.91)	NA	5.2 (0.87)
2011L-2624	Peel	4.3 (0.94)	2.3 (0.85)	NA	4.1 (0.84)
G1091	Peel	3.2 (0.92)	1.8 (0.88)	NA	5.7 (0.70)

R^2 values are shown in brackets

Table 3.5 Average biofilm formation of six strains of *L. monocytogenes* in cantaloupe extracts under different conditions.

Age of biofilm (days)	Cantaloupe extract	Average biofilm formation of *L. monocytogenes* (log CFU/coupon)			
		50 mg/ml		2 mg/ml	
		10°C	22°C	10°C	22°C
1	Flesh	2.2 ± 0.1^{aA}	5.5 ± 0.2^{aC}	2.0 ± 0.1^{aA}	4.1 ± 0.1^{aB}
4	Flesh	3.8 ± 0.1^{bA}	7.0 ± 0.1^{bC}	3.5 ± 0.1^{bA}	5.5 ± 0.2^{bB}
7	Flesh	5.2 ± 0.1^{cB}	7.0 ± 0.1^{bC}	4.0 ± 0.2^{bA}	5.6 ± 0.1^{bB}
1	Peel	2.1 ± 0.18^{aA}	6.0 ± 0.10^{aC}	2.1 ± 0.04^{aA}	4.3 ± 0.12^{aB}
4	Peel	4.1 ± 0.07^{bA}	6.9 ± 0.09^{bC}	3.5 ± 0.02^{bA}	5.3 ± 0.07^{bB}
7	Peel	5.7 ± 0.08^{cB}	7.1 ± 0.06^{bC}	4.2 ± 0.13^{bA}	5.3 ± 0.09^{bB}

Numbers in the same column of the same extract sharing different lowercase letters or numbers in the same raw sharing different upper case letters are significantly different.

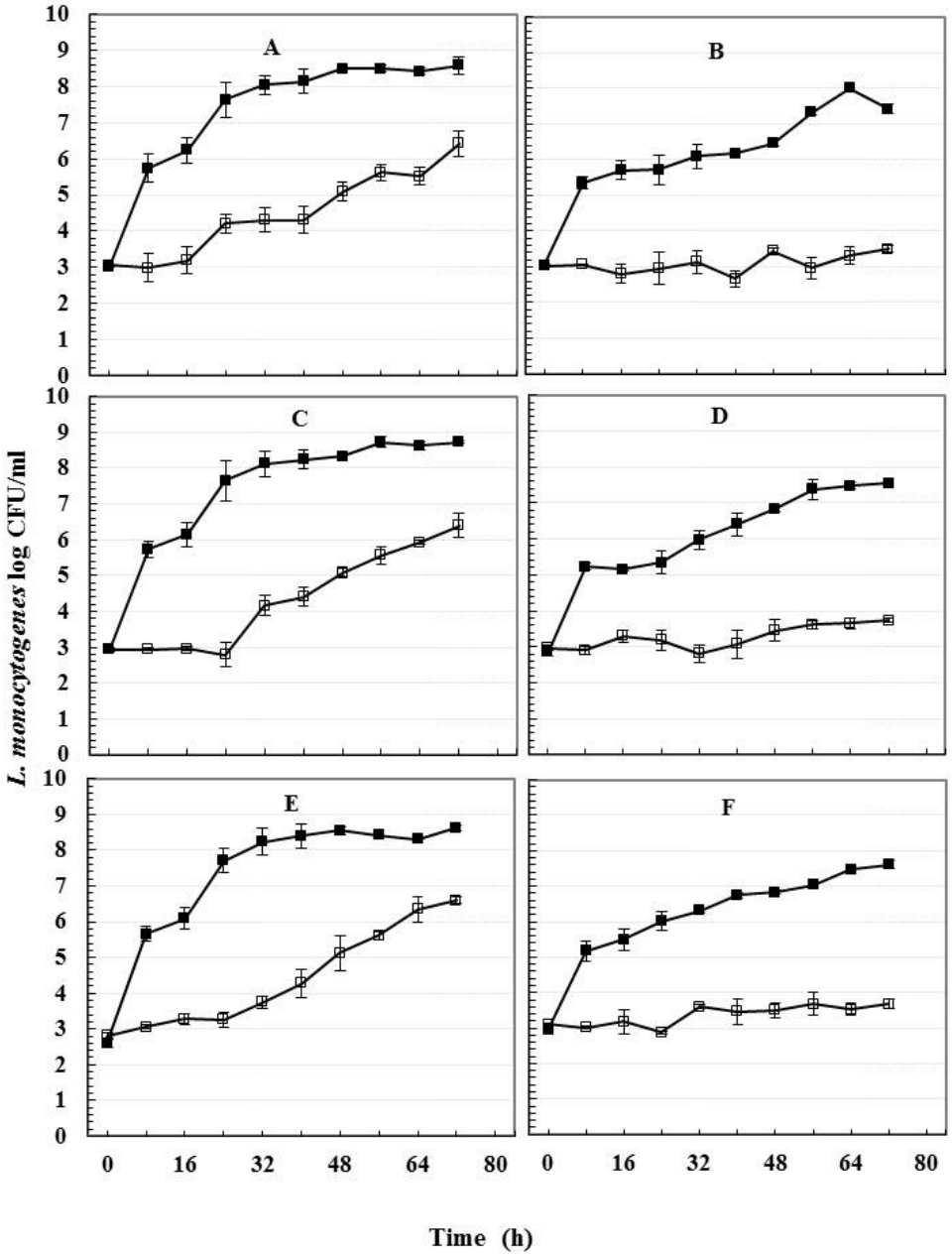

Figure 3.1 Growth of *L. monocytogenes* Bug600 (A, B), 2011-2625 (C, D) and G1091 (E, F) in 50 mg/ml (A, C, E) and 2 mg/ml (B, D, F) of cantaloupe flesh extract at 22°C (■) and 10°C (□).

Error bars indicate standard error.

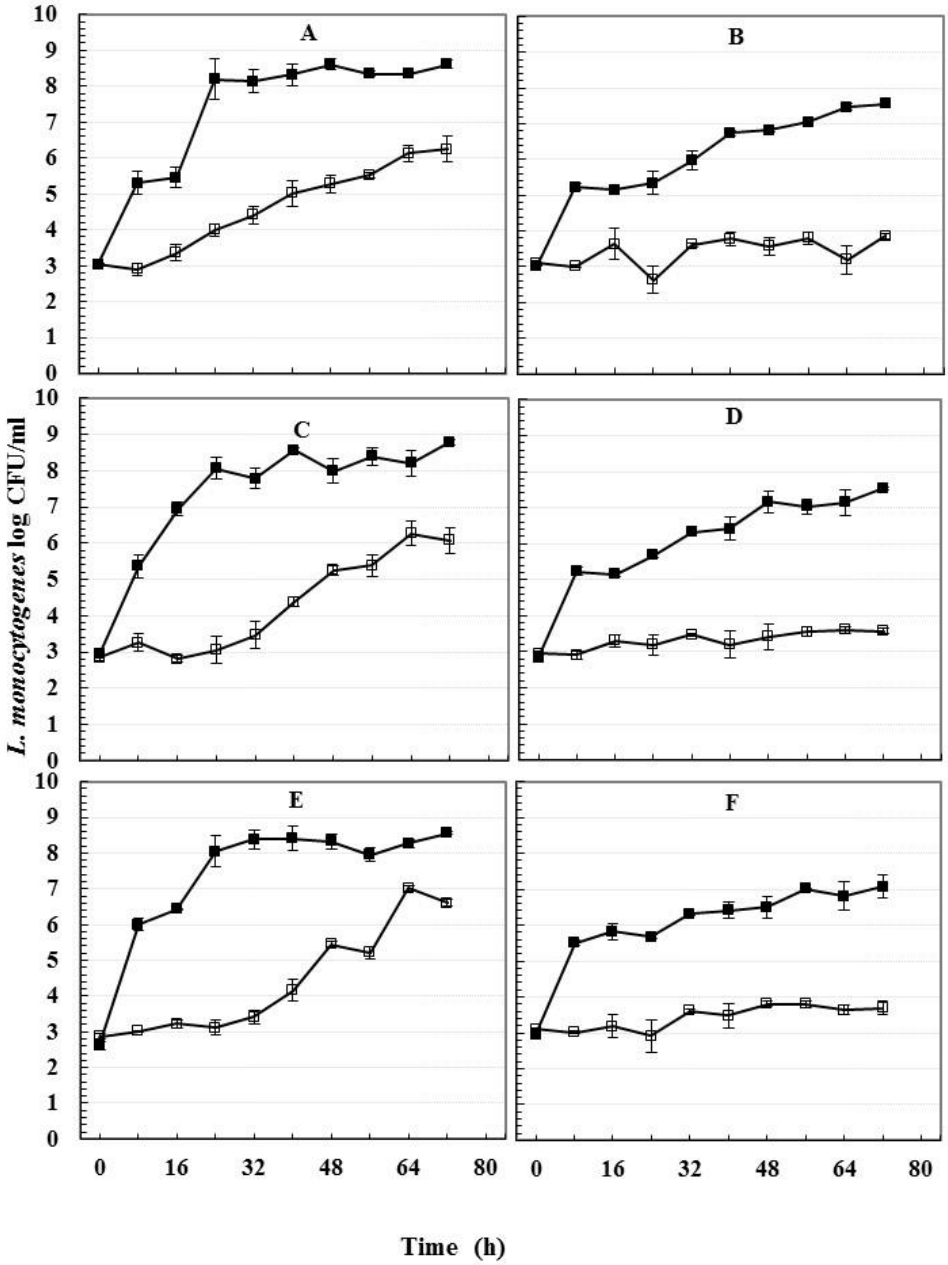

Figure 3.2 Growth of *L. monocytogenes* Bug600 (A, B), 2011-2625 (C, D) and G1091 (E, F) in 50 mg/ml (A, C, E) and 2 mg /ml (B, D, F) of cantaloupe peel extract at 22°C (■) and 10°C (□).

Error bars indicate standard error.

