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Green tea extract assisted low-temperature pasteurization to inactivate enteric viruses in juices

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ABSTRACT

The current popularity of minimally processed foods is an opportunity for natural antimicrobial agents to be combined with mild heat treatments to act synergistically in reducing viral foodborne pathogens. Viral inactivation by heat-treatments (at 25, 40, 50 and 63 °C for 30 min) combined with aged green tea extract (aged-GTE) was initially evaluated in phosphate buffered saline (PBS) against murine norovirus (MNV-1) and hepatitis A virus (HAV) by cell culture, and against human norovirus by *in situ* capture RT-qPCR. The combination of aged-GTE and heat treatment at 50 °C for 30 min exerted strong antiviral activity, reducing by more than 5 log MNV-1 infectivity in PBS. Heating at 40 °C for 30 min reduced the binding of norovirus to porcine gastric mucine (PGM) to 41.5% and the addition of aged-GTE further decreased the binding to 4.7%. Additionally, the reduction of MNV-1 and HAV infectivity was investigated in two different types of juices exposed to mild heat treatments alone, and combined with aged-GTE. The addition of aged-GTE increased to more than 4 log the inactivation of MNV-1 in juices exposed to 50 °C for 30 min. However, this synergistic effect of aged-GTE combined with heat treatments was not observed for HAV in any of the juices. Aged-GTE, then, could be considered as an additional control measure to improve the food safety of mild heat pasteurized juices.

1. Introduction

Over the last decade, infections caused by human enteric viruses have affected the population, with human norovirus and hepatitis A virus (HAV), being the most relevant viral pathogens from a food safety perspective (EFSA, 2018; Harrison and DiCaprio, 2018). Infections caused by human enteric viruses are mainly associated with a development of gastroenteritis. This is the case with noroviruses, which cause vomiting, diarrhea and nausea. When the infection occurs in a compromised population (elderly or immunocompromised people), it can even lead to death (Goller et al., 2004). Clinical manifestations of HAV are even more severe, accounting for 0.5% of the mortality due to viral hepatitis (WHO, 2017). Enteric viruses have received less attention than other foodborne pathogens, so strategies to guarantee their control are necessary, not only to ensure food safety and reduce the number of human infections, but also to reduce the direct health costs of nearly $5 billion per year in the case of norovirus (Bartsch et al., 2016).

Vegetables and berries are currently among the most implicated food categories associated with enteric virus outbreaks, since they are generally consumed fresh or only mildly treated (*e.g.* frozen or freeze-dried berries, blanched vegetables), increasing the risk of infection. Those foods are susceptible to contamination due to the use of irrigation water contaminated with fecal matter or to a lack of proper hygiene among food handlers (Carter, 2005; Kokkinos et al., 2017). In industrialized countries, norovirus is known to be responsible for most of the foodborne outbreaks caused by produce consumption (Callejón et al., 2015; Machado-Moreira et al., 2019).

Measures based on the application of heat as an effective method for pathogen inactivation are currently applied by the food industry to control bacteria and yeast (Stumbo, 2013). However, heat inactivation of enteric viruses, particularly norovirus and HAV in food, has not been fully explored (Bartsch et al., 2019; Bozkurt et al., 2015; Chen et al., 2020; Shao et al., 2018). Several studies showed that heat treatments provoke a conformational change of the capsid resulting in the reduction of initial titers, but this effect depends on the type of enteric virus, heat-treatment conditions, and composition of processed foods. For example, fat, sugar and protein present in food could protect viruses from inactivation (Deboosere et al., 2004; Hirneisen et al., 2010).

A wide variety of food items, including beverages and juices, are typically preserved by heat treatments that are characterized by the combination of two parameters: temperature and time. On a

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microbiological perspective, among different thermal processes, pasteurization is used to control pathogen bacteria by exposing the product at temperatures typically ranging between 70 and 85 °C, while sterilization aims to destroy of all viable microorganisms by reaching temperatures above 100 °C (Ramesh, 1999) and thus prolonging the shelf-life of the product. Pasteurization is one of the most trusted and widely used process applied in the food industry for food preservation (Peng et al., 2017). However, high temperature treatments negatively impact food quality by destroying active biological components (vitamins and antioxidant compounds), and by adversely affecting color, taste, and texture, finally decreasing both the nutritional and the sensory value of foods (Koskiniemi et al., 2013; Zang et al., 2013).

