Human intestinal enteroids and predictive models validate the operational limits of sanitizers used for viral disinfection of vegetable process wash water

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ABSTRACT

Vegetables are globally associated with a considerable number of foodborne outbreaks caused by viral infections, specifically human norovirus. In fresh produce industry, washing represents a critical step for food safety as process wash water (PWW) needs to be maintained at appropriate microbial quality to prevent water-mediated cross-contamination. This study aimed to explore the disinfection efficacy of chlorine (free chlorine, FC), chlorine dioxide (ClO_2) and peracetic acid (PAA) in PWW against infectious human norovirus and Tulane virus (TV). First, we tested the extent of TV inactivation in baby leaf, bell pepper, and vegetables mix PWW and monitored the viral decay by cell culture. Then, inactivation kinetics were defined for infectious human norovirus exposed to FC, ClO_2 and PAA in baby leaves PWW using the human intestinal enteroids (HIE) system. Finally, kinetic inactivation models were fitted to TV reduction and decay of sanitizers to aid the implementation of disinfection strategies. Results showed that >8 $\log_{10}$ human norovirus and 3.9 $\log_{10}$ TV were inactivated by 20 ppm FC within 1 min; and by 3 ppm ClO_2 in 1 min (TV) or 5 min (norovirus). PAA treatment at 80 ppm reduced ca. 2 $\log_{10}$ TV but not completely inactivated the virus even after 20 min exposure, while 5 min treatment prevented norovirus replication in HIE. TV inactivation in PWWs was described using an exponential decay model.

Taking these data together, we demonstrated the value of applying the HIE model to validate current operational limits for the most commonly used sanitizers. The inactivation kinetics for human norovirus and TV, along with the predictive model described in this study expand the current knowledge to implement post-harvest produce safety procedures in industry settings.

1. Introduction

Human noroviruses are the leading cause of sporadic cases and outbreaks of acute gastroenteritis worldwide and the most common cause of foodborne illness in the United States (Ahmed et al., 2014; Burke et al., 2021; Havelaar et al., 2015). In Europe, human noroviruses are the third most frequently reported causative agent of outbreaks being responsible for the 6.3 % of total foodborne cases (EFSA and ECDC, 2022). Noroviruses are transmitted to humans through multiple routes, even though the fecal-oral route is the primary pathway of disease transmission (Ahmed et al., 2014; De Graaf et al., 2016; Ramani

et al., 2014). Moreover, the low infectious dose, the high shedding concentration, the resistance to common disinfectants (e.g., alcohols, QUATs, chlorine, ozone) and the environmental stability facilitate viral spread through droplets, contaminated food, water, and fomites (Glass et al., 2009; Kotwal and Cannon, 2014; Lopman et al., 2012; Randazzo et al., 2018; Teunis et al., 2008).

The 2021 EFSA report informed 19 norovirus foodborne outbreaks (FBOs) associated with "crustaceans, shellfish, molluscs and products thereof" accounting to 147 reported cases, and 3 norovirus FBOs associated with "vegetables and juices and other products thereof" with 263 cases (EFSA and ECDC, 2021). In Europe, norovirus was associated with

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the 54.3 % number of cases related to contaminated fresh produce (Aiyedun et al., 2021). Moreover, the increased consumption of fresh produce in Europe and North America, as measures of improved diets, correlates with the increased fresh produce-related outbreaks of microbial origin. Specifically, produce may be contaminated by norovirus through several exposure pathways being irrigation water and infected handlers the most commonly reported sources (Chandrasekaran and Jiang, 2018; Li et al., 2018; Souza et al., 2020; Truchado et al., 2021a,b). The enhanced risk of contamination in those food items is also explained by the presence of histo-blood group antigens (HBGA)-like carbohydrates described in oysters and vegetables to which norovirus specifically binds (Esseili et al., 2019; Le Guyader et al., 2006; Xiang et al., 2016; Zhang et al., 2020).

