CENTER FOR PRODUCE SAFETY

CPS 2023 RFP – FINAL PROJECT REPORT

Project Title:
Improving methods for the assessment of infectious human enteric virus survival in produce

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Objectives:

1. Optimize the currently standardized methods for the recovery of infectious enteric viruses from leafy greens and berries.
2. Optimize capsid-integrity molecular assays to better estimate infectious human norovirus (HuNoV) and hepatitis A virus (HAV).
3. Investigate persistence of infectious, capsid-protected genome equivalents (GE), and total GE HuNoV and HAV on harvested lettuce and berries under different post-harvest conditions.

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FINAL REPORT

Summary of Findings and Recommendations

Key Findings

1. **Improved Concentration/Detection Methods**
    - The project developed optimized, user-friendly methods for detecting and quantifying human norovirus (HuNoV) and hepatitis A virus (HAV) in leafy greens and berries.
    - The optimized method based on ultrafiltration (Concentrating Pipette, CP) performed well across matrices, reduced turnaround time, avoided ultracentrifugation, preserved infectivity, and matched or outperformed ISO and FDA standard methods.
    - Salivary gland cell line does not offer advantages over the human intestinal enteroids for the assessment of infectious HuNoV occurrence in contaminated foods in real scenarios.

2. **Molecular Tools and Risk Assessment**
    - Capsid-integrity PMAxx RT-qPCR assays were shown to better approximate viral infectivity.
    - PMAxx ISO-Long RT-qPCR assays with long amplicons reduced false positives from inactivated viruses and better correlated with infectivity during storage at 4°C and 21°C.
    - However, PMAxx RT-qPCR assays are not suitable for evaluating disinfection efficacy and should complement, not replace, infectivity assays.

3. **Viral Persistence and Storage Effects**
    - Viral stability is matrix- and temperature-dependent.
    - Higher stability on blueberries than strawberries for HAV, HuNoV GII, and MNV (HuNoV surrogate) during refrigerated and ambient storage.
    - Higher stability for HAV and MNV on spinach than lettuce.
    - Refrigeration offers limited viral reduction starting at 7 days.
    - Higher temperatures accelerate viral decay (e.g., 21°C), while freezing (−20°C) prolongs infectivity.
    - Decay of viral genome detection of UV-inactivated viruses occurs ~2× faster than infectious viruses.

4. **Sanitizer Efficacy**
    - Treatments with free chlorine (FC), chlorine dioxide (ClO_2), and peracetic acid (PAA), applied at doses and contact times representative of commercial produce washing, achieved only modest reductions in viral contamination on leafy greens.
    - PAA showed the highest efficacy against HAV (~0.8 log reduction).
    - No significant differences among disinfectants were observed for HuNoV and MNV.

5. **Limitations of Molecular Methods**
    - Capsid-integrity assays improve on standard RTqPCR but cannot reliably predict infectivity after disinfection.
    - Correlation with infectivity is not universal; caution is needed when interpreting molecular results, and complementary infectivity assays remain essential for accurate risk evaluation.

Recommendations for the Produce Industry

1. **Adopt Improved Detection Tools**
   - Adopt the CP-based concentration method when rapid, high efficiency virus recovery is needed.
   - Use capsid integrity molecular assays (PMAxx + ISO-Long) as a complementary tool for estimating risk from naturally contaminated samples.

2. **Tailor Post-Harvest Storage Strategies**
   - Consider the type of produce and storage conditions:
     - Blueberries: monitor carefully due to higher viral persistence.
     - Refrigeration: modest viral reduction; implement additional control measures.
     - Freezing: preserves viral infectivity; assess downstream processing to mitigate risk.

3. **Choose Sanitizers Strategically**
   - Recognize that sanitizer washes (free chlorine, chlorine dioxide, and PAA) have limited efficacy against enteric viruses on leafy greens; focus on preventing contamination before harvest through good agricultural practices rather than removing viruses post-harvest.
   - The produce industry should evaluate and improve disinfection treatments—including dose, contact time, and alternative disinfectants—to ensure more reliable inactivation of viruses during post-harvest washing.

4. **Continuous Monitoring and Risk Assessment**
   - Use capsid-integrity assays for screening, but rely on infectivity assays for risk-critical decisions, particularly after disinfection treatments.
   - Incorporate new die-off data into risk assessments and shelf-life modeling.

Abstract

Viral contamination of fresh produce, particularly with human norovirus (HuNoV) and hepatitis A virus (HAV), is a major food safety concern, as these pathogens can cause widespread outbreaks and severe illness. Standard detection methods, including ISO and FDA RTqPCR protocols, detect viral genomes without assessing infectivity, creating challenges when positive results do not necessarily indicate a risk of disease. This project evaluated improved methodological approaches for HAV and HuNoV recovery, infectivity assessment, and survival characterization on leafy greens and berries. Key objectives included: (i) optimizing user-friendly detection methods, (ii) optimizing capsid integrity RTqPCR assays to better approximate infectivity, and (iii) investigating viral stability under post-harvest storage and disinfection practices.

An ultrafiltration-based method using the Concentrating Pipette (CP) demonstrated the most consistent and highest viral recoveries across all matrices tested, outperforming or matching ISO and FDA reference methods. The CP method also showed reduced processing time, eliminating ultracentrifugation, and preservation of viral infectivity, making it suitable for routine use and ulterior combination with infectivity assessment. Novel capsid integrity PMAxx RTqPCR assays employing longer amplicons (ISO Long) improved discrimination between infectious and heat inactivated viruses and reduced false positive

genome detection, although they were not suitable for evaluating disinfectant efficacy. Virus persistence studies revealed viral stability was matrix- and temperature-dependent. Infectious HAV, HuNoV GII, and murine norovirus (MNV) exhibit considerable stability on produce, with greater persistence on blueberries than strawberries and high stability during frozen storage (−20°C). Genomic decay of UV inactivated viruses occurred approximately twice as fast as that of infectious viruses. Evaluations of free chlorine, chlorine dioxide, and peracetic acid washes showed limited reductions in infectious viruses, reaffirming that post-harvest sanitizers have minimal impact on enteric virus removal. Overall, the study provides improved tools for virus detection and infectivity estimation, new data on viral survival in post-harvest scenarios, and evidence supporting prevention-focused strategies to mitigate viral contamination in the produce supply chain.

Background

Although food safety has significantly improved over recent decades, foodborne diseases continue to be a major global cause of illness and death. According to the CDC, each year approximately one in six individuals becomes ill after consuming contaminated food or beverages, and around 3,000 people die from foodborne infections. Fresh produce—including fruits, berries, and vegetables—has frequently been linked to foodborne disease outbreaks in industrialized nations, with viruses ranking among the top 3 biological hazards associated with produce (1,2). As a result, foodborne viruses have become an increasing concern for the produce industry, especially as fruit and vegetable consumption is widely promoted for maintaining a healthy, balanced diet. Among the many viral agents that can contaminate food and water, human noroviruses (HuNoV) and hepatitis A virus (HAV) account for most of the well-documented foodborne viral outbreaks, some of them of large dimensions (3–6).

Detection of viral pathogens in fresh produce remains challenging due to the typically low concentrations of contaminating viruses, the limited recovery efficiencies of current extraction methods, the lengthy analysis times, and the reliance on standardized molecular assays that quantify only viral genome equivalents (GE), which do not necessarily reflect infectious virus particles (3,7). Current standardized protocols for detecting HuNoV and HAV in leafy greens and soft fruits, published by ISO (8,9) and FDA (10), involve the elution of viruses from the food surface followed by concentration using polyethylene glycol (PEG) precipitation or ultracentrifugation, respectively. Although these methods are designed to provide adequate viral recovery, acceptable efficiencies are not consistently achieved. Moreover, they require sophisticated, non-portable equipment and are time-consuming, indicating that further refinement and innovation are still needed. Despite some previous studies have described alternative methods with varying improvements in the recovery of viral pathogens (11–14), most studies do not compare their performance to both reference methods. But more important, current methods target viral genomes by RTqPCR and do not inform on viral infectivity.

For both HuNoV and HAV, detection of infectious wildtype viruses on naturally contaminated foods has remained a challenge for decades, and despite cell-culture adapted strains obtained for HAV and some progress made in the development of cell culture systems to propagate HuNoV using human intestinal enteroids (HIEs) (15), key enabling methodologies are highly needed and still to be developed. Some of these efforts using HIEs have been funded by previous and ongoing CPS grants (16,17). Despite being widely accepted that enteric viruses may remain infectious for periods exceeding the shelf lives of produce products, only a small number of studies have addressed specific survival of HAV on leafy greens and berries (reviewed by (18,19)), and to our knowledge no published studies exist on HuNoV survival on these items, as measured by real infectious viruses. Instead, most data come from the use of surrogate viruses or capsid-integrity RTqPCR assays (20). Several capsid-integrity RTqPCR assays have been developed by us

and others, mostly based on the use of intercalating dyes and suitable to be used on produce and water sources (21–26). Most assays have proven useful to better estimate infectivity reduction during certain controlled inactivation/disinfection processes, but the significance of signals detected, even after treatment or in naturally contaminated samples, still challenges data interpretation in terms of health risk, indicating that further refinement of capsid integrity RTqPCR assays is still required. Regarding development of additional cell culture systems to determine HuNoV infectivity, a report published in 2022 described susceptibility of a manageable and affordable system based on salivary gland (SG)-derived continuous cell lines to HuNoV replication (27). The study showed consistent replication of HuNoV GII.4 purified from stool specimens, with over 1,000-fold increase in viral titer in the supernatant from 6 to 96 hours post-infection.

The main aim of this project was to optimize current HuNoV and HAV detection methods in leafy greens and berries, to increase their efficiency, reduce their turnaround time and provide estimates of viral infectivity. Two alternative virus concentration protocols (aluminum hydroxide adsorption-precipitation method and InnovaPrep Concentrating Pipette) were tested and compared to the FDA and ISO reference protocols. The project also refined capsid-integrity assays to estimate viral infectivity and explored the applicability of salivary-gland cell lines for detecting infectious HuNoV. Finally, improved procedures were applied to investigate persistence of HuNoV and HAV on harvested lettuce and berries under different post-harvest scenarios, as of real infectious viruses, capsid-protected genomes, and total viral genomes.

Research Methods and Results

Objective 1. Optimize the currently standardized methods for the recovery of infectious enteric viruses from leafy greens and berries.

Task 1.1. Optimize HuNoV and HAV concentration and extraction procedures, and analytical comparison with current standardized FDA and ISO protocols

Methods

Virus stocks, cells and preparation of pooled viral stock suspension

The cell-adapted cytopathogenic HAV strain pHM175 43c was propagated in FRhK-4 cells (ATCC CRL-1688). Mengo virus (MGV) strain MC0 was cultured in Buffalo Green Monkey (BGM) cells (Cytion 302158). For both viruses, infected monolayers were frozen at –80 °C upon reaching generalized cytopathic effect and subjected to three freeze-thaw cycles to release intracellular virions. Lysates were then centrifuged at 3,000 × g for 20 min, and the resulting supernatants were stored at –80 °C until use. Infectious titers of the viral stocks were determined using the Tissue culture infectious dose ($TCID_{50}$) assay. Stool specimens containing HuNoV GI.P11_GI.6, GII.P31_GII.4 Sydney 2012, and GII.P17/GII.17 from patients with gastroenteritis were obtained from previously characterized outbreaks (28). Ten percent (wt/vol) suspensions were prepared in phosphate-buffered saline (PBS), vortexed for 1 min, clarified at 2,000 × g for 15 min, and aliquoted before storage at –80 °C. HAV, HuNoV GI, and HuNoV GII titers were quantified by RT-qPCR following ISO 15216-1:2017 guidelines. A mixed viral pool was then prepared by combining the three viruses to a final concentration of 10^6 GE/mL each in PBS, and aliquots were stored at –80 °C for subsequent spiking experiments necessary for method comparison.

Experimental contamination of food samples

Leafy greens [romaine lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), baby leaf (red and green batavia lettuce, red lollo lettuce, spinach and arugula)] and berries [strawberries (*Fragaria × ananassa*), raspberries (*Rubus idaeus*), blueberries (*Vaccinium caesariense*)] were purchased from local

supermarkets. Each sample was weighted to 25 g according to the ISO 15216:2017 guidelines. For samples analyzed using the FDA method, 50 g portions were used instead. Samples were transferred into mesh filter bags and inoculated at a level of 10^5 GE/25 g, through dropwise inoculation across 10–20 spots on the sample surface. After drying for 1 hour in a laminar flow hood, samples were processed for virus concentration. Each experiment included three artificially contaminated replicates for each condition tested, along with an unspiked sample used as a negative control.

Virus concentration methods

A total of 4 virus concentration methods were used. Two alternative protocols were adapted: aluminum hydroxide adsorption-precipitation ($AlCl_3$) and ultrafiltration-based Concentrating Pipette Select (CP InnovaPrep, USA), and they were compared to the current ISO and FDA reference protocols.

a) ISO method

Briefly, 25 g samples were eluted in 40 mL of Tris/glycine/beef extract (TGBE) buffer (pH 9.5) and 10 µL of MGV stock were added as process control virus. In the case of soft fruits, 30 units of pectinase from *Aspergillus niger* were added. The samples were incubated on a rocker at room temperature (RT) for 20 min, then clarified by centrifugation at 10,000 × g for 30 min at 4 °C. Supernatants were collected for virus concentration step, using PEG-NaCl precipitation method and were resuspended in 500 µL of PBS. For berries, an additional purification with 500 µL chloroform/butanol was performed, followed by centrifugation at 10,000 × g for 15 min at 4 °C and the aqueous phase was collected. The eluates were stored at –80 °C or used for RNA extraction.

b) FDA method

Elution was carried out using 50 g of sample and 55 mL of Glycine Buffer (pH 7.6) for leafy greens, or 30 mL of Glycine Beef Extract (pH 9.5) supplemented with 100 units of pectinase for berries. Prior to elution, 10 µL of MGV stock were added as a process control. Samples were then incubated on a rocking platform at RT for 15 min and clarified by centrifugation at 9,000 × g for 30 min (leafy greens) or 12,000 × g for 15 min (berries). Supernatants were concentrated by ultracentrifugation at 170,000 × g at 4 °C for 60 min (leafy greens) or 45 min (berries). Resulting pellets were resuspended in 280 µL of Glycine buffer for leafy greens. For berry samples, pellets were resuspended in 600 µL of PBS, mixed with 800 µL of chloroform, and centrifuged at 3,000 × g for 5 min at 4 °C. The aqueous phase was collected and either stored at –80 °C or immediately processed for RNA extraction.

c) Aluminum hydroxide adsorption-precipitation method ($AlCl_3$)

Elution and clarification steps were performed following the ISO protocol and further diluted in PBS to a final volume of 100 mL to proceed with the previously described protocol (29,30). Briefly, after adjusting the pH to 6, 1 mL of $AlCl_3$ was added, then the pH was adjusted back to 6 and the mixture was centrifuged at 1,700 × g for 20 min. The resulting pellet was resuspended in 5 mL of 3% beef extract and centrifuged at 4,900 × g for 10 min. Finally, the pellet was resuspended in 1 mL of PBS and either stored at –80 °C or used immediately for RNA extraction.

d) Ultrafiltration (CP)

Elution and clarification steps were performed following the ISO protocol. The pH of eluted samples was adjusted to 7 and then concentrated through ultrafiltration using a CP Select™ Concentrating Pipette device (InnovaPrep, Drexel, MO, USA) with “single-use” hollow fiber Concentrating Pipette Tips (CPT) (Ultra, 100 kDa; cat. CC08003, InnovaPrep) and PBS Elution Buffer (PBS 0.0075% Tween 20; cat. HC08000, InnovaPrep) to obtain a final volume of approximately 1 mL. Concentrated samples were kept at –80 °C until use or directly subjected to RNA extraction.

