INFLUENCE OF WATER ANTIMICROBIALS AND LOW TEMPERATURE STORAGE ON MS2 BACTERIOPHAGE AND *ESCHERICHIA COLI* O157:H7/O26:H11 ON STRAWBERRIES

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ABSTRACT OF THE THESIS

Influence of Water Antimicrobials and Low Temperature Storage on MS2 Bacteriophage and *Escherichia coli* O157:H7/O26:H11 on Strawberries

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Foodborne illnesses caused by viruses and STEC contaminated fresh produce are currently a world concern. Strawberries are delicate and could be contaminated at any point during the farm to fork continuum. Typically, fresh strawberries would be stored at refrigerated temperature and not washed until immediately prior to serving. In contrast, strawberries are typically washed prior to freezing at -40 °C or even lower temperature. Washing and low temperature storage are two common practices utilized by commercial processors and in-home to enhance safety and quality of strawberries. The efficacy of two combining practices: washing with a commercial water antimicrobial and freezing/refrigeration were investigated.

In the present study, MS2 bacteriophage was used as a surrogate for Norovirus and Hepatitis A virus. The microorganisms were spot inoculated onto strawberries to

achieve 6.6 log PFU/g. The inoculated strawberries were washed with tap water, electrolyzed water or 50 ppm free chlorine solution for 90 seconds prior to and after storage. After initial washing, the strawberries were separately stored at −20 °C and −80 °C for 30 days, and at 4 °C for 2 days. Change in MS2 populations on strawberries was evaluated by plaque assay on pre-determined sampling days. The same experiment was conducted independently using *E. coli* O157:H7 and *E. coli* O26:H11.

The results showed that washing with antimicrobials provided a significant decrease of MS2 and STEC populations ($p < 0.05$). The log reduction of MS2 was approximately 1 log PFU/g and that of STEC was 2.5 log CFU/g. Frozen storage had minor effect on inactivating MS2, resulting in 0.5 log PFU/g reduction at the end of storage. In contrast, freezing successfully reduced the population of *E. coli* O157:H7/O26:H11 on strawberries and the maximum reduction was up to 3 log CFU/g achieved by the electrolyzed water treatment. The second wash conducted following storage provided an additional 1 log reduction in the population of MS2 and STEC. The influence of refrigeration on populations of MS2 and STEC was similar to frozen storage.

Collectively, population of MS2 bacteriophage did exhibit remarkable decrease following exposure to water antimicrobials and low temperature. STEC were more susceptible to these conditions. The utilization of multiple hurdles may result in greater reduction of MS2 and STEC. Based on results of the present study, it is recommended that water antimicrobials are used particularly when strawberries are washed using an immersion process.

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DEDICATION

To my parents,

Bolin Huang and Yinhua Zhu

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

Fresh and frozen strawberries have become an increasingly popular food item globally (Simpson, 2018). Meanwhile, in recent years foodborne outbreaks related to strawberries have caused thousands of illnesses and huge economic loss. Most of the outbreaks are caused by Hepatitis A, Norovirus and Shiga-toxin producing *E. coli*. Product contamination can occur during any point along farm to table continuum. Contaminated soil, irrigation water and human hands can all serve as vehicles of microbial transmission (Palumbo et al., 2013).

There are limited methods to inactivate foodborne pathogens associated with strawberries that will not adversely influence organoleptic and sensory characteristics. For fresh strawberries, temperature control is the primary method to limit microbial growth and pathogen outgrowth. Washing or other processing of fresh strawberries is not recommended until immediately prior to serving since moisture will encourage spoilage and shorten the shelf life (Mitcham and Mitchell, 2002). Consumers usually use tap water to wash strawberries and may only rinse them to remove soil or dirt. The washed strawberries may be placed in a refrigerator if not consumed and subsequently washed a second time immediately prior to serving.

Frozen strawberries are typically produced commercially, although preparation of fresh strawberries for home-freezing is also common. During commercial processing, strawberries are washed by using water antimicrobials before quick freezing at -40°C

or at even lower temperatures (Arthey, 1995). The calyx of strawberries is typically removed; therefore, consumers usually do not wash frozen strawberries but directly use or consume. However, consumers may wash frozen strawberries to facilitate thawing. A typical bag of retail commercially frozen strawberries contains multiple servings, in some instances it is possible that multiple freeze-thaw cycles occur which may provide an opportunity for outgrowth of microorganisms.

Washing using water antimicrobials and low temperature storage are methods that may inactivate and inhibit the growth of microbes on strawberries. Chlorine is commonly used as a water antimicrobial since it is inexpensive and effective. However, chlorine is now seen as counter to sustainability efforts being implemented by the food industry. Neutral electrolyzed water is one alternative to the use of chlorine. Multiple researchers have demonstrated the efficacy of electrolyzed water in reducing the number of pathogens including viruses on various types of food. (Gulati, 2001; Izumi, 1999; Kim, 2000; Issa-Zacharia, 2011).

In this study, MS2 bacteriophage was used as a surrogate for Hepatitis A virus and Norovirus. The efficacy of electrolyzed water (50 ppm free chlorine), 50 ppm free chlorine solution and tap water as a control in inactivating MS2 bacteriophage and Shiga-toxin producing *E. coli* serotypes O157:H7 and O26:H11 on strawberries were compared prior to and after low temperature storage. Two typical freezing temperatures (-80 °C and -20 °C) and refrigeration temperature (4 °C) were used to equate to commercial and home storage of frozen and fresh strawberries, respectively.

The combined influence of freezing and washing was investigated.

2. HYPOTHESIS AND OBJECTIVE

The hypothesis of this research is that using water antimicrobials prior to and after low temperature storage will significantly reduce the level of MS2 bacteriophage and STEC on fresh and frozen strawberries. The multiple hurdles may improve reduction in MS2 and STEC on strawberries.

The objectives of this research are:

1. To evaluate the efficacy of water antimicrobials and refrigerated temperature on inactivating MS2 bacteriophage on fresh strawberries during 2 days storage
2. To evaluate the efficacy of water antimicrobials and frozen temperature on inactivating MS2 bacteriophage on frozen strawberries during 30 days storage
3. To evaluate the efficacy of water antimicrobials and refrigeration temperature on inhibiting *E. coli* serotypes O157:H7 and O26:H11 on strawberries during 2 days storage
4. To evaluate the efficacy of water antimicrobials and frozen temperature on inhibiting *E. coli* serotypes O157:H7 and O26:H11 on strawberries during 30 days storage

3. LITERATURE REVIEW

3.1 Fresh/frozen strawberry industry

Strawberries have become a popular fruit all over the world. Strawberries contain an abundance of vitamins and antioxidants including ascorbic acids, anthocyanin and other phytochemicals. These chemical compounds may be linked to maintaining health. Researches have reported that consuming strawberries may lower blood pressure, support the function of immune system, and reduce the risk of cardiovascular disease (Hannum, 2004).

Unfortunately, strawberries are a rather delicate fruit and are susceptible to physical damage and rapid spoilage. Fresh strawberries have a relatively short shelf life of about one week under refrigerated storage (Gol et al., 2013). There are limited ways to extend the shelf life of fresh strawberries since traditional washing prior to storage is not recommended as it exasperates spoilage. Applying edible coatings on the surface of fresh strawberries has been shown to prolong shelf life, but the cost and the sensory change diminishes the utility of the method (Vu et al., 2011; Del-Valle et al., 2005). In general, cold storage remains one of the best methods for extending the shelf life of strawberries.

Fresh strawberries can be frozen using IQF (Individual Quick Freezing) technology to extend the shelf life. Frozen strawberries have similar nutritive value as fresh strawberries and are an excellent source of vitamin C (Miller and Knudson, 2014).

Frozen strawberries have a shelf life of 2-3 years; enhancing ability for global distribution and in-home storage.