With the advent of "hurdle technology" and the demand for green food-processing technologies, the use of natural antimicrobials is often combined with mild processing techniques in order to minimize the severity of food processing and, at the same time, achieve the inactivation of foodborne pathogens (Del Nobile et al., 2012). This results in cost savings, maintenance of food safety, and preservation of nutritional and sensory attributes.

The combination of different hurdles such as antimicrobial agents and thermal treatments results in pronounced inactivation efficacy due to additive or synergistic effects. As hurdle effect, the overall inactivation will not just be the sum of the different preservative factors (additive effect), but it might be even greater given the synergistic activity of the treatments (synergistic effect) (Gurtler et al., 2019; Leistner and Gorris, 1995). In some cases, especially with juices, this combination of technologies has been described as chemically-assisted low-temperature pasteurization (Essia Ngang et al., 2014; Gurtler et al., 2019). A recent study investigating the antiviral activity of natural extracts and thermal treatments on foodborne viruses refers to the combined effect as heat sensitization (Patwardhan et al., 2020).

Among several antiviral compounds with demonstrated antiviral activity, green tea extract (GTE) was selected because it has successfully been applied as a natural sanitizer or added to edible coatings or films to control human enteric viruses (Falcó et al., 2018; Randazzo et al., 2018; Randazzo et al., 2017).

The aim of the present study was to investigate by cell-culture methods the effects of different mild heat treatments (at 25, 40, 50, or 63 °C for 30 min) and GTE, both separately and in combination, against enteric virus, specifically HAV and murine norovirus (MNV-1), a norovirus surrogate. Nonetheless, norovirus replication in cell-culture has recently become available (Costantini et al., 2018; Ettayebi et al., 2016); its implementation is far from routine due to technical and economic issues. A variety of approaches based on capsid integrity coupled with RT-qPCR have been reported (reviewed by Randazzo et al., 2018), including pre-treatments with nucleases, proteolytic enzymes, and viability dyes, as well as saliva or porcine gastric mucine (PGM) assays to bind norovirus. Thus, mild heat treatment sensitization of human norovirus by GTE was investigated by an *in situ* capture RT-qPCR (ISC-RT-qPCR).

Finally, the synergistic effect of heat treatments and GTE in two different juices was also investigated.

2. Material and methods

2.1. Virus propagation, cell lines and clinical sample

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 quantified 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).

A fecal sample containing human norovirus GI.4 strain was kindly provided by Dr. Buesa (University of Valencia, Spain). One part of fecal sample was suspended in 9 parts of PBS containing 2 M $NaNO_3$ (Panreac), 1% beef extract (Conda), and 0.1% Triton X-100 (Fisher Scientific) (pH 7.2), vortexed and centrifuged at 1000 $\times g$ for 5 min. The supernatant was stored at −80 °C in aliquots.

2.2. Assessment of MNV-1 and HAV infectivity exposed to GTE assisted low-temperature pasteurization by cell culture

GTE (Naturex SA, France) at 10 mg/ml was dissolved in PBS pH 7.2 and stored for 24 h (aged-GTE) as previously suggested (Falcó et al., 2018) to increase antiviral activity. Aged-GTE was mixed with an equal volume of MNV-1 and HAV suspensions (*ca.* 6 or 5 log $TCID_{50}$/ml, respectively) followed by incubation at 25, 40, 50 or 63 °C for 30 min. Thereafter, Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) was added to stop the reactions as previously reported (Randazzo et al., 2017). Each experiment included a cytotoxicity control (aged-GTE without viral inocula) and viral suspensions exposed to different heat treatments without aged-GTE. Each experimental condition was performed in triplicate. Antiviral activity of aged-GTE was estimated by comparing the number of infectious viruses on suspensions without aged-GTE and on the aged-GTE-treated virus suspensions. The decay of MNV-1 and HAV titers was calculated as $\log_{10}$ (N_x/N_0), where N_0 is the infectious virus titer for GTE-untreated samples and N_x is the infectious virus titer for aged-GTE-treated samples.

