Antiviral activity of aged green tea extract in model food systems and under gastric conditions

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Abstract

Aged-green tea extract (GTE) is known to reduce the infectivity of hepatitis A virus (HAV) and murine norovirus (MNV), a human norovirus surrogate, *in vitro* and in washing solutions. Initially, the effect of aged-GTE was evaluated on virus like particles (VLPs) of human norovirus (HuNoV) genogroup I (GI) by a porcine gastric mucine (PGM)-enzyme-linked immunosorbent assay (ELISA) and transmission electron microscopy (TEM), and on HuNoV GI suspensions by an *in situ* capture-RT-qPCR method, suggesting that HuNoVs are very sensitive to aged-GTE treatment at 37 ºC. Moreover, the potential application of aged-GTE was evaluated using model foods and simulated gastric conditions. Then, aged-GTE samples prepared in orange juice, apple juice, horchata, and milk, respectively, were individually mixed with each virus and incubated overnight at 37 ºC. Aged-GTE at 5 mg/ml in apple juice reduced MNV infectivity to undetectable levels and from 1.0 to 1.8 log in milk, horchata and orange juice. Aged-GTE at 5 mg/ml in orange juice, apple juice, horchata and milk reduced HAV infectivity by 1.2, 2.1, 1.5, and 1.7 log, respectively. Additionally, aged-GTE at 5 mg/ml in simulated intestinal fluid reduced MNV titers to undetectable levels and reduced HAV infectivity by ca. 2.0 log. The results show a potential for aged-GTE as a suitable natural option for preventive strategies for foodborne viral diseases.

Keywords: Hepatitis A virus; human norovirus; simulated gastric fluid; green tea extract; food model systems

1. Introduction

Foodborne pathogens are a matter of increasing concern to consumers, regulatory bodies, and the food industry (WHO, 2015). Food is, in fact, a vehicle for the transmission of disease agents, most notably pathogenic bacteria and enteric viruses. Epidemiologically significant foodborne viruses include human noroviruses (HuNoV), hepatitis A virus (HAV), and hepatitis E virus (HEV) among others (EFSA, 2016, 2017; WHO, 2015). Globally, it is estimated that foodborne pathogens cause 600 million foodborne illnesses annually, mainly due to infectious agents causing diarrheal diseases (550 million), with HuNoVs being responsible for 120 million cases attributed to food and water (WHO, 2015). For most of these viruses there are no licensed antivirals. Consequently, there is an urgent need for foodborne virus therapeutics, particularly for HuNoV. In this sense, there is a great interest in moving towards natural antiviral and antimicrobial compounds. Natural plant extracts potentially have multiple functionalities, not only to increase the safety and enhance the quality of food products, but also to act as natural antivirals (reviewed by D'Souza (2014)). Over the last two decades, a great deal of effort has been directed toward identifying natural products, mainly of plant origin, to control foodborne viruses. For instance, several natural compounds have been reported to exhibit virucidal activity and have been evaluated against HuNoV surrogates (Li et al., 2013; Ryu et al., 2015). However, even if many natural compounds have already been characterized for their antiviral activity, limited information is available for their use in food applications (Fabra et al., 2016; Li et al., 2012; Sanchez et al., 2015). Additionally, reports on the antiviral activity of natural plant extracts within model food systems and under simulated gastric conditions are still limited (Joshi et al., 2015) .

Green tea extract (GTE), from *Camellia sinensis L.*, has demonstrated antiviral effects against murine norovirus (MNV), a human norovirus surrogate, and HAV at 25 °C and 37 °C *in vitro* and in food applications (Falcó et al., 2018; Marti et al., 2017; Randazzo et al., 2017). GTE contains large amounts of catechins which contribute greatly to its health benefits (Yilmaz, 2006); (Steinmann et al., 2013). Additionally, recent studies showed that the activity of epigallocatechin-3-gallate (EGCG) and GTE against enteric viruses is due to catechins derivatives; thus, the antiviral activity of GTE is enhanced by preparing the GTE solution 24 h before its use (aged-GTE) (Falcó et al., 2018). However, for GTE to be used as a therapeutic antiviral agent, its effectiveness in complex food matrices and gastrointestinal fluids that mimic digestion needs to be further explored.

