RESEARCH NOTE

RUNNING TITLE

Survival of foodborne pathogens in deciduous fruit postharvest processing environments

TITLE

Effect of temperature and nutrient concentration on survival of foodborne pathogens in deciduous fruit processing environments for effective hygiene management

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KEYWORDS: Growth dynamics, foodborne pathogens, cross-contamination, hygiene management, fruit postharvest processing

Abstract

Temperature and good sanitation practices are important elements to control growth of microorganisms. Fresh produce is stored at various temperatures to ensure quality and prolong shelf-life. If foodborne pathogens are able to survive and grow at fresh produce storage temperatures, then further control strategies are needed to inactivate pathogens. The aim of this study was to determine how temperatures associated with deciduous fruit processing and storage facilities (0.5, 4 and 21°C) impact on the growth and/or survival of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Staphylococcus aureus* at different nutrient conditions (nutrient-rich and nutrient-poor) and on simulated contact surfaces (vinyl coupons). Information on the growth and survival of foodborne pathogens under specific deciduous processing and storage temperatures (0.5°C) is not known. All pathogens, except *E. coli* O157:H7 were able to survive on vinyl coupons under all temperature conditions. *Listeria monocytogenes* proliferated under both nutrient conditions independent of temperature. *Staphylococcus aureus* was shown to be least affected by nutrient conditions. The prospect of foodborne pathogen survival on vinyl coupons, a model system to study surfaces in fruit preparation and storage environments, indicates the potential for cross-contamination of deciduous fruit products under poor sanitation conditions. Foodborne pathogens that are able to proliferate and survive at different temperatures under different nutrient conditions could lead to fruit cross-contamination. In addition, temperature mismanagement which would allow pathogen proliferation in contaminated packhouses and storage environments is a potential concern. Therefore, proper hygiene and sanitation practices, removal of possible contaminants, and proper food safety management systems need to be in place to ensure food safety assurance.

There is a global increase in the number of foodborne diseases associated with various kinds of food products. Fruit and vegetables, mainly consumed raw, are an important source of foodborne pathogens which historically lead to large outbreaks (*23*). Deciduous fruit, specifically, accounted for 20 outbreaks in the United States of America (USA) alone from 1998 to 2014 resulting in eight deaths (*8*). Deciduous fruit can become contaminated during the pre- or post- harvest stage of fruit production. Sources include handling and contact surfaces within packing, processing and storage facilities (*10, 13*) that can harbour microorganisms (*16*) and allow transferal to food (*18, 20*) with possible survival which can potentially lead to product cross-contamination. Deciduous fruit packing and storage facilities do have the potential to harbour foodborne pathogens if sanitation programmes are not up to standard (unpublished data). Foodborne bacterial pathogens do not require a constant high nutrient source to survive for extended periods of time (*20*). This may lead to build-up of pathogen concentrations in food or storage facilities and contact surfaces (*1*). Foodborne pathogens have been shown to adhere to deciduous fruit (*11*), food contact surfaces (*12, 14, 22*) as well as form biofilms on different types of inert surfaces (*12, 14, 22*). However, if hazardous control points and sanitation systems are adequately implemented, the presence and survival of foodborne pathogens could be minimized.

The most effective hurdle technology approach is correct storage temperature combined with effective sanitation and proper handling practices. Storage temperatures differ depending on the commodity type and the commodity-specific processing or packaging systems being employed. Understanding how foodborne pathogens react under different temperature in which fruit are exposed and different nutrient conditions that can occur due to fruit residues that could be present on fruit contact surfaces is key to effective food safety management systems. Deciduous fruit processing occurs at 21°C, with short term fruit holding at the packhouse, processing facility, retail and the consumers home occurring at 4°C and are commercially stored

prior to distribution at 0.5°C for extended periods of time. Foodborne pathogens attached to deciduous fruit are able to survive throughout the distribution network at different temperatures (*11*).

The aim of this study was to determine how temperature and nutrient conditions affect the growth of a group of important human-health relevant foodborne pathogens including *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* under simulated fruit packing, storage and export conditions in order to assess the potential impact within the fruit environment and supply chain. To the authors' knowledge, although growth and survival of these pathogens under refrigeration and room temperature has been extensively studied (*3*, *4*, *5*, *6*, *7*, *21*) understanding how *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium and *S. aureus* grow and survive under specific commodity associated conditions like 0.5°C is key to understanding the cross-contamination potential of the facility.

