

MICROBIAL LOAD AND SANITATION OF FRESH BLUEBERRY PACKING LINES IN GEORGIA

by

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(Under the Direction of Jinru Chen)

ABSTRACT

This study determined the hygienic conditions of selected surfaces of fresh blueberry packing lines and evaluated the ease of biofilm accumulation on, and cleanability of, materials commonly found in the blueberry packing environment. Packing line hygiene was assessed by enumerating total aerobes, total yeasts and molds and total coliforms and determining the incidence of fecal coliforms and enterococci in surface swab samples. Twelve fecal coliforms isolated from the six packing lines were used in the sanitation study. Results showed that berry lugs, rubber belts on color sorters, and immature berry disposing areas had significantly higher ($P<0.05$) microbial counts than the other sites sampled. The amount of biofilms accumulated on polypropylene surfaces was significantly higher than that on the other surfaces tested in the study. Ozonated water was significantly more effective in removing biofilms than a quaternary ammonium compound which were significantly more effective than chlorine dioxide, followed by sodium hypochlorite.

INDEX WORDS: Blueberry, packing line, environmental hygiene,

fecal coliforms, fruit quality, abiotic surfaces, sanitizers

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DEDICATION

To my sister and brother-in-law.

Your unconditional love and encouragement has always meant the world to me. Love you both.

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TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.....	v
LIST OF TABLES.....	vii
LIST OF FIGURES.....	ix
CHAPTER	
1 INTRODUCTION.....	1
2 LITERATURE REVIEW.....	3
3 MICROBIAL LOADS ON SELECTED FRESH BLUEBERRY PACKING LINES..	34
4 EASE OF BIOFILM ACCUMULATION, AND EFFICACY OF SANITIZING TREATMENTS IN REMOVING THE BIOFILMS FORMED, ON COUPONS MADE OF MATERIALS COMMONLY USED IN BLUEBERRY PACKING ENVIRONMENT.....	64
5 CONCLUSIONS.....	93
APPENDIX	
A. SUSCEPTIBILITY OF SELECTED FECAL COLIFORMS TO ANTIBIOTICS, WITH OR WITHOUT THE PRE-EXPOSURE TO SUBLETHAL CONCENTRATIONS OF CHLORINE DIOXIDE.....	94

LIST OF TABLES

	Page
Table 3.1 Overall mean populations of total aerobes, total yeasts and molds, and total coliforms on different sample sites and packing lines and at sampling points.....	56
Table 3.2 Mean populations of total aerobes, total yeasts and molds, and total coliforms from samples collected from 6 different packing lines at different sampling times	57
Table 3.3 Mean populations of total aerobes on different sites of individual packing lines.....	58
Table 3.4 Mean populations of total yeasts and molds on different sample sites of individual packing lines.....	59
Table 3.5 Mean populations of total coliforms on different sites of individual packing lines.....	60
Table 3.6 Number and percentage of samples tested positive for enterococci and fecal coliforms.....	61
Table 4.1 Fecal coliforms used in the study.....	88
Table 4.2 Overall mean value of biofilm mass accumulated by two- strain mixtures of fecal coliforms, isolated from different fresh blueberry packing lines, on coupons of various materials after 7 days of incubation at 10°C	89
Table 4.3 Percentage of biofilm remaining after sanitizing treatment.....	90
Table 4.4 The effect of various sanitizers in removing biofilms from various coupons.....	91
Table A.1 Minimum inhibitory concentrations of various antibiotics against selected fecal coliforms preadapted in sub lethal concentrations of antibiotics.....	106

Table A.2 Number of tested fecal coliforms completely inhibited by a changed or unchanged minimal inhibitory concentrations of antibiotics.....	108
Table A.3 Changes in the susceptibility of selected fecal coliforms to various antibiotic as affected by preadaptation in sub-lethal concentrations of chlorine dioxide.....	109
Table A.4 Number of pre adapted fecal coliform isolates with changed or unchanged susceptibility to various compared to non-adapted cells according to the break points.....	111

LIST OF FIGURES

	Page
Fig. 4.1 Ten selected sampling sites on each packing line.....	63

CHAPTER 1

INTRODUCTION

The demand for blueberries has exceeded its supply during the past decade, primarily as a result of the positive health benefits associated with blueberry consumption (Beattie, Crozier, & Duthie, 2005; Szajdek & Borowska, 2008). Blueberries are among the most popular berries in the retail market, sold in numerous forms such as fresh, frozen, and processed fruits (Strik, 2007). The United States ranks first in the production of blueberries in the world, producing over 236.5 million Kg in 2016 (Eklund, 2017). Georgia ranks fourth in the United States in total production and second in total acreage, yielding a farm gate value of \$283 million, which makes blueberries the state's economically most important fruit crop (Stubbs, 2017). Approximately, 52% of the total harvested blueberry crop was sold as fresh fruit in the U.S. in 2016 (Brazelton, Young.K, & Bauer, 2017). At present, fresh blueberries are available in the market throughout the year, sourced from different regions of the world based on growing seasons (Lin, Variyam, Allshouse, & Cromartie, 2003).

Fresh blueberries that are ready to be sent to the market do not undergo any treatment or washing before shipping, due to the belief that washing will promote mold growth and shorten the shelf life of the product (Sy, McWatters, & Beuchat, 2005). Additionally, washing would also remove the visually appealing wax layer ("bloom") from the fruit surface that is generally associated with fresh blueberries by the consumer. Since fresh berries are consumed raw or minimally processed, they have the potential to serve to transmit foodborne illnesses. Despite the healthy outcomes of regular blueberry consumption, foodborne illnesses have been linked to fresh

blueberries contaminated with pathogenic microorganisms in a few cases (Palumbo, Harris, & Danyluk, 2013). For example, a multistate outbreak of *Salmonella* Muenchen infection associated with eating blueberries was reported in 2009 (Huang & Chen, 2014). An outbreak of hepatitis A infection in New Zealand was linked to the consumption of fresh blueberries which were likely contaminated by the food handlers (Calder et.al, 2003).

This research aims at improving the microbial safety of fresh blueberries by filling knowledge gaps on the hygiene conditions of fresh blueberry packing lines.

The objectives of this study were:

1. To evaluate the microbial loads on selected fresh blueberry packing lines
2. To assess the ease of biofilm accumulation, and efficacy of sanitizing treatments in removing the biofilms formed on coupons made of materials commonly used in blueberry packing environments.

CHAPTER 2

LITERATURE REVIEW

Growing demand for fresh fruits and vegetables

The demand for fresh fruits and vegetables is on the rise in the U.S (Naanwaab & Yeboah, 2012). Increase in purchasing power, consumer awareness and growth in public knowledge about the benefits of consuming fruits and vegetables make them an increasingly popular choice in many diets (Nzaku, Houston, & Fonsah, 2010). Fresh produce can be a significant source of vitamins, minerals, and fiber, which are key components of healthy diets (Monaghan & Hutchison, 2010). It is well known that an adequate intake of fruit and vegetables promotes health as it is important in the prevention of non-communicable diseases such as cardiovascular disease, obesity and cancer (Slavin & Beate, 2012). In the U.S., the per capita consumption of fresh fruit and vegetables is 52.6 and 53.5 Kg respectively, in 2016 (Statista, 2018).

Among fresh fruits, there has been a constant increase in the popularity of berries as they contain significant dietary sources of bioactive compounds such as tannins, indoles and ascorbic acid (Yang & Kortessniemi, 2015). Moreover, berries are considered high-valued specialty crops, as they are capable of earning higher returns per unit of land than could be achieved with traditional agricultural products (Sobekova, Thomsen, & Ahrendsen, 2013). The demand for fresh and processed blueberries has substantially increased over the past two decades (Kaiser, 2010). In the last decade, blueberries have become more popular due to their well-known health benefits and nutritional value (Ștefănescu, Eșianu, Laczkó-Zöld, Tudor, & Dogaru, 2017). In addition, blueberries are by far the most consumed berry by volume (Gutierrez, 2014). It is reported that

per capita, blueberry consumption in the United States grew more than 600% in the past 20 years (Villata, 2018).

Blueberries and their health benefits

Blueberries are included in the family Ericaceae, genus *Vaccinium*, with approximately 450 species worldwide (Cortés-Rojas, Mesa-Torres, Grijalba-Rativa, & Pérez-Trujillo, 2016). Blueberries can be diploids, tetraploids and hexaploids, meaning they have 2, 4 or 6 sets of chromosomes, respectively (Bruederle et.al 1991). They have a shallow adventitious root system and grow best in acidic (pH 4.5-5.2) well drained soils with an organic matter content ranging from 3-20% (Smith & Harris, 2017). Blueberries are native to North America and have a notable antioxidant capacity, three times greater than strawberries or raspberries (Saftner, Polashock, Ehlenfeldt, & Vinyard, 2008). Blueberries are spherical in shape, and their size ranges from 0.7 to 1.5 cm in diameter (Vega-Gálvez et al., 2009). The characteristic color of blueberries is due to the water-soluble anthocyanins (Routray & Orsat, 2011).

Promotion of the potential health benefits of blueberries has resulted in a surge in the production and consumption of berries (Qu, Bradley, & Rumble, 2018). Blueberries contain vitamins A, C, and E, as well as carbohydrates, protein, fiber, and fat, and also contain organic acids and polyphenolic substances (Szajdek & Borowska, 2008). These nutraceutical compounds in blueberries have been linked to a number of health benefits, such as their capability to reduce blood sugar, serum cholesterol, and triglyceride levels (Potter & Coneva, 2018). Additional health benefits of blueberries are linked to their effect on reducing the loss of age-related motor skills and memory and improving urinary tract and visual health (Seeram & Shukitt-hale, 2016). Blueberries are rich in antioxidant, anti-aging and anticarcinogenic properties, as they are high in phenolic compounds, flavonoids, and phenolic acids (Beattie, Crozier, & Duthie, 2005).

Blueberry production

The United States ranks first in the world production of blueberries in the world with a yield of over 521.6 million pounds in 2016 (Eklund, 2017). Nationwide, blueberry acreage has increased by 73%, from 21,618 ha in 2007 to 37,554 ha in 2016 (USDA, 2018). The state of Georgia ranks second in the nation in total acreage (18.2%) and fourth in total production with a farm gate value of \$283 million in 2016 (Stubbs, 2017). It was the fourth largest crop in Georgia in 2016 with a production of 70 million pounds (USDA, 2017).

Four types of blueberries, native to North America, namely, highbush or northern highbush (*Vaccinium corymbosum*), southern highbush (*Vaccinium darowii*), rabbiteye (*Vaccinium virgatum*) and lowbush (*Vaccinium angustifolium*) are cultivated today and are commercially harvested and sold in the United States (Skrovankova, Sumczynski, Mlcek, Jurikova, & Sochor, 2015). Highbush and rabbiteye are the cultivated blueberries while the lowbush species is often marketed as wild blueberry (Strik, 2007). Potter & Coneva (2018) reported that among these types, rabbiteye and southern highbush blueberries are the most important one in Georgia, as they are the easiest and most productive type that can be grown. Rabbiteye is native to South Georgia, north Florida and south-east Alabama. They require low to moderate chilling temperatures and are better adapted to mineral soils (Fonsah, Massonnat, Wiggins, Stanaland, & Ed Smith, 2016). Ripening of rabbiteye blueberries starts in late May-late July in southern Georgia (Krewer & Nesmith, 2006). Southern highbush blueberries are crosses between the highbush blueberry (northern type) and native southern blueberries. Southern highbush blueberries are very early ripening and have commercial potential for the April and May market window in South Georgia. Southern highbush cultivars with a low winter chilling requirement and bloom early, so they are best adapted to south

Georgia. They are generally more difficult to grow than rabbiteyes, but their fruit ripens very early and brings a good price.

At present, blueberries are available in retail stores all year long and are sourced from different regions of the United States or the world depending on growing seasons (Lin, Variyam, Allshouse, & Cromartie, 2003). Blueberries are sold as fresh, frozen or processed fruits in the market. For fresh consumption, they can be sold either through 1) U-pick (customer harvested) or on-farm sales (grower harvested) and 2) fresh sale through local stores or distributed to distant locations (Strik, 2007). Fresh blueberries have been increasingly favored by U.S. consumers over frozen blueberries (Pollack & Perez, 2008). The percentage of blueberries sold in the fresh market has increased sharply due to the rapid growth of consumer demand for fresh fruits. Approximately 52% of the blueberries harvested are sold to the market as fresh fruit (Brazelton, Young, & Bauer, 2017).

Postharvest handling of blueberries

The ability to deliver a quality product to the market and ultimately to the consumer commands buyer attention and gives the grower a competitive edge. Proper postharvest cooling and handling can help ensure that quality is maintained until the product reaches the consumer. According to Forney (2009) the most crucial factor in the post-harvest handling of blueberries is storage at appropriate temperature. In general, most blueberries are most rapidly cooled by forced air. Higher storage temperatures will result in loss of firmness and that affects the final market quality of the product (Paniagua, East, Hindmarsh, & Heyes, 2013). Mature blueberries for the fresh market are usually sent to packinghouses after harvest and cooled at 0°C to remove field heat before packing (Watson, Treadwell, Sargent, & Brecht, 2016). It is also reported that cooling blueberries to 5°C before packing reduces microbial activity, especially when blueberries are not

washed (Forney, 2009). Soots, Maksarov, & Olt (2014) mentioned that machine-harvested blueberries need post-harvest processing technology that includes many steps. The less blueberries are processed and the shorter the chain to the consumer, the better it is for blueberry quality (Forney, 2009).

In a blueberry packinghouse, packing starts by dumping the harvested berries from lugs onto the packing line. The berries are moved through a lifting conveyor to a blower cleaner which removes leaves, sticks, peat and other small berries from the fruit. The blueberries then go through the color sorter. The idea of sorting is to separate immature, soft and off-colored blueberries.

After being sorted, the berries are rolled onto a grading/inspection belt where workers manually remove additional immature and soft fruit missed by the color sorter from the packing line. The poor-quality berries removed at the manual sorting area can be used as industrial berries if necessary. After manual sorting, fresh market berries are transferred through conveyors before being packed in clam shell boxes (Xu, Takeda, Krewer, & Li, 2015). Blueberries for the fresh market do not undergo any treatment or washing before being shipped to the market because of that washing will promote mold growth and reduces the shelf life of the product (Sy, McWatters, & Beuchat, 2005).

Food safety issues associated with blueberries

Despite their nutritional characteristics, berries are identified as one of the top ten riskiest foods by a consumer advocate group (Klein, Witmer, Tian, & DeWaal, 2009). Typically, blueberries intended for the fresh market are not washed (Gallardo et al., 2018). Blueberries that are not washed or treated prior to market may become contaminated from a variety of sources including, improper sanitation and contaminated irrigation water (Han & Selby, 2004).