While generic measures to disrupt norovirus transmission rely on adhering to hand hygiene practices, enhancing environmental cleaning and excluding sick staff from work (Barclay et al., 2014; Bhatta et al., 2020), in produce industry settings, water used for handling and processing fresh fruit and vegetables is critical to prevent contamination of the final product (JEMRA, 2021). A huge consumption of water (2 to 11 m^3/t of vegetables) is typically used for washing, rinsing, or conveying produce and it must be safe and of adequate sanitary quality (CFR, 2022; Manzocco et al., 2015). Based on the European Commission Notice (EC, No. 2017/C 163/01), water used for final washing of fresh fruit and vegetables during postharvest processing operations should be potable (intended for human consumption, Council Directive 98/83/EC) or adequately cleaned to prevent contamination. During the working day the quality of the process water deteriorates mostly due to the accumulation of organic matter (e.g. soil, dust and microorganisms) coming from the produce (Manzocco et al., 2015). This is why the microbial quality of process wash water (PWW) needs to be maintained to prevent pathogen (e.g., *Salmonella*, pathogenic *Escherichia coli*, *Listeria monocytogenes*, human norovirus) cross-contamination of the produce during washing (Gombas et al., 2017; Maffei et al., 2017). The commercial sanitizers most commonly used to maintain the microbiological quality of PWW include sodium hypochlorite (FC, free chlorine), chlorine dioxide (ClO_2) and peroxyacetic acid (PAA), which operational limits have been either recommended or enforced by scientific studies, guidelines and regulations. Targeting the residual concentration of 20 mg/L chlorine (Tudela et al., 2019), 80 mg/L PAA (21 CFR173.315, 2012), or 3 mg/L ClO_2 (FDA, 2019) has been claimed as effective to maintain the microbial quality of PWW. However, maintaining sufficient sanitizer residuals is a challenge because of variable characteristics of PWW (e.g., organic load, pH, temperature, and reaction time) that affect the final efficacy of the disinfection process (Banach et al., 2015; Gil et al., 2009; Srinivasan et al., 2020). Moreover, there is the need to specifically evaluate the efficacy of sanitizers against viral contamination, also because disinfection of virus is likely more variable and less effective compared to bacteria (Lin et al., 2020). In addition, research on the efficacy of sanitizers to inactivate human norovirus in PWW has been stymied by the inability to propagate it in cell lines (Estes et al., 2019).

Despite the recent development of an in vitro model based on stem cell-derived human intestinal enteroids (HIEs) for human norovirus replication (Costantini et al., 2018; Estes et al., 2019; Ettayebi et al., 2016), its complexity has limited its extended experimental application, especially in food and environmental virology. Thus, cultivable surrogates are commonly used to infer inactivation of the actual pathogen. Among others, Tulane virus (TV) is considered an appropriate surrogate for human norovirus because it recognizes HBGAs as cellular receptors (similar to human norovirus), and it is genetically more related to human norovirus than other caliciviruses (Cromeans et al., 2014; Farkas, 2015; Polo et al., 2018). The presence of HBGA-like carbohydrates on produce that act as specific binding moieties requires special attention when investigating viral contamination on this commodity as described for lettuce (DiCaprio et al., 2012; Esseili et al., 2019; Xiang et al., 2016). Moreover, TV has been indicated as an accurate surrogate model for studying inactivation profiles of human norovirus given its resistance to free chlorine (Cromeans et al., 2014).

In this study, we investigated the inactivation of TV in response to FC, ClO_2 and PAA treatments at recommended operational limits for the disinfection of different types of PWW (i.e. baby leaf, bell pepper and vegetables mix). Moreover, inactivation kinetics of infectious human norovirus were determined in baby leaf PWW using the novel human intestinal enteroids system. Finally, infectivity data sets were used to develop a predictive inactivation model for the implementation of disinfection strategies in produce industry settings.

2. Materials and methods

2.1. Viruses and cell lines used in the study

Human norovirus and TV inactivation in PWW were assessed by determining viral replication on HIE and LLC-MK2 cells, respectively.

Human GII.4 Sydney[P16] norovirus-positive fecal sample was provided by Prof. Buesa (University of Valencia, Spain). A 10 % fecal filtrate was prepared in phosphate buffered saline (PBS) and stored at −80 °C in aliquots until the time of testing.

Three-dimensional HIE derived from human jejunal biopsy (J2 cell line) were provided by Prof. Mary K. Estes (Baylor College of Medicine, Houston, TX). Undifferentiated 3D HIEs and differentiated monolayers were maintained and produced as originally described by Ettayebi et al. (2016), Zou et al. (2019) and the modifications included in Carmona and Randazzo (2023). The commercial IntestiCult™ Organoid Medium Human media (STEMCELL Technologies Inc.) was used for maintaining HIE (Ettayebi et al., 2021). To determine human norovirus infectivity, RT-qPCR was used to quantify the amount of norovirus RNA from input virus and from HIE monolayers at 1 h post-infection (hpi) and at 48 hpi. To this end, two sets of 96-well plates with 100 % confluent 4–6 day-old-differentiated HIE monolayers were inoculated in triplicate for each sample and incubated at 37 °C for 1 h. After the inoculum was removed, monolayers were washed twice with Complete Media without Growth Factors (CMGF−) and 100 μL of Organoid Medium Human containing 500 μM sodium glycochenodeoxycholate was added to each well. For each set of infections, one 96-well plate was immediately frozen at −80 °C (1 hpi) and the second plate was incubated at 37 °C and 5 % CO_2 for 48 h and then frozen (48 hpi).