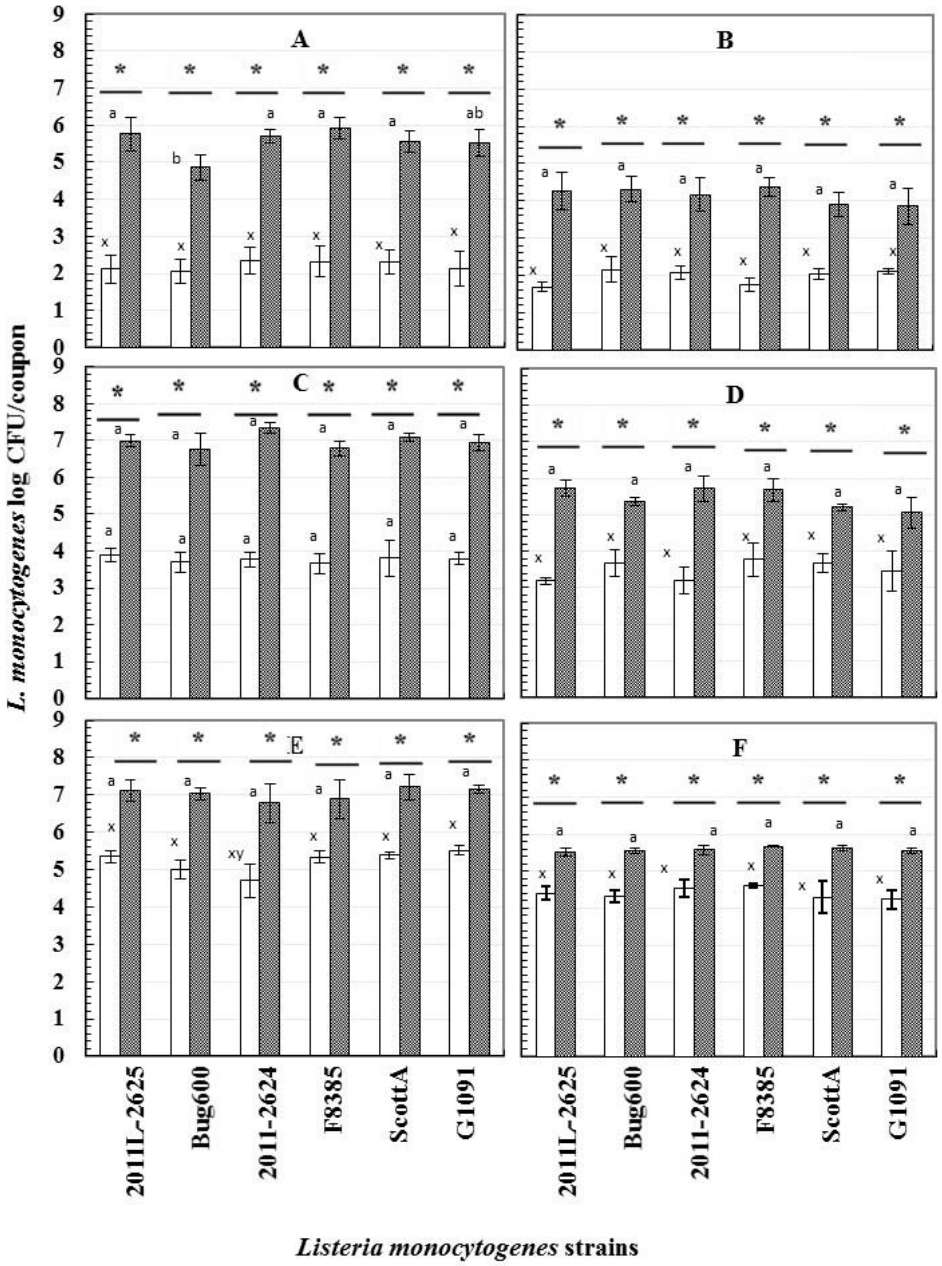

Figure 3.3 Biofilm formation by six strains of *L. monocytogenes* on the stainless steel surface in 50 mg/ml (A, C, E) and 2 mg /ml (B, D, F) of cantaloupe flesh extract at 22°C (▩) and 10°C (□) in 1 day (A, B),4 days (C, D) and 7 days (E, F).

Bars with different lowercase letters indicate significant differences between biofilms formed at the same temperature and * indicate significant difference between biofilms belong to the same strain formed at different temperature based on Tukey's Honestly Significant Difference test ANOVA test ($P < 0.05$). Error bars indicate standard error.

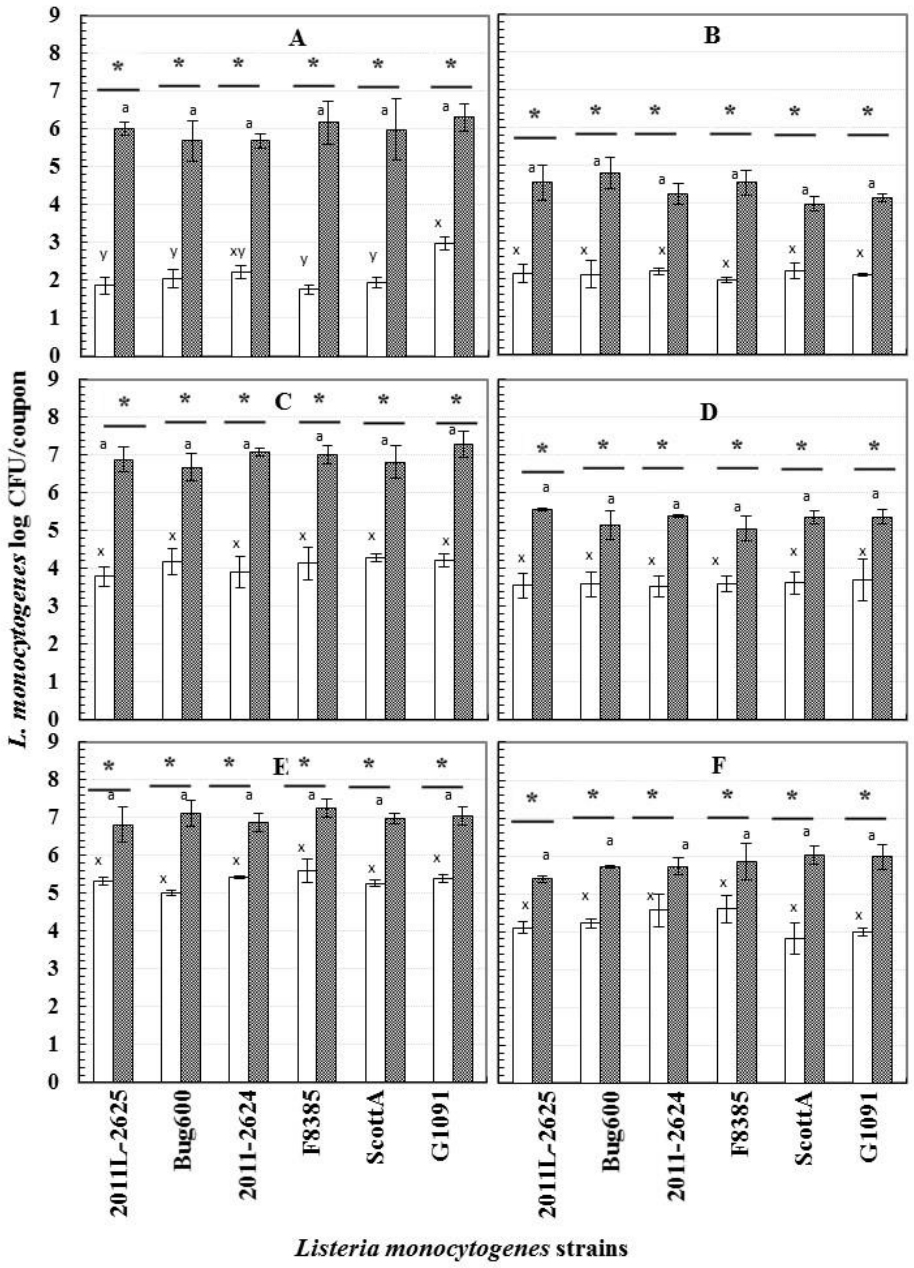

Figure 3.4 Biofilm formation by six strains of L. monocytogenes on the stainless steel surface in 50 mg/ml (A, C, E) and 2 mg /ml (B, D, F) of cantaloupe peel extract at 22°C (▨) and 10°C in 1 day (A, B), 4 days (C, D) and 7 days (E, F).

Bars with different lowercase letters indicate significant differences between biofilms formed at the same temperature and * indicate significant difference between biofilms belong to the same strain formed at different temperature based on Tukey's Honestly Significant Difference test ANOVA test ($P < 0.05$). Error bars indicate standard error.

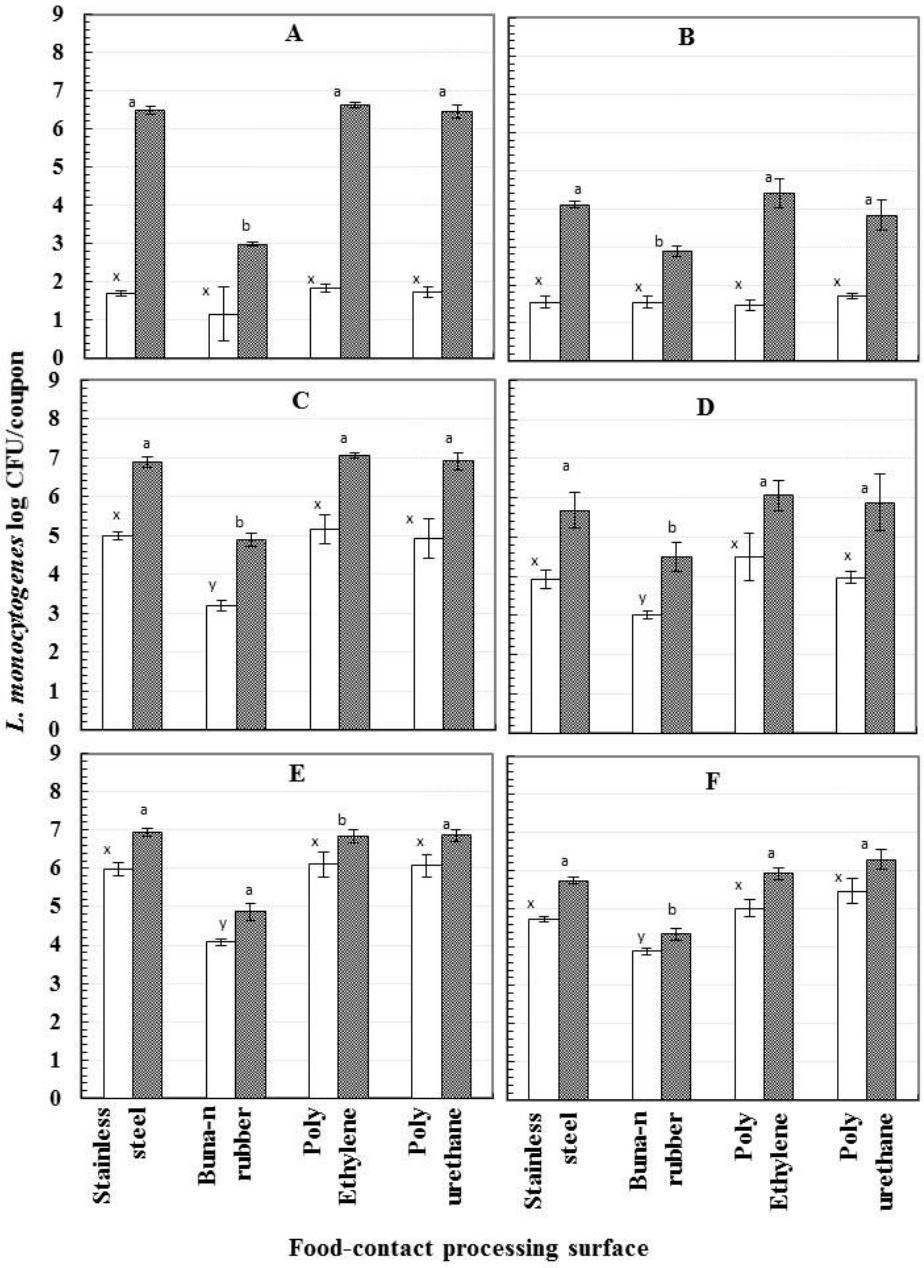

Figure 3.5 Biofilm formation by *L. monocytogenes* 2011L-2625 on four food-contact processing surfaces in 50 mg/ml (A, C, E) and 2 mg /ml (B, D, F) of cantaloupe flesh extract at 22°C (▩) and 10°C in 1 day (A, B), 4 days (C, D) and 7 days (E, F).