The consumption and the production of fresh and frozen strawberries continues to increase. In the United States, the average annual per capita consumption of fresh strawberries in 2012 was approximately 3.6 kg; with frozen strawberries accounting for approximately 1 kg (USDA, 2013). The production of strawberries in the United States in 2017 was over 1.56 million tons; making the United States a global production leader accounting for around one third of the total world's strawberry production (USDA, 2018). With the development of modern technology and transportation, strawberries are available for purchase from local markets, supermarkets, and even online outlets. The entire strawberry industry is expanding, which also brings food safety challenges from the farm to the table. Foodborne outbreaks associated with strawberries have caused thousands of illnesses and huge economic losses. The predominant microbial food safety concerns associated with strawberries to date have been Hepatitis A, Norovirus, and *Escherichia coli* O157:H7 (Table 1).

Table 1. Examples of some outbreaks associated with fresh and frozen strawberry in USA (Marler, 2016).

Commodity	Microorganism	Year	States
Fresh strawberry	Cyclospora	1997	Multi
	Escherichia coli O26:H11	2006	Massachusetts
	Norovirus	2007	Georgia
	Norovirus	2007	California
	Escherichia coli O157:H7	2011	Oregon
Frozen strawberry	Hepatitis A	1990	Georgia
	Hepatitis A	1997	California
	Hepatitis A	2016	Multi

3.2 Norovirus and Hepatitis A virus

3.2.1 Prevalence and outbreaks

Norovirus and Hepatitis A virus (HAV) are non-enveloped RNA foodborne viruses. They account for over half of the foodborne illness every year in the world. In 2016, they caused over 10,000 illness and 20 deaths. Most of the cases were not severe, however large numbers of individuals were impacted. The infectious dose of human norovirus can be as few as 18 viral particles (Teunis et al.,2008). The typical pathway of virus transmission is via the fecal-oral route. Fecal particles containing virus may come in contact with food through exposure to contaminated hands, soil, water, and flies. Individuals infected through consumption of contaminated food may

develop gastrointestinal symptoms including vomiting and diarrhea, which passes more viral particles into the environment. In closed areas such as a cruise ship or airplane the fecal to oral cycle may result in hundreds of illness in a very short time (USDA-NIFA Food Virology Collaborative NoroCORE, 2017).

Fresh produce is one of the major vehicles of virus transmission. Among all norovirus outbreaks, outbreaks associated with fresh produce accounts for over 15%, and the number is increasing (Greig and Ravel, 2009). Table 2 lists several foodborne virus outbreaks associated with fruits and vegetables during the past two decades. Fresh produce can be contaminated in the field through application of contaminated irrigation water, soil and fertilizers. Infected workers and food handlers may also contaminate fresh produce during harvest and post-harvest stages (Carter, 2005). Unlike other types of food which may undergo thermal processing before consumption, fruits and vegetables are usually served without thermal processing or perhaps even washing process. As a result, the risk of infections associated with consumption of raw contaminated fresh produce may be greater than for other types of food.

Table 2. Foodborne virus outbreaks associated with fruits and vegetables in recent two decades (Radin, 2012).

Commodity	Microorganism	Year	Country	No. of cases
Raspberry	Norovirus	2001	Sweden	30
Lettuce	Hepatitis A	2001	Sweden	80
Blueberry	Hepatitis A	2002	New Zealand	39
Green onion	Hepatitis A	2003	USA	600
Raspberry	Norovirus	2005	France	75
Raspberry	Norovirus	2005	Denmark	276
Raspberry	Norovirus	2006	Sweden	43
Tomato	Norovirus	2007	Sweden	400
Raspberry	Norovirus	2009	Finland	200
Lettuce	Norovirus	2010	Denmark	260
Strawberry	Norovirus	2012	Germany	11000
Strawberry	Hepatitis A	2016	USA	143

Frozen fruits or vegetables may also be contaminated with viruses. In fact, several serious outbreaks were caused by frozen berries imported from other countries. In 2012, a Norovirus outbreak related to imported frozen strawberries from China affected over 11,000 people in Germany (Mäde et al., 2013). In 2016, 143 people from 9 states in the USA became infected with Hepatitis A after eating smoothies containing contaminated frozen strawberries imported from Egypt (CDC, 2016).

Polluted irrigation water was suspected to be the source of contamination in those outbreaks. Concern associated with use of polluted irrigation water in developing countries remains. The structure of the virus enables it to withstand extreme environments such as low temperature and low pH for extended periods (Seymour and Appleton, 2001). Once a viral particle has attached onto the rough surface of strawberries it could persist for the duration of the shelf life (Verhaelen et al.,2012).

3.2.2 Surrogate for Norovirus and Hepatitis A virus

In 2016, growing human norovirus on human intestinal epithelial cells in vitro cultivation system was reported for the first time (Ettayebi et al.,2016). Culturing human norovirus outside of the human body was not possible previously. The method requires a high degree of technical skill and significant monetary resources. Typically, surrogates for Norovirus and Hepatitis A virus are used to study the behavior of foodborne virus. The most widely used surrogates are murine norovirus, feline calicivirus, and different types of bacteriophage such as MS2 and Phi174 (Belliot et al., 2008; Richards, 2012). The response of MS2 to disinfectants, environmental stresses, and the suitability as a surrogate for human norovirus has been evaluated (Bae and Schwab, 2008; Solomon et al., 2009).

To quantify the viral particles, plaque assay and PCR technology were used. Plaque assay was conducted based on the ability of bacteriophage infecting specific host bacteria cells. A host bacterial lawn is formed on an agar layer and samples containing bacteriophage are spotted on the lawn. After incubation, bacteriophage

propagate on host cells, which develop plaques (the circular area of lysed cells). Each plaque could be considered as one viral particle and the total number represents the population of bacteriophages in the original sample (Cormier and Janes, 2014).

3.3 STEC: prevalence and outbreaks

Escherichia coli (*E. coli*) normally exist in the intestines of animals including humans. *E. coli* as inhabitants of the gastrointestinal track serve an important role in the gut microbiota and have essential functions and contribute to human health and well-being. However, some *E. coli* serotypes are foodborne pathogens that endanger public health. Among them, Shiga toxin-producing *E. coli* (STEC) could produce shiga toxin which can cause severe debilitating disease. Symptoms of STEC infection include bloody diarrhea, vomiting and fever. Although most people experience mild infection and usually recovered after one week, some people suffer hemolytic uremic syndrome (HUS) that result in kidney failure and even death (Mead and Griffin, 1998). CDC estimates that each year STEC causes 265,000 illness, 3,600 hospitalizations and 30 deaths in the United States (CDC,2018).

The most commonly identified STEC serotype in the United States is *E. coli* O157:H7. Even at low cell numbers, *E. coli* O157:H7 still have the ability to cause diseases (Lim et al., 2010). *E. coli* O157:H7 can be transmitted by various types of food including ground beef, dairy products, fresh produce, and nuts. The major recognized reservoir of *E. coli* O157:H7 is cattle, so the consumption of incompletely cooked ground beef is a leading cause O157 infection (Ma et al., 2014). However, a

greater number of O157 outbreaks are related to fresh produce consumption. Research has shown that compared to non-pathogenic bacteria, *E. coli* O157:H7 exhibit stronger adhesion to surface and internal tissues of fresh produce and survive plant defense response (Berger et al., 2010). *E. coli* O157:H7 may spread into the environment through feces, tissues, and animal carcass, which makes controlling *E. coli* O157:H7 very difficult (Beuchat, 1996).

Besides *E. coli* O157:H7, non-O157 STEC have gained increased attention in recent years. Common non-157 STEC identified in the United States are O26, O45, O103, O111, O121 and O145 which are also call the “Big six”. These non-O157 STEC may cause similar symptoms as O157 but were typically not screened in the past. Serotype O26 are the second most isolated STEC from outbreaks after O157 and also rank second behind O157 in the cause of HUS (Mathusa et al., 2010).

