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Effect of (−)-epigallocatechin gallate at different pH conditions on enteric viruses

Irene Falcó [a, b], Walter Randazzo [a, b], Laura Gómez-Mascaraque [b], Rosa Aznar [a, b], Amparo López-Rubio [b], Gloria Sánchez [a, b, *]

[a] Department of Microbiology and Ecology, University of Valencia, Av. Dr. Moliner, 50, 46100, Burjassot, Valencia, Spain
[b] Food Safety and Preservation Department, IATA-CSIC, Avda. Agustin Escardino 7, 46980, Paterna, Valencia, Spain

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ABSTRACT

Epigallocatechin gallate (EGCG), a flavonoid from green tea, is said to have extensive antimicrobial activity in a wide range of food spoilage or pathogenic fungi, yeast and bacteria. In this work, the antiviral activity of EGCG was assessed against hepatitis A virus (HAV) and murine norovirus (MNV), a human norovirus surrogate, at different temperatures, contact times and pH conditions by cell-culture methods. EGCG was effective in reducing the titers of HAV and MNV in a dose-dependent manner at neutral pH and 25 and 37 °C, while no effect was reported at 4 °C. HAV and MNV infectivity was completely removed after overnight treatment with EGCG at 2.5 mg/mL at 37 °C. Furthermore, results also revealed that EGCG was very effective inactivating MNV and HAV at neutral and alkaline pH but was ineffective at pH 5.5. Results from cell-culture assays and viability RT-qPCR assays indicated that EGCG did not dramatically affect viral capsid, which instead may suffer subtle alterations of proteins. Moreover, HPLC/MS analysis of catechin solutions at different pHs indicated that antiviral activity was most likely due to catechin derivatives rather than EGCG itself, given the evolution of these compounds at the various pH conditions tested. These findings suggest that green tea catechins appear to be a suitable natural option for foodborne viral reduction.

1. Introduction

Nowadays, foodborne viral outbreaks are a growing concern for food safety authorities. Indeed, enteric viruses, in particular human noroviruses, which cause gastroenteritis, are the leading causes of foodborne illnesses in industrialized countries (Control & Prevention, 2013; EFSA, 2015). Moreover, hepatitis A virus (HAV) is considered as a re-emerging pathogen and is responsible for about half the total number of human hepatitis infections diagnosed worldwide (Sprenger, 2014). Norovirus and HAV can be transmitted directly from person-to-person, but also indirectly via virus-contaminated food (mainly associated with shellfish, soft fruits, leafy greens, and ready-to-eat meals), water, and surfaces.

Due to their low infectious dose (10–100 viral particles) (Teunis et al., 2008; Yezli & Otter, 2011) and to their stability and resistance to inactivation processes, the development of alternative methods for the viral decontamination of food has been recently promoted by public authorities (WHO, 2013). Amongst them, promising results have been reported for many natural compounds tested as antivirals *in vitro*, but when they were evaluated in food model systems or food applications, the viral decay was somewhat limited (Bozkurt, D'Souza, & Davidson, 2015; D'Souza, 2014; C. Sánchez, Aznar, & Sánchez, 2015). Many factors could be responsible for such decrease in efficacy such as the interaction of the active compound or the virus with food matrices, the pH, the water activity, etc.

From the commercially available natural extracts, green tea extract (GTE) has demonstrated inhibitory properties against foodborne bacteria (Perumalla & Hettiarachchy, 2011) and more recently against norovirus surrogates as well (Ueda et al., 2013). Chemically, GTE mainly contains catechins, a group of flavonoids with antioxidant properties (Yilmaz, 2006). Specifically, epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), gallocatechin (GC), catechin (C) and epigallocatechin gallate (EGCG) have been found to be the main catechins present in GTE (Kajiya

* Corresponding author. Food Safety and Preservation Department, Institute of Agrochemistry and Food Technology (IATA-CSIC), Av. Agustín Escardino, 7, 46980, Paterna, Valencia, Spain.
E-mail address: gloriasanchez@iata.csic.es (G. Sánchez).

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et al., 2004). These bioactive compounds possess many health benefits (Singh, Shankar, & Srivastava, 2011), including protective effects against infections (Steinmann, Buer, Pietschmann, & Steinmann, 2013), cardiovascular and neurodegenerative diseases (Fu, Zhen, Yuskavage, & Liu, 2011), inflammation and arthritis (Singh, Akhtar, & Haqqi, 2010) and cancer (Larsen & Dashwood, 2010). EGCG and ECG are the most abundant compounds in GTE and they have showed strong antimicrobial (Shimamura, Zhao, & Hu, 2007; Veluri, Weir, Bais, Stermitz, & Vivanco, 2004) and antiviral (Dhiman, 2011; Savi, Barardi, & Simões, 2006; Xiao, Yang, Shi, Liu, & Chen, 2008) activities, even when encapsulated within chitosan electrosprayed microcapsules (Gómez-Mascaraque, Soler, & Lopez-Rubio, 2016) or applied as hand sanitizer formulations (Zhang, Yang, Yang, Wu, & Wu, 2016). Due to their autooxidation and varying degree of polymerization, catechins show diverse structural features (Li, Taylor, Ferruzzi, & Mauer, 2012), which result in different binding modes and inhibitory effects. Evidences clearly showed a pH dependent effect on the antiviral activity of EGCG. For example, it was reported that EGCG at neutral pH inactivates herpes simplex virus (HSV), an enveloped virus, but it was ineffective below pH 7.4. In contrast, when EGCG was oxidatively coupled to form dimers with one or two gallate moieties, the antiviral activity at acid pH was substantially increased (Isaacs et al., 2011).