Material and Methods

Cultures. American Type Culture Collection cultures were used (*E. coli* O157:H7 ATCC 35150, *L. monocytogenes* ATCC 19115, *S.* Typhimurium ATCC 14028 and *S.* a*ureus* ATCC 12600) were used and maintained lyophilized at -70°C with subcultures on standard 1 nutrient agar (Merck, Johannesburg, South Africa) prepared 24h prior to use. Cultures were used to inoculate five replicates of 100 ml tryptone soy broth (TSB) (Merck, South Africa) for each pathogen and subsequently incubated at 37°C for 18h to achieve 8 $\log_{10}$ cfu/ml. Cultures were centrifuged at 6500 *g* and washed twice with sterile distilled water and finally re-suspended into 1% (w/v) peptone buffered water (PBW). Cultures were serially diluted to obtain a concentration of 6 $\log_{10}$ cfu/ml, which was used to inoculate the nutrient-rich broth (NRB) and nutrient-poor broths (NPB) to achieve a 3 $\log_{10}$ cfu/ml final concentration. In addition, cultures that were diluted to 5 $\log_{10}$ cfu/ml were used to inoculate a 12.25 cm^2 vinyl coupon to achieve

a final concentration of 3 $\log_{10}$ cfu/coupon to determine the survival of each pathogen on a nutrient-free surface. Concentrations were enumerated by serial dilution and subsequent manual plating in duplicate onto Baird-Parker medium for *S. aureus*, Oxford *Listeria* Selective Agar for *L. monocytogenes*, Levine Eosine-Methyl Blue Agar for *E. coli* O157:H7 and Xylose Lysine Deoxycholate Agar (all supplied by Merck, South Africa) for *S.* Typhimurium.

Growth of foodborne pathogens under nutrient -rich and -poor conditions. Forty-eight sterile 100 ml TSB (1.5 g peptone and 0.5 g sodium chloride per 100 ml) representing NRB and 48 sterile 0.3% (w/v) TSB (0.15 g peptone and 0.05 g sodium chloride per 100 ml) representing NPB, due to a ten-fold reduction in nutrient content, were both divided into four sets of twelve. Three sets of broth were individually inoculated with 500 µl of 6 $\log_{10}$ cfu/ml of each pathogen resulting in 3 $\log_{10}$ cfu/ml, which was confirmed. Each set was subsequently incubated at 0.5°C, 4°C, 21°C for six days (representing weekly intensive cleaning), with daily agitation at 230 rpm for 5 minutes. Daily, 1 ml of the solution was removed from all 48 broths and serially diluted with subsequent plating onto selective media as previously stated. Following 24h incubation, counts were recorded and transformed to $\log_{10}$ (x+1) cfu/ml.

Growth of foodborne pathogens on nutrient-free vinyl coupons. Vinyl coupons of 3.5 x 3.5 cm were dip sterilized in 70% ethanol for 1 min and subsequently allowed to air-dry in the laminar flow cabinet. Sixty-three vinyl coupons were subsequently spot inoculated with 50 µl of 5 $\log_{10}$ cfu/ml of each culture. Spot inoculation was carried out to ensure that cultures did not mix. Coupons were air dried in a laminar flow for 15 minutes. Subsequent to drying the concentration per coupon of each pathogen was enumerated by serial dilution and plating and confirmed to be 3 $\log_{10}$ cfu/coupon. The 63 coupons were divided into three sets according to temperature for incubation, placed into sterile storage containers and stored at 0.5°C, 4°C and 21°C for six days, representing weekly intensive cleaning. Three coupons per day per temperature were removed from each storage temperature and placed into sterile 100 ml

containers. Five grams of sterile 0.55 µm glass beads with 1 ml 0.1% PBW was added to the 12.25 cm^2 vinyl coupon in a sterile container and subsequently shaken at 5 kHz for 10 min. Subsequently, 8 ml of 0.1% PBW was further added to the container in which the coupon was shaken. The solution was then further diluted as necessary and plated onto selective media as described previously. Following 24 h incubation, counts were recorded and transformed to $\log_{10}$ (x+1) cfu/coupon.

