International Journal of Food Microbiology 257 (2017) 176–182

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Inactivation of viruses and bacteria on strawberries using a levulinic acid plus sodium dodecyl sulfate based sanitizer, taking sensorial and chemical food safety aspects into account

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ARTICLE INFO

Keywords:
Human norovirus
Hepatitis A virus
Salmonella
E. coli
Inactivation
Decontamination
Sanitizer
Chemical residue
Fruits

ABSTRACT

The efficacy of levulinic acid (LVA) in combination with sodium dodecyl sulfate (SDS) in removal of foodborne viruses, enteric bacterial pathogens and their surrogates on fresh strawberries was investigated. Inoculated strawberries were treated with potable water, sodium hypochlorite solution (50 ppm), 0.5% LVA plus 0.5% SDS solution, and 5% LVA plus 2% SDS solution respectively for 2 min, followed by spray-rinsing with potable water. Water washing removed at least 1.0-log of the tested viral and bacterial strains from the strawberries' surfaces. The 50 ppm chlorine wash induced 3.4, 1.5 and 2.1-log reductions for hepatitis A virus (HAV), murine norovirus-1 (MNV-1) and MS2 bacteriophage, respectively. In comparison, the tested bacterial strains showed uniform reductions around 1.6-log CFU/ml. The 0.5% LVA plus 0.5% SDS wash induced 2.7, 1.4 and 2.4-log reductions for HAV, MNV-1 and MS2, which were comparable with the reductions induced by chlorine ($P > 0.05$). For bacteria, over 2.0-log reductions were obtained for *Enterococcus faecium, Listeria monocytogenes* and *Salmonella*, while *Escherichia coli* O157:H7 and *Escherichia coli* P1 showed reductions of 1.9 and 1.8-log CFU/ml. Higher concentration of LVA plus SDS showed no significantly higher reductions ($P > 0.05$). Sensory tests of washed strawberries and chemical residue analysis of LVA on strawberries after washing were also performed. In conclusion, this study demonstrates good performance of 0.5% LVA plus 0.5% SDS to reduce the levels of enteric pathogens if present on strawberries without altering taste and introducing chemical safety issues.

1. Introduction

Foodborne diseases are a major concern to global health, and estimated to cause approximately 600 million cases each year and 420,000 associated deaths (WHO, 2015). Foodborne viruses, human norovirus (HuNoV) and hepatitis A virus (HAV), are recognized as the most important foodborne pathogens regarding the number of outbreaks and people affected (Koopmans and Duizer, 2004). Viruses are small microorganisms that cannot replicate but survive well and remain infectious in the environment (Seymour and Appleton, 2001). Foodborne outbreaks associated with viruses are particularly related with minimally processed food, which have no inactivation steps during manufacture to control the overall microbial safety (Sivapalasingam et al., 2004). Strawberries present many specific nutritional characteristics and are harvested throughout the fruiting season usually by manual picking (Delbeke et al., 2015). After minimal processing (including washing, freezing or freeze-drying), they are often dispatched into the global market and used as ingredients in cereal, smoothie and ready-to-eat cakes (Hjertqvist et al., 2006). Thus, once the berries are contaminated, multi-state and cross country outbreaks can easily happen from one single contamination source. HAV and HuNoV outbreaks associated with soft fruits have been reported in the past years (Cotterelle et al., 2005; Guzman-Herrador et al., 2014; Hutin et al., 1999; Maunula et al., 2009; Swinkels et al., 2014). A major outbreak including over 10,000 cases related to norovirus in frozen strawberries occurred in Germany in 2012 (Bernard et al., 2014). Another outbreak related to HAV in frozen strawberries has been reported in 8 states in the United States (FDA, 2016). Virus contamination is most likely due to lack of hygiene, which is also a source of contamination for enteric pathogenic bacteria such as *Salmonella* and shiga-toxin producing *E. coli* (STEC). For bacterial pathogens, due to the low pH (3.2 to 4.2) usually no growth occurs on berries (Knudsen et al., 2001), but some pathogens

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http://dx.doi.org/10.1016/j.ijfoodmicro.2017.06.023
Received 16 January 2017; Received in revised form 16 June 2017; Accepted 23 June 2017
Available online 23 June 2017

such as *Salmonella* and *E. coli* O157:H7 are tolerant to acidic conditions and may survive (Delbeke et al., 2015; Han and Linton, 2004). One STEC O157 outbreak has been traced to the consumption of strawberries (Laidler et al., 2013). Although to our knowledge no *Salmonella* outbreak was linked to berries, *Salmonella* was ranked, together with HuNoV, as a priority pathogen to be considered in control of food safety of berries by EFSA (EFSA, 2014).