2.3. Assessment of human norovirus infectivity exposed to GTE assisted low-temperature pasteurization by in situ capture RT-qPCR (ISC-RT-qPCR)

Suspension of human norovirus GI.4 was mixed with an equal volume of aged-GTE (10 mg/ml), followed by incubation at 25, 40, 50 or 63 °C for 30 min. Control samples were virus suspensions added with PBS only and run under the same experimental conditions. ISC-RT-qPCR was performed as previously described by Falco et al. (2019). Briefly, treated and untreated norovirus 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, to each well 100 μl of lysis buffer from the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.) was added. Then, viral RNA was extracted using the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.) according to the manufacturer's instructions. RNA samples were analyzed in duplicate by 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). Standard curve was generated using the Public Health England (PHE) reference material for microbiology for norovirus GI (batch number 0122-17) consisting of a quantified viral preparation. Amplification was performed for 1 cycle of 55 °C for 1 h, 1 cycle of 95 °C for 5 min, and 45 cycles of 95 °C for 15 s, 60 °C for 1 min and 65 °C for 1 min. Norovirus GI suspension without aged-GTE treatment and maintained at room temperature was used as positive control and considered as 100% of binding. The binding percentages of samples exposed to heat treatment alone and combined with aged-GTE were calculated with respect to the positive control. Norovirus GI suspension treated at 99 °C for 5 min was used as negative control (no binding expected). Each sample was analyzed in triplicate and the mean values and SD were calculated.

2.4. Effect of aged-GTE on heat-treated juices

Two commercial juices, J1 (containing strawberry, carrot, beetroot and apple at pH 4.10) and J2 (apple juice, pH 3.75) were obtained from a local supermarket. Juices were artificially inoculated with MNV-1 (*ca.* 6 log $TCID_{50}$/ml) and HAV (*ca.* 6 log $TCID_{50}$/ml) and mixed, in equal volumes, with aged-GTE (10 mg/ml), and treated for 30 min at 25, 40, 50, and 63 °C. Controls consisted of juices only (cytotoxicity controls), and inoculated juices without the addition of aged-GTE. DMEM supplemented with 10% FCS was used to stop the reaction. Each sample

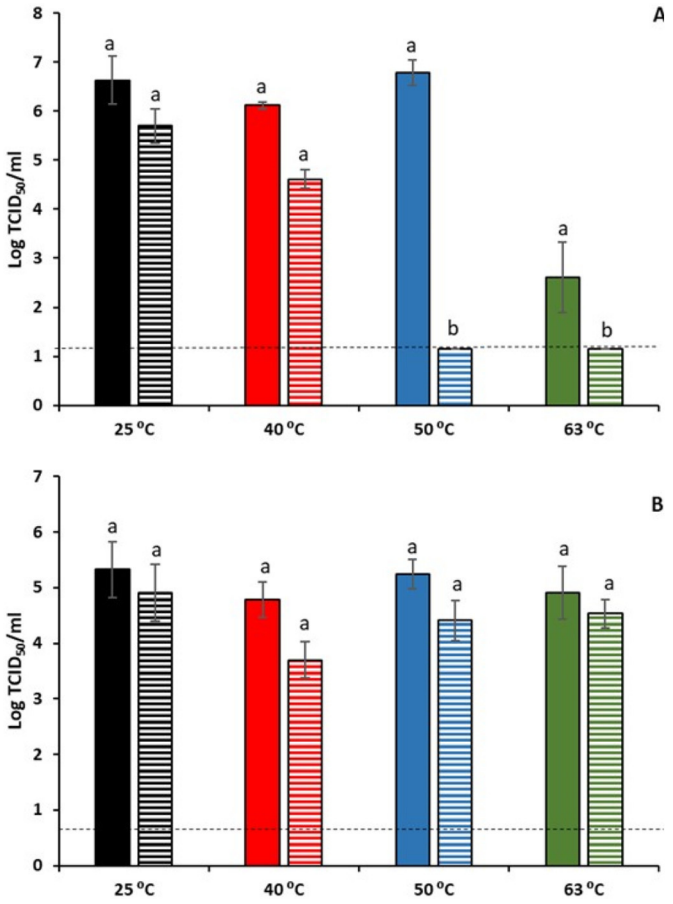

Fig. 1. Reduction of murine norovirus (MNV-1) (A) and hepatitis A virus (HAV) (B) titers (log $TCID_{50}$/ml) exposed to different mild heat treatments (30 min) alone (filled bars) and combined with aged-GTE (5 mg/ml) (stripped bars). Dashed line depicts the detection limit. Each column represents the average of triplicates. Different letters denote significant differences between mild heat treatment alone and combined with aged-GTE for each temperature and for each virus ($p < 0.05$).

was run in triplicate. The antiviral effect was evaluated as described above.