Bars with different lowercase letters indicate significant differences between biofilms formed at the same temperature based on Tukey's Honestly Significant Difference test ANOVA test ($P < 0.05$). Error bars indicate standard error.

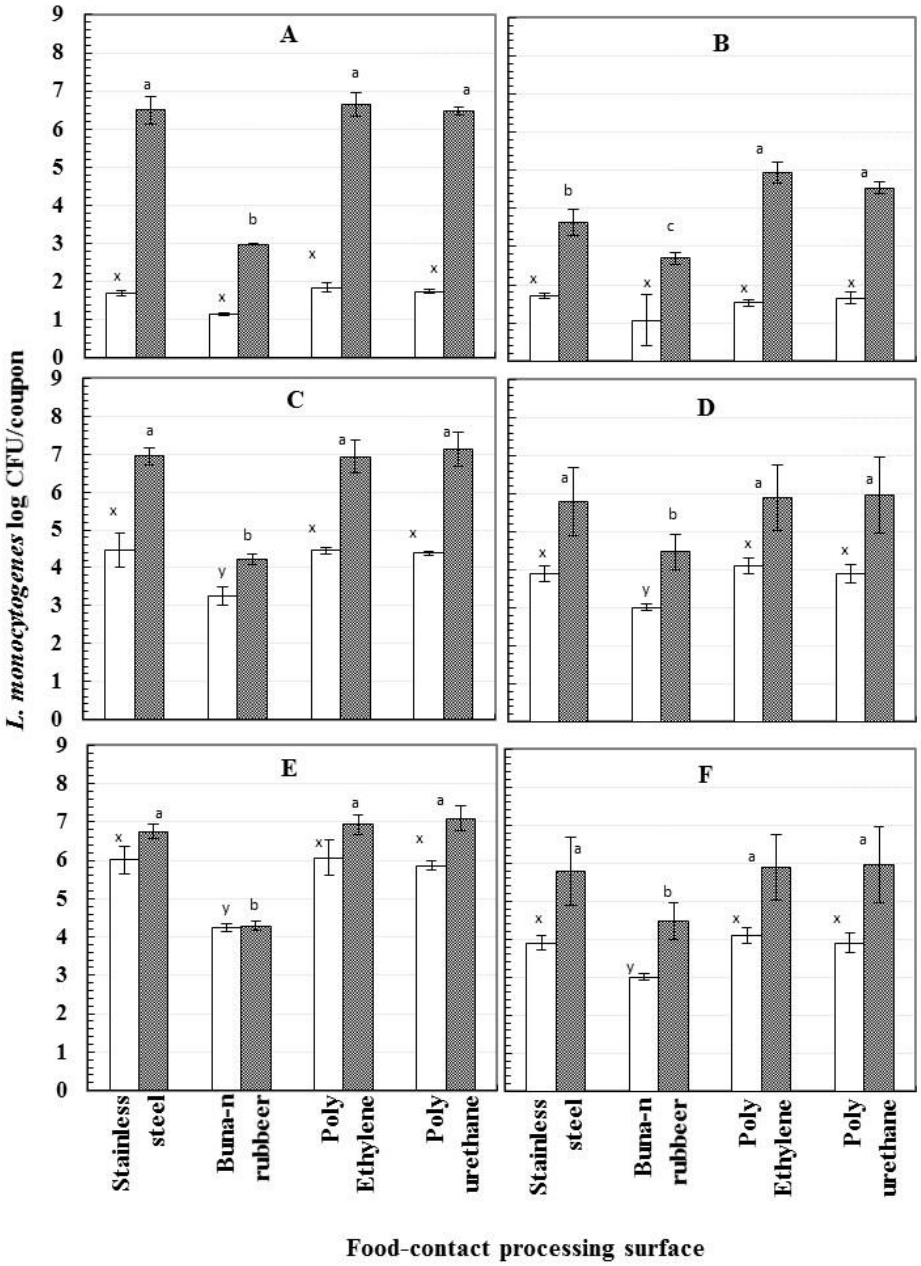

Figure 3.6 Biofilm formation by *L. monocytogenes* 2011L-2625 on four food-contact processing surfaces in 50 mg/ml (A, C, E) and 2 mg /ml (B, D, F) of cantaloupe peel extract at 22°C (▩) and 10°C in 1 day (A, B),4 days (C, D) and 7 days (E, F).

Bars with different lowercase letters indicate significant differences between biofilms formed at the same temperature based on Tukey's Honestly Significant Difference test ANOVA test ($P < 0.05$). Error bars indicate standard error.

CHAPTER IV

GROWTH AND BIOFILM FORMATION BY *SALMONELLA* SPP. IN CANTALOUPE FLESH AND PEEL EXTRACTS ON FOUR FOOD-CONTACT SURFACES AT 22°C AND 10°C

4.1 Abstract

Salmonella spp. is the pathogen that is responsible for the highest number of outbreaks in cantaloupe industry. The goal of this study was to examine the growth and biofilm formation of *Salmonella* spp. on four different food-contact processing surfaces in cantaloupe flesh and peel extracts at 22°C and 10°C. The growth of *Salmonella* spp. was greater in high concentration of cantaloupe extract and high temperature. In 50 mg/ml of cantaloupe flesh or peel extract, the growth of *Salmonella* spp. was increased by 5 log CFU/ml in 24 h at 22°C and 1 log CFU/ml in 72 h at 10°C. In 2 mg/ml of cantaloupe flesh or peel extracts, the growth of *Salmonella* spp. was increased by 3.5 log CFU/ml in 56 h at 22°C, but there was no change in 72 h at 10°C. The biofilm production of *Salmonella* spp. was greater at high concentration of cantaloupe extract and high temperature but no major differences ($P < 0.05$) were found among the strains tested. In 50 mg/ml cantaloupe extract, *Salmonella* spp. produced 6-7 log CFU/coupon biofilm in 4-7 days at 22°C and approximately 4 log CFU/coupon in 7 days at 10°C. In 2 mg/ml of cantaloupe extract, *Salmonella* spp. produced 5-6 log CFU/coupon biofilms in 4-7 days at 22°C and 3-4 log CFU/coupon in 7 days at 10°C. Biofilm formation by *Salmonella*

01A4754 was lowest on buna-n rubber compared to stainless steel, polyethylene and polyurethane surfaces under the majority of conditions tested. Overall, these findings show that *Salmonella* spp. can grow rapidly and form biofilms on different cantaloupe processing surfaces the presence of low concentrations of residue from cantaloupe flesh or peel.

4.2 Introduction

Salmonella enterica is a Gram-negative, non-spore forming, rod shape bacteria (Yates, 2011). Presence of *Salmonella* spp. in food is recognized as a potential human health hazard (Jones et al., 2008). Among over 2500 serovars of *S. enterica*, four serovars (*S.* Typhimurium, *S.* Enteritidis, *S.* Newport and *S.* Javaiana) account for almost half of the human isolates that are reported in the USA (Braden, 2006).

The CDC estimates that *Salmonella* causes approximately 1.2 million foodborne illnesses in the USA, with 19,000 hospitalizations and 450 deaths annually (Kuchler, 2016). Even though, eggs and poultry products are the main sources for *Salmonella* infection (Painter et al., 2013), plant-based food products are responsible for a significant number of *Salmonella* outbreaks in the USA. For example, in last few years, the CDC investigated many *Salmonella* outbreaks associated with various plant-based food products such as alfalfa sprouts, pistachios, cucumber, bean sprouts, and mangoes. This situation is further evident in the findings of Wells and Butterfield (1997), who reported that 33.9% of fresh and 28.1% of rotten fruit and vegetable samples that were obtained from local supermarkets in Somerset and Middlesex counties, New Jersey, USA were positive for *Salmonella* spp.

Fruits and vegetables such as cantaloupe, cilantro, broccoli, cauliflower, lettuce, tomato, and water melon have been reported as vehicles of *Salmonella* (Buck et al., 2003). As a fruit grown close to the ground, cantaloupe has a high risk of contamination with pathogens that are associated with soil and animals. Cantaloupe may also be contaminated during harvesting or processing from contaminated water and equipment (Castillo et al., 2004). *S.* Poona, *S.* Newport, *S.* Javiana are the highly associated are the serovars of *Salmonella* Spp. with melon related outbreaks. (Zarecki et al., 2013).

Control of bacterial biofilms in the food industry is essential due to their due to their unfavorable effects on food safety and human health (Menon, 2016). Pathogenic bacteria such as *S. enterica*, *L. monocytogenes*, and *Escherichia coli* form biofilms on various surfaces as their predominant form of living (Bernbom et al., 2011; Vestby et al., 2009). Biofilm protect *Salmonella* cells and allow them to survive in harsh environmental conditions that can be encountered during food processing (Bridier et al., 2011). Such biofilms that are formed on food contact and non-food contact surfaces can act as a source of cross-contamination, thus leading to serious food safety hazards (Carpentier and Cerf, 1993).

Growth and biofilm formation of *Salmonella* spp. is well characterized in the standard microbiological growth media which contain optimal level of all the necessary nutrients. In cantaloupe processing environments, *Salmonella* spp. cells are exposed to nutrients that are leaked from cantaloupe flesh or peel. These juices can form deposits on the food contact and non-food contact surfaces when improperly washed and may support the growth and biofilm formation of *Salmonella S*pp.. Therefore, understanding the critical factors governing the growth and biofilm formation in the cantaloupe processing

environment will enhance food safety. The main objectives of the present study were to examine the effect of strain, temperature, nutrient level and food-contact surface on the growth and biofilm formation of *Salmonella* spp. in cantaloupe flesh and peel extracts.

