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High pressure treatment and green tea extract synergistically control enteric virus contamination in beverages

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ABSTRACT

Consumers are driving food production toward the use of natural preservatives and minimal processing technologies. Green tea extract (GTE) at low concentration could be combined with high pressure processing (HPP) for reduced treatment times and quality impact on foods in a hurdle concept for synergistic effects on foodborne viral pathogens, specifically human norovirus and hepatitis A virus (HAV). Viral inactivation by HPP (at 300, 400, and 500 MPa for 5 min) combined with 3.3 mg/mL aged-GTE was initially evaluated in buffer (PBS) against murine norovirus (MNV), a culturable human norovirus surrogate, and HAV. Furthermore, human norovirus inactivation was evaluated by the novel human intestinal enteroid system (HIE) and a capsid integrity binding assay (ISC-RT-qPCR). HPP treatment completely inhibits human norovirus GII.4 infectivity when applied at 500 MPa alone and at 400 MPa combined with aged-GTE. Additional experiments investigated the reduction of MNV and HAV infectivity in apple and horchata juices exposed to combined aged-GTE and HPP treatments. Results demonstrated that the addition of aged-GTE to the juices exposed to HPP significantly inactivated MNV and HAV at reduced holding pressure time. This synergistic effect of aged-GTE combined with HPP treatments represents a hurdle technology that could be exploited as a control measure to improve the food safety of beverages.

1. Introduction

The consumption of vegetables and fruits is strongly advised to reduce the risk of diseases and functional declines (Temple, 2000; Willett, 1994), and nowadays juices, smoothies, and beverages are the most common consumers' choice for their intake. However, human norovirus and HAV can survive on vegetables and fruits for prolonged period, thus produce retains a moderate to relevant risk of infection as it is generally consumed fresh or only mildly processed (Escudero et al., 2012; Sun et al., 2012). Moreover, vegetables, fruits and products thereof are adversely affected by common thermal treatments which severely alter active biological components and sensory properties (Koskiniemi et al., 2013; Zang et al., 2013).

The use of natural extracts exerting antimicrobial activity has been adopted in food production as most of them are generally recognized as safe (GRAS), approved by food safety agencies, and confidently accepted by consumers. Green tea extract (GTE), a derivative of the tea plant (*Camellia sinensis* L.) with a high content of polyphenolic compounds (e.g., catechins), is considered to be safe (ANS et al., 2018), and has demonstrated antimicrobial activity against a wide range of pathogens including human enteric viruses (Falcó et al., 2019; Seo et al., 2016). Interestingly, controlled storage conditions (24 h at 25 °C) significantly increased GTE antiviral activity (referred to as aged-GTE), likely due to the increasing accumulation of catechin derivatives (Falcó et al., 2018).

In the last decades, high hydrostatic pressure processing (HPP), a nonthermal processing technique, has emerged as a promising technology to preserve a variety of food products, such as fruit jams, orange juice, salsa, ready-to-eat meats, and oysters. In addition, inactivation studies demonstrated HPP effective to control viral pathogens, including HAV and human norovirus surrogates (Govaris & Pexara, 2021; Huang et al., 2016; Kingsley et al., 2007; Pan et al., 2016; Sánchez et al., 2011).

In the absence of a robust *in vitro* cell culture system for human norovirus (Manuel et al., 2018; Moore et al., 2015), the effect of HPP has been demonstrated by conducting clinical trial studies (Leon et al., 2011) or using gnotobiotic pig models (Fangfei et al., 2015). To overcome ethical issues and generate a large amount of dataset, most recent research relies on surrogates, such as murine norovirus (MNV), feline calicivirus (FCV) and Tulane virus (TV) (Cannon et al., 2006; Chen et al., 2005; Cromeans et al., 2014; Li, Ye, et al., 2013). However, the actual inactivation of human norovirus cannot be directly predicted using

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surrogates without introducing bias (Li et al., 2013).

Alternatively, approaches based on capsid integrity coupled with quantitative reverse transcription real-time PCR (RT-qPCR) have been reported, including porcine gastric mucine (PGM) assays to selectively bind infectious human norovirus (Dancho et al., 2012; Li & Chen, 2015). However, this technique provides an indirect estimation for viral reduction that may not exactly resemble actual infectivity.