Since blueberries are generally consumed raw or only minimally processed without a pasteurization step, microbial contaminants can easily reach the consumers (Popa, Hanson, Todd, Schilder, & Ryser, 2007). Four outbreaks linked to the consumption of contaminated fresh blueberries were reported during the period between 1983 to 2010 (Palumbo, Harris, & Danyluk, 2013). In 1984, an outbreak of listeriosis likely associated with fresh blueberries was reported in Connecticut (Popa, Hanson, Todd, Schilder & Ryser, 2007). In New Zealand, in 2002, an outbreak of hepatitis A was linked to the consumption of raw blueberries, which were likely contaminated by infected food handlers or fecally contaminated groundwater (Calder et al., 2003). Additionally, according to the Foodborne Outbreak Online Database, in June 2009 a confirmed outbreak of *Salmonella* Muenchen on blueberries caused 14 people to become ill (CDC, 2013). These outbreaks highlight the need for more effective control measures in the blueberry industry.

Regulations for produce safety

The Food Safety Modernization Act (FSMA) of 2011 aims to strengthen the food system by developing food safety standards and shifting the focus to a prevention-based food safety culture in the United States. After the legislation was finalized in 2014, all fresh produce growers, with an average of more than \$25,000 to \$250,000 in annual produce sales were given four years to bring their businesses into compliance with most of the provisions. Farmers were granted an additional two years to become compliant with water quality requirements, while businesses with annual produce sales from \$250,000 to \$500,000 will have three years to become compliant. The FSMA rule for produce carried a number of changes to food safety practices in the Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption (FDA, 2013).

Among these, audit requirements for USDA Good agricultural practices (GAP) specified that water applied to crops is required to meet the standards of the 2012 Environmental Protection

Agency's Recreational Water Quality criteria for all produce except sprouts. For water unable to meet the EPA microbial level standards, it allows provisions for irrigation water to be sanitized through a timed process of natural microbial die-off. This option was suggested in the comment period for the rule, and required specific time intervals between irrigation and harvest, and harvest and end of storage (Ribera, Yamazaki, Paggi, & Seale James., 2016). The FSMA does require that soil amendments of animal origin, such as raw manure must be applied in a manner that does not contact covered produce during application, and any potential for subsequent contact is minimized (FSMA, 2015). The final FSMA rule requires that fresh produce identified as likely having been disturbed by animals must be excluded from harvest. However, the rule allows for animals for intrude into outdoor growing areas, and also does not stipulate a mandatory length of time between animal grazing in fields and harvest of fresh produce. FSMA requires those areas used for growing produce to be monitored during the growing season and immediately prior to harvest if there is a reasonable probability animal intrusion will contaminate the crop. The FSMA rule also established standards for worker health and hygiene management practices, with requirements for proper usage of handwashing and toilet facilities and prevention of contact by sick workers. Workers who handle fresh produce are required to have food safety education, including on-the-job training combined with experience (FSMA, 2015).

Monitoring of hygiene conditions in packing environments

Detecting and/or quantifying pathogen contamination in the packing environment is difficult because of low population levels, low prevalence, and a non-uniform distribution of pathogens (Ray, 2004). Thus, levels of microbial indicator species are often used to predict product safety and hygiene conditions under which a product has been processed and/or handled (Heredia et al., 2016). Commonly used microbial indicators include total aerobic bacteria,

enterococci, fecal coliforms, total coliforms and yeasts and molds (Castro-Ibáñez, Gil, Tudela, & Allende, 2015). As no single hygiene indicator is ideal, use of a variety of indicators is recommended when assessing food safety and sanitation of the food production environment (Cárdenas, Molina, Heredia, & García, 2013).

Fecal coliforms

Fecal coliforms, a subset of total coliforms, are differentiated by their ability to ferment lactose with the production of acid and gas at 44.5°C within 24 h. Fecal coliforms have been more accurately termed “thermotolerant” coliforms because they differ from total coliforms by higher optimal growth temperature and not necessarily origin. Fecal coliforms are present in raw foods of animal origin and in plant foods from contaminated soil and water. Their presence in heat-processed and ready-to-eat foods is probably because of improper sanitation after heat treatment before packaging and may indicate possible fecal contamination and presence of enteric pathogens (Ray, 2004).

Fecal coliforms are detected by using a high incubation temperature ($44.5 \pm 0.2^{\circ}\text{C}$ or $45.0 \pm 0.2^{\circ}\text{C}$) for 24 h in selective broths containing lactose. Lactose fermentation, with the production of gas, is considered a presumptive positive test (Francy, Myers, & Metzker, 1993). A significant correlation between the presence of enteric pathogens (*Salmonella* and *E. coli* O157:H7) and counts of fecal coliforms isolated from leafy greens was observed (Castro-Ibáñez et al., 2015).

Enterococci

Enterococci occur in all human feces and in the colons of mammals at a concentration of approximately 10^6 - 10^7 organisms/g fecal matter (Layton, Walters, Lam, & Boehm, 2010). The lifespan of enterococci is weeks to months, similar to that of most enteric pathogens (Dubin & Pamer, 2017). Enterococcus testing is considered in addition to *E. coli* as it may provide more

accuracy at a low additional cost (Dubin & Pamer, 2017). Enterococci have been used as an indicator of fecal contamination in soils (Byappanahalli & Fujioka, 2004), on plants (Byappanahalli, Shively, Nevers, Sadowsky, & Whitman, 2003), and in packinghouses (Abadias, Usall, Anguera, Solsona, & Viñas, 2008; Heredia et al., 2016).

Contamination of food contact surfaces in packinghouse environments

Postharvest interventions implemented in the packinghouse are centered around minimizing or controlling the spread of microorganisms on the walls, utensils, and on the surface of the packing lines (Zoellner, Al-mamun, Grohn, Jackson, & Worobo, 2018). Moreover, packing lines are considered as a potential source of microbial contamination (Heredia et al., 2016). Sanitization practices may vary greatly among packinghouses as it depends on the type of product, procedure or sanitizers used (Donlan, 2002). Poor maintenance of the packing line could exacerbate the load of bacterial pathogens within or on packing line surfaces due to insufficient processing or sanitation practices (Alum, Akanele, Chukwu, & Ahudie, 2016).

Poor hygienic design of a packing line could cause food safety issues during or after the processing. For example, areas that cannot be easily cleaned could harbor microorganisms, protecting them from the effects of sanitizers (Marchand et al., 2012). These areas that are more prone to collection of microorganisms, called niche points, could result in survival and growth of microorganisms, as evidenced by repeated detection during monitoring the food processing environment for foodborne pathogen (Donlan, 2002).

Biofilms

Biofilms are communities of microorganisms adherent to a surface and/or one another and surrounded by a self-produced extracellular polymeric substance (EPS) matrix (Srey, Jahid & Ha, 2013). Development of biofilms consists of five steps: (1) reversible attachment of planktonic

bacteria cell to a surface, (2) irreversible attachment by production of extracellular polymers by the bacteria and/or by adhesion interacting with the surface, (3) development of biofilm structure, (4) development of microcolonies, and (5) dispersion of cells from the biofilm (Brooks & Flint, 2008; Schlegelová, Babák, & Holasová, 2010).

The reversible attachment of bacterial cells to the surface occurs by interaction forces including van der Waals, hydrophobic and electrostatic forces (Chmielewski & Frank, 2003). Then, the cells are irreversibly attached to the surface by hydrogen bonding, dipole-dipole interaction, and ionic covalent bonding (Brooks & Flint, 2008). The irreversibly attached cells use the nutrients from the surrounding environment to grow and divide, forming microcolonies (Kumar & Anand, 1998). EPS is also produced by the attached cells during this stage, which assist cells to firmly attach to the surface and to trap nutrients from the environment (Van Houdt & Michiels, 2010). In addition to the continuous attachment of cells, EPS forms a complex biofilm architecture (Tachikawa, Yamanaka, & Nakamuro, 2009).

As the biofilm matures, the bacterial cells growing in the biofilm are detached, and the released cells move to another surface (Brooks & Flint, 2008). Nikolaev & Plakunov (2007) reported that biofilm formation could be affected by various environmental factors including, pH, salinity, osmolarity, oxygen partial pressure, accessible nutrient sources, hydrophobicity of the phase interface, and the force and type of liquid motion around surface.

Bacteria within a biofilm are more resistant to sanitizer treatments because of limited availability of key nutrients (Donlan, 2002). This resistance is attributed to the conditions associated with biofilms including; reduced diffusion, physiological changes due to reduced growth rates and production of enzymes which degrade antimicrobial substances (Simões, Simões, & Vieira, 2010). In the food industry, attachment of bacteria to the food or on the surfaces of

equipment can lead to illness, outbreaks and economic losses (Khelissa, Abdallah, Jama, Faille, & Chihib, 2017; Shi & Zhu, 2009). Biofilm-associated organisms can easily withstand the attack of commonly used natural and pharmacological agents (Marchand et al., 2012; Sadekuzzaman, Yang, Kim, Mizan, & Ha, 2017).

During the formation of biofilm, EPS produced by bacterial cells trap nutrients and may develop complex architecture with pedestal-like structures, water channels and pores. Cell surface structure plays a role in biofilm formation (Van Houdt & Michiels, 2010). Donlan (2002) reported that cell surface polymers such as fimbriae, proteins, curli, exopolysaccharides and flagella are essential in the attachment, thus leading to the formation of biofilm. Fimbriae and other proteins make cell surfaces hydrophobic and contribute to attachment to hydrophobic substrata, while exopolysaccharide and lipopolysaccharides contribute to attachment to hydrophilic surfaces (Donlan 2002).

Effect of surface topography

The characteristics of surfaces such as chemical composition, roughness, morphology or configuration, and surface hydrophobicity or hydrophilic properties, play an important role in bacterial attachment to surfaces (Donlan, 2002; Fletcher & Loeb, 1979). Fresh blueberry packing lines are made of various materials including stainless steel, rubber, polyurethane, polyvinyl chloride and/or polypropylene. It was reported that bacteria attach with different efficiencies depending upon surface topography (Garrett, Bhakoo, & Zhang, 2008). Sadekuzzaman, Yang, Kim, Mizan, & Ha (2017) found that the *E. coli* O157:H7, *S. Enteritidis* and *S. Typhimurium* in biofilms attach better to stainless steel surfaces compared with rubber surfaces. Rubber and plastic surfaces including polyurethane, polypropylene, high-density polyethylene and polyvinyl chloride are hydrophobic, with little or no surface charge (Sinde & Carballo, 2000). Stainless steel

and polyethylene terephthalate are hydrophilic with either positive or neutral surface charges (Donlan, 2002; Khangholi & Jamalli, 2016). Hydrophobic cells adhere strongly to hydrophobic surfaces, whereas hydrophilic cells adhere strongly to hydrophilic surfaces with opposite surface charge (Giaouris, Chapot-Chartier, & Briandet, 2009).

Control of biofilms

The practice of cleaning, using effective detergent or mechanical force is of great significance to avoid accumulation of particulates and bacterial cells on surfaces and subsequent biofilm formation (Chmielewski & Frank, 2003). However, cells in biofilms have greater resistance against cleaning and sanitizing chemicals than do planktonic cells (Gilbert & McBain, 2001). Ryu and Beuchat (2005) reported that biofilm cells of *E. coli* O157:H7 on the surface of stainless steel were more resistant to chlorine than the planktonic cells.

Extracellular polymeric substances produced by bacterial cells serve as protective barriers against antimicrobial treatments (Costerton et al., 1987). In addition, polysaccharides produced by biofilm cells confer resistance to bacterial cells against cleaning or sanitizing chemicals by inhibiting penetration of biocides into the biofilm structure (Meyer & Wallis, 1997). Therefore, it is important to implement cleaning procedures that remove organic materials before applying disinfectants which inactivate and kill the microorganisms on the surface or after detachment (Zottola and Sasahara 1994). Routine cleaning programs use detergents which are composed of mixtures of ingredients and solubilize the foods and other organic matter present on the surfaces (Schmidt, 2012). The most commonly used sanitizers are chlorine-based sanitizers, quaternary ammonium compounds, ozone, and peracetic acid, among others (Bridier, Briandet, Thomas, & Dubois-Brissonnet, 2011).

Chlorine based sanitizers

(a) Chlorine dioxide

Chlorine dioxide (ClO_2) is used in the food industry due to its efficacy in killing microorganism and its cost effectiveness (Joshi, Mahendran, Alagusundaram, Norton, & Tiwari, 2013). According to FDA (1998), aqueous ClO_2 can be employed to sanitize equipment (maximum of 200 $\mu\text{g/ml}$), whole fresh fruits, whole vegetables, shelled beans and peas with cuticles intact (not exceeding 5 $\mu\text{g/ml}$), as well as peeled potatoes with a maximum wash concentration of 1 $\mu\text{g/ml}$. ClO_2 does not react with ammonia to form chloramines, and more importantly, its oxidizing capacity is 2.5 times greater compared with chlorine (Hubbs, Amundsen, & Olthius, 1981). The bactericidal efficacy of ClO_2 was found to be effective at pH 6 to 10 (Grunert, Frohnert, Selinka, & Szewzyk, 2018). ClO_2 is less corrosive than the other chlorine sanitizers because of the low concentration, which is essential for its effectiveness (Bohner & Bradley, 1991). ClO_2 is generated at the site where it is intended to be used as it is highly unstable in nature (Gordon, Kieffer, & Rosenblatt, 1972).

The mode of action of ClO_2 has not been completely determined, although Sharma & Sohn, (2012) reported that ClO_2 is highly reactive against amino acids in viral proteins, thus altering proteins to result in changes in outer membrane permeability. Kang et al. (2007) reported that the permeability of the outer membrane in *E. coli* was affected by ClO_2 and it was also influenced by cell growth conditions, since starved cells caused the membrane-associated cations, calcium, and magnesium to be more accessible to ethylenediaminetetraacetic acid. The disadvantages of ClO_2 are that it is unstable and explosive when concentrated, and it decomposes at temperatures greater than 80°C when exposed to light (Beuchat, Pettigrew, Tremblay, Roselle, & Scouten, 2005).

(b) Sodium hypochlorite

Chlorine is more effective in the undissociated form (HOCl) than the dissociated form (OCl⁻) (Fukuzaki, 2006). Sodium hypochlorite is a salt of hypochlorous acid, which hydrolyzes in water, producing the hypochlorite ion, as described in the reaction below:



Sodium hypochlorite is commercially available at different concentrations (1-16% by weight of available chlorine, but usually between 5 and 15%) and is commonly known as bleach. Several modes of action of sodium hypochlorite have been proposed (1) disruption of protein synthesis; (2) oxidative decarboxylation of amino acids to nitrites and aldehydes; (3) reactions with nucleic acids, purines, and pyrimidines; (4) unbalanced metabolism after the destruction of key enzymes; (5) induction of DNA lesions with an accompanying loss of DNA-transforming ability; (6) inhibition of oxygen uptake and oxidative phosphorylation, coupled with leakage of some macromolecules; (7) formation of toxic N-chloro derivatives of cytosine; and (8) creation of chromosomal aberrations (Estrela et al., 2002).