Quantitative detection of recovered viruses using RT-qPCR
Nucleic acids were extracted from 300 µL of the concentrated samples using the Maxwell RSC Pure Food GMO and Authentication Kit and the Maxwell® RSC 16 instrument (Promega Corporation, Madison, US). Quantification of HAV, HuNoV GI, GII and MGV RNA was performed using RT-qPCR with the UltraSense One Step quantitative RT-PCR kit (Invitrogen SA), using the primers and probes outlined in ISO 15216:2017. When indicated, primers and probes outlined in the FDA protocol were also used. All reactions were conducted using a CFX96™ Real-Time PCR System (Bio-Rad). Occurrence of inhibition was estimated by comparing average viral titers obtained for the process control virus on undiluted RNA and 10-fold diluted RNA. Inhibition was ascertained when difference in average titers was higher than 0.5 log_{10}, and if that occurred, viral titers were inferred from the 10-fold RNA dilution. Standard curves for each target were generated from triplicates of 5 ten-fold serial dilutions of synthetic DNA fragments (gblocks IDT).

Estimation of Limit of Detection (LoD)
Leafy greens and berries were spiked with three different doses: 200 GE/g, 40 GE/g and 8 GE/g, for each of the three viral targets (HAV, HuNoV GI and GII). Three samples were spiked per dose, and each sample was extracted twice and analyzed by ISO and FDA RT-qPCR assays. The LoD95 was calculated using PODLoD v10 calculation program to estimate the POD (probability of detection) function and the LOD of a qualitative microbiological measurement method (31).

Detection of recovered viruses using infectivity assays
Samples were filtered through cellulose acetate membrane filters with pore size 0.22 µm (Corning Costar® Spin-X®). HAV infectivity was determined using the $TCID_{50}$ assay in FRhK-4 cell monolayers in 96-well plates. For HuNoVs, infectivity was tested on HIEs (15). Briefly, samples were two-fold diluted before infecting differentiated (5-7 days) jejunal J2 monolayers in triplicate wells in 2 separate 96-well plates. One plate was immediately frozen at –80 °C (1 hpi) and the second plate was incubated at 37 ºC for 72 hours and then frozen (72 hpi). Infection of each sample was performed in triplicate wells. RNA was extracted from each well using the NucleoSpin™ RNA Virus kit (Macherey-Nagel) and viral GE were quantified by RTqPCR. Samples were considered infectious when mean fold increase in viral GE between 1 and 72 hpi was ≥3.

Data and statistical analysis
Viral recoveries were calculated by dividing the virus recovered from each sample by the total amount of viruses inoculated in each sample, using the formula % Recovery = Measured Recovered Virus /Total Inoculated Virus x 100. Statistical analysis was performed using GraphPad Prism version 10.2.3 and significance was defined as $p < 0.05$.

Results
Comparison of performance of different concentration methods
The performance of the $AlCl_3$ and CP concentration protocols was evaluated in comparison with the ISO and FDA reference methods. Six food matrices were tested—three leafy greens (romaine lettuce, spinach, and baby leaf) and three berry types (strawberries, raspberries, and blueberries)—each inoculated with HAV and HuNoV genogroups I (GI; GI.P11_GI.6) and II (GII; GII.P31_GII.4 Sydney 2012) at 10^5 GE per 25 g for each target virus. As expected, no viral RNA was detected in any uninoculated control samples. In terms of turnaround time, the ISO method required the longest processing time (3 h 15 min), followed by the $AlCl_3$ protocol (2 h), the FDA method (1 h 30 min to 2 h), and the ultrafiltration-based CP method, which was the fastest (1 h 15 min). MGV process-control recoveries were satisfactory across all assays, ranging from 7.14–39.92% for leafy greens and 4.02–62.26% for berries. For leafy greens, overall MGV recoveries were significantly higher with the FDA and CP methods compared with ISO and $AlCl_3$, with no

variation among leafy-green types (**Figure 1**). For berries, CP yielded significantly higher MGV recoveries than all other methods. While the CP protocol showed no differences across berry types, the other three methods exhibited matrix-dependent variability, highlighting the consistent performance of the CP approach across produce categories. Inhibition was observed only in a single strawberry sample processed using the ISO method.

As for recoveries of the three target viruses, the CP method showed better performance than ISO method in 6/6 matrices; better (3/6) or equal (2/6) than FDA; and better (5/6) or equal (1/6) than $AlCl_3$ (**Figure 2A-F**). The $AlCl_3$ method only outperformed ISO method for baby leaves, strawberries and blueberries, and performed equal (3/3) and worse (3/3) than FDA (**Figure 2A-F**). While $AlCl_3$ method showed different recoveries for some viral targets in spinach, baby leaves and strawberries (**Figure 2B-D**), the CP method showed homogeneous recoveries for the 3 target viruses in all 6 matrices. When considering mean recoveries for each of the two produce categories (**Figure 2G-H**), mean recoveries of reference and novel protocols on leafy greens/berries were 14.0/3.5%, 50.9/8.4%, 26.5/24.7%, and 64.1/32.5%, for ISO, FDA, $AlCl_3$ and CP, respectively. Highest recoveries were obtained for FDA and CP methods for leafy greens and for $AlCl_3$ and CP methods for berries. CP showed homogeneous recoveries within each matrix, emphasizing its robustness across viruses with distinct physicochemical properties (mean recoveries of 77.6±22.6% for lettuce, 29.0±11.2% for spinach, 85.8±120.9% for baby leaf, 27.3±8.3% for strawberries, 33.8±7.8% for raspberries, and 36.5±4.0% for blueberries). Importantly, recoveries were particularly interesting for berries, considering that, as opposed to the ISO and FDA protocols, our CP method did not include a chloroform/butanol treatment, which has been shown to be required to eliminate all organic compounds and improve molecular detection. The other alternative protocol compared in the current study, the AlCl3 method, was the workflow with fewer technical challenges and showing a good turnaround time-cost ratio, but displayed a higher matrix-dependent variability (Figure 3), outperforming the FDA method only for strawberries and blueberries, and with mean recoveries of 22.5±6.4% for lettuce, 11.7±9.0% for spinach, 45.2±26.0% for baby leaf, 38.2±24.8 for strawberries, 7.3±8.0% for raspberries, and 28.6±10.1% for blueberries. Altogether these results indicate that the ultrafiltration-based CP concentration method provides satisfactory recoveries for all matrices without requiring ultracentrifugation equipment and allowing virus concentration in a low turnaround time (1h 15 min).

The performance of the ultrafiltration CP method was further evaluated by determining the LoD95 for each virus across two types of leafy greens (romaine lettuce and fresh spinach) and two types of berries (strawberries and blueberries). The resulting LoD95 values were compared with those obtained for both reference methods and are presented in **Table 1**. Except for HAV and HuNoV GII in fresh spinach, the LoD95 values of the CP method were within or below the limits established by the ISO and FDA methods. Overall, LoD95 values for the CP method combined with ISO RTqPCR assays ranged from <8 to 777 GE/g and were consistently lower for romaine lettuce and blueberries than for fresh spinach and strawberries. LoD95 values were generally slightly higher when combined with FDA RTqPCR assays. For the HuNoV GI target, LoD95 values were <8 GE/g across all matrices.

Preservation of viral infectivity using the ultrafiltration CP method

Numerous studies have underscored the limitations of methodologies that rely exclusively on viral RNA detection, thereby emphasizing the imperative to develop analytical approaches capable of verifying the presence of infectious viruses in food, an objective of immeasurable importance. Whether the virus concentration step is coupled with infectivity assays or with capsid-integrity molecular assays, it remains essential to ascertain that the concentration procedure itself does not compromise viral infectivity. We analyzed the effect of the CP concentration method on viral infectivity using the HAV and HuNoV GII (GII.P17/GII.17). Two different stool suspensions were used for leafy greens and berries. Portions of 25 g of leafy greens were spiked with a dose of $1x10^6$ $TCID_{50}$ of HAV or $1.4x10^9$ GE of a 10% stool suspension of HuNoV GII. Portions of 25 g of berries were spiked with a dose of $1x10^6$ $TCID_{50}$ or $8.5x10^6$ GE of HuNoV GII.

After virus concentration using the CP protocol, concentrates were filtered through 0.22 µm and used to infect FRhK-4 cells or HIEs. Infectious HAV was recovered from all types of foods, with mean % recoveries of 43.9±14.3, 39.5±20.0, 77.1±22.0 and 66.8±38.0 for lettuce, spinach, strawberries and blueberries, respectively (**Figure 3**). Infectivity of HuNoV was confirmed by infecting HIEs and observing 25-fold (1.4 log_{10}) and 4,000-fold (3.6 log_{10}) increases in GE titers from 0 to 72 hpi in lettuce and strawberries, respectively (**Figure 4**). Since the sensitivity threshold for the HIE assay is high, ranging between 10^3-10^5 GE/well (15,32–36), our results suggest that a notable proportion of infectious HuNoVs was recovered during the concentration protocol. The use of different stool suspensions for lettuce and strawberries further demonstrates that infectivity is preserved during the process.

Altogether, these results demonstrate that the ultrafiltration-based CP method consistently outperforms or matches the reference methods across multiple vegetable matrices and viral targets, while offering important operational advantages including substantially reduced processing time and avoidance of ultracentrifugation equipment. However, these advantages come at a substantially higher consumable and equipment cost compared with PEG precipitation or aluminum-based workflows. Ultrafiltration concentrates viruses primarily through physical size-exclusion rather than through exposure to harsh chemical conditions or extreme pH, both of which can compromise viral integrity. Moreover, it operates efficiently under neutral pH and does not require the addition of polyvalent salts, which may negatively affect the performance of the cell-culture infectivity assays. Altogether, these results are particularly relevant, given the need to confirm infectivity of viruses on contaminated food products potentially linked to human cases and for risk assessment studies.

Task 1.2. Develop a method using a salivary gland (SG)-derived continuous cell line to detect and quantify infectious HuNoV

The main aim of this task was to study the use and suitability of salivary gland (SG)-derived ductal (NS-SV-TT-DC) continuous cell line for the detection of infectious HuNoV, according to a work published by Ghosh et al (27), which described the ability of this cell line to support replication of two NoV GII strains (GII.4 Sydney and GII.4 77.1) from filtrate fecal samples.

Methods

Virus stocks and cells

Stool specimens containing HuNoV GI.3, GII.2, GII.4, GII.17 and untyped GII from patients with gastroenteritis were obtained from previously characterized outbreaks occurring during 2017-2019 (28) or outbreaks occurring in 2025, provided by the Public Health Agency of Barcelona. Ten percent (wt/vol) suspensions were prepared in PBS, vortexed for 1 min, and further processed using three different processes: (i) filtration as described by Zou et al (37), (ii) serial centrifugation as described by Ghosh et al (27), and (iii) serial centrifugation plus purification of vesicle-cloaked virions using the MagCapture™ Exosome Isolation kit PS (Wako), as described by Ghosh et al (27). NS-SV-TT-DC cells were kindly provided by Prof Altan-Bonnet (NHLBI) and Prof Jay Chiorini (NIDCR) and were maintained in Keratinocyte Growth Medium-2 (KGM-2) Bullet Kit (Lonza, CC-3107).

Infection protocol

Cells were seeded on 96-well plates, in duplicate, and infected when confluent. Briefly, cells were washed twice with PBS and infected with 100 µL of stool sample per well. After a 4-h-incubation at 37 °C, cells were washed twice with PBS and 100 µL of KGM-2 +/- supplementation with 20 µM of ruxolitinib were added. Infection of each sample was performed in triplicate wells. One plate was immediately frozen at –80 °C (4 hpi) and the second plate was incubated at 37 °C for 96 hours and then frozen (96 hpi). RNA was extracted from each well using the NucleoSpin™ RNA Virus kit (Macherey-Nagel) and viral GE were

quantified by RTqPCR. Samples were considered infectious when mean fold increase in viral GE between 1 and 72 hpi was significantly ≥3.

Cytotoxicity assay
Sensitivity of NS-SV-TT-DC cells to toxicity from concentrated berries was determined using the Viability Cell Proliferation Reagent WST-1 (Roche), following the manufacturer's instructions. Cells were processed following the infection protocol using serial 2-fold dilutions of strawberry concentrates obtained by different concentration methods as inoculum. Triplicate wells were included per condition and the experiment was repeated twice. Strawberry concentrates which had been tested negative for all viral targets were used.

Results
Attempts to obtain efficient replication were performed numerously without obtaining robust reproducible results. Total tests included a collection of 16 archived HuNoV stool samples (1 GI.3, 6 GII.2, 4 GII.4, 2 GII.17 and 3 untyped GII) and two fresh samples (GII.17). Three different stool purification methods were tested: (i) filtration (ii) serial, and (iii) serial centrifugation plus purification of vesicle-cloacked virions. Addition of ruxolitinib (ruxo) in the post-infection media, which has been described to enhance replication of HuNoV in 3D HIEs (38), was also explored. Results are summarized in **Table 2**. Unfortunately, despite successful replication was observed in 4/39 challenges, results were not consistent for a specific genotype or a specific experimental condition, and reproducibility of results was low.