Most studies aiming to determine the antiviral activity of natural compounds have been performed by artificially adding a known amount of the selected compound to a given viral suspension, determining the reduction in the infectious titer after subjecting the treated sample to designated conditions, and applying statistical procedures to determine the significance of virus decay. Obviously, this implies the use of virus strains that may be propagated in cell cultures and enumerated through infectivity, thus greatly restricting the range of viruses to be used in these studies. This is extremely relevant for human norovirus, since only very recently, a human norovirus culture system using enteroids cells has been developed (Ettayebi et al., 2016), but there are limitations that need to be overcome before this assay can be routinely used. Until then, evaluation of the efficacy of natural compounds on human norovirus is still performed using norovirus surrogates such as feline calicivirus (FCV), murine norovirus (MNV), and Tulane virus (TV). Moreover, a novel approach to assess human norovirus and HAV infectivity by combining intercalant dyes and RT-qPCR (Moreno, Aznar, & Sánchez, 2015; Randazzo, López-Gálvez, Allende, Aznar, & Sánchez, 2016; Sánchez, Elizaquível, & Aznar, 2012) has been recently developed with the potential to be used for inactivation studies.

In the present work, the antiviral activity of EGCG was assessed against enteric viruses at different temperatures, contact times and pH conditions by cell-culture methods. Furthermore cell-culture results were compared to results obtained by viability RT-qPCR. Moreover, HPLC analysis of the catechin solutions at different pHs were performed to correlate the antiviral effect of EGCG and its derivatives formed at the various tested conditions and, thus, be able to explain the different antiviral activity displayed.

2. Material and methods

2.1. Viral strains, cell lines and infections

HAV, HM-175/18f strain (ATCC VR-1402), was propagated and assayed in FRhK-4 cells (kindly provided by Prof. A. Bosch, University of Barcelona, Spain). Murine norovirus, MNV-1 strain, was propagated and assayed in RAW 264.7 cells (kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA). Semi-purified stocks were subsequently produced from the same cells by centrifugation of infected cell lysates at 660×*g* for 30 min. Infectious viruses were enumerated by determining the 50% tissue culture infectious dose ($TCID_{50}$) with eight wells per dilution and 20 μl of inoculum per well using the Spearman-Karber method (Pintó, Diez, & Bosch, 1994).

Faecal suspension of human norovirus genogroup II genotype 4 (GII.4 variant Den Haag 2006b) was kindly provided by Dr. J. Buesa, University of Valencia, Spain. Norovirus stool sample was suspended (10%, wt/vol) in phosphate-buffered saline (PBS) containing 2 M $NaNO_3$ (Panreac, Spain), 1% beef extract (Conda, Spain), and 0.1% Triton X-100 (Fisher Scientific, USA) (pH 7.2) and pelleted at 1000 × g for 5 min. The supernatant was stored at −80 °C in aliquots.

2.2. Effect of EGCG on HAV and MNV suspensions

EGCG from green tea (Sigma-Aldrich, CAS number 989-51- 5, Spain) was dissolved in PBS (pH 7.2) to obtain concentrations of 0.25 and 2.5 mg/mL. Each EGCG solution was mixed with an equal volume of HAV and MNV suspensions (ca. 6–7 log $TCID_{50}$/mL), followed by incubation at 4, 25 and 37 °C in a shaker for 2 or 16 h (overnight incubation). Ten-fold dilutions of EGCG-treated and untreated virus suspensions were inoculated into confluent FRhK-4 and RAW monolayers in 96-well plates. Then, infectious viruses were enumerated by cell culture assays as described above. Each treatment was done in triplicate. Positive controls were virus suspensions added with PBS only. The decay of HAV and MNV titers was calculated as $\log_{10}(N_x/N_0)$, where N_0 is the infectious virus titer for untreated samples and N_x is the infectious virus titer for EGCG-treated samples.

2.3. Pre-treatment with EGCG prior to virus infection

Ninety six-well cell culture plates were seeded with RAW 264.7 or FRhk-4 cells. After 24 h or 72 h, RAW 264.7 or FRhk-4 cells, respectively, cell media was removed and washed two times with PBS pH 7.2. Cell lines were treated for 1 h with 0.1 and 1 mg/mL (0.2 and 2 mM, respectively) of EGCG in PBS pH 7.2. Then, EGCG was removed from the 96-well plate and the cells were washed twice with PBS. Untreated and EGCG-treated monolayers were then infected to ten-fold dilutions of MNV and HAV. Infectious viruses and effectiveness of the treatments were calculated as described above.