Only very recently, a novel three-dimensional (3D) cell culture technique based on non-transformed stem cell-derived human intestinal enteroids (HIEs), which recapitulate the complexity and cell diversity of the gastrointestinal tract has been reported to be permissive towards human norovirus infection (Costantini et al., 2018; Ettayebi et al., 2016). However, the HIE system is still at the dawn of its application in food and environmental virology field (Estes et al., 2019; Overbey et al., 2021).

To understand and effectively manipulate various HPP control parameters for the maximum reduction of viral contamination in juices, the aim of this study was to (i) evaluate different HPP (at 300, 400 or 500 MPa for 5 min) and aged-GTE treatments, individually and in combination, on HAV, MNV and human norovirus infectivity; and, (ii) kinetically define the synergistic antiviral effect of HPP treatments and aged-GTE in apple and horchata juices contaminated with HAV and MNV.

2. Materials and methods

2.1. Virus strains, cell lines and clinical samples

MNV-1 (kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA) and HAV strain HM-175/18f (ATCC VR-1402) were propagated and assayed respectively in RAW 264.7 (also provided by Prof. H.W. Virgin) and FRhK-4 cells (ATCC CRL-1688) as previously described (Falcó et al., 2018). Infectious viruses were enumerated by determining the 50% tissue culture infectious dose ($TCID_{50}$/mL) in 96-well microtiter plates with eight wells per dilution and 20 μL of inoculum per well using the Spearman-Karber method.

Human GII.4 Sydney[P16] and GI.4 norovirus-positive fecal samples were kindly provided by Prof. Buesa (University of Valencia, Spain) and tested using human intestinal enteroids or *in situ* capture RT-qPCR (ISC-RT-qPCR) assay, respectively. A fecal filtrate of human norovirus GII.4 Sydney[P16] was prepared by passing clarified stool extracts through serial filters (5, 1, 0.45, and 0.22 μm) as described previously (Costantini et al., 2018) and stored at −80 °C in aliquots until the time of testing on HIE.

Ten percent suspension of GI.4 norovirus-positive fecal sample was prepared in PBS containing 2M $NaNO_3$ (Panreac), 1% beef extract (Conda), and 0.1% Triton X-100 (Fisher Scientific) (pH 7.2), vortexed and centrifuged at 1000×*g* for 5 min. The supernatant was stored at −80 °C in aliquots.

2.2. Beverages, green tea extract, and high-pressure processing treatments

Two commercial beverages, J1 (apple juice, pH 3.75) and J2 (horchata, pH 4.10) were obtained from a local supermarket. Horchata drink is a traditional beverage from Valencia (Spain) made with soaked, ground, and sweetened tiger nuts.

GTE powder (Naturex SA, France) was dissolved in PBS (pH 7.2) at 10 mg/mL and stored for 24 h at room temperature (RT) for optimal antiviral activity (referred to as aged-GTE) (Falcó et al., 2018).

High-pressure processing treatments were performed in a pilot−scale unit (High-Pressure Food Processor, EPSI NV, Belgium) with a vessel operating pressure of 2.35 L and a maximum treatment pressure of 600 MPa. The pressure transmitting fluid was a mixture of water and ethylene glycol (70:30, v:v). The HPP treatments were applied in samples distributed in 300 μL PCR tubes, placed in polyethylene bags and heat-sealed (MULTIVAC Thermosealer). A constant holding temperature of 22 ± 1 °C was maintained during HPP treatments as the pressure transmitting fluid was automatically and continually refrigerated. After completing the treatment, the samples were immediately stored at −80 °C.

Two sets of experiments were carried out. Initially, MNV-1 and HAV suspensions (6 and 5 log $TCID_{50}$/mL, respectively), along with human norovirus GII.4 Sydney[P16] filtrate (5.5 log genomic copies (gc)/100 μL) and GI suspension (6 log gc/100 μL) were mixed with PBS or 3.3 mg/L aged-GTE and exposed to 0 (untreated control), 300, 400 and 500 MPa for 5 min.

Then, the kinetic inactivation of MNV-1 and HAV suspensions (6 and 5 log $TCID_{50}$/mL, respectively) in beverages was investigated for HPP treatments alone and combined with aged-GTE. To this end, viral suspensions were prepared in PBS (matrix control), in 3.3 mg/L aged-GTE solution, or in fruit juices (ratio 1:3). Finally, samples were either treated at 0 (untreated control), 300, and 400 MPa for increasing pressurization times (0, 5, 7.5, 10, and 12.5 min).