Sodium hypochlorite also rapidly becomes inactivated by organic material, which is inherent in raw produce (De Beer, Srinivasan, & Stewart, 1994). Sharma & Sohn (2012) observed a significant reduction in populations of *E. coli* O157:H7 on stainless steel by treatments with 50 and 100 µg/ml of free chlorine for 1 min. Some of the organic materials that inactivate chlorine include tyrosine, tryptophan, cystine, egg albumin, peptone, body fluids, tissues, microbes, and vegetable matter. The effectiveness of sodium hypochlorite decreases with increased pH. For example, it has been reported that 25 µg/ml available chlorine killed 99% of *Bacillus metiens* spores within 2.5 min at pH 6, 5 min at pH 8, and 465 min at pH 12.86 (Rudolph et al., 1941).

Quaternary ammonium compounds

Quaternary ammonium compounds (QACs) are positively charged surfactants which have been extensively used since the mid 1940s. They have a basic structure consisting of at least one hydrophobic hydrocarbon chain linked to a positively charged nitrogen atom, the other alkyl groups being short-chain substituents such as methyl or benzyl groups (Ying, 2004). QACs are active ingredients in commercial products for hard surface cleaning and disinfecting surfaces in households and in the food processing industry (Buffet-Bataillon, Tattevin, Bonnaure-Mallet, & Jolivet-Gougeon, 2012). The most commonly applied QACs are benzalkonium chloride, stearylalkonium chloride, cetrimonium chloride/ bromide (cetrimide) and didodecyl dimethyl ammonium bromide (Hegstad et al., 2010). Benzalkonium chloride has one long hydrocarbon chain that can vary in length from 8 to 18 carbons. Stearylalkonium chloride is alkyl benzyl-dimethyl-ammonium chloride with alkyl chain length of 18. The bromine and chlorine salts of cetrimonium are common QACs with an alkyl chain length of 16 (Zhang et al., 2015).

At higher concentrations, QACs are lethal to vegetative bacteria, yeasts and molds, algae and lipophilic viruses (Elkholly, Hegab, Ismail, & Hassan, 2016). QACs initiate their antibacterial mechanism of action by virtue of their cationic surfactant properties (Mulder et al., 2018). The positive charge allows them to interact with the negatively charged phospholipids that make up the cellular outer membrane for subsequent integration of the hydrophobic hydrocarbon tail into the hydrophobic bacterial membrane core. This action disrupts membrane integrity leading to leakage of cellular components and disturbance of membrane-bound proteins (Ferreira, Pereira, Pereira, Melo, & Simões, 2011; Peter Gilbert & Moore, 2005).

Ozone

Ozone is a tri-atomic oxygen molecule formed by addition of singlet oxygen to the oxygen molecule (Onyango, 2016). Ozone is unstable in both aqueous and gaseous form, and degrades to form hydroxyl, hydroperoxyl, and superoxide radicals (Joshi et al., 2013). Ozone as an additive to water, commonly referred to as ozonated water or aqueous ozone, has been used as an effective bactericidal agent. Efficacy of ozone against microorganisms is affected by ozone concentration, pH, and temperature (Joshi et al., 2013). Ozone has to be generated on site for use because it is unstable and decomposes in water. It can be used in the aqueous or gaseous form.

Several theories regarding the bacterial inactivation mechanisms by ozone have been reported. Giese & Christensen (1954) suggested that the bacterial surface was the primary inactivation site of ozone. Scott & Leshner (1963) proposed the site of ozone attack was at the double bonds of the unsaturated lipids in the cell envelope. Komanapalli & Lau (1998) reported that membrane components are the primary targets of ozone oxidation and suggested that subsequent reactions take place involving the intracellular components, protein and DNA. Two studies involving the inactivation mechanism(s) of ozone on *E. coli* showed that the primary inactivation mechanism was enzymatic in nature. Ingram & Haines (1949) observed a general destruction of the dehydrogenation enzyme systems and proposed that cellular death may have resulted from interference with the respiratory system.

Another possible inactivation site is the sulfhydryl bonds in the bacterial cell. Obadi, Zhu, Peng, Ammar, & Zhou (2016) suggested that oxidation of sulfhydryl groups (SH- to S-S) in the enzyme is the principal cause of cellular death. Other studies suggested that the sulfhydryl group in the membranes is the primary target of ozone attack (Mudd, Leavitt, Ongun, & McManus, 1969). Several factors could affect variation among inactivation mechanisms including strain of

microorganism, number of microorganisms treated, presence of ozone-demanding media components, method of ozone application and method and accuracy of ozone measurement (Khadre, Yousef, & Kim, 2001).

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CHAPTER 3

MICROBIAL LOADS ON SELECTED FRESH BLUEBERRY PACKING LINES

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Abstract

Foodborne outbreaks have been linked to the consumption of blueberries contaminated with pathogenic bacteria. The microbial quality of blueberries could be influenced by the conditions of hygiene prevailing during post harvest handling. This study evaluated the hygienic conditions of 6 selected fresh blueberry packing lines. Five of the packing lines were sampled twice and the remaining one was sampled once during the summer of 2015 and the summer of 2017. A delimited area (100 cm²) on each of the ten selected sites of the packing lines was swabbed with sterile sponges before the packing started (AM samples), during lunchtime break (NOON samples), and at the end of a packing day (PM samples). The sponges were rinsed with Dey-Engley neutralization broth which was subsequently sampled for total aerobes, yeasts and molds, total coliforms, fecal coliforms, and enterococci. Results showed that sample site and sampling time had a significant influence ($P \leq 0.05$) on total aerobic, yeast and mold, and total coliform counts. The PM samples had significantly higher ($P \leq 0.05$) total aerobic and yeast and mold counts than the NOON samples which had significantly higher ($P \leq 0.05$) counts than the AM samples. Forty-six out of the 310 (14.8%) collected samples tested positive for enterococci while 27 (8.7%) samples tested positive for fecal coliforms. Berry lugs, rubber belts on color sorters, and immature berry disposing areas had significantly higher ($P \leq 0.05$) microbial counts than the other sites. The study suggests that some sites along fresh blueberry packing lines could become contaminated by microorganisms during packing. Whether these contaminated sites will become a food safety concern depends on the incidence of pathogen presence in the microbial community and efficacy of routine sanitizing treatments.

Key words: Produce safety, blueberry, blueberry packing line, environmental hygiene

1. Introduction

The demand for blueberries has exceeded its supply during the past decade, primarily as a result of the positive health benefits of blueberry consumption (Beattie, Crozier, & Duthie, 2005; Szajdek & Borowska, 2008). The United States ranks first in the world in the production of blueberries, with a yield of over 593.6 million pounds in 2016 (Eklund, 2016). Blueberries are available to consumers as packed fresh, processed, or “U harvest” products (Zhao, 2007).

An estimated 52% of harvested blueberries was sold as fresh fruit in 2016 in the U.S (Brazelton, Young & Bauer, 2017). Blueberries for the fresh market do not undergo any treatment or washing in packinghouses before being shipped to the market due to the belief that washing will promote mold growth and shorten the shelf life of the product (Sy, McWatters, & Beuchat, 2005). Because of the absence of the treatment, blueberries have a potential risk of contamination by human pathogens. The lack of rigid microbial safety standards for fresh market berries could be a concern to consumers (Popa, Hanson, Todd, Schilder, & Ryser, 2007).

Despite their nutritional characteristics, berries are identified as one of the top ten riskiest foods by a consumer group (Klein, Witmer, Tian, & DeWaal, 2009). Four outbreaks have been linked to the consumption of contaminated fresh blueberries during the period of 1983 to 2013 (Palumbo, Harris, & Danyluk, 2013). An outbreak of listeriosis likely associated with fresh blueberry consumption was reported in Connecticut in 1984 (Zhang, Yan, Hanson, & Ryser, 2015). In New Zealand in 2002, a multi-district outbreak of Hepatitis A infection was likely caused by the consumption of fresh blueberries contaminated by pickers or irrigation water (Calder et al., 2003). In 2009, 14 cases of *Salmonella* Muenchen infection were linked to the consumption of fresh blueberries, but the trace back investigations failed to identify the source and mode of contamination (Centers for Disease Control and Prevention, 2013). A 2010 outbreak with 6 cases

of *S. Newport* infection in Minnesota was also linked to fresh blueberry consumption (Miller, Rigdon, Robinson, Hedberg, & Smith, 2013). Investigations by the Minnesota State Health Department identified a single grower in Georgia as the source of contamination.

Blueberries may become contaminated at any point along the farm-to-table value chain (Liu, Huang, & Chen, 2015). The hygienic condition in the fresh berry packing environments is a significant factor in the production of microbiologically-safe and wholesome products (Heredia et al., 2016). Under the Food Safety Modernization Act, the Produce Safety Rule enforces federal standards to ensure the safety of fresh fruits and vegetables (U.S. Food and Drug Administration, 2013). The Produce Safety Rule provides a framework for best practices to minimize the risk of pathogen contamination throughout the production process; however, knowledge gaps remain. To date, limited studies have been conducted to identify the risks among various components along the fresh produce production and supply chain (Bartz et.al., 2017). This study was undertaken to evaluate the hygienic conditions of selected fresh blueberry packing lines in Georgia.

2. Materials and methods

2.1. Sampling sites

Six fresh blueberry packing lines located at six different packing establishments (P1 to P6) in Georgia were selected for the study. Five of the establishments were visited twice on two separate packing days while the remaining one was visited once in the summers of 2015 and 2017. Ten selected sites on each packing line were sampled, which included the berry dumping site (site 1), the top (site 2) and bottom (site 3) of the in-feed conveyor belt, the rubber belt on the color sorter (site 4), the beginning (site 5) and end (site 6) of the manual sorting area, the beginning (site 7) and end (site 8) of the transfer conveyor belt to the packing area, immature berry disposing area

(site 9), and the berry harvest lug (site 10) (Fig. 3.1). Each site was sampled three times during each visit: before the packing started (AM sample), during lunchtime (NOON sample), and at the end of a packing day (PM sample).

2.2. Sampling procedure

A delimited area of 100 cm² on each site described above was swabbed using a speci-sponge (3.8 × 7.6 × 1.6 cm; Nelson Jameson Inc., Marshfield, WI) pre-moistened with 25 ml of Dey-Engley (DE) neutralizing broth (pH 7.0) (Becton, Dickinson and Company, Franklin Lakes, NJ) in a 18 oz whirl-pak sample bag (Nelson Jameson Inc). Each site was swabbed with 10 horizontal strikes and 10 vertical strikes using the force similar to what is used to remove dry blood from a surface. The sponges were immediately returned to the sampling bags containing DE broth and transported to the laboratory in a car cooler (Igloo, Katy, TX). The samples were analyzed immediately upon arrival to the laboratory.

2.3. Microbial enumeration

The sponge was hand-massaged for 1 min to release microbial cells into DE broth. Microbial cells in the DE broth were diluted in 0.1% phosphate buffered saline, and appropriate dilutions of each sample were surface-plated onto tryptic soy agar (TSA) for total aerobes, potato dextrose agar acidified with 10% tartaric acid (PDA) for yeasts and molds, MacConkey agar (MAC) for total and presumptive fecal coliforms, and Enterococcus agar (EA) for presumptive enterococci. All the samples were plated in duplicate. The agar plates for total aerobes, total coliforms, and presumptive enterococci enumeration were incubated at 37°C for 24 h. The MAC plates for presumptive fecal coliforms enumeration were incubated at 44.5°C for 24 h, and the PDA plates

were incubated at 25°C for 48-72 h. The microbial counts on each sampled site were expressed as log CFU/cm². All microbiological media used in the studies were purchased from Difco (Becton, Dickinson and Company) unless specified.

2.4. Confirmation of fecal coliform and enterococci

Colonies of presumptive fecal coliforms and enterococci with typical morphology were sub-cultured on MAC and EA, respectively, to obtain pure cultures. After overnight incubation at 37°C, cultures of presumptive enterococci on EA plates were transferred into brain heart infusion broth with 6.5% NaCl and incubated at 37°C for 24 h. The presumptive fecal coliform cultures on MAC plates after 24 h incubation at 44.5°C were transferred to EC broth with inverted durham tubes (Kimble Chase, Rockwood, TN) and triple sugar iron agar slants, and were incubated at 37°C for 24 h.

2.5. Statistical analysis

Data were analyzed with ANOVA for the split-plot design (with sampling time as main factor and sampling sites as sub plot factor) using the General Linear Model in SAS v. 9.4 (SAS Institute, Cary, NC). Significant differences ($P \leq 0.05$) between mean values of microbial counts collected from selected packing lines, and at different sampling sites and sampling times were determined based on a 95% confidence interval.

3. Results

3.1. Overall statistical analysis

The average counts of total aerobes, total yeasts and molds, and total coliforms from the 6 sampled packing lines were statistically similar (Table 3.1). On average, the PM samples had significantly higher ($P \leq 0.05$) numbers of total aerobes and yeasts and molds than the NOON samples. Different from the counts of total aerobes and yeasts and molds, the NOON samples had significantly higher mean total coliform counts than the PM samples. The AM samples had significantly lower microbial counts compared to the NOON and PM samples. The berry lug had the highest mean total aerobic counts as well as yeast and mold counts, followed by the rubber belt on the color sorter and the immature berry disposing area (Table 3.1). The rubber belt on the color sorter and immature berry disposing area also had significantly higher total coliform counts than the other sites sampled (Table 3.1).

3.2. Mean total aerobic counts

The mean total aerobic counts from each packing line at different sampling times are summarized in Table 3.2. On average, total aerobic counts in the NOON and PM samples of P1 and P6 were statistically similar ($P > 0.05$), but they were significantly higher ($P \leq 0.05$) than the counts in the AM samples. The PM samples of P2 and P3 had significantly higher total aerobic counts than those from the NOON samples which had significantly higher counts than the AM samples. However, the total aerobic counts in the AM sample of P4 were not significantly different from the NOON and PM samples. The PM samples of P5 were not available due to the early closing at the business day, but its NOON samples had significantly higher total aerobic counts than the AM samples.

Site 10 of P2 had significantly higher ($P \leq 0.05$) total aerobic counts than those on other sites, except site 4 of the same packing line (Table 3.3). The mean total aerobic counts on sites 9 and 10 of P3 were significantly higher than the other sites, except site 3. The counts on site 10 of P4 were higher than those on sites 6, 7 and 8. Site 1 of P5 had significantly higher total aerobic counts compared with the other sites, except sites 4 and 10. Site 9 of P6 had significantly higher mean total aerobic counts than other sites of the same packing line, except sites 1 and 10.

3.3. Mean total yeast and mold counts

Total yeast and mold counts in the NOON and PM samples of all sampled packing lines were statistically similar ($P > 0.05$), except for P2 (Table 3.2). These counts were significantly higher than the counts in the AM samples. The PM samples of P2 had significantly higher yeast and mold counts than those from the NOON samples which had significantly higher counts than the AM samples. The total yeast and mold counts in the NOON samples of P5 were significantly higher than the AM samples.

The mean yeast and mold counts on site 4 of P1 were significantly greater than those on sites 1, 5, 6, 8 and 9 of the same packing line (Table 3.4). Site 10 of P2 and P3 had significantly higher mean yeasts and mold counts than the other sites of the same packing lines. The counts on site 9 of P4 were significantly higher than those from site 5 and 7. Mean yeast and mold counts on sites 4 and 5 of P5 were significantly higher than those from sites 2 and 6. Site 9 of P6 had significantly higher total yeast and mold counts than those from sites 6, 7, 8 and 10.