4.3 Materials and methods

4.3.1 Bacterial strains and culture conditions

The working stock culture of *Salmonella* strains (Table 4. 1) were prepared by inoculating a colony grown on XLD agar onto tryptic soy agar slants that contained 0.6% yeast extract (TSAYE) and incubation at 37°C for 24 h. These working stocks were stored at 4°C for 4-5 weeks. Overnight culture of each strain was prepared by inoculation of 10 ml of tryptic soy broth that contained 0.6% yeast extract (TSBYE) from the working stocks and incubation at 37°C for 18 to 20 h with shaking at 150 rpm (Imperial III incubator, Lab-Line Instrument Inc., IL, USA).

4.3.2 Food-contact surfaces preparation

The food-contact surfaces (Table 4.2) that were used in this study were cut into 1 cm ×1.5 cm size coupons, soaked in an alkali detergent for 1 h and washed thoroughly with deionized water. Stainless steel coupons were autoclaved at 121°C for 15 min. Buna-n rubber, ultra-high molecular weight polyethylene and thermoplastic polyurethane coupons were sterilized by submersion in 100% alcohol for 15 min, followed by sterile water rinse to eliminate alcohol residue. Sterile coupons were surface dried inside a biosafety cabinet prior to use.

4.3.3 Preparation of cantaloupe extracts

Cantaloupe (*Cucumis melo* L. var. reticulatus) fruits were obtained from a local supermarket and washed under running tap water for 1 min. The peel was then removed and the flesh was deseeded. In order to prepare cantaloupe flesh and peel extracts, 100 g of cut cantaloupe flesh or peel was blended with 400 ml of deionized water for 30 seconds using a juice extractor (Hamilton beach). The blended flesh and peel extracts were then filtered using a filter bag (BA6141/STR) and centrifuged at a low speed (2500 × g) (Beckman, Model TJ-6 centrifuge) for 5 min to eliminate coarse particles. These cantaloupe extracts were autoclaved at 121°C for 15 min and stored at 22°C until needed. These cantaloupe extracts (200 mg/ml) were further diluted in physiological saline to obtain 50 mg/ml and 2 mg/ml of concentrations, respectively, based on the fresh weight used in their preparation.

4.3.4 Determination of physical/chemical properties of cantaloupe extracts

The pH of sterile cantaloupe flesh and peel extracts were determined using a calibrated pH meter (Accumet Basic, AB15 pH meter, Fisher Scientific) and brix values were determined using a refractometer (RHB-18ATC, GxPro). In order to determine the dry weight of cantaloupe flesh or and peel extract, 25 ml of each extract was transferred to a pre-weighed 50 ml centrifuge tube. These extracts were freeze dried (LABCONCO Corporation, Kansas City, MO, 64132) until a constant weight was obtained. The dry weight was calculated using following equation.

$$\text{Dry weight } \left(\frac{\text{mg}}{\text{ml}}\right) = \frac{(\text{Post dry weight of the tube} - \text{Weight of the empty tube})\text{mg}}{\text{Volume of extract (ml)}} \quad (4.1)$$

4.3.5 Evaluation of growth of *Salmonella* spp. in cantaloupe extracts at different temperatures

Growth of *Salmonella* 01A4754, H1256 and G4639 in 50 and 2 mg/ml of cantaloupe flesh and peel extracts at 22°C and 10°C was studied. The overnight cultures of these strains were prepared as described previously. One ml volumes of overnight cultures were centrifuged for 5 min (9000 ×g, MARATHON 21000R, Fisher Scientific). The resulting cell pellets were resuspended in 1 ml of physiological saline to obtain 9 log CFU/ml of stationary phase cells.

The 50 and 2 mg/ml of cantaloupe flesh or peel extracts were inoculated with *Salmonella* spp. cells in the stationary phase to yield an initial cell concentration of 3 log CFU/ml. The inoculated cantaloupe extracts were incubated at 10°C (VWR, Scientific Products, Sheldon manufacturing, INC., OR, 97113) and at room temperature (22°C). Increase in cell number was enumerated every 8 h for 72 h by serially diluting an aliquot from the cell suspensions in physiological saline and plating on tryptic soy agar that was enriched with 0.6% yeast extract (TSA-YE). These plates were incubated at 37°C (Imperial III Incubator, Lab line instrument Inc., IL, USA) for 48 h to obtain colony forming unit (CFU) counts.

4.3.6 Calculation of generation time

The generation time (g) was calculated from the slope of the line obtained in the semi logarithmic plot of exponential growth. The equation of g = 0.301/slope of the semi logarithmic plot of exponential growth was used to calculate the generation time as previously described (Penteado and Leitao, 2004a, b).

4.3.7 Evaluation of biofilm formation by different strains of *Salmonella* spp. in cantaloupe extracts at different temperatures

Six strains of *Salmonella* spp. were evaluated for their ability to form biofilm formation (Table 1) on stainless steel in 50 and 2 mg/ml of cantaloupe flesh and peel extracts at 22°C and 10°C. One ml from each overnight culture was centrifuged and the resulting cell pellets were resuspended in 1 ml of physiological saline. The 50 and 2 mg/ml of samples cantaloupe flesh or peel extracts were inoculated with stationary phase cells to obtain an initial cell concentration of 3 log CFU/ml. Sterile stainless steel coupons were placed in polystyrene 24-well plates with one coupon per well. Each well was filled with 2 ml of cell suspension that was prepared in cantaloupe flesh or peel extract. These coupons were incubated at 22°C or 10°C for 1, 4 or 7 days.

4.3.8 Enumeration of biofilms

To enumerate biofilm production of *Salmonella* spp. at the end of 1, 4 and 7 days, coupons were washed twice by adding 2.75 ml of physiological saline to remove loosely bound cells, coupons were then transferred into plastic tubes (15 ml-Tronado^{TM}) containing 5 ml of 0.1% peptone water with 0.02% Tween 80 and five sterile glass beads. Tubes were vortexed for 1 min (Vortex mixer, Labnet International, Inc, Edison, NJ, USA) and aliquots of detached cell suspensions were serially diluted in physiological saline. Each dilution was plated on TSA-YE and incubated at 37°C for 48 h.

4.3.9 Effect of the nature of the food-contact surface on biofilm formation by *Salmonella* 01A4754 in cantaloupe extracts at different temperatures

Stainless steel, buna-n rubber, ultra-high molecular weight polyethylene and thermoplastic polyurethane coupons were placed in 24-well plates with one coupon per

well. Two ml of *Salmonella* 01A4754 cell suspension at 3 log CFU/ml that was prepared in 50 and 2 mg/ml of cantaloupe flesh or peel extract were added to each well. These coupons were incubated at 22°C or 10°C for 1, 4 or 7 days. At the end of each incubation period, biofilm formation on the four different surfaces was enumerated as described previously.

4.3.10 Statistical analysis

A completely randomized design with a 2-way factorial structure and 4 replications was utilized. Tukey's honestly significant difference test ($P < 0.05$) was performed to determine different between treatments. For the comparison study of biofilm formation among 6 strains, srains and temperature were the two factors. For the comparison of average biofilm formation of *Salmonella* spp. in 1, 4 and 7 days, days and the concentration and temperature combinations were the two factors. Concentration and temperature were combined in to one factor due to lack of significant interaction. For the comparison of biofilm formation of *Salmonella* 01A4754 on different food contact surfaces, surfaces and temperature were the two factors ($P < 0.05$).

4.4 Results

4.4.1 Growth curves of *Salmonella* spp. in cantaloupe extracts

The growth of *Salmonella* spp. in cantaloupe flesh and peel extracts was slower or inhibited at 10°C as compared to 22°C. In 50 mg/ml cantaloupe flesh extract, the initial cell number (3 log CFU/ml) of *Salmonella* 01A4754, H1256 and G4639 was increased to 8 log CFU/ml in 24-32 h at 22°C and 4 log CFU/ml in 72 h at 10°C (Fig 4. 1 ACE). In 2 mg/ml cantaloupe flesh extract, the initial cell number of 3 log CFU/ml of *Salmonella*

01A4754, H1256 and G4639 was increased to 6.5 log CFU/ml in 56 h at 22°C but remained at 3 log CFU/ml in 72 h at 10°C (Fig 4.1 BDF).

A similar behavior as observed in cantaloupe flesh extract was also observed for the growth of *Salmonella* 01A4754, H1256 and G4639 in cantaloupe peel extract (Fig 4.2). In 50 mg/ml cantaloupe peel extract, the *Salmonella* 01A4754, H1256 and G4639 initial cell number of 3 log CFU/ml was increased to 8 log CFU/ml in 24-32 h at 22°C and 4 log CFU/ml in 72 h at 10°C (Fig. 4. 2ACE). For 2 mg/ml, the initial cell number of *Salmonella* 01A4754, H1256 and G4639 of 3 log CFU/ml was increased to 6.5 log CFU/ml in 56 h but did not change in 72 h at 10°C (Fig. 4. 2BDF).

The lag phase of *Salmonella* 01A4754, H1256 and G4639 were less than 8 h in 50 and 2 mg/ml cantaloupe flesh or peel extracts at 22°C. The lag phase was 24-32 h in 50 mg/ml or to >72 h in 2 mg/ml cantaloupe extracts at 10°C.

A large difference in generation time of *Salmonella* strains was observed between temperature of 22°C and 10°C. For example, in 50 mg/ml cantaloupe extract, the generation times were markedly increased by 13-15 h when the temperature was decreased from 22°C to 10°C. It is noteworthy that generation time of each strain in 2 mg/ml cantaloupe extract increased by 1-3 h beyond the corresponding generation time of the 50 mg/ml cantaloupe extract at 22°C. In 2 mg/ml of cantaloupe extract, no exponential growth was observed during the experiment, the generation time was not calculated for *Salmonella* spp. at 10°C.

4.4.2 Influence of strain and temperature on biofilm formation by *Salmonella* spp. on stainless steel surface in cantaloupe extracts

In 50 mg/ml cantaloupe flesh extract, *Salmonella* spp. produced approximately 5 log CFU/coupon biofilm in 1 d and 5.5-6.5 log CFU/coupon biofilm in 4 and 7 d at 22°C (Fig. 4. 3ACE). The corresponding biofilm production at 10°C was 1.5-4 log CFU/coupon less than that at 22°C ($P < 0.05$). In 2 mg/ml cantaloupe flesh extract, *Salmonella* spp. produced approximately 4 log CFU/coupon biofilm in 1 d and 5 log CFU/coupon biofilm in 4 and 7 d at 22°C. The corresponding biofilm production at 10°C was 1.5 - 3.5 log CFU/ml less than that at 22°C ($P < 0.05$) (Fig 4. 3BDF). No difference in biofilm production was observed among the *Salmonella* strains under all environmental conditions that were tested ($P < 0.05$).