In the present work, the antiviral activity of aged-GTE was initially assessed against virus-like particles (VLPs) of HuNoV. VLPs are morphologically and antigenically similar to the native infectious viruses and have been previously used to determine the antiviral activity of natural compounds (Li et al., 2012; Liu et al., 2018). HuNoV VLPs were treated with aged-GTE and analyzed by porcine gastric mucine (PGM)-ELISA binding assay and transmission electron microscopy (TEM). Furthermore, the effect of aged-GTE on HuNoV was evaluated by *in situ* capture RT-qPCR (ISC-RT-qPCR). Finally, antiviral activity of aged-GTE was evaluated in four food model systems and under simulated gastric conditions.

2. Materials and Methods

2.1. Clinical sample, virus propagation and cell lines

Fecal sample containing HuNoV genogroup I genotype 4 (kindly provided by Dr. J. Buesa, University of Valencia, Spain) was suspended (10%, wt/vol) in PBS containing

2 M $NaNO_3$ (Panreac, Barcelona, Spain), 1% beef extract (Conda, Madrid, Spain), 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.

MNV-1 (kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA) and HAV, strain HM-175/18f (purchased from ATCC VR-1402) were propagated and assayed in RAW 264.7 (kindly gifted by Prof. H. W. Virgin) and FRhk-4 cells (provided by Prof. A. Bosch, University of Barcelona, Spain), respectively. Cell lines and virus stocks were propagated as previously described (Randazzo et al., 2017). Infectious viruses were enumerated by determining the 50% tissue culture infectious dose ($TCID_{50}$) in 96-well microtiter plates with eight wells per dilution and 20 µl of inoculum per well using the Spearman-Karber method.

2.2. Aged-GTE preparation

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, (Falcó et al., 2018), from now on referred to as aged-GTE.

2.3. Binding of norovirus VLPs to porcine gastric mucine

Recombinant VLPs containing VP1 and VP2 proteins from the Norwalk GI.1 norovirus strain were produced as previously described (Allen et al., 2009). The PGM-ELISA binding assay was performed as described by Carmona-Vicente and collaborators (2016a) with a few modifications. Briefly, microtiter plates (Maxisorb, Life technologies) were coated with 10 µg/well of type III PGM (Millipore-Sigma) in carbonate-bicarbonate buffer pH 9.6 at 37 ºC for 1 h and then incubated overnight (ON) at 4 ºC. The following steps were performed at 37 ºC. Simultaneously, 10 µg/ml VLPs were incubated ON with aged-GTE at 0.5 and 5 mg/ml. ELISA plates were blocked

with 3% bovine serum albumin in PBS for 1 h. After washing with PBS with 0.05% Tween 20 (PBST) the VLP-GTE solutions were added to the plates and incubated for 1 h. Primary and secondary antibodies were diluted in PBST and incubated for 1 h each. The primary antibody was a rabbit anti-norovirus polyclonal antiserum (pAb) (Carmona-Vicente et al., 2016b) at a dilution of 1:2,000. The anti-rabbit horseradish peroxidase–labeled antibody IgG (Promega) was used as the secondary antibody at 1:10,000 dilution. The reaction was developed by the addition of OPD Sigma Fast (3,3',5,5'-tetramethyl-benzidine, Millipore-Sigma). Color development was stopped with 3M H_2SO_4 after 10 min. Absorbance was measured at 450 nm in microplate reader Multiskan FC (Thermo Scientific). After absorbance measurements the signal corresponding to the control VLPs (0 mg/ml GTE) was considered the 100% of the binding and the percentage of the treated VLPs calculated. Each sample was analyzed in triplicate and the mean values and standard deviation (SD) were calculated.