3.4. Mean total coliform counts

The total coliform counts in the NOON and PM samples of both P1 and P6 were statistically similar ($P>0.05$), but they were significantly higher than the counts in the AM samples (Table 3.2). On average, the PM samples of P2 had significantly higher total coliform counts than those from the NOON samples which had significantly higher counts than the AM samples. However, the NOON samples of P3 had significantly higher counts than the PM samples, followed by AM samples. The total coliform counts in the AM and PM samples of P4 were statistically similar, but they were significantly lower than the counts in the NOON samples. The AM and NOON samples of P5 had statistically similar total coliform counts.

On average, total coliform counts on site 4 of P1 were significantly higher compared with sites 5, 6 and 10 of the same packing line (Table 3.5). The total coliform counts on site 9 of P2 were significantly greater than those on sites 1, 5, 6, 7 and 8 of the same packing line. Site 10 of P3 and site 1 of P5 had significantly higher mean total coliform counts than the other sites on the same packing lines. Site 4 of P4 had significantly higher total coliform counts than the other sites of the same packing line, except site 9. The counts on site 1 of P6 were significantly greater than those on sites 2, 3, 5, 7 and 10 of the same packing line.

3.5. Prevalence of enterococci and fecal coliforms on all blueberry packing lines

Among the 310 samples analyzed in the study, 46 (14.8%) were positive for enterococci (Table 3.6). Site 4 had the highest incidence of enterococci (32.3%), followed by site 9 (19.4%). Sites 1, 2 and 5 had a similar incidence of enterococci at 16.1% which was greater than the 12.9% and 9.7% incidence on site 6, and sites 3, 7 and 8, respectively. Although higher in average aerobic and yeast and mold counts (Table 3.1), site 10 had the lowest incidence of enterococci (6.5%;

Table 3.6). Among the packing lines sampled in the study, P5 had the highest incidence of enterococci (30.0%), followed by P1 (21.7%). The lowest incidence of enterococci was observed on P2 (3.3%). The NOON samples had the highest incidence of enterococci (20.0%), followed by the PM samples (18.9%). The AM samples had the lowest incidence of enterococcus positive samples (6.4%).

A total of 27 out of the 310 sampled sites (8.7%) tested positive for fecal coliforms (Table 3.6). Site 4 had the highest incidence of fecal coliforms (22.6%), followed by site 9 (16.1%). Sites 5 and 8 had similar incidence levels of fecal coliforms at 9.7%, which was lower than the 12.9% incidence on site 6. A similar incidence of fecal coliforms was observed on sites 2 and 3 (6.5%). Similar to what was observed with the incidence of enterococci, site 10 had the lowest incidence of fecal coliforms (3.2%). Among packing lines, P5 had the highest incidence of fecal coliforms (30.0%), followed by P3 (11.7%) and P6 (10.0%). The lowest incidence of fecal coliforms was observed on P1, P2 and P4 (5.0%). The PM samples had the higher incidences of fecal coliforms (11.1%), followed by the NOON samples (9.1%). Approximately 6.4% of the AM samples tested positive for fecal coliforms.

4. Discussion

On November 27, 2015, the FDA issued the “Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption”, commonly known as the produce safety rule under the Food Safety Modernization Act (Ribera, Yamazaki, Paggi, & Seale James, 2016). The current study was undertaken to address the need of the blueberry industry. The data obtained in this research could assist blueberry growers and packers to set up self-administered guidelines to assess the food safety risks associated with their packing practices.

The overall hygiene condition of a packing facility could be reflected by the level of the microbial load found in that particular environment. A high microbial load usually signifies poor food safety management and/or ineffective sanitation practices. When this happens, improved environmental hygiene measures will have to be implemented. The blueberry packing facilities surveyed in the present study had robust sanitation routines, and the packing lines were sanitized daily, either at the beginning or the end of a packing day, with 4 different commercial sanitizers including ozonated water, quaternary ammonia compounds, chlorine dioxide and sodium hypochlorite. However, it is not clear whether berry lugs were cleaned and sanitized routinely.

It is commonly known that the incidence and level of foodborne pathogens on blueberries and in their packing environment are low. This situation has made the detection and quantification of pathogens extremely difficult (Meals, Harcum, & Dressing, 2013; Monaghan & Hutchison, 2010). Indicator organisms were, therefore, used in the present study to evaluate the overall hygiene conditions of blueberry packing lines. The most commonly used indicator organisms include fecal coliforms and enterococci, as they could indicate fecal contamination, possible presence of enteric pathogens or insufficient sanitary treatments (Cárdenas, Molina, Heredia, & García, 2013). Indicator bacteria have been used as a monitoring tool to assess the microbial quality of irrigation water (Gemmell & Schmidt, 2012). They have also been used to determine the microbial quality of fresh produce and environmental samples along the production chain (Ailes et al., 2008; Heredia et al., 2016; Johnston et al., 2005, 2006).

Several studies have demonstrated a strong positive correlation between the level of indicator organisms and the presence of enteric pathogens. An examination of microbial contamination of leafy greens grown in flood plains revealed a significant correlation between the

presence of enteric pathogens (*Salmonella* and *E. coli* O157:H7) and counts of fecal coliforms (Castro-Ibáñez, Gil, Tudela, & Allende, 2015). In a study involving pre-packed herbs, *E. coli* counts positively correlated with the presence of *Salmonella* or Shiga toxin producing *E. coli*, while coliform counts were only correlated with *Salmonella*, not to Shiga toxin producing *E. coli* (Ceuppens et al., 2015). Moniem et al. (2014) observed that the presence of *Salmonella* was correlated with *E. coli* and coliform counts in a study involving microbiological quality of strawberries.

However, there are limitations associated with the application of hygiene indicators, and low levels of correlation (Economou et al., 2013; Wilkes et al., 2009) or lack of correlation with the presence of pathogens (Garcia-Villanova Ruiz, Galvez Vargas, & Garcia-Villanova, 1987; Holvoet, Sampers, Seynnaeve, & Uyttendaele, 2014) was sometimes reported. Establishing an association between an indicator and pathogen can be problematic in studies with limited numbers of samples, because of variations in the pathogen population in samples analyzed (Payment & Locas, 2011). Therefore, a large sample size is essential for assessing the potential associations between the level of a hygiene indicators and the presence of enteric pathogens (National Research Council, 2010). Even though there is no specific relationship between indicator organisms and pathogens, it is generally assumed that a reduction in the number of indicator organisms will produce a similar reduction in the number of foodborne pathogens (Brown et al., 2000).

Data from the current study revealed that the total aerobic counts on sanitized surface of blueberry packing lines (in the AM samples) ranged from 0.91-2.57 log CFU/cm² (Table 3.2) with a mean value of 1.78 log CFU/cm² (Table 3.1). Few microbiological criteria for the assessment of hygiene conditions of food contact surfaces are available in the U.S. According to the U.S. Public Health Service, the viable microorganisms on cleaned and disinfected food contact surfaces

should not exceed 1 log CFU/cm² (Sagoo, Little, Griffith, & Mitchell, 2003). Lower than 1.3 log CFU/cm² of total aerobic counts and <1.0 log CFU/cm² of enteric bacterial counts were the recommended microbiological criteria for sanitized food contact and non-food contact surfaces in food service establishments (Cosby et al., 2008; Hendroid, Mendonca, & Sneed, 2004). This recommended standard seems stringent considering the results of that survey because food contact and non-food contact surfaces in only 4 out of 40 evaluated operations met the recommended standard. Bilgili (2008) stated that an aerobic bacterial count of 100 CFU/cm² (2 log CFU/cm²) is usually an accepted microbial limit for clean and disinfected food contact surfaces. Food contact surfaces containing 10⁰ to 10² CFU/cm², g, or mL of total aerobic count were considered by Fung and Goetsch (2004) to have a low level of contamination, 10³ to 10⁴ CFU/cm², g, or mL as an intermediate level of contamination, and 10⁵ to 10⁶ CFU/cm², g or mL as a high level of contamination (Olds, Fung, & Shanklin, 2005).

It was observed in the present study that the total aerobic and yeast and mold counts in the NOON and PM samples were significantly higher than in the AM samples (Table 3.1). This indicates that the packing line surfaces were getting contaminated during the packing process. Contamination of the surfaces may occur through the transfer of microorganisms from plant residue and soil that come along with fruits and/or improper hygiene and handling practices in the packinghouse environment (Portman, Frankish, & McAlpine, 2002). A study by Robbs et al. (1996) found that the bacterial composition on the surface of celery packing equipment was similar to that found on celery plants sampled at the packinghouse entrance.

It was observed in the current study that the belt on color sorters, the immature berry disposing areas, and the berry lugs had significantly higher mean microbial counts compared with the other sites of the 6 sampled packing lines (Table 3.1). The high microbial accumulation could

be attributed by the type of materials used at these sites of the packing lines. Different sites of fresh packing lines were made of various materials, including stainless steel, rubber, polyvinyl chloride, polyurethane, polypropylene and high-density polyethylene. The belts on the color sorter area, the immature berry disposing area, and berry lugs are made of rubber, polypropylene and high-density polyethylene, respectively, and all three types of materials are hydrophobic in nature. Numerous studies have shown that microorganisms tend to attach more rapidly and firmly to hydrophobic than hydrophilic surfaces (Adetunji, Kehinde, Bolatito, & Chen, 2014; Bendinger, Rijnaarts, Altendorf, & Zehnder, 1993; Fletcher & Loeb, 1979; Pawar, Rossman, & Chen, 2005). Furthermore, the surfaces of rubber belts ages and becomes cracked with repeated cleaning cycles, which increases their ability to entrap bacterial cells (Chmielewski & Frank, 2003; Mosteller & Bishop, 1993). The immature berry disposing area had continuous contact with plant residue and soil associated with pre-mature fruits, which could be a source of microorganisms (Marchand et al., 2012). Higher microbial counts on berry lugs could be caused by frequent exposure to the mechanical harvesting process and contact with freshly harvested fruits, as well as improper washing and sanitizing practices used in some of the packinghouse establishments sampled in the study (Moniem et al., 2014).

5. Conclusions

This study revealed that the microbial counts increased significantly on the surface of fresh blueberry packing lines as packing proceeded. A few sites such as the rubber belt on the color sorter area, the immature berry disposing area, and the berry lugs require special attention in terms of maintenance and sanitation treatment. Certain recommendations could be made in order to improve the hygiene conditions of blueberry packing lines. For example, i) routinely clean and

sanitize berry lugs and packing lines, ii) properly prepare and timely replace sanitizer solutions, iii) follow standard sanitation practice to keep the level of microbial counts on sanitized surfaces as low as possible, iv) keep berry contact surface in good condition, free of cracks and other physical defects, and v) frequently inspect packing lines during packing operations for residue build-up or cleaning needs.

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Table 3.1

Overall mean populations of total aerobes, total yeasts and molds, and total coliforms on different fresh blueberry sample sites and packing lines and at sampling points

	Total aerobes	Total yeasts & molds	Total coliforms
	log CFU/cm ²		
Sample sites (S; n=31)			
S1: Dumping area	2.55 ^C	2.28 ^{BC}	0.95 ^C
S2: Top of the infeed conveyor belt	2.39 ^{CD}	2.11 ^C	0.96 ^C
S3: Bottom of infeed conveyor belt	2.57 ^C	2.24 ^{BC}	0.96 ^C
S4: Rubber belt on color sorter	3.01 ^B	2.64 ^A	1.27 ^A
S5: Beginning of manual sorting area	2.47 ^{CD}	2.02 ^{CD}	0.84 ^C
S6: End of manual sorting area	2.18 ^D	1.73 ^D	0.75 ^C
S7: Beginning of transfer conveyor belt	2.40 ^{CD}	2.05 ^{CD}	0.76 ^C
S8: End of transfer conveyor belt	2.34 ^{CD}	2.09 ^C	0.94 ^C
S9: Immature berry disposing area	2.99 ^B	2.47 ^{AB}	1.22 ^{AB}
S10: Berry lugs	3.48 ^A	2.70 ^A	0.99 ^{BC}
Packing lines (P)			
P1: Packing line 1 (n=60)	2.54 ^A	2.07 ^A	1.32 ^A
P2: Packing line 2 (n=60)	2.79 ^A	2.41 ^A	0.99 ^A
P3: Packing line 3 (n=60)	2.49 ^A	2.19 ^A	0.53 ^A
P4: Packing line 4 (n=60)	2.69 ^A	2.15 ^A	0.69 ^A
P5: Packing line 5 (n=20)	2.37 ^A	2.87 ^A	0.48 ^A
P6: Packing line 6 (n=50)	2.79 ^A	2.10 ^A	1.53 ^A
Sampling time			
Before the packing started (n=110)	1.78 ^C	1.06 ^C	0.62 ^C
Lunchtime break (n=110)	3.03 ^B	2.77 ^B	1.23 ^A
End of the packing day (n=90)	3.21 ^A	3.01 ^A	1.05 ^B

^a Means followed by the same letters within a column and the same comparative category (sample sites, packing line or sampling time) are not significantly different based on 95% confidence intervals.

Table 3.2

Mean populations of total aerobes, total yeasts and molds, and total coliforms from samples collected from 6 different fresh blueberry packing lines at different sampling times

Sampling time \ Packing line (P)	P1	P2	P3	P4	P5	P6
log CFU/cm ²						
Aerobic bacteria						
Before the packing started	1.75 ^B	1.60 ^C	0.91 ^C	2.57 ^{AB}	2.30 ^B	1.81 ^B
Lunch break	2.72 ^A	3.17 ^B	3.11 ^B	3.01 ^A	2.45 ^A	3.41 ^A
End of the packing day	3.14 ^A	3.61 ^A	3.45 ^A	2.49 ^B	-	3.51 ^A
Yeasts and molds						
Before the packing started	0.95 ^B	0.99 ^C	0.35 ^B	0.73 ^B	2.53 ^B	1.24 ^B
Lunch break	2.65 ^A	2.45 ^B	3.11 ^A	2.99 ^A	3.20 ^A	2.74 ^A
End of the packing day	2.63 ^A	3.79 ^A	3.11 ^A	2.75 ^A	-	2.56 ^A
Total coliforms						
Before the packing started	0.81 ^B	0.44 ^C	0.22 ^C	0.68 ^B	0.50 ^A	1.02 ^B
Lunch break	1.64 ^A	1.05 ^B	1.04 ^A	0.95 ^A	0.47 ^A	1.86 ^A
End of the packing day	1.51 ^A	1.49 ^A	0.32 ^B	0.46 ^B	-	1.91 ^A

Mean values of each group of microorganisms followed by the same letters within a column are not significantly different based on 95% confidence intervals.