RNA was extracted from 100 μL sample using the Maxwell® RSC Instrument (Promega) and the Maxwell RSC Pure Food GMO and authentication kit (Promega) and eluted in 100 μL buffer. RNA was detected using the set of primers and probe recommended by the ISO 15216-1 (2017) and the RNA UltraSense One-Step quantitative RT-PCR system (Invitrogen) on LightCycler 480 instrument (Roche Diagnostics, Germany). Ten-fold serial dilutions of synthetic gBlock gene fragments (IDT) were included to quantify the RNA into genome equivalents (y = −3.56x + 40.664, R = 0.997). Positive and negative amplification controls were also included in each run. The limit of detection (LoD) resulted 8.97 gc/well for norovirus.

Tulane virus provided by Prof. Farkas (Louisiana State University, LA, US) was grown in LLC-MK2 cells (ATCC CCL-7) cultured in Opti-MEM (Gibco Life Technologies) supplemented with 2 % FBS and 1 % antibiotic cocktail (STR/Pen; Life Technologies) as described previously (Cromeans et al., 2014; Esseili et al., 2018), with the modifications reported in Randazzo et al. (2020) for titration. LLC-MK2 cells were used since a typical cytopathic effect could be observed within 3–5 days after TV infection (Farkas et al., 2008). Briefly, viral titer was determined by the 50 % tissue culture infectious dose ($TCID_{50}$) assay inoculating 20 μL of 10-fold serial dilutions of TV prepared in PBS on eight wells with 70–80 % confluent LLC-MK2 monolayers in 96 well plates. After incubation for 1 h at 37 °C, 180 μL Opti-MEM was added. After 3–5 days, cells showing cytopathic effect (CPE) were enumerated. The LoD resulted 15.8 $TCID_{50}$/mL.

2.2. Produce wash water and disinfection experiments

PWW samples were collected in the framework of a recent monitoring study involving three processing facilities (Cuevas-Ferrando et al., 2021; Sanchez et al., 2022). Specifically, PWW was collected from tanks used for washing of baby leaves (green and red pigmented baby romaine, rocket, baby spinach and lamb lettuce), bell peppers and a vegetables mix (tomato, pepper, cucumber, and onion mix). The produce:water ratio is indicated in Table 1. Two liters of water were collected from the washing tanks 4–5 h after production started and transferred to laboratory while maintained at 4 °C until used for the experiments.

PWW samples were characterized for physiochemical parameters (Table 1). Specifically, temperature, pH, oxidation-reduction potential (ORP), and electric conductivity (EC, μS/cm) were determined by a pH and redox multimeter (Crison, Barcelona, Spain), chemical oxygen demand (COD) was determined by Spectroquant NOVA 60 photometer following a standard photometric method, and turbidity was measured by a Turbiquant 3000R turbidimeter (Merck, Madrid, Spain).

Sodium hypochlorite (free chlorine, FC), ClO_2 (AGRI DIS®, STC S.L. U., Spain) and PAA (Citrocide® Plus, Citrosol, Spain) were tested as disinfectants against human norovirus and TV by performing batch scale experiments. A static experimental design was adopted in the study, without further addition of PWW to the system (Gómez-López et al., 2015). Initially, 200 mL of baby leaves, bell peppers and vegetables mix PWW were placed in sterile chlorine demand-free beakers and inoculated with TV at $5.6 \pm 0.2 \log_{10}$ TCID$_{50}$/mL. The actual concentration in the PWWs resulted to be 3.9 ± 0.5 logs, as it was determined in the PWWs at time 0. Then, FC, ClO_2 and PAA stock solutions were added to target the operational limits of 20, 3, and 80 ppm, respectively (21 CFR173.315, 2012; USDA, 2016; Tudela et al., 2019).

PWW were kept at 4 °C and continuously stirred to mimic the operating conditions of the commercial produce processing lines (e.g., water temperature and turbulence) (Gombas et al., 2017; Weng et al., 2016). Viral inactivation and concentration of disinfectants monitored at 0, 1, 5, 10, 15, and 20 min.

FC, ClO_2 and PAA concentrations were monitored using Kemio® (Palintest, Gateshead, UK) analytical platform based on chronoamperometry and the corresponding sensors (KEM21CLO, KEM21CDX, and KEM21PAA, respectively).

To determine viral inactivation, 1 mL aliquots were collected at each time point and neutralized by mixing at 1:10 ratio with Opti-MEM supplemented with 10 % fetal bovine serum (FBS).

Based on TV results, baby leaves PWW was selected as representative matrix to study the inactivation kinetics of human norovirus exposed to targeted operational limits of FC (20 mg/L), ClO_2 (3 mg/L), and PAA (80 mg/L) determined using the HIE system. Experiments were conducted as detailed for TV, inoculating human norovirus at $9.0 \pm 0.2 \log_{10}$ genomic copies (gc)/mL into 200 mL PWW and collecting aliquots at 0, 1, 5, 10, 15, and 20 min time points. CMGF− supplemented with 500 μM sodium glycochenodeoxycholate and 10 % FBS was used to neutralize the disinfectants.

A neutralization control was included in all experiments for both viruses.