Statistical Analysis. Each experiment (NRB, NPB and nutrient-free) for each pathogen was done with five replicates and all experiments were repeated once. Results obtained for each experiment were analyzed together per pathogen (n=10). Statistical analysis was performed on log values. Data were analysed using SAS 9.2 for Windows (SAS Institute Inc., Cary, USA). A one-way analysis of variance was used to determine the difference in pathogen counts. Means were analysed using the least significant difference (using the Fisher's Exact Test) at a 5% level of significance. Polynomial curves were fitted to all graphs and the curve's equations were displayed on the figures.

Results

Growth dynamics of foodborne pathogens at constant temperatures. When *E. coli* O157:H7 was incubated at 0.5°C, the highest concentration of the six-day trial was observed on Day 0, in both the NRB and NPB followed by an overall decrease in concentration from Day 0 to Day 6 (P<0.001) (Fig. 1A and 2A). In NPB *E. coli* O157:H7 decreased immediately following one day at 0.5°C, whereas in NRB a non-significant increase was seen (Fig. 1A and 2A). Under refrigeration (4°C) NRB conditions (Fig. 1A), *E. coli* O157:H7 was able to significantly increase in concentration but not under refrigeration NPB conditions (Fig. 2A). As expected *E. coli* O157:H7 was able to grow in both nutrient conditions at 21°C (Fig. 1A).

Listeria monocytogenes concentration demonstrated an overall increase at all constant temperatures under NRB conditions, but it occurred faster at 21°C, then at 0.5°C and 4°C (Fig. 1B). *Listeria monocytogenes* concentrations increased at 21°C and 4°C but were only maintained throughout the six-day period when under NPB growth conditions at 0.5°C and (*P<0.0001)* (Fig. 2B).

Salmonella Typhimurium concentrations under NRB conditions at a constant temperature of 0.5°C did not significantly differ throughout the study to the concentration upon inoculation (*P=0.9525),* however under NPB conditions there was a systematic decrease in pathogen concentration (*P=0.0005*) (Fig. 1C and 2C). At refrigeration (4°C) and room temperature (21°C) under NRB conditions there was a significant increase in concentration (Fig. 1C), but a systematic decrease under NPB conditions (Fig. 2C).

At 21°C, *S. aureus* concentrations both in the NRB and NPB broth all increased systematically (Fig. 1D and 2D); although in the NPB the increase to the highest concentration took more time. At refrigeration temperatures under NPB conditions and 0.5°C under NRB and NPB conditions there was an overall decrease in concentration (Fig. 1D and 2D). Under NRB refrigeration conditions there was no significant difference from Day 0 to Day 6 (*P=0.0153)* (Fig. 1D).

Growth dynamics of foodborne pathogens on vinyl coupons at constant temperatures. On the nutrient-free vinyl coupons all pathogens showed decreasing growth trends (Fig. 3). *Escherichia coli* O157:H7 incubated on vinyl coupons at 0.5°C, 4°C and 21°C did not demonstrate a significant increase or decrease (*P>0.5*) (Fig. 3). At 0.5°C, 4°C and 21°C *L. monocytogenes* concentrations recorded were the highest on Day 0 and then concentration systematically and significantly decreased until completion (*P<0.0001*) (Fig. 3).

Salmonella Typhimurium concentrations decreased when incubated at all three temperatures on the vinyl coupon but were still present following six days at all three temperatures, therefore

demonstrating the ability of the organism to survive even with the lack of nutrients (Fig. 3). Concentrations on Day 0 were the highest for all three temperatures (Fig. 3). *Salmonella* Typhimurium concentrations were maintained at the same level as Day 0 until Day 2 when incubated at 0.5°C (*P<0.0001*) or 21°C (*P=0.0011*) (Fig. 3). Thereafter concentrations decreased significantly when incubated at 0.5°C (*P<0.0001*) or 21°C (*P=0.0011*) (Fig. 3). At 4°C, *S.* Typhimurium concentrations decreased significantly on Day 1 when compared to Day 0, thereafter concentrations were maintained until Day 6 (*P<0.0001*) (Fig. 3).

Interestingly, *S. aureus* concentration were the most unaffected by the nutrient-free condition when at 0.5°C and 21°C, but not at 4°C. An overall decrease in *S. aureus* concentrations was observed on a nutrient-free vinyl coupon at all three temperatures and *S. aureus* was still detected on Day 6 from all three temperatures. When incubated at 0.5°C the smallest decrease in concentration was recorded for all pathogens under this temperature conditions (Fig. 3). A significant decrease in *S. aureus* concentration was observed following Day 1 at 4°C incubation, followed by a systematic and significant decrease to Day 6 (Fig. 3). At 21°C, *S. aureus* concentrations on the nutrient-free vinyl coupons followed a systematic decrease with the highest concentration recorded on Day 0 and lowest on Day 6 with an overall decrease of 60% (*P<0.0001)* (Fig. 3).