2.5. Statistical analysis

The statistical analysis was carried out by the post-hoc Tukey's method ($p < 0.05$) to compare and determine the difference among thermal treatments and a Student's *t*-test was used to compare average values of thermal treatments alone and combined with aged-GTE. Statistica software version 10 (StatSoft Inc., Tulsa, OK, USA) was used for statistical analyses.

3. Results and discussion

3.1. Antiviral activity of aged-GTE on MNV-1 and HAV at different temperatures

The effect of different mild heat treatments alone and combined with aged-GTE against MNV-1 and HAV are shown in Fig. 1. With respect to the control kept at room temperature (25 °C), MNV-1 infectivity was not reduced by heat treatments at 40 and 50 °C for 30 min ($p > 0.05$), while a significant reduction ($p < 0.05$) of 3.85 log $TCID_{50}$/ml was shown at 63 °C (Fig. 1A). This is in line with previously reported results, in which MNV-1 infectivity was significantly reduced after exposure to 55 °C due to major changes in the capsid surface (Brié et al., 2017). In contrast, heat treated HAV suspensions showed not significant differences ($p > 0.05$) compared to the control even when exposed at temperature as high as 63 °C for 30 min. This finding does not resemble a recent study that reported 6-D-values (referred as the time needed to decrease by 6 log the initial virus load) for heat inactivated HAV of 15.78 and 10.8 min at 60 and 65 °C, respectively, by using a linear model and of 11.89 and 3.99 min by using a Weibull model (Patwardhan et al., 2020). Further thermal inactivation studies have reported that the time required for a one log genome copies reduction (D-value) at 72 °C was of 0.88 and 3.33 min for HAV and human norovirus GII, respectively (Bozkurt et al., 2014; Wang and Tian, 2014).

Furthermore, the antiviral effect of aged-GTE was evaluated combined to different mild heat treatments. Aged-GTE at 5 mg/ml did not show any cytotoxicity effect on RAW 264.7 and FRhK-4 cells as determined by microscopical assessment of control cells (aged-GTE without viral inocula), according to a previous report (Randazzo et al., 2017). The titers of MNV-1 exposed to 50 and 63 °C and aged-GTE (5 mg/ml) were statistically different ($p < 0.05$) compared to the corresponding mild heat treatments alone, showing greater inactivations when the virus was exposed to the combined technology (Fig. 1A). A clear synergistic effect was observed when aged-GTE was combined with 50 and 63 °C treatments, resulting in undetectable infectious MNV-1 particles. Comparing the treatments, it is evident that a sharp reduction of infectious MNV was achieved by the only exposure at 63 °C (3.85 log $TCID_{50}$/ml) while a complete inactivation of the virus (> 5.82 log $TCID_{50}$/ml) was observed when aged-GTE was combined to the heat treatment at 50 °C. In line, a synergistic effect was reported for heat treatments coupled with natural compounds such as curcumin (0.015 mg/ml), gingerol (0.1 mg/ml) and grape seed extract (1 mg/ml) on Tulane virus, a recently proposed norovirus surrogate (Patwardhan et al., 2020).

On the other hand, HAV titers were not significantly reduced ($p > 0.05$) by any of the treatments applied independently or in combination (Fig. 1B).