In 50 mg/ml cantaloupe peel extract, *Salmonella* spp. produced approximately 5-6 log CFU/coupon biofilm in 1 d and 6-7 log CFU/coupon biofilm in 4 and 7 d at 22°C while the corresponding biofilm production at 10°C was 1.5- 3 log CFU/coupon less than that at 22°C ($P < 0.05$) (Fig. 4. 4ACE). In 2 mg/ml cantaloupe peel extract, *Salmonella* spp. produced approximately 4 log CFU/coupon biofilm formation 1 day and 5-6 log CFU/coupon biofilm formation in 4 and 7 d at 22°C while the corresponding biofilm production at 10°C was 1.5-3.5 logs less than at 22°C ($P < 0.05$) (Fig. 4. 4BDF).

Table 4. 5 shows that the average biofilm production of six strains of *Salmonella* spp. in 50 and 2 mg/ml cantaloupe flesh and peel extract gradually increased from 1 to 4 d ($P < 0.05$) but did not increase after 4 to 7 d at 22°C. In 50 and 2 mg/ml of cantaloupe extract, there were no significant differences between *Salmonella* biofilm cell count in 1 and 4 d but they were increased by 1.5-2.5 logs from 4 to 7 d at 10°C.

4.4.3 Biofilm formation by *Salmonella* on different food-contact surfaces in cantaloupe extracts

In 50 mg/ml cantaloupe flesh extract, *Salmonella* 01A4754 produced approximately 5 log CFU/coupon in 1 d or 6-7 log CFU/coupon biofilm in 4 and 7 d on stainless steel, polyethylene and polyurethane (Fig. 4. 5ACE). The corresponding biofilm formation on buna-n rubber was 2-3 log CFU/coupon less than the other three surfaces at 22°C ($P < 0.05$). In 50 mg/ml cantaloupe extract, *Salmonella* 01A4754 produced 1-2 log CFU/coupon biofilm on all four surfaces in 1 and 4 d at 10°C (Fig. 4. 5AC). In 50 mg/ml, *Salmonella* 01A4754 formed approximately 5 log CFU/coupon on stainless steel, polyethylene and polyurethane but was 2 log CFU/ ml less than other surfaces at 10°C ($P < 0.05$) (Fig. 4. 5E).

In 2 mg/ml cantaloupe flesh extract, *Salmonella* 01A4754 produced approximately 4 log CFU/coupon biofilm in 1 d or 5 log CFU/coupon biofilm in 4 and 7 d on stainless steel, polyethylene, polyurethane while the corresponding biofilm formation on buna-n rubber was 1-2 logs less than that on the other surfaces at 22°C ($P < 0.05$) (Fig. 4. 5 BDF). In 2 mg/ml cantaloupe flesh extract, *Salmonella* 01A4754 produced 2 log CFU/coupon biofilm on all four surfaces in 1 and 4 d at 10°C. In 2 mg/ml, *Salmonella* 01A4754 formed approximately 4 log CFU/coupon on stainless steel, polyethylene and polyurethane. *Salmonella* 01A4754 formed 2 logs less on buna-n rubber than on other surfaces at 10°C ($P < 0.05$).

As described above, a similar patterns of biofilm production of *Salmonella* 01A4754 in cantaloupe peel extract was observed on stainless steel, Buna-n rubber, polyethylene and polyurethane (Fig. 4. 6). For both 50 and 2 mg/ml cantaloupe peel extract, biofilm production of *Salmonella* 01A4754 on buna-n rubber was 1-3 logs less

than that on stainless steel, polyethylene and polyurethane at 22°C. At 10°C, it formed approximately 2 log CFU/coupon biofilm on all surfaces in 1 and 4 d include the exception.

4.5 Discussion

The behavior of foodborne bacterial pathogens with respect to their growth and biofilm formation greatly differs depending on the nature of the growth medium and environmental factors (Pan et al., 2010). There is a lack of information on the growth and biofilm formation of *Salmonella* spp. in cantaloupe extracts for developing predictive models (Penteado and Leitao, 2004a). Due to the increasing number of food outbreaks associated with the cantaloupe industry, there is a critical need to understanding the behavior and survival of foodborne bacterial pathogens such as *Salmonella* spp. in cantaloupe food processing environment (Bowen et al., 2006).

The pH of both cantaloupe flesh and peel extracts that were used in this study was 6.4-6.5 and optimal for *Salmonella* spp. growth. The brix value indicated that the cantaloupe flesh extract contained more soluble solids than the peel extract which was also reflected in the dry weight that was calculated for each extract. This was probably due to the high sugar content in cantaloupe flesh as compared to peel.

Data from the current study indicates that the 50 or 2 mg/ml of cantaloupe flesh and peel extracts enabled the rapid growth of *Salmonella* spp. The growth of *Salmonella* spp. was greater at 22°C when compared to 10°C in both concentrations of cantaloupe flesh and peel extracts. The length of lag phase of *Salmonella* strains was extended at 10°C as compared to 22°C in both concentrations of cantaloupe flesh or peel extracts. Factors such as size of the inoculum, physiological history of cells (whether cells were in

stationary phase or exponential phase) and the physiochemical nature of growth media (pH, temperature etc.) affect the length of the lag phase of a microbial growth curve (Rolfe et al., 2012). Moreover, when the inoculum was exposed to a large temperature fluctuation, the lag time of microorganisms was extended when compared to the lag time when there is a small fluctuation in temperature (Swinnen et al., 2004). This could be one reason for a longer lag phase in 50 or 2 mg/ml of cantaloupe extracts at 10°C as compared to 22°C.

In addition to the length of the lag phase, the growth rate of bacteria is also affected by the physiochemical nature of the growth environment. In this study it was observed that the generation time of all three strains of *Salmonella* spp. was increased with decreasing growth temperature. For example, in 50 mg/ml cantaloupe extracts, the generation time of *Salmonella* strains was increased from 1.5-2.5 h to 12-16 h which resulted in decreased growth rates of *Salmonella* spp. when the growth temperature was decreased from 22°C to 10°C. Similarly, an increase in generation time of *Salmonella* spp. and *L. monocytogenes* was observed with decreasing growth temperature in melon and papaya pulp (Penteado and Leitao, 2004a, b; Rezende et al., 2016). These findings indicate that the growth of *Salmonella* spp. is slower in places such as refrigerators and cold rooms as compared to other processing areas with higher temperature conditions in the presence of cantaloupe flesh and peel residue.

The persistence of biofilms on the food-contact surfaces is a problem for food industry since biofilms serve as reservoirs for the cross contamination of food products (Carrasco et al., 2012). There is potential correlation between the ability to form biofilms and the persistence of *Salmonella* strains in the food processing environments since

stronger producers of biofilms tend to persist in food processing environments when compared to low biofilm producers. In support of this hypothesis, Vestby et al. (2009) observed that the persistent *Salmonella* strains of fish meal-and feed processing plants formed more biofilms than the non-persistent *Salmonella* strains on polystyrene.

In this study, stainless steel was used to compare the biofilm formation of six *Salmonella* strains since it is the most commonly found food contact surface in food processing environments (Hilbert et al., 2003). Result of this study indicated that all *Salmonella* strains formed biofilms at very low concentrations of cantaloupe flesh or peel extracts while biofilm formation was prominent at 22°C as compared to 10°C. Similarly, Kroupitski et al. (2009) reported that *Salmonella* spp. formed more biofilm at 25°C as compared to 4°C. Also, Stepanović et al. (2004) and Karaca et al. (2013) reported that 20-30°C was the optimum temperature for the formation of *Salmonella* spp. biofilm compared to 5°C or 37°C. This could be due to the decrease in hydrophobicity of the *Salmonella* spp. cell surface with decreasing growth temperature, which reduces the cell attachment to hard surfaces (Chavant et al., 2002; Di Bonaventura et al., 2008). The cell motility is also reduced as temperature decreases from 22°C to 10°C, thus reducing cell attachment to hard surfaces in the initial stage of biofilm production (Di Bonaventura et al., 2008). The higher expression of *agfD* promoter at 20-30°C may also contribute to increased biofilm formation due to the induced production of polymers such as thin aggregative fimbriae and cellulose to produce stronger extra cellular matrices in biofilm (Gerstel and Römling, 2001).

The six strains of *Salmonella* spp. that belonged to three different serovars, did not differ in biofilm production under all environmental conditions tested. In contrast,

Vestby et al. (2009) found that *Salmonella* spp. strains differed in their ability to form biofilms depending on the serovar of the strain. For example, *S.* Agona, and *S.* Montevideo formed biofilms with higher counts as compared to *S.* Typhimurium. Similarly (Kroupitski et al., 2009) reported that *S.* Enteritidis, *S*. Virchow, *S.* Thompson, *S*. Typhimurium and *S*. Newport produced biofilms with more cells than *S.* Hadar, *S*. Poona and *S.* Amager.

Salmonella spp., may be exposed to different levels of nutrient fluctuations depending on the location in the cantaloupe processing plant. The biofilm formation by *Salmonella* spp. strains was significantly greater in 50 mg/ml compared to 2 mg/ml at 22°C. Therefore, biofilm formation of *Salmonella* spp. was affected by the level of available nutrients in the standard growth media. For example, Stepanović et al. (2004) reported that *Salmonella* spp. had a greater biofilm forming capacity in diluted TSB as compared to TSB. Similarly, Patel and Sharma (2010) observed that *S. enterica* attachment to cut lettuce pieces is enhanced in 1/10 TSB as compared to TSB. One possible reason for this behavior could be the higher expression of *agfD* promoter which was also highly expressed during biofilm production in *Salmonella* spp. in nutrient limiting conditions (Kroupitski et al., 2009; Römling et al., 2000). However, the present findings show that the behavior of *Salmonella* spp. in cantaloupe extract may not always correlate with its behavior in standard microbiological media.