Discussion

Contamination with *S. aureus* is often a result of human contact as it is a resident microorganism of 50% of humans' noses, throats and skins (*9*). *Staphylococcus aureus* was able to survive up to six days at 0.5°C, 4°C and 21°C on a vinyl surface. Survival on dry plastic surfaces is possible up to 1097 days (*3*) due to the organism being especially tolerant to desiccation. Gram positive organisms are more robust and hardy when compared to Gram negative organisms (*19*) and are able to enter into a dormant state (*9*). Whiting *et al.* (*25*), demonstrated that following

approximately 100 days at 4°C, *S. aureus* was still present at 10% of the initial inoculum concentration ($10^{9.5}$ cfu/ml). Jackson *et al*. (*17*) found that *S. aureus* was the organism most isolated from domestic refrigerators tested (6.3%). Survival on a surface within a packing, processing or cold storage facility for an extended period of time allows for inoculum build up and possible cross-contamination. In addition, if there is a source of nutrients and/or an increase in water activity as a result of fruit juice or residues from damaged fruit during processing or packaging on the contact surface, proliferation of the organisms could result, leading to an increased risk of contamination (*20*).

Desai *et al*. (*15*) showed that *S. aureus* was able to transmit to skin up to 63 days post inoculation from a vinyl surface. This organism is also readily transferred from surfaces to food (without pressure) at a rate of 74% (*20*). Therefore, if *S. aureus* was present on a food contact surface it is possible that this could lead to cross-contamination of hands and/or food products. Under NRB conditions, concentrations of *S. aureus* can increase at 4°C, however not under NPB conditions. Under the nutrient-poorer fresh produce conditions like that of minimally processed lettuce, perilla leaf and sprouts artificially contaminated with *S. aureus* Tian *et al.* (*23*) demonstrated no significant change in concentration when stored at 4°C. On minimally processed vegetables, nutrient-rich and -poor conditions, *S. aureus* was able to proliferate at refrigeration temperatures. Therefore, demonstrating that under mild to extreme temperature abuse conditions, *S. aureus* could proliferate to a high enough concentration to produce toxins in food.

The recent *L. monocytogenes* outbreak associated with caramel apples in the USA was traced back to the apple packing facility (*23*). *Listeria monocytogenes*, a psychrotroph, is able to grow from 2°C and can survive freezing conditions (-18°C). As expected, *L. monocytogenes* was able to proliferate under all nutrient and temperature conditions, except on the vinyl surfaces. It was found that *L. monocytogenes* was the only one of the four foodborne pathogens tested in

this study that were able to exhibit growth at 0.5°C throughout the study period, and only under a high nutrient condition. Tienungoon *et al.* (*24*) demonstrated that *L. monocytogenes* was able to grow at 4°C. In addition, Jackson *et al.* (*17*) found that *L. monocytogenes* was present in 1.2% of all refrigerators investigated. The main hurdle technology for fresh and processed foods is the use of refrigeration temperatures, which in the case of *L. monocytogenes* would not be effective. In order to avoid health concerns for consumers it is essential to prevent the presence of this organism on all contact surfaces and on produce, as presence of a low concentration on a contact surface or food product can lead to cross-contamination and eventual proliferation even if the cold chain is effectively managed.

At ultra-low temperatures (0.5°C) *E. coli* O157:H7 showed a concentration decrease between 5 and 6 $\log_{10}$ cfu/ml, although survival was possible. Survival on the nutrient-free vinyl surface by *E. coli* O157:H7 was not possible following six days at 4°C and 21°C. However, survival was possible at 0.5°C and therefore *E. coli* O157:H7 present could be transferred in a potential cross-contamination event. Jensen *et al.* (*18*) demonstrated that *E. coli* O157:H7 can be transferred from contact surfaces to fresh produce (*18*) and meat (*21*) at different rates. Therefore, the presence of these pathogens on food preparation surfaces could lead to the contamination of food. This in turn could lead to the proliferation or maintenance of the pathogen, thereby implying a potential health hazard especially if temperature abuse takes place at any point through the supply chain.