Toxicity of food concentrates on the NS-SV-TT-DC cell line was tested by inoculating cells with strawberry concentrates obtained by CP, ISO and FDA methods. Concentrates obtained by the CP method showed a stronger cytotoxicity in this cell line as compared to the ISO and FDA protocols, which only showed toxicity on undiluted concentrates (**Figure 5**). Overall, these results indicate that NS-SV-TT-DC does not offer advantages over the HIE replication system for the assessment of infectious virus occurrence in contaminated foods in real scenarios.

Objective 2. **Optimize capsid-integrity molecular assays to better estimate infectious human norovirus (HuNoV) and hepatitis A virus (HAV).**

Task 2.1. Optimize performance of PMAxx viability RTqPCR assays to improve correlation with infectivity

Capsid-integrity RTqPCR assays based on PMAxx (21,24,39,40) consistently show that complete avoidance of genome amplification is not always achieved, suggesting that assay conditions should still be improved. Efficiency of most tested crosslinkers in preventing amplification of RTqPCR targets is highly affected by amplicon length (22,41), and RTqPCR targets for HAV and HuNoV in current standardized procedures range only between 84-97 bp for FDA protocol and 86-174 bp for ISO protocol. The aim of this task was to develop novel HAV and HuNoV PMAxx viability RTqPCR assays with ×2-4-fold increase in amplicon lengths (ISO-Long) to optimize correlation with infectivity and reduce detection of false positive samples.

Methods
ISO-Long RTqPCR assays
Novel RTqPCR assays were designed to increase amplicon length between ×2-4 fold (ISO-Long) as compared to the reference assays included in the ISO and FDA protocols. Assays used Forward (Fw) primer and probe from ISO protocol and only Reverse (Rv) primer was replaced. For HAV, the new ISO-Long RTqPCR used the previously published Rv primer (42). For HuNoV GI and GII, Rv primers were designed in this project based on previously published primers (43). Confirmation that ISO-Long GI and GII primer sets could react against all different HuNoV genotypes was proved *in silico*. A complete summary of primers

and probes used in this Task, including Long-ISO RTqPCR assays, is shown in **Table 3**. RT-qPCR assays were performed using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) and the One Step PrimeScript™ RT-PCR kit (Takara Bio). To optimize amplification conditions, various temperature gradients, extension times, and combinations of primers and probes were evaluated. For the Long-ISO HAV RT-qPCR assay, Fw primer, Rv primer, and probe concentrations of 625 nM, 1125 nM, and 312 nM, respectively, were used. The temperature program consisted of 1 h at 55 °C, 5 min at 95 °C, and 45 cycles of 15 s at 95 °C, 1 min at 60 °C, and 1 min at 65 °C. For HuNoV ISO-Long assays, Fw primer, Rv primer, and probe concentrations of 500 nM, 900 nM, and 250 nM, respectively, were used. The temperature program consisted of 1 h at 55 °C, 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 2 min at 55 °C. The LoD of each RTqPCR assay was determined by running a series of dilutions synthetic DNA fragments with 4−10 replicates per dilution. Information on the standard curve parameters of the reference and newly developed RTqPCR assays is shown in **Table 4**.

Viral capsid integrity assay

A working solution of PMAxx™ Dye 4 mM (Biotium) was prepared in water. Briefly, samples were placed in DNA LoBind 1.5 mL tubes (Eppendorf), and the PMAxx™ was added to 300 µL of each sample at 100 µM final concentration along with 0.5% Triton 100-X and then incubated in the dark at RT for 30 min at 150 rpm. Later, samples were photoactivated for two periods of 15 min (with a 15-min incubation at RT between them) using a Led-Active Blue system (GenIUL), and nucleic acid extraction was performed using the Maxwell® RSC instrument (Promega), following the manufacturer's instructions. The samples were ultimately eluted in a final volume of 100 µL.

Validation experiments

The suitability of PMAxx capsid-integrity assays was initially assessed using HAV stocks and HuNoV GII stool suspensions inactivated by heat treatment. Samples were ten-fold diluted in PBS and subjected to heat inactivation at 99 °C for 5 min, while infectious controls were kept at RT for the same duration. Then, performance of HAV, HuNoV GI and GII PMAxx RTqPCR assays were validated to be used on produce sample concentrates obtained following the Innovaprep CP method developed in Task 1.1. Two types of leafy green CP concentrates were used to validate PMAxx RTqPCR assay for HAV, and fresh strawberry CP concentrates were used to validate PMAxx RTqPCR assays for HuNoV GI and GII.

Results

The use of the newly developed PMAxx RTqPCR assays based on ISO-Long amplicons was preliminary validated for HAV and GII using infectious and heat-inactivated viruses (5 min at 99 °C) diluted in PBS (**Figure 6**). For both targets, the use of ISO-Long RTqPCR assays resulted in complete absence of signal in samples containing inactivated virions, confirming its improvement compared to combining PMAxx with ISO or FDA RTqPCR assays, which are based on a very short amplicon.

Further validation of the technique was performed by mixing infectious and inactivated viruses in different produce samples concentrated using the Innovaprep CP method developed in Task 1.1 (**Figure 7**). For HAV, an overall better performance to discriminate infectious and inactivated viruses was observed for ISO-Long PMAxx RTqPCR assay, especially in spinach, where the assay resulted in a complete absence of signal (**Figure 7A-B**). ISO PMAxx RTqPCR assay allowed a better discrimination than FDA. Similar results were observed for HuNoV GI and GII, with no differences observed between ISO and FDA, and a better discrimination power for Long-ISO PMAxx RTqPCR assay, again resulting in complete absence of signal in most cases (**Figure 7C-D**). Overall, reduction in false positives or even complete loss of signal was obtained in samples containing heat-inactivated viruses for all 3 viral targets.

Task 2.2. Develop a specific RT-Loop-mediated isothermal amplification (RT-LAMP) viability assay

In RT-LAMP, the amplification of the genetic material of the virus occurs at a constant temperature and, therefore, tests based on RT-LAMP can be carried out anywhere with basic resources, as they only require a heat block or a water bath set to a single temperature. In addition, end-point colorimetric readouts are also possible through the detection of reaction by-products, enabling results to be evaluated by naked eye, making it easier to adapt for *in situ* applications. In this project, the suitability of RT-LAMP assays to replace standard RTqPCR molecular assays was explored.

Methods

Development of RT-LAMP assays for HAV, HuNoV GI and GII

Development of assays included the comparison of several previously described primers for HAV (44), HuNoV GI (45,46), and HuNoV GII (45–47); the comparison of three different mastermixes (Superscript IV RT-LAMP Master Mix, Invitrogen #A51801; WarmStart RT-LAMP Kit, NEB #E1700S; and WarmStart Colorimetric RT-LAMP Kit, NEB #M1800S); and the comparison of two different readout methods (end-point colorimetric versus SYTOTM 9 real-time fluorescent detection). The reaction mixture was incubated using a CFX96™ Real-Time PCR System (Bio-Rad) at 65 °C for 30 min, 95 °C for 2 min, and then at 4 °C until further analysis. Performance was tested using RNA extracts obtained from cell culture-adapted HAV stocks and HuNoV GI and GII clinical specimens, and synthetic double-stranded gBlock DNA molecules. All reactions included one reaction without RNA/DNA template as a negative control.

Results

For HuNoV, the LoD of RT-LAMP was higher for GII than for GI (**Table 5**), but in all cases, higher than the LoD observed for RTqCR assays. A minimum of $1.6x10^4$ GE/rxn and $3.8x10^2$ GE/rxn were required to provide a positive result for HuNoV GI and GII, respectively. Despite many attempts to lower the LoD of the assays, they were not comparable to the LoD observed for any of the RTqCR assays (**Table 4**), indicating that RT-LAMP assays would not align with industry acceptance criteria. Similar results were obtained for HAV, with LoD significantly higher than RTqPCR assays.

Objective 3. Investigate persistence of infectious, capsid-protected genome equivalents (GE), and total GE HuNoV and HAV on harvested lettuce and berries under different post-harvest conditions.

Task 3.1 Enteric virus die-off rate on berries and leafy greens in post-harvest storing conditions

Methods

Virus stocks and cells

The cell-adapted cytopathogenic HAV strain pHM175 43c was propagated and titrated by $TCID_{50}$ infectivity assay in FRhK-4 cells as described in Task 1.1. Stool specimens containing HuNoV GI.3 and GII.17 from patients with gastroenteritis were obtained from previously characterized outbreaks (28). Ten percent (wt/vol) suspensions were prepared in PBS, vortexed for 1 min, clarified at 2,000 × g for 15 min, and aliquoted before storage at –80 °C. HAV, HuNoV GI, and HuNoV GII titers were quantified by RTqPCR following ISO 15216-1:2017 guidelines. Murine Norovirus (MNV) was not included in the original proposal, but it was added due to its relevance as a surrogate for HuNoV and its wide use in the literature. MNV-1 was propagated and titrated by $TCID_{50}$ infectivity assay in RAW 264.7 cells (both kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA). When indicated, viruses were inactivated by incubating them under UV light for 15 min.

Stability experiments during storage

Locally purchased strawberries (*Fragaria × ananassa*), raspberries (*Rubus idaeus*), romaine lettuce (*Lactuca sativa*) and spinach (Spinacia oleracea) were used in the study. Dilutions of viruses in PBS were inoculated onto the surfaces of each 25 g portion of fresh produce by distributing 100-150 µL over 10-15 spots. For berries experiments, inoculation doses were of 4.05×10^5 TCID50/25 g of HAV, 5.74×10^6 GE/25 g for HuNoV GI, 8.55×10^6 GE/25 g for HuNoV GII, and 2.06×10^5 TCID50/25 g of MNV. For leafy green experiments, inoculation doses were of 4.37×10^5 TCID50/25g of HAV, 1.44×10^9 GE/25 g for HuNoV GII, and 2.06×10^5 TCID50/25 g of MNV. Inoculated samples were air-dried in a laminar flow hood for 60 min. Batches of three samples were stored at 4 °C and 21 °C and analyzed at the following time points: 0, 1, 4 and 7 days. For strawberries and blueberries, batches of three samples were also stored at –20 °C during 2h, 2 days, 30 days and 90 days. Each experiment included one inoculated sample which served as a negative control.

Virus extraction and titration

Viruses were recovered from produce using the Innovaprep CP method optimized in Task 1.1. HAV, HuNoV GI and HuNoV GII targets were quantified by molecular methods comparing the 3 different RTqPCR methods (ISO, FDA and ISO Long) either using RTqPCR alone or in combination with PMAxx. Quantification of infectious HuNoV GII was performed using HIEs, and infectious HAV and MNV were quantified by $TCID_{50}$ assays on FRhK-4 and RAW264.7 cells, respectively.

Data and statistical analysis

Virus survival was calculated as $\log_{10}$ (Nt/N0), where N0 is the virus titer recovered from fresh berries and Nt is the virus titer recovered at various time intervals after storage. Statistical analysis was performed using GraphPad Prism version 10.2.3. ANOVA tests (Kruskal-Wallis) were used to compare titers versus initial data; Student t-test (unpaired) was used to compare titers obtained by different methods on the same sample. Correlation analysis was performed to compare the level of reduction measured by the infectivity assay and the molecular assays. Significance was defined as $p < 0.05$.

Results

Stability of infectious viruses on berries stored at 4 °C and 21 °C

Persistence of HAV, MNV, HuNoV GI and HuNoV GII viruses at 4 °C and 21 °C as measured by infectivity assays is shown in **Figure 8**. These temperatures were selected to represent cold storage and to mimic survival of viruses during berries growth at 21 °C. Data on HuNoV GI infectivity could not be measured due to lack of sensitivity of the HIE assay for this particular genotype. As expected, all three targets, HAV, MNV and HuNoV GII, were more stable at 4 °C as compared to 21 °C (**Fig 8A-B** and **8E-F** vs **8C-D** and **8G-H**). Viruses were also more stable on blueberries than on strawberries. HAV only showed a weak but significant reduction in viral titers on strawberries after 4 days at 4 °C (0.6±0.3 $\log_{10}$) and 21 °C (1.0±0.4 $\log_{10}$), but not on blueberries, showing a high stability. MNV infectivity was also stable through 7 days on blueberries but showed a significant log reduction of 2.1±0.3 at 4 days at 21 °C and 2.2±0.3 at 7 days on strawberries. HuNoV GII infectivity using HIEs was more efficient on strawberries than on blueberries; data also showed a higher stability on blueberries than strawberries, where it significantly decreased at 7 days at 4 °C and at 4 days at 21 °C.

Stability of infectious viruses on berries during frozen storage (–20 °C)

Persistence of viruses was also investigated during freezing up to 90 days as measured by infectivity assays, and results are shown in **Figure 9**. All three targets (HAV, MNV and HuNoV GII) showed a high stability for the whole period, with only HAV significantly reducing infectivity after storage on frozen strawberries for 90 days (2.5±0.2 $\log_{10}$), but not on blueberries.

Correlation between molecular and infectivity assays on berries

Stability of viruses on strawberries at the three tested temperatures was also characterized using capsid-integrity molecular assays developed in Task 2.1 Samples were extracted with and without PMAxx treatment and quantitated using the three RTqPCR assays (ISO, FDA and ISO-Long). Results are shown in **Figure 10**. Although not consistent for the tested temperatures, PMAxx pretreatment allowed discrimination of viruses with damaged capsids at the latest timepoints, especially at low temperatures of 4 °C (**Figure 10A**) and –20 °C (**Figure 10C**), but not at 21 °C (**Figure 10B**). Reductions of viral genome concentrations when compared to time 0 were especially marked at 21 °C for HuNoV GII and HAV (**Figure 10B**), but this effect was not observed for HuNoV GI, which showed higher stability when tested for all three methods.

To assess with precision the correlation between molecular and infectious measurements, linear regression analyses were performed using log reductions obtained by the different molecular assays and infectivity measurements. This approach was done for HAV, and results are shown in **Figure 11**. For experiments which did not involve a freezing step (4 °C and 21 °C), results indicate that addition of PMAxx increases correlation parameters as compared to RTqPCR alone, confirming its use as to improve infectivity estimates (**Figure 11A-B**). R^2 coefficients were slightly stronger for PMA_FDA (0.98) and PMA_ISOLong (0.96) assays. For experiments involving a freezing step (–20 °C), correlation parameters were not good (**Figure 11C-D**), indicating a high dependance on the mechanism underlying virus loss of infectivity.

