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Influence of water antimicrobials and storage conditions on inactivating MS2 bacteriophage on strawberries

Licheng Huang, Xin Luo, Jingwen Gao, Karl R. Matthews*

Department of Food Science, Rutgers University, New Brunswick, NJ 08901, USA

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ABSTRACT

Foodborne illnesses caused by norovirus contaminated fresh produce remain a food safety concern worldwide. In the present study, the impacts of commercial and home processing conditions of strawberries were evaluated for inactivation of the MS2 bacteriophage. MS2 was used as a surrogate of norovirus and was spot inoculated onto strawberries to achieve 6.6 log PFU/g. The inoculated strawberries were washed with tap water, electrolyzed water, or 50 ppm chlorine for 90 s prior to and after storage. After initial washing, the strawberries were separately stored at −20 °C and −80 °C for 30 days. Change in MS2 populations on strawberries was evaluated by plaque assay method on day 1, 15, and 30 for −20 °C and −80 °C groups. The results showed that washing strawberries prior to storage resulted in a significant decrease (approximately 1 log PFU/g) of MS2 population regardless of the treatment ($p < 0.05$). Frozen storage had minor effects on inactivating MS2, which resulted in approximately a 0.5 log PFU/g reduction at the end of storage. Washing frozen berries in electrolyzed water or 50 ppm chlorine on day 30 resulted in an additional 1 log PFU/g decrease in MS2 compared to water alone. These results suggest that washing strawberries with a chemical antimicrobial prior to and post frozen storage may enhance microbial safety.

1. Introduction

Noroviruses annually account for over 50% of foodborne illnesses in the world. Each year, millions of people suffer gastroenteritis the result of consuming food contaminated with viruses resulting in huge economic loss in part associated with medical costs and product recalls (USDA-NIFA Food Virology Collaborative NoroCORE, 2017). Strawberries, which usually do not undergo heat treatment or processing before serving, can serve as a vehicle of virus transmission from the farm to the fork. 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). Pick-your-own (PYO) farming operations continue to be a popular means for consumers to purchase fruits and vegetables that are at peak freshness. Infected customers at PYO operations and workers could potentially contaminate the strawberries during bare hand harvest. Irrigation water polluted with human feces harboring viruses could result in large scale contamination of crops, for example, strawberries (Palumbo et al., 2013). In 2012, a norovirus outbreak related to imported frozen strawberries from China affected over 11,000 people in Germany (Mäde et al., 2013). Since frozen strawberries usually have a long shelf-life and may be imported from other countries, it is often difficult to identify the actual source of contamination which may help prevent more illnesses from the same source.

Norovirus is a non-enveloped RNA virus which has the ability to persist for extended periods in extreme environments such as low temperature and low pH (Seymour and Appleton, 2001). The rough surface of strawberries and raspberries is conducive for viral particle attachment, permitting viruses to persist throughout the shelf-life of the product (Verhaelen et al., 2012).

Temperature control is the most common and critical way to ensure the safety and quality of strawberries. Washing or other processing is not encouraged until the fresh strawberry is ready to be served since moisture will encourage spoilage and shorten the shelf-life. Typically, retail food establishments and consumers do not wash frozen strawberries prior to use. During commercial processing strawberries are typically processed using water that contains an antimicrobial (e.g., chlorine, electrolyzed water) prior to undergoing Individual Quick Freezing [IQF] (Casteel et al., 2009; Huang et al., 2008). This may be of further importance depending on the source of water used by the food facility (Lee et al., 2018). Freezing fresh strawberries at home is a simple process; strawberries should be washed, not soaked, allowed to dry, placed in freezer containers or bags, and then placed into a freezer (−20 °C).

* Corresponding author.
E-mail address: matthews@sebs.rutgers.edu (K.R. Matthews).

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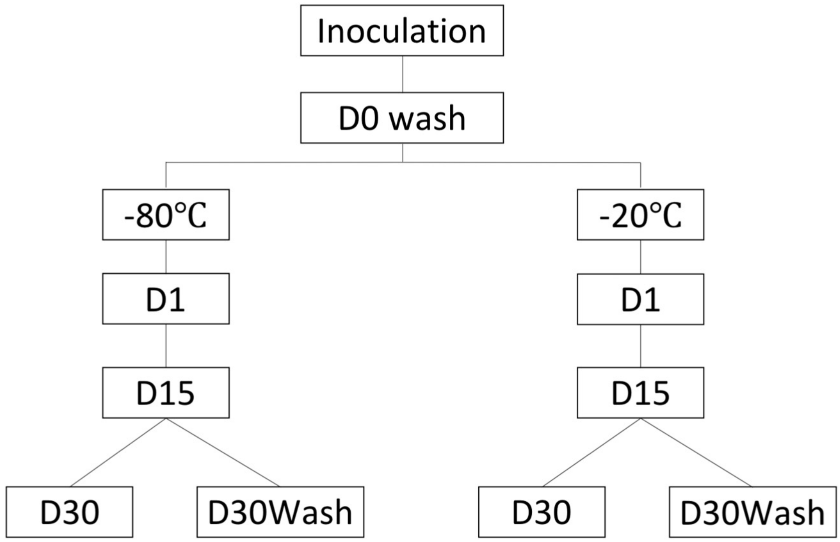

Fig. 1. Flow diagram of freezing and storage conditions.