2.4. Transmission electron microscopy

TEM was used to determine any structural and/or morphological changes of HuNoV GI.1 VLPs treated with aged-GTE compared to non-treated VLPs. PBS 7.2 or aged-GTE at 1 and 10 mg/ml was mixed with equal volumes of VLPs at 100 µg/ml to give final concentrations of 0.5 and 5 mg/ml of aged-GTE and 50 µg/ml of VLPs. The mixtures were then incubated ON at 37 ºC. The treated VLPs of HuNoV GI.1 were applied to glow-discharged carbon-coated grids and negatively stained with 2 % uranyl acetate. Images were recorded with Gatan 1k CCD camera in a FEI Tecnai 12 electron microscope operated at 120 kV.

2.5. ISC-RT-qPCR

ISC-RT-qPCR was performed as previously reported (Wang and Tian, 2014; Wang et al., 2014) with some modifications. Briefly, each well was coated with 100 µl of PGM

(100 µg/ml) in carbonate-bicarbonate buffer (pH 9.6) at 37 ºC for 1 h and then incubated ON at 4 ºC. Simultaneously, suspensions of HuNoV GI were mixed with aged-GTE at 0.5 and 5 mg/ml ON at 37 ºC.

After being washed 5 times with 300 µl of PBS containing 0.05% Tween 20 and 0.3% BSA (PBSTB), the wells were blocked with 300 µL of 3% BSA in PBS at 37 ºC for 2 h. The wells were washed 5 times with PBSTB, and 100 µl of HuNoV-GTE samples and controls were added to the microplate and incubated at 37 ºC for 1 h. HuNoV GI suspensions without aged-GTE treatment or treated at 99 ºC for 5 min were used as a positive and negative control, respectively. Finally, after washing 5 times with PBSTB, each well was added with 100 µl of lysis buffer from NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co.).

Then, viral RNA was extracted using the same kit 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 (ISO 15216-1, 2017) using the LightCycler 480 instrument (Roche Diagnostics, Germany). A standard curve for HuNoV GI, was generated by amplifying 10-fold dilutions of viral RNA by RT-qPCR in quintuplicates, and the numbers of PCRU were calculated. 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. The quantification corresponding to the control HuNoV suspension (0 mg/ml GTE) was considered the 100% of the binding and the percentage of the treated HuNoVs calculated. Each sample was analyzed in triplicate and the mean values and SD were calculated.

2.6. Effect of aged-GTE on food model systems

MNV and HAV suspensions (ca. 4 log $TCID_{50}$/ml) were mixed with equal amounts of aged-GTE in orange juice (pH 2.6), apple juice (pH 3.8), "horchata de chufa" (a local drink speciality, with a composition of 83.5% of water, 10% of sugar and 2.2% of fat; pH 6.8) purchased from a local grocery store, or 2% reduced fat milk (Difco, CAS number 2021-04-13). Final concentrations of aged-GTE were 2.5 and 5 mg/ml. Samples were incubated at 37ºC ON in a shaker (180 rpm). Then, the effect of aged-GTE was neutralized with DMEM supplemented with 10% fetal calf serum (FCS). Positive controls were MNV and HAV suspensions added with PBS pH 7.2 under the same experimental conditions. Each treatment was run in triplicate. Confluent RAW 264.7 and FRhK-4 monolayers in 96-well plates were used to evaluate the effect of aged-GTE on food model systems. Antiviral activity of aged-GTE was estimated by comparing the number of infectious viruses on the aged-GTE treated virus suspensions and suspensions without aged-GTE. The decay of MNV and HAV titers was calculated as $\log_{10} (N_x/N_0)$, where N_0 is the infectious virus titer for untreated samples and Nx is the infectious virus titer for aged-GTE treated samples.