Various materials such as stainless steel, glass, polyurethane, polyethylene and buna-n rubber are used in food processing and retail environments as food contact surfaces (Di Bonaventura et al., 2008; Joseph et al., 2001). Therefore, evaluation of the ability of *Salmonella* spp. to form biofilms on such surfaces is very important. In this

study, it was observed that *Salmonella* 01A4754 can form biofilm on stainless steel, buna-n rubber, polyethylene, polyurethane surfaces in the presence of cantaloupe flesh or peel residue. However, the result of this study showed that under most of the environmental conditions that were tested *Salmonella* formed less biofilm on buna-n rubber as compared to stainless steel, polyethylene or polyurethane. This finding confirms by previous research by Ronner and Wong (1993) who reported that *L. monocytogenes* and *Salmonella* form significantly less biofilm on buna-n rubber when compared to stainless steel. Surfaces such as glass and stainless steel are hydrophilic while rubber and plastic are hydrophobic (Stepanović et al., 2004). Bonsaglia et al. (2014) reported that *L. monocytogenes* biofilm production is enhanced on hydrophilic materials as compared to hydrophobic materials. However, in this study, biofilm formation of *Salmonella* 01A4754 on polyurethane and polyethylene were similar to stainless steel despite them being hydrophobic materials. This suggest that not all hydrophobic materials discourage biofilm formation by *Salmonella* spp.

In summary, these findings show that the cantaloupe flesh and peel extracts at very low concentrations support the growth and biofilm production of *Salmonella* spp. Growth rate and biofilm production increased with increasing concentration and temperature. Therefore, removal of cantaloupe residue from the food-contact surfaces is very important to prevent growth and biofilm formation by *Salmonella* spp. No major differences were found among the *Salmonella* strains tested for the biofilm formation in cantaloupe extracts. Biofilm production of *Salmonella* 01A4754 on buna-n rubber was less as compared to stainless steel, polyurethane and polyethylene surfaces in cantaloupe

extracts. Overall, the result illustrates the importance of effective cleaning and sanitization of cantaloupe processing surface to minimize food safety risks.

Table 4.1 *Salmonella* spp. strains used in this study

S. enterica Subsp. *enterica*	Serovar	Isolation source	Obtained from
01A4754	Poona	Cantaloupe	UGA
00A3279	Poona	Cantaloupe	UGA
01A242	Poona	Cantaloupe	UGA
00A3208	Poona	Cantaloupe	UGA
H1256	Stanley	Alfalfa sprout outbreak	CDC
G4639	Montevideo	Tomato outbreak	CDC

UGA, University of Georgia
CDC, Center for Disease Control, USA

Table 4.2 Description of food-contact surfaces tested in this study.

Material	Thickness (inch)	Manufacturer	Used for
Stainless steel (304 #4)	0.018	Thyssen Krup	Table tops, equipment surface
Nitrile buna-n rubber	0.031	Warco Nitrite	Conveyer belt
Thermoplastic polyurethane	0.01	Habasit	Conveyer belt
Ultrahigh molecular weight polyethylene	0.125	Sibe Automation	Cutting boards, Conveyer belt, Table tops

Table 4.3 Physical and chemical properties of cantaloupe extracts

Physical/chemical property	Cantaloupe flesh extract	Cantaloupe peel extract
pH	6.4	6.5
Brix (°B)	1.6	0.6
Fresh weight (mg/ml)	200	200
Dry weight (mg/ml) *	1.7 ± 0.3	6.9± 0.3

*Results expressed as a mean of four repetitions and standard deviation.

Table 4.4 Generation time (g) in hours for *Salmonella* spp. in cantaloupe extracts

Salmonella strain	Cantaloupe extract	Generation time of *Salmonella* Spp. (h)			
		50 mg/ml		2 mg/ml	
		10°C	22°C	10°C	22°C
01A4754	Flesh	15.9 (0.78)	2.2 (0.93)	NA	3.7 (0.98)
H1256	Flesh	16.3 (0.46)	1.4 (0.96)	NA	3.2 (0.94)
G4639	Flesh	12.9 (0.74)	2.1 (0.86)	NA	3.6 (0.98)
01A4754	Peel	16.1 (0.62)	2.6 (0.88)	NA	4.2 (0.95)
H1256	Peel	12.5 (0.91)	1.6 (0.96)	NA	3.7 (0.99)
G4639	Peel	13.3 (0.69)	2.6 (0.83)	NA	4.5 (0.99)

R^2 values are shown in brackets

Table 4.5 Average biofilm formation of six strains of *Salmonella* spp. in cantaloupe extracts at 10°C and 22°C

Age of biofilm	Cantaloupe extract	Average biofilm formation (log CFU/coupon)			
		50 mg/ml		2 mg/ml	
		10°C	22°C	10°C	22°C
1	Flesh	1.8 ± 0.2^{aA}	4.7 ± 0.2^{aC}	1.7 ± 0.2^{aA}	3.8 ± 0.2^{aB}
4	Flesh	2.2 ± 0.2^{aA}	6.7 ± 0.2^{bC}	1.9 ± 0.2^{aA}	4.9 ± 0.2^{bB}
7	Flesh	4.6 ± 0.2^{bB}	5.9 ± 0.2^{bC}	3.2 ± 0.2^{bA}	4.8 ± 0.2^{bB}
1	Peel	1.8 ± 0.3^{aA}	5.5 ± 0.3^{aC}	1.8 ± 0.3^{aA}	4.3 ± 0.3^{aB}
4	Peel	1.7 ± 0.3^{aA}	6.6 ± 0.3^{bC}	2.1 ± 0.3^{aA}	5.4 ± 0.3^{bB}
7	Peel	4.2 ± 0.3^{bA}	6.8 ± 0.3^{bC}	3.5 ± 0.3^{bA}	5.7 ± 0.3^{bB}

Numbers in the same column of the same extract sharing similar lowercase letters or numbers in the same raw sharing similar upper case letters are not significantly different.

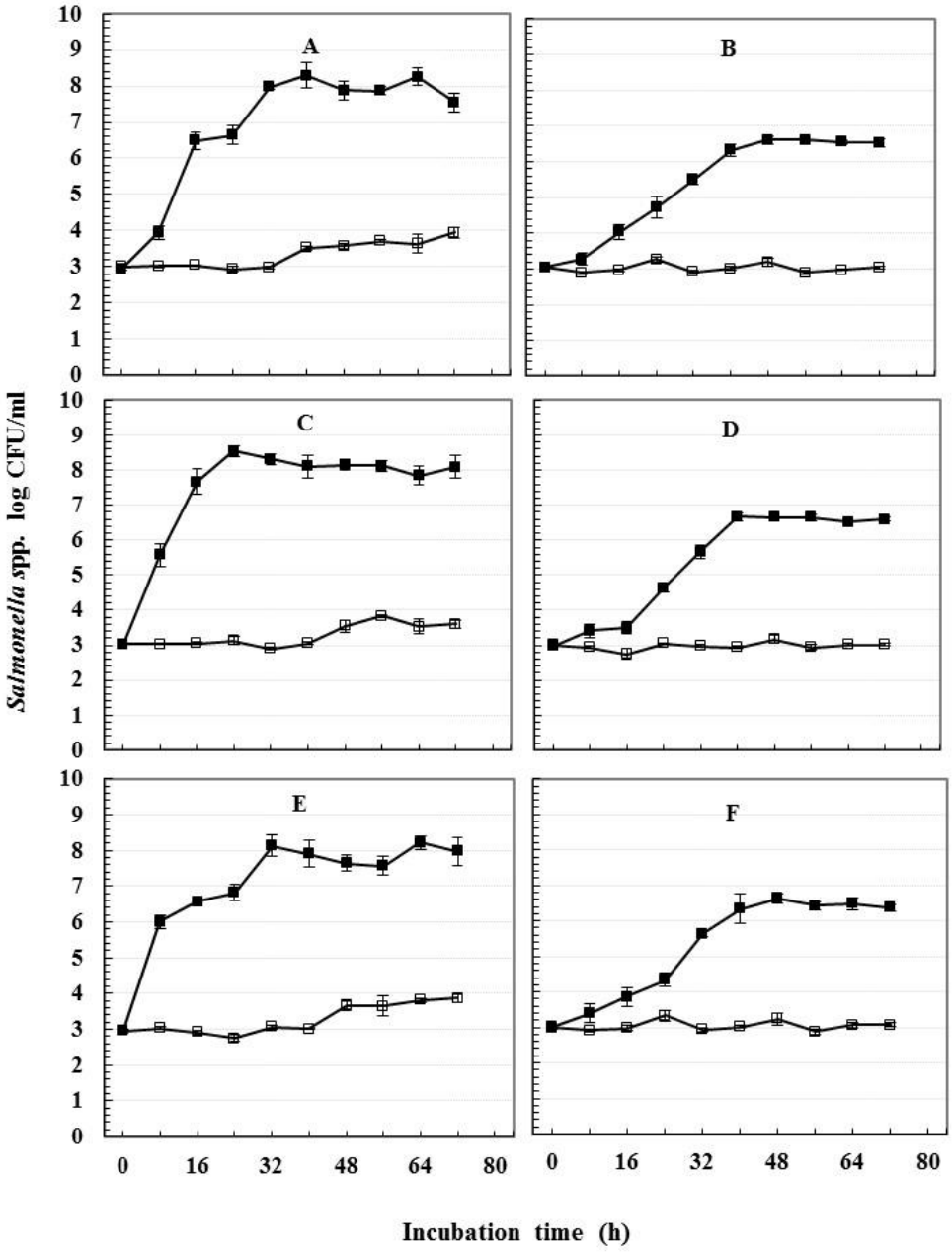

Figure 4.1 Growth of *Salmonella* 01A4754 (AB), H1256 (CD) and G4639 (EF) in 50 mg/ml (ACE) and 2 mg/ml (BDF) of cantaloupe flesh extract at 22 °C (■) and 10 °C (□).

Error bars indicate standard error.