2.3. Model development

TV infectivity data were used for predictive inactivation modelling. Experimental data from specific combinations of PWW and water disinfectant types were selected for survival modelling according to the availability of enough data points to perform the fitting process. The type of sanitizer influenced the type of data obtained in each trial. Data were fitted using an exponential decay model defined by the equation:

$$y = a - (a - b) \cdot e^{-ct}$$

where y is viral infectivity (log S), a is the smallest value that y can take, which matches with the value associated to the horizontal asymptote of the curve; b is the value of y at $t = 0$; t is time; c is a value proportional to the relative rate of y change with respect to the increase of t, and t is the time.

2.4. Statistical analysis

All data were compiled from three independent experiments with three technical replicates for each experimental condition. Significant differences in mean infectivity were determined by using one-way ANOVA followed by Dunnett's multiple comparisons test. Differences in means were considered significant when the p was <0.05. GraphPad Prism version 8 (GraphPad Software) software was used for statistical analyses and data representation.
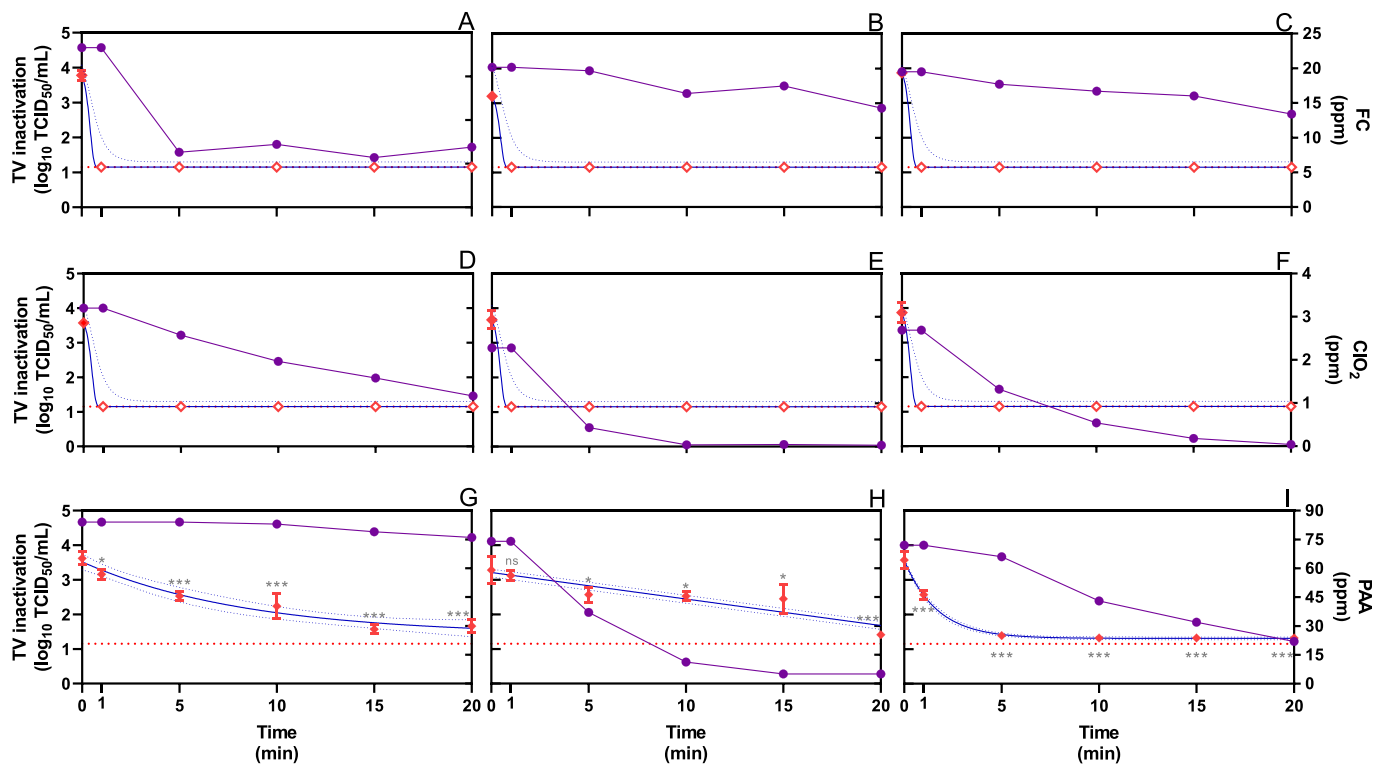
3. Results and discussion

3.1. Use of chemical sanitizers for viral disinfection of produce wash waters

Our study shows that $>8 \log_{10}$ human norovirus and $>3.9 \log_{10}$ TV are inactivated by 20 ppm FC (LoD = 15.8 TCID$_{50}$/mL for TV, and 8.97 gc/well for norovirus) within 1 min; and by 3 ppm ClO_2 in 1 min (TV) or 5 min (norovirus). In addition, PAA treatments at 80 ppm reduced ca. 2 $\log_{10}$ TV but not completely inactivated the virus even after 20 min exposure, while 5 min treatment prevented norovirus replication in HIE.