Strawberries can be contaminated with pathogenic *E. coli* in the fields. Depending on farming practice the fruit may be in contact with soil and many strawberry farms lack effective fences to prevent animal intrusion. In 2011, an *E. coli* O157:H7 outbreak occurred in Oregon and that was traced to strawberries. The outbreak caused 15 illness, 6 hospitalizations and 2 deaths (Laidler et al., 2013). Investigation and laboratory testing showed the strawberries were contaminated by deer feces. In 1995, an *E. coli* O157:H7 outbreak associated with deer jerky occurred only 60 miles away from the strawberry farm linked to the outbreak; indicating a possible relationship between these two outbreaks (Gurtler et al., 2014). In 2006,

there was an O26 outbreak traced to strawberries in Massachusetts which caused 5 illness (Luna and Mody, 2010). Compared to Norovirus and Hepatitis A virus, STEC contamination on strawberries is not very common. Nevertheless, efforts to prevent outbreaks linked to STEC must be implemented (e.g., fence to keep animals out).

3.4 Efforts to approach microbial safety of fresh produce

3.4.1 Water antimicrobials

FDA 2017 food code indicates that raw fruits and vegetables shall be thoroughly washed in tap water to remove soil and other contaminants (FDA, 2017). Water antimicrobials are not required but recommended to enhance microbial safety of fresh produce. Chlorine is the most widely used water antimicrobials by the food industry due to its low cost and efficacy in controlling microorganisms. Chlorine can not only be applied on food commodities, but it is also used to sanitize equipment and processing environment. The concentration of free chlorine used in fresh produce ranges from 50 ppm to 200 ppm (Stopforth et al., 2008). High concentrations (over 500 ppm) have been used to treat high risk foods such as alfalfa sprouts. However, researchers indicated that higher concentrations and longer contact time may not achieve better inactivation of pathogens (Jaquette et al., 1996; Taormina and Beuchat, 1999). Numerous studies evaluated the efficacy of chlorine and chlorine-based antimicrobials on inactivating pathogens but results were mixed. For instance, Lukasik et al. (2003) reported that using 50 ppm free chlorine to wash strawberries resulted in a 2 log PFU/g reduction of MS2 bacteriophage, but only 1.3 log CFU/g reduction of

E. coli O157:H7.

There are some negative aspects associated with chlorine. Chlorine may react with organic compounds which would decrease the level of free chlorine. The new chemicals formed by these reactions such as trihalomethanes can negatively influence human health and the environment (Dunnick and Melnick, 1993). Electrolyzed water is often suggested as a substitute for the use of chlorine. It can be produced by passing diluted salt solution through an electrolytic cell with which the anode and cathode are separated by a membrane (Huang, 2008). There are three main types of electrolyzed water based on pH value: acidic, neutral and basic electrolyzed water. Among them, neutral electrolyzed water has less smell and is stable near neutral pH, which will cause less damage to processing equipment and food materials (Izumi, 1999). The active compounds of electrolyzed water are hypochlorous acid (HOCl) and hypochlorous acidic ion (ClO-). Those compounds have high oxidative potentials which disrupt microbial membranes. The efficacy of NEW in reducing the number of pathogens on various types of food is at least equal to chlorine solution (Gulati,2001; Izumi,1999; Kim,2000; Issa-Zacharia,2011). For example, Park et al. (2001) reported that electrolyzed water with 45 ppm free chlorine successfully reduced 2.5 log CFU/g *E. coli* O157:H7 and *Listeria monocytogenes* on lettuce. Overall, electrolyzed water could be an effective alternative to chlorine in many circumstances.

Other water antimicrobials include organic acid, hydrogen peroxide, and Quaternary ammonium compounds (QAC). Their efficacy greatly depends on the type

of food commodities and microbial population (Rodgers et al., 2004). Yu et al. (2001) compared the efficacy of NaOCl (100 ppm and 200 ppm), acetic acid (2% and 5%), Na3PO4 (2% and 5%), and H2O2 (1% and 3%) on the reduction of *E. coli* O157:H7 on strawberries. Results showed that that the 3% H_2O_2 treatment achieved 2.2 log CFU/g reduction while all other solutions had less than 2 log CFU/g reduction. Another study showed that the combination of peroxyacetic acid and hydrogen peroxide could reduce the number of FCV (surrogate for norovirus) on strawberries by 3 log PFU/g, but the concentration is much higher than the recommended concentration (Gulati et al., 2001). These water antimicrobials each have distinct advantages and disadvantage associated with their use.

3.4.2 Cold storage

Cold storage is essential for maintaining the quality of fresh produce. Fruits such as strawberries have a very short shelf life and refrigeration is a key factor to maximize shelf life. However, refrigeration cannot eliminate the risk from pathogens. Researchers reported that while most of foodborne pathogens excluding *Listeria monocytogenes* may not grow under refrigeration, they can persist on food for a long time period and their survival could even be enhanced as response to additional stress (Zhao et al., 1993). Nguyen et al. (2014) observed significant decline in the population of *E. coli* O157:H7 and *Salmonella* on whole strawberries after storage at refrigeration temperature for 7 days, but the population of both pathogens remained high.

Freezing can negatively impact microorganisms through both physical and

chemical effects and may result in genetic changes (Archer, 2004). There have been very few outbreaks linked to frozen food, suggesting freezing may improve microbial safety. Studies indicated the efficacy of freezing on killing pathogens such as *Listeria monocytogenes* depends on the temperature, bacterial strain, and chemical compounds of the food (El-kest and Marth, 1992). Researchers reported that freezing (−20 °C, 24 h) ground beef contaminated with different strains of *E. coli* O157:H7 led to a decrease in population by 0.62- 2.52 log CFU/g (Sage and Ingram, 1998). The wide range of inactivation suggests that freezing is not a promising way to achieve food safety.

Most of outbreaks related to frozen food were caused by foodborne viruses (e.g., Norovirus, Hepatitis A). Many researchers showed the resistance of virus against freezing and frozen storage (Archer, 2004; Baert et al., 2009; Butot et al., 2008). Ice could serve as vehicles for Norovirus transmission and an outbreak on a cruise ship that sickened 200 people was traced to contaminated ice (Khan et al., 1994). Frozen berries are very susceptible to Norovirus and Hepatitis A. Usually the contamination occurred before freezing process and the viral particles are able to survive freezing and frozen storage for a very long time. Limited studies in the literature have focused on the impact freezing has on bacterial and viral survival.

3.4.3 Commercial, retail and home practice

A wide variety of studies have focused on the influence of different stresses on controlling microorganisms on food. The results sometimes are not consistent and

even contradictory. This may in part be the result of conducting the studies under different conditions (strains, temperature, inoculation method, personnel, equipment, etc.). When it comes to commercial, retail, and home practices; laboratory-based studies may not reflect actual practices. Most of studies only paid attention to one type of treatment or process. Table 3 shows some common practices and risk assessment for enteric viruses on fruits and vegetables. The studies were in agreement that viruses are difficult to inactivated using a single commercial process. However, developing an effective and systemic approach that could be applied commercially, in retail and home to ensure food safety may not be feasible (Butot et al., 2008). A combination of processes (e.g., washing and freezing) could lead to greater microbial reduction or have undesired effects on microbes and negatively influence sensory or nutrition characteristics. Implementing a series of stresses is called the “hurdle effect” and it is used to effectively control microbial growth in food, and it arouses more and more attention (Leistner, 2000).

In order to provide guidance to commercial, retail and in-home processors studies are required that addresses each of those operations. For example, in preparation of commercial frozen strawberries the key steps include removal of calyx, washing, freezing, and frozen storage. The influence of these steps on inhibition and inactivation of pathogens on strawberries should be studied continuously and collectively. Washing with water antimicrobials may have limited effect on inactivation of viruses on strawberries but combined with freezing the virus population may

decrease providing some degree of safety (Huang et al., 2019).