-: No samples were taken

Table 3.3

Mean populations of total aerobes on different sites of individual fresh blueberry packing lines

Packing lines (P)\Site (S)	Mean aerobic counts (log CFU/cm ²)									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
P1	2.00 ^D	2.10 ^{BCD}	2.76 ^{ABCD}	2.92 ^{ABC}	2.24 ^{ABCD}	2.02 ^{CD}	3.10 ^A	3.00 ^{AB}	2.41 ^{ABCD}	2.82 ^{ABCD}
P2	2.17 ^{DE}	2.42 ^{DE}	2.07 ^E	3.62 ^{AB}	2.73 ^{CD}	2.52 ^{DE}	2.52 ^{DE}	2.44 ^{DE}	3.19 ^{BC}	4.24 ^A
P3	2.35 ^{CD}	2.28 ^{DE}	2.74 ^B	2.42 ^C	2.19 ^{EF}	2.15 ^F	2.35 ^{CD}	2.32 ^{CD}	2.74 ^B	3.38 ^A
P4	2.71 ^{ABC}	2.77 ^{AB}	2.74 ^{ABC}	2.88 ^{AB}	2.84 ^{AB}	2.18 ^{BC}	2.06 ^{BC}	1.85 ^C	3.35 ^A	3.53 ^A
P5	3.29 ^A	2.73 ^C	1.97 ^E	2.98 ^{ABC}	2.79 ^{BC}	1.25 ^F	2.38 ^D	1.89 ^E	1.41 ^F	3.05 ^{AB}
P6	3.43 ^{AB}	2.23 ^{DE}	2.75 ^{CD}	3.28 ^{BC}	2.21 ^{DE}	2.34 ^{DE}	1.90 ^E	2.22 ^{DE}	3.94 ^A	3.63 ^{AB}

Mean values in the same row followed by the same letters are not significantly different based on 95% confidence intervals.

Table 3.4

Mean populations of total yeasts and molds on different sample sites of individual fresh blueberry packing lines

Packing lines (P)/Site (S)	Mean yeast and mold counts (log CFU/cm ²)									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
P1	1.84 ^{CDE}	2.27 ^{ABC}	2.74 ^{AB}	2.89 ^A	1.25 ^{DE}	1.14 ^E	2.28 ^{ABC}	2.00 ^{BCD}	1.91 ^{BCDE}	2.41 ^{ABC}
P2	1.76 ^C	2.10 ^{BC}	2.21 ^{BC}	2.67 ^B	2.20 ^{BC}	2.06 ^{BC}	2.03 ^{BC}	2.22 ^{BC}	2.78 ^B	4.10 ^A
P3	2.44 ^B	1.79 ^E	1.87 ^E	2.22 ^C	2.05 ^D	1.79 ^E	2.07 ^D	2.05 ^D	2.19 ^C	3.42 ^A
P4	2.41 ^{AB}	2.02 ^{ABC}	2.05 ^{ABC}	2.47 ^{AB}	1.97 ^{BC}	2.00 ^{ABC}	1.67 ^C	2.06 ^{ABC}	2.55 ^A	2.35 ^{AB}
P5	3.22 ^A	2.24 ^{BC}	2.59 ^{ABC}	3.43 ^A	3.25 ^A	2.02 ^C	3.12 ^{AB}	2.74 ^{ABC}	2.92 ^{ABC}	3.13 ^{AB}
P6	2.71 ^A	2.37 ^{AB}	2.18 ^{ABC}	2.73 ^A	2.24 ^{ABC}	1.55 ^{CD}	1.80 ^{BC}	1.85 ^{BC}	2.85 ^A	0.77 ^D

Mean values in the same row followed by the same letters are not significantly different based on 95% confidence intervals.

Table 3.5

Mean populations of total coliforms on different sites of individual fresh blueberry packing lines

Packing lines (P)/Site (S)	Mean coliform counts (log CFU/cm ²)									
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
P1	1.42 ^{AB}	1.56 ^A	1.56 ^A	1.68 ^A	0.85 ^B	0.89 ^B	1.27 ^{AB}	1.41 ^{AB}	1.61 ^A	0.93 ^B
P2	0.40 ^C	1.09 ^{AB}	1.10 ^{AB}	1.18 ^{AB}	0.94 ^{BC}	0.67 ^{BC}	0.82 ^{BC}	0.89 ^{BC}	1.73 ^A	1.09 ^{AB}
P3	0.06 ^D	0.52 ^{BC}	0.44 ^C	0.49 ^C	0.58 ^{BC}	0.53 ^{BC}	-	0.44 ^C	0.64 ^B	1.58 ^A
P4	0.80 ^{BC}	0.58 ^{BC}	0.69 ^{BC}	1.33 ^A	0.48 ^C	0.40 ^C	0.62 ^{BC}	0.56 ^{BC}	0.99 ^{AB}	0.49 ^C
P5	1.33 ^A	0.19 ^{CD}	-	0.72 ^B	0.59 ^{BC}	-	0.75 ^B	0.47 ^{BC}	-	0.77 ^B
P6	2.14 ^A	1.38 ^{CD}	1.40 ^{BCD}	1.97 ^{AB}	1.53 ^{BC}	1.64 ^{ABC}	1.15 ^{CD}	1.65 ^{ABC}	1.58 ^{ABC}	0.90 ^D

Mean values in the same row followed by the same letters are not significantly different based on 95% confidence intervals.

- Detection limit <0.06 log CFU/cm²

Table 3.6

Number and percentage of samples tested positive for enterococci and fecal coliforms

	Enterococci			Fecal coliforms		
	Number of positives	No. of samples analyzed	%	Number of positives	No. of samples analyzed	%
Sample sites						
S1	5	31	16.1	0	31	0.0
S2	5	31	16.1	2	31	6.5
S3	3	31	9.7	2	31	6.5
S4	10	31	32.3	7	31	22.6
S5	5	31	16.1	3	31	9.7
S6	4	31	12.9	4	31	12.9
S7	3	31	9.7	0	31	0.0
S8	3	31	9.7	3	31	9.7
S9	6	31	19.4	5	31	16.1
S10	2	31	6.5	1	31	3.2
Total	46	310	14.8	27	310	8.7
Packing lines						
P1	13	60	21.7	3	60	5.0
P2	2	60	3.3	3	60	5.0
P3	5	60	8.3	7	60	11.7
P4	10	60	16.7	3	60	5.0
P5	6	20	30.0	6	20	30.0
P6	10	50	20.0	5	50	10.0
Total	46	310	14.8	27	310	8.7
Sampling time						
AM	7	110	6.4	7	110	6.4
NOON	22	110	20.0	10	110	9.1
PM	17	90	18.9	10	90	11.1
Total	46	310	14.8	27	310	8.7

Figure Legend

Fig 3.1. Ten selected sites on each fresh blueberry packing line.

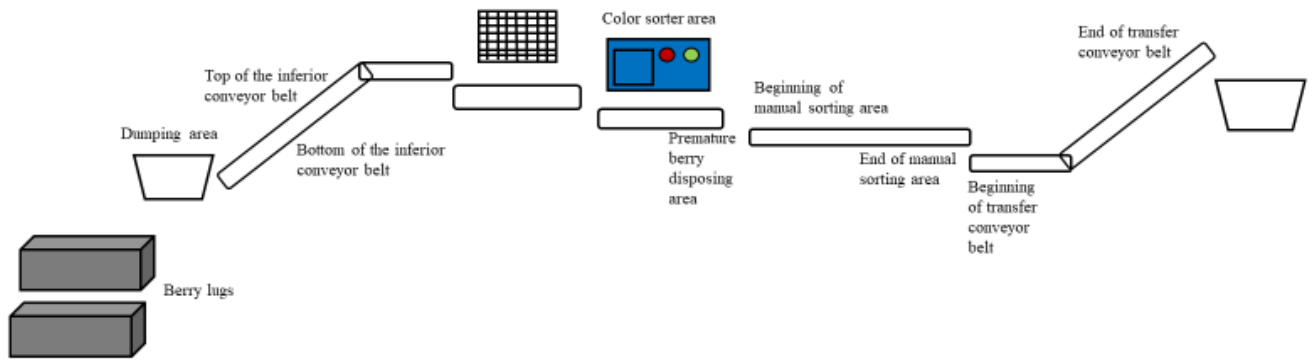


Fig. 3.1

CHAPTER 4

EASE OF BIOFILM ACCUMULATION, AND EFFICACY OF SANITIZING TREATMENTS
IN REMOVING THE BIOFILMS FORMED, ON COUPONS MADE OF MATERIAL
COMMONLY USED IN BLUEBERRY PACKING ENVIRONMENT

Gazula, H., H. Scherm, C. Li, F. Takeda, Wang, P., and J. Chen. 2019. *Food Control* 104: 167-173. Reprinted here with permission of publisher.

Abstract

The biofilm-forming ability of six two-strain mixtures of fecal coliforms, isolated from six blueberry packinghouses, was assessed at 10°C for 7 days on coupons made of materials commonly used for fresh blueberry packing lines, lugs and clamshells, including stainless steel, rubber, polyvinyl chloride, polypropylene, polyurethane, high-density polyethylene and polyethylene terephthalate. Coupons with developed biofilms were treated for 1 min at room temperature with sanitizers commonly used by blueberry packers including 5 ppm chlorine dioxide, 3 ppm ozonated water, 200 ppm of a quaternary ammonium compound or 200 ppm sodium hypochlorite, and residual biofilms on treated coupons was quantified using the crystal violet binding assay. On average, the amount of biofilms accumulated on polypropylene coupons was significantly greater ($P \leq 0.05$) than on the other coupons used in the study. Biofilms formed on polyvinyl chloride and rubber coupons were statistically similar ($P > 0.05$), but were significantly lower than those on polypropylene and polyurethane coupons and significantly higher than those on high-density polyethylene, stainless steel and polyethylene terephthalate coupons. Ozonated water had significantly higher efficiency in biofilm removal than the quaternary ammonium compound which had significantly higher efficiency than chlorine dioxide, followed by sodium hypochlorite. On average, significantly more residual biofilm mass was found on rubber coupons after the sanitizing treatments. The amounts of residual biofilms on polypropylene, polyvinyl chloride and high-density polyethylene coupons were statistically similar, but were significantly higher than those on polyurethane and stainless steel coupons. The evaluated sanitizers had different efficacies in biofilm removal. These results emphasise the importance of selecting proper chemical sanitizers (with high efficacies in microbial inactivation and biofilm removal) and surface materials (with low tendency in attracting microbial attachments).

for fresh market blueberry packing lines because these decisions may effect the hygiene conditions of fresh market blueberry packing lines as well as microbial quality of the fruits.

Key words: Biofilm, fecal coliform, abiotic surfaces, sanitizers

1. Introduction

Fresh blueberries are highly valued for their antioxidants, flavonoids, anthocyanins and other nutrients (Ortiz, Marín-Arroyo, Noriega-Domínguez, Navarro, & Arozarena, 2013). As a result, the consumption of fresh blueberries has increased rapidly in the United States (Huang & Chen, 2014). In spite of their nutritional characteristics, blueberries were linked to outbreaks of human gastrointestinal infections (Palumbo, Harris, & Danyluk, 2013). In 1984, an outbreak of listeriosis likely associated with the consumption of fresh blueberries was reported in Connecticut (Zhang, Yan, Hanson, & Ryser, 2015). In 2009, *Salmonella* Muenchen contaminating fresh blueberries was linked to a multistate outbreak of human gastrointestinal infections (CDC, 2013). In 2010, an outbreak of *Salmonella* Newport infection was caused by the consumption of contaminated fresh blueberries (Miller, Rigdon, Robinson, Hedberg, & Smith, 2013).

Mature blueberries for the fresh market are usually sent to packinghouses after harvest and cooled at 0°C to remove field heat before packing (Watson, Treadwell, Sargent, & Brecht, 2016). The packing process starts from dumping the harvested blueberries from lugs onto the packing line. The berries are moved by a lifting conveyor to a blower that removes leaves, sticks, small berries and other debris from the berries. The blueberries then go through the color sorter to remove immature, soft and off-colored berries. Sorted berries roll onto a grading/inspection belt where workers manually remove additional immature and soft fruit missed by the color sorter from the packing line. Finally, one or multiple conveyors drop the blueberries into a hopper atop of a clamshell filling machine for packaging (Xu, Takeda, Krewer, & Li, 2015).

Fresh blueberry packing lines are made of various materials including stainless steel, rubber, polyurethane, polyvinyl chloride and/or polypropylene. Berry lugs are made of high-density polyethylene, while packing clamshells are made of polyethylene terephthalate. Possible

attachment of bacterial cells to these materials is a concern of blueberry packers, as bacterial cells adhering to the materials, if not effectively and promptly removed by sanitizing treatments, can form biofilms (Koutsoumanis & Sofos, 2004; Mafu et.al., 2011). The biofilms on fruit contact surfaces could serve as a source of contamination and adversely impact fruit quality and safety (Pérez-Rodríguez, Valero, Carrasco, García, & Zurera, 2008). Biofilms are difficult to control, thus, effective sanitation to eliminate microorganisms from blueberry-contact surface is critical for enhancing the shelf life of blueberries and preventing berry-related outbreaks of infections.

The objectives of this study were to assess the formation of biofilms by fecal coliforms, isolated from blueberry packing lines, on coupons made of materials commonly used for fresh blueberry packing lines, lugs, and clamshells and evaluate the efficacy of commercial sanitizers, routinely used by blueberry packers, in removing the biofilms formed on selected coupons.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Twelve fecal coliform isolates were used in the study, and these fecal coliforms were isolated from various sites of six different fresh market blueberry packing lines in separate packing facilities of Georgia in the summer of 2015 and 2017 (Gazula et al., 2019, Table 1). The blueberry packing lines where the fecal coliforms were isolated had sanitation routines, and the packing lines were sanitized daily, either at the beginning or the end of a packing day, with different commercial sanitizers. The fecal coliforms stored at -80°C in tryptic soy broth (Becton Dickinson, Franklin Lakes, NJ USA) with 15% (v/v) glycerol (Fisher Scientific, Hampton, NH USA) were retrieved and subcultured twice on Luria-Bertini no salt (LBNS) agar (Becton Dickinson) at 37°C for 24h. Genomic DNA was subsequently extracted and a section of 16S rDNA was amplified using primers

27F (5'-AGAGTTTGATCMTGGCT CAG) and 1492B (5'-TACGGYTACCTTGTTACGACTT). Amplified PCR products were submitted to Eurofins Genomics, a Eurofins MWG Operon company (Louisville, KY) for purification and sequencing. The identifies of the 12 fecal coliform isolaes were determined by comparing the 16S rDNA sequences with those in the NCBI database (Table 4.1). Further charactering analysis uisng the PCR assays described by Low et al. (2006) revealed that most of the isolates did not carry some of the fimbriae genes commonly found in *Escherichia coli*, however, colony morphotypes of these isolates suggest that they are potential biofilm formers (Table 1).