Altogether these results indicate that capsid-integrity molecular assays provide an improvement over RTqPCR assays alone, but their correlation with infectivity titers is not universal for all mechanisms underlying virus inactivation, suggesting that despite they may reduce detection of false positive signals in naturally contaminated samples, this may only represent a low proportion of cases.

Stability of viruses on leafy greens stored at 4 °C and 21 °C

Persistence of HAV, MNV, HuNoV GI and HuNoV GII was also investigated on two types of leafy greens stored at 4 °C and 21 °C. HAV and MNV infectious viruses were determined by infectivity assays, and HAV and HuNoV GII were also measured by capsid-integrity molecular detection using Long-ISO RTqPCR assays (**Figure 12).** For HuNoV, spiking dose was of 1.4×10^9 GE/25g. Die-off of HAV infectivity showed a similar pattern on lettuce and spinach, with reductions at 7 days ranging between 0.46-0.67 log at 4 °C and 0.50-0.63 log at 21 °C (**Figure 12 A-D**). MNV, however, showed a different behavior on lettuce and spinach, with 1.04-1.21 log reductions on lettuce and no reduction at all on spinach (**Figure 12 A-D**). Detection of viral genomes through capsid integrity RTqPCR assay (Long ISO), confirmed the ability to significantly reduce quantification of non-infectious viruses at 7 days (**Figure 12 E-L**). As opposed to MNV, HuNoV GII stability was similar on lettuce and spinach.

Die-off rate of viral genomes coming from inactivated viruses on strawberries

Because most virological analyses of food samples rely on direct RTqPCR, the persistence of viral genomes from inactivated viruses on contaminated foods became a relevant question during the project. Although this aspect was not included in the initial proposal, additional experiments were conducted to assess and compare the stability of viral genomes from infectious and inactivated viruses on berries. For this purpose, both infectious and UV-inactivated viruses (HAV, HuNoV GI, and HuNoV GII) were spiked onto 25 g portions of strawberries at the following doses: 8.1×10^4 $TCID_{50}$/25 g for HAV, 1.15×10^6 GE/25 g for HuNoV GI, and 1.71×10^6 GE/25 g for HuNoV GII. Sets of three samples were stored at 21 °C and analyzed at 0-, 4-, and 7-days post-contamination. Levels of viral genomes were determined using either ISO RTqPCR or ISO-Long RTqPCR. Estimates of T_{90} values (time required for viral titer to decrease by 90%) from these results (**Figure 13**) indicate that decay of UV-inactivated viruses is faster than for infectious viruses (approx. 2-fold difference in T90 when using ISO RTqPCR) (**Table 6**). T90 values ranged between 7.8-9 days

for infectious viruses and 4.0-4.9 days for genomes from UV-inactivated viruses. Increases in amplicon size allowed an even faster assessment of genome decay, suggesting a better correlation with genome degradation.

Task 3.2 Efficacy of chlorine, chlorine dioxide and peracetic acid washes for viral disinfection of leafy greens

Methods

Virus stocks and cells (see Task 3.1)

Batch scale disinfection experiments

Locally purchased romaine lettuce (*Lactuca sativa*) and spinach (*Spinacia oleracea*) were used in the study. Dilutions of viruses in PBS were inoculated onto the surfaces of each 25 g portion of fresh produce by distributing 100-150 µL over 10-15 spots. Inoculation doses were of 4.37×10^5 $TCID_{50}$/25 g of HAV, 1.44×10^9 GE/25g for HuNoV GII, and 2.06×10^5 $TCID_{50}$/25g of MNV. Inoculated samples were air-dried in a laminar flow hood for 60 min. Leafy greens were washed with different disinfectants at the following concentrations: chlorine dioxide (ClO_2, 2.37 ppm, pH 4.42), free chlorine (FC, 21.3 ppm, pH 5.43), and peracetic acid (PAA, 81 ppm, pH 3.78). Unwashed samples and samples washed with water alone were included as controls. Washing step used 240 mL of wash water per 25 g of product for 5 min.

Virus extraction and titration (see Task 3.1)

Results

Efficacy of washes to eliminate infectious viruses

Data on viral infectivity are presented in **Figure 14**. PAA showed slightly higher efficacy against HAV for both types of vegetables, although the average viral reduction was of 0.77±0.16 log compared to unwashed samples. No significant differences were observed for any of the washing procedures for MNV on lettuce (**Figure 14A**), nor for HuNoV GII on HIEs either (data not shown). Despite the lack of statistical differences for any of the disinfectants, overall log reductions observed for MNV were higher than for HAV, suggesting a higher susceptibility to disinfectants: 2.15±0.85 for ClO_2, 1.70±1.1 for FC and 1.28 ±0.35 for PAA. Results on spinach could not be satisfactory obtained for MNV due to technical difficulties in titrating spinach concentrates in RAW264.7 cells.

Correlation between infectivity assays and capsid-integrity molecular assays

Performance of the different molecular RTqPCR assays used to quantify remaining genomes for HuNoV GI and HuNoV GII and HAV, with and without PMAxx pretreatment is shown in **Figure 15**. Viral genome concentrations measured by RTqPCR assays including PMAxx pretreatment to reduce detection of inactivated viruses were slightly lower than for those without PMAxx pretreatment, but differences were not significant, suggesting that none of the disinfectants had produced a large impact on viral capsid integrity. Similar to section 3.1, a close correlation analysis was performed for HAV between log reductions measured by molecular assays and those determined by infectivity assays, but no significant correlation was observed for any type of assay, suggesting that neither PMAxx pretreatment nor the increase in the amplicon length improved correlation with infectivity to assess the efficacy of disinfection treatments.

Outcomes and Accomplishments

1. Overall, the project provided critical data on (i) optimized and user-friendly methods for the detection and quantification of HuNoV and HAV in leafy greens and berries, (ii) the development of capsid-integrity RT-qPCR assays to better approximate viral infectivity, and (iii) the persistence and die-off of infectious HAV and NoV under post-harvest storage conditions, as well as the efficacy of different sanitizers for viral inactivation.
2. The study demonstrated that methodological factors, including the concentration approach used, significantly influence virus recovery and detection. The Concentrating Pipette (CP)-based method showed good performance across matrices, reduced turnaround time, eliminated the need for ultracentrifugation, and preserved viral infectivity, making it suitable for routine applications. Importantly, the performance characteristics of the CP method were directly compared to current ISO and FDA standard methods, demonstrating that it matches or outperforms these reference protocols while offering operational advantages for routine testing.
3. To improve infectivity estimation using molecular tools, novel PMAxx-based capsid-integrity RTqPCR assays employing extended amplicon lengths (ISO-Long) were developed for HAV, HuNoV GI, and HuNoV GII. These assays enhanced suppression of signal from heat-inactivated virions and reduced false positive detection compared to ISO and FDA standard assays. However, their correlation with infectivity was context-dependent and did not extend to disinfection assessments. RT-LAMP assays evaluated for HAV and HuNoV were insufficiently sensitive for industry or regulatory applications.
4. Comprehensive persistence studies demonstrated that viral stability is strongly matrix- and temperature-dependent. Infectious HAV, HuNoV GII, and MNV exhibit substantial stability on produce under post-harvest conditions. Viruses persisted longer at 4 °C than at 21 °C, and stability was consistently higher on blueberries than strawberries. Freezing at −20 °C for up to 90 days did not significantly reduce infectivity for most viruses, highlighting the resilience of enteric viruses in frozen supply chains. Additionally, persistence of viral genomes from inactivated viruses on contaminated foods was also investigated, finding that genomic decay of UV-inactivated viruses occurred approximately twice as fast as that of infectious viruses.
5. The results confirmed that sanitizing treatments using FC, ClO_2, and PAA can reduce viral contamination in leafy greens, although their efficacy varies depending on the virus and overall inactivation rates were very low. PAA showed higher efficacy against HAV (≈0.8 log reduction), while no significant differences were observed among disinfectants for MNV (HuNoV surrogate), suggesting that disinfectant selection should consider the target virus.
6. Importantly, the study highlights that current molecular and viability PCR approaches, including capsid-integrity assays, provide an improvement over RTqPCR alone but still have limitations in reliably predicting viral infectivity, particularly after disinfection treatments. Their correlation with infectivity titers is not universal for all inactivation mechanisms, and while they may reduce false-positive signals in naturally contaminated samples, these cases likely represent only a small proportion. Therefore, caution should be exercised when interpreting molecular detection results, and complementary infectivity assays are recommended for accurate risk evaluation.

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APPENDICES

Publications and Presentations

Publications *(in preparation)*

A El Boubekri, I Girón-Guzmán, MI Costafreda, A Blanco, C Fuentes, AM Aparicio, A Diaz-Reolid, RM Pintó, A Bosch, A Pérez-Cataluña, G Sanchez and S Guix. **Enhanced virological analysis of leafy greens and berries using an ultrafiltration method.** *To be submitted to International Journal of Food Microbiology.*

Method for extraction, concentration and quantification of hepatitis A virus and norovirus using an ultrafiltration-based method coupled with capsid-integrity real-time RT-PCR. *SOP in preparation to be published in protocols.io.*

A El Boubekri, I Girón-Guzmán, MI Costafreda, A Blanco, C Fuentes, AM Aparicio, A Diaz-Reolid, RM Pintó, A Bosch, A Pérez-Cataluña, G Sanchez and S Guix. **Stability of enteric viruses in berries and leafy greens during freezing and storage.** *Manuscript in preparation to be submitted to Foods.*

Presentations

- This work has been presented at the annual Center for Produce Safety's Research Symposium in Denver 2024 (poster) and in San Diego 2025 (2 min talk and poster).
- A report highlighting the project was published in the CPS e-newsletter in January 2025 (https://www.centerforproducesafety.org/resources-library/research-seeks-more-efficient-accurate-virus-detection)
- This work was presented at the European IAFP Symposium on Food Safety, as a poster in Madrid 6-8 May 2025. Authors: A El Boubekri, A Diaz-Reolid, MI Costafreda, A Blanco, C Fuentes, AM Aparicio, I Girón-Guzmán, RM Pintó, A Bosch, A Pérez-Cataluña, G Sanchez and S Guix. Title: Enhanced virological analysis of leafy greens and berries using an ultrafiltration method.
- This work was presented as a 10-min oral presentation at the IX INSA-UB Workshop in Barcelona, November 2025. Authors: A El Boubekri, A Diaz-Reolid, MI Costafreda, A Blanco, C Fuentes, AM Aparicio, I Girón-Guzmán, RM Pintó, A Bosch, A Pérez-Cataluña, G Sanchez and S Guix. Title: Enhanced virological analysis of leafy greens and berries using an ultrafiltration method.
- Final results will be presented at the 2026 CPS Research Symposium in Nashville, TN.

Budget Summary

This project received a total of $326,230 in research funds, and all funds were spent to implement the project successfully ($175,474 by University of Barcelona, and $150,756 by IATA-CSIC).

Tables 1–6 and Figures 1–15 (see below)

Table 1. Limits of detection at a 95% confidence (LoD_{95}) for each extraction/concentration method expressed in genome equivalents (GE)/g, using ISO and FDA RTqPCR protocols for detection.

		HAV			HuNoV GI			HuNoV GII		
	Concentration	ISO	FDA	CP	ISO	FDA	CP	ISO	FDA	CP
ISO RTqPCR	Romaine Lettuce	156.0	<8[a]	29.9	13.3	<8[a]	<8[a]	436.0	35.2	13.3
	Fresh Spinach	121.0	110.0	293.0	13.3	21.1	<8[a]	178.0	178.0	777.0
	Strawberries	221.0	22.4	106.0	18.4	<8[a]	<8[a]	94.4	<8[a]	63.7
	Blueberries	178.0	178.0	29.9	13.5	<8[a]	<8[a]	197.0	178.0	<8[a]
FDA RTqPCR	Romaine Lettuce	227.4	21.1	51.8	<8[a]	<8[a]	<8[a]	860.9	120.7	66.8
	Fresh Spinach	287.6	146.4	848.2	<8[a]	<8[a]	<8[a]	196.6	237.0	4150.9
	Strawberries	452.3	214.3	51.8	<8[a]	<8[a]	<8[a]	<8[a]	<8[a]	<8[a]
	Blueberries	130.9	18.41	16.96	<8[a]	<8[a]	<8[a]	13.3	<8[a]	<8[a]

[a] Estimated values. It was not possible to determine values because the LoD was not reached.

Table 2. Results of HuNoV replication in NS-SV-TT-DC cell line.

NoV Sample	Genotype	Stool purification method	Ruxo	Log_{10} GE/well (4 hpi)	Log_{10} GE/well (96 hpi)	Fold increase	Replication
NOV63	GI.3	Filtration	-	4.28 ± 0.18	3.19 ± 0.13	-0.08	Neg
H557	GII.2	Filtration	-	3.61 ± 0.35	3.43 ± 0.04	0.56	Neg
		Centrifugation	-	3.61 ± 0.35	3.76 ± 0.03	2.60	Neg
		Centrifugation	**+**	**3.88 ± 0.02**	**4.29 ± 0.31**	**3.31**	**Pos***
		Centrifugation/ vesicle purif.	+	3.12 ± 0.40	2.71 ± 0.00	0.27	Neg
NOV57	GII.2	Centrifugation	-	<LOD	<LOD	NA	NA
		Centrifugation	+	<LOD	<LOD	NA	NA
NOV64	GII.2	Centrifugation	-	<LOD	<LOD	NA	NA
		Centrifugation	+	<LOD	<LOD	NA	NA
DPL	GII.2	Centrifugation	-	3.23 ± 0.10	3.90 ± 0.09	4.66	Neg
		Centrifugation	+	3.57 ± 0.18	3.87 ± 0.30	2.33	Neg
MGP	GII.2	Centrifugation	-	2.25 ± 0.27	2.66 ± 0.11	2.25	Neg
		Centrifugation	**+**	**2.83 ± 0.09**	**3.25 ± 0.54**	**5.64**	**Pos***
AMV	GII.2	Centrifugation	-	4.06 ± 0.09	4.00 ± 0.08	0.88	Neg
		Centrifugation	+	4.16 ± 0.14	4.31 ± 0.15	1.43	Neg
NOV71	GII.4	Filtration	-	3.56 ± 0.15	3.49 ± 0.26	0.91	Neg
ICP	GII.4	Centrifugation	-	3.00 ± 0.12	3.28 ± 0.19	2.04	Neg
		Centrifugation	**+**	**2.42 ± 0.17**	**3.86 ± 0.10**	**26.50**	**Pos***
REG	GII.4	Centrifugation	-	1.90	1.78	0.77	Neg
		Centrifugation	**+**	**<LOD**	**2.53 ± 0.12**	**19.77**	**Pos**
IHM	GII.4	**Centrifugation**	**-**	**2.31 ± 0.47**	**3.35 ± 0.08**	**5.88**	**Pos***
		Centrifugation	+	2.99 ± 0.26	3.40 ± 0.14	2.26	Neg
NOV6	GII.17	Filtration	-	5.07 ± 0.28	5.32 ± 0.06	1.54	Neg
		Centrifugation	-	6.17 ± 0.15	6.27 ± 0.17	1.28	Neg
		Centrifugation/ vesicle purif.	+	3.42 ± 0.27	3.92 ± 0.07	2.80	Neg
NOV29	GII.17	Filtration	-	5.19 ± 0.02	5.36 ± 0.16	1.53	Neg
		Centrifugation	-	4.08 ± 0.04	3.97 ± 0.08	0.78	Neg
		Centrifugation	+	4.29 ± 0.05	4.28 ± 0.08	1.00	Neg
A1.1	GII.17	Filtration	-	3.00 ± 0.21	2.63 ± 0.03	0.38	Neg
		Filtration	+	2.93 ± 0.16	2.76 ± 0.26	0.78	Neg
		Centrifugation	-	3.52 ± 0.02	3.18 ± 0.08	0.38	Neg
		Centrifugation	+	3.42 ± 0.02	3.32 ± 0.18	0.85	Neg
B1.2	GII.17	Filtration	-	3.70 ± 0.09	3.41 ± 0.10	0.52	Neg
		Filtration	+	3.79 ± 0.21	3.56 ± 0.02	0.54	Neg
		Centrifugation	-	4.00 ± 0.14	3.68 ± 0.13	0.47	Neg
		Centrifugation	+	4.04 ± 0.10	3.96 ± 0.14	0.84	Neg
HVH667	GII	Filtration	-	4.62 ± 0.16	4.38 ± 0.08	0.56	Neg
HVH668	GII	Filtration	-	4.73 ± 0.08	4.66 ± 0.10	0.87	Neg
HVH687	GII	Filtration	-	4.59 ± 0.07	4.58 ± 0.03	0.98	Neg