Table 3. Common practices and risk assessment on enteric viruses in fruits and vegetables (adapted from Koopmans and Duizer, 2004).

Food product	**Process**	**Virus inactivation (log_{10})**	**Risk for the consumer if viruses present***
Carrot	Storage at 4 °C for 4 days	HAV > 4 Rotavirus < 1	Variable
Green onions	Storage at 4 °C for 15 days	Poliovirus < 1	High
Lettuce	Storage at 4 °C for 9 days	HAV < 2 Poliovirus < 2	Medium
Lettuce	Washing (5 min potable water)	HAV < 1	High
Lettuce and strawberries	Gamma irradiation (3 kGy)	HAV = 1	Medium
Raspberries	4 °C for 9 days	Poliovirus < 1	High
Strawberries	Freezing	Poliovirus < 2	Medium

Food product	**Process**	**Virus inactivation (log_{10})**	**Risk for the consumer if viruses present***
Strawberries	Washing with 2 ppm ClO_2	HAV < 1	High
Strawberries	Washing with 200 ppm chlorine	Poliovirus< 2	Medium
Strawberry purée and sliced green onions	High hydrostatic pressure (375 MPa, 5 min)	HAV > 4	Negligible

*Suggested risk based on published literature concerning reported outbreaks and the effectiveness of various processing methods to inactivate enteric viruses. Negligible risk: product highly unlikely to contain infectious viruses; treatment results in at least 4 log inactivation of common foodborne viruses. Medium risk: product may contain infectious viruses in numbers that may cause disease; treatment results in approximately 2 log inactivation of common foodborne viruses. High risk: products in which the level of viruses is likely to be high enough to cause disease in healthy individuals; treatment results in less than 1 log inactivation of common foodborne viruses. Variable risk: treatment results in significant differences in inactivation of several common foodborne viruses.

4. MATERIALS AND METHODS

4.1 Preparation of inoculum

Escherichia coli MS2 bacteriophage (ATCC 15597-B1) and its *E. coli* host (ATCC 15597) were obtained from ATCC as freeze-dried stock. Tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks) was used to rehydrate the freeze-dried stock and to culture the bacteriophage and its *E. coli* host. The *E. coli* broth culture was streaked on a tryptic soy agar (TSA, Difco, Becton Dickinson, Sparks, MD) plate and incubated at 37 °C for 20 h. A single colony from the plate was transferred to 10 ml of TSB and incubated at 37 °C for 20 h. For each experiment, 20 mL of MS2 (9.5 log PFU/mL) was propagated by the Adams agar-overlay method (Interscience Publishers, Inc., New York, 1959) according to the ATCC instructions. The phage was purified by filtering through a 0.22 µm pore size filter (Acrodisc® Syringe Filters with Supor® Membrane, Pall Laboratory) and stored at 4 °C for 2 days prior to use.

Nalidixic acid resistant *E. coli* O157:H7 strain and kanamycin resistant *E. coli* O26:H11 strain were provided by Dr. Joshua Gurtler (Eastern Region Research Center, USDA, Wyndmoor, PA). The strains were stored in TSB broth with 20% glycerol at −80 °C. Prior to each experiment, a single colony on tryptic soy agar (TSA; Difco, BD) plates supplemented with nalidixic acid (100 µg/mL) or kanamycin (30 µg/mL) was transferred to 30 mL of TSB broth which contained nalidixic acid(100 µg/mL) or kanamycin (30 µg/mL),respectively. Inoculum cultures were incubated at 37 °C for 20 h with shaking. On experiment day, the inoculum was centrifuged at 4500 rpm for

10 min (Allegra™ 21R, Beckman Coulter) and washed using 0.1% sterile peptone water (SPW; Difco, BD). Approximately 9 log CFU/mL of *E. coli* O157:H7 and *E. coli* O26:H11 culture was achieved and suspended in 30mL tap water.

4.2 Strawberry samples and inoculation

Strawberries (average weight: 22 g ± 5 g) were purchased from a local supermarket the day prior to the experiment and kept at 4 °C. The calyx of each strawberry was carefully removed using aseptic technique on the day of experiment. An aliquot of 100 µL purified MS2 (9.5 log PFU/mL) was spot inoculated on each strawberry and allowed to dry in a biosafety cabinet for 1 h, achieving an average initial population of 6.6 log PFU/g. A total 147 strawberries were inoculated per experiment. For experiments using STEC, strawberries were spot inoculated with 3 spots (20 µL each) of each STEC culture to achieve an initial population of 5-6 log CFU/g. All inoculated strawberries were dried in a laminar flow biosafety cabinet for 1h.

4.3 Washing and storage

Three stainless steel sinks were cleaned using commercial sanitizer (Steramine quaternary sanitizer, Edwards-Councilor, Virginia Beach, VA). Each sink was filled with 24 L of tap water, neutral electrolyzed water (ProduceMaxx, SteriloxTM; free chlorine 50 ppm - 55 ppm) or chlorine solution (50ppm free chlorine). The water temperature was measured and kept constant at 24 °C. Forty-eight inoculated strawberries were gently washed in each sink for 90 seconds. After washing, the strawberries were dispensed into 3 steam pans marked as W (water), P (electrolyzed water), and C

(chlorinated water) which indicated three washing treatments.

For each treatment, strawberries were stored at two frozen temperatures: −80 °C and −20 °C for 30 days and refrigerated temperature 4 °C for 2 days. Fig. 4.1 is a schematic showing the processing of strawberries. Strawberries were separately placed in pans and contact with each other was avoided, which was similar to idea of individually quick frozen (IQF) commercially. On day 2 (D2), 6 strawberries stored at 4 °C were washed in 3 L of the same type of solution as the first-time washing. On day 30 (D30) six strawberries stored at -80 °C or -20 °C were washed in water alone or the antimicrobial treatments.