Generally, the inactivation of viruses on fresh fruits and vegetables using antimicrobial agents alone is not highly successful (Baert et al., 2009). Chlorine is commonly used in processing water since it is inexpensive and effective in inactivating foodborne pathogens in the process water. Electrolyzed water has become a popular substitute for chlorine in water used for processing of fresh fruits and vegetables. Neutral electrolyzed water (NEW) has little off-odor and is stable near neutral pH which will do less damage to the equipment and food contact surfaces (Izumi, 1999). Multiple researchers have demonstrated the efficacy of NEW in reducing the number of pathogens including virus on various types of food (Gulati et al., 2001; Huang et al., 2008; Issa-Zacharia et al., 2011; Izumi, 1999; Kim et al., 2000).

It is difficult to culture human norovirus outside of the human body. In 2016, human norovirus was cultured on human intestinal epithelial cells successfully for the first time (Ettayebi et al., 2016); regardless the method is not well adopted. Bacteriophages such as MS2 are commonly used as a surrogate for foodborne viruses in research on water and food systems (Dawson et al., 2005). The response of MS2 to disinfectants and environmental stress, 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, the plaque assay and PCR are usually used, but the former assay requires no specialized equipment.

In the present study, MS2 was used as a surrogate for norovirus in evaluating the effects of washing and freezing strawberries on inactivation of the virus. The efficacy of electrolyzed water and 50 ppm chlorine in inactivating MS2 inoculated on strawberries were compared before and after thirty days of frozen storage. Two typical freezing temperatures (−80 °C and −20 °C) were used to equate to commercial and home preparation/storage of frozen strawberries, respectively.

2. Method

2.1. Bacteriophage and 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.

2.2. Inoculation of strawberries

Strawberries (average weight: 22 ± 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.

2.3. Washing and freezing

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, Sterilox™; free chlorine 50–55 ppm) or chlorine solution (50 ppm 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 s. 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. Strawberries were stored at two different temperatures: −80 °C and −20 °C. Fig. 1 is a schematic showing the processing of strawberries. Strawberries were separately placed in pans to avoid contact with other strawberries, which was similar to placement used for individually quick frozen (IQF) commercially. On day 30 (D30) six strawberries stored at −80 °C or − 20 °C were washed in water alone or the antimicrobial treatments.

2.4. Microbiological analysis

Whole strawberries (n = 3) were sampled to determine the initial population of MS2 on the strawberries as well as the MS2 population after each treatment and storage condition. Dey/Engley neutralizing broth (Criterion, Hardy Diagnostics, Santa Maria, CA) was used to neutralize any residual chlorine on the strawberries (1:5 dilution). Samples were homogenized for 1 min (easyMIX™, BioMerieux). An aliquot of 1 ml was collected and serially diluted in 0.1% peptone water (Difco, Becton Dickinson, Sparks, MD).

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.

The population of aerobic bacteria was determined using a direct plating method, aerobic plate count (APC). To maximize the detection of aerobic bacteria, 500 μl of each dilution was spread plated on the surface of a TSA plate and incubated overnight at 37 °C.

2.5. Statistical analysis

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

3. Results

3.1. Efficacy of initial wash treatment

Fig. 2 shows the initial population of inoculated MS2 and the remaining population on strawberries after first washing. Based on microbiological analysis the initial numbers of inoculated MS2 on strawberries were 6.6 log PFU/g. All three treatments significantly ($p < 0.05$) reduced (approximately 1 log) the number of MS2 on strawberries. Treatment with 50 ppm chlorine was the most effective, resulting in a log reduction of 1.2 ± 0.0 log PFU/g. Although treatment with electrolyzed water resulted in a 0.9 ± 0.1 log reduction in MS2, it was not significantly different from tap water or chlorine ($p > 0.05$).

3.2. Influence of freezing and frozen storage on MS2

The freezing of strawberries at −80 °C or −20 °C had a similar effect on MS2 inactivation across treatment groups (Fig. 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.