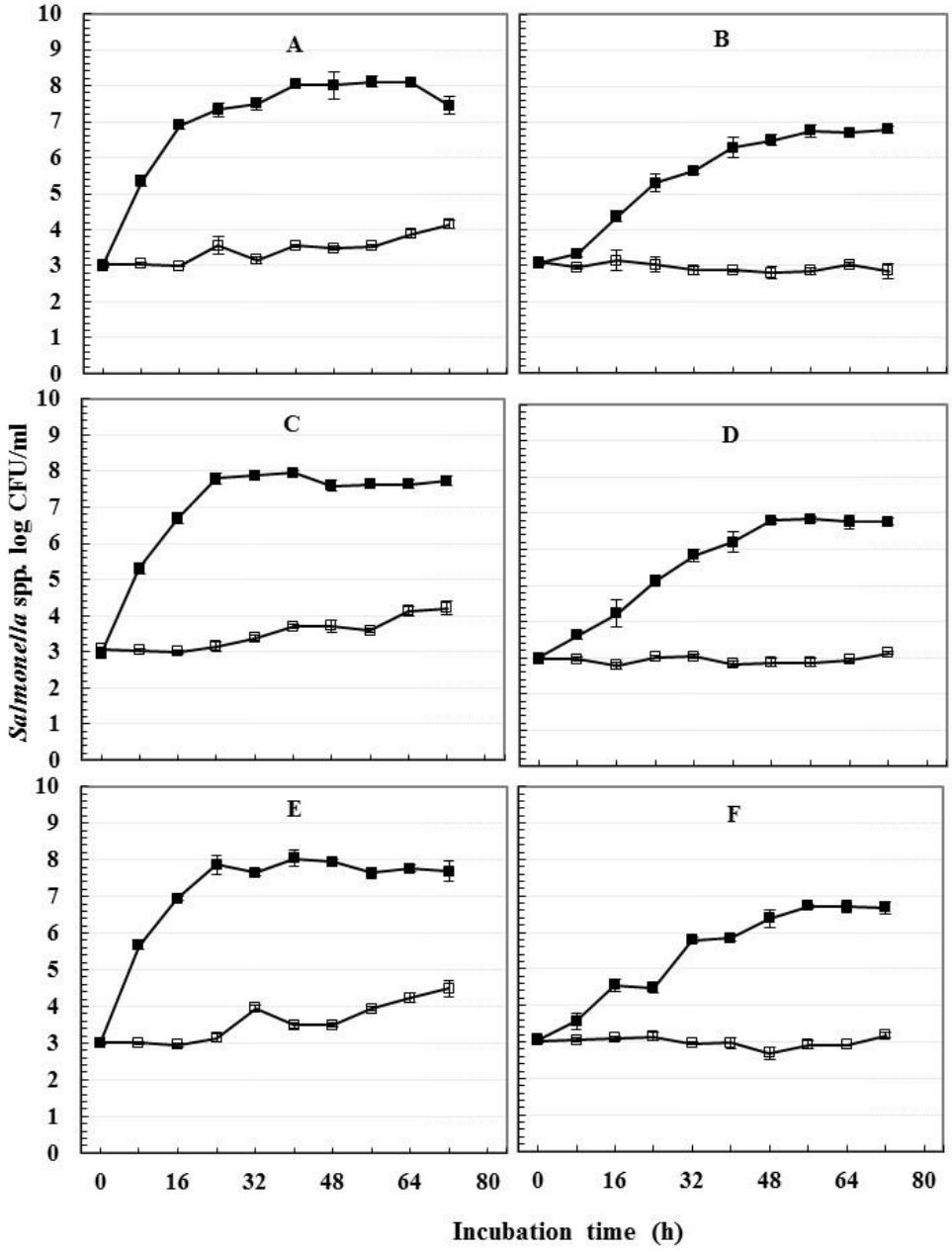

Figure 4.2 Growth of *Salmonella* 01A4754 (AB), H1256 (CD) and G4639 (EF) in 50 mg/ml (ACE) and 2 mg/ml (BDF) of cantaloupe peel extract at 22 °C (■) and 10 °C (□).

Error bars indicate standard error.

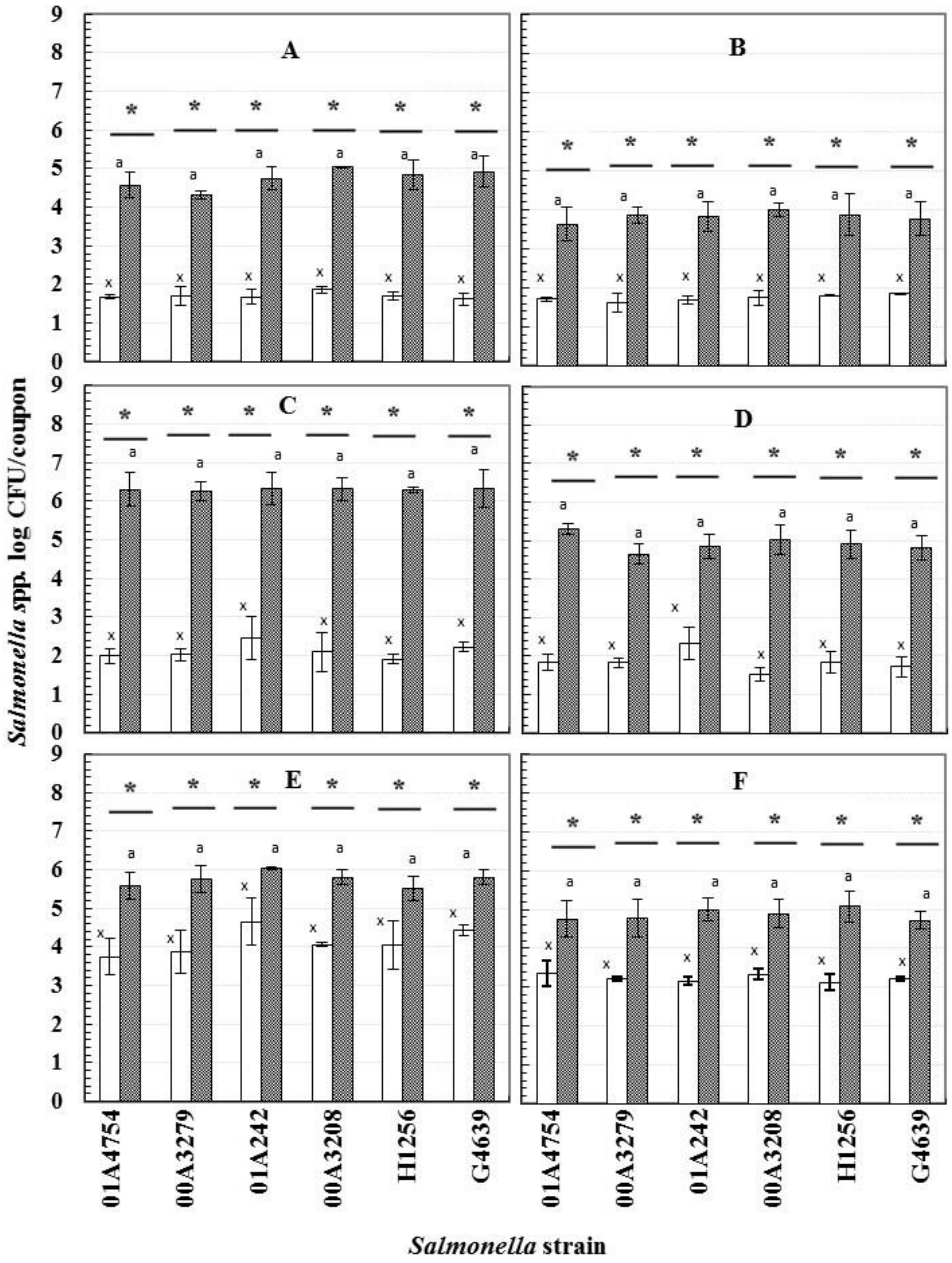

Figure 4.3 Biofilm formation by six strains of *Salmonella* spp. on stainless steel surface in 50 mg/ml (ACE) and 2 mg/ml (BDE) of cantaloupe flesh extract at 22°C (▨) and10°C (□) in 1 day (AB),4 days (CD**)** and 7 days (EF).

Bars with different lowercase letters indicate significant differences between biofilms formed at the same temperature and * indicate significant difference between biofilms belong to the same strain formed at different temperature based on Tukey's Honestly Significant Difference test ANOVA test ($P < 0.05$). Error bars indicate standard error.

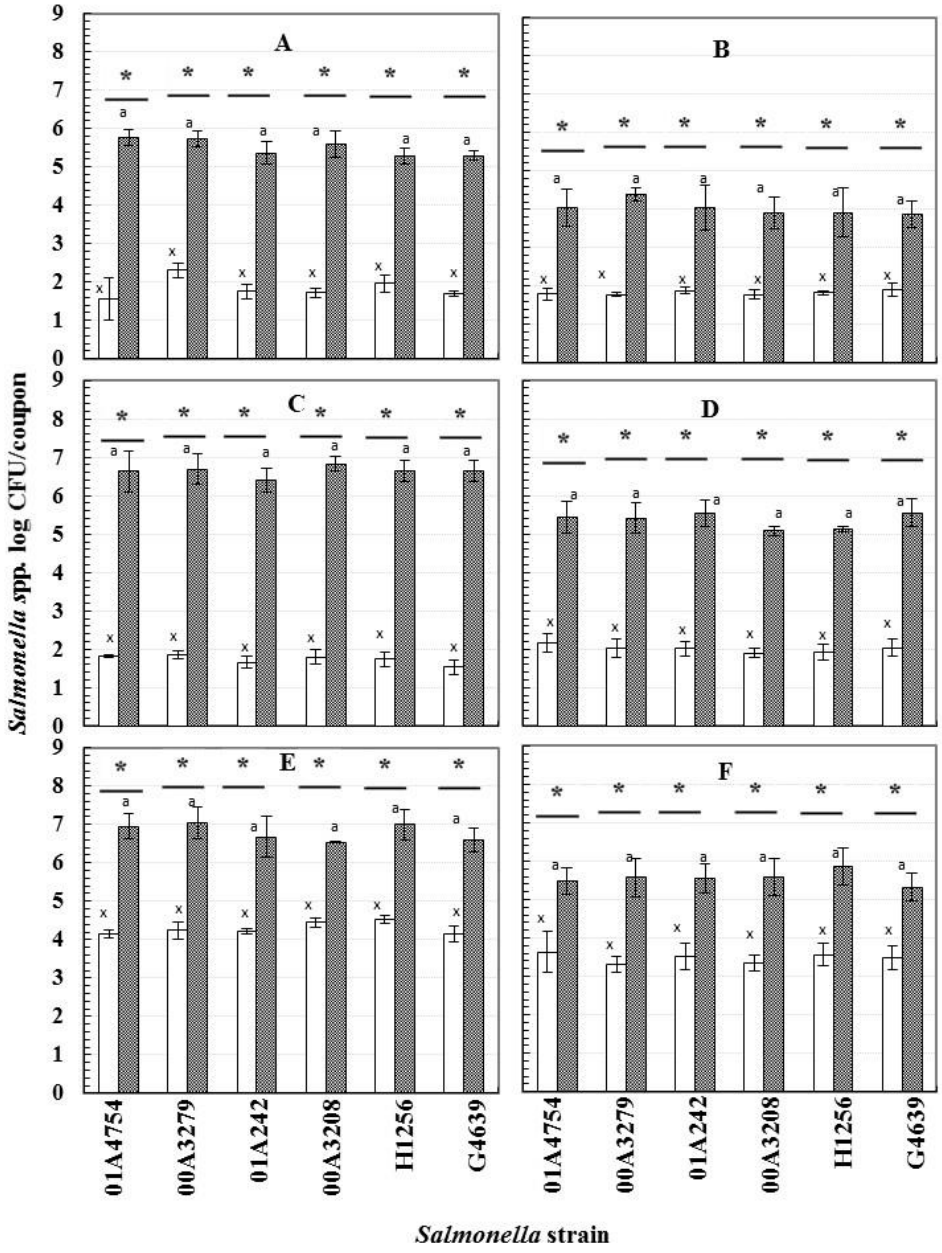

Figure 4.4 Biofilm formation by six strains of *Salmonella* spp. on stainless steel surface in 50 mg/ml (ACE) and 2 mg/ml (BDE) of cantaloupe peel extract at 22°C (▨) and10°C (□) in 1 day (AB),4 days (CD**)** and 7 days (EF).