2.7. Effect of aged-GTE under gastric conditions

Determination of the antiviral activity of aged-GTE was assayed on different solutions of simulated digestion fluids. Simulated salivary fluid (SSF; pH 7.0), simulated gastric fluid (SGF; pH 3.0) and simulated intestinal fluid (SIF; pH 7.0) were prepared as previously described by Minekus et al. (2014). A concentration of 10 mg/ml of GTE was dissolved in each fluid and stored for 24 h at RT. Stocks of MNV and HAV with titers ca. 5 log $TCID_{50}$/mL were mixed in equal proportions in each solution (SSF, SGF and SIF) obtaining a final concentration of 5 mg/mL of aged-GTE. Samples were incubated in a shaker (180 rpm) at 37 ºC during 2 minutes for SSF and 2 h for SGF and SIF. Treatments were neutralized by adding DMEM containing 10% FCS. Positive

controls were virus suspensions added with PBS and with each simulated digestion fluids without aged-GTE under the same experimental conditions. Infectious viruses were quantified and effectiveness of the treatments was calculated as described above.

2.8. Data analysis

Results from three replicates of the treatments and controls were statistically analysed using ANOVA with STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA) and Tukey's test on a completely randomized design. A P value <0.05 was deemed significant.

3. Results

3.1 Effect of aged-GTE on the binding ability of HuNoV VLPs to PGM

In order to explore the effect of aged-GTE on HuNoV, the binding ability of HuNoV VLPs was tested by PGM-binding ELISA after treatment with aged-GTE at 0.5 and 5 mg/ml. The results show a significant reduction, close to 50%, in the binding of the VLPs to the PGM after treatment at both aged-GTE concentrations (data not shown).

3.2 Effect of aged-GTE on the morphology of HuNoV VLPs

In order to investigate whether the effect of aged-GTE on VLPs is due to the denaturation of viral capsid proteins or to morphological changes, the morphology of HuNoV GI.1 VLPs before and after treatment with aged-GTE was examined by TEM. The untreated samples of VLP presented assemblies with three different morphologies. Most were isometric particles with a diameter of 23 nm (Fig. 1A, black arrow) that were compatible with the icosahedral VLP with a T=1 architecture. A few isometric particles presented a higher diameter of 40 nm that must have corresponded to icosahedral T3 VLPs (Fig 1A, white star). A certain background of smaller assemblies that could be

associated with capsomers were also observed (Fig 1A, white arrow). Treatment with aged-GTE at 0.5 mg/ml dramatically decreased the number of VLPs observed per microscopic field (Fig. 1B). Interestingly aged-GTE at 5 mg/ml completely abolished the presence of VLPs, as was observed by TEM. The background of the untreated VLPs revealed the presence of smaller capsomers that could have been unassembled VP1 dimers (Fig. 1A, white arrow) that were less present in the 0.5 mg/ml aged-GTE treatment, indicating that the aged-GTE was not destructuring the VLPs into VP1 dimers but more likely affecting the VP1 structure itself.

3.4 ISC-RT-qPCR

Additionally, the binding ability of HuNoV to PGM was tested by ISC-RT-qPCR after treatment with aged-GTE at 0.5 and 5 mg/ml and heating at 99 ºC (Fig. 2). Aged-GTE at 0.5 mg/ml reduced the binding of HuNoV GI to PGM approximately 65 %, while aged-GTE at 5 mg/ml and heating completely eliminated HuNoV GI binding.