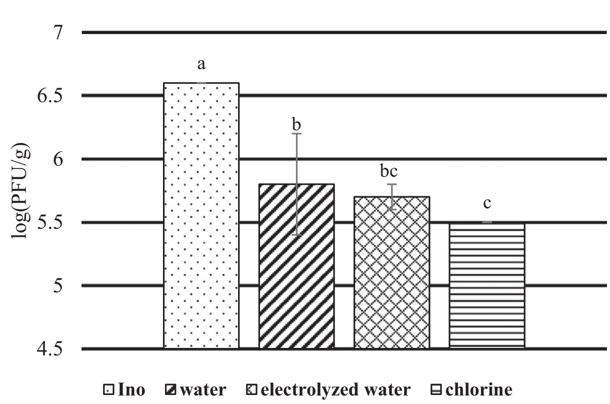

Fig. 2. Populations of MS2 phage on strawberries after inoculation and after washing with 24 l of tap water, electrolyzed water or chlorine solution for 90 s; different letters indicate statistical difference ($p < 0.05$).

3.3. Aerobic plate count

The population of aerobic bacteria on strawberries was < 3 log CFU/g prior to treatment. Regardless of treatment the population of aerobic bacteria on strawberries remained relatively constant (data not shown).

4. Discussion

Consumers and retail food establishments typically do not wash frozen strawberries prior to use in food preparation. Washing frozen strawberries in conjunction with thawing may influence the taste and flavor of the strawberries (Moraga et al., 2006). Regardless, washing frozen strawberries prior to consumption may enhance microbial safety of the strawberries. Commercially prepared frozen fruits and vegetables are typically washed with a water antimicrobial (e.g., chlorinated water, ≤50 ppm) as a drench or spray prior to freezing. 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 to1 log (Casteel et al., 2009). Another study treated leafy vegetables (lettuce and cabbage) with 200 ppm chlorine resulted in a reduction of 2.9 log on MS2 (Allwood et al., 2004). In the present study, washing strawberries with 50 ppm chlorine achieved a reduction of approximately 1 log PFU/g. During home processing, only tap water is used for washing. Guidance for in home preparation of strawberries intended for freezing typically indicates berries should be washed using running tap water prior to freezing (Harris and Mitcham, 2007).

Freezing may have little impact on reducing population of active virus on berries (Baert et al., 2009). Similar results were shown 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, 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). 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 (Fig. 3A and B). The level of inactivation of MS2 was < 1 log PFU/g regardless of the wash treatment prior to freezing.

This study differs from previous research addressing the behavior of MS2 on strawberries since the influence of washing with a chemical antimicrobial before and after freezing and different freezing temperatures was investigated. The combined effect of frozen storage and washing with a chemical antimicrobial was investigated to determine whether inactivation of MS2 would be enhanced. A maximum 2.5 log PFU/g decline in MS2 was achieved after exposure to a wash, freeze, 30 days' frozen storage, and second wash treatment. Using a chemical antimicrobial in wash water was beneficial in achieving greater inactivation of MS2 following 30 days of frozen storage, which