2.2. Coupons for biofilm formation

Coupons (2×5 cm²) made of stainless steel (Gauge type 316 and 2B finish; Impulse Manufacturing, Inc; Dawsonville, GA), buna-n-rubber (0.317 cm and 70-Duro; Dixie Packing and Gasket Co., Lithia Springs, GA), polyvinyl chloride (2P100/05/0 G White; Universal Belting Resource, Carmichaels, PA), polypropylene (U97/2E-AS White; Universal Belting Resource), polyurethane (U92/2E-AS Matt White; Universal Belting Resource), high-density polyethylene (Universal Belting Resource) and polyethylene terephthalate (Advanced Extrusion Inc; Rogers, MN) were used in this study. The stainless steel and rubber coupons were washed with an alkaline detergent, Sparkleen (Fisher Scientific) and then rinsed with deionized water before being autoclaved at 121°C for 15 min, while the other coupons were decontaminated with 3% sodium hypochlorite (Fisher Scientific) solution for 15 min and rinsed three times with sterile water.

A loop of inoculum from each fecal coliform culture on LBNS agar described above was transferred to 9mL of LBNS broth. The inoculated cultures were incubated at 37°C for 16h. After

incubation, an equal volume of cells of the two fecal coliforms isolated from an individual packing line were combined to form a two-strain mixture.

2.3. Biofilm formation by fecal coliform isolates

Five milliliters of each two-strain mixture of fecal coliforms was added to 200 mL of sterile LBNS broth (1:40 dilution) in a sterile glass container (18 cm×13 cm×4 cm; Pyrex ware, World Kitchen LLC, Rosemont, IL) in which the coupons were immersed. The mixture of fecal coliforms in the broth cultures was allowed to develop biofilms on the coupons at 10°C for 7 days. The glass container was covered with aluminum foil during biofilm development to prevent the broth from evaporating. Uninoculated LBNS broth was used as the control.

2.4. Quantification of biofilms

After 7 days of incubation, developed biofilms were quantified using the crystal violet binding assay (Adetunji, Kehinde, Bolatito, & Chen, 2014) with some modifications. The coupons with developed biofilms were washed three times, each with 5 mL of sterile distilled water. Biofilm mass was fixed with 5 mL of 95% ethanol (VWR International, LLC, Radnor, PA) for 10 min at room temperature. The fixed samples were air-dried for 10 min and then stained for 15 min with 2% crystal violet (Fisher Scientific). Excess stain was rinsed with running tap water, and the coupons were then air-dried. Each of the dried coupons was placed on a sterile Petri dish, and 5 mL of ethanol: acetone (80:20) mixture was used to solubilize the crystal violet. The solubilized stain was then pipetted into a cuvette (Fisher Scientific). Absorbance was measured at 550 nm using a spectrophotometer (Thermo Electron Corporation, Waltham, MA). In the event that the absorbance values were above the dynamic range of the spectrophotometer (3.0 optical density

units), the samples were diluted with an equal volume of ethanol:acetone mixture until the absorbance value fell within-range absorbance values (Kaplan et al., 2012). The final absorbance values of diluted samples were calculated by multiplying the within-range absorbance values with the dilution factors (Srinivasan, Pamula, Pollack, & Fair, 2003). The absorbance of the negative control was subtracted from the absorbance values of test sample.

2.5. Biofilm control by sanitizers

Four commercial sanitizers routinely used by blueberry packers were used in the study: chlorine dioxide (ClO_2) (Selective Micro Technologies, Dublin, OH), quaternary ammonium compound (QAC) (Astro Products, Fitzgerald, GA), ozonated water (OW, generated in the laboratory) and sodium hypochlorite (NaOCl) (Fischer Scientific). The working solutions of OW and ClO_2 were 3 ppm and 5 ppm, respectively, whereas the concentration of QAC and NaOCl was 200 ppm. All sanitizer solutions were prepared as per the manufacturers' instructions. The ozone was generated using a 1 KNT generator (Oxidation Technologies, Inwood, IA) and a small, commercial industrial "D" oxygen cylinder (Airgas USA LLC, Kennesaw, GA) with oxygen (95% purity) at an output pressure of 34,474 P. OW at a final concentration of 3 ppm was generated by bubbling the ozone into deionized water at a controlled flow rate of 4 liters/min. After the required ozone concentration was reached, OW was collected immediately into a 1-liter beaker, covered with a lid, and used within 15 min of production.

Coupons with developed biofilms were rinsed three times with sterile distilled water as described above and then submersed in 200 mL of each sanitizing solutions mentioned above for 1 min at room temperature to ensure that all sides of the coupons were exposed to the sanitizer. After the treatments, coupons were immediately immersed in 200 mL of double strength Dey-

Engley neutralizing broth (Becton Dickinson) for 10 min, followed by 2 h of drying at 60°C. The residual biofilm mass on various coupons was subsequently quantified using the crystal violet binding assay as described in section 2.4. The efficacy of the treatments in biofilm removal is expressed as the ratio of absorbance of residual biofilm mass to the absorbance of untreated control biofilm mass at 550 nm.

2.6. Statistical analysis

Each experiment was repeated once, with duplicate treatments in each replication. Data were analyzed using general linear model procedure of SAS v. 9.4 (SAS Institute Inc., Cary, NC). Significant differences in biofilm mass formed by different fecal coliform mixtures on various contact surfaces and the efficacy of different sanitizers in removing the biofilms formed by the mixtures of fecal coliform isolates from various coupons were determined using Fisher's Least Significant differences based on 95% confidence intervals.

3. Results

3.1. Biofilm formation

On average, the amount of biofilms accumulated on polypropylene coupons was significantly higher ($P \leq 0.05$) than the other coupons used in the study (Table 4.2). Biofilms formed on polyvinyl chloride and rubber coupons were statistically similar, but were significantly lower than those on polypropylene and polyurethane coupons and significantly higher than those on high-density polyethylene, stainless steel and polyethylene terephthalate coupons. Biofilm mass on the latter two surfaces was statistically similar, but was significantly lower than that on other surfaces used in the study.

3.2. Biofilms removal

Significantly more ($P \leq 0.05$) residual biofilm mass was found on rubber coupons (53.1%) after the sanitizing treatments (Table 4.3). Residual biofilm masses on polypropylene, polyvinyl chloride and highdensity polyethylene surfaces were statistically similar ($P > 0.05$), but they were significantly higher than those on polyurethane and stainless steel surfaces. The latter had significantly lower residual biofilm mass (34.1%) compared to other surfaces after the sanitizing treatments. The OW had significantly higher efficiency in biofilm removal (36.9% of biofilm remaining) than QAC which had significantly higher efficiency than ClO_2 , followed by NaOCl (50.8% remaining) in removing biofilms.

3.3. Efficacies of different sanitizing treatments in removing biofilms from individual types of coupons

The ClO_2 treatment removed significantly more ($P \leq 0.05$) biofilms from stainless steel and polyurethane than from other surfaces, with 34.3% and 38.4% of the biofilm mass left after the sanitizing treatments (Table 4.4). The residual biofilms on high-density polyethylene and polypropylene surfaces were statistically similar ($P > 0.05$) after the ClO_2 treatment, but they were significantly higher than those on stainless steel, polyurethane and polyvinyl chloride surfaces and significantly lower than the residual biofilm on rubber surface. Treatment with ClO_2 was least effective in removing the biofilms on rubber surfaces (with 62.3% residual biofilms), compared to the other surfaces used in the study. Similarly, the OW and QAC treatments were also significantly more effective in removing the biofilms from stainless steel surface (with 19.6% residual biofilms) than from other surfaces used in the study. Rubber surface retained the greatest amount of residual biofilms (with 52.2% residual biofilms) after the OW treatment, and rubber along with

polypropylene surface had the highest amount of residual biofilms of 49.5% and 52.0%, respectively after the QAC treatment. Although NaOCl was most effective in removing the biofilms from polypropylene surface compared to the other surfaces used in the study, 40.1% of the biofilm mass remained after the treatment. The efficiency of NaOCl in biofilm removal from rubber and polyvinyl chloride surfaces was statistically similar, but the sanitizer was significantly less effective in removing biofilms from polypropylene surface and significantly more effective in removing biofilms from high-density polyethylene, stainless steel and polyurethane surfaces with 54.4–57.1% of biofilm mass remaining after the treatments.

The OW treatment was significantly more effective ($P \leq 0.05$) than the other three treatments in removing the biofilms from high-density polyethylene, stainless steel, and polyurethane surfaces (Table 4.4). ClO_2 was significantly less effective compared to all the other three sanitizers in biofilm removal from rubber surfaces. Treatment with OW and NaOCl was significantly more effective than ClO_2 and QAC in removing the biofilms from polypropylene surface. Furthermore, similar ($P > 0.05$) amount of residual biofilm mass was found on polyvinyl chloride surface after all four sanitizing treatments.

4. Discussion

This study found that, on average, significantly more biofilms were formed by fecal coliforms on polypropylene, and significantly less biofilms on stainless steel and polyethylene terephthalate, coupons (Table 4.1). It is well-known that the properties of abiotic surfaces play a key role in attracting bacterial cells for attachment (Shi & Zhu, 2009). Among the seven types of surface materials used in the study, rubber, polyurethane, polypropylene, high-density polyethylene and polyvinyl chloride are hydrophobic, with little or no surface charge (Sinde & Carballo, 2000).

Stainless steel and polyethylene terephthalate are, on the other hand, hydrophilic with either positive or neutral surface charges (Donlan, 2002; Khangholi & Jamalli, 2016). Garrett, Bhakoo, & Zhang (2008) reported that bacterial attachment to surfaces is also influenced by cell surface topography. The surfaces of most bacterial cells are negatively charged and possess hydrophobic surface structures such as fimbriae, adhesive proteins and/or lipopolysaccharides (Brooks & Flint, 2008; Palmer, Flint, & Brooks, 2007). These extracellular appendages play an important role in the attachment of bacterial cells to biotic and abiotic surfaces during biofilm development (Fletcher & Loeb, 1979). Hydrophobic interactions between cell surfaces and the substratum enable bacterial cells to overcome electrostatic repulsive forces and subsequently attach to these surfaces (Donlan, 2002). Hydrophobic cells adhere strongly to hydrophobic surfaces, whereas hydrophilic cells adhere strongly to hydrophilic surfaces with opposite surface charge (Giaouris, Chapot-Chartier, & Briandet, 2009). The observations made in the present study were consistent with those of Pagedar, Singh, & Batish (2010), Pawar, Rossman, & Chen (2005) and Adetunji et al., (2014) who observed that bacterial cells attach in high numbers to hydrophobic surfaces compared to hydrophilic surfaces.

It was observed in the present study that rubber surfaces were relatively more difficult, and stainless steel surfaces were relatively easier, to sanitize compared with the other surfaces used (Table 4.3). These observations are consistent with what was reported in previous literatures (Bang et al., 2014; Somers & Wong, 2004). Wong & Ronner (1993) observed that biofilms developed by *Salmonella* Typhimurium on rubber surfaces were much more resistant to detergent and non-detergent sanitizer treatments than those on stainless steel. Sadekuzzaman, Yang, Kim, Mizan, & Ha (2017) found that the resistance of *E. coli* O157:H7, *Salmonella* Enteritidis and *Salmonella* Typhimurium in biofilms on stainless steel surfaces was significantly lower than that

on rubber surfaces. Bremer, Monk, & Butler (2002) reported a significant difference in the efficacy of NaOCl treatment against cells attached to the stainless steel surface than those attached to a conveyer belt made of polyvinyl chloride/polyester.

Four different commercial sanitizers routinely used by the blueberry packers were evaluated in this study (Gazula et.al., 2019). Among the four sanitizers, OW had significantly higher efficiency in biofilm removal than QAC which in turn had significantly higher efficiency than ClO₂, followed by NaOCl (Table 4.2). Consistent with the results of this study, OW treatment was found to be more effective than chlorine in removing *P. putidas* biofilms on stainless steel (Dosti, Guzel-Seydim, & Greene, 2005). Baig, Meng, Saingam, & Xi (2018) reported that bacterial extracellular polymeric substance (EPS) contains polysaccharides, proteins, humic acids, lipids and nucleic acid that are reactive to ozone. Ozone can oxidize EPS into different organic compounds including carboxylic acids, aldehydes, ketones and benzaldehydes because of its selective and strong oxidant nature (Hoigné & Bader, 1983). It was reported that ozone could cleave the glycosidic bond of polysaccharides by inserting oxygen into the anomeric C-H bond, which yields aldonic acid lactones (Pan, Chen, Chang, & Grtazi, 1981). Ozone could also cause the oxidation of hydroxyl groups at C2, C3 or C6 in polysaccharides to produce carbonyl groups (Katai & Schuerch, 1966). However, a major constraint to use of OW as a sanitizer is that it decays faster in water than in air/gaseous form and may lead to the production of highly reactive species including hydroperoxyl ($\cdot\text{HO}_2$), hydroxyl ($\cdot\text{OH}$) and superoxide ($\cdot\text{O}_2$) radicals (Brodowaska, Nowak, & Smigielski, 2010). Furthermore, prolonged use of ozonated water may break down or corrode materials such as rubber and stainless steel (Postma, 2013).

QACs are cationic detergents, commonly known as surfactants or surface active agents (Gerba, 2015). These surfactants may interact with bacterial cellular components such as proteins

and lipids (Chatterjee & Moulik, 2005). Jennings et.al. (2014) reported two possible mechanisms of biofilm removal by QAC. The polycationic compounds in QAC could interact with the EPS in biofilms, leading to the dispersion and subsequent killing of released bacterial cells. QAC could also perturb the biofilms through electrostatic interactions, and kill the cells within the biofilm matrix, ultimately resulting in biofilm degradation due to cell death (Ganewatta et al., 2015). Consistent with the current study, Nguyen & Yuk (2013) reported that QAC was more effective than NaOCl in removing *Salmonella* biofilms from a stainless steel surface. QAC was also shown to be more effective than chlorine in removing biofilms formed by *Staphylococcus* on stainless steel and polycarbonate surfaces (Frank & Chmielewski, 1997).

Despite being widely studied, the actual mechanism of action of ClO₂ on biofilms is not fully known (Jang, Szabo, Hosni, Coughlin, & Bishop, 2006). ClO₂ reacts primarily with the aldehyde groups of reducing end units of cellulose, a major component contributing to biofilm formation by bacterial cells, oxidizing them to carboxyl groups (Lemeune, Jameel, Chang, & Kadla, 2004). In the present study, treatment with ClO₂ was more effective than NaOCl in biofilm removal (Table 4.3), which is consistent with a recent study which found that ClO₂ was more effective than NaOCl in removing biofilms formed by *E. coli* on polystyrene and stainless steel surfaces (Meireles, Ferreira, Melo, & Simões, 2017). The present study also found that ClO₂ was less effective than OW and QAC in control of biofilms (Table 4.3). Jang et al. (2006) suggested that the inefficiency of ClO₂ to reach the deep layer inside a biofilm could be due to multiple factors, such as molecular diffusion limitations, biofilm density and reactive depletion of ClO₂ molecules.