2.4. Effect of pH on the antiviral activity of EGCG

In order to elucidate the effect of pH on the antiviral activity of EGCG, virus suspensions were ten-fold diluted in PBS at different pHs and incubated with a EGCG solution at 0.25 and 2.5 mg/mL prepared in PBS at different pHs (5.5, 6.5, 7.2, 8.0 and 8.5). Samples were further incubated at 37 °C in a water-bath shaker at 150 rpm for 2 h. Infectious viruses and effectiveness of the treatments were calculated as described above.

2.5. Efficacy of EGCG on human norovirus and HAV using viability RT-qPCR

Human norovirus and HAV suspensions were overnight incubated with 2.5 mg/mL of EGCG at 37 °C in a shaker. Positive controls were virus suspensions added with PBS only. To assess virus infectivity, a viability-RT-qPCR procedure recently developed was applied (Moreno et al., 2015; Randazzo et al., 2016). Briefly, one-hundred microliters of EGCG-treated virus and un-treated virus were added to PMAxx 50 μM (Biotium, USA) and 0.5% Triton X-100

Table 1
The effect of EGCG on the infectivity of murine norovirus (MNV).

[EGCG] (mg/mL)	37 °C (log $TCID_{50}$/mL)				25 °C (log $TCID_{50}$/mL)				4 °C (log $TCID_{50}$/mL)			
	2 h		Overnight		2 h		Overnight		2 h		Overnight	
	Recovered titer	R	Recovered titer	R	Recovered titer	R	Recovered titer	R	Recovered titer	R	Recovered titer	R
0	6.74 ± 0.26A		4.87 ± 0.43A		6.28 ± 0.26A		6.32 ± 0.12A		6.32 ± 0.25A		6.32 ± 0.36A	
0.25	6.32 ± 0.12B	0.42	3.71 ± 0.12B	1.16	6.07 ± 0.21A	0.21	5.87 ± 0.07B	0.45	6.24 ± 0.31A	0.80	6.16 ± 0.07A	0.16
2.5	4.20 ± 0.00C	2.54	<1.15C	>3.72	6.01 ± 0.09A	0.27	4.32 ± 0.17C	2.00	6.32 ± 0.53A	0.00	6.01 ± 0.09A	0.31

R: reduction.
Within each column for each temperature and time, different letters denote significant differences between treatments ($P < 0.05$).

Table 2
The effect of EGCG on the infectivity of hepatitis A virus (HAV).

[EGCG] (mg/mL)	37 °C				25 °C				4 °C			
	2 h (log $TCID_{50}$/mL)		Overnight (log $TCID_{50}$/mL)		2 h (log $TCID_{50}$/mL)		Overnight (log $TCID_{50}$/mL)		2 h (log $TCID_{50}$/mL)		Overnight (log $TCID_{50}$/mL)	
	Recovered titer	R	Recovered titer	R	Recovered titer	R	Recovered titer	R	Recovered titer	R	Recovered titer	R
0	6.49 ± 0.14A		5.39 ± 0.09A		5.82 ± 0.00A		5.51 ± 0.26A		6.32 ± 0.12A		6.15 ± 0.28A	
0.25	6.32 ± 0.25A	0.17	3.45 ± 0.35B	1.94	5.82 ± 0.33A	0.00	3.78 ± 0.09B	1.73	6.03 ± 0.19B	0.29	5.70 ± 0.33AB	0.45
2.5	5.36 ± 0.26B	1.13	<1.15C	>4.24	5.20 ± 0.00B	0.62	<1.15C	>4.36	6.03 ± 0.07B	0.29	5.74 ± 0.14B	0.41

R: reduction.
Within each column for each temperature and time, different letters denote significant differences between treatments ($P < 0.05$).

and incubated in the dark at room temperature for 10 min at 150 rpm. Thereafter, samples were exposed to light for 15 min using a Led-Active Blue system (Geniul, Spain). Then, viral RNA was extracted using the NucleoSpin® RNA virus kit (Macherey-Nagel GmbH & Co., Germany) according to the manufacturer's instructions. As a control, EGCG-treated virus and untreated virus without PMAxx-Triton pretreatment was included. RNA samples were analyzed in duplicate by RT-qPCR using the RNA UltraSense One-Step quantitative RT-PCR system (Invitrogen, USA) and the set of primers and probe recommended by the ISO 15216 (ISO 15216-1, 2017) using the LightCycler 480 instrument (Roche Diagnostics, Germany).