Ruxo: ruxolitinib; GE: genome equivalents; LOD: Limit of detection of the assay (1.25 Log_{10} GE/well); NA: Not available; * significant increase from 4h to 96h (unpaired t-test; $p<0.05$)

Table 3. Primer and probe sequences for RTqPCR assays used in this study to detect HAV, HuNoV GI and GII.

RTqPCR	Virus	Primer /Probe	Sequence 5'-3'	Position	Size (pb)
ISO	HAV	HAV68 (Fw) HAV240 (Rv) HAV150(-) (P)	TCACCGCCGTTTGCCTAG GGAGAGCCCTGGAAGAAAG FAM-CCTGAACCTGCAGGAATTAA-MGB	68-241[a]	174
	GI	QNIF4 (Fw) NV1LCR (Rv) TM9 (P)	CGCTGGATGCGNTTCCAT CCTTAGACGCCATCATCATTTAC FAM-TGGACAGGAGATCGC-MGBNFQ	5291–5376[b]	86
	GII	QNIF2 (Fw) COG2R (Rv) QNIFs (P)	ATGTTCAGRTGGATGAGRTTCTCWGA TCGACGCCATCTTCATTCACA FAM-AGCACGTGGGAGGGCGATCG-TAMRA	5012–5100[c]	89
FDA	HAV	GAR2F GAR1R GARP	ATAGGGTAACAGCGGCGGATAT CTCAATGCATCCACTGGATGAG FAM-AGACAAAAACCATTCAACGCCGGAGG-BHQ1	448-508[a]	90
	GI	COG1F COG1R COGP	CGYTGGATGCGNTTYCATGA CTTAGACGCCATCATCATTYA FAM-AGATYGCGATCYCCTGTCCA-BHQ1	5291–5376[b]	86
	GII	COGP1b COG2F COG2R	FAM-AGATCGCGGTCTCCTGTCCA-BHQ1 CARGARBCNATGTTYAGRTGGATGAG TCGACGCCATCTTCATTCACA	5003-5100[c]	98
ISO-Long	HAV	HAV68 (Fw) HAVRvLong HAV150(-) (P)	TCACCGCCGTTTGCCTAG GCCGCTGTTACCCTATCCAA FAM-CCTGAACCTGCAGGAATTAA-MGB	68-463[a]	396
	GI	COG2P E_RevShort TM9 (P)	FAM-TGGGAGGGCGATCGCAATCT-BHQ1 TTCCCAACCCANCCRTTRTACAT FAM-TGGACAGGAGATCGC-MGBNFQ	5291-5674[b]	384
	GII	QNIF2 (Fw) G_RevShort QNIFs (P)	ATGTTCAGRTGGATGAGRTTCTCWGA CCACCTGCATANCCRTTRTACAT FAM-AGCACGTGGGAGGGCGATCG-TAMRA	5012-5389[c]	378

[a] Positions according to HAV HM175 reference strain (Genbank M14707).
[b] Positions according to Norwalk virus GI reference strain (Genbank M87661).
[c] Positions according to Lorsdale virus GII reference strain (Genbank X86557).

Table 4. Parameters of the standard curves and limit of detection (LoD, genomic copies per reaction, GE/rxn) for the different RTqPCR assays.

Target	Assay	Amplicon length (bp)	Slope	Intercept	Efficiency	R^2	LoD (GE/rxn)
HAV	ISO	174	-3.456	39.805	94.7 %	0.999	12.0
	FDA	90	-3.315	38.532	100.3 %	0.994	3.2
	ISO-Long	396	-3.383	39.394	97.5 %	0.996	3.4
GI	ISO	86	-3.317	39.441	100.2 %	0.997	7.4
	FDA	85	-3.240	41.006	103.5 %	0.995	86.2
	ISO-Long	384	-3,830	43,792	82,42 %	0.982	69.8
GII	ISO	89	-3.607	41.173	89.3 %	0.994	19.7
	FDA	98	-3.280	39.549	101.8 %	0.999	88.9
	ISO-Long	378	-3,616	40.59	90.1 %	0.998	87.8

Table 5. Limit of Detection of RT-LAMP assay for HuNoV GI and GII.

	GI		GII	
	GE/rxn	**RT-LAMP**	**GE/rxn**	**RT-LAMP**
RNA	1.6×10^4	+	3.8×10^4	+
	1.6×10^3	-	3.8×10^3	+
	1.6×10^2	-	3.8×10^2	+
	1.6×10^1	-	3.8×10^1	-
gBlock	2.0×10^7	+	2.0×10^7	+
	2.0×10^6	+	2.0×10^6	+
	2.0×10^5	+	2.0×10^5	+
	2.0×10^4	-	2.0×10^4	+
	2.0×10^3	-	2.0×10^3	+
	2.0×10^2	-	2.0×10^2	-

Table 6. T90 values (days) of HAV, HuNoV GI and HuNoV GII genomes on strawberries at 21 °C, as measured by direct RTqPCR assay (ISO and ISO-Long).

	ISO RTqPCR			**ISO-Long RTqPCR**		
	HAV	**HuNoV GI**	**HuNoV GII**	**HAV**	**HuNoV GI**	**HuNoV GII**
Infectious	9.0	8.9	7.8	9.0	9.1	8.0
UV-inactivated	4.6	4.9	4.0	2.0	ND	3.3

ND: Not determined

Figure 1. Recovery of Mengo virus (MGV) process control from different types of leafy greens **(A)** and berries **(B)** using 4 concentration methods. Plotted values are means of 3-4 repetitions ± standard deviation. Asterisk (*) denotes significant differences between food items using the same method ($p < 0.05$, Kruskal-Wallis test). Capital letters above indicate significant differences in mean recoveries between methods ($p < 0.05$, One-way ANOVA).

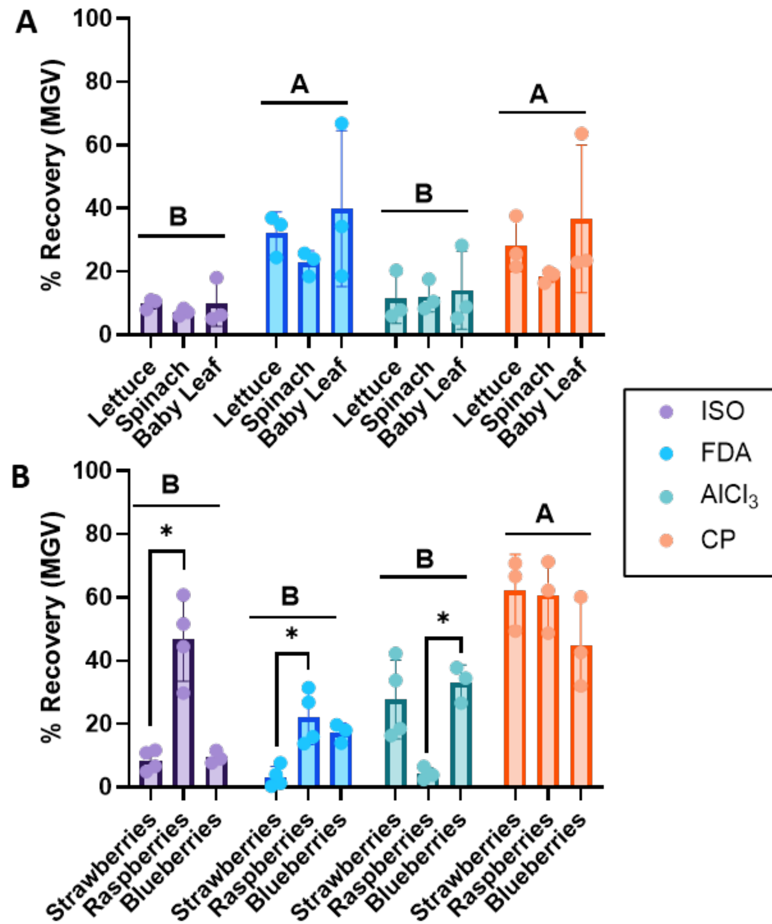


Figure 2. Recovery of HAV, HuNoV GI and HuNoV GII (10^5 GE/25 g) from fresh romaine lettuce **(A)**, spinach **(B)**, baby leaf **(C)**, fresh strawberries **(D)**, raspberries **(E)** and blueberries (**F**), using the reference ISO and FDA methods, the $AlCl_3$ precipitation and ultrafiltration CP methods. Plotted values are means ± standard deviation (n = 3). Asterisk (*) denotes significant differences between targets using the same concentration method ($p < 0.05$; Kruskal-Wallis test). Capital letters above indicate significant differences in mean recoveries between methods ($p < 0.05$, One-way ANOVA test). **(G)** Box plot of mean recovery for all types of leafy greens and all viral targets for each of the 4 methods. Whiskers indicate 10-90 percentiles. Different letters above whiskers indicate significant differences ($p < 0.05$; One-way ANOVA test). **(H)** Box plot of mean recovery for all types of berries and all viral targets for each of the 4 methods. Whiskers indicate 10-90 percentiles. Different letters above whiskers indicate significant differences ($p < 0.05$; One-way ANOVA test).

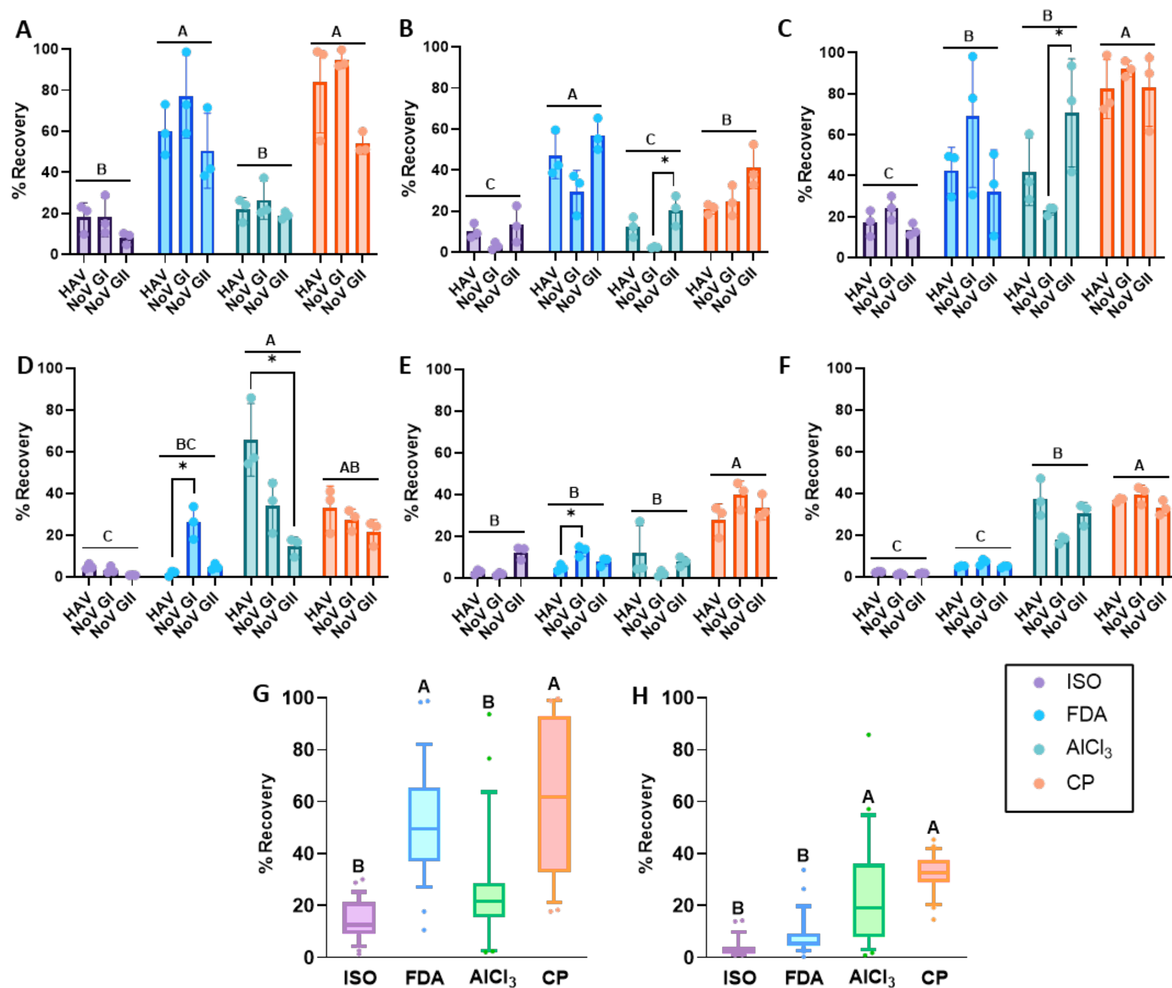


Figure 3. Recovery of HAV from different food matrices, using the CP ultrafiltration method, as measured by cell-culture $TCID_{50}$ assay. Box plot include mean recoveries from 4-8 replicates. Whiskers indicate 10-90 percentiles.