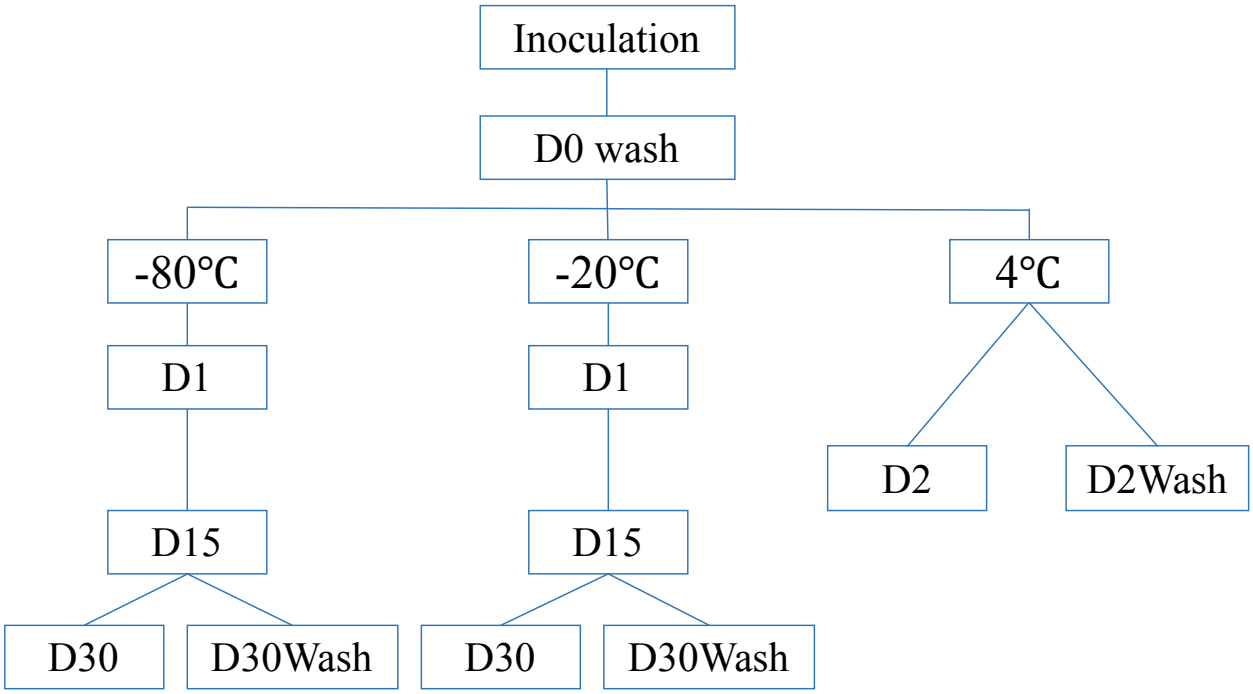


Fig. 4.1. Flow diagram of washing and storage process

4.4 Microbiological analysis

Whole strawberries (n = 3) were sampled to determine the initial and after treatment population of MS2 bacteriophage or *E. coli* O157:H7 and *E. coli* O26:H11 after each treatment and storage condition. Fig. 4.1 shows the pre-determined date of sampling. Dey/Engley neutralizing broth (Criterion, Hardy Diagnostics) was used to neutralize any residual chlorine on the strawberries (1:5 dilution according to the weight of sample). Samples were homogenized for 1 min (easyMIX™, BioMerieux). An aliquot of 1 ml was collected and diluted in 0.1% peptone water (Difco, Becton Dickinson).

To quantify the number of MS2, the plaque assay method was used. *E. coli* host was incubated in TSB at 37 °C for 20 h and added into molten TSA at 43 °C. After mixing, 5 mL of the molten TSA MS2 mixture was layered onto TSA plate to form a bacterial lawn on the top. A 25 µL aliquot of each sample was dispensed onto the solidified agar. After drying for 30 min, the plates were incubated overnight at 37 °C. Plaques were counted and total PFU/g was calculated.

To quantify the number of *E. coli* O157:H7 and *E. coli* O26:H11, an aliquot of 100 µL serial (1:10) diluted sample was spread plated on TSA plates supplemented with nalidixic acid (100 µg/mL) or kanamycin (30 µg/mL). The plates were incubated at 37 °C for 20 h. Colonies were counted, and total CFU/g was calculated.

4.5 Statistical analysis

The MS2 bacteriophage experiments and the *E. coli* experiments were independently replicated two times. The mean values of the population of MS2 bacteriophage/ *E. coli* on strawberries were compared by one-way ANOVA (Duncan's post hoc analysis) using a SAS software (university edition, SAS Institute Inc., USA).

5. RESULTS

5.1 Efficacy of initial wash treatment

Based on microbiological analysis the initial numbers of inoculated MS2 on strawberries were 6.6 log PFU/g. Fig. 5.1 shows the remaining MS2 population on strawberries after initial wash treatment. The three wash treatments were tap water, electrolyzed water and chlorine solution. All three treatments significantly ($p < 0.05$) reduced (approximately 1 log PFU/g) the number of MS2 on strawberries. The chlorine treatment provided the best effect on inactivating MS2 bacteriophage on strawberries, which was 1.2 log PFU/g. Electrolyzed water treatment resulted in 0.9 log PFU/g reduction, which was better than tap water treatment, but there was no statistical difference ($p < 0.05$).

The initial population of *E. coli* O157:H7 inoculated on strawberries was 6.0 log CFU/g and *E. coli* O26:H11 was 5.3 log CFU/g. Figure 5.2 shows the remaining STEC population on strawberries after the initial wash treatment. Two serotypes of STEC showed similar survival patterns on strawberries after wash treatment. The tap water treatment reduced the number of both *E. coli* O157:H7 and *E. coli* O26:H11 by 1.5 log CFU/g. Using water antimicrobials led to nearly an additional 1 log CFU/g reduction than using tap water alone. The maximum log reduction of *E. coli* O26:H11 achieved was 2.8 log CFU/g when using a chlorine solution.

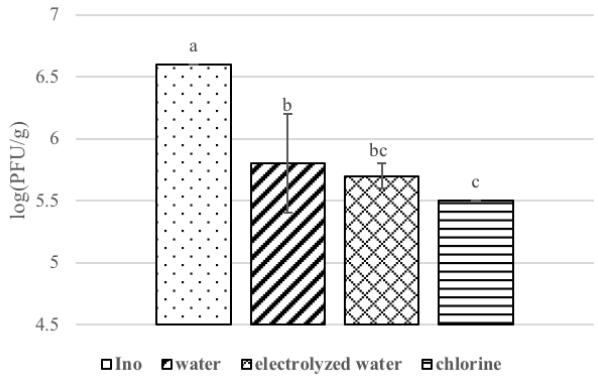


Fig. 5.1. Populations of MS2 on strawberries after inoculation and after washing with tap water, electrolyzed water or chlorine solution for 90 s; different letters indicate statistical difference ($p < 0.05$).

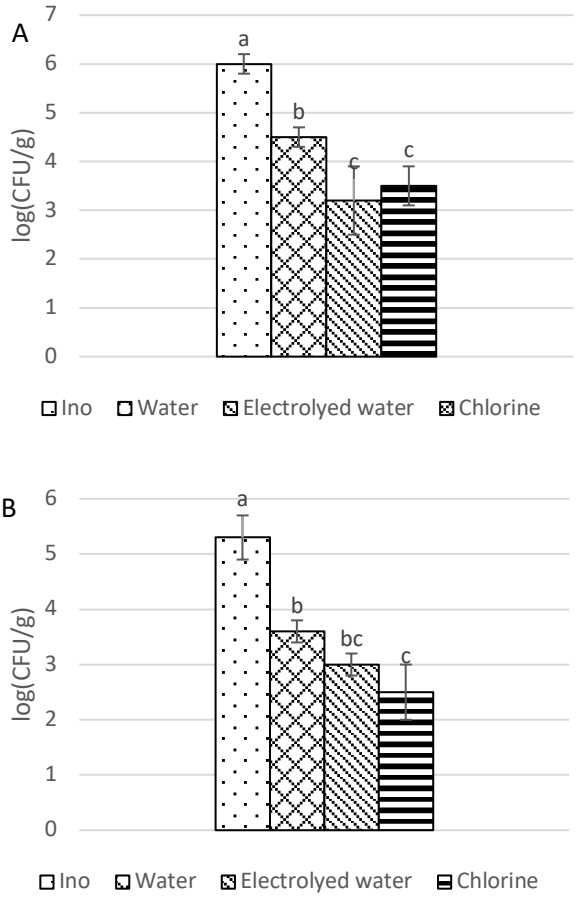


Fig. 5.2. Populations of *E. coli* O157:H7(A) and *E. coli* O26:H11(B) on strawberries after inoculation and after washing with tap water, electrolyzed water or chlorine solution

for 90 s; different letters indicate statistical difference ($p < 0.05$).