There is a need to investigate innovative control measures that are capable to deliver safer berries to use in a variety of ready-to-eat foods. Reduction of microbial surface contamination may involve washing of strawberries at the postharvest stage in sanitizing solutions (Hirneisen et al., 2011). Taking into account the strict regulation on chlorine wash in European countries (Banach et al., 2015), alternative sanitizers are in need to be investigated. Levulinic acid (4-oxovaleric acid, LVA) and sodium dodecyl sulfate (SDS) are FDA approved flavoring substance and multipurpose food additive, respectively (FDA, 2015a; FDA, 2015b). The combined use of LVA and SDS solution has shown to be effective in inactivating norovirus surrogates and bacterial pathogens on stainless steel and food surfaces (meat, cantaloupe, lettuce etc.) (Bolton et al., 2013; Cannon et al., 2012; Webb et al., 2013; Zhao et al., 2009). However, the antimicrobial effect of this sanitizer against HAV and *Salmonella*, *E. coli* O157:H7, *Listeria monocytogenes* on soft fruit such as strawberries has not been investigated. Given the limitation of using pathogens at industrial scale, information on surrogate strains are necessary to validate in due time the laboratory-scale findings in the industrial process. Non-pathogenic *E.coli* strain and *Enterococcus faecium* are commonly used as surrogates for enteric bacterial pathogens (Bianchini et al., 2014; Keeling et al., 2009) and MS2 bacteriophage could be used as indicator for foodborne viruses (Casteel et al., 2009). Murine norovirus (MNV-1), a laboratory scale cultivable norovirus, is frequently used in activation studies as a surrogate virus for HuNoV considering the difficulties in norovirus cultivation (Ettayebi et al., 2016; Jones et al., 2015; Park and Sobsey, 2011).

The objectives of the present study were to investigate the efficacy of LVA plus SDS wash in removal of both foodborne viruses and enteric pathogenic bacteria and their surrogates on strawberries. In addition, not only the reduction of the microbial load of above mentioned pathogens or surrogates by the sanitizer solution was studied, but also the presence of any chemical residue and associated chemical safety issue or sensory interference potentially resulting from the sanitizer wash was assessed.

2. Materials and methods

2.1. Bacterial strains and inoculum preparation

Three strains of *E. coli* O157:H7 (ATCC 700728, BRMSID 188 and LFMFP 846), *L. monocytogenes* (LMG 23192, LMG 23194, LMG 26484) and *Salmonella enterica* (serovars Thompson RM1987 and Typhimurium SL1344, LFMFP 883) were used. All *E. coli* O157:H7 strains were *stx*-negative strains in order to be able to perform experiments in the biosafety level 2 laboratory. LFMFP strains are part of the Laboratory of Food Microbiology & Food Preservation culture collection at the Faculty of Bio-Science Engineering of Ghent University. LMG strains are part of the Belgian Coordinated Collection of Micro-organisms (BCCM) situated at the Laboratory of Microbiology at the Faculty of Sciences of Ghent University. LFMFP 846 was derived from strain *E. coli* O157:H7 EH 1434 (obtained from Prof. Piérard, EHEC Reference Laboratory at the University hospital in Brussels). *E. coli* O157:H7 BRMSID 188 was obtained from Dr. Susan Bach (Agri-Food Canada). *Salmonella enterica* Typhimurium strain LFMFP 683 was an environmental isolate, provided from Prof Venter from Pretoria University, South Africa and deposited to LFMFP-Ghent University culture collection. *Salmonella enterica* (serovars Thompson RM1987 and Typhimurium SL1344) were obtained from Dr. Maria Brandl (ARS, USDA). A single strain of *Enterococcus faecium* (ATCC 8459) and *E.coli* P1 (ATCC BAA-1427) were used as bacterial surrogate strains.