3.2. Antiviral activity of aged-GTE and mild heat treatments on norovirus

In the current study, the use of a PGM binding assay to infer viral capsid integrity was investigated by applying an ISC-RT-qPCR technique on norovirus exposed to mild heat treatments alone and combined with aged-GTE at 5 mg/ml (Fig. 2). Human norovirus GI.4 suspension treated at 25 °C for 30 min along with aged-GTE reduced viral binding by more than 95%. Previous results have shown that overnight

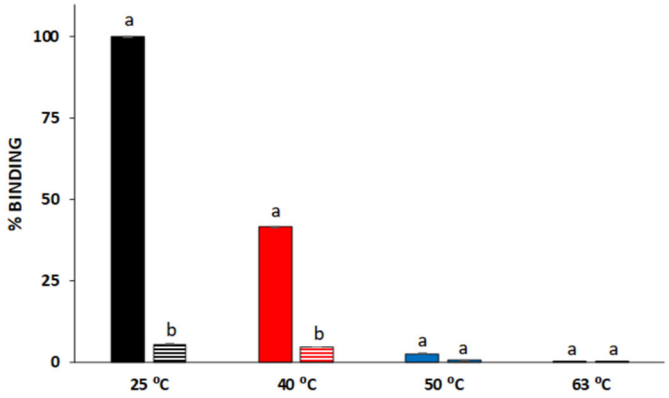

Fig. 2. Effect of different mild heat treatments alone (filled bars) and combined with aged-GTE (5 mg/ml) (stripped bars) on human norovirus GI. Norovirus GI binding ability to PGM was analyzed by ISC-RT-qPCR. Each bar represents the average of triplicates.
Different letters denote significant differences between heat treatments alone and combined with aged-GTE for each temperature ($p < 0.05$).

incubation with aged-GTE at 5 mg/ml at 37 °C completely prevented norovirus binding to PGM (Falco et al., 2019).

The binding of human norovirus exposed to 40 °C for 30 min was reduced by 41.5%, while the addition of aged-GTE resulted in an enhanced effect with an average binding of only 4.7%. Minimal residual binding was observed at 50 °C (2.5%), and complete prevention of binding was achieved with the addition of aged-GTE. Treatment at 63 °C for 30 min determined the complete loss of binding capacity (Fig. 2). This is consistent with a previous report showing an average of 6% norovirus binding to PGM-magnetic beads after a 1 min treatment at 64 °C (Dancho et al., 2012). In contrast, D-values of 100, 25, 3.33, and 0.57 min have been reported at 56, 63, 72 and 100 °C, respectively, for human norovirus GII using ISC-RT-qPCR (Wang and Tian, 2014).

Finally, using the novel stem cell-derived human enteroids replication model, noroviruses were inactivated by heating at 60 °C for as little as 15 min (Ettayebi et al., 2016).

3.3. Combined effect of heat treatment and GTE on juices

GTE has been investigated for its potential as a natural antimicrobial to preserve the quality and enhance the safety of different types of foods (Nikoo et al., 2018). In the current study, following the principles of hurdle technology, we tested a multiple simultaneous preservation approach based on the use of aged-GTE and mild heat treatments to assess the inactivation kinetics of MNV-1 and HAV in artificially contaminated fruit juices. Although fruit juices do not represent a high risk matrix from a viral food safety perspective, human enteric viruses have been associated to the consumption of juices (Frank et al., 2007). Epidemiological investigations suggest that unpasteurized and inadequate heat-treated juices, contaminated fruits, contaminated water and environmental contamination are the responsible of outbreaks along with person to person transmission (Frank et al., 2007; Visser et al., 2010).

In PBS, titers of MNV-1 decreased according to the exposure to increasing temperature.

By combining mild heat treatments at 40, 50 or 63 °C with aged-GTE (3.3 mg/ml), MNV-1 titers were lower than those resulting from the thermal treatment alone (Fig. 3A, B, and Table S1). These differences resulted in reduction of 1.31 and 2.90 log $TCID_{50}$/ml, and undetectable infectious MNV-1 particles for thermal treatments at 40, 50 and 63 °C, respectively, and they were statistically significant ($p < 0.05$), finally suggesting a relevant synergistic effect ($p < 0.05$) of aged-GTE combined with mild heat treatments.

No synergistic effect ($p > 0.05$) was observed when aged-GTE was added to juices and treated at 40 °C for 30 min. MNV-1 titers decreased by 0.69 log $TCID_{50}$/ml in J1, and no reduction was observed in J2 (Table S1, Fig. 3A and B), indicating a protective effect of juice compounds. Similar protective effects have been observed when aged-GTE was prepared in milk (Falco et al., 2019). However, a statistically significant synergistic effect ($p < 0.05$) between aged-GTE (3.3 mg/ml) and heat treatments resulted in undetectable infectious MNV-1 particles for the treatments at 50 and 63 °C for juice 2 and at 63 °C for juice 1.