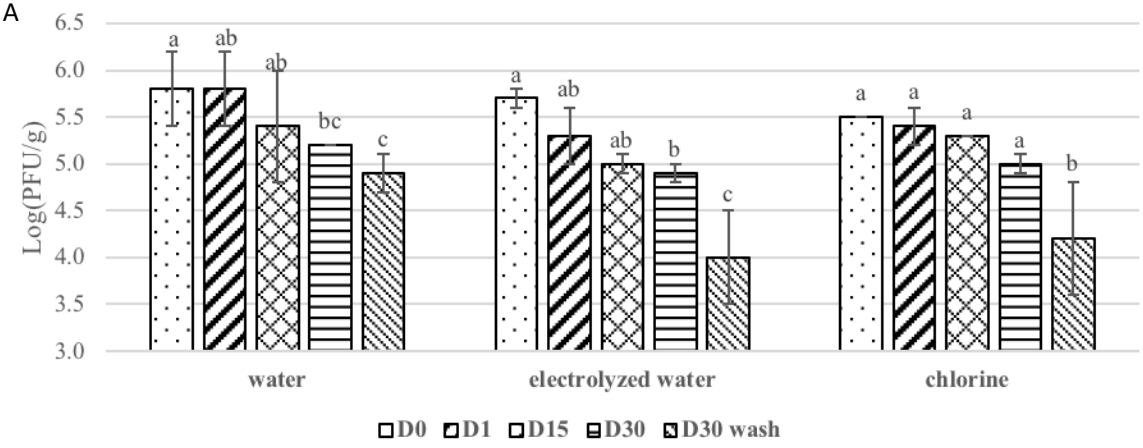
5.2 Effect of freezing and frozen storage

The freezing of strawberries at -80 °C or -20 °C had a similar effect on MS2 inactivation across treatment groups (Fig. 5.3). A 0.5 log PFU/g reduction occurred following 30 days of frozen storage. The process of freezing (D0 compared to D1) appeared to have little influence on MS2 activity. On day 30 frozen strawberries were either washed or not washed to determine the effect of an additional stress (frozen storage) on inactivation of MS2. Washing strawberries in water alone after frozen storage failed to significantly reduce MS2 associated with the berries compared to no washing. In contrast, washing frozen strawberries in electrolyzed water or 50 ppm chlorinated water achieved a significant reduction ($p < 0.05$) compared to no washing.

Freezing exhibited a significant effect on the inactivation of *E. coli* serotypes O157:H7 and O26:H11 (Fig. 5.4). On Day 1, the population of STEC decreased approximately 1 log CFU/g in all treatment groups. The electrolyzed water treatment provided the greatest reduction up to 3 log CFU/g. Treatment using chlorine solution and tap water appeared to have no noticeable difference on reducing STEC populations in association with freezing. There was no significant difference between the remaining population of the two serotypes of *E. coli* on strawberries evaluated under the same conditions. However, the process of freezing of strawberries at −20 °C resulted in an additional 1 log CFU/g reduction of *E. coli* O157:H7 across all treatment groups compared to freezing at −80 °C.

Prolonged frozen storage appeared to have a negative effect on survival of STEC. Strawberries were washed a second time on Day 30. Based on microbiological analysis 2.2 log CFU/g *E. coli* O157:H7 remained on strawberries stored at −80 °C and washed a second time using tap water while 1.4 log CFU/g remained for strawberries held at -20 °C and washed a second time. The population of STEC was below 1 log CFU/g for strawberries treated with electrolyzed water or chlorine following frozen storage. Overall, the greatest reduction in population of STEC occurred when the process of washing in electrolyzed water, freezing and storage at −80 °C was followed by a second wash in electrolyzed water. STEC population was below detection limit under this process, which led to a 5 log CFU/g reduction.

A



B

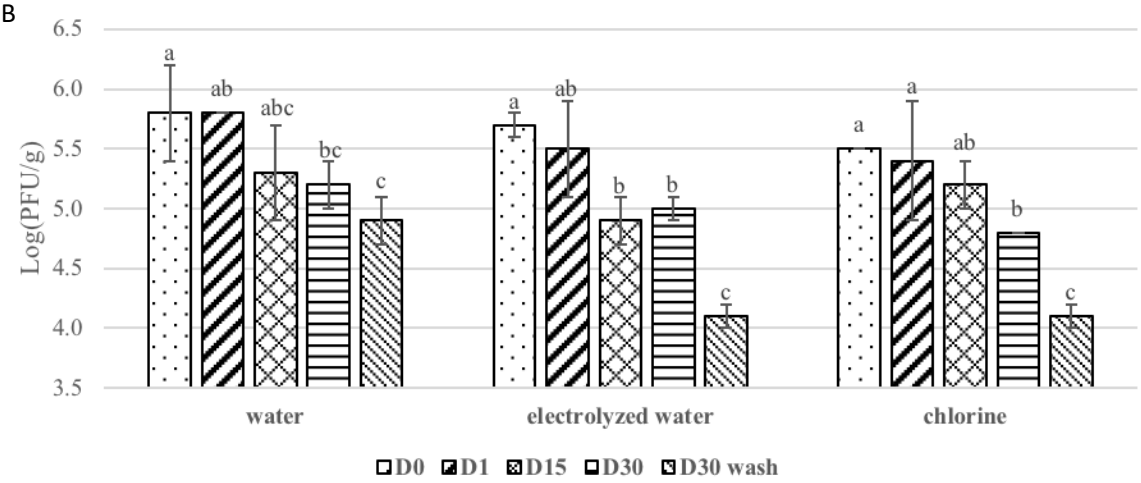
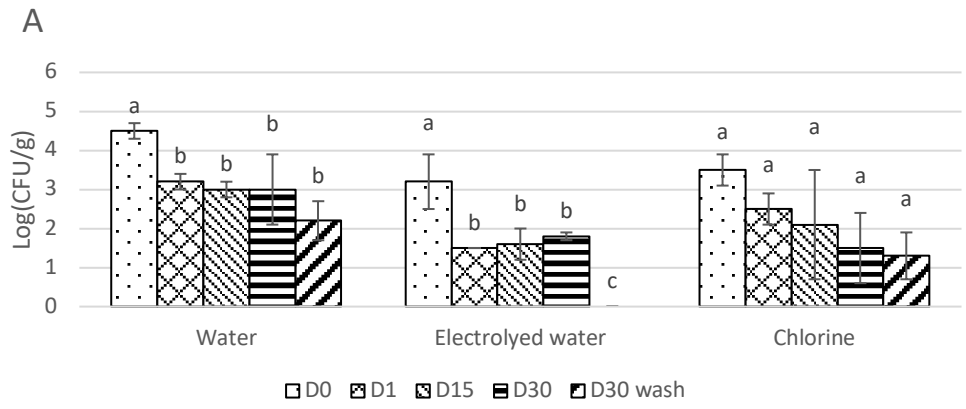
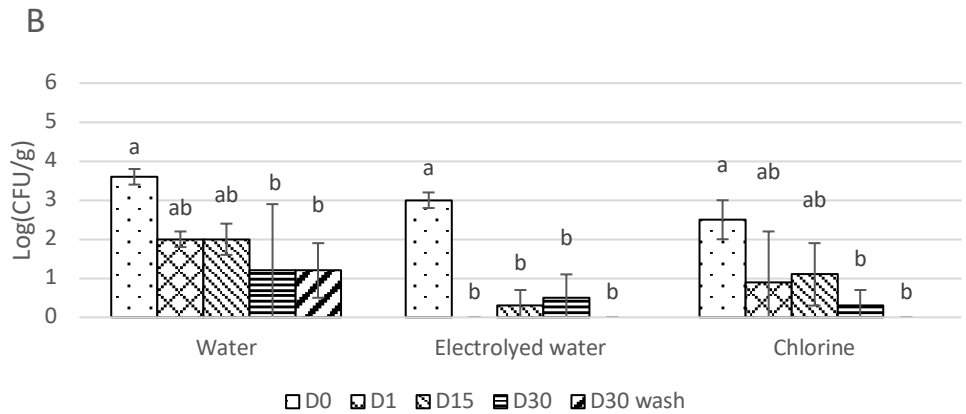


Fig. 5.3. Populations of MS2 phage on strawberries stored at -80°C (A) and -20°C (B). Different letters indicate significant differences within the same treatment group($P<0.05$).