Each experimental condition was performed in triplicate and each experiment included a cytotoxicity control (aged-GTE and juices without viral inocula).

2.3. Maintenance, differentiation and infection of human intestinal enteroids

Three-dimensional HIEs 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 described previously (Costantini et al., 2018) using IntestiCult™ Organoid Medium Human media (STEMCELL Technologies Inc.).

To determine human norovirus infectivity, RT-qPCR was used to determine 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 with 100 μL of human norovirus samples (HPP treated and untreated) and incubated at 37 °C for 1 h. After the inoculum was removed, monolayers were washed twice with complete media without growth factors (CMGF−, prepared as previously described, Costantini et al., 2018) and 100 μL of Organoid Medium Human (ODM, as in Costantini et al., 2018) 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 using the Maxwell® RSC Instrument (Promega) and the Maxwell RSC Pure Food GMO and authentication kit (Promega) with the modifications described elsewhere (Pérez-Cataluña et al., 2021). Finally, the RNA was detected and quantified in duplicate (see Section 2.5).

2.4. Assessment of human norovirus binding ability

In situ capture RT-qPCR (ISC-RTqPCR) was performed as previously described by Falcó et al. (2019). Briefly, treated and untreated human norovirus GI suspensions were added to a 96-well plate previously coated with type III porcine gastric mucine (PGM, Sigma Aldrich, 100 μg/mL) and incubated at 37 °C for 1 h. After washing, 100 μL of lysis buffer from the NucleoSpin® RNAvirus kit (Macherey-Nagel GmbH & Co.) was added to each well. Then, viral RNA was extracted using the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions. RNA samples were analysed in duplicate by RT-qPCR (see Section 2.5). Human norovirus GI suspension without aged-GTE and HPP treatments was used as positive control and considered as 100% of binding. The binding percentages of samples exposed to HPP treatment alone and combined with aged-GTE were calculated with respect to the positive control. Human norovirus GI suspension treated at 99 °C for 5 min was used as negative control.

2.5. Detection and quantification of human norovirus by RT-qPCR

Norovirus RNA was detected by GI/GII TaqMan RT-qPCR using the RNA UltraSense One-Step quantitative RT-PCR system (Invitrogen) and the set of primers and probe recommended by the ISO 15216-1 (2017) using the 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 (human norovirus GI: $y = -3.5106x + 37.329$, $R = 0.999$; human norovirus GII: $y = -3.56x + 40.664$, $R = 0.997$). Positive and negative amplification controls were also included in each run.

2.6. Statistical analysis

All data were compiled from three independent experiments with three technical replicates for each variable. The decay of MNV-1, HAV, human norovirus GI and GII titers was calculated as $\log_{10}$ S (N/N0), where N0 is the HPP-untreated virus titer and N is the treated one (either HPP or aged-GTE, or both). 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, USA) software was used for statistical analyses and data representation.

3. Results and discussion

3.1. Effect of HPP and aged-GTE on HAV, MNV and human norovirus

The effect of HPP treatments at 0, 300, 400 and 500 MPa for 5 min on HAV, MNV, and human norovirus GII.4 Sydney[P16] infectivity was initially evaluated in buffer (PBS) and aged-GTE (3.3 mg/mL) solutions.

HAV, MNV and human norovirus GII.4 Sydney[P16] inactivation were assessed by cell culture methods (Fig. 1). Overall, no significant viral reductions were observed for HAV, MNV and human norovirus exposed to 300 MPa HPP treatment. The combined effect of aged-GTE with 300 MPa HPP significantly ($p < 0.05$) affected both HAV and human norovirus GII.4 Sydney[P16] infectivity with inactivation of 2.37 log $TCID_{50}$/mL and 0.91 log gc/100 μL, respectively, while MNV infectivity was not impacted.

The aged-GTE synergistic antiviral effect was even more explicit combined with 400 MPa HPP as complete inactivation was observed for HAV and human norovirus GII.4 Sydney[P16]. MNV was inactivated at 400 MPa, even without the addition of aged-GTE (Fig. 1B). Increasing the pressure to 500 MPa, all the three virus were completely inactivated, either when exposed to the HPP treatment alone or combined with aged-GTE.

HAV inactivation rates observed in our study are in line with the viral titer reduction from approximately 10^7 PFU/ml to nondetectable levels after 450 MPa HPP as previously reported (Kingsley et al., 2002).