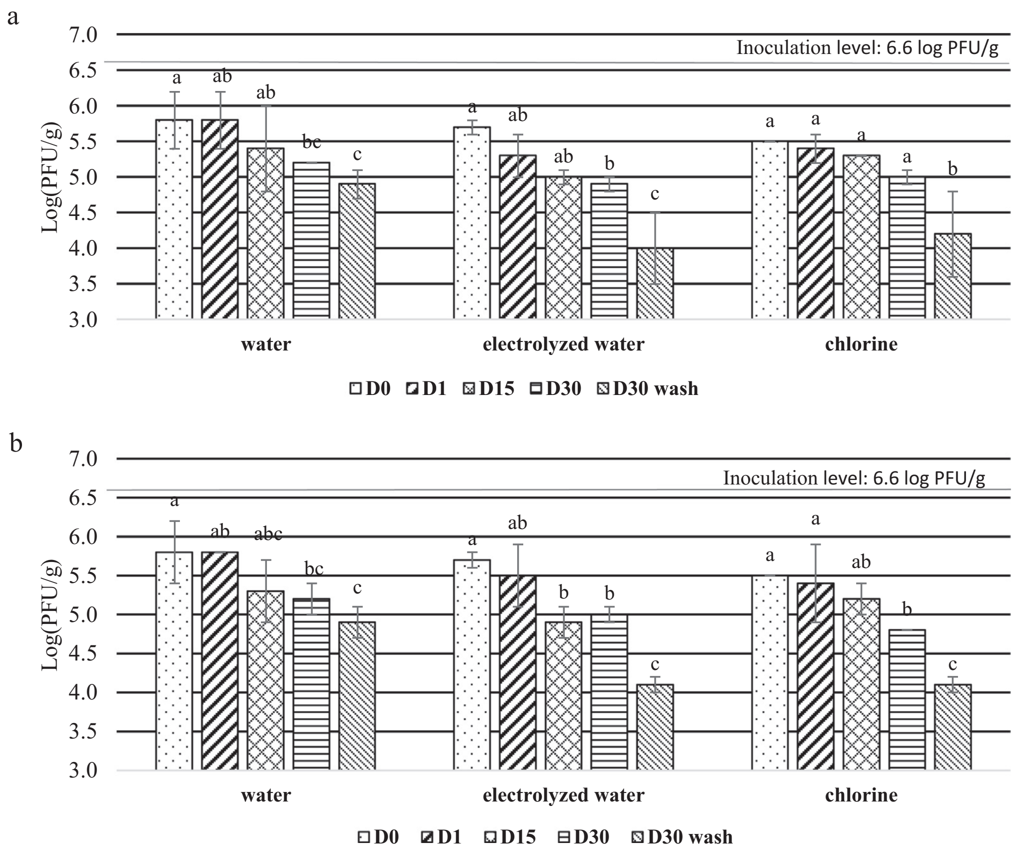

Fig. 3. Populations of MS2 phage on strawberries stored at −80 °C (a) and −20 °C (b). Different letters indicate significant differences within the same treatment group.

gave an additional 1 log PFU/g reduction of MS2 compared to no wash treatment after frozen storage (Fig. 3). Washing with tap water alone failed to achieve a significantly greater inactivation of MS2. Based on results of the present study, utilization of a chemical antimicrobial in the wash water may enhance the microbial safety of frozen strawberries (Zhou et al., 2017). Although under conditions evaluated in the present study tap water alone had a marginal effect in inactivation of MS2 on frozen strawberries; washing frozen strawberries under running water prior to consumption in the home or in food establishments is recommended since some reduction was achieved. Utilizing a chemical antimicrobial in-home processing may be difficult since such agents are often available to only commercial outlets such as restaurants and food vendors.

In this study, electrolyzed water and 50 ppm chlorine provided similar levels of MS2 inactivation. Both antimicrobials have high oxidation ability to inactivate pathogens including viruses (Girard et al., 2016). The treatments did not significantly reduce the population of commensal microbiota (≤3 log CFU/g) on strawberries. Ability of the treatments to control yeast and mold was not investigated. Different foodborne pathogens and viruses may show various survival patterns against disinfectants (Lukasik et al., 2003).

The sensitivity of norovirus could be different when present in various foods or aqueous solutions. The survival of MS2 in various aqueous solutions was evaluated; survival was better in buffer held at 4 °C than at −80 °C for 8 days. Interestingly, when held for a much longer time period the loss of active MS2 was less at −80 °C compared to 4 °C (Olson et al., 2004). That study also indicted the quality and type of buffer/water would influence the inactivation of MS2. Extrapolation of virus in water or buffered systems to a food system (e.g., strawberries) may not be relevant. For example, the attachment and viability of a norovirus surrogate differed for strawberries, raspberries, and blueberries (Palumbo et al., 2013). The inoculation of hepatitis A virus, norovirus, rotavirus, and feline calicivirus onto blueberries, raspberries, and strawberries, finding that freezing failed to significantly reduce the activity of any of the viruses, with the exception of feline calicivirus on strawberries which may be due to the low pH of strawberries (Butot et al., 2008). At room temperature, the population of murine norovirus declined rapidly on strawberries while remaining active on raspberries after several days of storage. When held at refrigerated temperature, there was no significant change of murine norovirus population on strawberries or raspberries (Verhaelen et al., 2012). Collectively, the results of those studies and the present study demonstrate the ability of viruses to persist on berries and in water systems. Novel processing techniques such as UV and HPP have been studied in recent years, which may provide more effective methods to inactivate viruses on fresh produce (Fino and Kniel, 2008; Kovač et al., 2012; Lacombe et al., 2017; Schultz et al., 2012). Unfortunately, those treatments may have limited utility; not being suitable for certain commodities or for small volume processing.

5. Conclusion

Under the conditions evaluated in the present study, washing strawberries using a water antimicrobial prior to and after frozen storage will significantly reduce the population of active viral particles (approx. 2.5 log PFU/g). These results underscore the importance of using a water antimicrobial when preparing strawberries for consumption particularly in retail food establishments. Consumers are limited in types of water antimicrobials that can be used in the home. In retail food operations this may be particularly important where frozen berries may be used in preparation of smoothies or milkshakes and other foods not intended to go through a thermal process.

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