Bars with different lowercase letters indicate significant differences between biofilms formed at the same temperature and * indicate significant difference between biofilms belong to the same strain formed at different temperature based on Tukey's Honestly Significant Difference test ANOVA test ($P < 0.05$). Error bars indicate standard error.

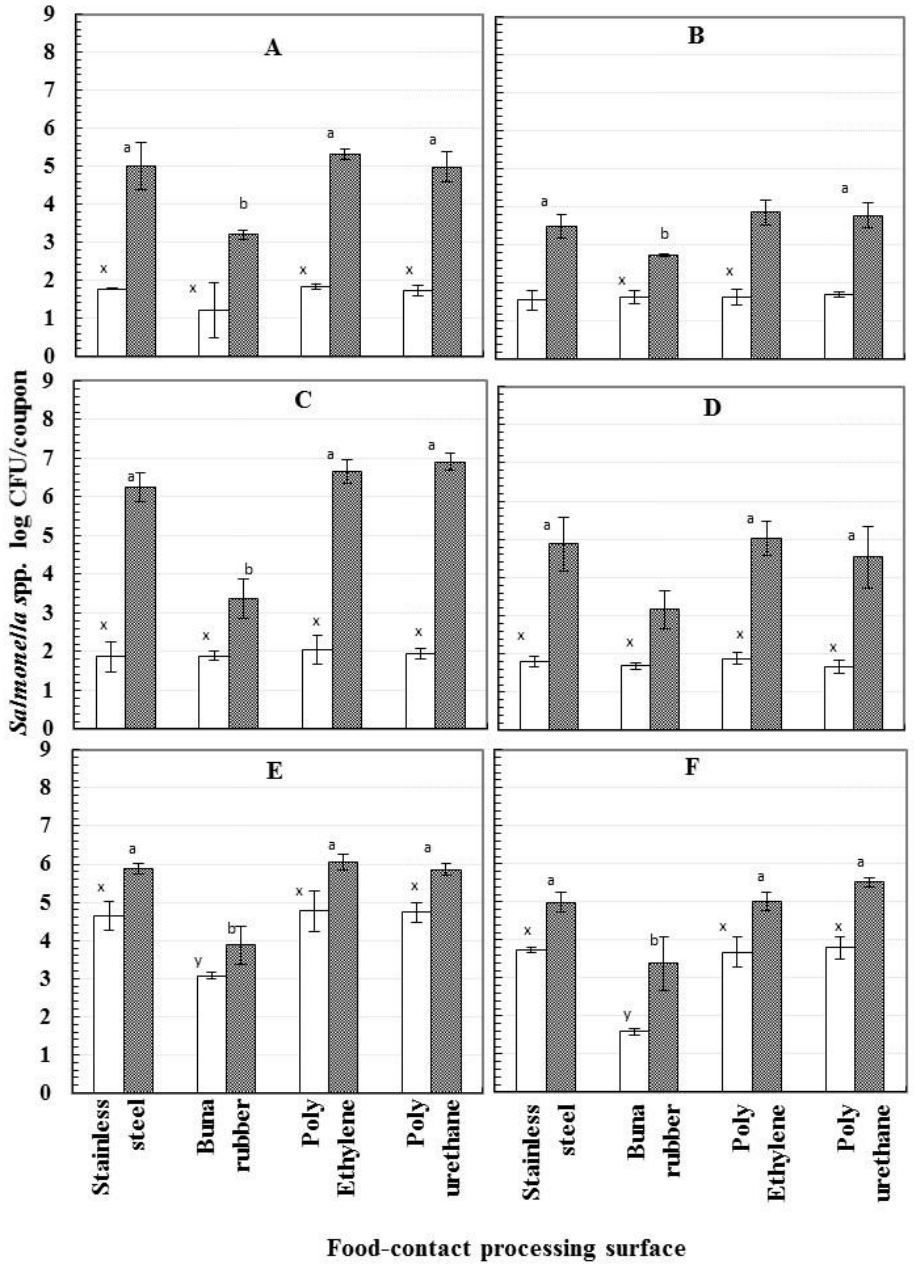

Figure 4.5 Biofilm formation by *Salmonella* 01A4754 on different surfaces in 50 mg/ml (ACE) and 2 mg/ml (BDE) of cantaloupe flesh extract at 22°C (▩) and 10°C (□) in 1 day (AB),4 days (CD**)** and 7 days (EF).

Bars with different lowercase letters indicate significant differences between biofilms formed at the same temperature based on Tukey's Honestly Significant Difference test ANOVA test ($P < 0.05$). Error bars indicate standard error.

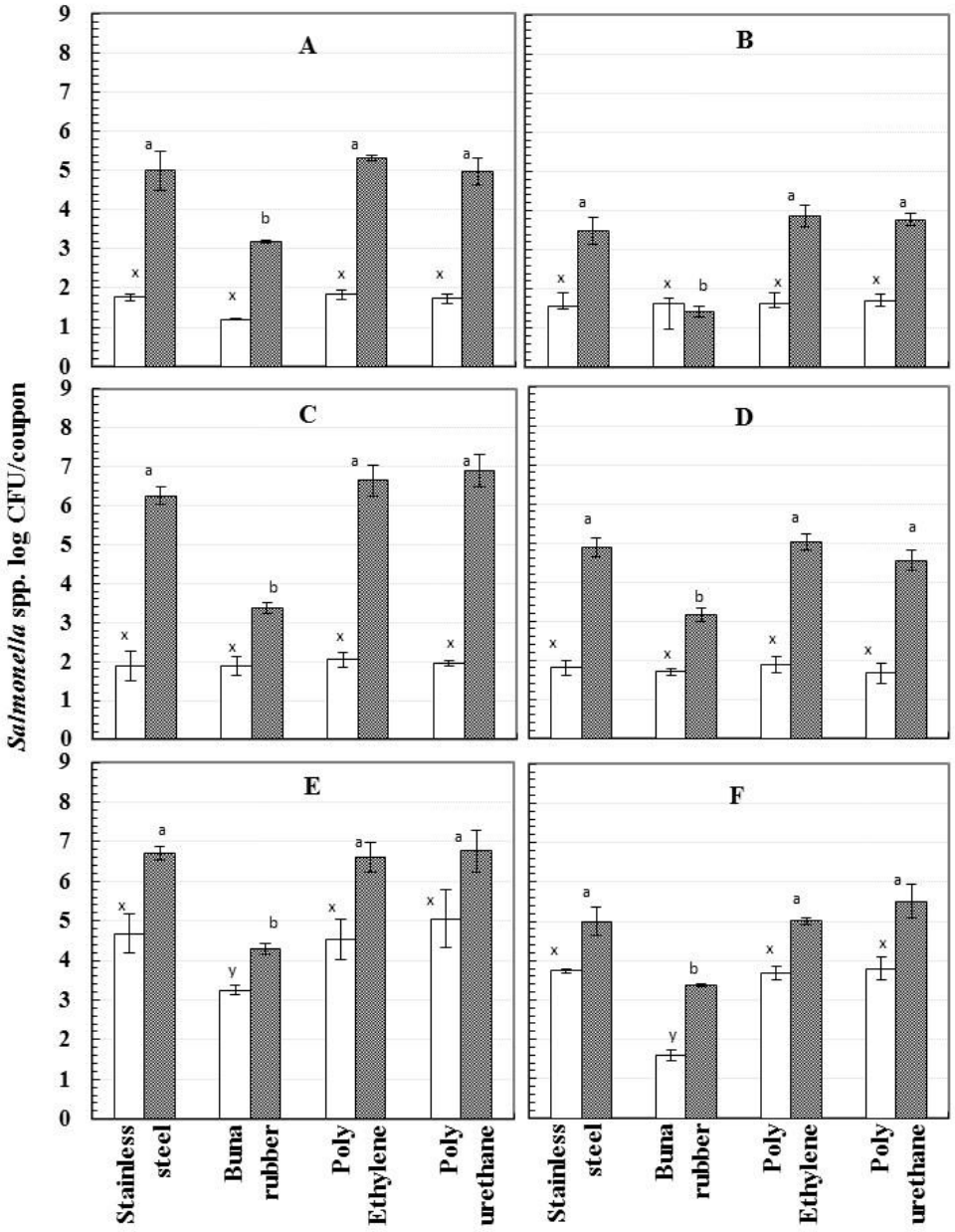

Figure 4.6 Biofilm formation by *Salmonella* 01A4754 on different surfaces in 50 mg/ml (ACE) and 2 mg/ml (BDE) of cantaloupe peel extract at 22°C (▩) and 10°C (□) in 1 day (AB),4 days (CD**)** and 7 days (EF).

Bars with different lowercase letters indicate significant differences between biofilms formed at the same temperature based on Tukey's Honestly Significant Difference test ANOVA test ($P < 0.05$). Error bars indicate standard error.

CHAPTER V

SUMMARY AND CONCLUSION

Two foodborne bacterial pathogens, *L. monocytogenes* and *Salmonella* spp., are often associated with cantaloupe. Critical factors governing how *L. monocytogenes* and *Salmonella* spp. establish, survive and persist in the food processing environment is essential for developing the effective steps for eliminating their contamination. These findings illustrate that temperature, nutrient level and food-contact surface determines the growth and biofilm formation by diverse strains of *L. monocytogenes* and *Salmonella* in cantaloupe flesh and peel extracts. The growth and biofilm formation of *L. monocytogenes* and *Salmonella* strains were increased with increasing temperature and with increasing cantaloupe flesh and peel extract concentration. No major difference in biofilm formation was observed among the six strains of *L. monocytogenes* or six strains of *Salmonella* spp. tested. *L. monocytogenes* and *Salmonella* spp. formed less biofilm on buna-n rubber compared to stainless steel, polyurethane and polyethylene surfaces containing cantaloupe extract. A very low concentration of nutrients leaked from cantaloupe flesh or peel can induce growth and biofilm formation in *L. monocytogenes* and *Salmonella* spp. on different food-contact surfaces. These findings illustrate the importance of appropriate cleaning and sanitization measures for cantaloupe processing surfaces to prevent the conditions for growth and subsequent biofilm formation by *L. monocytogenes* and *Salmonella* spp. which can lead to dangerous food safety risks.

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