Comparing MNV, the reference surrogate model for human norovirus, with the actual pathogen, some differences on the inactivation patterns could be noted. Specifically, 400 MPa HPP treatment caused the complete inactivation of MNV, while infectious norovirus exposed to the same condition replicated on HIE system. Cromeans et al. (2014) reported similar findings for MNV, which resulted completely inactivated after 1 min at 400 MPa HPP treatment. Using the HIE cell culture system for human norovirus, our results demonstrate that 5 min HPP treatment at 500 MPa, but not at 400 MPa, completely inhibits human norovirus GII.4 infectivity (Fig. 1B). Interestingly, both HAV and human norovirus showed similar inactivation profiles at 400 and 500 MPa as well as the synergistic effect of aged-GTE at 300 and 400 MPa.

Only human norovirus GII.4 Sydney[P16] was tested using HIE system as none of the available biobanked human norovirus GI fecal samples (n = 25) showed replication on HIE monolayers (data not shown). Thus, the ISC-RT-qPCR technique based on viral binding to PGM was alternatively used to infer human norovirus GI.4 capsid integrity.

Human norovirus GI binding to PGM following HPP treatments with or without 3.3 mg/mL aged-GTE was completely prevented as resulted from ISC-RT-qPCR assays (Fig. 2). Also, a binding decrease to 17% was observed due to the aged-GTE exposure only. Previous results showed that human norovirus GI exposed to 5 mg/mL aged-GTE for 30 min reduced viral binding by more than 95% (Falcó et al., 2020), thus current observations are in line with reported data, despite experimental differences (e.g., GTE concentration, exposure time).

These data together do not come to a univocal conclusion, especially for HPP treatments at lower pressure condition. For example, human norovirus GII.4 infectivity was negligibly affected following 300 MPa HPP treatment, while human norovirus GI completely lost its binding ability at the same condition. Those differences may be explained by either the diverse resistance to HPP inactivation of GI and GII genogroups included in the study, as demonstrated for heat-treated GII genotypes (Tan et al., 2022), and the varied sensitivity of the HIE and ISC-RT-qPCR assays used to test for viral infectivity. Using a porcine gastric mucin conjugated magnetic beads (PGM-MBs) binding assay, human norovirus GI.1 reduced its binding by 4.7 $\log_{10}$ following 600 MPa HPP treatment, while only 0.45 $\log_{10}$ reductions were measured for 300 MPa treatment (Dancho et al., 2012).