The strains, stored at − 80 °C on glass beads, were revived and subcultured twice in 10 ml of brain heart infusion broth (BHI; Oxoid, UK) at 37 °C for 24 h before use. A cocktail of three *E. coli* O157:H7 strains was prepared by combining same amount (e.g. 1 ml) of each strain before inoculation. The final cell population was determined by plating appropriate dilutions in Buffered Peptone Water (BPW; Oxoid) on Cefixime-Tellurite Sorbitol Mac Conkey Agar (CT-SMAC; Oxoid). *L. monocytogenes* and *Salmonella* cocktails were prepared following the same procedure and plated out on Agar Listeria Ottavani & Agosti medium (ALOA; Biolife, Italy) and Xylose Lysine Desoxycholate Agar (XLD; Oxoid), respectively. Slanetz and Bartley medium (SB; Oxoid) was used to enumerate *E. faecium*, and Rapid *E. coli* 2 Agar (REC; Bio-Rad, France) was used for *E.coli* P1.

2.2. Viral strains and inoculum preparation

2.2.1. HAV and $TCID_{50}$ method

Fetal rhesus kidney (FRhK-4, ATCC CRL1688) cells was used to determine the titer of HAV (ATCC, VR-1402). Cells were resuscitated and passaged in Dulbecco's Modified Eagle's Medium (ATCC, 302002) supplemented with 10% Fetal Bovine Serum (FBS) (ATCC, 30-2020) and 1% Penicillin/Streptomycin 100 X (Sigma, P0781). HAV was propagated on FRhK-4 cultured in 175 cm^2 tissue culture flask at a multiplicity of infection (M.O.I) of 0.05–0.1 followed by incubation at 37 °C with 5% CO_2 for 1 to 2 h to allow the adsorption of the virus into the cells. The adsorption was stopped by adding 25 ml of EMEM (ATCC, 302003) supplemented with 2% FBS and 1% Penicillin/Streptomycin 100 X (Sigma, P0781).

The titer of HAV ($TCID_{50}$/ml) was determined by 50% tissue culture infective dose ($TCID_{50}$). Briefly, monolayer FRhK4 cells were seeded onto 96-well plates 24–48 h at 37 °C with 5% CO_2 prior to $TCID_{50}$ titration. Ten-fold serial dilutions of HAV samples were prepared in PBS. Old media in 96-well plate was removed and monolayer FRhK4 cells were inoculated with 20 µl per well and 8 wells per dilution followed by the incubation of the plate for 1 to 2 h at 37 °C with 5% CO_2 for virus adsorption into the cells. After incubation, 150 µl of HAV infection medium was added in each well and the plate was incubated for 14 days at 37 °C with 5% CO_2 (Butot et al., 2009). The cytopathic effect was recorded for each well and the $TCID_{50}$ calculated by the Spearman and Käber algorithm as described in Hierholzer and Killington (1996).

2.2.2. MNV-1 and plaque assay

Mouse macrophage cell line (RAW 264.7; ATCC TIB-71TM) was used for MNV-1 infectivity assay. Cells were resuscitated and passaged in growth medium DMEM (Dulbecco's Modification of Eagle's Medium plus 10% Fetal Bovine Serum (FBS), 1% L-glutamine, 1% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) and 1% Penicillin/Streptomycin) and incubated at 37 °C with 5% CO_2.

MNV-1 strain CW1, Passage 7, (originally supplied by Prof. H. W. Virgin, Washington University, St. Louis, MO, USA), was propagated on RAW 264.7 cells cultured in 125 cm^2 tissue culture flasks at a M.O.I. of 0.05 and stored at − 80 °C until use. The titer of MNV-1 (PFU/ml) was determined by plaque assay, as described by Wobus et al. (Wobus et al., 2004). Briefly, RAW 264.7 cells were seeded onto 6-well plates at a density of 2×10^6 viable cells per well 24 h prior to plaque assay. Ten-fold serial dilutions of MNV-1 samples were prepared in complete DMEM growth media, and 500 µl of diluted sample was inoculated into each well after the old media was removed. Plates were incubated for 1 h at room temperature. Afterwards, the inoculum was aspirated and the cells were overlaid with 1.5% SeaPlaque agarose (Cambrex, Rockland, ME) in MEME (Minimum Essential Medium Eagle supplemented with 10% FBS, 1% HEPES, 1% Penicillin/Streptomycin, and 2% L-glutamine). Plates were incubated at 37 °C with 5% CO_2 for 48 h, a second overlay of 1.5% SeaPlaque agarose in MEME with additional 1% neutral red was added to visualize plaque. Visible plaques were counted