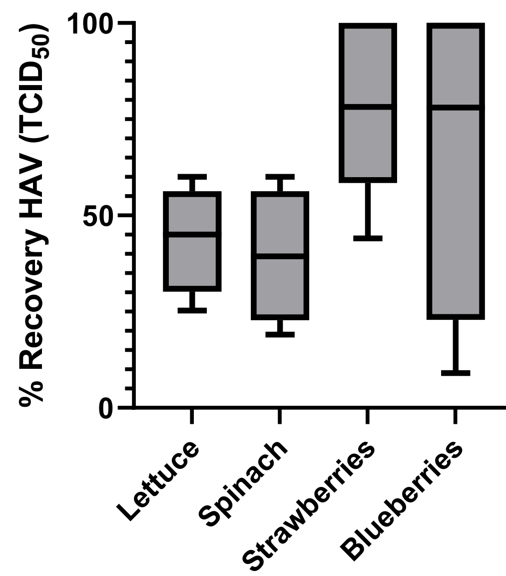


Figure 4. Recovery of HuNoV GII from Romaine lettuce and strawberries, using the CP ultrafiltration method, as measured by HIEs infectivity assay. Plot indicate titers of GE per well at 1 hpi and 72 hpi of each individual sample. Numbers above each set of samples indicate average fold increase (log_{10}).

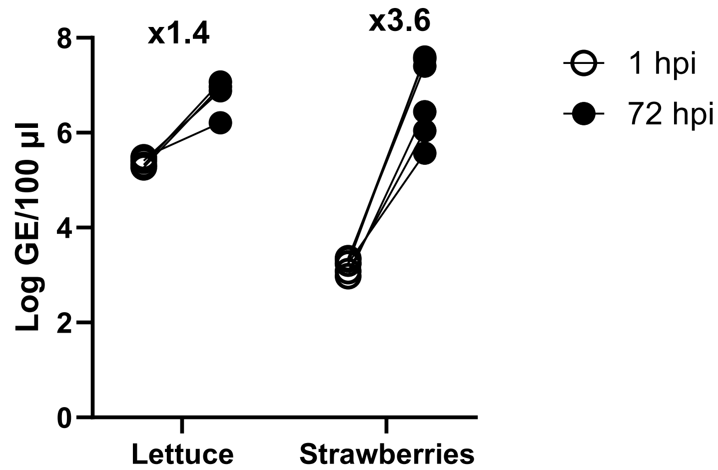


Figure 5. Effect of berry concentrates obtained by the ISO, FDA or CP concentration methods on NS-SV-TT-DC cell viability. Dotted line indicates the maximum level of cytotoxicity after treating cells with 1% Triton X-100.

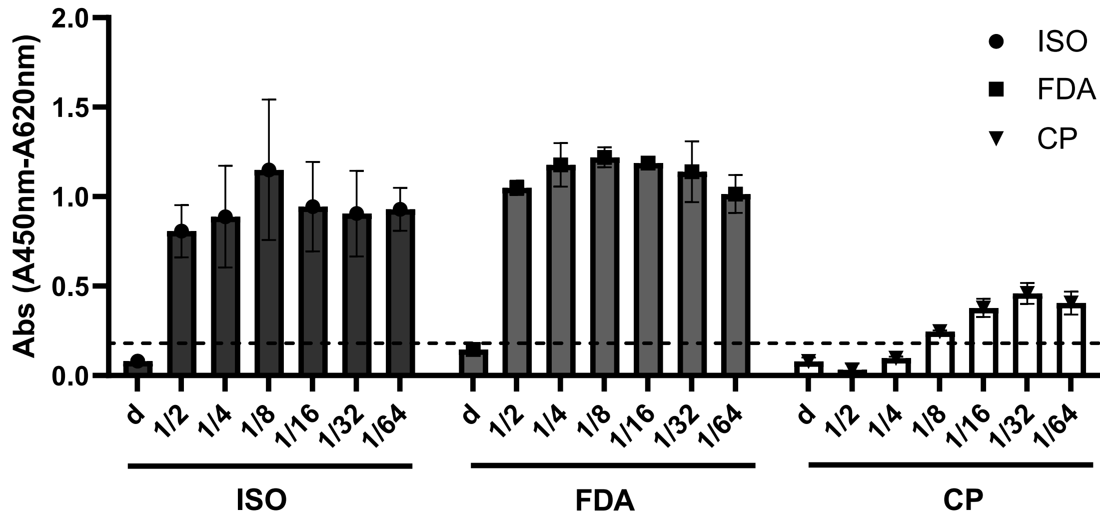


Figure 6. Validation of novel PMAxx capsid-integrity assays for HAV **(A)** and HuNoV GII **(B)** on infectious and heat-inactivated viruses (5 min at 99 °C) diluted in PBS, using the three RTqPCR assays based on ISO, FDA and ISO-Long primers/probes. Numbers above bars denote $\log_{10}$ reductions between infectious and inactivated samples. Dotted line indicates the LoD of the ISO-Long RTqPCR assays.

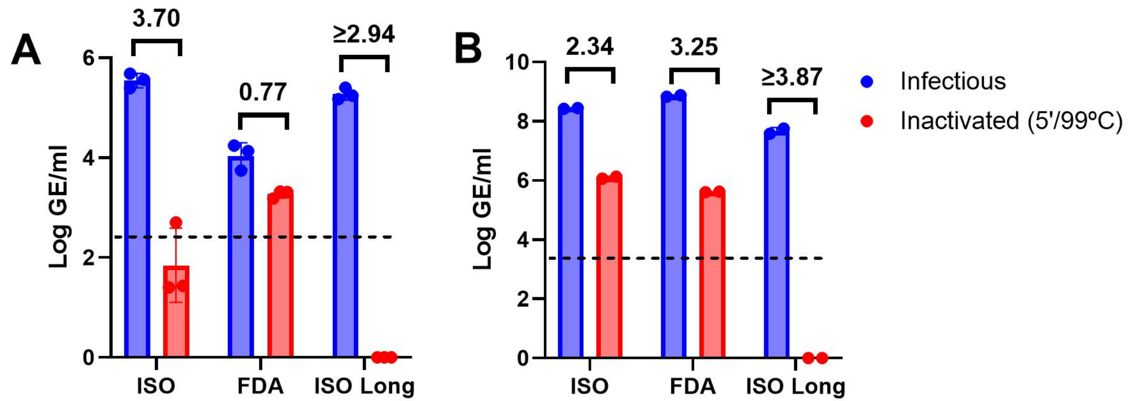


Figure 7. Performance of PMAxx viability RTqPCR to discriminate between infectious and inactivated (5 min at 99 °C) HAV on lettuce **(A)** and spinach **(B)**, HuNoV GI on strawberries **(C)** and HuNoV GII on strawberries **(D)**, using the ISO RTqPCR assay, the FDA RTqPCR assay and the modified ISO-Long RTqPCR assays. Number above bars indicate the mean $\log_{10}$ reduction. Dotted line indicates the LoD of the ISO-Long RTqPCR assays.

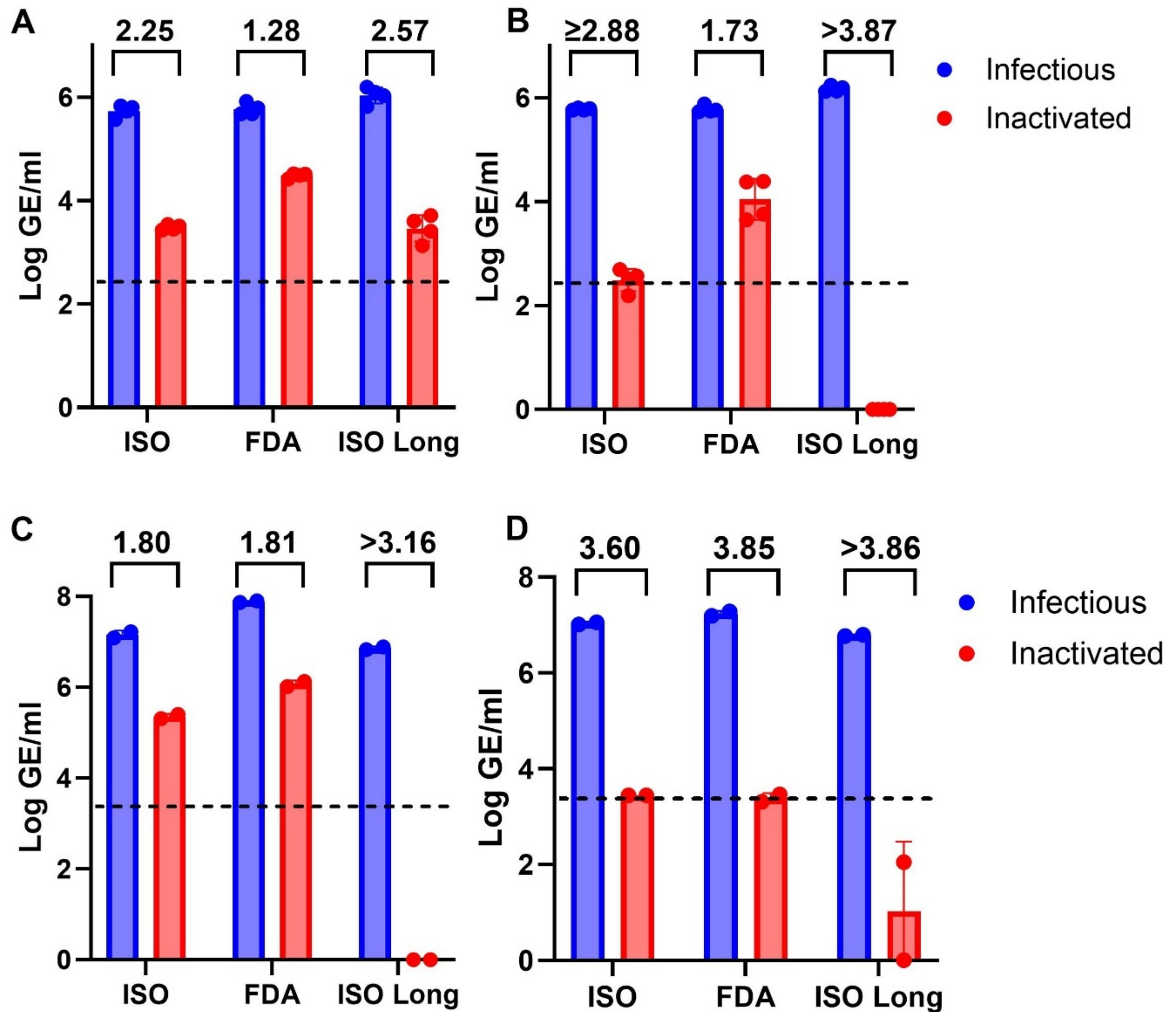


Figure 8. Persistence of infectious HAV, MNV and HuNoV GII on strawberries **(A-D)** and blueberries **(E-H)** stored at 4 °C (**A-B** and **E-F**) and 21 °C (**C-D** and **G-H**) up to 7 days. **(A, C, E, G)** HAV and MNV infectivity as measured by $TCID_{50}$ assays on FRhK-4 and RAW264.7 cells, respectively. **(B, D, F, H)** HuNoV GII infectivity as measured by Human Intestinal Enteroids assay. Black shading indicates genome equivalents (GE) per well at 1 hpi and grey at 72 hpi. Values above the bars represent the number of replicas showing viral replication as measured by a ≥3-fold (0.47 log_{10}) increase in viral titer from 1h to 72h. Asterisk (*) denotes significant differences (Kruskal-Wallis) as compared to samples analyzed at 0 days ($p < 0.05$).

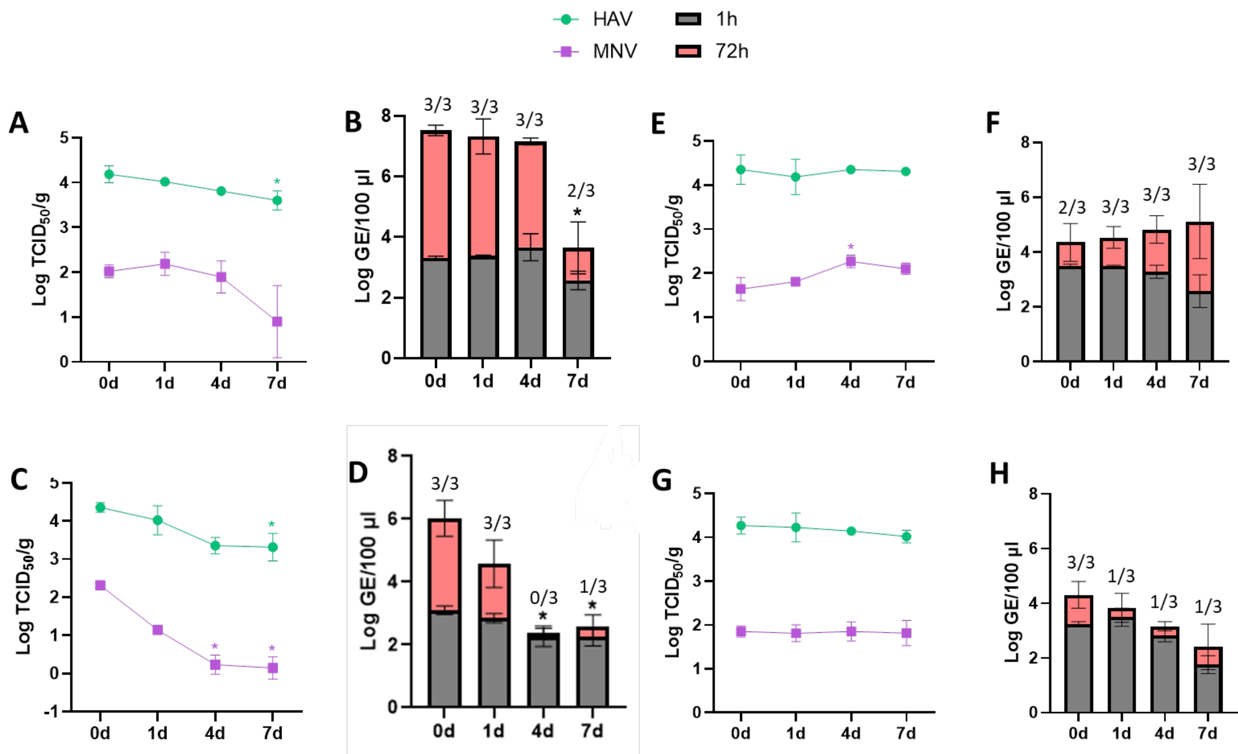


Figure 9. Persistence of infectious HAV, MNV and HuNoV GII on strawberries **(A-B)** and blueberries **(C-D)** stored at –20 °C up to 90 days. **(A, C)** HAV and MNV infectivity as measured by $TCID_{50}$ assays on FRhK-4 and RAW264.7 cells, respectively. **(B, D)** HuNoV GII infectivity as measured by Human Intestinal Enteroids assay. Black shading indicates genome equivalents (GE) per well at 1 hpi and grey at 72 hpi. Values above the bars represent the number of replicas showing viral replication as measured by a ≥3-fold (0.47 log_{10}) increase in viral titer from 1h to 72h. Asterisk (*) denotes significant differences (Kruskal-Wallis) as compared to samples analyzed at 0 days without freezing (WTF) ($p < 0.05$).