Results showed that 20 ppm FC inactivated $>3.9 \log_{10}$ TV regardless of the type of PWW assayed (Fig. 1), and a similar kinetic resulted for infectious human norovirus in baby leaves PWW from replication experiments in HIE (Fig. 2). The antiviral effect of FC was likely instantaneous as none infectious viral particle was detected after 1 min (Figs. 1A–C, 2).

With regard to ClO_2 disinfection, our original data indicates that ca. 3 ppm ClO_2 completely inactivated TV within 1 min in all types of tested PWW (Fig. 1D–F), while human norovirus replication in HIE was prevented after 5 min of treatment, indicating $>8 \log_{10}$ inactivation (Fig. 2).

Following PAA treatment for 20 min, infectious TV was recovered in all types of PWWs, even though at concentrations bordering the limit of detection and only for some of the technical replicates (Fig. 1G–I). More in detail, PAA reduced TV titer by ca. 2 $\log_{10}$ TCID$_{50}$/mL in baby leaves (Fig. 1G) and bell pepper (Fig. 1H) PWWs after 15 and 20 min, respectively, while a similar reduction was observed in vegetables mix PWW after 5 min (Fig. 1I). On the contrary, infectious human norovirus was recovered after 1 min PAA treatment in baby leaves PWW, while viral replication in HIE was completely prevented for longer exposure times

Table 1
Physiochemical characterization of produce wash water. Data represent mean ± SD of three measurements.

PWW	Produce: water ratio	pH	ORP	EC	COD	Turbidity	Temperature
Baby leaves	1.7	7.8 ± 0.1	755 ± 17	1499 ± 5	132 ± 1.0	19 ± 7	8 ± 2
Bell pepper	120	3.8 ± 0.2	410 ± 39	765 ± 54	1496 ± 437	112 ± 67	14 ± 3
Vegetables mix	10	7.3 ± 0.3	218 ± 51	879 ± 155	166 ± 16.7	19 ± 2	14 ± 1

Abbreviations and units: PWW, produce wash water; ORP, oxidation-reduction potential (m/V); EC, electric conductivity (μS/cm); COD, chemical oxygen demand (mg/L); Turbidity (NTU, nephelometric turbidity unit); temperature expressed in Celsius degrees (°C).

Fig. 1. Decay of 20 ppm FC, 3 ppm ClO_2 and 80 ppm PAA and TV inactivation in baby leaves (A, D, and G), bell pepper (B, E, and H), and vegetables mix (C, F, and I) PWW. The mean concentration of sanitizers (violet lines) is plotted on the secondary y-axes, and TV load (red points) on the primary y-axes. Fitted exponential decay models for FC, ClO_2, and PAA are plotted as blue continuous lines with confidence bands as dashed blue lines. Hollow symbols represent viral concentrations below the sensitivity limit of the cell culture assay ($TCID_{50}$/mL, dashed red lines). For each time point, differences in viral load means were considered significant for $*p < 0.05$; $***p < 0.0001$; ns, not significant compared with the nontreated sample at initial time point. Error bars indicate SDs.
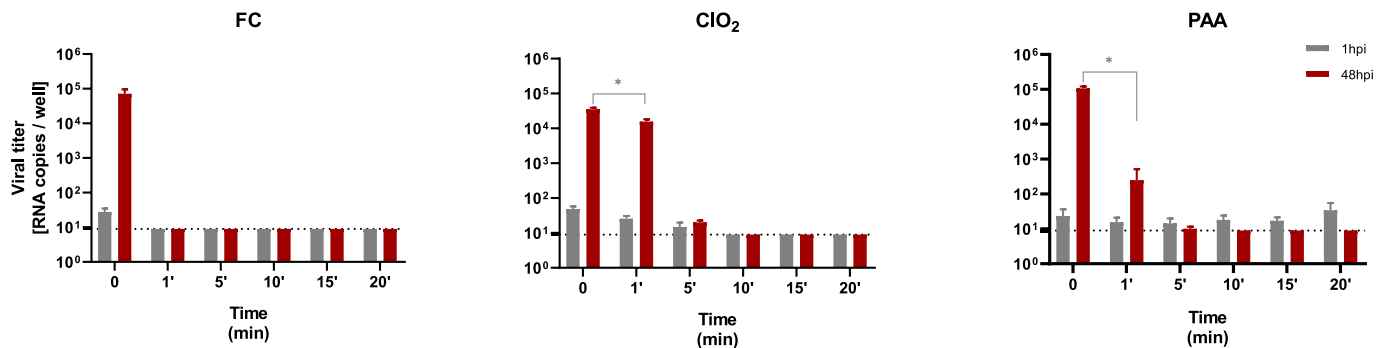
Fig. 2. Human norovirus inactivation in response to 20 ppm FC, 3 ppm ClO_2 and 80 ppm PAA treatments in baby leaves PWW. Bars show replication of human norovirus in human intestinal enteroids at 1 (grey bars) and 48 (red bars) hours post-infection (hpi). Error bars indicate SDs. Differences in mean replication titers were considered significant for $*p < 0.05$. hpi, hours postinfection. Dashed lines indicate the limit of detection of the assay (8.97 gc/well).