3.5 Antiviral activity of aged-GTE in model food systems

The reduction of MNV and HAV titers in food models after ON incubation with aged-GTE (2.5 and 5 mg/ml) at 37ºC is shown in Table 1. Orange juice significantly ($p < 0.05$) reduced MNV infectivity by 0.8 log compared to PBS control. Aged-GTE at 2.5 mg/ml in horchata, orange juice, and apple juice reduced MNV titers by 1.2, 0.4 and 1.2 log, respectively; no significant differences were reported in milk. Aged-GTE at 5 mg/ml in milk, horchata, orange juice and apple juice reduced MNV titers by 1.0, 1.9, 1.2 log, and to undetectable limits, respectively. Infectivity of HAV treated with aged-GTE at 2.5 mg/ml in milk, horchata, orange juice and apple juice was reduced by 0.9,

1.2, 1.0 and 1.2 log, respectively (Table 1) while aged-GTE at 5 mg/ml reduced HAV infectivity by 1.2, 2.1, 1.5, and 1.7 log, respectively.

3.6 Antiviral activity of aged-GTE under simulated gastric conditions

Initially, the infectivity of MNV and HAV was evaluated on the three fluids (Fig. 3). The MNV titers were 5.9 ± 0.3, 5.4 ± 0.1, and 5.4 ± 0.1 log $TCID_{50}$/ml and the HAV titers were 4.7 ± 0.1, 4.5 ± 0.5, and 4.5 ± 0.0 log $TCID_{50}$/ml, SSF, SGF and SIF respectively. Aged-GTE at 5 mg/ml prepared in SSF (pH 7.0) reduced MNV infectivity by 0.7 log, while a 1.5 log reduction was reported after 2 min at 37 ºC for HAV. Additionally, aged-GTE at 5 mg/ml reduced virus infectivity by 3.1 and 2.2 log for MNV and HAV, respectively, under SGF conditions (pH 3.0, 37 ºC, 2 h). Moreover, aged-GTE at 5 mg/ml reduced MNV and HAV infectivity to undetectable levels and by 2.0 log, respectively, under SIF conditions (pH 7.0, 37 ºC, 2 h).

4. Discussion

GTE and EGCG have been shown to be highly effective in reducing the titers of MNV and HAV at neutral and alkaline pHs, where the antiviral activity was found to be concentration-, temperature- and exposure time-dependent (Falcó et al., 2017; Gómez-Mascaraque et al., 2016; Randazzo et al., 2017). Moreover, a previous study demonstrated that storage of the GTE solutions for 24 h at 25 ºC increased the amount of the antiviral active compounds as a consequence of the degradation and epimerization reactions of polyphenols of GTE (Falcó et al., 2017; Falcó et al., 2018). In the present study, for the first time, we evaluated the effects of aged-GTE on VLPs of HuNoVs GI by PGM ELISA and on HuNoV GI suspensions by ISC-RT-qPCR. PGM contains multiple histo-blood group antigens that have been recognized as receptors or co-receptors for HuNoVs (Tian et al., 2005). Our results indicate that 0.5 mg/ml aged-

GTE impairs the binding of HuNoVs to histo-blood group antigens (HBGAs) present in PGM in a way similar to that of the higher concentration (5 mg/ml). It can be argued that the PGM-ELISA binding assay shows binding activity of non-VLP noroviral proteins present in the suspensions after aged-GTE treatments, since the P-domains of norovirus VP1 are enough to bind HBGAs (Tan et al., 2004); thus, the PGM ELISA binding assay would probably be underestimating the antiviral effect of aged-GTE. Additionally, TEM analysis showed that aged-GTE caused structural damage to the HuNoV VLPs with an important reduction of structured VLPs at 0.5 mg/ml and total abolition of VLPs at 5 mg/ml.

ISC-RT-qPCR based on PGM has been used successfully to estimate the inactivation of HuNoVs treated by heating, high-pressure processing, chlorine and ethanol (Dancho et al., 2012; Wang and Tian, 2014). In parallel, our study indicated that aged-GTE at 5 mg/ml abolished HuNoV GI binding to PGM, while at 0.5 mg/ml some viral particles were still able to bind its receptors, suggesting that HuNoV may be very sensitive to aged-GTE treatment. Furthermore, we demonstrated that the ISC-RT-qPCR method could be used to indirectly indicate the infectivity of HuNoV after treatment with natural compounds.