2.6. HPLC analysis

In order to elucidate the presence of green tea catechins and their derivatives in the different incubation media, EGCG solutions (2.5 mg/mL) were prepared in PBS at the different pH and analyzed through HPLC-MS. For this purpose, an Agilent 1290 HPLC system (USA) equipped with an Acquity BEH C18 (Waters, USA, 50 mm × 2.1 mm, 1.7 μm of particle size) LC-column was used, following a method adapted from Gómez-Mascaraque, Sanchez, and López-Rubio (2016). The injection volume was 10 μL. Eluent A was water and eluent B methanol (Fisher Chemical, USA), both slightly acidified with 0.1% of formic acid (Fisher Chemical, USA). The flow rate was 0.4 mL/min and the elution gradient started with 10% of eluent B during 2 min, followed by 100% eluent B for 13 min, and 10% eluent B for the last 7 min. A TripleTOF™ 5600 system with a DuoSpray™ source operating in the negative mode was used for detection (AB SCIEX, USA). The parameter settings used were: ion spray voltage −4500 V, temperature 400 °C, curtain gas 25 psi, ion source gas 50 psi. Data were evaluated using the XIC manager in the

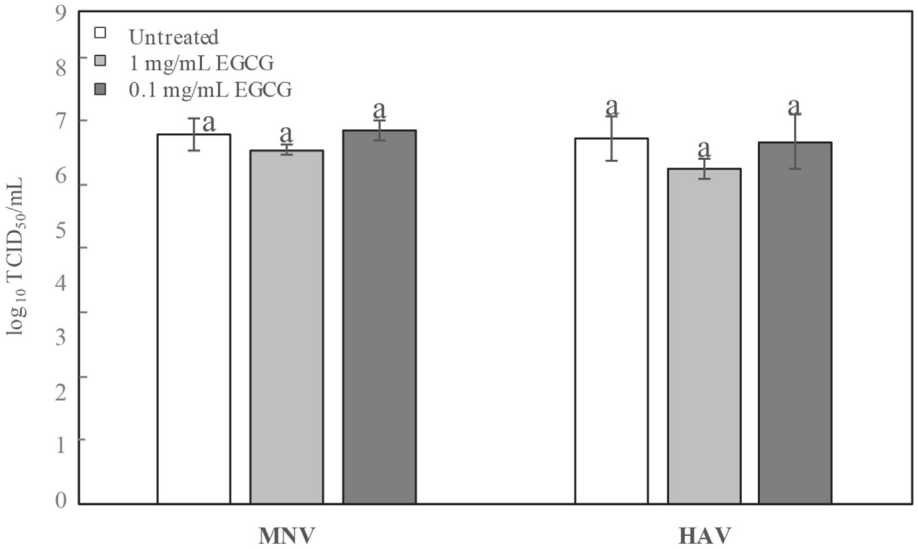

Fig. 1. Effect on murine norovirus (MNV) and hepatitis A virus (HAV) titers after pretreatment of RAW and FRhk-4 cells with EGCG at 0.1 and 1 mg/mL. Titers are the means ± standard deviations of results of three replicates.