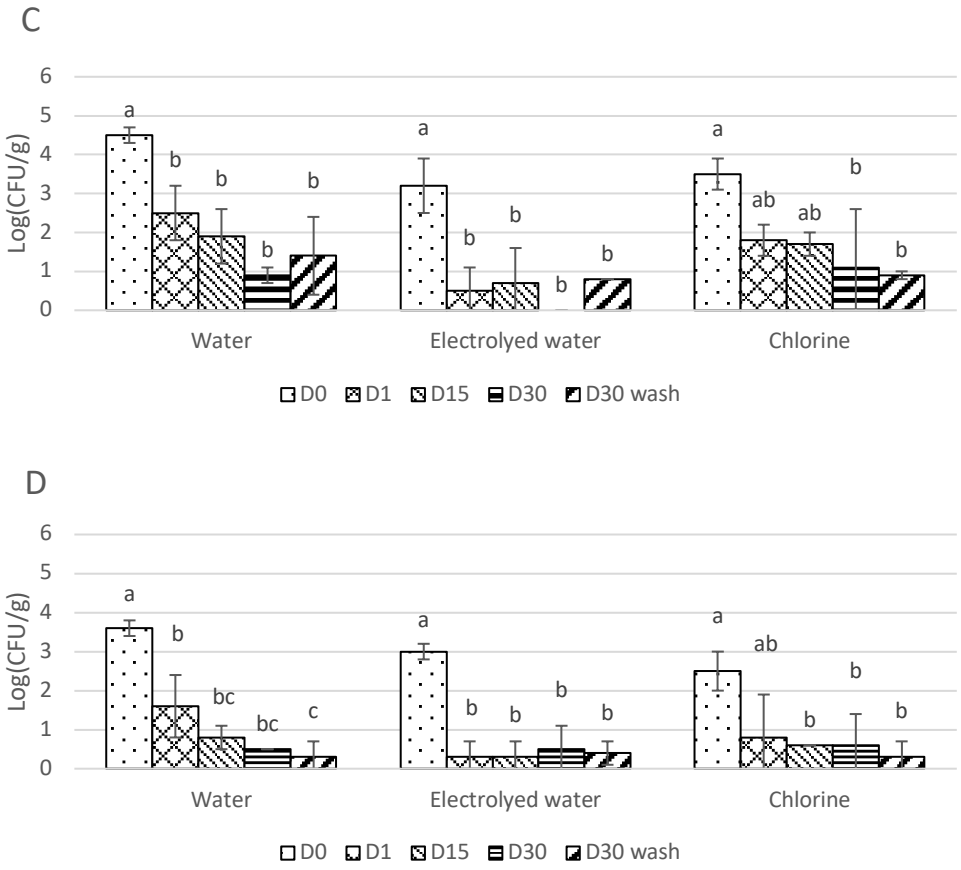


Fig. 5.4. Populations of *E. coli* O157:H7 on strawberries stored at -80°C (A); *E. coli* O26:H11 *at* -80°C (B); *E. coli* O157:H7 at -20°C (C); *E. coli* O26:H11 *at -20°C* (D). Different letters indicate significant differences within the same treatment group($P<0.05$).

5.3 Effect of refrigeration

After the initial washing, a subset of strawberries was placed in a refrigerator at 4 °C and stored for 2 days. Except for the chlorine treatment, there was an approximate 1 log PFU/g reduction of MS2 on Day 2 (Fig. 5.5). The second washing using water antimicrobials achieved a 0.5 log PFU/g reduction; level of MS2 on tap water treated berries remained constant. At the end of process, the population of MS2 for all three treatment groups of strawberries remained high (>4.5 log PFU/g).

The log CFU/g reduction of *E. coli* O157:H7 and O26:H11 on strawberries following washing in electrolyzed water on Day 2 was 2.6 and 1.2, respectively (Fig. 5.6). The chlorine and tap water treatments resulted in <1 log CFU/g reduction after 2 days storage. The second washing on Day 2 had little further influence in the reduction of STEC populations. The greatest number of STEC remained on strawberries washed with water alone followed by chlorine and then the electrolyzed water treatment.

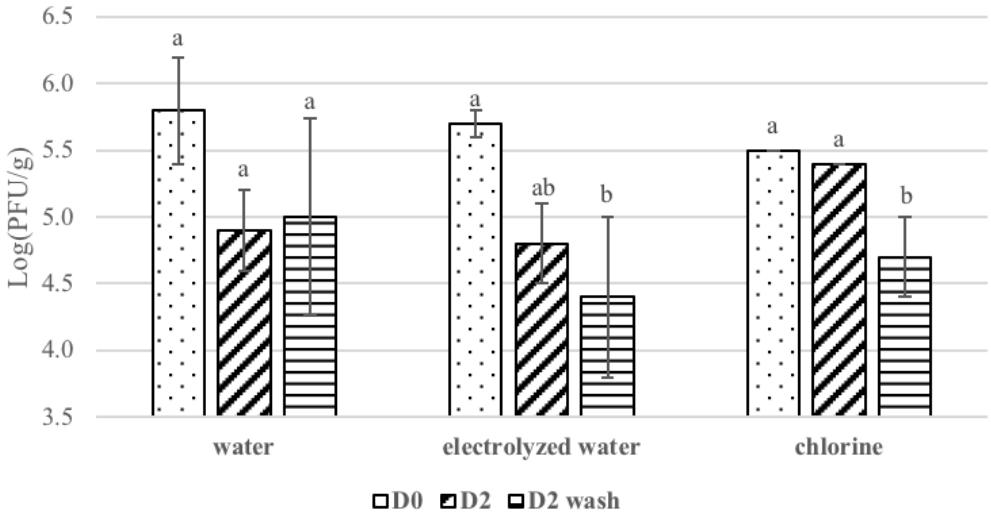
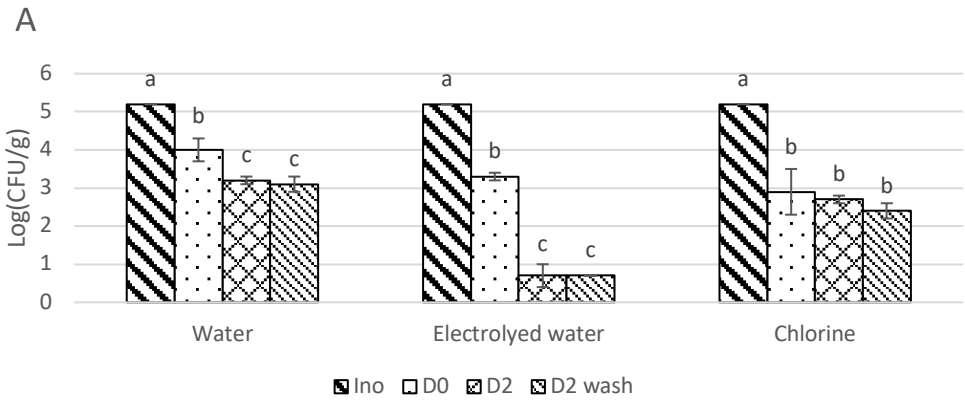


Fig. 5.5. Populations of MS2 on strawberries stored at 4 °C. Different letters indicate significant differences within the same treatment group(P<0.05).



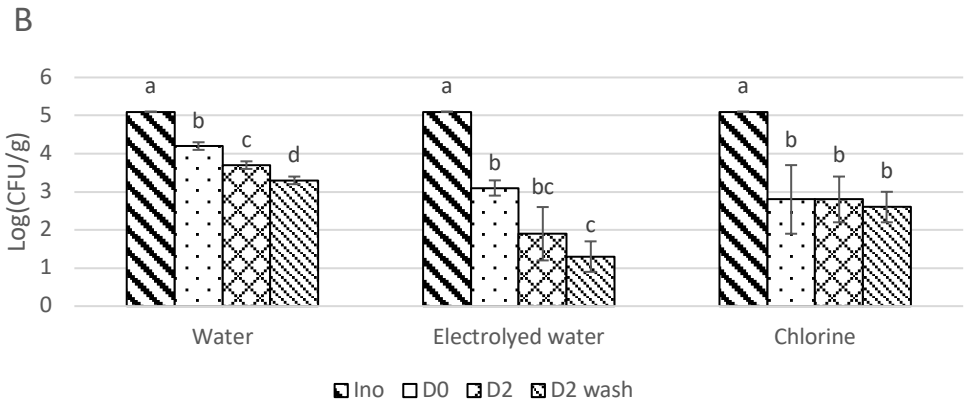


Fig. 5.6. Populations of *E. coli* O157:H7(A) and *E. coli* O26:H11(B) on strawberries stored at 4 °C; Different letters indicate significant differences within the same treatment group($P<0.05$).