(Fig. 2).

To our knowledge, this is the first report incorporating infectivity data of genuine human norovirus and a surrogate (TV) to evaluate the efficacy of sanitizers for PWW disinfection. Human norovirus and TV demonstrated similar inactivation patterns for FC, while slight differences were observed for ClO_2 and PAA disinfection. Under the experimental conditions of this study, it seems that the human pathogen is more resistant to ClO_2 and more sensitive to PAA treatment than the tested surrogate. However, it is difficult to make a direct quantitative comparison between the viruses due to the differences of the infectivity assays (e.g., amount of initial viral titer, dilutions used for cell infections, titration method and limit of detection). TV has been considered one of the most accurate surrogates for human norovirus to evaluate the efficacy of sanitizers, and more recently TV infectivity was

directly compared to human norovirus replication in the HIE model (Cromeans et al., 2014; Escudero-Abarca et al., 2022; Randazzo et al., 2020). These studies concluded that the infectivity assays for human norovirus and TV performed similar for sodium hypochlorite inactivation, but not for an antiviral plant extract (e.g., green tea extract).

In order to comprehensively describe viral inactivation in PWW, important test variables should be considered in disinfection assays, such as type of water, type and concentration of sanitizers, contact time, experimental design (static vs continuous disinfection), type of viruses, and viral titration method. Unfortunately, a wide variety of experimental conditions has been used in the literature, making these results very difficult to compare across the different studies. Results obtained in the present work indicate that the effectiveness of the sanitizer is dependent on both the stability of the sanitizer concentration in the

water and the efficacy of the sanitizer to inactivate viruses under specific conditions (see Section 3.2). Similar findings have been previously described by Srinivasan et al. (2020), who indicated that maintenance of the residual concentration of the sanitizer is critical in controlling pathogen inactivation and preventing cross-contamination but this will depend on the stability of the sanitizer in the process water.

Our results for viral sensitivity to chlorine are in general agreement with the results reported in previous studies (Costantini et al., 2018; Dunkin et al., 2017a,b; Fuzawa et al., 2019). Fuzawa et al. (2019) reported that TV lost its ability to bind to its receptor after exposure to FC at 29 ppm over 1 min, which is in line with our measurements. Dunkin et al. (2017a,b) observed 4 log_{10} MS2 infectivity reduction exposed to ca. 1.5 ppm FC within 3 min in whole-leaf and chopped romaine PWWs, while complete inactivation was not achieved in shredded iceberg PWW within 15 min. As well, human norovirus replication in the permissive HIE model has been reported to be completely prevented following FC treatment at $\geq$50 ppm (Costantini et al., 2018). In contrast, other studies found that FC concentrations lower than 500 ppm (Tian et al., 2013) or 2000 ppm (Hirneisen and Kniel, 2013) did not inactivate TV in solution. The discrepancies could be most likely explained by the different experimental designs adopted and water characteristics.

Regarding ClO_2 disinfection, CT (concentration x time) values retrieved from the literature ranged from 0.06 to 10 ppm $\times$ min to achieve a 4 log_{10} enteric viral removal in several water matrices, including drinking and wastewater (Ge et al., 2021). Kingsley et al. (2014) reported that human noroviruses are resistant to 350 CT as resulted by molecular assays, which is notably higher than the ClO_2 inactivation observed of our study. However, the RNA titration does not quantitate the loss of virus infectivity (Shaffer et al., 2022), which has been done in our study.

We observed TV and human norovirus resistance to PAA disinfection. Fuzawa et al. (2020) also found that 10 ppm PAA for 30 min were needed to achieve a 2 log_{10} reduction in buffer solutions (equal to CT_{99} = 300, where CT_{99} = 99 % reduction). The authors explain that such resistance might be due to the significant aggregation of TV in PAA solutions observed by transmission electron microscopy. It has been shown that viral aggregation protects virions from the effect of disinfectants, especially with more reactive ones (e.g., chlorine) (Gerba and Betancourt, 2017; Mattle et al., 2011). In our case, this may prevent the optimal interactions between PAA and the surface area of TV virion, and/or the decrease of the PAA diffusion inside aggregated virions. We now report that higher PAA concentration of $\approx$80 mg/L (CT_{99} = 394) was not even sufficient to achieve a complete ($\approx$2.7 log_{10}) TV inactivation in PWW within 20 min. This hypothesis might not apply to human norovirus tested in the HIE model, since fecal suspensions were filtered before being inoculated in PWW, which may have facilitated the disaggregation of viral particles, making them more susceptible to disinfection. This could help explaining the higher sensitivity of human norovirus to PAA treatment compared to TV.