Salmonella spp. also demonstrated the ability to be transferred from different surfaces to fresh produce at different rates (*18*). *Salmonella* Typhimurium on vinyl coupon surfaces were able to survive for the six-day period. *Salmonella* spp. were shown to survive up to 100 h following inoculation with a high inoculum concentration (*20*). Cross-contamination with *S.* Enteritidis from an inoculated stainless steel surface to cucumber and roast chicken fillet slices took place at a 50 to 65% and 32 to 49% transfer rates, respectively (*20*). Therefore, if *S.* Typhimurium is

present on a food preparation surface it poses a potential cross-contamination situation of food products coming into contact with the surface. The ability of *S.* Typhimurium to proliferate under NRB conditions at constant (4°C and 21°C) temperatures indicates a level of potential to compromise product integrity under conditions of temperature mismanagement. During 2012, *Salmonella* spp. was the most important bacterial pathogen causing the most foodborne disease outbreaks, mainly being associated with fruit, fish and chicken (*2*).

In deciduous storage facilities at 0.5°C, surfaces should remain clean, residue and waste free due to effective facility sanitation, much like the vinyl coupons with no nutrients present. In this scenario, none of the pathogens are able to proliferate. Therefore, if cross-contamination occurs on a contact surface, temperature will limit the growth of all four pathogens if the surface remains nutrient-free. If the scenario changes and food residues are found to build up on contact surfaces, then a high nutrient (NRB or NPB) scenario is created. Our study demonstrates that *L. monocytogenes* is able to grow at 0.5°C irrespective of the concentration of nutrients present. When nutrients are optimum *S.* Typhimurium and *S. aureus* are able to survive at 0.5°C, but the pathogen concentrations decrease if the nutrients become limited. *Escherichia coli* O157:H7 concentrations decrease at 0.5°C irrespective of the nutrient concentration available. This emphasizes the importance of facility sanitation and ensuring that all contact surfaces are clean.

In conclusion, all four pathogens could persist on the test surfaces used to simulate typical export storage and distribution conditions. The fact that these organisms can persist under these conditions implies that cross-contamination of fruit has the potential to occur, and that certain foodborne pathogens can survive and even proliferate when temperature abuse or mismanagement takes place. It is therefore important to ensure that these pathogens do not become a resident on any surface within the packing, processing or storage environments. This study therefore shows the importance of proper food safety management programs which include adequate sanitation systems. Future research should focus on different food surface

contact materials and transfer potential within the distribution and storage environments. In addition, this study did not take relative humidity or properties of potentially nutrients on the contact surfaces into account, this is a limitation of the study and needs to be addressed with future studies.

Acknowledgements

This research was made possible by the funding and support of the Water Research Commission of South Africa solicited project co-funded by the Department of Agriculture, Forestry and Fisheries "*An investigation into the link between water quality and microbiological safety of fruit and vegetables from the farming to the processing stages of production and marketing"* (WRC Project No K5/1875/4). The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF. Dr S. Duvenage would like to acknowledge the NRF/DST Centre of Excellence for financial assistance. The authors would like to acknowledge Mr F.J. Duvenage for laboratory assistance and Mrs A. Lombard for editorial support.

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Figure Legends

FIGURE 1: Survival of *Escherichia coli* O157:H7 (A), *Listeria monocytogenes* (B), *Salmonella* Typhimurium (C) and *Staphylococcus aureus* under varying temperature conditions in nutrient -rich broths.

Data points with the same letter represent no significant difference between values within the temperature range per pathogen at the 0.05 significance level. Error bars represent the standard deviation. Polynomial curves were fitted to all graphs and illustrated with dotted lines, with equations for each displayed.

FIGURE 2: Survival of *Escherichia coli* O157:H7 (A), *Listeria monocytogenes* (B), *Salmonella* Typhimurium (C) and *Staphylococcus aureus* under varying temperature conditions in nutrient -poor broths.

Data points with the same letter represent no significant difference between values within the temperature range per pathogen at the 0.05 significance level. Error bars represent the standard deviation. Polynomial curves were fitted to all graphs and illustrated with dotted lines, with equations for each displayed.

FIGURE 3: Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* Typhimurium and *Staphylococcus aureus* under varying temperature conditions on nutrient-free vinyl coupons.

Data points with the same letter represent no significant difference between values within the temperature range per pathogen at the 0.05 significance level. Error bars represent the standard

deviation. Polynomial curves were fitted to all graphs and illustrated with dotted lines, with equations for each displayed.