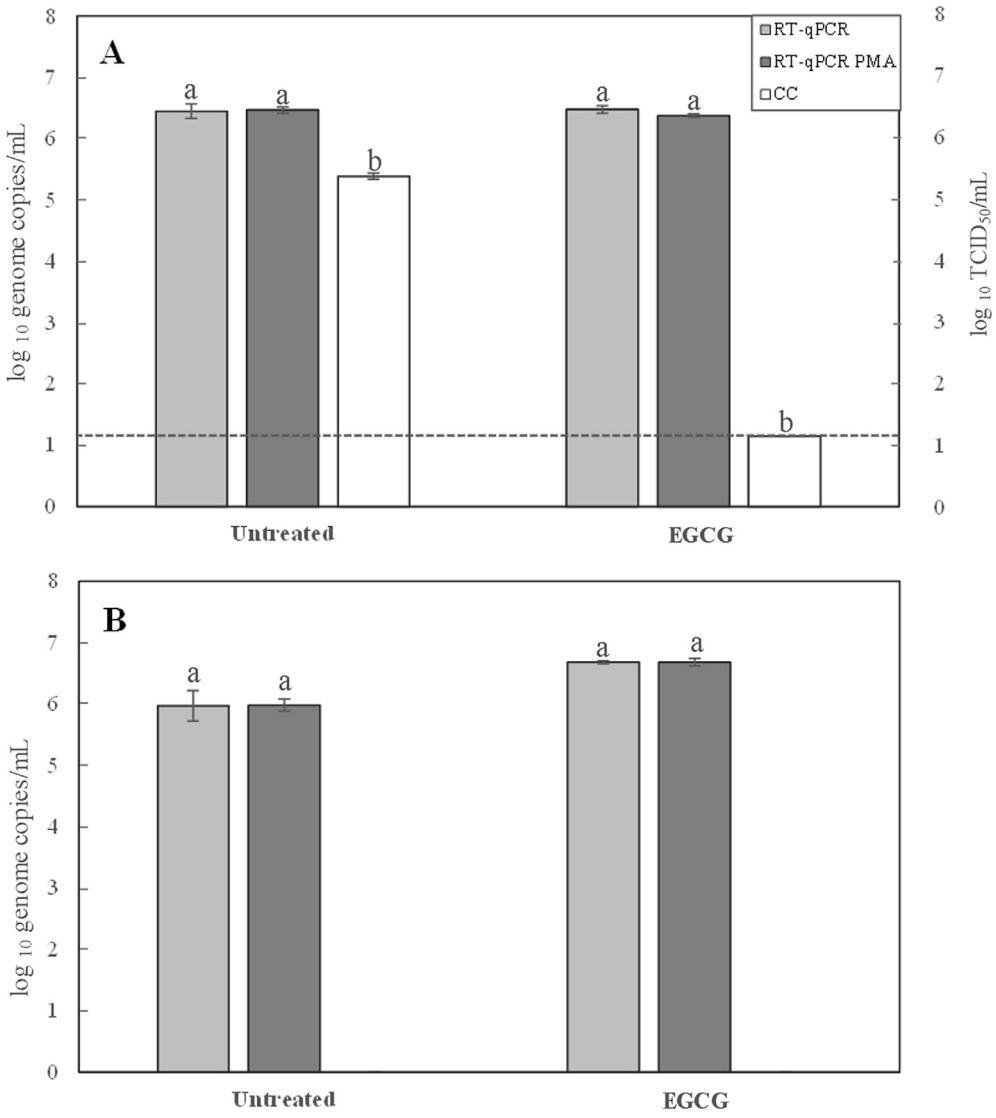

Fig. 2. Titers of HAV (A) after overnight treatment with 2.5 mg/mL EGCG as determined by infectivity ($TCID_{50}$) and by molecular methods (RT-qPCR alone and PMAxx-Triton-RT-qPCR) and NoV (B) by molecular methods (RT-qPCR alone and PMAxx-Triton-RT-qPCR).
Dashed lines indicate the detection limit for the $TCID_{50}$ assay. Titers are the means ± standard deviations of results of three replicates.

PeakViewTM software (version 2.2).

2.7. Statistical analysis

A statistical analysis of experimental data was performed using the Student's *t*-test with a significance level of $P < 0.05$ (Microsoft Office Excel; Microsoft Professional Plus 2010).

3. Results and discussion

3.1. Effect of EGCG on the infectivity of MNV and HAV

This study clearly demonstrates that EGCG was effective in reducing the titers of MNV and HAV in a dose-dependent manner, where increasing concentrations of EGCG showed increased reduction in viral titers. Incubation of MNV and HAV with EGCC at concentrations of 0.25 mg/mL for 2 h at 4, 25 and 37 °C slightly decreased the titer of both viruses (Tables 1 and 2) while significant differences ($p < 0.05$) were observed after overnight (ON) incubation at 25 and 37 °C. Moreover, EGCG at 2.5 mg/mL reduced HAV titers to undetectable levels after ON incubation at 25 and 37 °C, while MNV was completely inactivated after ON incubation at 37 °C only.

Currently, efficacy of flavonoids has been mainly evaluated on norovirus surrogates (reviewed by D'Souza, 2014; D. Li, Baert, & Uyttendaele, 2013; Ryu et al., 2015) and information about their efficacy on HAV is somewhat limited. Moreover, a number of studies have assessed the efficacy of green tea catechins on norovirus surrogates. Su and D'Souza (2013) reported that L-epicatechin at 0.5 mM reduced by 1.40 $\log_{10}$ FCV infectivity while no effect was reported on MNV. Moreover, Oh et al. (2013) evaluated the effect of EC, EGCG, EGC, and ECG against FCV. Among the catechins tested, EGCG exhibited the most effective antiviral activity. In line with these results, EGCG at 100 μM and ECG at 150 μM had the most potent antiviral activity against FCV and MNV (Seo et al., 2016).

As for other natural compounds (reviewed by Sánchez & Aznar, 2015) EGCG exerted the strongest effect at 37 °C, although it was still active at room temperature, fact that could facilitate its final application in the food industry. To this end and based on the time-