NaOCl was significantly less effective in removing biofilms compared with the other three sanitizers used in the study (Table 4.3). This result corroborates the observations of Swaraj,

Kumar, Harinath, & Rao (2013) that NaOCl was less effective than OW in removing *Bacillus pumilis* biofilm cells from polystyrene surface. Møretrø et al. (2009) compared the efficacies of nine different sanitizers including alkyl amino acetate, didiecy methyl ammonium chloride, benzalkonium chloride, glutaraldehyde, hydrogen peroxide, peracetic acid, persulphate, ethanol, acetic and malic acid against *Salmonella* biofilms and reported that hypochlorite had the lowest effect whereas QAC had an intermediate effect. Gray, Wholey, & Jakob (2013) attributed the mechanism of NaOCl to the formation of toxic oxidative species. These oxidative species combine with protein to inhibit oxidative phosphorylation, and damage both nucleic acids and proteins. It was also reported that NaOCl depolymerizes polysaccharides through chlorinolysis of glycosidic bonds (Fukayama, Tan, Wheeler, & Wei, 1986). The lower efficacy of NaOCl in biofilm removal observed in the present study could be caused by the poor penetration of NaOCl into biofilms, a phenomenon associated with reactive neutralization of the active chlorine in the outermost regions of the biofilm matrix. It is believed that chlorine is consumed by reaction with the organic matter in the surface layers of the biofilm faster than its rate of diffusion into biofilm interior (De Beer, Srinivasan, & Stewart, 1994).

In the current study, none of the sanitizing treatments completely removed the biofilms from the surface materials used (Table 4.3). This could be because of the insufficient contact time between the sanitizers and biofilms (Weber, Rutala, & Sickbert-Bennett 2007). Previous study has shown that prolonging the contact between sanitizer and treated surface increased the efficacies of QAC and NaOCl treatments (Wong, Townsend, Fenwick, Trengove, & O'Handley, 2010). Furthermore, bacterial cell wall components such as peptidoglycan, fimbriae, capsules and S-layer could affect the diffusion of chemical compounds within a biofilm (Habimana et al., 2011).

5. Conclusion

Significantly more biofilms were formed on polypropylene surfaces and significantly less biofilms on polyethylene terephthalate and stainless steel surfaces than on the other surfaces used in the study. Overall, rubber surface was relatively more difficult, and the stainless steel surface was relatively easier, to sanitize compared with the other surfaces used in the study. OW was significantly more effective, and NaOCl was significantly less effective, in biofilm removal. These results emphasize the importance of selecting proper chemical sanitizers and surface materials for fresh blueberry packing lines because these decisions will affect the hygiene conditions of fresh berry packing lines as well as the microbial quality of the fruits.

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Table 4.1

Fecal coliform isolates used in the study

Two-strain mixture from	Isolate ID	Year of Isolation	Site of isolation of the packing line	Fimbriae Genes of <i>E. coli</i> 0157:H7 ^a				Colony morphotype ^b
				<i>Sfma</i> Fimbria protein precursor	<i>ycbq</i> Fimbriae like adhesin	<i>csgA</i> curli protein	<i>IpFA</i> Long polar fimbriae	
Packing line 1	<i>Klebsiella</i> F5	2015	Transfer belt to packing area	-	-	-	-	Mucoid
	<i>Pantoea</i> F8	2015	Transfer belt to packing area	-	-	-	-	Mucoid
Packing line 2	<i>Klebsiella</i> F35	2015	Berry lug	-	-	-	-	Mucoid
	<i>Klebsiella</i> F252	2015	Premature berry/ debris disposing site	-	-	-	-	Mucoid
Packing line 3	<i>Enterobacter</i> F219	2015	Manual sorting area	-	-	-	-	Pdar
	<i>Enterobacter</i> F112	2015	Premature berry/ debris disposing site	-	-	-	-	Bdar
Packing line 4	<i>Klebsiella</i> F272	2015	Infeed conveyor belt	-	-	-	-	Mucoid
	<i>Enterobacter</i> F174	2015	Packing area	-	-	-	-	Bdar
Packing line 5	<i>Klebsiella</i> F329	2015	Rubber belt of color sorter	-	-	-	-	Mucoid
	<i>Raoultella</i> F354	2015	Premature berry/ debris disposing site	-	-	-	-	-
Packing line 6	<i>Klebsiella</i> F390	2017	Rubber belt of color sorter	-	-	-	-	Mucoid
	<i>Raoultella</i> F406	2017	Premature berry/ debris disposing site	-	-	-	-	-

^a Primer sequences derived from *E. coli* 0157:H7 Sakai (Low et.al., 2006).^b Bdar: Brown dry and rough; Pdar: Pink, dry and rough; and rdar: Red, dry and rough colonies

Table 4.2

Overall mean value of biofilm mass accumulated by two-strain mixtures of fecal coliforms, isolated from different fresh blueberry packing lines, on coupons of various materials after 7 days of incubation at 10°C.

	Biofilm mass (A_{550})
On coupons ($n = 84$) with material of:	
High-density polyethylene	0.480 ^D
Stainless steel	0.240 ^E
Rubber	0.898 ^C
Polypropylene	5.502 ^A
Polyurethane	1.177 ^B
Polyvinyl chloride	0.927 ^C
Polyethylene terephthalate	0.171 ^E

Mean values followed by the same letter within a comparative category (contact surface materials or fecal coliform isolates) are not significantly different ($P \leq 0.05$).

Table 4.3

Efficacy of sanitizing treatments on biofilm removal.

	A ₅₅₀ of residual biofilm mass:A ₅₅₀ of untreated control biofilm mass
On coupons (<i>n</i>= 288) made of:	
High-density polyethylene	45.1 ^B
Stainless steel (Gauge Type 316)	34.1 ^D
Rubber (70-Duro)	53.1 ^A
Polypropylene	46.5 ^B
Polyurethane	41.8 ^C
Polyvinyl chloride	46.4 ^B
Sanitizing treatments (<i>n</i>=288) using:	
Active chlorine dioxide	48.2 ^B
Ozonated water	36.9 ^D
Quaternary ammonium cation	42.0 ^C
Sodium hypochlorite	50.8 ^A

Mean values followed by the same letter within a comparative category (coupons, or chemical sanitizers) are not significantly different ($P \leq 0.05$).

Table 4.4

The effect of various sanitizers in removing biofilms from various coupons by different sanitizers.

	ClO ₂	OW	QAC	NaOCl
A ₅₅₀ of residual biofilm mass:A ₅₅₀ of untreated control biofilm mass				
On coupons (n=72) made of:				
High-density polyethylene	53.6 ^{B,a}	29.6 ^{E,c}	40.3 ^{C,b}	56.7 ^{A,a}
Stainless steel (Gauge type 361)	34.3 ^{D,b}	19.6 ^{F,d}	25.3 ^{D,c}	57.1 ^{A,a}
Rubber (60-70 Duro)	62.3 ^{A,a}	52.2 ^{A,b}	49.5 ^{AB,b}	48.3 ^{B,b}
Polypropylene	55.6 ^{B,a}	38.3 ^{C,b}	52.0 ^{A,a}	40.1 ^{C,b}
Polyurethane	38.4 ^{D,b}	34.3 ^{D,c}	39.9 ^{C,b}	54.4 ^{A,a}
Polyvinyl chloride	45.1 ^{C,a}	47.4 ^{B,a}	44.8 ^{BC,a}	48.5 ^{B,a}

The mean values followed by a different uppercase letter within a column and comparative category (coupons and fecal coliform isolates) are significantly different ($P \leq 0.05$).

The mean values followed by a different lowercase letter within a row are significantly different ($P \leq 0.05$).

CHAPTER 5

CONCLUSIONS

Results from the studies described in Chapters 3 and 4 can be summarized as following:

1. Evaluation of hygiene conditions of selected fresh blueberry packing lines showed that sample site and sampling time had a significant influence ($P \leq 0.05$) on total aerobic, yeast and mold, and total coliform counts. The PM samples had significantly higher total aerobic and yeast and mold counts than the NOON samples which had significantly higher counts than the AM samples. Forty-six out of the 310 (14.8%) collected samples tested positive for enterococci while 27 (8.7%) samples tested positive for fecal coliforms. Berry lugs, rubber belts on color sorters, and immature berry disposing areas had significantly higher microbial counts than the other sites. This study suggests that some sites along fresh blueberry packing lines could become contaminated by microorganisms during packing and require special attention in terms of maintenance and sanitation treatment.
2. Biofilm-forming ability of six two-strain mixtures of fecal coliforms, isolated from fresh blueberry packing environments, on coupons made of materials commonly used for fresh blueberry packing lines, lugs, and clamshells indicated that the amount of biofilms accumulated on polypropylene coupons were significantly higher ($P \leq 0.05$) than that on all other coupons used in the study. Biofilms formed on polyvinyl chloride and rubber coupons were statistically similar, but were significantly lower than those on polypropylene and polyurethane coupons and significantly higher than those on high-density polyethylene, stainless steel and polyethylene terephthalate coupons. Evaluation

of efficacy of commercial sanitizers, routinely used by blueberry packers, in removing the biofilms formed on selected coupons showed that ozonated water had significantly higher efficiency in biofilm removal than a quaternary ammonium compounds which had significantly higher efficiency than chlorine dioxide, followed by sodium hypochlorite. On average, significantly more residual biofilm mass was found on rubber coupons after the sanitizing treatments. These results emphasize the importance of selecting proper chemical sanitizers and materials for fresh blueberry packing lines because these decisions will affect the hygiene conditions of fresh berry packing lines as well as the microbial quality of the fruits.

APPENDIX A

SUSCEPTIBILITY OF SELECTED FECAL COLIFORMS TO ANTIBIOTICS, WITH OR WITHOUT THE PREEXPOSURE TO SUBLETHAL CONCENTRATIONS OF CHLORINE DIOXIDE

Abstract

Adaptive exposure to sub-lethal concentrations of sanitizers was previously reported to lead to the development of cross resistance in bacteria to antibiotics. This study was undertaken to determine whether pre-exposure of selected fecal coliforms to sublethal concentrations of a chemical sanitizer, chlorine dioxide, would alter their susceptibility to antibiotics. Selected fecal coliforms ($n = 12$) were adapted in $\frac{1}{2}$ or $\frac{1}{4}$ of manufacturer recommended concentration of chlorine dioxide. The susceptibility of adapted and non-adapted cells to 13 different antibiotics was determined by observing the changes in their minimal inhibitory concentrations (MIC). Results showed that preadaptation to sub-lethal concentrations of chlorine dioxide, in general, did not change or decreased the MICs of 13 antibiotics included in the study against selected fecal coliform isolates. There were two exceptions, whereby preadaptation increased the MICs of kanamycin against two of the fecal coliform isolates and of nalidixic acid against one of the fecal coliform isolates.

Keywords: Fecal coliforms, Antibiotic Resistance, Sanitizers, Adaptation,

1. Introduction

Chemical sanitizers are widely used in food production environments for decontamination of food contact surfaces and prevention of microbial contamination (Hricova, Stephan, & Zweifel, 2008; Møretrø, Høiby-Pettersen, Habimana, Heir, & Langsrud, 2011). Russell (2002) reported that the frequent use of sanitizers may impose a selective pressure and contribute to the emergence of resistant microorganisms in food environments. Each sanitizer has its manufacturer's recommended working concentration and treatment protocols (Cruz & Fletcher, 2012). Application of sanitizers at lower than recommended dosage may allow some bacterial cells to evade the sanitization treatment (Araújo, Lemos, Mergulhão, Melo, & Simões, 2011). It has been shown that repeated exposure to sub-inhibitory concentrations of sanitizers has resulted in enhanced tolerance to chloramphenicol, erythromycin and ciprofloxacin for some bacterial species (Braoudaki & Hilton, 2004a; Condell et al., 2012; Mavri & Smole Možina, 2013). Langsrud, Sundheim, & Holck (2004) observed that *Escherichia coli* adapted to sub-inhibitory concentrations of benzalkonium chloride had elevated tolerance to chloramphenicol. In a study by Alonso-Hernando, Capita, Prieto, & Alonso-Calleja (2009), *Salmonella* Typhimurium adapted to sub-inhibitory concentrations of acidified sodium chlorite had increased resistance to streptomycin, erythromycin, rifampicin and chloramphenicol.

At present there are comparatively few reports of cross-resistance between clinically used antibiotics and chlorine dioxide (ClO₂), a chemical sanitizer commonly used by the food industry. The objective of this study was to determine whether the pre-exposure of fecal coliforms to sub-lethal concentrations of chlorine dioxide would alter the susceptibility of the fecal coliforms to the antibiotics used in human medicine.

2. Materials and Methods

2.1. *Fecal coliform isolates*

Fecal coliforms ($n = 12$) isolated from different fresh blueberry packing facilities during the summer of 2015 to 2017 were used in this study. The fecal coliform isolates from frozen storage were retrieved and sub cultured twice on tryptic soy agar (Becton Dickinson, Franklin Lakes, NJ) and the inoculated agar dishes were incubated at 37°C for 24 h. A single colony of each fecal coliform culture was transferred to 9 mL of tryptic soy broth (Becton Dickinson) and the broth cultures were incubated at 37°C for 16 h.

2.2. *Preadaptation studies*

Commercial chlorine dioxide (ClO₂) (Selective Micro Technologies, OH USA) was used in the study. Sub-lethal concentrations of ClO₂ were prepared by diluting the 5 ppm ClO₂ solution to 2.5 and 1.25 ppm with sterile distilled water. All the sanitizer solutions were prepared on the day of the experiment.

The fecal coliform cultures were diluted to 10^5 CFU per mL of Mueller-Hinton broth and each diluted culture (1 mL) was mixed with an equal volume of or 2.5 ppm ClO₂ with a vortexer, resulting in a fecal coliform cell suspension in MH broth containing 2.5 or 1.25 ppm of ClO₂. The fecal coliform cells were adapted in the sub-lethal concentration of ClO₂ at room temperature for 1 min before being mixed with an equal volume of Dey-Engley (DE) neutralizing broth. The minimal inhibitory concentrations (MICs) of adapted and non-adapted cells of the fecal coliforms were determined subsequently.

2.3. MICs of sanitizer-adapted vs. non-adapted cells

Adapted and non-adapted cells of each fecal coliform culture in the DE neutralizing broth were centrifuged for 5 min at 4,200 g. The supernatant was removed, the cell pellet was rinsed twice with 1 mL of sterile deionized water, and 0.5 mL of the resulting bacterial suspension was added into a series of 4.5 ml of MH broth supplemented with an appropriate concentration (2-fold dilution series) of each of the 13 antibiotics including ampicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, sulfisoxazole, tetracycline, streptomycin, trimethoprim, ciprofloxacin, cefazolin, doxycycline, amoxicillin-clavulanic acid, and nitrofurantoin (Sigma Aldrich, MO USA).