In the present study, the increase of RNA genomic copies following

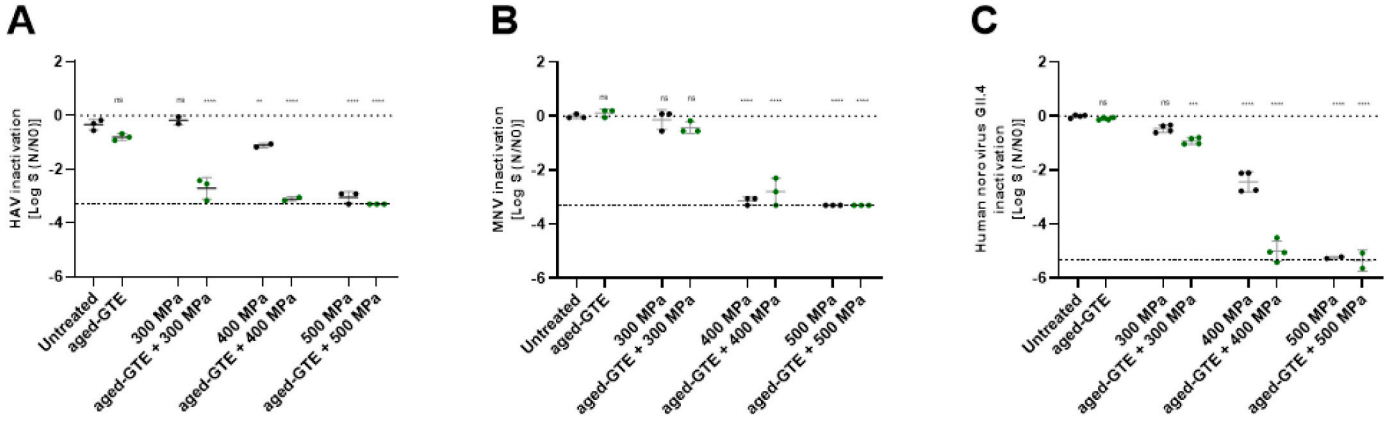

Fig. 1. Effect of 5 min HPP treatments alone (black dots) and combined with aged-GTE (3.3 mg/mL, green dots) on hepatitis A virus (HAV; A), murine norovirus (MNV; B), and human norovirus GII.4 (C) infectivity tested in FRhK, RAW and human intestinal enteroids (HIE) cultures. Experiments were carried out in buffer medium (PBS). Data are represented as $\log_{10}$ S (N/N0), where N0 is the infectious HPP-untreated virus titer and N is the treated one (either HPP or aged-GTE, or both). Dashed line indicates the limit of quantification of the assay. Abbreviations: ns, not significant; ***, $p < 0.001$; ****, $p < 0.0001$.

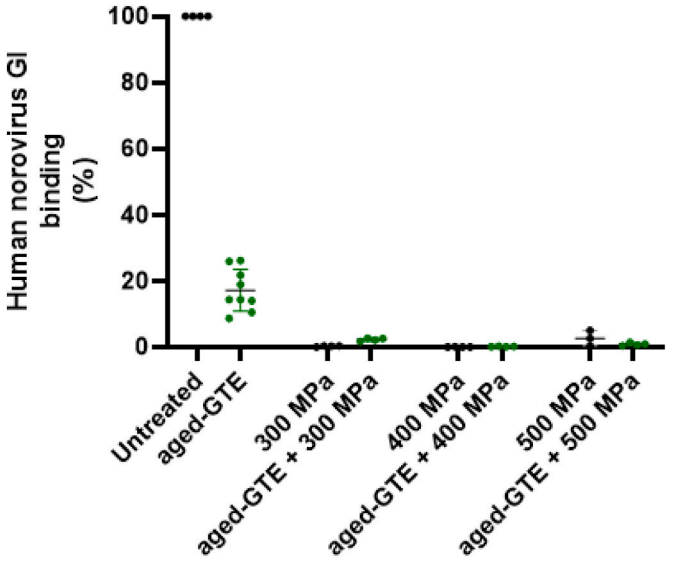

Fig. 2. Effect of HPP treatments alone (black dots) and combined with aged-GTE (3.3 mg/mL, green dots) on human norovirus GI binding ability assayed in buffer medium (PBS) by ISC-RT-qPCR. All treatments determined significant differences with respect to the untreated control ($p < 0.0001$).

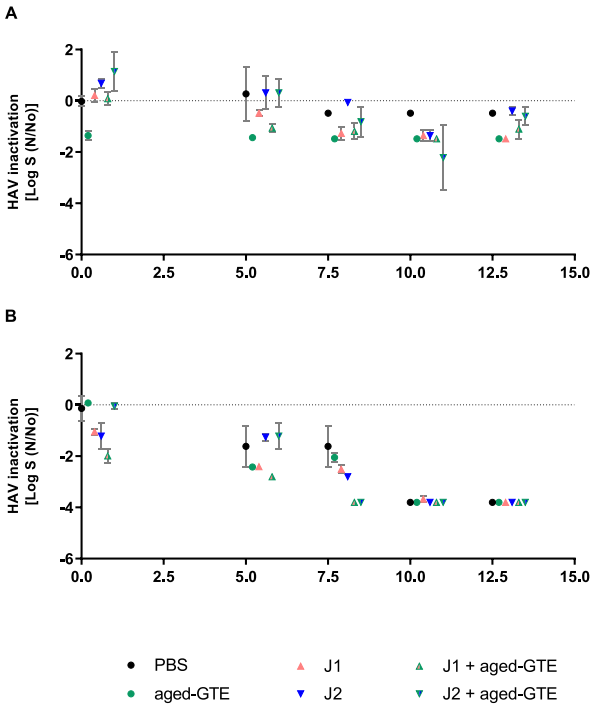

Fig. 3. Effect of 300 MPa (A) and 400 MPa (B) HPP treatments alone and combined with aged-GTE (3.3 mg/mL) on hepatitis A virus (HAV) in juices. Leg end: PBS, reference substrate; aged-GTE, aged-Green Tea Extract; J1, apple juice; J2, horchata.

replication on HIE would serve as a more robust and definitive approach than the ISC-RT-qPCR assay. Moreover, our HIE replication results are consistent with a clinical trial for human norovirus GI inactivation in oysters that demonstrated that 400 MPa HPP treatment did not completely inactivate the virus in seeded oysters and resulted in infection among the subjects, while 600 MPa HPP completely prevented participants from the infection (Leon et al., 2011).