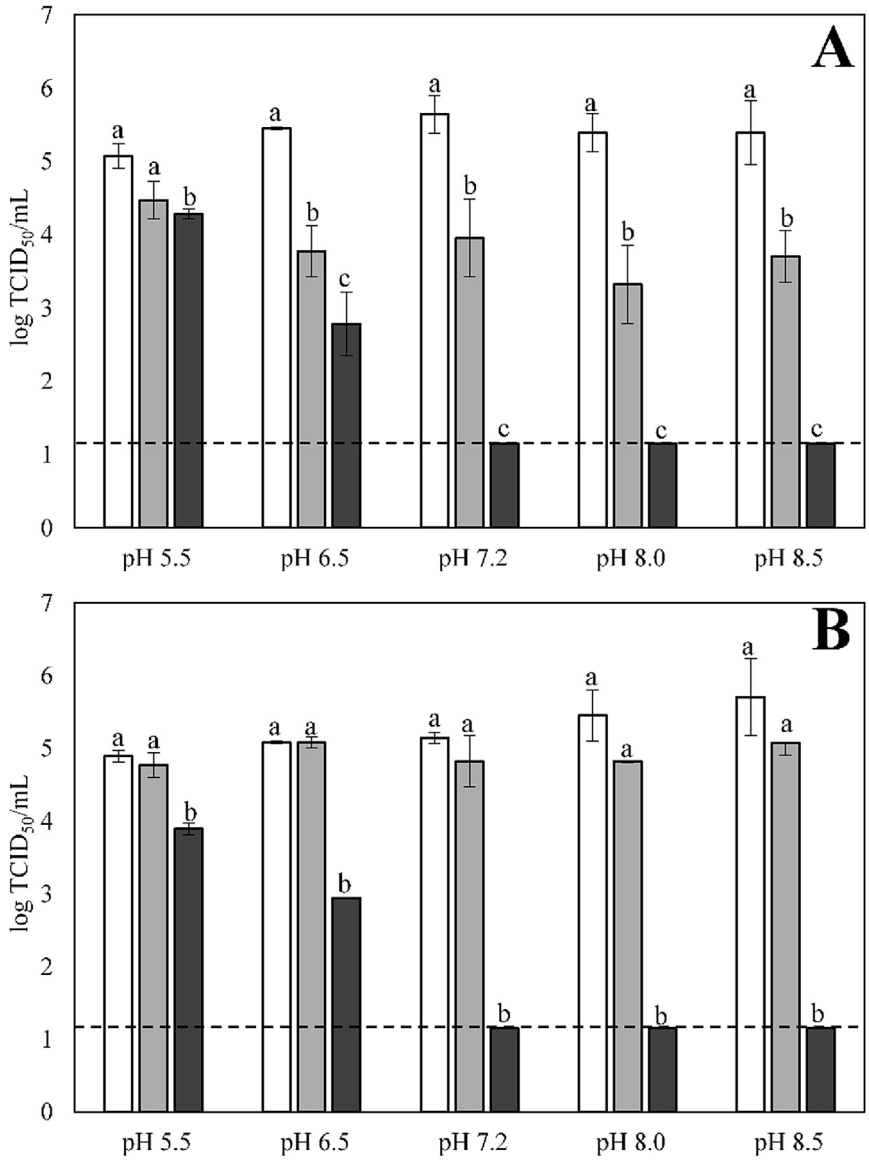

Fig. 3. Effect of pH on the activity of EGCG against MNV (A) and HAV (B).
White bars indicate untreated samples, greys bars indicate 0.25 mg/mL EGCG and black bars 2.5 mg/mL EGCG. Dashed lines indicate the detection limit for the $TCID_{50}$/mL assay. Titers are the means ± standard deviations of results of three replicates.

and concentration-dependent effects of EGCG against enteric viruses, these natural compounds are promising alternatives to be used in food industry not only to limit cross contamination when applied at room temperature, but also to retain activity after ingestion being active as well at 37 °C (Su & D'Souza, 2011). Interestingly, our results showed that EGCG was more efficient on HAV than MNV at room temperature, in line with results obtained with grape seed extract (GSE) (Su & D'Souza, 2011), which also contains proanthocyanidins in the form of monomeric phenolic compounds, such as catechin, epicatechin and epicatechin-3-*O*-gallate (reviewed by Perumalla & Hettiarachchy, 2011).

Moreover, as green tea catechins have GRAS (Generally Recognized as Safe) status and was very effective against HAV and MNV at 25 and 37° C this may facilitate either the use of EGCG or green tea extracts in food applications applied as natural hand sanitizers (Zhang et al., 2016), encapsulated as food ingredients (Gómez-Mascaraque, Sanchez, et al., 2016) or incorporated within packaging structures (Muriel-Galet, Cran, Bigger, Hernández-Muñoz, & Gavara, 2015; Wrona, Cran, Nerín, & Bigger, 2017) to control virus cross-contamination of food-contact surfaces.

3.2. *Effect of EGCG on RAW and FRhk-4 cells*

Pretreatments of cells with the EGCG were performed primarily to determine the ability of the EGCG to inactivate cellular receptor(s) (Ryu et al., 2015). Our results showed no effects of EGCG pre-treatment on both FRhK-4 and RAW 264.7 cells at 2 mM after 1 h (Fig. 1). Moreover, when infectious MNV and HAV titers obtained on untreated RAW and FRhk-4 monolayers were compared with EGCG-treated monolayers no differences were observed (Fig. 1). EGCG was previously reported to reduce MNV titers by only 34.54% after pre-treatment at 0.3 mM when incubated for 24 h (Seo et al., 2016). Hence, these results suggest that neither EGCG has an effect on blocking the host cell receptors nor with the attachment of the viral surface protein to the host cells.