The broth tubes with inoculated fecal coliforms were incubated at 37°C for 16-20 h. The lowest concentration of an antibiotic that inhibited the growth of bacteria after the overnight incubation was regarded as the MIC. The MICs of antibiotics for sanitizer-adapted and non-adapted cells of fecal coliforms were compared. Susceptibility categorization was assessed according to current susceptibility and resistant breakpoints advised by the Clinical and Laboratory Standards Institute (CLSI, 2017)

3. Results

The MICs of various antibiotics against the fecal coliforms with or without the preadaptation in sub-lethal concentrations of ClO₂ are shown in Table A.1. Results suggest that the preadaptation process did not change the MICs of ciprofloxacin and trimethoprim against the 12 fecal coliform isolates used in the study (Table A.2). However, a decrease in the MICs of cephalothin and tetracycline was observed with four of the fecal coliform isolates, whilst the MICs against the other eight fecal coliform isolates remained the same. A decrease in the MICs of gentamycin and

streptomycin was observed with three of the fecal coliform isolates and of cefazolin and doxycycline with six of the isolates, respectively, while the MICs remained the same for the rest of the fecal coliform isolates used in the study. The other three antibiotics, ampicillin, chloramphenicol, and nitrofurantoin each had a decreased MIC against two, seven or nine isolates used in the study. Preadaptation with sub-lethal concentrations of chlorine dioxide increased the MICs of kanamycin against two of the fecal coliform isolates and decreased the MICs against four fecal coliform isolates. The MIC of nalidixic acid increased for one of the fecal coliform isolates and decreased for nine of the fecal coliform isolates.

The susceptibility of selected fecal coliforms to various antibiotics as affected by the preadaptation in sub-lethal concentrations of ClO₂ are shown in Table A.3. It was observed that the preadaptation process, in the large majority of cases either did not change or decreased the susceptibility of the fecal coliforms to antibiotics changing from resistant/intermediate resistant to sensitive or resistant to intermediate resistant. In only two incidences, the increase in MIC changed the susceptibility of two fecal coliform isolates from sensitive to resistant/intermediate resistant (Table A.4).

4. Discussion

In this study, it was observed that the preadaptation with sub-lethal concentrations of chlorine dioxide generally did not decrease the susceptibility of fecal coliform isolates to antibiotics. Alonso-Hernando et al., (2009) reported that *Salmonella enterica* serotype Enteridis preadapted with chlorine dioxide did not change its susceptibility to gentamicin, amoxicillin-clavulanic acid, ciprofloxacin and tetracycline. Soumet, Fourreau, Legrandois, & Maris (2012) observed that pre-exposure of *Escherichia coli* to quaternary ammonium cation decreased the MIC of ciprofloxacin,

nalidixic acid, trimethoprim and ampicillin by 4-fold, whereas MIC of gentamycin and sulfamethoxazole remained the same. The exposure of *E. coli* to sub-inhibitory concentrations of sodium hypochlorite did not change its susceptibility to several antibiotics including ampicillin, gentamicin, ciprofloxacin (Capita, Riesco-Peláez, Alonso-Hernando, & Alonso-Calleja, 2014).

It was suggested that oxidizing agents such as chlorine dioxide generate hydroxyl radicals which may interact with the metabolic pathways in microbial cells, leading to decreased susceptibility to antibiotics (Ikai et al., 2013). The mechanism of antimicrobial action of ClO₂ against *E. coli* includes inhibiting the activity of intracellular β -D-galactosidase, resulting in the increase of permeability of outer and cytoplasmic membranes (Ofori, Maddila, Lin, & Jonnalagadda, 2017). Disruption of the cytoplasmic membrane causes efflux of intracellular components and eventual decrease in the susceptibility to antibiotics (Delcour, 2009).

In this study, it was observed that the preadaptation with sub-lethal concentrations of chlorine dioxide generally did not decrease the susceptibility of fecal coliform isolates to antibiotics. In the current study, it was observed that preadaptation with sub-lethal concentrations of ClO₂ did not enhance the resistance of fecal coliforms to antibiotics tested, with only two exceptions (Table A.4). In contrast to what was found in the present work, a study involving a poultry isolate of *Salmonella enterica* serotype Typhimurium repeatedly exposed to increasing sub-lethal concentrations of chlorine dioxide resulted in a 1.13-fold increase in the MIC of streptomycin (10 μ g) compared with unexposed strains (Alonso-Hernando et al., 2009). The authors did not explain the possible linkage between the sub-lethal concentration of sanitizer and acquisition of antibiotic resistance.

The mechanism of cross resistance between chlorine dioxide and antibiotics has not yet been determined. It has been suggested that the possible linkage between resistance to antibiotics and

sanitizers might be due to common resistance mechanisms such as changes in the cell surface, efflux or enzymatic inactivation of the compound before it reaches its target site, changes in surface permeability, modification or overproduction of the target site, and acquisition of alternative metabolic pathways to those inhibited by the drug (IFT, 2006). Changes in the cell surface induce a reduction in cell permeability and do not allow chemically unrelated molecules into the resistant cells, thus playing a vital role in the establishment of resistance to a variety of antimicrobial agents (Nikaido, 1996). The role of lipopolysaccharides as a permeability barrier in Gram-negative bacteria has been well documented (Stickler, 2002). There have also been a number of reports of reduced sanitizer efficacy following changes in other components of the outer membrane ultrastructure (Braoudaki & Hilton, 2004b) including proteins (Winder et al., 2000), fatty acid composition and phospholipids (Boeris, Domenech, & Lucchesi, 2007).

Efflux pumps decrease the intracellular concentration of toxic compounds, including sanitizers (Borges-Walmsley and Walmsley 2001, Levy 2002, McKeegan et al. 2003, Piddock 2006, Poole 2001). Bacterial efflux pumps actively transport many antibiotics out of the cell and are major contributors to the intrinsic resistance of Gram-negative bacteria. When overexpressed, efflux pumps can also confer high levels of resistance to previously clinically useful antibiotics.

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Table A.1

Minimum inhibitory concentration ($\mu\text{g/ml}$) of various antibiotics against selected fecal coliforms exposed to sub-lethal concentrations of chlorine dioxide.

Antibiotic	Exposure	ClO ₂ conc.	Fecal coliform isolates (F) from packing facilities (P)											
			P1		P2		P3		P4		P5		P6	
			F5	F8	F35	F252	F219	F112	F272	F174	F329	F354	F390	F406
Ampicillin	W	2.5 ppm	32	16	16	16	16	16	32	16	16	8	32	16
	W	1.25 ppm	32	8	16	16	16	16	32	16	16	8	32	8
	W/O		32	16	16	16	16	16	32	16	16	8	32	16
Cefazolin	W	2.5 ppm	16	16	8	16	16	32	16	32	64	16	16	16
	W	1.25 ppm	16	16	8	16	16	32	16	32	64	16	16	16
	W/O		32	32	8	64	32	32	16	32	64	16	32	32
Cephalothin	W	2.5 ppm	16	16	4	16	16	32	16	32	64	16	16	16
	W	1.25 ppm	16	16	4	16	16	32	16	32	64	16	64	16
	W/O		128	32	8	128	32	32	16	32	128	16	128	32
Chloramphenicol	W	2.5 ppm	32	32	8	128	64	64	16	64	64	8	32	32
	W	1.25 ppm	64	32	8	128	64	64	16	64	64	8	64	32
	W/O		128	32	8	128	128	64	64	128	128	16	128	32
Ciprofloxacin	W	2.5 ppm	8	16	16	8	8	16	8	16	16	8	8	16
	W	1.25 ppm	8	16	16	8	8	16	8	16	16	8	8	16
	W/O		8	16	16	8	8	16	8	16	16	8	8	16
Doxycycline	W	2.5 ppm	16	8	16	32	32	32	16	32	16	8	16	16
	W	1.25 ppm	16	16	16	16	32	16	16	32	8	16	16	16
	W/O		16	16	16	32	32	64	16	64	16	16	16	16

Gentamycin	W	2.5 ppm	8	8	8	8	8	8	8	16	8	8	8	8
	W	1.25 ppm	8	8	8	8	8	8	8	16	8	8	8	8
	W/O		8	16	16	8	8	16	8	16	8	8	8	8
Kanamycin	W	2.5 ppm	8	8	8	8	8	8	8	8	8	8	8	8
	W	1.25 ppm	8	8	16	8	8	8	32	8	8	8	8	8
	W/O		8	16	8	16	8	8	16	16	16	8	8	8
Nalidixic acid	W	2.5 ppm	32	64	16	<i>64</i>	64	32	32	64	128	32	32	64
	W	1.25 ppm	64	8	32	<i>64</i>	64	64	32	64	64	16	64	8
	W/O		64	64	16	128	64	64	64	64	128	32	64	64
Nitrofurantoin	W	2.5 ppm	32	<i>16</i>	<i>16</i>	<i>16</i>	<i>16</i>	<i>16</i>	32	<i>16</i>	<i>16</i>	8	32	<i>16</i>
	W	1.25 ppm	32	<i>16</i>	<i>16</i>	<i>16</i>	<i>16</i>	<i>16</i>	32	<i>16</i>	<i>16</i>	8	32	<i>16</i>
	W/O		32	32	64	64	32	32	32	32	64	16	32	32
Streptomycin	W	2.5 ppm	16	8	16	8	8	8	32	16	16	8	16	8
	W	1.25 ppm	16	8	16	8	16	8	32	16	16	8	16	8
	W/O		16	8	16	16	16	16	32	16	16	8	16	8
Tetracycline	W	2.5 ppm	16	8	16	32	32	32	16	32	16	8	16	8
	W	1.25 ppm	16	16	16	<i>16</i>	32	<i>16</i>	16	32	8	16	16	16
	W/O		16	16	16	32	64	64	16	64	16	16	16	16
Trimethoprim	W	2.5 ppm	32	16	16	8	8	8	16	16	64	32	32	16
	W	1.25 ppm	32	16	16	8	8	8	16	16	64	32	32	16
	W/O		32	16	16	8	8	8	16	16	64	32	32	16

W- with exposure in sub-lethal concentration of sanitizer solution.

W/O- without exposure in sub-lethal concentration of chlorine dioxide; control samples.

Increased antibiotic MICs are highlighted in bold.

Decreased antibiotic MICs are highlighted in italics.

Table A.2

Number of tested fecal coliforms (out of 12) completely inhibited by a changed or unchanged minimal inhibitory concentration (MIC) of antibiotics.

Antibiotics	No. of fecal coliform isolates completely inhibited by		
	Increased MIC	Unchanged MIC	Decreased MIC
Ampicillin	0	10	2
Cefazolin	0	6	6
Cephalothin	0	4	8
Chloramphenicol	0	5	7
Ciprofloxacin	0	12	0
Doxycycline	0	6	6
Gentamycin	0	9	3
Kanamycin	2	6	4
Nalidixic acid	1	2	9
Nitrofurantoin	0	3	9
Streptomycin	0	9	3
Tetracycline	0	4	8
Trimethoprim	0	12	0

Table A.3

Changes in the susceptibility of selected fecal coliforms to various antibiotic as affected by exposure to sub-lethal concentrations of chlorine dioxide.

Antibiotic (Break points)	Exposure	ClO ₂ conc.	Fecal coliform isolates from packing facilities (P)											
			P1		P2		P3		P4		P5		P6	
			F5	F8	F35	F252	F219	F112	F272	F174	F329	F354	F390	F406
Ampicillin ≤8, 16 , ≥32	W	2.5 ppm	R	I	I	I	I	I	R	I	I	S	R	I
	W	1.25 ppm	R	S	I	I	I	I	R	I	I	S	R	S
	W/O		R	I	I	I	I	I	R	I	I	S	R	I
Cefazolin ≤16, - , ≥32	W	2.5 ppm	S	S	S	S	S	R	S	R	R	S	S	S
	W	1.25 ppm	S	S	S	S	S	R	S	R	R	S	S	S
	W/O		R	R	S	R	R	R	S	R	R	S	R	R
Chloramphenicol ≤8, 16 , ≥32	W	2.5 ppm	R	R	S	R	R	R	I	R	R	S	R	R
	W	1.25 ppm	R	R	S	R	R	R	I	R	R	S	R	R
	W/O		R	R	S	R	R	R	R	R	R	I	R	R
Doxycycline ≤4, 8 , ≥16	W	2.5 ppm	R	I	R	R	R	R	R	R	R	I	R	R
	W	1.25 ppm	R	R	R	R	R	R	R	R	I	R	R	R
	W/O		R	R	R	R	R	R	R	R	R	R	R	R
Gentamycin ≤4, 8 , ≥16	W	2.5 ppm	I	I	I	I	I	I	I	R	I	I	I	I
	W	1.25 ppm	I	I	I	I	I	I	I	R	I	I	I	I
	W/O		I	R	R	I	I	R	I	R	I	I	I	I
Kanamycin ≤16, 32 , ≥64	W	2.5 ppm	S	S	S	S	S	S	S	S	S	S	S	S
	W	1.25 ppm	S	S	S	S	S	S	I	S	S	S	S	S
	W/O		S	S	S	S	S	S	S	S	S	S	S	S

Nalidixic acid ≤16, -, ≥32	W	2.5 ppm	R	R	S	R	R	R	R	R	R	R	R	R
	W	1.25 ppm	R	S	R	R	R	R	R	R	R	S	R	S
	W/O		R	R	S	R	R	R	R	R	R	R	R	R
Nitrofurantoin ≤32, 64, ≥128	W	2.5 ppm	S	S	<i>S</i>	<i>S</i>	S	S	S	S	<i>S</i>	S	S	S
	W	1.25 ppm	S	S	<i>S</i>	<i>S</i>	S	S	S	S	<i>S</i>	S	S	S
	W/O		S	S	I	I	S	S	S	S	I	S	S	S
Tetracycline ≤4, 8, ≥16	W	2.5 ppm	R	<i>I</i>	R	R	R	R	R	R	R	<i>I</i>	R	<i>I</i>
	W	1.25 ppm	R	R	R	R	R	R	R	R	<i>I</i>	R	R	R
	W/O		R	R	R	R	R	R	R	R	R	R	R	R

W- with exposure in sub lethal concentration of chlorine dioxide.

W/O- without exposure in sub-lethal concentration of chlorine dioxide; control samples.

Increased antibiotic susceptibilities are highlighted in bold.

Decreased antibiotic susceptibilities are highlighted in italics.

MIC breakpoints for ciprofloxacin are not available and not included in the table.

Exposure did not change the MIC of ciprofloxacin, streptomycin and trimethoprim and these antibiotics are excluded from the table.

MIC breakpoints for antibiotics described by the Clinical and Laboratory Standards Institute (CLSI) except for streptomycin, for which the breakpoint was described by the US Food and Drug Administration.

S-Sensitive, I-Intermediate and R-Resistant

Table A.4

Number of exposed fecal coliform isolates (out of 12) with changed or unchanged susceptibility to various antibiotics compared with unexposed cells according to the breakpoints.

Antibiotic	No. of exposed fecal coliform isolates		
	With increased susceptibility	With unchanged susceptibility	With decreased susceptibility
Ampicillin	0	10	2
Cefazolin	0	6	6
Chloramphenicol	0	10	2
Doxycycline	0	9	3
Gentamycin	0	9	3
Kanamycin	1	11	0
Nalidixic acid	1	8	3
Nitrofurantoin	0	9	3
Tetracycline	0	8	4

Exposure did not change the MIC of ciprofloxacin, streptomycin and trimethoprim and these antibiotics are therefore excluded from the table.