A previous study demonstrated that human norovirus GII.4 was completely inactivated by exposure to 1.75 mg/mL aged-GTE for 1 h at 21 °C (Randazzo et al., 2020). Data from the present study did not show differences between 3.3 mg/mL aged-GTE- and un-treated samples (Fig. 1C), indicating that the antiviral activity of the tested natural compound is not instantaneous.

Collectively, our data showed that aged-GTE exerted a synergistic antiviral effect resulting in an enhanced viral inactivation compared to the HPP treatment alone.

3.2. *Kinetic inactivation of enteric viruses contaminating juices exposed to HPP and aged-GTE*

To comprehensively study the response of enteric viruses to HPP treatments in food matrices, apple (J1) and horchata (J2) beverages were artificially contaminated with HAV and MNV and subjected to 300 and 400 MPa HPP treatments for increasing time intervals (0, 5, 7.5, 10, and 12.5 min). Moreover, the synergistic antiviral effect of aged-GTE and HPP was considered testing also juice samples enriched with 3.3 mg/mL aged-GTE. Horchata drink was included in the study as a traditional beverage from Valencia (Spain) exerting beneficial nutritional properties (Selma-Royo et al., 2022). However, its production in sub-urban areas and the typical flood irrigation may undermine its food safety.

Results showed that 300 MPa HPP treatment did not considerably affected neither HAV nor MNV infectivity in juices. To describe HAV inactivation at 300 and 400 MPa, both time ($P_{time}<0.001$) and matrix ($P_{matrix}<0.001$) variables resulted statistically significant. A protective effect of J2 was observed for HAV showing lower inactivation rates as compared to either J1 and PBS control (Fig. 3A). Prolonged 400 MPa HPP treatments for 10 min or more resulted in complete inactivation regardless of the matrix (Fig. 3B), while the aged-GTE enrichment of juices shorten to 7.5 min the time required for HAV inactivation.

MNV titers showed limited infectivity reduction at 300 MPa HPP with 0.83 $\log_{10}$ reduction as maximum, beside statistical significant differences were observed for time ($P_{time} = 0.0131$) and matrix ($P_{matrix} = 0.0017$) variables (Fig. 4A). The kinetic MNV inactivation at 400 MPa HPP treatment is showed in Fig. 4B and viral inactivation due to increasing time exposures is sharply represented for PBS and aged-GTE solutions. Moreover, the synergistic effect of aged-GTE enriched juices and HPP is observed at 7.5 min treatment and for extended HPP times as compared to pure juices. Time ($P_{time}<0.001$) and matrix ($P_{matrix}<0.001$) variables resulted statistically significant to describe MNV kinetic inactivation at 400 MPa HPP. Also, a protective effect was observed for MNV exposed to 400 MPa in J2, and more markedly in J1, where the addition of aged-GTE determined up to 3.98 log viral decrease (Fig. 4B).

Similarly, HAV infectivity was reported to be significantly inactivated (4.3 log PFU/mL) in mashed strawberry by 375 MPa HPP treatment (Kingsley et al., 2002). On the contrary, DiCaprio et al. (2019) observed that 400 MPa HPP treatment did not significantly affect the human norovirus GII.4 RNA as resulted from a PGM-MB binding assay.

Overall, our kinetic inactivation data corroborate the different sensitivity of the two viruses to aged-GTE, which was previously described to affect HAV more than MNV (Falcó et al., 2018).

The synergistic effect of natural compounds and food processing technologies has been described for heat treatments coupled with curcumin, gingerol, grape seed extract or GTE on Tulane virus, MNV or HAV (Falcó et al., 2020; Patwardhan et al., 2020). Our data support previous reports concluding that the overall inactivation is not the sum of the effects of individual treatments, but it is greater because of their synergistic impact on microorganisms (Leistner & Gorris, 1995). Specifically, we demonstrated that viral inactivation in aged-GTE enriched juices occurs at reduced holding pressure times.