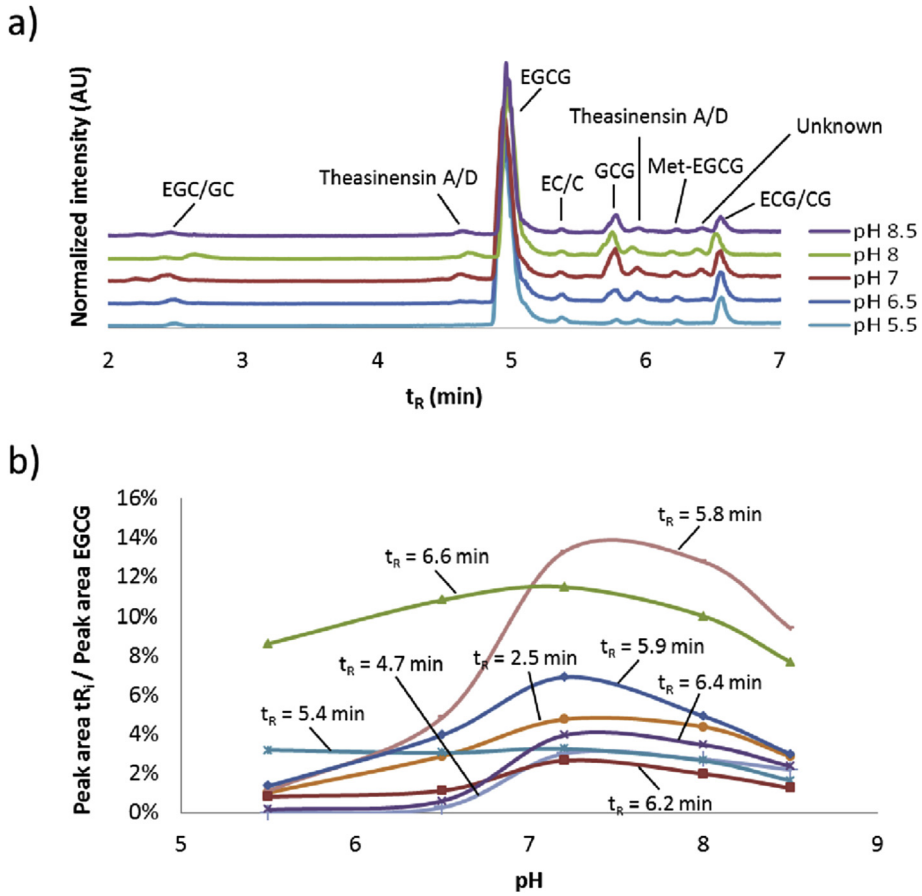

Fig. 4. Chromatograms of EGCG solutions after incubation at different pHs (a) and variation in the relative areas of the peaks at different retention times (t_R) with respect to EGCG (t_R = 4.9 min) (b).

3.3. Monitoring HAV and human norovirus inactivation by viability RT-qPCR assays

Molecular methods that measure HAV capsid integrity, such as the viability dye pre-treatment prior to RT-qPCR have been explored under different inactivation processes (Coudray-Meunier, Fraisse, Martin-Latil, Guillier, & Perelle, 2013; Fuster et al., 2016; Moreno et al., 2015; Sánchez et al., 2012). In our study, average levels of HAV inactivation after overnight treatment with 2.5 mg/mL of EGCG was evaluated both by $TCID_{50}$ assays and by two molecular assays, and compared (Fig. 2A). Molecular assays included RT-qPCR alone and RT-qPCR after PMAxx and Triton pre-treatment in order to assess capsid integrity. EGCG at 2.5 mg/mL only affected the infectious titer, with infectivity reduced below the detection limit (Fig. 2A). Despite this high effect on infectivity, no effects were observed in any of the genome copy numbers calculated by RT-qPCR alone or PMAxx/Triton-RT-qPCR. These results suggest that it is possible that viral capsids suffer structural alterations that render them non-infectious without causing holes through which PMAxx can get inside. Similar results have previously reported for other inactivation processes, whereas PMA did not completely prevent detection of HAV inactivated by high pressure processing or chlorine (Fuster et al., 2016; Sánchez et al., 2012).

Similarly, as human norovirus cannot be routinely cultivated, surrogates or viability PCR (Randazzo et al., 2016) has been used to assess human norovirus infectivity. In this case only RT-qPCR and PMAxx/Triton-RT-qPCR were evaluated, and likewise no effects were observed in any of the genome copy numbers calculated by both assays, indicating that PMAxx/Triton pre-treatment did not predict the infectivity of human norovirus after EGCG treatment (Fig. 2B). Therefore further studies needs to be performed with human norovirus and EGCG when the new culture system using enteroids cells will become routinely available (Ettayebi et al., 2016).

3.4. Effect of pH on the antiviral activity of EGCG

The results revealed that EGCG has a pH dependent antiviral effect for both viruses, showing greater inactivation rates at basic

Table 3
Green tea catechins and derivatives identified in the HPLC/MS spectra of EGCG solutions.