We tested both viruses in PWW, which is a complex matrix (e.g., organic load), whereas experiments in suspension (oxidant demand-free buffer) provided different results. In oxidant demand-free buffer, Lim et al. (2010) reported that CT values of 0.314 and 0.247 were required for reducing 4 log_{10} MNV using FC and ClO_2, respectively. These values are significantly lower than those applied in our study for either human norovirus or TV, and the difference might be explained by the diverse oxidant demand of the matrices. This hypothesis is also supported by Escudero-Abarca et al. (2022) findings on the variable susceptibility of TV and norovirus to sodium hypochlorite suspensions and an alcohol-based sanitizer applied at different conditions of soil load. Thus, our data in PWW with variable amount of organic load are relevant and predictive of real-world disinfection efficacy.

This is the first study reporting on human norovirus inactivation in PWW by chemicals sanitizers using the HIE model. The HIE model allowed for the confirmation of successful neutralization (viral binding occurred at 1 hpi), as well as for norovirus inactivation exposed to

sanitizers. To date, only few studies investigating chlorine inactivation of infectious human norovirus have been performed by means of controlled clinical trial (Keswick et al., 1985) or using the in vitro HIE system (Costantini et al., 2018; Escudero-Abarca et al., 2022). The clinical trial study found that adding 10 ppm of FC in drinking water failed to protect all volunteers from infection (Keswick et al., 1985), while 50 ppm FC was reported to prevent norovirus replication in the HIE model (Costantini et al., 2018).

Our data do not provide quantitative data on the degree of norovirus inactivation, which is a limitation of the method yet to be overcome. Moreover, the complexity and the cost associated to the human norovirus replication in HIE, along with the limited amount of positive fecal samples restricted the number of inactivation experiments and biological/technical replicates. Indeed, the experimental design included several dilution steps such as the spiking of human norovirus stool suspensions in a large volume of PWW (200 mL), the neutralization needed to block sanitizers effect and avoid cytotoxicity, and the dilution with infectious media to infect HIE. As a consequence, a high input of infectious virus is required to carry out those experiments which is not always available. It is important to note that our experiments were performed using human norovirus GII.4 Sydney[P16] strain, which has been reported to replicate in the HIE at high rates compared to other genogroups/genotypes (Costantini et al., 2018; Ettayebi et al., 2016). However, it has been demonstrated by molecular methods that noroviruses exhibit genogroup-dependent resistance to FC and PAA (Dunkin et al., 2017a,b). Thus, survival models describing human norovirus inactivation in response to PWW sanitation, specifically adjusted for different genogroups infectiousness, should be considered in future works.

Studies exploring viral inactivation in a continuous washing system, in which water, produce and sanitizers are continuously introduced to the system, might provide a more relevant information for real-world scenarios, and preliminary results have been recently published using MS2 phage or *Escherichia coli* (Abnavi et al., 2021; Falcó et al., 2023). However, such studies are difficult to be performed using genuine human norovirus, and surrogates will be likely used to build scientific evidence in these complex disinfection systems.

It is remarkable that several authors reported that concentrations of sanitizers (10–25 ppm FC, 3 ppm ClO_2, and 80 ppm PAA) similar to those used in this study effectively inactivated foodborne bacterial pathogens (e.g., Shiga-toxigenic *E. coli*, *Salmonella enterica*, and *Listeria monocytogenes*) in PWW (Gu et al., 2020; Francisco López-Gálvez et al., 2020; Truchado et al., 2021b). Ultimately, our data and those of others on bacterial inactivation, provide compelling evidence that FC and ClO_2 unlike PAA at recommended operational limits might represent effective mitigation strategies to effectively prevent microbial cross-contamination in PWW.

3.2. Decay of sanitizers in PWW

The effectiveness of sanitizers is dependent upon many factors such as concentration of agent, reaction time, temperature, and organic load (Lin et al., 2020; Srinivasan et al., 2020). Thus, a proper concentration of sanitizers (e.g., 10 to 25 ppm FC, 30 to 80 ppm PAA) should be targeted and maintained to ensure an effective washing process (Gombas et al., 2017).

In our study, we monitored the decay of sanitizers which differed among the PWW (Fig. 1). Specifically, FC sharply decreased in baby leaves PWW after 5 min, but not in bell peppers and vegetables mix PWW. On the contrary, ClO_2 completely decayed in bell peppers and vegetables mix PWWs, while residual concentrations (<1 mg/L) were measured after 20 min in baby leaves. PAA showed a marked decay in bell pepper PWW but not in baby leaves PWW (Fig. 1). The difference in the decay of sanitizers could be related to the varied physiochemical composition of PWW (e.g., EC, COD, turbidity) (Table 1), even though resulting TV inactivation patterns were similar for a given sanitizer

tested in different PWW (Fig. 1). Most of the inactivation has been observed within the first minute of contact time, when the highest concentration of sanitizers was present in the PWW. This most likely is a result of the static experimental design adopted in the study, in which additional PWW with higher organic loads was not added to the system (Gómez-López et al., 2015). Also, this might explain the similar patterns observed for each sanitizer in different types of PWW.