Many natural compounds have shown promising antiviral effects when tested *in vitro*; however, when evaluated in food applications (i.e., sanitizing solutions or incorporated in food packaging), the viral inactivation rate was reduced (Li et al., 2012; Randazzo et al., 2017; Sanchez et al., 2015). Thus, the potential application of natural compounds as antivirals needs to be evaluated in model food systems and under gastrointestinal conditions. For both MNV and HAV, aged-GTE significantly reduced ($p < 0.05$) viral infectivity in the four model food systems evaluated, except for MNV in milk treated with aged-GTE at 2.5 mg/ml. Several factors could be responsible for the decrease in

efficacy, such as the interaction of the active compounds or the viruses with food matrices, especially the fat, protein, or sugar content (Joshi et al., 2017; Joshi et al., 2015; Li et al., 2012). Apple juice (rich in carbohydrates) and milk (rich in proteins and lipids) have been used as model food systems in some studies that evaluated the efficacy of natural antivirals (Joshi et al., 2017; Joshi et al., 2015). When aged-GTE (5 mg/ml) was prepared in apple juice (pH 6.8), MNV titers were reduced to undetectable levels after 24 h and by 1.7 log for HAV. When aged-GTE was prepared in milk, its effectiveness decreased significantly, with only 1 log reduction of HAV and MNV infectivity. These results are in agreement with previous studies (Joshi et al., 2017; Joshi et al., 2015), where blueberry proanthocyanidins (B-PAC) and GSE retained their antiviral activity in apple juice, though their antiviral effect decreased in milk. For instance, aged-GTE (5 mg/ml) prepared in milk reduced MNV titers by 1.0 log (Table 1) after 24 h at 37 ºC, while B-PAC at the same experimental conditions (5 mg/ml, 24 h, 37 ºC) decreased MNV titers by 0.8 log (Joshi et al., 2017). For HAV, aged-GTE at 5 mg/ml reduced HAV titers by 1.2 log in milk (Table 1) while similar inactivation rates (0.8 log) were reported for GSE at 4 mg/ml tested under the same experimental conditions (Joshi et al., 2015). Although horchata contains 2.2% fat, 5 mg/ml aged-GTE in hochata reduced MNV and HAV infectivity by ca. 2 log, resulting in a potential carrier of natural antivirals.

In order to use natural compounds as antivirals, it is particularly important to assess the maintenance of their antiviral activity under conditions encountered during consumption and transition through the gastrointestinal tract. Interestingly, when aged-GTE was added to SSF, SGF, and SIF solutions, significant reductions of MNV and HAV infectivity were recorded. In particular, aged-GTE (5 mg/ml) prepared in SIF reduced MNV infectivity to undetectable levels and by ca. 2 log for HAV (Fig. 3). These results

are consistent with the inactivation rates reported for GSE and PAC-B in SIF. GSE prepared in SIF reduced MNV and HAV titers by 1.7 and 1.4 log, respectively (Joshi et al., 2015), while PAC-B prepared in SIF reduced MNV infectivity to undetectable levels (Joshi et al., 2017). Moreover, aged-GTE prepared in SGF (pH 3.0) reduced MNV infectivity to a lesser extent compared to aged-GTE in SIF (pH 7.0). One plausible reason could be derived from the fact that aged-GTE is very effective in inactivating MNV at neutral and alkaline pHs, but less effective at pH 5.5, and this has been correlated to the formation of catechin derivatives (Falcó et al., 2018).

Overall, the results of the evaluation of aged-GTE in model food systems and simulated gastric conditions, could help in moving toward the development of sustained-released products containing aged-GTE for consumption. In addition, this study suggests that exposure to intestinal and gastric fluids maintains the antiviral activity of aged-GTE, but future studies should involve animal feeding studies with aged-GTE to determine its antiviral effects.