6. DISCUSSION

The strawberry is a delicate and fragile fruit. Treatments including washing may damage the sensitive tissues of the strawberry and residual moisture may result in promotion of mold growth. There are numerous publications on novel methods to inactivate foodborne pathogens in strawberry puree, juice, or slices (Huang et al., 2013; Gurtler et al., 2011; Duan et al., 2009; Hsu et al., 2014). However, limited approaches are available to enhance the microbial safety of whole fresh and frozen strawberries during commercial, retail, and in-home processing and handling. For whole fresh or frozen strawberries, low temperature storage and washing prior to consumption/freezing are standard approaches to minimize microbial outgrowth on strawberries. During commercial preparation of frozen strawberries, water antimicrobials are typically used as a drench or spray prior to freezing. During home or retail processing only tap water may be used for washing. Guidance for in-home preparation of strawberries indicates they should be washed using running tap water (Huang et al., 2014; Parish et al., 2003). Although frozen strawberries are usually not washed prior to consumption, washing may enhance microbial safety especially when thawing is intended.

Chlorine is commonly used as a water antimicrobial by the food industry and it has been proven to be effective and economical (Beuchat, 1997). The usage of chlorine in fruits is usually in low free chlorine concentration ranging from 50 ppm to 200 ppm (Stopforth et al., 2008). According to FDA CFR title 21 part 173, the residual amount of

chlorine after washing cannot exceed 3 ppm and the fruits must be rinsed. Usage of higher chlorine concentrations (> 50 ppm) is not recommended. One study simulated the commercial washing of strawberries and found that even at 200 ppm the reduction of MS2 was limited to 1 log PFU/g (Casteel et al., 2009). Another study treated leafy vegetables (lettuce and cabbage) with 200 ppm chlorine resulted in a reduction of 2.9 log PFU/g on MS2 (Allwood et al., 2004). Others reported that using 50 ppm free chlorine to wash strawberries resulted in a 2 log PFU/g reduction of MS2, but only 1.3 log CFU/g reduction of *E. coli* O157:H7 (Lukasik et al., 2003). Dawson et al. (2005) found that 2 log PFU/g MS2 could be removed from strawberries using 100 ppm chlorine. In the present study, washing strawberries with 50 ppm free chlorine achieved an approximately 1 log PFU/g reduction on MS2 and over 2.5 log CFU/g reduction on *E. coli* O157:H7/O26:H11.

Electrolyzed water is a potential alternative to the use of chlorine. The present study is in agreement with previous studies demonstrating that electrolyzed water was equal to or better than chlorine in inactivating viruses and pathogens on strawberries. Hung et al. (2010) treated strawberries with electrolyzed water for 1 min and achieved a 1.17 log CFU/g reduction on *E. coli* O157:H7. In another study, *E. coli* and fungi on strawberries were reduced by 1.0 to 1.5 log per strawberry, respectively, after treatment with acidic electrolyzed water (Koseki et al., 2004). In the present study, compared with chlorine, electrolyzed water showed similar efficacy in inactivation of MS2 and *E. coli* O157:H7/O26:H11. One reason for similarity in efficacy may be that

free chlorine level (50 ppm) of these two solutions were the same. A previous study showed that the maximum inactivation of MS2 occurred at pH 6-8 and at 5-35 °C (Feng et al., 2003), so the neutral pH of electrolyzed water used in this study could possibly achieve better inactivation of MS2 than chlorine which was basic.

Freezing may have little impact on reducing population of active virus on berries (Baert et al., 2009). Similar results were reported by other studies on foodborne pathogens on frozen strawberries (Knudsen et al., 2001). Commercially prepared frozen strawberries are typically IQF processed to achieve best quality. The temperature of IQF depends on the cryogen used and could be -40 °C or even lower temperature (Modise et al., 2008). Home processed frozen berries are stored at -20 °C which is the typical temperature of a home freezer. The shelf life for frozen strawberries can be 12 to 24 months and viruses can remain active after extended periods of frozen storage. Research demonstrates that 25% of MS2 remained active in water after 290 days of storage at -80°C (Olson et al., 2004). Others reported that MS2 along with other virus surrogates for Norovirus or Hepatitis A virus were very stable on fresh produce under frozen temperature storage (Baert et al., 2009; Butot et al., 2008). Those results suggest that virus contaminated strawberries even after long-term low temperature storage may represent a potential human health risk. In this study, inactivation of MS2 was evaluated 24 h (D1) after freezing of the strawberries, which encompasses the influence of the freezing process. The population of MS2 on D1 remained nearly identical to D0 whether strawberries were frozen at -80 °C or -

20 °C. These results suggest that the process of freezing has minimal influence on inactivation of MS2 associated with strawberries. In contrast, there was a collective decrease in active MS2 associated with strawberries frozen for 30 d. The level of inactivation of MS2 was < 1 log PFU/g by frozen storage regardless of the wash treatment prior to freezing.

The influence that freezing has on STEC has been extensively reported, but the results are often conflicting. Knudsen et al. (2001) reported that the population of *E. coli* O157:H7 decreased only 0.7 log from a starting population of 7 log CFU per strawberry following storage at -20 °C for 30 days. In contrast, another study indicated there was a 3 log CFU/g reduction of *E. coli* O157:H7 on strawberries (inoculation level: 4.3 log CFU/g) after 3 days' storage at -20 °C (Yu et al., 2001). A possible explanation for these results is the difference between inoculation level and handling methods. Under conditions evaluated in this study, the population of *E. coli* serotypes O157:H7 and O26:H11 decreased dramatically from prior to freezing through the end of 30 days frozen storage. The remaining population of STEC associated with the two antimicrobial treatment groups were < 1 log CFU/g. Sample enrichment was not conducted to determine whether STEC were completely inactivated. However, STEC populations remained elevated on strawberries treated with water alone.

Refrigerated storage of fresh strawberries had only limited effect on reducing the population of MS2 and STEC. The findings of this study are consistent with previous studies suggesting bacterial growth is limited on strawberries held at refrigeration

temperature(Delbeke et al., 2015; Verhaelen et al., 2012). In this study, although experiments were not conducted to quantify mold growth during refrigerated storage, no mold growth was present based on visual examination. Limited anti-mold effect of electrolyzed water and chlorine on strawberries has been reported (Udompijitkul et al., 2007).

Most of the previous studies focused only on the influence of storage temperature and did not investigate the combined effect of temperature and with washing with or without a water antimicrobial. Exposure of the microorganism to multiple stresses has in the present study may have facilitated inactivation. Washing before storage (initial washing) resulted in significant reduction of MS2 and STEC regardless of treatment. Importantly, washing after storage (second washing) provided additional inactivation and may improve safety.

7. CONCLUSIONS

Under the conditions evaluated in the present study, washing strawberries using a water antimicrobial prior to and after storage can significantly reduce the population of MS2 and *E. coli* serotypes O157:H7 and O26:H11. Low temperature storage failed to inactivate MS2 but had a significant effect on reducing the population of STEC. The findings underscore the importance of using a water antimicrobial when preparing strawberries prior to consumption/storage particularly in retail food establishments. It is also recommended that running tap water be used to wash fresh/frozen strawberries in the home since commercial water antimicrobials are not available for in home use.

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