Combining aged-GTE with 40 °C treatment reduced MNV-1 infectivity in J1 by 0.69 log $TCID_{50}$/ml, and the increase of 10 °C in thermal regimen (to 50 °C) led to a reduction of 2.69 log $TCID_{50}$/ml viral titers (Table S1 and Fig. 3B). A similar inactivation pattern was observed in J2. For the sake of comparison, infectivity of MNV-1 in J1 and J2 was reduced by 1.81 and 1.38 log $TCID_{50}$/ml when heat treatment at 50 °C was applied alone.

In line with the results obtained by applying aged-GTE at 5 mg/ml, no significant difference ($p > 0.05$) was observed when HAV in PBS was treated with aged-GTE (3.3 mg/ml) and combined with mild-heat treatments (Fig. 4A and B, Table S2). When HAV was inoculated in J1, no viral reduction was reported after treatments at 40, 50, and 63 °C, while a maximum reduction of 0.78 log $TCID_{50}$/ml was reported in PBS, indicating a protective effect of juice on viral particles exposed to mild heat treatments. In J2, treatments at 40, 50, and 63 °C alone reduced

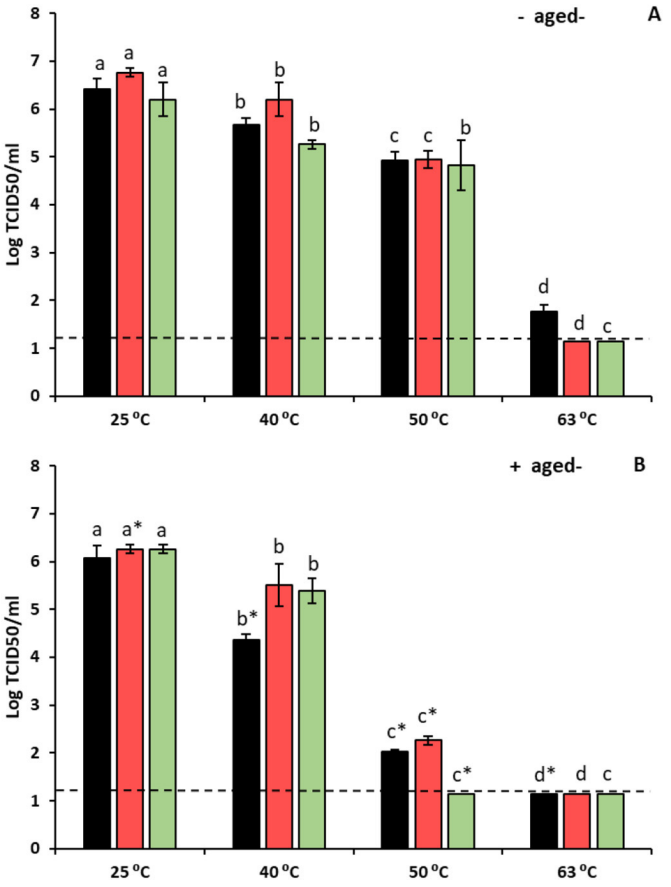

Fig. 3. Murine norovirus (MNV-1) titers (log $TCID_{50}$/ml) exposed to different mild heat treatments in PBS (black bars), commercial juice 1 (red bars) and commercial juice 2 (green bars) alone (A) and combined with aged-GTE (3.3 mg/ml) (B).
Dashed lines depict the detection limit. Each column represents the average of triplicates. Different letters denote significant differences among heat treatments for each matrix ($p < 0.05$). Asterisks denote significant differences among heat treatment alone (panel A) and combined with aged-GTE (panel B) for each temperature and for each matrix ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HAV infectivity by 1.37, 0.62, and 1.44 log $TCID_{50}$/ml (Fig. 4A, Table S2), most probably due to the combined effect of ingredients or low pH and temperature. The addition of aged-GTE to the juices did not significantly improve the antiviral effect of mild heat treatments against HAV (Fig. 4B, Table S2).