Overall data on the decay of sanitizers and residues are relevant for implementing effective sanitation management and avoiding the presence of disinfection by-products (Tudela et al., 2019).

3.3. Model describing Tulane virus inactivation in PWW

Modelling approaches for microbiological food safety have evolved to characterize the inactivation of microorganisms as a function of relevant intrinsic and extrinsic factors (Allende et al., 2022). The model was fit to data presented in Fig. 1 and selected on the bases of its level of adjustment to the experimental dataset. The estimated model parameters are presented in Table S1.

An exponential decay model was used to explain the TV inactivation in PWW exposed to FC and ClO_2 (Fig. 1A–F) on one hand, and PAA (Fig. 1G–I), on the other hand. The parameterisation selected for the inactivation of TV by FC and ClO_2, describes the rapid inactivation of TV by these sanitizers in PWW even at low concentrations, indicating the high oxidative capacity of tested disinfectants (Fig. 1). Moreover, as 1 min time points were below LoD, TV inactivation kinetic can even be faster than the one described by the model, which should finally be interpreted as conservative.

In the case of PAA, the parameterisation selected indicates 2.3 and 2.0 log_{10} TV inactivation in pepper and vegetables mix PWW, which makes this sanitizer less suitable to maintain the microbiological quality of the water (Fig. 1G–I).

Survival kinetics models of the Chick-Watson-Hom's kind have been reported to reflect the biological response in static disinfection (Peleg, 2021). As an example, Abnavi et al. (2021) modelled the disinfection kinetics of *E. coli* in batch- and continuous-wash processes taking into account the loss of efficacy of FC in the presence of organic load. Also, Dunkin et al. (2017a,b) reported that the incomplete gamma Hom function effectively represents MS2 infectivity reduction in whole leaf and shredded iceberg PWWs sanitized with FC, while Hom-Power law model was the best fit for chopped romaine PWW. The reported data reveals that a 3-log_{10} reduction of infectious virions is achieved at CT values of <0.1 for MNV and 2.5 for MS2 in whole-leaf wash water. As well, the model-predicted CT value of 22 for 3-log_{10} gene copy reduction of human norovirus GII in whole-leaf wash water, which has been demonstrated in this study to be sufficient to prevent the replication of 9-log_{10} infectious virions in HIE. However, this data should be considered with caution as the infectious:genomic copies ratio for human norovirus has not been defined yet. These mechanistic models use mathematical expressions that best describe the inactivation activity of biocides. In this study a different approach was followed using a statistical model which applied an exponential mathematical expression to describe the viral inactivation. The inactivation processes studied in this study is a nonlinear phenomenon that is appropriately modelled by a nonlinear regression equation. Moreover, the selection of a nonlinear regression model versus mechanistic models is mostly due to the relevant attributes as parsimony, easy interpretation, prediction, and flexibility of nonlinear regression models, which can adopt a variety of shapes using data-driven methods of successive approximations (Bates and Watts, 2007).

On an all-embracing perspective, researchers, risk assessors, risk managers and food safety agencies (e.g., European Food Safety Authority, EFSA) indicate the lack of data as a factor that limits risk assessments. Our study provides a modelling approach based on experimental dataset that could be combined with a wide range of risk assessment tools to provide appropriate risk mitigation and control

options. Such expanded models should account for the effects of multiple factors and determining their parameters/coefficients, either experimentally or in simulations, would be the priority of future research.

4. Conclusions

In the present study, we define viral inactivation kinetics of infectious human norovirus and predictive models of TV in response to chlorine, chlorine dioxide and peracetic acid treatments in process wash water. The increase of human norovirus RNA genomic copies following replication on human intestinal enteroids served as a robust and definitive approach -compared to the RNA detection by RT-qPCR alone- to support the effectiveness of FC and ClO_2 applied in PWW at the operational limits specifically defined for each processing line by the food business operators.

Our data are of interest for implementing produce industry practices, specifically washing processes, where targeting effective sanitizer residuals to prevent viral cross-contamination could be extremely challenging. The inactivation kinetics and the predictive model presented in this study expand the current knowledge to improve produce food safety.

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CRediT authorship contribution statement

Ana Allende: Conceptualization, Funding acquisition, Writing – review & editing. **José Antonio Férez-Rubio:** Formal analysis. **Juan Antonio Tudela:** Formal analysis. **Rosa Aznar:** Writing – review & editing. **Maria Isabel Gil:** Conceptualization, Writing – review & editing. **Gloria Sánchez:** Conceptualization, Writing – review & editing. **Walter Randazzo:** Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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