Based on the effects of aged-GTE against MNV and HAV, with reduced effectiveness in model food systems, encapsulation strategies (Falcó et al., 2017; Gómez-Mascaraque et al., 2016) to protect aged-GTE from food matrices may be of great interest for optimal antiviral activity as well as time-released in the intestinal tract.

Acknowledgements

This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO) (RYC-2013-12442) and the Spanish National Institute for Agriculture and Food Research and Technology (INIA) co-financed by the European Social Fund (Project RTA2014-00024-C03). Financial support has been co-sponsored by the European Regional Development Fund. JRD was supported by the “Ramon y Cajal”

348 Young Investigator Grants and WR by a postdoctoral fellowship from Generalitat
349 Valenciana (APOSTD/2018/150).
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Table 1. Murine norovirus (MNV) and hepatitis A virus (HAV) titers (log $TCID_{50}$/ml) after treatments with aged-GTE prepared in different model food systems and incubated overnight at 37 ºC. Each treatment was done in triplicate. Within each column for each model food system, different letters denote significant differences between treatments ($p < 0.05$).

Food models	Aged-GTE (mg/ml)	MNV		HAV	
		Recovered titers	Log reduction	Recovered titers	Log reduction
PBS		4.45 ± 0.12 $_a$	-	4.57 ± 0.13 $_a$	-
Milk	0	4.41 ± 0.38 $_a$	-	4.45 ± 0.22 $_a$	-
	2.5	4.32 ± 0.13 $_a$	0.08	3.57 ± 0.13 $_b$	0.88
	5	3.37 ± 0.31 $_b$	1.04	3.28 ± 0.29 $_b$	1.17
Horchata	0	4.45 ± 0.22 $_a$	-	4.52 ± 0.26 $_a$	-
	2.5	3.20 ± 0.00 $_b$	1.25	3.32 ± 0.22 $_b$	1.20
	5	2.57 ± 0.22 $_c$	1.88	2.37 ± 0.29 $_c$	2.15
Orange juice	0	3.62 ± 0.19 $_b$	-	4.08 ± 0.00 $_a$	-
	2.5	3.20 ± 0.00 $_c$	0.42	3.07 ± 0.33 $_b$	1.00
	5	2.45 ± 0.22 $_d$	1.17	2.62 ± 0.26 $_c$	1.46
Apple juice	0	4.53 ± 0.33 $_a$	-	4.28 ± 0.14 $_a$	-
	2.5	3.20 ± 0.00 $_b$	1.25	3.07 ± 0.22 $_b$	1.21
	5	< 1.15 $_c$	>3.38	2.53 ± 0.07 $_c$	1.75

Figure legends

Figure 1. Representative TEM field showing negatively stained untreated VLPs (A) and VLPs treated with aged-GTE (0.5 mg/ml) (B). The white star indicates a 40 nm putative T3 symmetry VLP. The black arrow points to a 23 nm putative T1 symmetry VLP. The white arrow points to VP1 capsomer. The scale bar indicates 200 nm.

Figure 2. Effect of aged-GTE on the binding of human norovirus GI to PGM analyzed by ISC-RT-qPCR. Each bar represents the average of triplicates.
Asterisks show statistical differences ($p<0.05$)

Figure 3. Reduction of murine norovirus (MNV) (A) and hepatitis A virus (HAV) (B) titers (log $TCID_{50}$/ml) after treatments with aged-GTE (5 mg/ml) prepared in simulated salivary fluid (SSF; 2 min at 37 ºC), simulated gastric fluid (SGF; 2 h at 37 ºC) and simulated intestinal fluid (SIF, 2 h at 37 ºC)
*Black: Control (virus in simulated fluids); grey: aged-GTE prepared in simulated fluids
**Each column represents the average of triplicates. Each bar represents the average of triplicates. Within each column for each virus, different letters denote significant differences between treatments ($P < 0.05$).
Solid line depicts the detection limit.