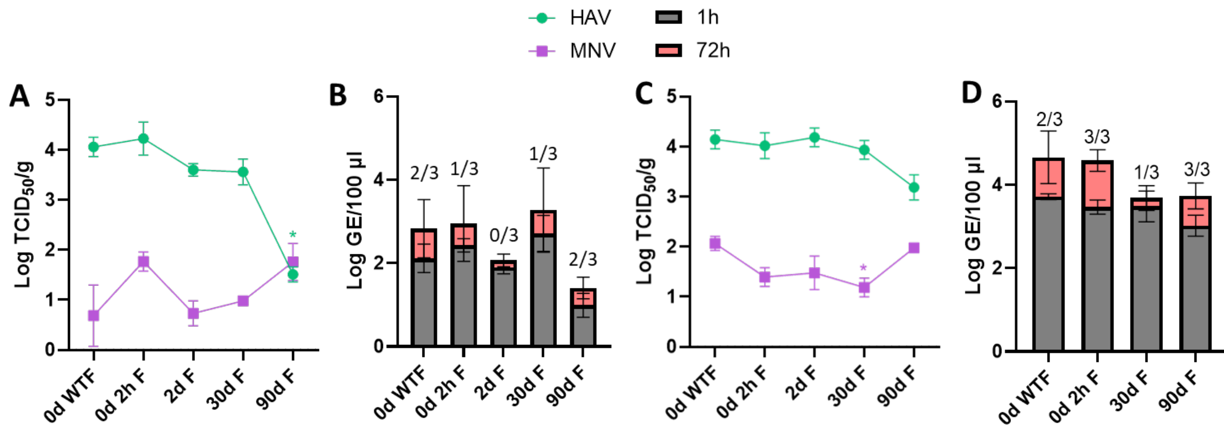
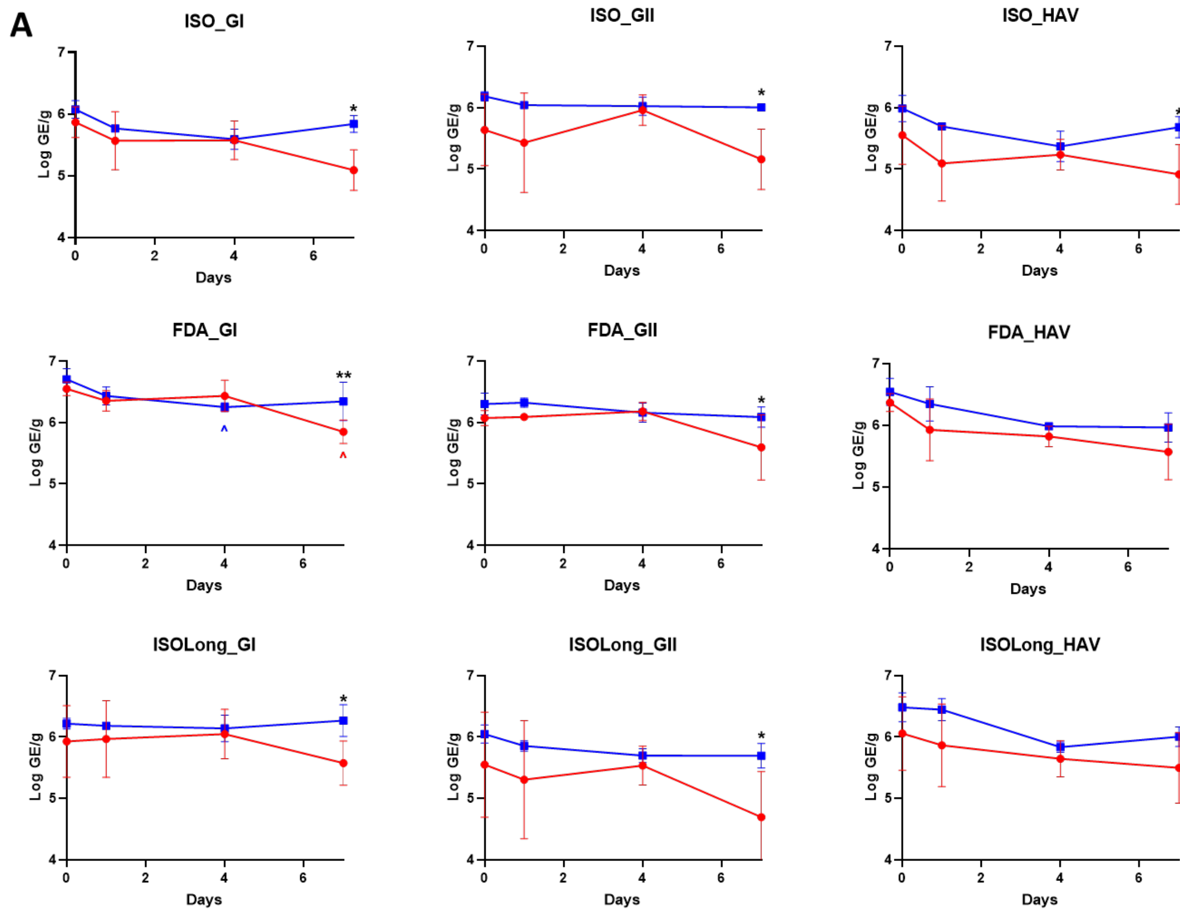
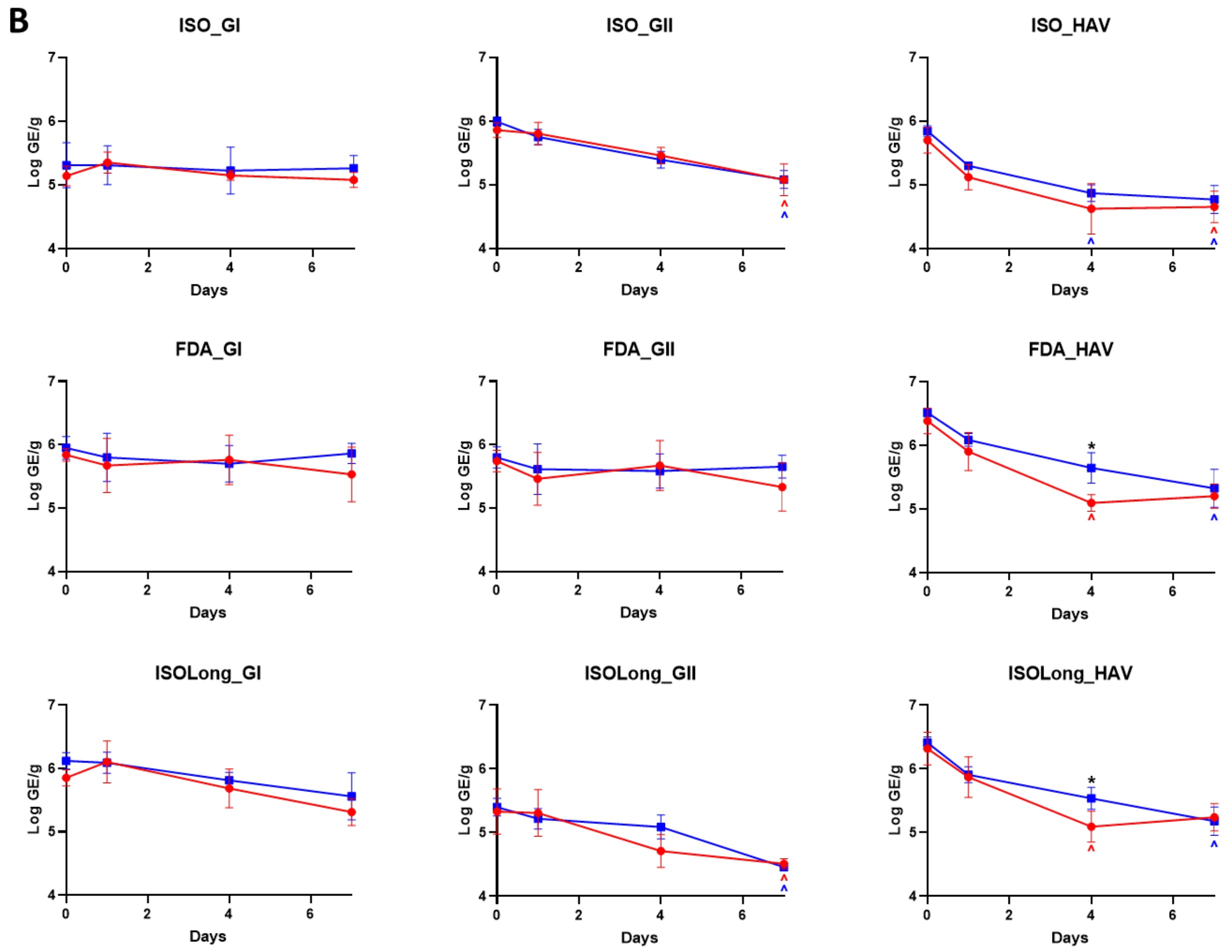


Figure 10. Persistence of HuNoV GI, HuNoV GII and HAV on fresh strawberries stored at 4 °C **(A),** 21 °C **(B)** and –20 °C **(C)** using molecular RTqPCR assays based on ISO, FDA and ISOLong primers, either with (red) or without (blue) PMAxx pretreatment. Data are shown as mean and error bars represent standard deviation. Black asterisk (*) indicates significant differences (Kruskal-Wallis) due to PMAxx pretreatment for each time point. Red and blue symbols (^) indicate significant differences versus samples analyzed at 0 days (One-way ANOVA, $p < 0.05$).

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ISO_GI
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Log GE/g
Days

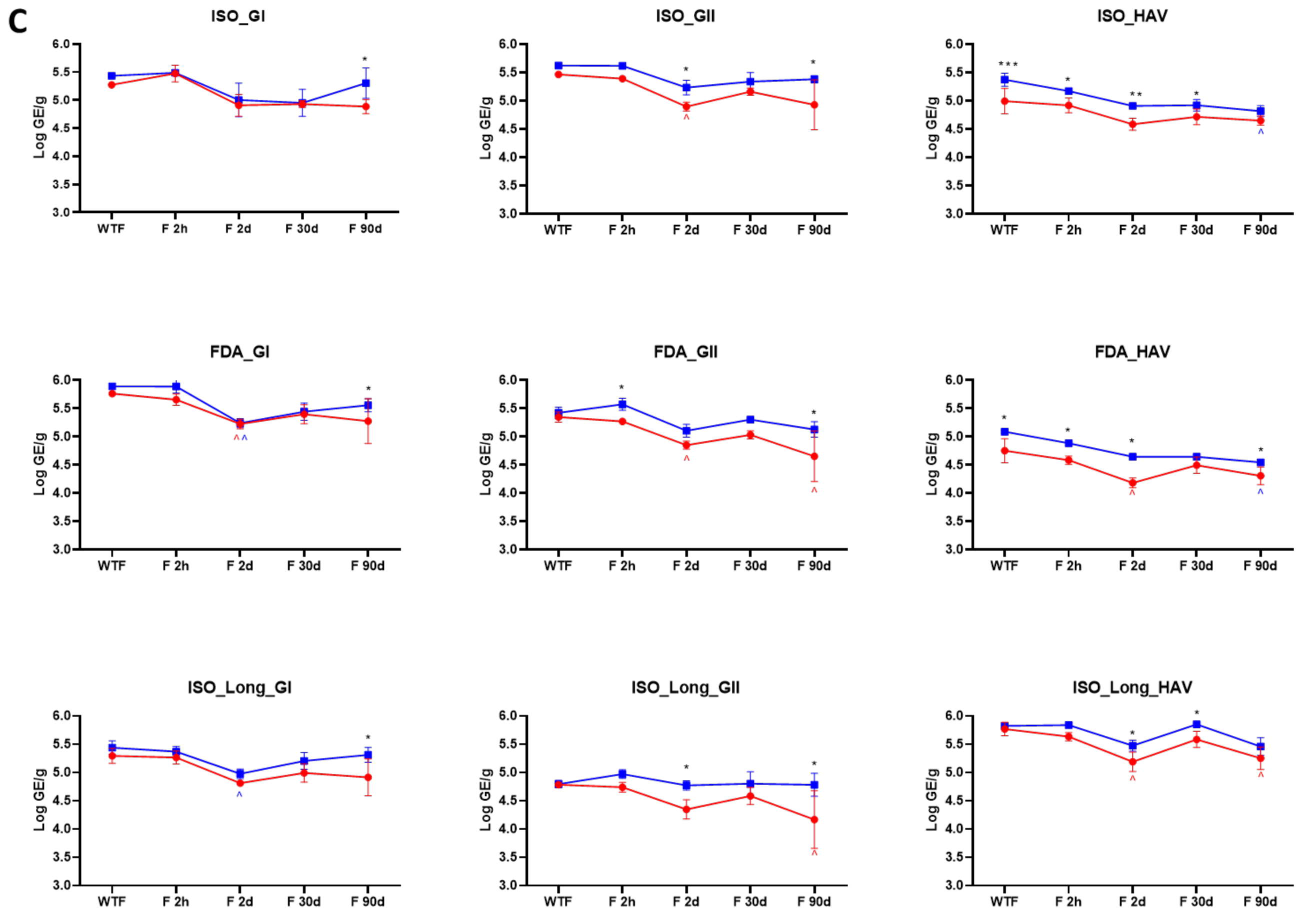
C
ISO_GI
ISO_GII
ISO_HAV
FDA_GI
FDA_GII
FDA_HAV
ISO_Long_GI
ISO_Long_GII
ISO_Long_HAV
Log GE/g
6.0
5.5
5.0
4.5
4.0
3.5
3.0
WTF
F 2h
F 2d
F 30d
F 90d

Figure 11. Correlation analysis between HAV log reductions measured by molecular RTqPCR assays (ISO [blue], FDA [orange] and ISOLong [red]) either RTqPCR alone **(A, C)** or in combination with PMAxx assay **(B, D)**, and infectivity assays on strawberries. Panels **A-B** correspond to experiments performed at 4 °C and 21 °C, and panels **C-D** to –20 °C.