Describing the antiviral mode of action of aged-GTE was not among the goals of this study, even though previous works may contribute to formulate some hypotheses. Combining cell-culture and viability RT-

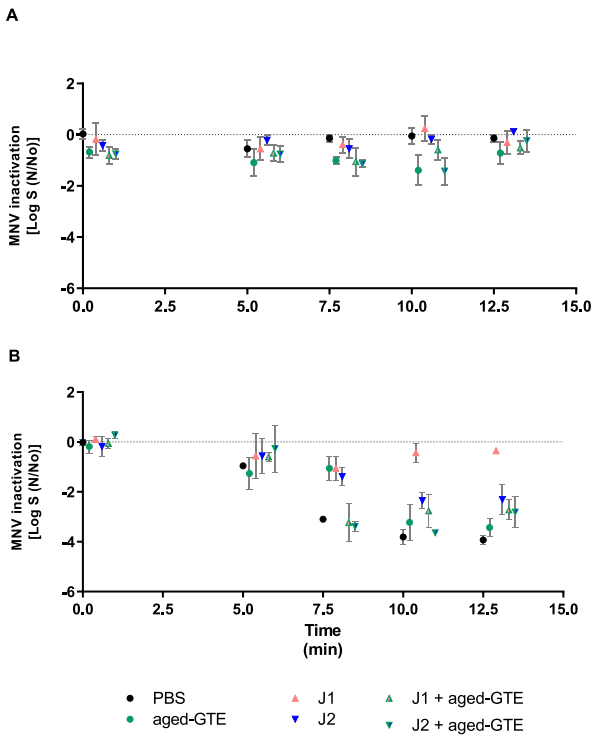

Fig. 4. Effect of 300 MPa (A) and 400 MPa (B) HPP treatments alone and combined with aged-GTE (3.3 mg/mL) on murine norovirus (MNV) in juices. Legend: PBS, reference substrate; aged-GTE, aged-Green Tea Extract; J1, apple juice; J2, horchata.

qPCR assays, we previously demonstrated that epigallocatechin gallate, the mayor flavonoid in GTE, does not dramatically affect viral capsid, which may be exposed to structural alterations instead. While causing the loss of viral infectivity, such damage did not affect nucleic acids, which can be detected by either RT-qPCR and viability RT-qPCR (Falcó et al., 2017). The increased antiviral effect observed in this study when combined treatments are applied may be due to the damage on viral capsid determined by HPP that allow the entry of aged-GTE which finally interfere with the viral RNA. GTE oxidising activity (Falcó et al., 2018) or its function as inhibitor due to histo-blood group antigen blocking potential described for other natural compounds (Ruoff et al., 2022) are additional possible inactivation mechanisms that are still pending to be tested.

All these data together strongly support the feasibility of the addition of natural compounds with antiviral activity to reduce the operating conditions (e.g., temperature, pression, time) of processing technologies to finally preserve food quality while guarantying food safety.

4. Conclusions

The combined use of natural antimicrobials and HPP treatments has been increasingly adopted to preserve food quality while guarantying food safety. However, no information was available on the HPP inactivation of common enteric viral pathogens such as HAV, human norovirus or its surrogates (e.g., MNV) in beverages combined with natural compounds. Using the recently reported HIE cell culture system and a capsid integrity binding assay, we demonstrated the synergistic inactivation effect of HPP and aged-GTE against human norovirus. This is the first report on human norovirus inactivation by HPP tested using the novel HIE model, demonstrating its suitability also for food and environmental studies. Moreover, HAV and MNV contaminating apple and horchata juices enriched with aged-GTE were significantly inactivated at reduced holding pressure time. Altogether, the results of the present study proved that antimicrobial natural compounds and HPP have been combined to increase the inactivation of foodborne viral pathogens in juices. Opportunities exist for the further exploitation of this hurdle technology in a whole array of food products, which could benefit from viral elimination (e.g., berries puree).

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Declaration of interests

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.

CRediT authorship contribution statement

Irene Falcó: Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Walter Randazzo:** Methodology, Formal analysis, Resources, Writing – original draft, Writing – review & editing. **Ana Pérez:** Methodology. **Antonio Martínez:** Formal analysis, Writing – review & editing. **Dolores Rodrigo:** Formal analysis, Writing – review & editing. **Gloria Sánchez:** Conceptualization, Resources, Writing – review & editing, All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Data availability

Data will be made available on request.

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