The antiviral mode of action of GTE has not been considered in this study, even though it would have contributed to explain the differences observed between MNV and HAV exposed to mild heat treatments alone and combined with the natural extract. As well, the antiviral effect of aged-GTE combined with mild heat treatments on norovirus in juices should have been further investigated by additional approaches other than PGM binding assay. Viability RT-qPCR (Chen et al., 2020; Randazzo et al., 2016) have been recently described as a rapid method for inferring norovirus inactivation. However, PMAxx-RT-qPCR assay was not able to infer the viability of norovirus and HAV exposed to epigallocatechin gallate, one of the main antiviral compound in GTE (Falcó et al., 2017). Thus, this molecular approach was not considered in the framework of this study. Similarly, norovirus inactivation by chemicals (Costantini et al., 2018) and aged-GTE (Randazzo et al., 2020) has been recently investigated by *in vivo* replication on human intestinal enteroids, but the feasibility of the technique in complex matrix remains to be explored. Finally, the study did not consider any

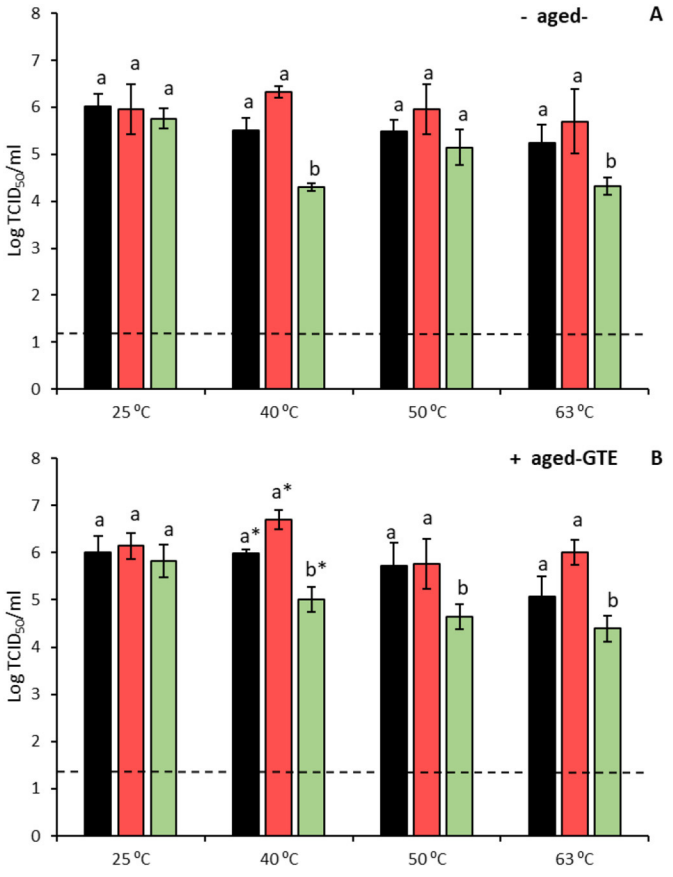

Fig. 4. Hepatitis A virus (HAV) titers (log $TCID_{50}$/ml) exposed to mild heat treatments in PBS (black bars), commercial juice 1 (red bars) and commercial juice 2 (green bars) alone (A) and combined with aged-GTE (3.3 mg/ml) (B). Dashed lines depict the detection limit. Each column represents the average of triplicates. Different letters denote significant differences among heat treatments for each matrix ($p < 0.05$). Asterisks denote significant differences among heat treatment alone (panel A) and combined with aged-GTE (panel B) for each temperature and for each matrix ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

quality or sensorial determination on juices to further extend our findings to a broader perspective.

4. Conclusions

Combinations of natural compounds and conventional food manufacturing treatments are increasingly popular. Enhanced antiviral activity from heat treatment was observed on MNV-1 and human norovirus when aged-GTE was incorporated into the solution. The combined use of natural antimicrobials and heat treatments has been proven successful in improving bacterial inactivation in juices reviewed by Gurtler et al. (2019); however, no information was available on the thermal inactivation kinetics of MNV-1 and HAV in juices combined with natural compounds. Altogether, our results demonstrate that aged-GTE enhances the inactivation of MNV-1 in juices after a mild heat treatment at 50 °C for 30 min. The results of the present study reveal that the addition of aged-GTE could be a suitable option to ensure food safety in mildly heat-treated juices, exerting a synergistic and greater antiviral activity against MNV-1 than the thermal treatment applied alone.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2020.108809.

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