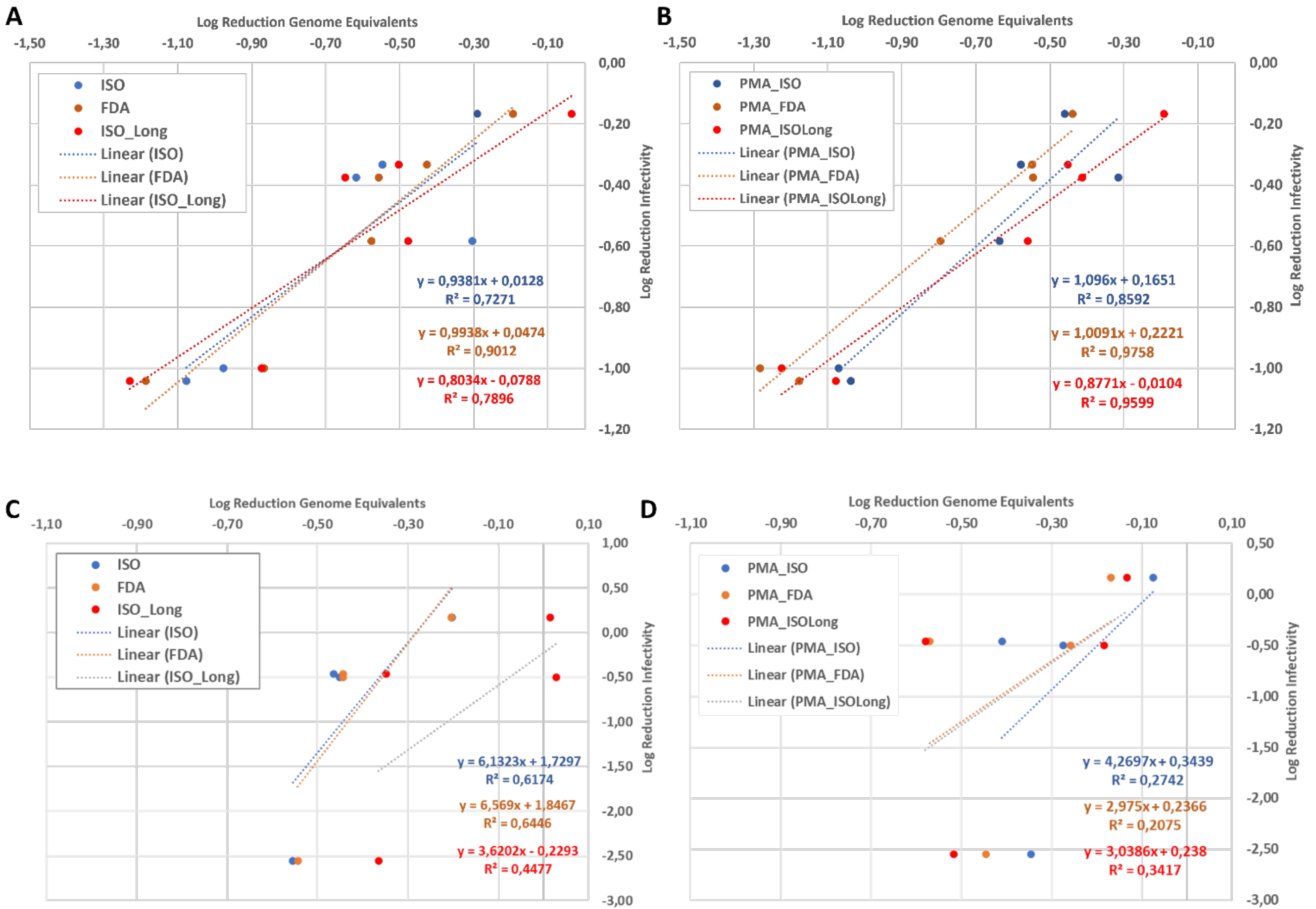

Figure 12. Persistence of HAV, MNV and HuNoV GII on leafy greens. **(A-D)** Survival of infectious HAV and MNV on lettuce **(A, B)** and spinach **(C, D)** stored at 4 °C **(A, C)** and 21 °C **(B, D)** up to 7 days. HAV and MNV infectivity as measured by $TCID_{50}$ assays on FRhK-4 and RAW264.7 cells, respectively. Colored asterisk (*) denotes significant differences (Kruskal-Wallis) as compared to samples analyzed at 0 days ($p < 0.05$). **(E-L)** Stability of viral genomes determined by capsid-integrity assays using ISO-Long RTqPCR, for HAV **(E-H)** and HuNoV GII **(I-L)**. Black asterisk (*) indicate significant differences (Kruskal-Wallis) due to PMAxx pretreatment for each time point. Red and blue symbols (^) indicate significant differences versus samples analyzed at 0 days (One-way ANOVA, $p < 0.05$).

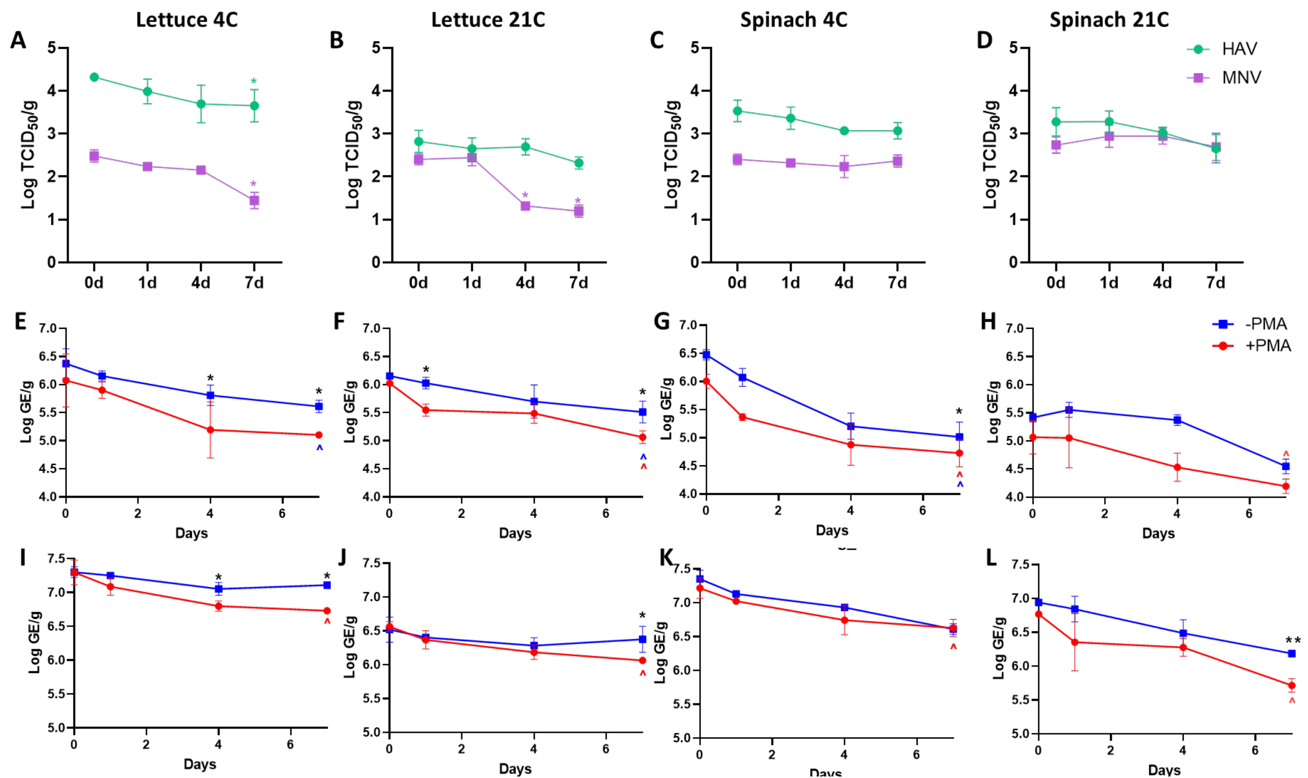

Figure 13. Persistence of viral genomes from infectious (blue) of UV-inactivated viruses (red) on strawberries stored at 21 °C, as measured by RTqPCR using ISO primers **(A-C)** or ISO-Long primers developed in the Project **(D-F)**. Viral targets included HAV **(A, D)**, HuNoV GI **(B, E)** and HuNoV GII **(C, F)**. Dotted line indicates the LoD of the RTqPCR assays.

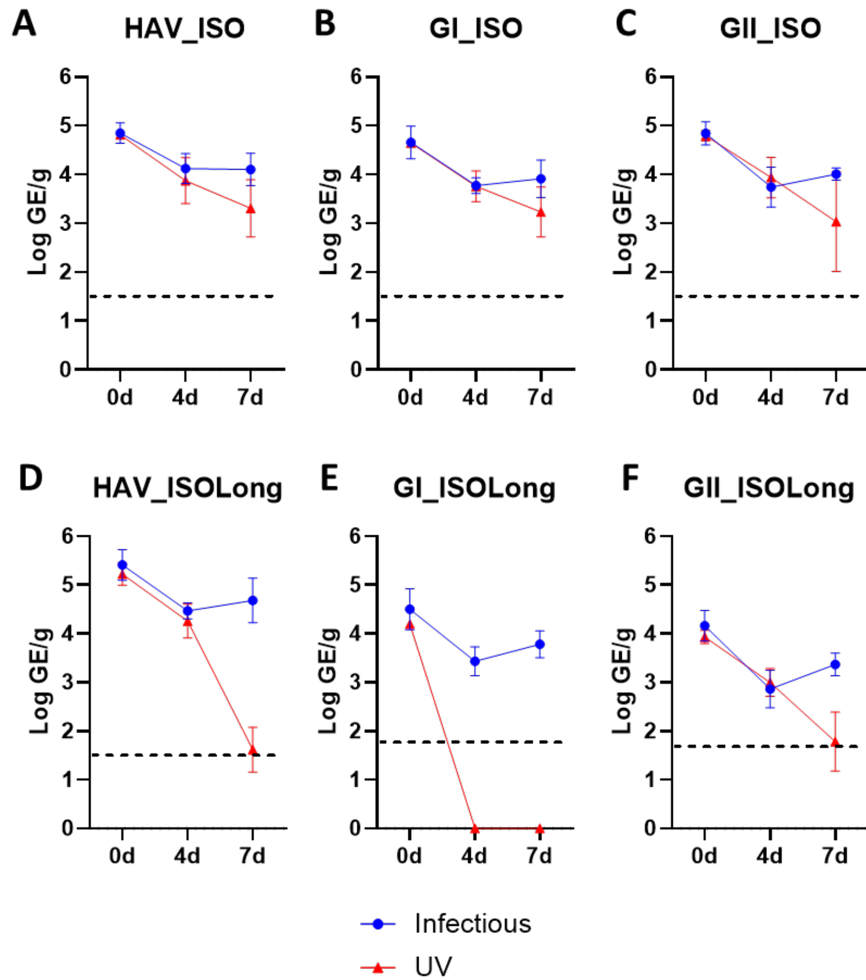

Figure 14. Effect of washes and disinfectants on infectious HAV and MNV on lettuce **(A)** and spinach **(B)**. Box plots represent data from 2 experiments for lettuce and 1 experiment for spinach, each containing 3 biological replicates. Asterisk (*) denotes statistical differences (Kruskal-Wallis test, $p < 0.05$).

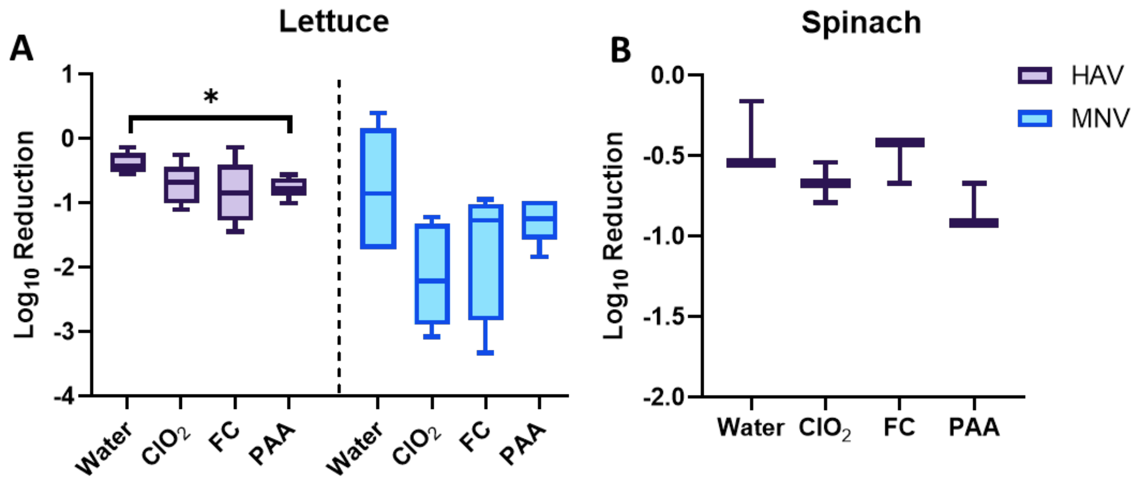

Figure 15. Performance of different molecular RTqPCR assays based on ISO, FDA and ISO-Long with and without PMAxx treatment, to quantify HAV (A, B), HuNoV GI (C, D) and HuNoV GII (E, F) levels on lettuce **(A, C, E)** and spinach **(B, D, F)** washed with ClO_2, FC and PAA, as compared to infectivity assays on lettuce (**G, H**).