Retention time (min)	*m/z*	Compound (*)
2.5	305 $[M-H]^-$	EGC/GC
4.7	913 $[M-H]^-$	Theasinensin A/D
4.9	457 $[M-H]^-$; 915 $[2M-H]^-$	EGCG
5.4	289 $[M-H]^-$	EC/C
5.8	457 $[M-H]^-$; 479 $[M+Na-2H]^-$	GCG
5.9	913 $[M-H]^-$	Theasinensin A/D
6.2	471 $[M-H]^-$; 493 $[M+Na-2H]^-$	Methylated EGCG
6.4	525	Unknown
6.6	441 $[M-H]^-$; 463 $[M+Na-2H]^-$	ECG/CG

(*) Abbreviations: EGC: epigallocatechin; GC: gallocatechin; EGCG: epigallocatechin gallate; EC: epicatechin; C: catechin; GCG: gallocatechin gallate; ECG: epicatechin gallate; CG: catechin gallate.

and neutral pH (Fig. 3). For instance, EGCG at 2.5 mg/mL had almost no effect against MNV and HAV at pH 5.5, while complete inactivation was observed above pH 6.5. Similarly, antimicrobial activity of EGCG has been found to be pH dependent for herpes simplex virus (Isaacs et al., 2011) or *Candida albicans* (Hirasawa & Takada, 2004). The great instability of EGCG in slightly alkaline solutions gives rise to a number of different isomers and derivatives which may be responsible, at least partially, of the observed and reported antiviral activity. This pH dependent behaviour has to be considered for further developments, such as in sanitizer formulations containing EGCG (Zhang et al., 2016).

3.5. Identification of catechins and their derivatives

A total of 9 green tea catechins and their derivatives were identified in the chromatograms of the EGCG solutions after 2 h of incubation at different pH conditions (Fig. 4A). The identification was based on the values of m/z obtained from the mass spectra is summarized in Table 3. As expected, the relative concentration of these compounds after incubation was different depending on the pH of the buffer solution, due to the degradation and epimerization reactions which are known to take place in aqueous solutions (Wang, Zhou, & Jiang, 2008) and whose kinetics are pH-dependent (Zimeri & Tong, 1999). Fig. 4B shows the relative content of each catechin or catechin derivative with respect to EGCG after 2 h of incubation at different pHs, as detected by HPLC/MS.

In general, the concentration of the identified compounds relative to the concentration of EGCG increased with the pH up to 7.2, slightly decreasing at higher pHs. Two exceptions to this general tendency were epicatechin/catechin (EC/C, $t_R = 5.4$ min), which remained almost constant, and epicatechin gallate/catechin gallate (ECG/CG, $t_R = 2.5$ min) which experienced little variations with the pH, suggesting that the rate of degradation of these catechins relative to that of EGCG was similar at all pHs. For the rest of the compounds in Fig. 4, their presence (relative to EGCG) was greater at alkaline pHs. Indeed, some of these compounds have been reported as derivatives or degradation products from EGCG, like methylated EGCG (Suzuki et al., 2003) and theasinensin A/D (Sang, Lee, Hou, Ho, & Yang, 2005; Yoshino, Suzuki, Sasaki, Miyase, & Sano, 1999), as well as its epimer GCG. As EGCG is unstable in aqueous solution, the presence of its epimer and degradation products was not unexpected. In fact, in light of the results, the increased antiviral activity of the EGCG solutions at neutral and alkaline pHs could be attributed to the presence of EGCG derivatives instead of to EGCG itself. This hypothesis is in agreement with the results previously published by Isaacs et al. (2011), who reported that EGCG showed stronger antiviral activity against herpes simplex virus at neutral and alkaline pHs than at acidic pHs. Moreover, they found that EGCG digallate dimers, such as theasinensin A, exhibited antiviral activities both at acidic and neutral pHs. Given that the relative concentration of the theasinensin A/D (and other catechin derivatives) detected in the EGCG solutions increased from acidic to neutral pH, a plausible explanation for the increased antiviral activity detected against MNV and HAV at neutral and alkaline pHs would be that the degradation or epimerization products from EGCG, which include the aforementioned dimers, were responsible for the antiviral activity which had been attributed to the EGCG solutions. From Fig. 4 it can be observed that the compound which exhibited the greatest change in relative concentration with the pH was GCG, suggesting that the EGCG epimer could be potentially exerting an antiviral effect on MNV and HAV. Further studies with the individual EGCG derivatives should be done to ascertain which molecules are the main responsibles for the observed antiviral activity.

4. Conclusions

The effect of EGCG on MNV, a norovirus surrogate, and HAV was investigated. It was found that EGCG significantly decreased the MNV and HAV infectivity in a dose-dependent manner. Its antiviral activity was also found to vary with the temperature and the pH. EGCG was very effective against HAV and MNV at 25 and 37 °C and at neutral and alkaline pHs, while no effect was reported at 4 °C or pH 5.5. Furthermore, the HPLC/MS analysis of EGCG solutions at different pHs suggested that the observed changes in antiviral activity might be attributed to the presence of EGCG derivatives such as its epimer GCG or its digallate dimers theasinensins A/D. Overall, our findings highlight the potential of green tea catechins as a natural alternative to reduce viral contamination either as natural disinfectant, incorporated in food-contact surfaces or to be used as a therapeutic antiviral agent.

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